Site-Specific Neutrophil Migration and CXCL2 Expression in Periodontal Tissue

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Abstract

The oral microbial community is the best-characterized bacterial ecosystem in the human host. It has been shown in the mouse that oral commensal bacteria significantly contribute to clinically healthy periodontal homeostasis by influencing the number of neutrophils that migrate from the vasculature to the junctional epithelium. Furthermore, in clinically healthy tissue, the neutrophil response to oral commensal bacteria is associated with the select expression of the neutrophil chemokine CXCL2 but not CXCL1. This preliminary study examined the contribution of commensal bacteria on neutrophil location across the tooth/gingival interface. Tissue sections from the root associated mesial (anterior) of the second molar to the root associated distal (posterior) of the second molar were examined for neutrophils and the expression of the neutrophil chemokine ligands CXCL1 and CXCL2. It was found that both the number of neutrophils as well as the expression of CXCL2 but not CXCL1 was significantly increased in tissue sections close to the interdental region, consistent with the notion of select tissue expression patterns for neutrophil chemokine expression and subsequent neutrophil location. Furthermore, mice gavaged with either oral *Streptococcus* or *Lactobacillus* sp. bacteria induced a location pattern of neutrophils and CXCL2 expression similar to the normal oral flora. These data indicate for the first time select neutrophil location and chemokine expression patterns associated with clinically healthy tissue. The results reveal an increased inflammatory load upon approaching the interproximal region, which is consistent with the observation that the interproximal region often reveals early clinical signs of periodontal disease.

Keywords: bacteria, cell signaling, chemokines, epithelia, gingiva, histochemistry

Introduction

Neutrophils represent a key component of the innate defense system that protects clinically healthy periodontal tissue from disease. Individuals that have congenital deficiencies in either neutrophil numbers (Attström and Schroeder 1979; Page et al. 1987; Carrassi et al. 1989; Hart et al. 1994; Deas et al. 2003; Hajishengallis et al. 2015) or transit (leukocyte adhesion deficiency 1 and 2) or have an induced neutropenia by chemical induction with antimitotic agents such as cyclophosphamide (Attström and Schroeder 1979; Sallay et al. 1984; Hemmerle and Frank 1991; Yoshinari et al. 1994) can develop periodontitis. Likewise, studies have shown that knockout mice that are defective in neutrophil transit also develop periodontitis (Niederman et al. 2001; Yu et al. 2007; Hajishengallis et al. 2011). Consistent with the key contribution of neutrophils in protection from periodontal disease, the periodontium contains a highly orchestrated expression of select innate host defense mediators that facilitate the transit of neutrophils from the highly vascularized gingival connective tissue to the gingival crevice (Moughal et al. 1992; Nylander et al. 1993; Gemmell et al. 1994; Tonetti et al. 1994; Tonetti 1997), where they form a “wall” between the host tissue and the dental plaque biofilm (Tonetti et al. 1994). Conversely, the prolonged presence of

neutrophils in gingival tissue (Nussbaum and Shapira 2011) or the failure to downregulate orchestrated neutrophil transit, as was observed in *del-1*^{-/-} mice, results in an increase in neutrophil numbers in gingival tissue and a significant increase in periodontal bone loss (Eskan et al. 2012).

Therefore, neutrophil homing to the gingival crevice is highly regulated, such that under conditions of periodontal health, the appropriate quantity of neutrophils are present to

