Chemokine-like receptor 1-positive cells are present in the odontoblast layer in tooth tissue in rats and humans

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Abstract. Cluster of differentiation (CD)44 is a marker of dental pulp stem cells and is involved in odontoblast differentiation and calcification. Chemokine-like receptor 1 (CMKLR1), also known as chemerin receptor 23 (ChemR23) is also expressed in odontoblasts and dental pulp stem cells and is involved in inflammation suppression and tooth regeneration. Resolvin E1, a bioactive lipid, is a CMKLR1 ligand that mediates the chemerin-CMKLR1 interaction and suppresses pulpal inflammation. The present study clarified the intracellular and tissue localization of CD44 and CMKLR1 by immunohistochemical staining of normal pulp and pulp with pulpitis from 12-week-old male Wistar rat teeth or human teeth. In addition, the localization of CD44 and CMKLR1 in human dental pulp stem cells was observed by immunofluorescence staining. The present study also examined the involvement of resolvin E1 in inhibiting inflammation and calcification by western blotting. CD44- and CMKLR1-positive cells were confirmed in the odontoblast layer in normal dental pulp of rats and humans. CD44 was mainly localized in the cell membrane and CMKLR1 was mainly found in the cytoplasm of human dental pulp stem cells. CMKLR1 was also confirmed in the odontoblast layer in rats and humans with pulpitis but CD44 was not present. Following treatment of dental pulp stem cells with lipoteichoic acid, which imitates Gram-positive bacterial infection, resolvin E1 did not suppress the expression of cyclooxygenase-2 or of the odontoblast differentiation marker, dentin sialophosphoprotein. Furthermore, resolvin E1 induced the differentiation of dental pulp stem cells into odontoblasts even in the presence of the inflammatory stimulus.

Introduction

Cluster of differentiation (CD)44 is a cell-surface glycoprotein in cell-cell interactions. Its expression has been confirmed in a number of cells including lymphocytes, cancer cells and mesenchymal stem cells (1-3). The authors previously revealed that CD44 is involved in odontoblast differentiation (4,5). Chemokine-like receptor 1 (CMKLR1), also known as chemerin receptor 23 (ChemR23), has been implicated in tooth development and in the inhibition of inflammation (6). Therefore, the present study aimed to determine the detailed localization of CD44 and CMKLR1 in teeth and to clarify changes in CD44 and CMKLR1 expression in pulpitis.

A number of studies have investigated inflammation of the dental pulp (7-10); however, further clarification of the mechanism of pulp inflammation is expected to lead to more reliable treatment of pulpitis. Suppression of pulpal inflammation and induction of remineralization have also been investigated (7,11-13) and are considered to be important in dental treatment to preserve the dental pulp or to regenerate the tooth structure (14-16). When dental pulp is infected with bacteria as a result of dental caries or periodontitis and irreversible pulpitis develops, removal of dental pulp is often performed (17). To remove pulp and perform root canal treatment, the tooth structure must be opened; however, the loss of tooth material leads to a higher risk of fracture or tooth extraction (18). By preserving uninfected pulp as much as possible, it is possible to prevent invasion of bacteria into the tooth interior, which is one of the functions of the pulp. Preserving or regenerating dental pulp is therefore thought to extend the life of a tooth. The present study focused on CMKLR1 because it is involved in tooth development and inflammation. A deeper understanding of CMKLR1 may be critical to suppressing inflammation in dental pulp.

CMKLR1 is present in ameloblasts and odontoblasts during tooth development and chemerin and CMKLR1 is expressed

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Abbreviations: ChemR23, chemerin receptor 23; CMKLR1, chemokine-like receptor 1; COX-2, cyclooxygenase-2; DAB, 3,3'-diaminobenzidine tetrahydrochloride; DSPP, dentin sialophosphoprotein; EDTA, ethylenediaminetetraacetic acid; LTA, lipoteichoic acid; RIPA, radioimmunoprecipitation assay

Key words: cluster of differentiation 44; chemerin receptor 23, chemokine-like receptor 1, dental pulp stem cells, odontoblast differentiation

in differentiating tooth epithelial and mesenchymal cells and has an important role in tooth development (19). Furthermore, chemerin/CMKLR1 interactions suppress excessive inflammation and promote tissue regeneration in non-dental tissues (20,21). CD44 is a transmembrane glycoprotein with various biological functions and is a marker for mesenchymal stem cells (22,23). It is also strongly expressed in odontoblasts during tooth development (24,25). CD44-positive cells in teeth are localized at the tip of the immature root and at the coronal pulpal corner. CD44 is also expressed in some odontoblasts and is involved in calcification (26).

