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REVIEW

The NADPH oxidase NOX2 plays a role in periodontal pathologies

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Abstract Oxidative stress plays an important role in periodontal health and disease. The phagocyte nicotinamide adenine dinucleotide phosphate oxidase NOX2 is most likely one of the key sources of reactive oxygen species (ROS) in periodontal tissues. This review will discuss three clinical aspects of NOX2 function. We will first focus on oral pathology in NOX2 deficiency such as chronic granulomatous disease (CGD). CGD patients are thought to suffer from infections and sterile hyperinflammation in the oral cavity. Indeed, the periodontium appears to be the most common site of infection in CGD patients; however, as periodontitis is also common in the general population, it is not clear to which extent these infections can be attributed to the disease. Secondly, the role of oxidative stress in periodontal disease of diabetic patients will be reviewed. Diabetes is indeed a major risk factor to develop periodontal disease, and increased activity of leukocytes is commonly observed. Enhanced NOX2 activity is likely to be involved in the pathomechanism, but data remains somewhat preliminary. The strongest case for involvement of NOX2 in periodontal diseases is aggressive periodontitis. Increased ROS generation by leukocytes from patients with aggressive periodontitis has clearly been documented. This increased ROS generation is to be caused by two factors: (1) genetically enhanced ROS generation and (2) oral pathogens that enhance NOX function. NOX enzymes in the oral cavity have so far received little attention but are likely to be important players in this setting. New therapies could be derived from these new concepts.

Keywords Aggressive periodontitis · Chronic granulomatous disease · Diabetes · Reactive oxygen species

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Introduction

The term "periodontal diseases" refers to inflammatory as well as recessive alterations of the gingiva and periodontium. Clinically, a gingivitis presents as inflammation of the gums that appear swollen and red, and bleed on touch. Gingivitis is reversible by simple interventions like oral hygiene measures but progresses, if untreated, to a periodontitis that involves permanent loss of soft tissue attachment and alveolar bone. Untreated periodontitis is progressive and finally leads to tooth mobility and loss. Gingivitis and periodontitis are plaque-elicited diseases caused by specific microorganisms via local opportunistic infections in an otherwise systemically healthy host. However, genetic and metabolic factors may play a direct role in their etiologies or enhance the pathology.

There is a longstanding suspicion that reactive oxygen species, ROS, are involved in periodontal diseases. Over

the last years, it has become increasingly clear that a major source of ROS is the NOX family of ROS-generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidases [1]. NOX enzymes are electron transporters that accept electrons from NADPH inside the cell and transport them across the membrane to attach it to oxygen. This reaction leads to the generation of superoxide and downstream ROS. The NOX family consists of seven different genes, coding for seven isoforms referred to as NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2. In this review, we will focus on the present knowledge of the role of NOX2, the phagocyte NADPH oxidase, in periodontal diseases. Indeed, this enzyme has been known for a long time; thus, clinical and scientific data on this topic are available.

Periodontal pathologies in chronic granulomatous disease

Chronic granulomatous disease (CGD) is caused by NOX2 deficiency. It is a rare genetic disorder characterized by recurrent severe infections due to the inability of neutrophils and macrophages to mount a respiratory burst and kill invading bacteria. The most common bacterial and fungal infections include pneumonia, lymphadenitis, liver abscesses, osteomyelitis, septicemia, and otitis media [2].

Until now, oral complications of CGD have rarely been investigated. A first report of a CGD case was published by Wolf and Ebel [3]. The male patient presented with severe gingivitis and ulcerous lesions in the buccal mucosa and palate. Oral hygiene measures improved his condition greatly, suggesting that the oral symptoms are more likely associated to the accumulation of plaque than to the CGD. A further case report described a 5-year-old boy diagnosed with CGD [4]. At his initial dental examination, the patient presented severe gingival inflammation and pronounced marginal erythema. Extensive attachment loss with furcation involvement and gingival recessions, as well as tooth mobility, were evident in all remaining teeth. Four teeth had already been lost due to advanced periodontal disease. The patient was diagnosed with "generalized pre-pubertal periodontitis," which the authors suggested to add to the conditions associated with CGD.