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maintain control of dental plaque bacterial growth and yet not elicit tissue damage. However, most studies that have examined neutrophil homing into host tissue are concerned with inflammation associated with disease states. Several of these recent studies demonstrated a highly orchestrated and select expression of different host neutrophil chemotaxis receptor ligands when called in response to infection (Borregaard 2010; McDonald et al. 2010). These studies revealed that the plethora of both host and bacterial factors that can facilitate neutrophil recruitment do not represent host redundancy as once thought (Sadik et al. 2011). Rather through distinct patterns of temporal and spatial expression, neutrophil chemoattractants are effectively employed to direct neutrophils to the site of damage or infection (Sadik et al. 2011). For example, the 2 commonly studied CXCR2 chemokine ligands in the mouse, CXCL1 (Gro alpha, KC), and CXCL2 (Gro beta, MIP-2) were shown to have differing affinities for the CXCR2 receptor, resulting in a hierarchy of neutrophil chemotaxis and activation (Lee et al. 1995; Wuyts et al. 1996). Furthermore, the kinetics of expression and tissue expression profiles in several different models of infection revealed that these CXCR2 ligands display both differences in temporal and spatial responses, suggesting differing functional roles for these ligands in different disease models (Rovai et al. 1998; Ritzman et al. 2010; Mei et al. 2012).

However, the contribution of different chemokine ligands to the neutrophil homing process in clinically healthy tissue is not fully understood. Numerous cell types are capable of secreting chemokines, including epithelial cells, endothelial cells, and macrophages (Kornman et al. 1997), although the cell types responsible for chemokine secretion in the junctional epithelial tissue have not been identified. It has been shown that CXCR2 is required for neutrophil location in clinically healthy mouse gingival tissue and that CXCL1 and CXCL2 are differentially regulated by commensal bacteria (Tsukamoto et al. 2012; Zenobia et al. 2013). In this preliminary work, it is demonstrated that neutrophil location and CXCL2 expression, but not CXCL1 expression, was significantly higher when examined in gingival tissue sections taken closest to the root associated interdental region. Furthermore, single oral commensal bacterial species when introduced to germ-free mice were sufficient to induce both site-specific neutrophil location and CXCL2 expression. These data are consistent with the notion of select tissue expression patterns for neutrophil location and provide a mechanism by which the interproximal area is more prone to increased inflammation and early signs of periodontal disease.

Materials and Methods

Animals

All animal procedures were in compliance with established federal and state policies and were approved by the institutional animal care and use committees. Animals consisted of germ-free C3H/Orl mice (Charles River Laboratories International) and were maintained in isolators at the Royal Veterinary College, University of London, as previously described (Zenobia et al. 2013).

Oral Microbial Colonization

Streptococcus sp. and *Lactobacillus murinus* bacteria were collected from oral swabs of the mouse oral cavity from strain-matched specific pathogen-free mice as previously described (Hajishengallis et al. 2011). Oral colonization was accomplished in germ-free mice by gavage on 3 separate occasions every other day for a week, with each bacteria (10^9 cfu/ml), suspended in 2% carboxymethylcellulose as originally described by Baker et al. (2000). After completion of the bacterial gavage method, oral samples were taken for analysis by swabbing the oral cavity with sterile swabs for 30 s, 1 d after the last gavage, and subjected to plate count analysis. It was found that $>10^6$ cfu of *Streptococcus* and *Lactobacillus* spp. were cultured from the oral cavity of the respective mice at both time points. This approach yielded 4 groups of mice used in this study (total number of mice used, $N=12$): germ-free mice ($n=3$), specific pathogen-free mice ($n=3$), germ-free mice gavaged with *Streptococcus* sp. ($n=3$), and germ-free mice gavaged with *L. murinus* ($n=3$).

Immunohistochemistry to Detect Neutrophils and Expression of CXCR2 Chemokine Ligands

