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Ultrastructural damage in *Streptococcus mutans* incubated with saliva and histatin 5

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ABSTRACT

Objective: To study the ultrastructural alterations induced in *Streptococcus mutans* (ATCC 25175) incubated with saliva, saliva plus histatin 5 and histatin 5.

Methods: *S. mutans* incubated with saliva histatin 5 or a combination of both were morphologically analyzed and counted. The results were expressed as $(CFU)ml^{-1}$. Ultrastructural damage was evaluated by transmission electron microscopy. Ultrastructural localization of histatin 5 was examined using immunogold labeling. Apoptotic cell death was determined by flow cytometry (TUNEL).

Results: A decrease in the bacteria numbers was observed after incubation with saliva, saliva with histatin 5 or histatin 5 compared to the control group ($p < 0.0001$). Ultrastructural damage in *S. mutans* incubated with saliva was found in the cell wall. Saliva plus histatin 5 induced a cytoplasmic granular pattern and decreased the distance between the plasma membrane bilayers, also found after incubation with histatin 5, together with pyknotic nucleoids. Histatin 5 was localized on the bacterial cell walls, plasma membranes, cytoplasm and nucleoids. Apoptosis was found in the bacteria incubated with saliva (63.9%), saliva plus histatin 5 (71.4%) and histatin 5 (29.3%). Apoptosis in the control bacteria was 0.2%.

Conclusions: Antibacterial activity against *S. mutans* and the morphological description of damage induced by saliva and histatin 5 was demonstrated. Pyknotic nucleoids observed in *S. mutans* exposed to saliva, saliva plus histatin 5 and histatin 5 could be an apoptosis-like death mechanism. The knowledge of the damage generated by histatin 5 and its intracellular localization could favor the design of an ideal peptide as a therapeutic agent.

1. Introduction

Dental caries remains a major oral health problem in most industrialized countries. The World Health Organization estimated that nearly 80% of the world's population suffers from tooth decay (WHO, 2009). Colonization of the oral surface by pathogenic microorganisms (Kolenbrander et al., 2006), especially by *Streptococcus mutans,* is the major etiologic agent involved in human dental caries (Simón & Mira, 2015). The main cariogenic character of *S. mutans* is its ability to form biofilms in the oral cavity (Krzysciak, Jurczak, Koscielniak, Bystrowska, & Skalniak, 2014; Senadheera & Cvitkovitch, 2008). Bacterial coaggregation is essential for the orchestrated and highly ordered development of multi-species biofilms, which are microbial communities present on a wide variety of surfaces. Biofilms are associated with an extracellular matrix that includes several types of biopolymers (Abee, Kovács, Kuipers, & van der Veen, 2011).

Bacterial growth within biofilms confers protection from antibiotics due to the reduced penetration of several antimicrobial agents into their inner structure (Costerton et al., 2003). Microorganisms included within biofilms are therefore extremely difficult or impossible to eradicate (Alhede et al., 2009; Van Gennip et al., 2009). Thus, the oral cavity represents a unique environment for a wide array of microbial

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species that inhabit the dental plaque and requires multiple types of defenses to prevent infection (Melino, Santone, Di Nardo, & Sarkar, 2014).

Saliva contains proteins that range from 3 kD to over 100 kD and are secreted by three pairs of major salivary glands (parotid, submandibular and sublingual) as well as many minor salivary glands. Saliva exerts important protective functions, such as physical cleansing, which acts together with a number of innate and adaptative immune components (Phattarataratip et al., 2011), (Fábián, Fejérdy & Csermely, 2008a, 2008b). Human saliva contains various forms of anti-microbial polypeptides that play vital roles in combating invading foreign pathogens, (Lamkin & Oppenheim, 1993), promoting wound healing (Oudhoff et al., 2008), and supporting apoptosis (Rudney, Staikov, & Johnson, 2009). Various protein components have been identified in all the major glandular secretions, including secretory IgA, proline-rich proteins (acidic, basic and glycosylated families), amylase, statherin, histatins, lysozyme, lactoferrin and lactoperoxidase, which form the key components of the innate defense system in the oral cavity (Azen & Oppenheim, 1973; Anders & Ole, 1985; Bennick & Connell, 1971; Eckersall & Beeley, 1981; Edgerton & Koshlukova, 2000; Hay, 1973; Hay & Oppenheim, 1974; Kauffman, Bennick, Blum, & Keller, 1991; Levine et al., 1987; Oppenheim, Hay, & Franzblau, 1971; Oppenheim et al., 1988). On the other hand, cystatins and mucins have been identified in submandibular/sublingual secretions (Bobek & Levine 1992; Bobek, Tsai, Biesbrock, & Levine 1993; Lamkin, Migliari, Yao, Troxler, & Oppenheim 2001: Levine et al., 1987).

