Research article

The potential role of neutrophil extracellular traps (NETS) in periodontal disease—A scanning electron microscopy (SEM) study

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Abstract: Background: Oral biofilms are the major etiological agents of periodontal pathologies. Neutrophils are the first line of defense in fighting the pathogens in the biofilm. One of the mechanisms by which they operate is neutrophil extracellular traps (NETs), which are a novel mechanism of defense in the presence of pathogenic plaque. The present study was carried out to investigate their role in periodontal disease. Methodology: Following institutional ethical committee approval, ten gingival (biopsy) samples from individuals with periodontitis were collected during periodontal flap surgery and processed and examined under scanning electron microscope (SEM). Results: SEM examination revealed that the excised gingival tissues displayed an intricate meshwork of NETs. Conclusion: NETs may be one of the means of defense against periodontal infections, although further research is necessary to understand the exact nature of their role.

Keywords: plaque; neutrophils; NETs; gingival biopsy; periodontitis

1. Introduction

Plaque bacteria and their metabolites are the primary cause of the infectious illness periodontitis. The immunological response to the pathogenic bacteria is crucial in this process [1]. Gingivitis and periodontitis are caused by bacteria in plaque, which function as an inflammatory agent [2]. Fine particles and a lobulated or rod-shaped nucleus are features of neutrophils, and colorless or reddish cytoplasm is present. It acts bactericidal and through phagocytosis and chemotaxis [3] and neutrophils are influenced by chemokines when pathogens attack the body. They quickly assemble close to the

infection site, pass through the vessel wall into the infected tissue, and phagocytose pathogenic microorganisms kill them by releasing bactericidal particles [4].

In a manner different from apoptosis and necrosis, neutrophils can be stimulated by a variety of conditions to create neutrophil extracellular traps (NETs) to eliminate pathogenic microorganisms [5]. Brinkmann first described NETs as bactericidal traps, disarming and promoting the elimination of extracellular bacteria [6]. NETs resemble web-like structures, that are composed of decondensed nuclear chromatin fibers combined with various antimicrobial compounds, including histones and antimicrobial peptides (AMPs) from azurophilic granules, which are specific granules and tertiary granules (gelatinase) released out of the neutrophil after activation. These AMPs were found to be effective not only against bacterial species, but also against viruses, fungi and other microorganisms [7,8].

NETs have also the capable of capturing, neutralising, and killing a variety of pathogens, and act as a physical barrier to stop the spread of microbes and raise the concentration of antimicrobial effectors in the area [9–11]. NETs have also recently been discovered to control B cell activity in the spleen [12] and to contribute to a number of sterile disorders, including autoinflammation and autoimmune disease, in addition to infection. More and more organisms have been found to lead to the formation of NETs, including bacteria, fungi, viruses, and protozoan parasites. In both people and animal models, deficiencies resulting in reduced NET formation increased susceptibility to opportunistic infections, suggesting antimicrobial defense may be an important role played by NET formation.

Peripheral blood neutrophils are eventually recruited from the bloodstream into the site of the infection. Naturally present in the oral cavity, neutrophils attach to the endothelial cells through the well integrated interaction with selectin and integrin receptors. Later on, by extravasation they abandon the bloodstream and migrate from the gingival sulcus into the oral cavity. In the presence of infection, neutrophils are the first of the immune cells to arrive at the site through periodontal tissues and into gingival crevices as part of normal immune control. Additionally, oral neutrophils have been found to show different chemotactic and antimicrobial functions compared to circulating neutrophils [13,14]. Moreover, neutrophils have been known to contribute to a considerable extent to the periodontal tissue breakdown in the inflammatory process. Whether or not NETs are critical to this destruction is yet unclear, although the phenomenon has been observed with oral neutrophils in a number of studies as well as the role of NETs in periodontal disease. Whether their role is limited to the early stages of the disease or continues beyond is not very clear. Although, blood samples, immunohistochemistry, plaque samples and more have been widely investigated, very few studies have examined NETs in periodontal tissue specimens [15–17]. The presence or absence of NETs in these specimens would provide a valuable insight to understanding the phenomenon in a better manner.