All mice were euthanized between age 11 and 12 wk. Germ-free mice gavaged with *Streptococcus* sp. and germ-free mice gavaged with *L. murinus* were euthanized 1 d after the gavage procedure. Three mice per group were dissected and the mouse heads, which included the mandible and maxilla, were prepared for immunohistochemistry. Tissues were fixed in 4% paraformaldehyde for 2 to 3 d, rinsed with 70% ethanol, and demineralized in ethylenediaminetetraacetic acid–cacodylate decalcification solution. Tissues were processed according to standard histological procedures and embedded in paraffin. Each mandible/maxilla combination was sectioned using serial sectioning in a coronal orientation (5 mm) from mesial to distal using a microtome and mounted as numbered serial sections on charged glass slides (Fisher Scientific), 2 sections per slide. This resulted in approximately 100 sections per tooth, and 50 slides per tooth. Immunohistochemistry was performed on mouse mandible and maxilla tissues. Every fourth slide of serially sectioned mouse mandible/maxilla was stained, resulting in approximately 24 stained sections per mouse for each primary antibody on 12 slides (Fig. 1). Tissues were stained as previously described (Zenobia et al. 2013) using a modified procedure utilizing the Vectastain Elite ABC kit (PK-6104 for rat immunoglobulin G (IgG) or PK-6101 for rabbit IgG; Vector Laboratories Inc.). In brief, tissues were deparaffinized in xylene and rehydrated using decreased graded dilutions of ethanol. Tissue specimens were blocked by incubation in 1.5% H_2O_2 in methanol solution for 30 min. Primary antibodies neutrophil elastase (sc-71674; Santa Cruz Biotechnology), Gro alpha (CXCL1; ab17882; Abcam), and Gro beta (CXCL2; ab9950; Abcam) were used with biotinylated secondary antibodies against rabbit or rat primary antibodies, as appropriate, and slides were developed using a 3,3'-diaminobenzidine peroxidase substrate kit (Vector Laboratories Inc.). Positive controls included staining in wild-type tissues, where immunolocalization

Figure 1. Diagram of the sectioning and sample selection (not to scale), as well as an example of the area analyzed for data collection. JE, junctional epithelium; OE, oral epithelium.

of target proteins was well characterized. Negative controls were performed without a primary antibody (Fig. 2A).

Data Analysis

The neutrophils were evaluated by direct count in the junctional epithelium (JE) and closely associated tissue (Fig. 2B, C). CXCL1 and CXCL2 were examined across the tooth, evaluating staining intensity as previously described (Zenobia et al. 2013). All slides were blindly scored for the number of neutrophils or expression levels of each chemokine on a scale of relative staining intensity from 0 (no stain) to 3 (strongest staining) (Zenobia et al. 2013). An example of CXCL2 staining intensity across the tooth surface with the associated staining intensity score is shown in Figure 2D–F. Groups of mice were evaluated for similarities between the pattern of expression, using quadratic analysis and mean analysis for each parameter using R software (<http://www.r-project.org>; R Project for Statistical Computing) for statistical analysis and graphics. Three different mice were examined per group; a total of 12 teeth were studied for the *Streptococcus* sp. and *Lactobacillus* sp., and 9 teeth were studied for the specific pathogen-free and germ-free controls.

Results

Serial Sectioning of the Mouse Molar to Determine Tissue-Specific Neutrophil and Chemokine Expression Patterns

It was previously shown that commensal colonization significantly increases the number of neutrophils expressed in the JE

(Tsukamoto et al. 2012; Zenobia et al. 2013). This result clearly demonstrated that oral commensal colonization of the oral cavity has a direct effect on the periodontal innate defense status. However, to our knowledge, the effect of oral commensal bacteria on the location of neutrophils in healthy gingival tissue has not been investigated. Furthermore, it is not known whether individual oral commensal bacterial species can significantly modulate neutrophil numbers or their location pattern across the tooth surface. Therefore, gingival tissue was obtained from serial sections of the second molar in groups of specific pathogen-free mice, germ-free mice, and germ-free mice gavaged with either *Streptococcus* sp. or *Lactobacillus* sp. This method of sectioning allowed visualization of the junctional epithelial tissue across the tooth from the root associated anterior (mesial) to root associated posterior (distal) areas (Fig. 1).