These separate studies of CD44 and CMKLR1 indicate that CD44 is important for odontoblast differentiation and tooth mineralization and that CMKLR1 acts in the regulation of pulpal inflammation. Moreover, CD44 and CMKLR1 are localized in odontoblasts and undifferentiated mesenchymal cells of teeth. However, it remains unclear whether the two receptors, CD44 and CMKLR1, coexist in odontoblasts or undifferentiated mesenchymal cells. Furthermore, it is unclear how CD44 and CMKLR1 interact and participate in the induction of odontoblast differentiation, tooth calcification and inhibition of pulpitis. The present study investigated the localization of CD44- and CMKLR1-expressing cells in teeth and also observed changes in their expression during inflammation in pulpitis.

Materials and methods

Tissue samples from animals. Previous studies have attempted animal models of pulpitis in mice and rats (27,28). The present study generated a rat pulpitis model based on these studies. A total of five 12-week-old male Wistar rats (250-300 g) were purchased from Japan SLC, Inc., and maintained at 23±2˚C and a 60±5% humidity with 12 h light/dark cycles and free access to sterilized food and water. Animal experiments were approved by the ethics committee of Asahi University (approval number: 21-009 and 22-045). General anesthesia was induced with 6% isoflurane (MSD Animal Health) and maintained at 2%. Limbs and tails of rats under anesthesia were taped. The occlusal surfaces of the bilateral maxillary first molars were observed under a dissecting microscope and opened with a 1:5 speed-up contra angle TorqTech attachment with a round burr (Morita Corp.) to create cavities and expose the pulp (Fig. S1A). After the treatment, the animals were monitored and, if there were signs of pain, the endpoint of comfort treatment was considered. However, such signs were not observed in this experiment. At 24 h after pulp exposure, rats were deeply anaesthetized by intraperitoneal injection of 8% chloral hydrate (400 mg/kg) without signs of peritonitis. After confirming loss of consciousness, perfusion fixation was performed with cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) while bleeding (29-31). Cardiac arrest was confirmed in rats after perfusion fixation. Micro computed tomography confirmed that the hole in the treated tooth reached the pulp. (Fig. S1B). Excised maxillae were immersed in 10% EDTA-2Na (Dojindo Laboratories, Inc.) solution and decalcified at 4˚C for 4 weeks. Paraffin embedding was performed by immersing the specimen in absolute ethanol and xylene and then adding dissolved paraffin according to the general method. Horizontal 5-μm sections were prepared using a microtome (REM-700; Yamato Kohki Industrial Co., Ltd.). Some sections were stained with 0.03% toluidine blue to confirm histological morphology.

Tooth samples. Human teeth were extracted from patients for orthodontic treatment after obtaining their permission. Teeth were placed in 10% formalin neutral buffer solution (FUJIFILM Wako Pure Chemical Corporation) and stored at 4˚C. Three normal upper wisdom teeth and two wisdom teeth with irreversible pulpitis were used. One of the affected teeth was the lower right wisdom tooth of a 55-year-old male (Fig. 4A) and the other was the upper right wisdom tooth of a 36-year-old male (Fig. 4B), which had to be extracted due to irreversible pulpitis associated with dental caries. The study was approved by the Asahi University Research Ethics Committee (approval no. 31020) and was conducted according to the Declaration of Helsinki. Teeth were immersed in 10% EDTA-2Na (Dojindo Laboratories, Inc.) solution and decalcified at 4˚C for 4 weeks. Thereafter, teeth were immersed in EDT-X (neutral decalcifying solution, FALMA, Inc.) at room temperature for an additional 4 weeks. Tissues were then embedded in paraffin and horizontal 5-μm sections were prepared using a sliding microtome (REM-700; Yamato Kohki Industrial Co., Ltd.).

Immunohistochemistry. Serial 5-μm paraffin sections were deparaffinized using xylene and serial ethanol dilutions and then stained with hematoxylin for 20 min and then eosin for 20 min at room temperature. Endogenous peroxidase was inactivated by treatment with 3% hydrogen peroxide solution for 10 min at room temperature. Sections were then washed with PBS, treated with 1% BSA for 30 min at room temperature and incubated with anti-CD44 (anti-homing receptor, cloneA020: MilliporeSigma) antibody at 1:200 dilution for 24 h at room temperature. Antigen retrieval for CMKLR1 antigen was performed using a microwave rapid sample processor MI-77 (Azumaya) with settings of 80˚C, 20 min and an output of 6. Sections were then incubated with anti-chemokine-like receptor 1 polyclonal antibody (anti-CMKLR1; Cayman Chemical Company) diluted at 1:100, followed by incubation with peroxidase-labelled goat anti-rat IgG (Bethyl Laboratories, Inc.) or Nichirei Histofine R (Nichirei Biosciences Inc.) diluted at 1:500 for 30 min. DAB staining was then performed and the nuclei were counterstained with hematoxylin for 1 min at room temperature.