The present research was thus attempted with the objective of assessing the potential role of NETs in periodontitis by examining surgically excised gingival tissue specimens under SEM.

2. Materials and methods

Ten individuals (five males and five females) with periodontitis between the ages of 32 and 45 years were selected based on the inclusion and exclusion criteria outlined below. Diagnosis of periodontal disease was made based on the American Academy of periodontology (AAP) and European Federation of Periodontology (EFP) diagnostic guidelines (2018). Patients with Periodontal abscesses, periodontitis linked to endodontic lesions, systemic disorders or conditions including

pregnancy/breast feeding, antibiotic medication within the last six months, steroid therapy, and/or radiation therapy were excluded from the study. Ethical approval was obtained from the institutional ethics committee (VEF-P14052016) and each participant gave their free and informed consent.

2.1. Experimental approaches: Biopsy procedure

The selected patients first underwent nonsurgical periodontal therapy as a part of phase 1 treatment, which included scaling and root planing and plaque control measures. The surgical periodontal therapy was planned following re-evaluation of the periodontal condition after ascertaining there was no resolution of the inflammation and persistent periodontal pockets. Gingival tissue was excised as part of the internal bevel incision of the flap procedure. The excised tissue was immediately washed twice in 50 mL of saline while being pushed side to side for 20 seconds to remove everything in contact with the epithelial surface but not adhering to it. The tissues (biopsies) were discarded if the excision took more than 15 seconds in case blood coagulation may have occurred. After that, the biopsies were prepared for examination using SEM.

2.2. SEM analysis

The samples were postfixed with 1% osmium tetroxide for two hours. The postfixed samples were critical-point dried, evaporated in an increasing concentration of ethyl alcohol, and thereafter flashed with gold. Carl Zeiss Sigma 300 Scanning Electron Microscope was used to investigate the samples (Germany's Carl Zeiss AG produces this).

3.   Results and discussion

All of the samples had an intricate network of NETs as shown in the SEMs of varying magnifications. The NETs formed a three-dimensional web, where only their threads (indicated as C) were clearly distinguishable. Interestingly, these threads appeared as thick bundles in higher magnifications (Figure 1). Portions of epithelial tissue with granulation tissue were also visible (indicated as D). Abundant polymorphonuclear neutrophils (PMNs), some disrupted/lost (indicated as A) and some intact (indicated as B) appeared to be entangled in the crevicular web of NETs. The entrapment of bacteria by the NETs was not visible. Higher magnifications revealed that the threads of NETs were simply a superimposition of NET fibers with the characteristic dimensions positioned closely abreast.

Research has shown that NETs may ensnare gram-positive bacteria, bind fungus and other microbes to destroy them in-situ without the need for phagocytosis, and contain cationic qualities that enable attachment to gram-negative bacteria. However, it is still possible that such a well planned procedure represents, at least in some instances, a type of deliberate cell death [18]. The enormous amount of imprisoned bacteria raises the possibility that, in comparison to NETs [19], phagocytosis may only be a minor player in the massive amounts of distributed crevicular bacteria that are eliminated.

The phenomenon of NETs has been suggested to occur as a part of the first line of defense activity of the neutrophil. Despite a surge in publications on NETs in the last five years, there are still many unanswered questions about their biophysiology. It is still debatable whether or not these traps still persist and exert their antimicrobial activity long after the programmed cell death of the neutrophil,