In a larger cohort, patients with CGD presented a significantly higher prevalence of oral ulcerations and gingival inflammation in comparison to control subjects [5]. These findings were supported by a report from the same year on five 17- to 32-year-old patients diagnosed with CGD [6]. All presented ulcerative lesions on the palate and alveolar mucosa. Detailed examination of the periodontium revealed that three of the five patients showed gingivitis, one patient had localized early periodontitis, and a further one had generalized early-to-moderate periodontitis. Despite the limited number of patients, the severity of the periodontal disease increased with age and local etiologic factors such as poor fillings or crowns. In spite of the leukocyte defects, none of the patients was diagnosed with juvenile, severe, or rapidly progressing periodontitis [6].

In a more recent survey of 368 patients with CGD, only nine were diagnosed with gingivitis and/or mild periodontitis [7]. No cases of oral infections were reported in a Japanese survey of 221 patients with CGD [8]. Equally, no oral infections were found over a 25-year observation period in the dental records from a series of pediatric patients with CGD [9]. Carnide and coworkers [10] reported that out of 18 patients with pneumonia, which is the most common manifestation of CGD, only two had gingival abscesses. Recently, a prospective controlled multicenter study was conducted in Italy on clinical symptoms and disease progress in 60 patients with CGD [2]. For the 1-year follow-up, 47 patients were available. Interestingly, stomatogingivitis was present in 35% of the patients and had an incidence of 0.12 per year. Thus, stomatogingivitis appears to be the most common organ manifestation in CGD patients, more frequent than pneumonia (0.09/year), GI tract infections (0.06/year), and septicemia (0.026/year).

In conclusion, most studies have shown that gingivitis and oral ulcers are common in patients with CGD, but periodontitis, which is commonly found in patients with leukocyte adhesion deficiency type I, seems unusual in CGD patients [11]. However, the available evidence is incomplete. Most reports are cohort studies that lack a control group. Periodontal diseases show also a high prevalence in epidemiological studies of the healthy population. Especially adolescents are known for a high prevalence of gingivitis [12]. In a national survey, only 1.5% of 15-year-old Germans had healthy oral tissues, 85.5% of the adolescents had gingivitis, and 13.4% periodontitis [13]. In the same survey, 26.2% of the 35- to 44-year-old adult population presented with gingivitis and 73.2% with periodontitis. Thus, the lack of control groups makes the data on CGD patients very difficult to interpret. Indeed, a simple comparison with the general population would even suggest a lower rate of periodontal disease in CGD patients. This is probably not the case, but it points toward methodological weaknesses of the presently available evidence.

NOX enzymes and diabetes

Strong evidence exists on the relationship between diabetes mellitus and periodontal disease. Diabetes patients show greater prevalence, incidence, severity, and extent or

progression of periodontal disease in comparison to non-diabetic patients [14]. Furthermore, it seems that severe periodontitis increases the risk of poor glycemic control and that, in return, periodontal treatment has a positive effect [15–17].

The mechanisms by which diabetes influences the periodontal tissues share common characteristics with those involved in its classic complications such as retinopathy, nephropathy, macrovascular disease, and altered wound healing. The subgingival microbiota between diabetic and non-diabetic patients with periodontitis is very similar, suggesting that alterations of the host immunoinflammatory response to several pathogens play a predominant role [18, 19]. Early studies have shown that diabetes leads to impaired adherence, chemotaxis, and phagocytosis of neutrophils, which are the first line of host defense [20, 21]. While these initial studies suggested hypofunctional neutrophils, most studies suggest hyper-responsive phagocytes, resulting in increased production of pro-inflammatory cytokines and mediators in the gingival crevicular fluid [22, 23].

Recently, it has been shown that hyperglycemia may cause periodontal destruction through several pathways. For example, the non-enzymatic reaction of glucose may lead to both extra and intracellular accumulation of irreversible advanced glycated end products, which can modulate the cellular function and alter the tissue structure [24, 25].