Cells. Human dental pulp stem cells were obtained from Lonza Group Ltd. Stem cells were cultured at 37˚C in Dental Pulp Stem Cell Growth Medium (Lonza Group Ltd.) in humidified air containing 5% CO_2.

Western blotting. Whole-cell extracts were obtained using lysis buffer (10X RIPA buffer; Cell Signaling Technology, Inc. supplemented with 1 mM PMSF and 1X protease inhibitors. Total protein concentration in the lysates was assayed using Pierce^{TM} 660 nm Protein Assay Reagent (Thermo Fisher Scientific Inc.). A 10 μg protein sample was separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride membranes. After blocking with 5% non-fat milk for 1 h at room temperature,

membranes were then incubated with anti-DSPP mouse monoclonal (cat. no. sc-73632; 1:200; Santa Cruz Biotechnology Inc.), anti-CD44 mouse monoclonal (cat. no. 5640; 1:1,000; Cell Signaling Technology, Inc.), anti-COX-2 rabbit polyclonal (cat. no. SAB4200576; 1:500; MilliporeSigma), or β-actin (cat. no. 5441; 1:10,000; MilliporeSigma) antibodies for 1 h at room temperature. A peroxidase-conjugated secondary antibody (anti-rabbit IgG; cat. no. 7074; 1:1,000; or anti-mouse IgG; cat. no. 7076; 1:1,000; Cell Signaling Technology, Inc.) for 1 h at room temperature, and chemiluminescence (Clarity™ Western ECL substrate; Bio-Rad Laboratories, Inc.) were then used to visualize immunoreactive proteins. Images were acquired using a Light-Capture II instrument (Atto Co., Ltd.). Band intensity was semi-quantified by densitometry using a CS Analyzer 3.0 (Atto Co., Ltd.). The abundance of the target protein relative to the abundance of β-actin was calculated.

Immunofluorescence staining. Samples were blocked with ready-to-use Immunofluorescence Blocking Buffer (Cell Signaling Technology, Inc.) for 1 h. The blocking solution was aspirated and anti-mouse CD44 monoclonal (cat. no. 5640, 1:400; Cell Signaling Technology, Inc.) and anti-rabbit CMKLR1 polyclonal (1:200; Cayman Chemical) primary antibodies were then added at the same time and incubated overnight at 4˚C. Fluorescence-labelled secondary anti-mouse IgG(H+L), F(ab')2 Fragment (Alexa Fluor R555 conjugate; cat. no. 4409, 1:500; Cell Signaling Technology, Inc.) and anti-rabbit IgG(H+L), F(ab')2 Fragment (Alexa Fluor R488 conjugate; cat. no. 4412, 1:500; Cell Signaling Technology, Inc.) were added, incubated in the dark for 1 h and counterstained with DAPI (cat. no. 4083, 1:2,000; Cell Signaling Technology, Inc.) for 5 min in the dark at room temperature. After dehydration and encapsulation, observation was performed using an LSM710 confocal laser microscope (Zeiss GmbH) and images were acquired with LSM780 software ZEN (version 2012 SP1; Zeiss GmbH).

Statistical analysis. Semi-quantitative western blotting data are presented as the mean ± standard deviation from three independent experiments and were evaluated using one-way analysis of variance followed by Dunnett's multiple comparison. $P<0.05$ was considered to indicate a statistically significant difference.

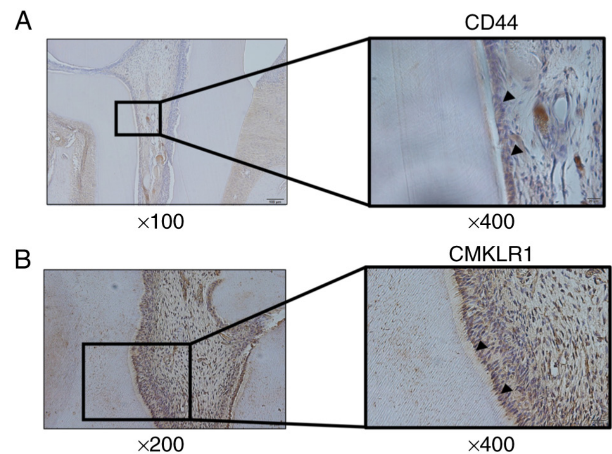

Figure 1. Localization of CD44 and CMKLR1 in rat tooth tissue. Immunohistochemical staining of CD44 in normal rat dental pulp. (A) The odontoblast layer shows anti-CD44 antibody positivity. (B) Immunohistochemical staining of CMKLR1 in normal rat dental pulp. The odontoblast layer shows anti-CMKLR1 antibody positivity. Arrowheads indicate representative CD44- or CMKLR1-positive cells in the odontoblastic layer. CD44, cluster of differentiation 44; CMKLR1, chemokine-like receptor 1.