which has been suggested to be anywhere between a few minutes to hours based on the type of NETs: vital, nuclear or mitochondrial [20].
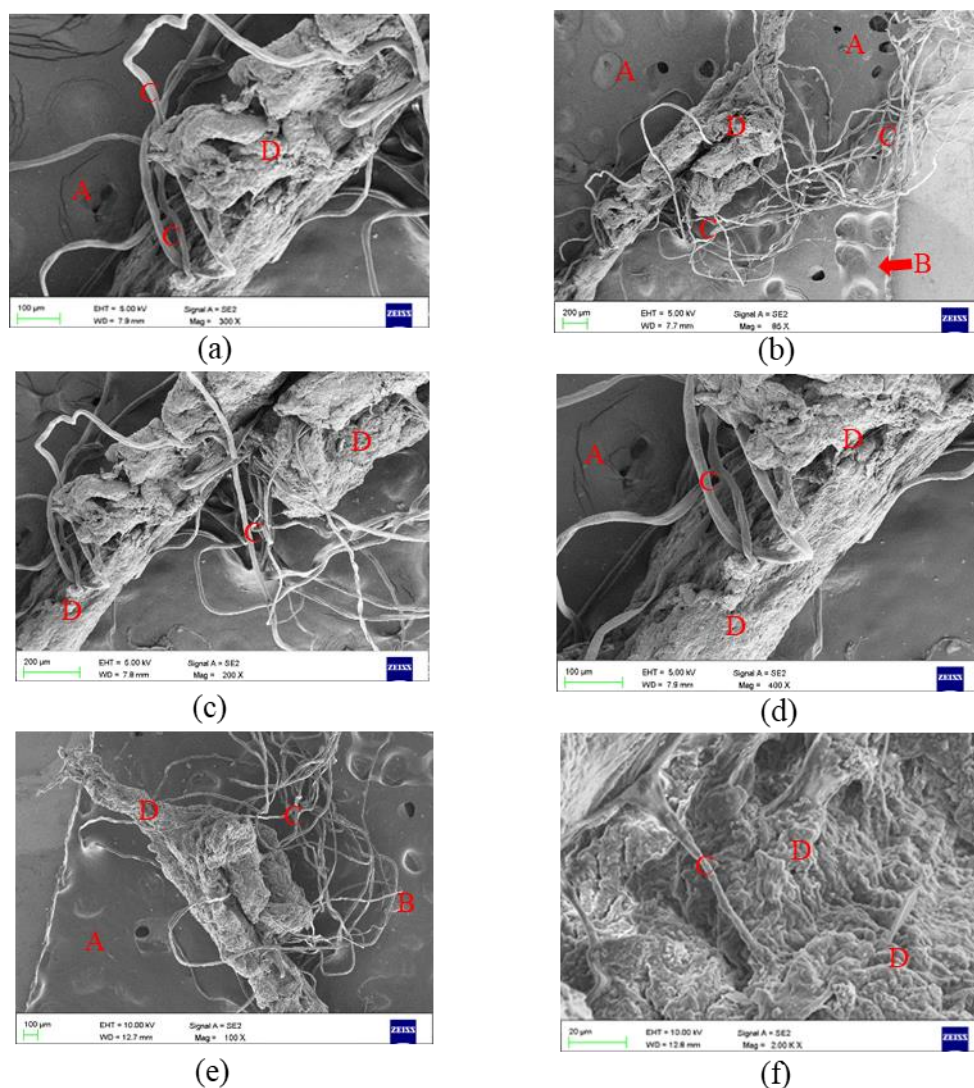

Figure 1. SEM photographs of various magnifications showed: Hollowed out/empty spaces (A) indicating emptied/disrupted neutrophils [*(a), (b), (d) and (e)*] with areas of intact neutrophils (B) [*(b) and (e)*] observed in a network/meshwork of extracellular neutrophil traps (C) [*(a) to (f)*] with portions of epithelium and granulation tissue (D) [*(a) to (f)*].

Neutrophil extracellular traps also have a strong bactericidal capacity, although certain bacteria such as fusobacterium species and group A streptococci can withstand them by producing extracellular DNase [21–23]. NETs contain a huge number of highly potent proteolytic enzymes, thereby increasing the possibility of periodontal damage [19,24]. In our study, ideally speaking, if NETs were able to eliminate the periodontal pathogens, phase 1 therapy including scaling and root planing should have resulted in a complete resolution of the periodontal inflammation. On the contrary, inflammation persisted necessitating periodontal surgery. Tissue specimens excised during the surgical procedure

