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Morphological and functional adaptations of *Fusobacterium nucleatum* exposed to human neutrophil Peptide-1

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ABSTRACT

Background and objective: We recently demonstrated that *Fusobacterium nucleatum* can resist to human neutrophil peptide (HNP)-1 by decreasing its membrane permeability and increasing its proliferation and biofilm formation. In this continuation study, we aimed to further evaluate and explain these resistance properties by determining the morphological and functional adaptations of *F. nucleatum*, using transmission electron microscopy (TEM).

Materials and Methods: Cultures of the type strain of *F. nucleatum* (ssp. *nucleatum* ATCC 25586) and two clinical strains (ssp. *polymorphum* AHN 9910 and ssp. *nucleatum* AHN 9508) were incubated without (0 μg/ml) or with four different test concentrations of recombinant HNP-1 (1, 5, 10 and 20 μg/ml). Membrane morphology and thickness, and cell (visualized by TEM), planktonic growth (measured in colony forming units), and biofilm formation (measured as total mass) were analyzed. Scrambled HNP-1 was used in planktonic growth and biofilm formation studies as a negative control.

Results: TEM analyses revealed a decrease in the outer membrane surface corrugations and roughness of the strain AHN 9508 with increasing HNP-1 concentrations. In higher concentrations of HNP-1, the strain AHN 9910 showed thicker outer membranes with a number of associated rough vesicles attached to the outer surface. Intracellular granules became increasingly visible in the strain ATCC 25586 with increasing peptide concentrations. With increased concentrations of HNP-1, planktonic growth of the two clinical strains was significantly enhanced ($\boldsymbol{P < 0.001}$) and of the type strain significantly suppressed ($P < 0.01$). HNP-1 decreased the biofilm formation of the two clinical strains, AHN 9910 ($P < 0.01$) and 9508 ($\boldsymbol{P < 0.001}$) significantly. Scrambled HNP-1 showed no effect on planktonic growth or biofilm formation of the tested strains.

Discussion: *F. nucleatum* has the ability to withstand the lethal effects of HNP-1, and the ultrastructural changes on bacterial membrane and cytoplasm may play role in this adaptive process.

1. Introduction

Fusobacterium nucleatum is an anaerobic filamentous gram-negative rod, which belongs to the phylum *Fusobacteria*. It belongs to the resident oral indigenous microbiota and is often isolated from mixed cultures of aerobic, facultative, and anaerobic bacterial species. In addition to its role in formation of oral biofilms, *F. nucleatum* is among the most common bacteria implicated in clinical infections of other body sites such as colorectal carcinoma [1]. Recent research has focused on *F. nucleatum* as an important causative factor in the development of intrauterine infection and thus preterm birth [2,3]. In mixed cultures, *F. nucleatum* obtains the synergistic potency to induce infection and to produce tissue irritants [4–7]. Attachment to and invasion of host cells [6,8], production of endotoxins (lipopolysaccharides) [9,10], and proteolytic enzyme activities [11] are significant examples of *F. nucleatum* virulence capabilities. Moreover, the species has the capability to trigger the release of interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α from different cell types in order to stimulate periodontal tissue destruction [12]. Besides, *F. nucleatum* was found to indirectly increase the levels of some proteolytic enzymes in

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periodontal tissues, i.e., matrix metalloproteinases [13–16] which are responsible for the degradation of extracellular matrix components of the periodontium; thereby participating in the process of periodontal tissue destruction [17].

Antimicrobial peptides (AMPs) are unique and diverse host defense molecules composed of cysteine-rich proteins of molecular weight ranging between 2000 and 6000 Da. With such a small molecular weight and net positive charge, defensins (subfamily of AMPs) are able to attach, invade, kill, or inactivate a variety of microorganisms [18]. In humans, two types of defensins are found: α-defensins (human neutrophilic peptides; HNPs), which consist of six subtypes, and β-defensins with four subtypes. When defensins bind electrostatically to the negatively charged surface molecules on the bacterial lipid bilayer membranes, voltage-regulated channels are created in the cell membrane, facilitating the passage of antimicrobial and cytotoxic molecules [19]. AMPs are characterized by their specificity, i.e., they selectively interact with bacterial cells and have the ability to maintain the host cells without being significantly affected [20]. There are several potential pathways by which bacteria resist the host antimicrobials. Among these are the inactivation of defense mechanisms of AMPs by the expression of bacterial proteases and peptidases [21]; the secretion of designated binding proteins [22], and the reduction of penetration and invasion of AMPs into bacterial cytoplasm by formation of biofilms [23,24]. Our study group recently demonstrated that, in *in vitro* conditions, the incubation of *F. nucleatum* with HNP-1 decreases its membrane permeability and increases both planktonic and established biofilm growth [25]. Up to our knowledge, the susceptibility and the resistance of *F. nucleatum* to HNPs are only partly covered in the current literature, and the exact mechanism of this resistance still remains unknown.

