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Original article

Actinobacillus actinomycetemcomitans-induced periodontal disease in mice: patterns of cytokine, chemokine, and chemokine receptor expression and leukocyte migration

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Abstract

Although the pathogenesis of periodontal disease (PD) is not well known, cytokines, chemotactic factors and inflammatory cells are certainly involved in the disease outcome. Here, we characterized the evolution of the PD induced by *Actinobacillus actinomycetemcomitans* in mice, showing that oral inoculation of these bacteria leads to the migration of leukocytes to periodontal tissues and marked alveolar bone resorption. We found the expression of pro-inflammatory and Th1-type cytokines including TNF-α, IFN-γ and IL-12 in periodontal tissues after infection with *A. actinomycetemcomitans*, from the early stages after infection and throughout the course of the disease. Similar kinetics of expression were found for the chemokines CCL5, CCL4, CCL3 and CXCL10 and for the receptors CCR5 and CXCR3, all of them linked to the Th1-type pattern. The expression of the Th2-type mediators IL-10, CCL1 and their receptors CCR4 and CCR8 was detected only after 30 days of infection, determining a time-dependent mixed pattern of polarized immune response. The chemokine expression was correlated with the presence of polymorphonuclear leukocytes, macrophages, CD4 and CD8 lymphocytes, and B cells in the inflammatory infiltrate. Interestingly, during the predominance of the Th1-type response, a sharp increase in the number of inflammatory cells and intense bone loss was seen. By contrast, after the increased expression of Th2-type mediators, the number of inflammatory cells remained constant. Our data demonstrate that mice subjected to oral inoculation of *A. actinomycetemcomitans* represent a useful model for the study of PD. In addition, our results suggest that expression of cytokines and chemokines can drive the selective recruitment of leukocyte subsets to periodontal tissues, which could determine the stable or progressive nature of the lesion.

Keywords: Experimental periodontal disease; *Actinobacillus actinomycetemcomitans*; Cytokines; Chemokines; Chemokine receptors; Cell migration; Inflammation

1. Introduction

Periodontal disease (PD), a chronic inflammatory disease of the attachment structures of the teeth, is one of the most significant causes of tooth loss in adults and the most prevalent form of bone pathology in humans, besides being a modifying factor of the systemic health of patients. The bacterial biofilm attached to the surface of the tooth in close association with periodontal tissues, is the etiologic factor of this disease. The biofilm hosts some typical periodontopathogens, such as *Actinobacillus actinomycetemcomitans*, one of the main etiological agents of localized aggressive human periodontitis and of a large number of cases of chronic periodontitis [1]. The virulence factors of this pathogen include leucotoxins, immunosuppressive factors, and a high capacity to invade the host cells [2–4]. In vitro, *A. actinomycetemcomitans* induces the expression of several cytokines and chemok-

Abbreviations: PD, periodontal disease; pi, post infection; PMN, polymorphonuclear leukocyte; RPA, RNAse protection assay; Th, T helper lymphocyte.

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ines by different cell types [5,6], which are possibly involved in mediating the migration of leukocytes to the affected tissue.

Indeed, the development of PD seems to be related to the extension of the inflammatory cell infiltrate to the deeper periodontal tissues [7]. Polymorphonuclear leukocytes (PMNs), the first cell type found in PD lesions, appear to provide the primary immune protection [8,9]. When the aggressive stimulus is not eliminated, it may lead to chronic PD, characterized by a cellular infiltrate dominated by mononuclear cells. Macrophages are outnumbered in chronic inflammatory lesions of PD and are thought to participate in the local immune response through presentation of antigen, killing of pathogens and production of inflammatory mediators [10,11]. Regarding lymphocytes, whereas T lymphocytes predominate in the established chronic lesion, the proportion of B cells and plasma cells increases with the progression of the disease [11–13].