Antimicrobial peptides (AMPs), a heterogenous group of molecules produced by various tissues, possess a broad-spectrum antimicrobial activity that significantly reduces infections. These AMPs are promising agents in controlling microbial growth due to their concentration and selective antimicrobial activity as well as their low rates of microbial resistance induction (Batoni, Maisetta, Brancatisano, Esin, & Campa, 2011; Tao et al., 2011). Histatins (HST) are a group of neutral and basic proteins rich in the amino acid histidine (18–28 mol%) (Lingstrom & Moynihan, 2003; Lamkin et al., 2001) that are present in the saliva at a concentration of 50–425 μM (Helmerhorst, Van't Hof, Veerman, Simoons, & Nieuw Amerongen, 1997). They are secreted by the parotid and sublingual glands in humans (Rijnkels, Elnitski, Miller, & Rosen, 2003) and in higher primates (macaques) (Sabatini, Warner, Saitoh, & Azen, 1989), and possess a broad-spectrum of antibacterial activity (De Smet & Contreras, 2005). Twelve salivary protein forms are found in saliva, including HST 1–3 and 5 (Pepperney & Chikindas, 2011). Different genes are present in HST 1 and 3, while HST 5 is a proteolytic cleavage of histatin 3 (Oppenheim et al., 1988). Histatin 5 has been shown to be a potential novel agent against oral candidiasis (Kavanagh & Down, 2004), yet limited research has been conducted to assess its anticaries potential (Adam & Chikindas, 2011). Histatin 5 exhibits some activity against cariogenic bacteria in a multispecies biofilm, but its effect on the morphology of *S. mutans* remains unstudied, and details on how histatin 5 penetrates bacterial membranes and interacts with DNA have not been elucidated.

The aim of this work was to study whether saliva and histatin 5 are able to induce ultrastructural damage on *Streptococcus mutans*. Additionally, ultrastructural localization of histatin 5 in the bacteria using Immunogold labeling was performed. The possible mechanism of death in bacteria incubated with saliva and histatin 5 is discussed.

2. Materials and methods

2.1. Saliva collection and processing

Six healthy volunteers (3 women and 3 men aged 25–35 years, all of whom were nonsmokers) were recruited for the study. The subjects exhibited no overt signs of gingivitis or caries. Written informed consent was obtained from all participants. To minimize circadian effects, saliva samples were collected between 9:00 and 11:00 AM. Subjects were instructed to refrain from eating or drinking at least one hour before the sessions. Salivary secretion was stimulated by chewing a piece of parafilm (25 cm^2 ~1 g) (Parafilm, American National Ca, Chicago, IL). A volume of 10 ml per individual was collected on ice, and the saliva of the six volunteers was mixed before centrifugation. Samples were centrifuged at 14,000 × *g* for 20 min at 4 °C (Siqueira, de Olveira, Mustacchi, & Nicolau, 2004). The supernatant was sterilized by ultrafiltration, first through a 0.45-μm pre-filter (Nuclepore Corp., Pleasanton, Ca.) and subsequently in small portions through sterile disposable 0.2-μm filters (Schleicher & Schull, Dassel, West Germany). Total saliva protein was determined using the Bradford method. The samples were concentrated by lyophilization, and the sterility of the saliva was verified by incubating a small portion anaerobically for 48 h at 37 °C.

Ethical Approval was given by the Ethics committee of the Medicine Faculty UNAM with the reference number C54-11.

2.2. Bacteria growth

Streptococcus mutans strain ATCC 25175 was used in all the experiments. The bacteria strain was subcultured twice from frozen stock prior to the experiments. Bacteria were cultured under microaerophilic conditions with a candle jar at 37 °C overnight in trypticase soy broth (TSB; Oxoid, Milan, Italy). Bacterial growth was monitored spectrophotometrically at 600 nm.

2.3. Bacteria assays

Bacteria were centrifuged twice with phosphate buffered saline at pH 5.2 (PBS pH 5.2) at 325 × *g* for 5 min. The supernatant was discarded, and the pellet was resuspended by vortexing in 1 ml PBS pH 5.2. The bacterial density was visually adjusted to a turbidity of 0.5 McFarland (1×10^8 colony forming units (CFU/mL)), diluted 1:10 with phosphate buffered pH 5.2 to yield 10^7 CFU/mL. Fifty microliters of this suspension was inoculated into each well of the microtiter plates to obtain a final concentration of 5×10^5 CFU/well.

2.4. Histatin 5

Lyophilized human histatin 5 (Sigma Aldrich Sigma St. Louis, MO; H6027) with the following Amino Acid Sequence:

[Asp-Ser-His-Ala-Lys-Arg-His-His-Gly-Tyr-Lys-Arg-Lys-Phe-His-Glu-Lys-His-His-Ser-His-Arg-Gly-Tyr] was reconstituted with 1 ml of (10 mM) PBS, pH 7.0, containing 0.9% NaCl.

2.5. Bacteria incubation with saliva, saliva plus histatin 5 and histatin 5

For experimental and control group assays, 50 μl of *Streptococcus mutans* (5×10^5 CFU/per well) was inoculated into 60 wells of the microtiter plates containing the following:

1) 375 μl (PBS pH 7.0) with 325 μg of whole saliva.
2) 373 μl/325 μg of saliva plus 2 μl/2 μg of histatin 5
3) 373 μl (PBS pH 7.0) plus 2 μl/2 μg of histatin 5
4) 375 μl (PBS pH 7.0)

The effect of saliva, saliva plus histatin 5, and histatin 5 on the growth of *S. mutans* was expressed as the CFU per plate. The microtiter plates were pre-incubated at 37 °C in a shaking water bath for 1 h (Mackay, Denepitiya, Iacono, Krost, & Pollock, 1984). To evaluate the bacterial viability, 10 μl of the specimens were diluted 1:1000, cultured on Mueller Hinton Agar (BD Bioxon) under microaerophilic conditions and incubated at 37 °C for 24 h.

After the pre-incubation period (60 min) all the specimens recovered from the wells of the microtiter plate were placed into Eppendorf tubes and centrifuged at 1 310 × *g* for 5 min. Supernatants

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