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Vitamin D reduces the inflammatory response by *Porphyromonas gingivalis* infection by modulating human β-defensin-3 in human gingival epithelium and periodontal ligament cells

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ABSTRACT

Periodontitis is a multifactorial polymicrobial infection characterized by a destructive inflammatory process. *Porphyromonas gingivalis*, a Gram-negative black-pigmented anaerobe, is a major pathogen in the initiation and progression of periodontitis; it produces several virulence factors that stimulate human gingival epithelium (HGE) cells and human periodontal ligament (HPL) cells to produce various inflammatory mediators. A variety of substances, such as vitamin D, have growth-inhibitory effects on some bacterial pathogens and have shown chemo-preventive and anti-inflammatory activity. We used a model with HGE and HPL cells infected with *P. gingivalis* to determine the influence of vitamin D on *P. gingivalis* growth and adhesion and the immunomodulatory effect on TNF-α, IL-8, IL-12 and human-β-defensin 3 production. Our results demonstrated, firstly, the lack of any cytotoxic effect on the HGE and HPL cells when treated with vitamin D; in addition, vitamin D inhibited *P. gingivalis* adhesion and infectivity in HGE and HPL cells. Our study then showed that vitamin D reduced TNF-α, IL-8, IL-12 production in *P. gingivalis*-infected HGE and HPL cells. In contrast, a significant upregulation of the human-β-defensin 3 expression in HGE and HPL cells induced by *P. gingivalis* was demonstrated. Our results indicate that vitamin D specifically enhances the production of the human-β-defensin 3 antimicrobial peptide and exerts an inhibitory effect on the pro-inflammatory cytokines, thus suggesting that vitamin D may offer possible therapeutic applications for periodontitis.

1. Introduction

Porphyromonas gingivalis, a Gram-negative black-pigmented anaerobe, is a key etiologic agent of periodontitis, especially its chronic form [1]. A number of factors are associated with the virulence of this oral anaerobe, including a variety of proteases, endotoxins, and collagenase [2], as well as the production of soluble and cell-bound proteases [3] that can degrade various tissue and plasma proteins and contribute to invasion of the periodontal tissues, a condition characterized by the destruction of tissue supporting the teeth [4]. *P. gingivalis* can adhere to a cell surface [5] and thus contribute to its establishment and colonization; one of the virulence factors is determined by its capacity to adhere to hard surfaces and soft tissues of the oral cavity [6]. *P. gingivalis* can also invade and survive within epithelial cells [7–9], and recent evidence suggests that in the early stages of infection in gingival epithelial cells it induces the production of reactive oxygen species established as secondary signaling molecules with a key role in diverse processes, including the immune response [10,11].

Human gingival epithelium (HGE) cells and human periodontal ligament (HPL) cells are two important components of periodontal soft tissue. The gingival epithelium has a stratified squamous structure that acts as an interface between the external environment, with its complex bacterial ecosystem, and the underlying periodontal tissue. Periodontal ligament cells are fibroblast-like cells characterized by collagen production but also possessing some osteoblastic features [12]; it is a structure connecting teeth to the alveolar bone and seems to actively participate in alveolar bone remodeling [13]. In addition to secreting different classes of inflammatory mediators in response to pathogen stimulation, both cells act as a mechanical barrier against bacterial invasion. *P. gingivalis* has developed different strategies to colonize and disrupt the structural and functional integrity of the gingival epithelium [14]. An important attribute associated with the virulence of *P. gingivalis* is its ability to inhibit or evade innate host responses, and this has led to the speculation that the strong association of this bacterium with diseased sites may be related to its ability to disrupt periodontal innate defense functions and facilitate untoward host interactions with the entire

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bacterial community [15]. On the other hand, *P. gingivalis*, and/or its components, is capable of inducing a strong proinflammatory cytokine response in the gingival epithelial and periodontal ligament cells in-vitro, which is correlated with the adhesive/invasive potential of *P. gingivalis* [12,16,17], and these mediators further activate the gingival epithelium and periodontal ligament cells to mount excessive host inflammatory responses resulting in disease progression. When the immune and inflammatory responses do not stop the progression of periodontal infection, uncontrolled secretion of cytokines occurs, leading to chronic inflammation and periodontal tissue destruction [18]. Due to the proximity of the periodontal ligament to the alveolar bone, the cytokine production by periodontal ligament cells might influence the processes of bone resorption in periodontal disease.