Oral Commensal Bacteria Significantly Affect Both the Number and Location of Neutrophils in Healthy Periodontal Tissue

The examination of neutrophils across the tooth in specific pathogen-free and germ-free mice revealed 2 distinctly different patterns of neutrophil location (Fig. 3). In specific pathogen-free mice or mice gavaged with either *Streptococcus* sp. or *Lactobacillus* sp., more neutrophils were in the root associated anterior (mesial) and posterior (distal) JE compared with the straight middle (buccal/lingual) portion of the tooth (Fig. 3A). In contrast, nearly identical numbers of neutrophils were found across the tooth surface in germ-free mice. This preliminary study demonstrated that commensal colonization, either by the indigenous oral microbiota or by gavage of select oral commensal species, selectively modulated neutrophil location across the tooth surface in healthy gingival tissue.

The significance of both the different location pattern of neutrophils across the tooth surface, from the root associated anterior (mesial) to root associated posterior (distal), as well as the total number of neutrophils found in each experimental group, was determined. A quadratic trend analysis was employed to determine whether the patterns of neutrophil location among all of the mouse experimental groups were significantly different. The analysis revealed that the neutrophil expression pattern across the tooth surface found in specific pathogen-free mice or mice that were gavaged with either *Streptococcus* sp. or *Lactobacillus* sp. was significantly different than that found in germ-free mice ($P = 0.05$) (Fig. 3B). Therefore, the presence of oral commensal bacteria significantly altered the location of neutrophils found next to the tooth surface. In contrast, no significant difference was found in the location of neutrophils when specific pathogen-free mice were compared with either of the mouse groups that were selectively gavaged with individual oral commensal species (Fig. 3B). However, the total quantity of neutrophils found in the JE in each group was significantly different ($P = 0.05$) (Fig. 3C). As reported previously (Tsukamoto et al. 2012; Zenobia et al. 2013), specific pathogen-free mice contained significantly more neutrophils than germ-free mice. This analysis also revealed, however, that the total numbers of neutrophils found

in the groups gavaged with either *Streptococcus* sp. or *Lactobacillus* sp. were significantly different from each other as well as the germ-free and specific pathogen-free groups ($P = 0.05$). Therefore, although mice gavaged with select oral commensal bacteria displayed a similar pattern of neutrophil location across the tooth surface, the total number of neutrophils found in the JE in gavaged mice was significantly affected by the oral bacterial species present.

Oral Commensal Bacteria Significantly Affect Both the Level and Location of CXCL2 but Not CXCL1 Expression in Clinically Healthy Tissue

It was previously demonstrated that the expression level of CXCL2, but not CXCL1, is modulated by oral commensal bacteria (Zenobia et al. 2013). Therefore, CXCL2 and CXCL1 expression levels were determined in groups of specific pathogen-free mice, germ-free mice, and germ-free mice gavaged with either *Streptococcus* sp. and *Lactobacillus* sp. in serial sections as described in Figure 1. Examination of CXCL2 stain intensity across the second molar in specific pathogen-free and germ-free mice revealed 2 distinctly different patterns (Fig. 4A). In specific pathogen-free mice, higher stain intensity was found in the root associated anterior (mesial) and posterior (distal) JE as opposed to the area midtooth (straight buccal/lingual) JE (Fig. 4A). In contrast, the stain intensity of CXCL2 was uniform in germ-free mice, exhibiting nearly identical stain intensity in all locations (Fig. 4A). Examination of germ-free mice gavaged with either *Streptococcus* sp. or *Lactobacillus* sp., revealed CXCL2 expression patterns similar to specific pathogen-free mice, indicating that the bacteria influenced the neutrophil expression pattern (Fig. 4A). This is in

Figure 2. Examples of immunohistochemistry staining. (A) Junctional epithelium (JE) of a negative control (no secondary antibody) specific pathogen-free (SPF) mouse also represents a stain intensity grade of 0. (B) JE of a neutrophil-stained SPF mouse. (C) JE of a neutrophil-stained germ-free mouse. (D) JE (mesial, anterior) of a black 6 SPF mouse stained with CXCL2, with a stain intensity of 2. (E) JE (midtooth) of a black 6 SPF mouse stained with CXCL2, stain intensity of 1. (F) JE (distal, posterior) of a black 6 SPF mouse stained with CXCL2, with a stain intensity of 2. Magnification, $\times 20$.