Transmission electron microscopy (TEM) is an imaging tool designed to image samples of ultrathin thickness (1–100 nm) at a high resolution contrast (atomic scale resolution) to enable the examiner to visualize the minute ultrastructural details of the specimen in a nanometer scale. In the present study, we hypothesized that the decreased membrane permeability and increased biofilm formation of *F. nucleatum*, which we observed in our previous study, are related with bacterial morphological adaptations detectable by TEM analyses. Therefore, the aims of this study were to expand our previous work [25] by examining the cell morphology (intracellular granules, outer membrane vesicles, and membrane surface characteristics) of *F. nucleatum* exposed to graded concentrations of HNP-1. Moreover, we re-evaluated the cell proliferation of *F. nucelatum* in the presence of both HNP-1 and scrambled HNP-1.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Three strains of *F. nucleatum* were used in this study: two clinical strains (ssp. *nucleatum* AHN 9508 and ssp. *polymorphum* AHN 9910) and the type strain (ssp. *nucleatum* ATCC 25586, which is also a clinical isolate from a cervico-facial lesion). The clinical strains, AHN 9508 from a gingival crevice sample fluid and AHN 9910 from saliva obtained from two children, were from the National Institute for Health and Welfare (THL), Helsinki, Finland. In all experimental steps, the study strains were inoculated on Brucella agar plates, supplemented with hemin (5 mg/l) and vitamin K1 (10 mg/l), and incubated in an anaerobic chamber (Whitley A35 Anaerobic Workstation, Don Whitley Scientific Ltd., West Yorkshire, UK) with an atmosphere of 10% H_2, 5% CO_2, and 85% N_2 at 37 °C for 3–5 days. When running an experiment, purely cultured colonies were collected from agar plates and immersed in Bacto-tryptone specific broth (TSB), supplemented with 25 mM KH_2PO_4, 4 mM $MgSO_4$ and 1% saccharose [26]. The bacterial suspensions in TSB were always kept in an anaerobic chamber for 24 h before starting any test in order to enhance bacterial growth phase. Optical densities of the strains were adjusted to 0.5 OD and at a wave length of 490 nm using spectrophotometer which was earlier calculated in terms of colony forming units (CFU) to be equal to 4×10^7 CFU/ml [27].

2.2. Preparations of defensin (HNP-1) suspension

Both HNP-1 and scrambled HNP-1 were commercially purchased (Genemed Synthesis, Inc. 6203 Wood Lake Center, San Antonio, TX 78244, USA). In all experiments, HNP-1 (2 mg) was taken out from −20 °C immediately before starting the experiment and was dissolved in 1 ml of 20% dimethyl sulfoxide to ensure optimum dissolution. The test concentrations of HNP-1 (1, 5, 10, and 20 μg/ml) and the control (0 μg/ml) were prepared. Each bacterial strain was incubated individually with the five graded concentrations of HNP-1 in triplicates. The peptide sequence of the HNP-1 was: AA: Ala-Cys-Tyr-Cys-Arg-Ile-Pro-Ala-Cys-Ile-Ala-Gly-Glu-Arg-Arg-Tyr-Gly-Thr-Cys-Ile-Tyr-Gln-Gly-Arg-Leu-Trp-Ala-Phe-Cys-Cys with Cys–Cys random cyclization. The sequence of the scrambled HNP-1 has the same amino acids but in a scrambled form, *i.e.*, Trp-Ile-Ala-Arg-Cys-Tyr-Arg-Ile-Thr-Gln-Arg-Cys-Pro-Arg-Cys-Leu-Gly-Cys-Ala-Tyr-Cys-Ala-Tyr-Ala-Gly-Cys-Ile-Glu-Ile-Gly, with random cyclization and different amino acid positions. Preparation of the five graded concentrations of the scrambled HNP-1 was performed exactly in the same way as those of the active HNP-1, and then tested on the three strains of *F. nucleatum*.

2.3. Planktonic growth measurements

To measure the planktonic growth of each *F. nucleatum* strain, the three study strains were first cultured on agar plates for 3–5 days and later in TSB for 24 h. Afterwards, each bacterial strain was adjusted to 0.5 OD at 490 nm and incubated with HNP-1 suspensions in an anaerobic atmosphere for another 24 h. Bacterial suspension of 10 μl was inoculated on agar plate and incubated in an anaerobic atmosphere for 3–5 days. Colonies were counted and bacterial growth was assessed as CFUs/ml. The rest of the bacterial suspensions were kept for TEM analyses.

2.4. Biofilm formation assay

Biofilm formation of the three study strains was tested for each strain individually using a 96-well plate and saliva coating as described before [28]. After blotting the saliva, suspensions of each OD-adjusted *F. nucleatum* strain, pre-incubated with the tested HNP-1 concentrations in anaerobic conditions for 48 h, were added to the wells in pentaplicate. After incubation, the wells were blotted on a paper towel and rinsed twice with PBS to remove unattached cells. Biofilm formation was assessed by analyzing the total biofilm mass [25], i.e., adherent bacterial cells were stained with 0.1% crystal violet and the microplate was incubated at room temperature for 15 min. To remove the excess stain, the wells were rinsed twice with PBS. In order to obtain homogeneous crystal violet dye in each well, 200 ml of 33% acetic acid was added to dissolve the bounded cells and the stains from the walls and the base of the wells, and the plates were incubated for 10 min before being ready to measure the OD by a spectrometer with the absorbance of 570 nm.

2.5. Sample preparations for TEM imaging

After the incubation of the OD-adjusted *F. nucleatum* strains