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Therapeutic effect of a topical CCR2 antagonist on induced alveolar bone loss in mice

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Background and Objective: Chemokines are known to regulate leukocyte trafficking, recruitment and infiltration in periodontal diseases. The study objective was to determine the effect of an experimental oral/topical chemokine (C-C motif) receptor 2 (CCR2)-antagonist treatment on alveolar bone loss in a mouse model of *Porphyromonas gingivalis*-induced periodontitis.

Material and Methods: Balb/C mice ($n = 41$) were randomly assigned to four groups. Group 1 was infected by *P. gingivalis* applied orally/topically for 5 wk. Group 2 was also infected and then treated with vehicle (aqueous methylcellulose) for an additional 4 wk. Group 3 was infected and orally/topically treated with CCR2 antagonist (10 mg/kg). Group 4 served as a noninfected, nontreated control group. Mice received intraperitoneal injections of Alizarin (30 mg/kg) and calcein (20 mg/kg) three times from the last day of infection to determine mineral deposition, reflecting bone dynamics. Mandibles were analysed by morphometry and confocal fluorescence microscopy.

Results: Alveolar bone loss was compared among groups using Tukey's test, and bone formation was qualitatively observed. Infected mice showed significantly greater alveolar bone loss than noninfected control animals (group 1 vs. 4, $p < 0.01$). Vehicle-treated mice (group 2) showed the largest area of alveolar bone loss ($p < 0.01$), while mice treated with the CCR2 antagonist showed the smallest area of alveolar bone loss and were similar to the control group (group 3 vs. 4, $p = 0.14$). Qualitative analysis of fluorescent dye uptake indicated increased bone formation in CCR2-antagonist-treated mice, suggesting an improved bone repairing process.

Conclusion: The results suggested that treatment with CCR2 antagonist inhibited alveolar bone loss and improved bone formation in this model. These data support further evaluation of CCR2 antagonist as a therapeutic target for the development of new treatment modalities on bacterially induced alveolar bone resorption.

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Inflammation and oxidative stress are known to be major components in the pathogenesis of several diseases, including atherosclerosis (1), asthma (2), multiple sclerosis (3), inflammatory arthritis (4) and periodontal diseases (5). In particular, periodontal disease is associated with microbial infection due to the build-up of an extremely diverse biofilm, with up to 100 cultivable species attached to the dental surface (6). As a chronic disease with persistent inflammatory host response, periodontal disease is typically characterized by the constant migration of polymorphonuclear

leukocytes, monocytes, lymphocytes, plasma and mast cells to specific gingival lesions in response to dental biofilm challenges (7). These primary proinflammatory events increase the flow of gingival crevicular fluid exudate, thus changing the local environment and allowing proteolytic and anaerobic species to predominate. As a result, the host immune response increases local tissue infiltration by macrophages and polymorphonuclear leukocytes, which in turn upregulate the production of cytokines and orchestrate a chronic proinflammatory response that ultimately results in alveolar bone resorption and tooth loss.

Among the different and complex signaling events derived during the onset of inflammation, chemokines are a family of potent chemotactic cytokines that regulate the trafficking and recruitment of leukocytes to infiltrate distant sites of developing inflammation (8). There are approximately 50 human chemokines that can be classified into two major families based on differences in their structure and function (9). They are divided into two major families, CC and CXC, based upon structural differences in arrangements of their N-terminal cysteine motifs (10). In general, the CC chemokine family is in charge of attracting mononuclear cells to sites of chronic inflammation. For example, the monocyte chemoattractant protein MCP-1 (also known as CCL2) is a well-characterized CC chemokine, which acts as a potent agonist for monocytes, memory T cells and basophils. Monocyte chemoattractant protein 1 can be produced by fibroblasts, endothelial cells, monocytes/macrophages, osteoblasts and mast cells (11). Monocyte chemoattractant protein 1 is recognized by chemokine (C-C motif) receptors 1 and 2 (CCR1 and CCR2) receptors, which are highly expressed on monocytes/macrophages (12). The interaction between these chemokine receptors on leukocyte surfaces (13) and their ligands results in diapedesis and the infiltration of these cells into tissues.