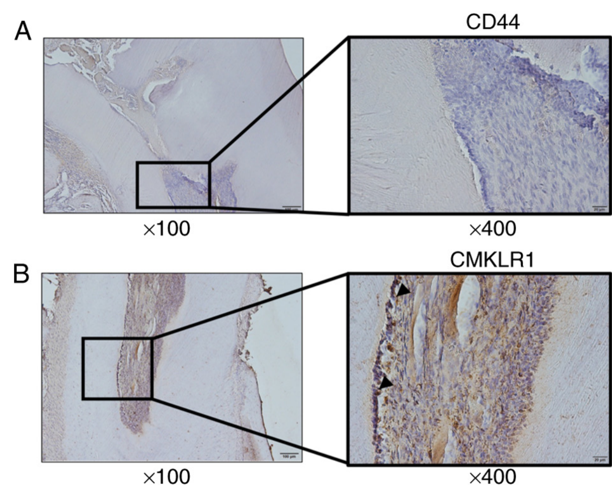

Figure 2. Localization of CD44 and CMKLR1 in rat pulpitis tissue. Immunohistochemical staining of CD44 in a rat pulpitis model. (A) Odontoblasts were not CD44-immunoreactive. (B) Immunohistochemical staining of CMKLR1 in a rat pulpitis model. Arrowheads indicate representative CMKLR1-positive cells in the odontoblastic layer. CD44, cluster of differentiation 44; CMKLR1, chemokine-like receptor 1.

Results

Localization of CD44 and CMKLR1 in rat teeth. CD44 and CMKLR1 localization in rat teeth was assessed by immunohistochemical staining. CD44 and CMKLR1 were both detected in odontoblasts, which connect to dentinal tubules in the enamel dentin junction (Fig. 1A and B). CD44- and CMKLR1-positive cells were confirmed in the odontoblast layer at sites such as the medullary horn and the base of the medulla. In pulp tissue that was inflamed by drilling a cavity in a tooth, the CD44-positive cells observed in normal tissue disappeared but CMKLR1-positive cells remained in the odontoblastic layer (Fig. 2A and B).

Localization of CD44 and CMKLR1 in human teeth. CD44 and CMKLR1 expression in human teeth were next verified. Immunohistochemical staining confirmed CD44- and CMKLR1-positive cells in the odontoblast layer around the pulp horn and at the base of the pulp bed, as in rat teeth (Fig. 3A and B). In odontoblasts from human pulpitis tissue, CD44-positive cells disappeared but CMKLR1-positive cells remained, as in rat inflamed pulp tissue (Fig. 4A and B).

Localization of CD44 and CMKLR1 in human dental pulp stem cells. The localization of CD44 and CMKLR1 in human dental pulp stem cells was confirmed by immunostaining. CD44 and CMKLR1 were both detected in different cytoplasmic sites in dental pulp stem cells but co-localization was not observed. CD44 was mainly localized in the cell

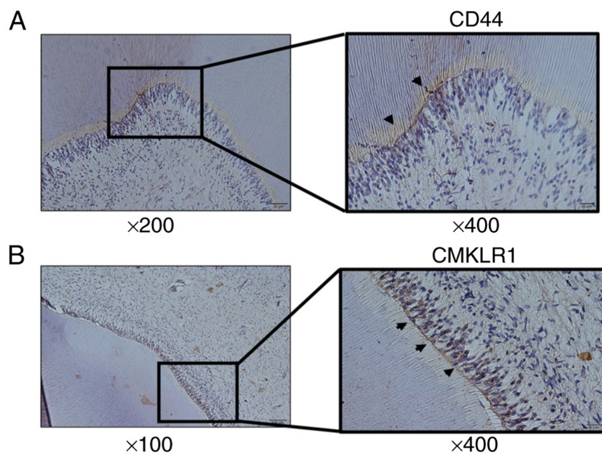

Figure 3. Localization of CD44 and CMKLR1 in human tooth tissue. Immunohistochemical staining of CD44 in human teeth. (A) Odontoblasts were CD44-immunoreactive. (B) Immunohistochemical staining of CMKLR1 in human teeth. Odontoblasts were CMKLR1-immunoreactive. Arrowheads indicate representative CD44- or CMKLR1-positive cells in the odontoblastic layer. CD44, cluster of differentiation 44; CMKLR1, chemokine-like receptor 1.