Figure 3. Examination of neutrophil migration across the tooth. (A) The average number of neutrophils, with 95% confidence intervals, for each slide of germ-free mice (germ-free), specific pathogen-free mice, germ-free mice gavaged with *Lactobacillus* sp., and germ-free mice gavaged with *Streptococcus* sp. (1–12), from the root associated mesial to the root associated distal of the second molar. (B) Quadratic trend analysis of neutrophil per mouse. (C) Mean neutrophil count per mouse. All groups were statistically significantly different from each other. Groups separated by shaded areas are statistically different at $P = 0.05$. 95% CI, 95% confidence interval; GF, germ-free.

Figure 4. Examination of CXCL2 expression levels across the tooth. **(A)** CXCL2 stain intensity (0 indicates no stain and 3 indicates heavy stain) across the tooth with 95% confidence intervals for each slide of germ-free mice, specific pathogen-free mice, germ-free mice gavaged with *Lactobacillus* sp., and germ-free mice gavaged with *Streptococcus* sp. (1–12) from the root associated mesial to the root associated distal of the second molar. **(B)** Quadratic trend analysis of CXCL2 stain intensity per mouse. **(C)** Mean CXCL2 stain intensity averaged per mouse (all were statistically significantly different from each other). Groups separated by shaded areas are statistically different at $P = 0.05$. 95% CI, 95% confidence interval; GF, germ-free.

contrast with CXCL1, in which the stain intensity was uniform across the tooth in all groups (germ-free mice, specific pathogen-free mice, germ-free mice gavaged with *Lactobacillus* sp., and germ-free mice gavaged with *Streptococcus* sp.; Fig. 5A).

The significance of the CXCL1 and CXCL2 expression patterns as well as their intensity levels in the different experimental groups was determined. A quadratic trend analysis revealed that the pattern of CXCL2 but not CXCL1 expression was significantly different ($P = 0.05$) when oral commensal bacteria were present in either specific pathogen-free mice or mice gavaged with either *Streptococcus* sp. or *Lactobacillus* sp. (Figs. 4B, 5B). An analysis of the mean values of the total stain intensity of CXCL2 in the gingival tissue revealed that both specific pathogen-free and mice selectively gavaged with oral bacteria contained significantly ($P = 0.05$) higher expression levels of CXCL2 compared with germ-free mice. However, each group that contained oral commensal bacteria was significantly ($P = 0.05$) different from each other such that the CXCL2 staining intensity in specific pathogen-free mice was greater in mice gavaged with *Streptococcus* sp., which was greater than mice gavaged with *Lactobacillus* sp. (Fig. 4C). In contrast, no significant differences were observed in the mean intensity of CXCL1 among the different experimental groups (Fig. 5C). The expression levels of CXCL1 and CXCL2 are consistent with our previous findings that CXCL2 expression

was greater in specific pathogen-free mice than germ-free mice, whereas CXCL1 expression was not significantly different (Zenobia et al. 2013). However, this analysis also revealed that the expression level of CXCL2, but not CXCL1, varied across the tooth surface and that the CXCL2 expression levels were modulated by individual species of oral commensal bacteria.

Discussion

Neutrophils are one of the most crucial components of the host immune system. Their role and the mechanisms of recruitment have been studied in health, disease, and host development (Christopher and Link 2007; Borregaard 2010; Tsukamoto et al. 2012; Zenobia et al. 2013). This study is consistent with the role of commensal bacterial influence on neutrophil location and reveals new information regarding neutrophil location in clinically healthy tissue. This study demonstrates the following. First, the location of the gingival tissue from the root associated anterior (mesial) of the second

molar to the root associated posterior (distal) was correlated with the location pattern of neutrophils and expression levels of CXCL2, but not CXCL1. Second, specific commensal bacteria could induce neutrophil location and CXCL2, but not CXCL1. However, it is important to realize that this study is preliminary as a result of the limited number of mice examined. Additional studies will further determine the extent and mechanisms by which site-specific neutrophil migration across the tooth surface occurs.