Chemokines, along with other proinflammatory mediators, have been of interest in periodontal research, although their biological relevance still remains largely undetermined. In humans, chemokines and adhesion molecules have been found to be differentially expressed in periodontal diseased tissues or related gingival crevicular fluid when compared with healthy sites (14,15), and MCP-1 expression seems to be confined to leukocytes infiltrating the connective tissue in gingival lesions (11). Untreated periodontitis patients show a trend towards high MCP-1 levels, which correlate with clinical severity (16), and periodontal therapy seems consistently to decrease MCP-1 levels in gingival crevicular fluid (17,18) and peripheral blood (19,20). However, to date, there is no experimental evidence of chemokine-mediated therapeutics in periodontal diseases. Since chemokines, such as MCP-1 and its interaction with receptors CCR1 and CCR2, play a key role during tissue infiltration and the onset and progression of inflammation in several inflammatory diseases, they may represent an attractive target for therapeutic intervention in periodontal diseases.

Different animal models of experimental periodontitis have been produced, in which oral mucosal inflammation and alveolar bone loss can be reliably induced in rodents by the direct application of oral bacteria (21). For example, *Porphyromonas gingivalis*, a gram-negative, black-pigmented oral microorganism that has been implicated as one of the major pathogens in the progression of periodontal disease, can induce local periodontal infection and alveolar bone loss (22). We used an experimental periodontitis model in Balb/C mice infected with *P. gingivalis* and maintained on a diet of macerated chow containing 30% dextrose in water. We aimed to determine the effect of a topically administered experimental CCR2 antagonist, JNJ-17166864, on alveolar bone loss resulting from *P. gingivalis*-induced experimental periodontal disease.

Material and methods

Animal protocol

All procedures were performed in accordance with animal welfare guidelines and approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. Balb/C mice were obtained at approximately 6 wk of age and maintained in standardized conditions of 12 h–12 h light–dark cycle (light from 07.00 to 19.00 h), a constant temperature of 25°C, regular mouse chow and water *ad libitum*. All animals were allowed a minimum of 1 wk to adapt to their new environment before any procedure was performed. During the experiment, all mice were fed with soft chow, obtained by macerating the regular chow with dextrose (30%, dissolved in water), to induce formation of dental plaque, facilitating bacterial adhesion.

Bacterial inoculum and drug preparation procedures

Aliquots of *P. gingivalis* strain A7436 were maintained in Wilkins-Chalgren anaerobe broth medium (WC broth; DSMZ, Braunschweig, Germany) containing 10% skim milk at −80°C. Aliquots were reconstituted on Anaerobic Reducible Blood Agar (Remel, Lenexa, KS, USA). For experiments, bacteria were grown anaerobically in an atmosphere of 5% CO_2, 10% H_2 and 85% N_2 at 37°C for 4–5 d. Bacterial suspensions were prepared from primary cultures at their log phase of growth, and concentrations were determined by spectrophotometry (Cecil Instruments Ltd, Cambridge, UK) with a measured optical density at 660 nm corresponding to 10^9 bacteria/mL. Finally, bacterial suspensions were centrifuged at 2150 *g* for 10 min and reconstituted according to the dose with methylcellulose (1%) as a carrier to facilitate bacterial application. The experimental CCR2 antagonist, JNJ-17166864 (OraPharma Inc., Warminster, PA, USA), was added to a 0.025% acetic acid solution, which was sonicated for 5 min to ensure complete dissolution, and then adjusted to 10 mg/kg with a 1% methylcellulose aqueous solution for mice in the treatment group. A vehicle control was prepared following the same protocol but omitting the experimental CCR2 antagonist compound.