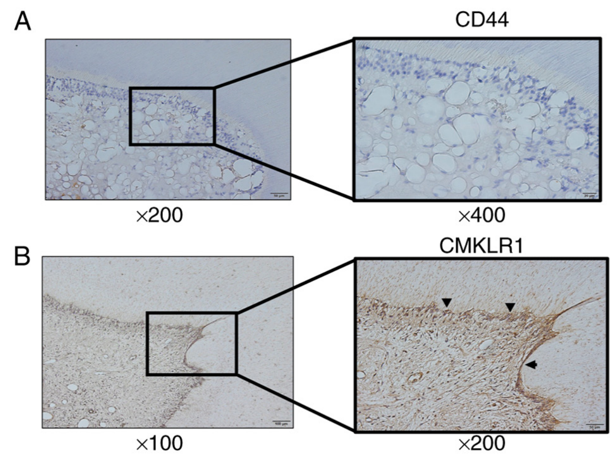

Figure 4. Localization of CD44 and CMKLR1 in human pulpitis tissue. Immunohistochemical staining of CD44 in human pulpitis. No CD44 immunoreactivity was observed (A) Immunohistochemical staining of CMKLR1 in human pulpitis (B) CD44, cluster of differentiation 44; CMKLR1, chemokine-like receptor 1.

membrane, while CMKLR1 was mainly detected in the cytoplasm (Fig. 5A and B).

Effect of resolvinE1 on human dental pulp stem cells. CMKLR1 regulates pulpal inflammation; therefore, the expression of cyclooxygenase-2 (COX-2) was examined to confirm whether stimulation with lipoteichoic acid (LTA), a major component of the Gram-positive bacterial cell wall (32,33), causes an inflammatory reaction in dental pulp stem cells. COX-2 expression was confirmed after stimulation with 10 to 50 μg/ml LTA (Fig. 6A and B). Resolvin E1, a bioactive lipid that interacts with chemerin/CMKLR1, is produced from ω-3 fatty acids and has an inflammatory convergence effect; it suppresses the increase in neutrophil infiltration in inflammatory exudate (34). Notably, treatment of dental pulp stem cells with resolvin E1 at 1 to 5 μM resulted in the expression of dentin sialophosphoprotein (DSPP), a differentiation marker for odontoblasts, but at 10 μM resolvin E1, DSPP expression ceased (Fig. 6C and D). Therefore, an optimum concentration of resolvin E1 is required to induce the differentiation of dental pulp stem cells into odontoblasts. Next, it was examined how the induction of DSPP expression is affected by the inflammatory response using low concentrations of resolvin E1 at optimum concentration. When the effect of LTA and resolvin E1 on inflammatory stimulation was examined, COX-2 expression was induced by LTA plus resolvin E1 but DSPP expression was not suppressed, while CD44 expression remained unchanged (Fig. 6E and F). This indicated that resolvin E1 may be able to induce the differentiation of dental pulp stem cells into odontoblasts even when infected with Gram-positive bacteria.

Discussion

Endodontic therapy in clinical dentistry is a treatment that attempts to preserve teeth by removing infected pulp, protecting the remaining pulp and maintaining an aseptic environment as far as possible. Therefore, preservation and regeneration of dental pulp are important areas of research in the field of endodontics. The control of pulpitis is essential for preserving pulp. The suppression of pulp inflammation and the induction of remineralization promote the preservation and regeneration of dental pulp. In previous research, we focused on CD44 and found that it is an important molecule for inducing the differentiation of dental pulp stem cells into odontoblasts. Other papers have shown that in tooth tissue, CD44 is expressed in the dental pulp tissue and odontoblast layer, which are in the process of differentiation (24-26). When considering the clinical application of research on the induction of differentiation of dental pulp stem cells into odontoblasts, it is possible to regenerate dentin by applying dental pulp capping to tissues that have been clinically inflamed. Considering this, several points are not yet clear: i) What is the localization of CD44 in tooth tissue when there is inflammation? ii) Are there molecules in tooth tissue that control inflammation and are involved in inducing differentiation of dental pulp stem cells into odontoblasts? If so, where are they located? Therefore, the present study focused on CD44, which is thought to be involved in inducing the differentiation of dental pulp stem cells into odontoblasts and CMKLR1, which is involved in the regulation of inflammation. The present study aimed to clarify their localization in dental tissues.

