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Neutrophil extracellular trap formation in supragingival biofilms

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ABSTRACT

Background: Oral biofilms are the causative agents of the highly prevalent oral diseases periodontitis and caries. Additionally, the host immune response is thought to play a critical role in disease onset. Neutrophils are known to be a key host response factor to bacterial challenge on host surfaces. Release of neutrophil extracellular traps (NETs) as a novel antimicrobial defense strategy has gained increasing attention in the past years. Here, we investigated the influx of neutrophils into the dental plaque and the ability of oral bacteria to trigger intra-biofilm release of NETs and intracellular proteins.
Methods: Supragingival biofilms and whole saliva were sampled from systemically healthy subjects participating in an experimental gingivitis study. Biofilms were analysed by immunofluorescence followed by confocal and fluorescence microscopy. Moreover, concentrations of cytokines and immune-associated proteins in biofilm suspensions and saliva were assessed by ELISA. Neutrophils obtained from blood were stimulated with twelve bacterial species isolated from cultured biofilms or with lipopolysaccharide to monitor NET formation.
Results: Neutrophils, NETs, neutrophil-associated proteins (myeloperoxidase, elastase-2, cathepsin G, cathelicidin LL-37), interleukin-8, interleukin-1β and tumor necrosis factor were detected within plaque samples and saliva. All tested bacterial species as well as the polymicrobial samples isolated from the plaque of each donor induced release of NETs and interleukin-8. The degree of NET formation varied among different subjects and did not correlate with plaque scores or clinical signs of local inflammation.
Conclusions: Our findings indicate that neutrophils are attracted towards dental biofilms, in which they become incorporated and where they are stimulated by microbes to release NETs and immunostimulatory proteins. Thus, neutrophils and NETs may be involved in host biofilm control, although their specific role needs to be further elucidated. Moreover, inter-patient variability suggests NET formation as a potential factor influencing the individual course of disease.

Introduction

Oral biofilms, also referred to as dental plaque, consist of numerous bacterial, fungal and viral species in an organized extracellular matrix. This structure creates a shield against antimicrobial substances and a protective environment for microbial growth (Zijnge et al., 2010). Endogenous control of biofilm growth in the oral cavity is exerted by different host defense mechanisms, which include the constitutive presence of antimicrobial peptides and the recruitment of immune cells to the adjacent gingival tissues (Dale and Fredericks, 2005; Silva et al., 2007). Nevertheless, oral microbes often overcome host defense, thus causing gingival and

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periodontal inflammation as well as occurrence of dental cavities (Fux et al., 2005; Sbordone and Bortolaia, 2003; Avila et al., 2009).

Within the gingival crevice and epithelium, neutrophils are found in large numbers and are considered a key protective cell type in the periodontal tissues (Scott and Krauss, 2012). In healthy individuals these granulocytes are equipped for phagocytosis and intracellular lysis of microorganisms. They represent the mainstay of immune defense against bacterial and fungal pathogens and are recruited from the bloodstream to the site of infection within minutes (Kolaczkowska and Kubes, 2013). In contrast, patients with acquired or congenital neutrophil deficiency suffer from frequent infections with bacteria or yeast as well as from severe periodontal destruction at early age (Moutsopoulos et al., 2014; Andrews and Sullivan, 2003). Chemoattractants such as interleukin-8 (IL-8), chemokines and anaphylatoxins promote tissue invasion and subsequent neutrophil-mediated clearance of microbes (Baggiolini and Clark-Lewis, 1992). Destruction of microorganisms is typically mediated by reactive oxygen species (ROS) and oxygen-independent lytic enzymes (Mantovani et al., 2011).

Upon challenge with microorganisms, neutrophils can release DNA, histones, intracellular granule content and antimicrobial peptides, all of which form an extracellular matrix structure recently termed neutrophil extracellular trap (NET), because it can trap and kill microbes (Brinkmann et al., 2004; Ermert et al., 2009). The process of release is being referred to as NETosis, a cellular reaction known to be preceeded by defined intracellular events, which trigger the activity of NADPH oxidase, myeloperoxidase and elastase, the generation of reactive oxygen species and the associated citrullination of histones (Papayannopoulos et al., 2010; Leshner et al., 2012; Remijsen et al., 2011). Next to their evident role in preventing the spread of invading pathogens, neutrophils and NETs can directly promote inflammation and were shown to induce damage to host cells, leading to their characterization as 'double-edged swords' (Cooper et al., 2013; Kaplan and Radic, 2012; Saffarzadeh and Preissner, 2013; Smith, 1994).