Previous studies have reported increased neutrophil and CXCL2, but not CXCL1, presence in specific pathogen-free versus germ-free mice (Tsukamoto et al. 2012; Zenobia et al. 2013). To our knowledge, the data presented in this study are the first to demonstrate the importance of tissue location with respect to neutrophil location and CXCL2 ligand expression. Although the expression pattern of CXCL2 is consistent with its contribution to the select location of neutrophils, other CXCR2 ligands may also play a role. CXCR2 has been shown to be an important component in maintaining periodontal homeostasis through CXCR2 ligands and resulting neutrophil migration (Yu et al. 2007; Zenobia et al. 2013). In this article, it is confirmed that both the CXCL2 expression level increases and neutrophil numbers increase in response to oral bacteria, but are also coordinately increased in a tissue site-specific manner. Furthermore, because select chemokines have been

shown to demonstrate antibacterial activity (Eliasson and Egesten 2008), it is possible that CXCL2 expression may also regulate oral bacterial numbers by direct antibacterial action. This novel finding expands our understanding of the tissue, demonstrating that not all areas of the JE elicit the same neutrophil location and CXCL2 expression. This provides further evidence that the host uses CXCR2 ligands to regulate its response to commensal colonization in a highly specific manner. In addition, the findings raise questions with regard to why the tissue location demonstrates differences in neutrophil numbers and CXCL2 ligand expression levels. There are several potential reasons for this, including the observation that the interdental region is more heavily colonized by bacteria and that the structure of the tissue in the interdental region, which has been found to differ from the buccal and lingual regions of humans, could be influencing the differential location of the neutrophils and CXCL2 (Csiszar et al. 2007).

This study also found that 2 mouse oral commensal bacteria, *Streptococcus* sp. and *Lactobacillus* sp., could induce a similar, if not identical, pattern of neutrophil location and CXCL2 expression as the whole specific pathogen-free community. However, there were significant differences in the total number of neutrophils and the CXCL2 expression levels among the experimental groups, demonstrating that single species colonization did not elicit the same intensity as the entire specific pathogen-free oral microbial community under the conditions of colonization and the time frame examined. To our knowledge, this study is the first to add commensal bacteria back into a germ-free mouse and examine the role of neutrophil location in the oral cavity. Additional studies are necessary to determine the contribution of the rate of bacterial colonization and growth as well as the host response to individual oral commensal bacteria on establishing periodontal tissue homeostasis.

The data presented provide the first evidence of the importance of tissue location when examining the periodontium and the host mediators involved in health. It was demonstrated that neutrophils and the associated CXCR2 ligand, CXCL2, were significantly increased toward the interproximal regions of the tooth. These data support the hypothesis that the interproximal area has an increase in stimulation of the innate host response to bacterial presence.

Figure 5. Examination of CXCL1 expression levels across the tooth. **(A)** CXCL1 stain intensity (0 indicates no stain and 3 indicates heavy stain) across the tooth with 95% confidence intervals for each slide of germ-free mice (germ-free), specific pathogen-free mice, germ-free mice gavaged with *Lactobacillus* sp., and germ-free mice gavaged with *Streptococcus* sp. (1–12) from the root associated mesial to the root associated distal of the second molar. No pattern difference is observed. **(B)** Quadratic trend analysis of CXCL1 stain intensity per mouse shows no significant difference between the groups of mice. **(C)** Mean CXCL1 stain intensity averaged per mouse shows no significant difference between the groups of mice. 95% CI, 95% confidence interval; GF, germ-free.

Author Contributions

A. Greer, contributed to conception, design, data acquisition, analysis, and interpretation, drafted the manuscript; K. Irie, A. Hashim, M.A. Curtis, contributed to data interpretation, critically revised the manuscript; B.G. Leroux, contributed to data analysis and interpretation, critically revised the manuscript; A.M. Chang, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript; R.P. Darveau, contributed to conception, design, data acquisition, analysis, and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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