The present study confirmed that CD44 and CMKLR1 were localized in odontoblasts in the pulp horn or pulp floor. CD44 and CMKLR1 were also expressed in dental pulp stem cells. CD44 was localized in the cell membrane and CMKLR1 was detected in the cytoplasm of dental pulp stem cells. Although it is unclear from immunohistochemical staining alone whether CD44 and CMLR1 are present in the cytoplasm or cell membrane of the odontoblast layer, it was evident that CD44 and CMKLR1 were expressed in the odontoblast layer in the present study. In pulpitis-affected teeth, odontoblast structures were disrupted and CD44 expression was not observed but CMKLR1 immunoreactivity remained in the odontoblastic layer. Furthermore, the expression of COX-2 was not suppressed by resolving E1, a bioactive lipid that acts on CMKLR1; however, in the inflammation model of dental pulp stem cells, the differentiation of dental pulp stem cells into

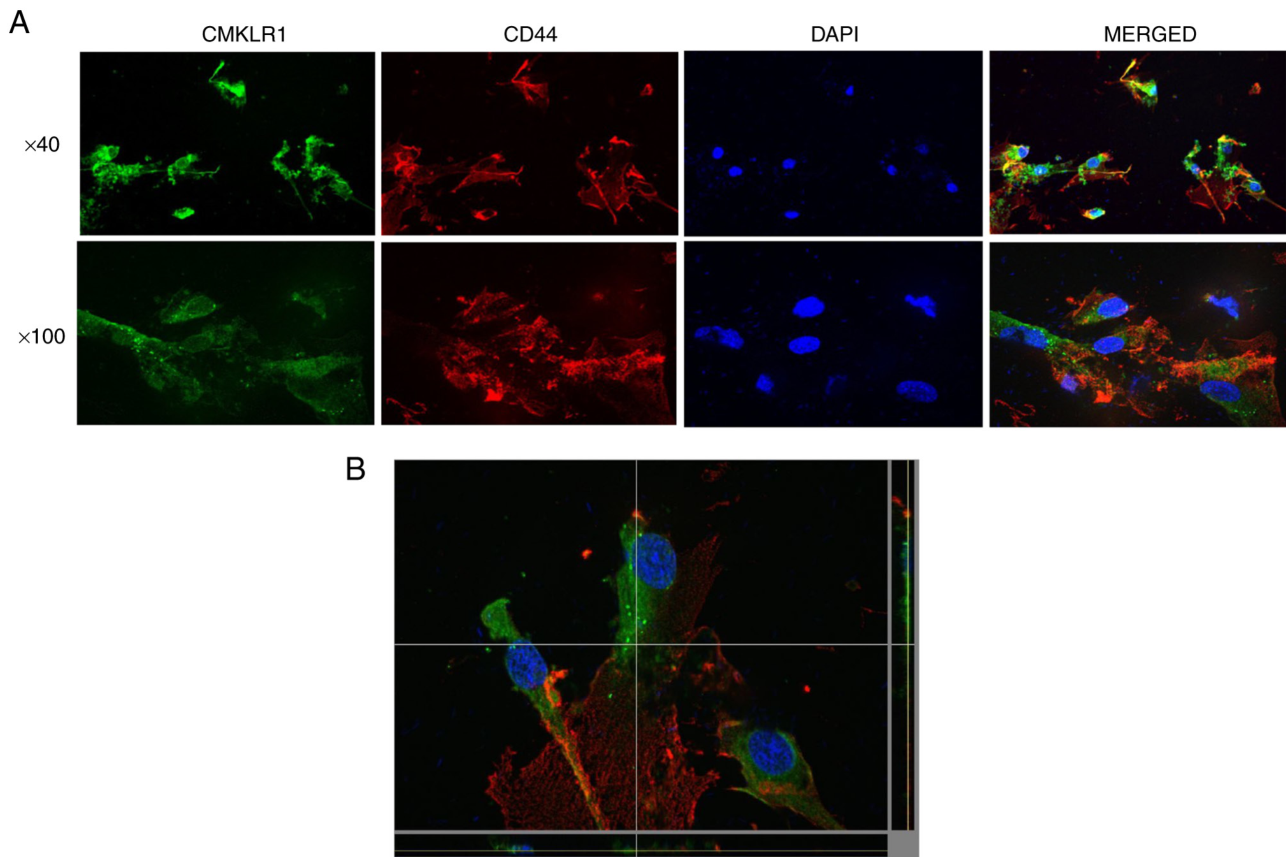

Figure 5. CD44 and CMKLR1 immunostaining in human dental pulp stem cells. (A) Localization of CD44 (red) and CMKLR1 (green) in dental pulp stem cells. Nuclei are stained with DAPI (blue). Localization of CD44 and CMKLR1 in cross-sections of dental pulp stem cells. (B) CD44 was mainly found in the cell membrane and CMKLR1 was mainly found in the cytoplasm. CD44, cluster of differentiation 44; CMKLR1, chemokine-like receptor 1.

odontoblasts was still induced. This indicated that targeting CMKLR1 may be useful for inducing the differentiation of dental pulp stem cells into odontoblasts in pulpitis.