Hyperglycemia is the major factor responsible for the activation of oxidative stress, which is the imbalance between the antioxidant defence and the production of ROS, leading to tissue damage [26, 27]. The main source of ROS is the NADPH oxidase family of enzymes, essential to the phagocyte bactericidal activity. Of particular interest is the NOX2 NAPDH oxidase, which is predominately expressed in phagocytes [28]. Recently, Karima et al. [29] have shown that neutrophils from diabetic subjects exhibit increased activity of protein kinase C, increased amounts of diglyceride, and enhanced phosphorylation of $p47^{phox}$ during cell stimulation, leading to increased O_2 stimulation. In addition, they reported a significant correlation between glycemic control and the severity of periodontitis, suggesting that the increased risk of periodontitis in diabetes is linked to increased inflammation and oxidative stress mediated by the neutrophil. The role of polymorphonuclear neutrophils in mediating diabetic tissue damage to the periodontium was investigated in a novel model of chronic hyperglycemia, the Akita mouse [30]. Exaggerated IL-6 response and reduced polymorphonucleocyte (PMN) chemotaxis was observed in the Akita mice as compared to the control mice (wild type, WT). Furthermore, intra-vital microscopy of the gingival vessels showed that leukocyte rolling and attachment to the vascular endothelium was enhanced in periodontal vessels of Akita mice. The authors suggested that chronic hyperglycemia predisposes to exaggerated inflammatory response and primes leukocytes for marginalization and superoxide production but not for transmigration. The leukocyte defects in hyperglycemia impairs the innate immune response to periodontal pathogens and increases the free radical load in the periodontal environment, thus contributing to periodontal tissue destruction.

Taken together, diabetes is a clear risk factor for the development of periodontal disease. And there are some indications that enhanced ROS generation by NOX enzymes might play a role. However, the evidence remains largely indirect.

NOX enzymes and aggressive periodontitis

Aggressive periodontitis (formerly termed juvenile periodontitis, early onset periodontitis, or rapidly progressive periodontitis) is characterized by a rapid and severe destruction of the periodontal tissues mostly in the first molar and the incisor teeth. Mainly, adolescents and young adults are concerned. Altered neutrophils functions, such as abnormalities in adherence, chemotaxis, superoxide generation, phagocytosis, and bactericidal activity are known to play a role in the prevalence, progression, and severity of aggressive periodontitis.

During the last decades, special attention has been given to the role of ROS in the pathogenesis of aggressive periodontal disease. Most studies on ROS production by neutrophils in periodontal disease are based on peripheral blood neutrophils.

After stimulation with *Staphylococcus aureus*, neutrophils from juvenile periodontitis patients exhibited greater ROS production than the matched control subjects [31–34]. Significant differences were found in FcγR-stimulated ROS production between periodontitis patients and healthy subjects, suggesting a hyperactivity of peripheral neutrophils in periodontitis [35–38]. Furthermore, in successfully treated patients with aggressive periodontitis, it was demonstrated that hyperactivity was present before and after treatment, thus supporting the hypothesis that ROS hyper-responsiveness is constitutional rather than reactive [39]. However, studies on ROS generation via zymosan/CR3 (complement receptor 3) failed to show an alteration of ROS generation in periodontal disease [40].

Several studies have been investigating the effect of the chemotactic peptide fMetLeuPhe on ROS generation in both chronic and aggressive forms of periodontal disease. Results are inconsistent with increased, decreased, and similar levels of ROS between periodontitis and periodontally healthy controls [41, 42]. Thus, the most consistent finding from studies on peripheral neutrophils in periodontitis is that

disease is associated with an increased ROS response to Fcγ stimulation. Interestingly, crevicular leukocytes of patients with periodontitis showed a heightened response compared to those from healthy subjects [43]. Comparison of cell responses isolated from blood with those from diseased, treated, and healthy tissues within patients suggested that ROS generation was lower in diseased sites [44]. It seems that neutrophils isolated from diseased sites are either inhibited from responding or that in vivo activation and ROS generation have reduced their ability to respond in vitro. Recently, peripheral neutrophils from chronic periodontitis patients and age-/sex-/smoking-matched healthy control subjects were assayed for total ROS generation and extracellular ROS release, with and without stimulation (Fcγ receptor and *Fusobacterium nucleatum*) [45]. Neutrophils from patients produced higher mean levels of ROS after Fcγ stimulation as compared to healthy individuals, but no differences were observed in unstimulated ROS generation. In contrast, patients' cells demonstrated greater baseline extracellular ROS release, thus suggesting that peripheral neutrophils from periodontitis patients exhibit hyperactivity after stimulation and hyperactivity in terms of excess ROS release, in the absence of exogenous stimulation. Recently, Johnstone et al. [46] compared the generation of oxygen radicals in peripheral PMNs from patients with refractory aggressive periodontitis (RAP), chronic periodontitis, and periodontally healthy subjects after stimulation with phorbol myristate acetate (PMA) and phagocytosis via the complement and Fcγ receptors. Increased phagocytosis and PMA-induced oxygen radical production was observed in the RAP group as compared to the two others, accounting in part for the continued periodontal breakdown observed in these patients despite periodontal treatment.