This host response to periodontopathogens protects against the infection, but the persistence of pathogens and the exacerbated immune response may render the protective roles of inflammatory cells dangerous to the host tissues. Inasmuch as the chemotactic factors produced in the lesions certainly are involved in the pathogenesis of PD, their identification is fundamental to guide the development of strategies for controlling the disease.

Chemokines, a family of chemotactic cytokines, attract leukocyte populations by means of their interaction with specific receptors that are members of the seven-transmembrane spanning G protein-coupled receptor family, selectively expressed in these cells [14]. Besides their chemoattractant activity, chemokines also are implicated in the polarization of the immune response, in leukocyte activation, and in the pathogenesis of several diseases [15,16]. Some chemokines have been found in diseased human periodontal tissues [17–20], but their role in the pathogenesis of PD remains unknown. We detected Th1-type chemokines and chemokine receptors in gingival biopsies of patients with aggressive periodontitis, and their expression may favor the migration of the IFN-γ-producing cells found in the lesions. Conversely, in chronic periodontitis there was predominance in the expression of Th2 chemokines, which correlated with the higher expression of IL-10 [20]. Therefore, the selective chemoattraction of T helper (Th) subsets could influence the clinical outcome of PD. In fact, the polarization of immune responses can determine the prognosis of several diseases [21–23], such as arthritis, that share several features with PD, such as the chronic nature of the inflammatory reaction with concomitant bone resorption [24].

The role of chemokines and cytokines in the orchestration of cellular traffic to periodontal tissues is not known, and data are lacking regarding the relevance and kinetics of their expression in the course of human PD. In addition, variables such as the different compositions of periodontal biofilm, the age at onset of disease and the genetic variability of hosts may hinder the interpretation of human studies. Consequently, experimental mouse models are useful to study PD, since they present several advantages including multiple strains with known genetic background, ease of handling, availability of experimental reagents, and the susceptibility to induction of PD [25–27].

Therefore, our aim was to characterize a mouse model of PD induced by *A. actinomycetemcomitans*. We investigated the expression patterns of chemokine, chemokine receptor and cytokine mRNA, and the kinetics of cell migration and alveolar bone loss throughout the course of experimental PD. We conclude that this murine model could be useful for testing hypotheses relevant to human PD.

2. Materials and methods

2.1. Mice

Experimental groups comprised 8-week-old male C57BL/6 mice, bred and maintained in the animal facilities of the Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto-USP. Throughout the period of the study mice were fed with sterile standard solid mice chow (Nuvital, Curitiba, PR), and sterile water. Mice colonies were free from the following periodontopathogens: *A. actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Eikenella corrodens*. The experimental protocol was approved by the local Institutional Committee for Animal Care and Use.

2.2. Induction of PD/periodontal infection

A. actinomycetemcomitans (ATCC 29522) was grown anaerobically in supplemented agar medium (TSBV) as previously described [28]. Initially the animals received a direct injection of 1×10^9 CFU of a diluted culture in 10 μl of PBS into the palatal gingival tissue of the second molar. Immediately after, 1×10^9 CFU of a diluted culture in 100 μl of PBS with 2% of carboxymethylcellulose (used to facilitate the retention of the bacterial suspension in the oral cavity) was placed in the oral cavity with a micropipette; and after 48 and 96 h only this procedure was repeated. This protocol represents the combination of the number of bacteria and the number of inoculations effective in promoting the colonization of the oral cavity by *A. actinomycetemcomitans* and the establishment of PD in 100% of the animals. The effectiveness of infection was confirmed by the detection of *A. actinomycetemcomitans* in periodontal tissues by polymerase chain reaction (PCR) (as previously described [28]) at all the times analyzed. Positive controls received a single injection of LPS (5 μg in 10 μl of PBS—*E. coli* LPS—Sigma Co., St. Louis, MO). Negative controls included sham-infected mice, which received PBS with carboxymethylcellulose solution without *A. actinomycetemcomitans*, and non-infected animals.

2.3. RNA extraction

Total RNA was extracted from gingival palatal tissues between the mesial of the first molar and the distal site of the