Experimental design

Balb/C mice ($n = 41$) received once daily ampicillin/kanamycin intraperitoneal administration (50 mg/kg of kanamycin and 25 mg/kg of ampicillin) for 4 d in order to suppress their normal oral microbiota and to facilitate subsequent bacterial colonization. After a wash-out period of 3 d, animals were randomly assigned to experimental groups as follows. Group 1 (positive control, $n = 11$) was infected daily with approximately 100 μL of 10^9 bacteria/mL in 1% methylcellulose by topical application into the oral cavity for 5 wk. Group 2 (vehicle group, $n = 11$) was also infected daily for 5 wk, but additionally treated twice daily with topical application of CCR2 vehicle only (0.1 mL of 1% aqueous carboxymethylcellulose) for an additional 4 wk. Group 3 (treatment group, $n = 11$) was also infected daily for 5 wk and then treated with 0.1 mL of the experimental CCR2 antagonist in 1% aqueous carboxymethylcellulose solution (10 mg/kg) applied twice daily by oral rinse, for an additional 4 wk. Group 4 ($n = 8$) was neither infected nor treated and served as a control group. Noninfected mice were kept in a separate room from the infected animals in the same conditions of light and temperature.

In order to analyse the time course of bone metabolism/formation, all mice in groups 2, 3 and 4 were given intraperitoneal injections of the fluorochromes Alizarin red (Sigma, St Louis, MO, USA; at 30 mg/kg) and calcein (Sigma; at 8 mg/kg), starting on the last day of the infection with Alizarin injection followed with two injections of calcein. Intraperitoneal injection of Alizarin and calcein offers a distinct sphere of usefulness; it is rapidly absorbed, rapidly fixed in the growing bone and the excess rapidly excreted, and the staining effects of these bone markers are precise and limited to a single layer of bone being laid down at the time of the circulation.

The time interval was 11 d between the first injection of bone marker (Alizarin red, 30 mg/kg) and the second injection (calcein, 8 mg/kg). The third injection (calcein, 8 mg/kg) was applied 10 d after the second.

These bone markers bind to calcium in the mineralization front of newly formed bone, providing three interlabel periods as shown in Figs 2–4.

Histomorphometric analysis

Mice from group 1 (positive control) were euthanized with 5 min exposure to CO_2 followed by cervical dislocation and the mandibles processed for cemento-enamel junction–bone crest morphometry to evaluate the area of alveolar bone loss after 5 wk of oral infection. Mouse mandibles were split into halves from the mid-line between the central incisors. One hemimandible was taken for morphometric analysis, and the other used for histological evaluation. For cemento-enamel junction–bone crest morphometric analysis, mandibles were defleshed by immersion in 3% hydrogen peroxide and stained with 1% methylene blue solution (Sigma-Aldrich, St Louis, MO, USA) to allow identification of the cemento-enamel junction and the alveolar bone crest. By using a stereomicroscope (Leica M420; Salzburg, Austria) with a video camera and computer, the surfaces of the mandibular dentition were recorded in a standardized manner by placing each jaw on a platform jig that provided uniform object-to-image capture geometry. The mandibular cemento-enamel junction–alveolar bone crest measurements were obtained on the recorded images by using ImageJ software (NIH, Bethesda, MD, USA). Digital measurements were done in a standardized manner by two examiners masked to the original treatment protocol. Hemimandibles were coded, and one examiner (S.P.B.), masked to the treatment conditions, analysed the tissues through a confocal laser scanning microscope (LSM5; Carl Zeiss, Thornwood, NY, USA) for bone deposition labeling with fluorochromes (Alizarin red and calcein). The molar regions of the mandible were embedded in acrylic resin and cut into 0.8-mm-thick sections. Sections were polished to obtain thinner sections of 30–50 μm. The area of bone formation (mineralization of the osteoid) was indicated by the distance between the two fluorescent markers injected on the last day of disease induction and during the 4 wk of drug treatment.