CD44 is a surface marker of dental pulp stem cells and is involved in the induction of their differentiation into odontoblasts. Our previous study revealed the following points: i) Even when dental pulp stem cells were induced to differentiate into odontoblasts by hyaluronic acid, a ligand for CD44, there was no change in the expression of CD44 (5); ii) shikonin (a naphthoquinone compound) induces differentiation of dental pulp stem cells into odontoblasts. However, knockdown of *CD44* in dental pulp stem cells does not induce their differentiation into odontoblasts (4); and iii) in the experiment using dental pulp stem cells, even when inflammatory stimulation was applied with LTA, there was no significant change in the expression of CD44 as detected by western blotting analysis. Considering these three points, even if the expression of CD44 in the odontoblastic layer disappears because of inflammation, it cannot be denied that CD44 is important for odontoblastic differentiation. In dental tissue, CD44-expressing cells are found in the apical portion of immature roots or in the odontoblast layer (26). CMKLR1 is involved in suppressing inflammation (6,21) and the action of chemerin-CMKLR1 is important for tooth development (19,35). However, the localization of CMKLR1 in teeth has not been clarified. The present study determined the detailed localization of CD44 and CMKLR1 in tooth tissue. CD44 expression was not observed in pulpitis, in which the pulp tissue was degenerated and the arrangement of the odontoblast layer was disturbed, but CMKLR1 expression was still confirmed in the degenerated odontoblast layer. It was also observed that CMKLR1 was expressed in dental pulp stem cells and was mainly localized in the cytoplasm. Furthermore, it was determined that resolvin E1, which acts on CMKLR1, did not suppress the induction of differentiation of dental pulp stem cells into odontoblasts even in the presence of an inflammatory stimulus.

The present study showed that resolvin E1 induced the differentiation of dental pulp stem cells into odontoblasts. This induction was also observed in the presence of an inflammatory stimulus. This indicated that resolvin E1 may induce odontoblast differentiation even in the presence of pulpitis. However, these findings were made in cell-based experiments and it is necessary to verify the inhibition of pulpitis and the regeneration of dentin by resolvin E1 treatment in an animal model of pulpitis.

The present study confirmed that CD44 and CMKLR1 are both present in odontoblasts of dental tissue. CD44 and CMKLR1 were both observed in the plasma membrane and CMKLR1 was also detected in the cytoplasm of dental pulp stem cells. In addition, although resolvin E1, which acts on CMKLR1, did not suppress the expression of COX-2, it induced the differentiation of dental pulp stem cells into odontoblasts. It is therefore hypothesized that resolvin E1 may be useful in inducing odontoblast differentiation in inflamed dental pulp. Although a direct association between CD44 and CMKLR1