revealed the presence of NETs, along with empty/vacant spaces indicating lost or destroyed neutrophils thereby suggesting that the traps remained in the periodontal tissues long after they were produced and even after the neutrophils producing them were dead. This could also be the cause of the continued periodontal destruction observed compelling the need for periodontal surgery to remove the traps. Many investigations about the removal of NETs have been published recently. While the investigations appreciated that the removal of NETs is essential for tissue homeostasis, the processes involved and time required in removing NETs are not well understood. In 2010, it was reported that NETs produced in vitro were stable for over 90 hours. DNase 1 is one of the mechanisms responsible for NET degradation, and the presence of DNase 1 inhibitors or anti-NET antibodies that also blocked the access of the enzyme would be responsible for the removal of impaired NETs in cases of autoimmune diseases such as systemic lupus erythematosus [25]. NETs are degraded by macrophages through lysosomic action. However, the whole specific nuclease pathway involved in this process remains difficult to find. A key to this process is that the mechanism of NET removal is similar to that of apoptosis, whereby macrophages do not release pro-inflammatory cytokines [26]. Recently it has been reported that NET degradation is increased in treated periodontitis patients, thus indicating that NET degradation contributes to a decreased pro-inflammatory state [27,28]. This is in contrast to the observations of our study wherein NETs structures were observed even weeks after the initial therapy in systemically healthy individuals.

Neutrophil extracellular traps, one of the primary mechanisms of PMN immune response in the oral cavity, are essential to the onset and progression of late-onset periodontitis [29]. The primary elements of NETs are proteins and chromatin. NETs have the ability to both increase and decrease inflammation, causing damage to tissues while increasing inflammation [29]. Periodontal bacteria have evolved a number of strategies to resist the antibacterial effects of NET as a result of their prolonged interactions with them. These mechanisms include NET DNA degradation, degradation of antibacterial proteins, and alteration of NET levels in the pocket environment [30]. Reducing NET levels, providing anti-inflammatory therapy, and prescribing broad-spectrum and targeted antibacterial medications are some options to minimize tissue destruction in periodontal disease. Thus, removal of the inflamed and diseased gingival tissue as part of the surgical periodontal therapy may have served to eliminate not only NETs, but also the ongoing tissue destruction occurring due to their continued presence.

The SEMs revealed a dense network of NETs in all of the samples. Isolated hollow areas of lost neutrophils adjacent to intact neutrophils were also observed. Interestingly, there was no evidence of bacteria or microorganisms trapped in the meshwork. This could possibly suggest that they may have been destroyed in the initial stages of NET production. The network of extracellular DNA strings that make up NETs bind harmful microorganisms, which could not be identified in the images in our study. Histones and a number of proteins found in neutrophil granules are linked to the bacteria that are trapped by the damaged DNA framework. It has been demonstrated that the neutrophil NADPH oxidase produced reactive oxygen species that are crucial for mediating NET released from a variety of stimuli, including a variety of pathogenic microorganisms [15]. At this point, SEM only helps in identifying the structure of the neutrophil traps without going into the specifics of the molecular configuration. Matrix metalloproteinase -8 (MMP-8), also known as neutrophil collagenase is generated by neutrophils and cannot be determined by SEM. Despite the fact that a variety of techniques have been employed to detect NETs in vitro and in vivo, a consensus on the best NET-specific assays is urgently required in the literature.

Both an excessive presence or absence of neutrophils in the tissue can lead to periodontitis, indicating how important neutrophil balance is in periodontal homeostasis. Thus the role of NETs is arguable, in terms of the part it plays in engaging the pathogenic bacteria (as evidenced by the SEM) or its more broader impact on the host's immune system and causing tissue destruction.

4. Conclusions

In conclusion, the excised gingival tissue samples in periodontitis patients showed features of NETs long after initial periodontal therapy, indicating that they may be a crucial defensive mechanism against periodontal infections. However, their continued presence in spite of destroyed neutrophils and lack of bacteria is a subject of further in-depth investigations to determine the precise nature of their role in periodontal disease.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Conflict of interest

The authors declare no conflict of interest.

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