Statistical analysis

A minimal sample size of four mice per group was calculated [power $(1 - \beta)$ of $>0.90\%$ with error threshold of $\alpha = 0.05$] based on reported alveolar bone area differences using a similar animal model of periodontal disease (23). We designed the experiment with eight control noninfected animals and 11 infected animals per group with different treatments; however, two animals from the noninfected group and one animal from group 2 (vehicle treated) died during the experimental phase. The continuous variable (alveolar bone loss) was described using means and standard errors, and their values were analysed using Tukey's test ($p < 0.01$). All analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC, USA).

Results

Representative photomicrographic images of each set of treatment conditions, illustrating the area corresponding to the cemento-enamel junction and alveolar bone crest, are shown in Fig. 1. The results of the morphometric analysis are shown in Table 1 and indicate that animals infected with *P. gingivalis* and killed immediately after the infection period (group 1) showed significantly higher alveolar bone loss in comparison to noninfected control animals (group 4), as shown by a more extensive cemento-enamel junction–alveolar crest area (0.67 ± 0.048 vs. 0.55 ± 0.027 mm^2, respectively, $p < 0.01$). Animals that were infected and then treated with vehicle for an additional 4 wk significantly showed the largest area of alveolar bone loss (group 2, 0.71 ± 0.070 mm^2, $p < 0.01$). Infected animals treated with the CCR2 antagonist (group 3) had the smallest cemento-enamel junction–alveolar crest area of 0.53 ± 0.046 mm^2, which was not statistically different from that of the noninfected control group (group 4, $p > 0.05$).

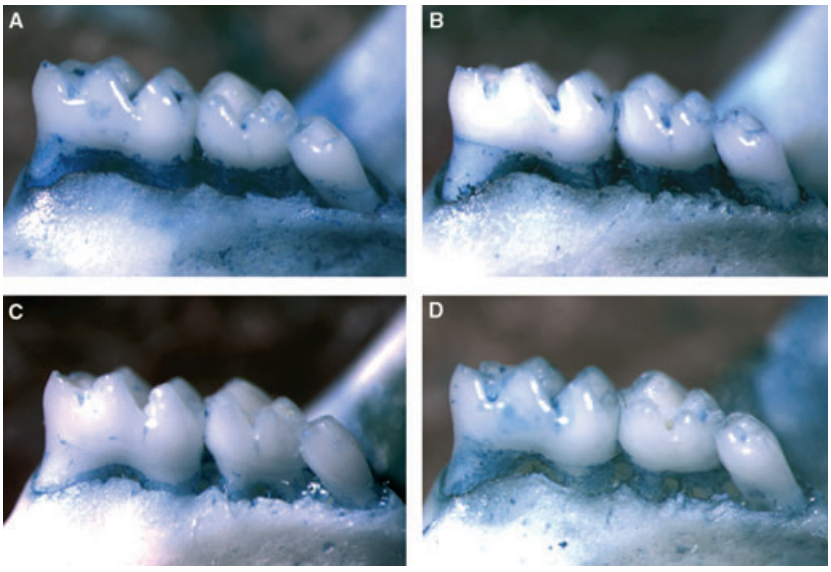

Fig. 1. Representative photomicrographic images for each set of treatment conditions. Tooth surfaces of posterior teeth are shown in defleshed hemimandibles. The area corresponding to the alveolar bone crest and cemento-enamel junction, evidenced by methylene blue staining, was analysed in control, noninfected mice (A), in mice infected with *P. gingivalis* for 5 wk (B), in *P. gingivalis*-infected and CCR2-antagonist-treated mice (C) and in *P. gingivalis*-infected and vehicle-treated mice (D). The cemento-enamel junction–alveolar bone area was quantified using National Institutes of Health ImageJ software.