The respiratory burst of neutrophils may be modulated by cytokines: For example, TNF-α can prime ROS generation by neutrophils from patients with chronic and aggressive periodontitis, as well as in periodontally healthy individuals [33, 37, 42, 47]. On the contrary, IL-8 can prime for fMLP-stimulated ROS production in healthy individuals and in chronic disease but not in rapidly progressive disease [33].

Genetic aspects in aggressive periodontitis

Periodontitis is not only caused by bacteria, but it is also a genetic disease. The identification of genes contributing to the pathogenesis of periodontitis has been the target of several studies of the last years [48, 49].

Recent data have shown that host hyperactivity has a genetic basis. In a blinded study, in 224 patients with confirmed diagnosis of aggressive periodontitis and 231 persons with a healthy periodontium, the genotypes for $p22^{phox}$ NADPH oxidase, FP, Fcα, and Fcγ receptors were analyzed from blood samples [50]. Statistically significant differences for NADPH oxidases $p22^{phox}$ C242T and the NA1 allele of Fcγ polymorphisms were found between aggressive periodontitis patients and control subjects. These results corroborate the neutrophils hyperactivation theory, in particular the increased release of superoxide. This further supports the hypothesis that it is not only bacteria but also the host genetic predisposition that causes the tissue damages in aggressive periodontitis. Furthermore, as the $p22^{phox}$ is an important component of the NADPH in osteoclasts [51], the combination of the increased PMN and osteoclast activity may lead to a more rapid and more severe bone loss in subjects with a genetic predisposition.

Microbiological aspects in aggressive periodontitis

Opsonized bacteria associated with periodontal disease are capable of stimulating peripheral neutrophils from patients with both chronic and aggressive forms of periodontal disease to generate enhanced ROS. In healthy subjects unopsonized *F. nucleatum* induced increased production of oxygen radicals, cytokines, and elastase from peripheral leucocytes activated in vitro [52]. *Porphyromonas gingivalis* is protected against oxidative stress by the cytoplasmic protein rubrerythrin [53]. These features allow *P. gingivalis* to proliferate in animals that possess a fully functional oxidative burst response. In other words, the host oxygen-dependent bactericidal system is not only ineffective in combating *P. gingivalis* infection but appears to exacerbate significantly the host tissue damage induced by the infection [53].

In conclusion, aggressive periodontitis is characterized by neutrophil hyperactivity with enhanced ROS generation. This hyperactivity seems to be determined by two factors: (1) genetic predisposition as evidenced by association with NOX polymorphisms and (2) oral pathogens that stimulate NOX2 and—at the same time—are resistant to its microbicidal activities.

Perspectives

As shown in this review article, it becomes increasingly clear that the phagocyte NADPH oxidase plays a role in oral health and disease. Enhanced ROS generation is clearly involved in the pathomechanisms of periodontal disease. Possibly, decreased ROS generation is also a risk factor, but this is less well documented. There are basically two ways in which the increased ROS generation in periodontal disease occurs: host factors, in particular genetic predisposition and altered metabolism (diabetes), and microorganisms of the oral cavity, which can directly or indirectly activate NOX2. These elements are relatively well under-

stood for the aggressive periodontitis. It is, however, very likely that they also participate in age-related development of peridontitis. Indeed, age-related periodontitis is an extremely frequent disease that affects the majority of the elderly population and is an important cause of tooth loss, caries, and subsequent malnutrition. Thus, understanding the involvement of NOX enzymes in this pathology will be an important challenge for the future.

In this review, we have focused on the phagocyte NADPH oxidase NOX2 in the oral cavity. It is, however, likely that other NOX enzymes also are important in this context. Indeed, NOX1 and NOX4 are highly expressed in the vascular system. NOX4 also plays an important role in fibroblasts and in osteoclasts. DUOX1 is thought to play a role in the host defence by salivary glands [54], and NOX1 is thought to contribute to radiation-induced salivary gland apoptosis [55]. Thus, future research will have to address also these other NOX isoforms, and their potential role in oral pathologies.

Presently, there are no NOX inhibitors available for clinical use; however, there are efforts to develop drugs in this domain. NOX2 inhibitors might find their place in the treatment of periodontal diseases.

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