To date it is well accepted that there is substantial variation in the individual predisposition to oral inflammatory disease, especially in regards to onset and severity. Albeit the reasons for these interindividual differences are presently unknown, it is very likely that host immune factors play an essential role in this context (Bartold and Van Dyke, 2013; Garlet, 2010; Kinane et al., 2011; Laine et al., 2012). However, despite their central role in first line defense against microbes, at present it is unclear whether and how neutrophils and the release of NETs influence the development of oral biofilm or alter its composition. In this study we addressed the question whether neutrophils are present within supragingival biofilms and investigated the potential of supragingival plaque inhabitants to trigger the release of NETs and antimicrobial molecules from neutrophils.

Methods

Study subjects

Pilot study group

A pilot study was performed with six healthy non-smoking subjects (26–50 years) with no or mild gingivitis (BoP $\leq$ 15%, PPD $\leq$ 3) and little to moderate dental plaque accumulation (Silness–Löe plaque index = 1–2). From this group, only supragingival plaque samples were taken. The pilot study was performed at The Forsyth Insitute (Cambridge, MA) and the participants signed informed consent as approved by the local Institutional Review Board (IRB no. 00000037).

Experimental gingivitis group

Fourteen healthy undergraduate volunteers (mean age = 24 $\pm$ 3 years) from the University of Bonn Dental School with no or minimal gingival inflammation and a plaque index of 0–1 participated in the experimental gingivitis study. Every subject was screened one week prior to the start of the study and instructed in oral hygiene measures to be conducted before start of the study. These were tooth brushing according to the modified bass technique and interproximal flossing in order to maintain or achieve a non-inflamed gingival status. All subjects received professional tooth cleaning. Exlusion criteria were periodontitis as assessed by pocket depth measurements (PPD $\geq$ 3 mm) and bleeding on probing (BoP $\geq$ 10%), smoking, pregnancy, acute or chronic diseases, allergic diatheses, extensive dental restaurations or untreated caries as well as the intake of antibiotics or nonsteroidal antiinflammatory drugs within three months prior to the study. All study participants provided written informed consent as approved by the ethics comittee of the University Hospital of Bonn (approval number 336/13).

Experimental gingivitis

At baseline all subjects presented with healthy gingival conditions and no signs of clinical inflammation. The experimental gingivitis study was carried out for 21 days followed by a 14 day period for the resolution of gingivitis (Loe et al., 1965; Jepsen et al., 2003). Study subjects were seen on days 0, 1, 3, 7, 14, 21 and 28 as well as 35 to examine gingival healing. BoP, plaque index and gingival crevicular fluid (GCF) volume were measured in each session to monitor the inflammation progress. BoP was assessed with a PCP 12 periodontal probe for a total of six sites per tooth. GCF was collected by inserting Perio Paper Strips (Oraflow Inc., NY, USA) into the sulcus at the mesio-buccal and mesio-palatal sites of premolars and first molar and held in place for 30 s. Immediate measurements were conducted in a calibrated Periotron 8000 (Oraflow Inc., NY, USA) and averages of the volume counts from each site were calculated. Plaque scores were monitored applying a fluorescent plaque revealer (Plaque Test, Ivoclar Vivadent, Ellwangen, Germany). Here, each buccal and palatal surface was subdivided into six zones, which were observed for positive or negative staining and percentages of stained zones were calculated in each subject (Rustogi Modified Navy Plaque Index) (Rustogi et al., 1992). On day 0, subjects had plaque scores of 0 and no signs of gingival inflammation. Experimental gingivitis was induced by discontinuing oral hygiene on upper premolars and upper first molars for three weeks. To ensure plaque accumulation, vacuum-formed splints covering the gingival margin of the included teeth were used by the subjects during brushing. These splints were individually fabricated on plaster models.

Oral sample collection

Pilot study group

Supragingival plaque was carefully collected from the buccal side using a curette and immediately embedded and frozen in a water-based matrix (Tissue-Tek® *O.C.T.*™ Compound, Alphen aan den Rijn, The Netherlands) on dry ice.

Experimental gingivitis group

Samples for microbial analysis were taken on days 0 and 21 with sterile paper points which were inserted into the gingival sulci for 30 s. Thereafter, they were streaked out onto Columbia agar plates with 5% sheep blood (Becton, Dickinson and Co., Heidelberg, Germany) and either incubated aerobically or anaerobically. After three weeks, the supragingival plaque was collected from the buccal side as described above. In both groups, plaque was collected