Table 1. Morphometric analysis

Treatment groups	Mean cemento-enamel junction–alveolar crest area (mm^2)	SD	*n**	$p < 0.01$†
Group 1, infected and killed at the end of week 5	0.672	0.048	11	b
Group 2, infected + sham (vehicle) treatment	0.708	0.070	10	c
Group 3, infected + CCR2-antagonist treatment (10 mg/mL)	0.525	0.046	11	a
Group 4, noninfected control group	0.551	0.027	6	a

*Group 4 originally had eight animals, but two died. Group 2 originally had 11 animals, but one died.
†Different letters indicate statistically significant differences between groups (Tukey's test, $p < 0.01$).

The qualitative evaluation of the bone dynamics observed in the labeling of new bone by calcein indicated bone formation after treatment with the CCR2 antagonist. Differences in bone deposition areas can be observed in Figs 2–4. Green (calcein) and red bands (Alizarin red) represent alveolar bone formed in the different treatment conditions during the experiment. The spacing between the Alizarin (red) and the calcein labels (green) is larger in the animals that were infected and subsequently treated with the CCR2 antagonist (Fig. 4) than in animals that either were not infected (Fig. 2) or were infected and treated with vehicle (Fig. 3).

Discussion

Chemokine receptors are seven-transmembrane G-protein-coupled receptors, which are widely expressed in

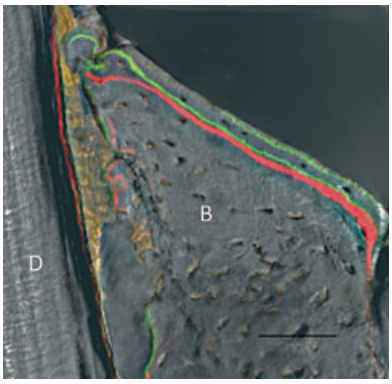

Fig. 2. Fluorescence confocal photomicrography of a control, noninfected mouse, showing uptake of calcium-binding fluorescent dyes in the mandibular first molar region. Alizarin was applied on the last day of *P. gingivalis* infection and calcein applied 11 d later and subsequently at 10 d. The area between the red (Alizarin red) and green (calcein) bands indicates bone formed in 4 wk. Abbreviations: B, alveolar bone; D, dentin. Scale bar represents 50 μm.

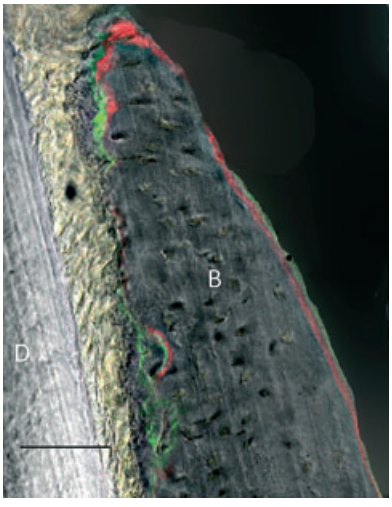

Fig. 3. Fluorescence confocal photomicrography of the first molar area of a mouse from the *P. gingivalis*-infected and subsequently vehicle-treated group, reflecting Alizarin and calcein uptake in the mandibular region. Abbreviations: B, alveolar bone; D, dentin. Scale bar represents 50 μm.

human tissues. Activation or inhibition of G-protein-coupled receptor signaling can affect many (patho)physiological processes. As such, they are potential targets in many diseases and represent possibly the most important target class of proteins for therapeutic

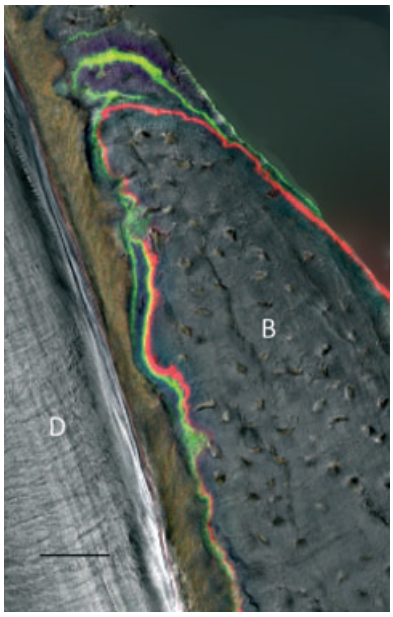

Fig. 4. Fluorescence confocal photomicrography of the molar region in animal infected with *P. gingivalis* and subsequently treated with CCR2 antagonist, showing bone formation as indicated by mineral dye uptake in the mandibular alveolar bone in the first molar region. Abbreviations: B, alveolar bone; D, dentin. Scale bar represents 50 μm.