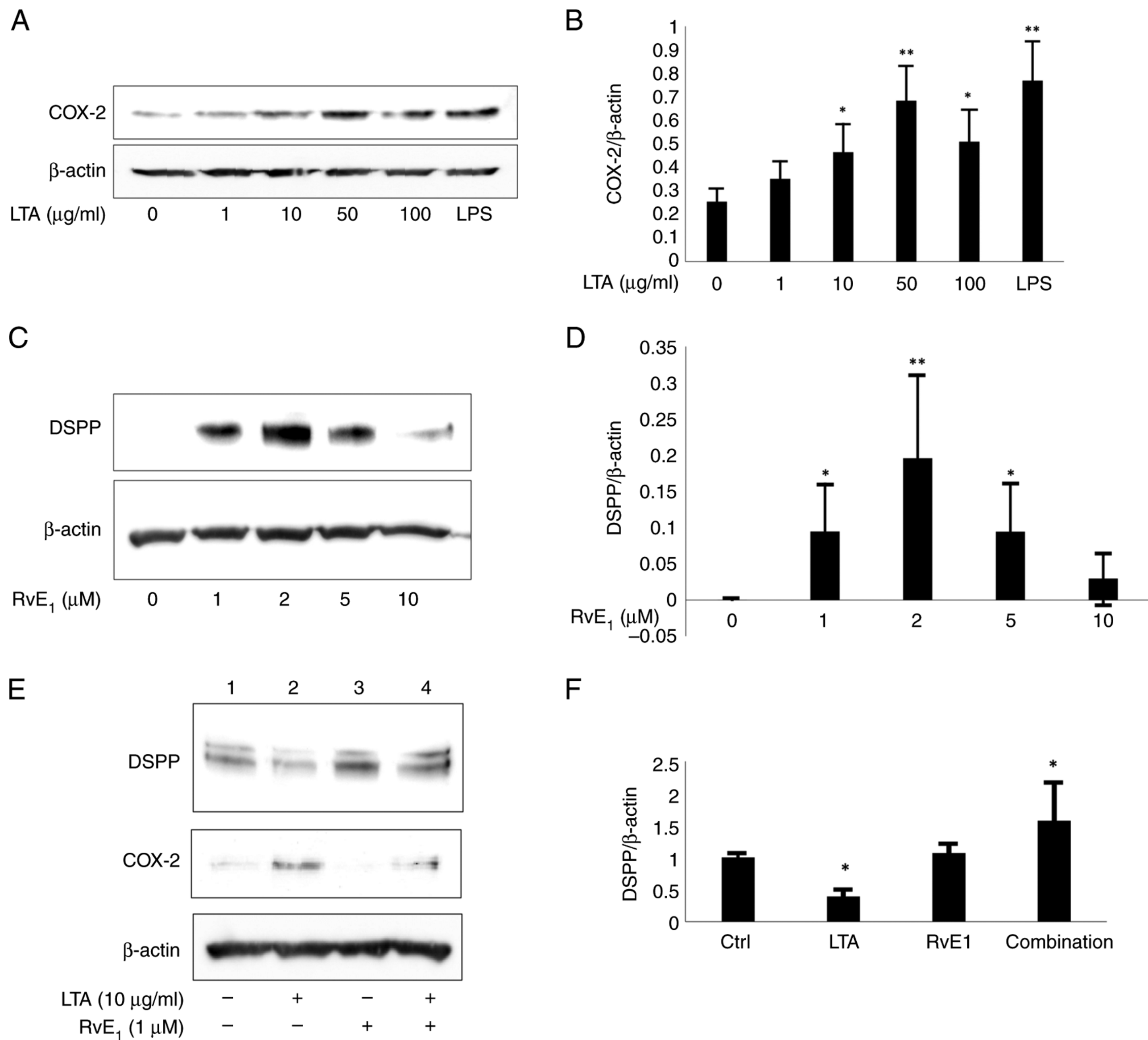

Figure 6. Ability of resolvin E1 to induce differentiation of dental pulp stem cells into odontoblasts. COX-2 was expressed after stimulation with 10 to 50 μg/ml LTA (A) The protein level of COX-2 in response to a negative control (lane 1) was semi-quantified relative to the level of β-actin. (B) Values were obtained from three independent experiments, with the COX-2 level in the negative control lane (lane 1) set to 1. *P<0.05, **P<0.01 (lane 1 vs. lane 2, 3, 4, 5 or 6). (C) When dental pulp stem cells were treated with 1-5 μM resolvin E1, DSPP was expressed, but when treated with 10 μM resolvin E1, DSPP expression ceased. (D) The protein level of DSPP in response to a negative control (lane 1) was semi-quantified relative to the level of β-actin. The negative control lane (lane 1) was set to 1. *P<0.05, **P<0.01 (lane 1 vs. lane 2, 3, 4 or 5). (E) COX-2 was expressed and DSPP expression was not suppressed after LTA and resolvin E1 (1 μM) treatment, while CD44 expression remained. (F) The protein level of DSPP in response to a negative control (lane 1) was semi-quantified relative to the level of β-actin. Values were obtained from three independent experiments, with the DSPP level in the negative control lane (lane 1) set to 1. *P<0.05 (lane 1 vs. lane 2, 3 or 4). COX-2, cyclooxygenase-2; LTA, lipoteichoic acid; DSPP, dentin sialophosphoprotein; CD44, cluster of differentiation 44.

was not determined in the present study and exploration of the interaction between CD44 and CMKLR1 is a future issue, CD44 and CMKLR1 may play an important role in the preservation and regeneration of dental pulp.

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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 on reasonable request.

Authors' contributions

DY performed the majority of the experiments and drafted the manuscript. NU contributed to the experimental design, performed some experiments and drafted the manuscript. YM performed some experiments and data analyses. NK participated in the study design and manuscript preparation

and critically revised the manuscript. SK contributed to the experimental conceptualization and data interpretation and critically revised the manuscript. DY and NU confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Asahi University Research Ethics Committee (approval no. 31020) and written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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