intervention drug discovery, making the G-protein-coupled receptor superfamily the most successful of any target class in terms of therapeutic benefit (2). Among chemokine receptors, CCR2 and its ligands (CCL2, CCL7, CCL8 and CCL13) have been particularly implicated in the pathogenesis of a number of diseases, including rheumatoid arthritis, multiple sclerosis and atherosclerosis (9,24). For example, CCL2 is elevated in the joints of rheumatoid arthritis patients and promotes the recruitment of monocytes and T cells into the synovial tissues (25). Expression of the CCR2 receptor is increased on monocytes/macrophages in patients with rheumatoid arthritis when compared with control patients (26). Likewise, studies in $CCR2^{-/-}$ knockout mice have shown reduced atherosclerotic lesion formation, suggesting that this receptor is important in recruiting monocytes/macrophages into the vessel wall, interfering with the pathogenic events of atherogenesis (27–29).

Notably, there are considerable similarities between periodontal disease, rheumatoid arthritis and atherosclerosis; while the etiologies of these diseases differ, the underlying pathogenic mechanisms related to chronic local accumulation/persistence of an inflammatory infiltrate are evidently comparable (30,31). In fact, it has been reported that the mRNA expression of different chemokine receptors, including CCR2, is up-regulated in highly infiltrated gingival lesions by B cells in humans (32). While it is true that monocyte recruitment and tissue infiltration by macrophages are desirable for controlling local infections such as periodontal disease, the chronicity of the inflammatory state produces a deleterious effect on the periodontium, which ultimately may lead to connective tissue breakdown and bone resorption. As the CCR2 signaling axis has been shown to regulate osteoclast function, antagonizing the receptors that mediate monocyte recruitment may potentially be one important factor in the inhibition of bone loss and the regeneration of bone seen in this oral infection model. CCR2 antagonists have been reported to be successful experimentally when applied in adjuvant-induced arthritis and collagen-induced arthritis *in vivo* models (33) and as a potential therapeutic agent when topically applied for treatment of dry-eye disease (34).

For the experimental CCR2 antagonist used in this study, the results in a murine model of oral infection with *P. gingivalis* suggest that the cemento-enamel junction–alveolar crest area of infected animals was significantly different from that of infected animals subsequently treated with this CCR2 antagonist ($p < 0.01$). Such results suggest that in vehicle-treated animals there was a progression in alveolar bone loss; however, when mice received topical therapy with the CCR2-antagonist formulation, bone formation could be observed. Vehicle-treated mice showed additional alveolar bone resorption corresponding to a bone area of 0.036 mm^2 during the drug vehicle treatment period; however, animals that were infected and subsequently treated with CCR2 antagonist showed bone formation corresponding to an area of 0.147 mm^2 (0.672–0.525 mm^2) during the same experimental period.

Appositional bands for both calcein and alizarin labeling periods indicate the temporal course of bone mineralization. As Alizarin- and calcein-labeled areas reflect newly formed bone, this is consistent with the view that bone lost during the infection period was regained after treatment with the CCR2 antagonist.

In conclusion, the findings of this study provide evidence that topically active CCR2 antagonist could interfere with *P. gingivalis*-elicited alveolar bone resorption and was also capable of promoting bone formation in this inflammatory alveolar bone loss model. Ultimately, we believe that additional studies should be designed to facilitate understanding of the limitations, as well as the full potential, of therapies targeting CCR2 antagonist for treatment of periodontal diseases.

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