Antimicrobial peptides are small cationic molecules of the innate immune response with a broad spectrum activity against pathogens, including those associated with periodontitis [19]. Human β-defensins (HBDs), important defensins in the oral cavity are expressed in response to Gram-positive and Gram-negative invasion [20–23]. These peptides exhibit antimicrobial properties [24,25] by binding to bacterial membrane components and leading to the formation of pores and cell lysis [26,27]. In addition to exerting antimicrobial activity, these peptides modulate the immune response [28]; some studies have reported a marked reduction in the amounts of HBD-3 in gingival crevicular fluid during periodontitis, which may be related to the ability of certain periodontopathogens to proteolytically inactivate the peptides or downregulate their expression [29–31]. Gingival epithelial cells have been reported to secrete several antimicrobial peptides either constitutively or in response to an infection [19,20]. The excessive use of antibiotics to manage periodontal diseases has led to the development of bacteria-resistant mutants. Therefore, as some patients do not respond adequately to conventional therapy, new therapeutic strategies with adjunctive antimicrobials need to be developed [32]. A variety of substances and biological factors have been suggested to reduce bacterial infection and promote periodontal healing and regeneration, suggesting the potential use of these compounds for the treatment and prophylaxis of infection in combination therapy with existing drugs [33–35].

Vitamin D, originally recognized as a vitamin needed in small amounts to affect the metabolism of calcium and phosphate, has been associated with various regulatory effects on cell proliferation and differentiation [36,37]. The role of vitamin D and its metabolites in the bone is well established. Since the function of vitamin D in the immune system may depend partly on its ability to alter cytokine signals [38,39], and current reports highlight the alteration of inflammatory mediators in gingival epithelia in periodontal disease, there is increasing awareness of its importance as a modulator of the immune response [40,41]. Since vitamin D exerts biological functions in many cells including intestinal and gingival epithelial cells [42,43], and hypothesizing that its immunomodulatory effects may influence the degree of the local response to infection and that it may have beneficial effects for *P. gingivalis*-associated periodontal diseases, its intrinsic influence on the inflammatory response of HGE and HPL cells appears to be significant. HGE and HPL cells participate in the immune response in the oral cavity and can produce cytokines that increase the inflammatory response and promote normal communication. In an effort to understand the mechanisms underlying the effects of vitamin D, we evaluated its influence on the antibacterial activity against a periodontal pathogen, *P. gingivalis*, and the anti-inflammatory effects in HGE and HPL cells.

In the present study, we investigated the effects of vitamin D on *P. gingivalis* growth and adherence to human gingival epithelium and periodontal ligament cells and the immunomodulatory effect of vitamin D on tumor necrosis factor (TNF)-α, interleukin (IL)-8, IL-12 and HBD-3 production. We found that vitamin D works with primary gingival epithelium and periodontal ligament cells to modulate cytokine responses to *P. gingivalis* through the induction of HBD-3.

2. Materials and methods

2.1. Bacterial strain and reagents

P. gingivalis (Pg) from the American Type Culture Collection (ATCC 33277) was grown anaerobically at 37 °C for 2 to 3 days in trypticase soy (TS) broth (30 g/l) containing 1 g/l yeast extract (Difco), 1 g/l glucose, 0.5 g/l potassium nitrate, 1 ml/l sodium lactate (Sigma L-1375), 0.5 g/l sodium succinate and 1 g/l sodium fumerate; after autoclaving, filter-sterilized supplements were added (0.4 g/l sodium carbonate; 0.005 g/l hemin [σ H-2250]; 0.4 g/l cysteine; and 0.001 g/l vitamin K [σ M-5625]). Mid logarithmic-phase cells were employed; bacteria were washed twice in sterile phosphate buffered saline (PBS). Following the final resuspension, *P. gingivalis* was diluted to an OD600 nm in sterile PBS, which corresponded approximately to10^6 colony-forming units (CFU)/ml, to obtain the multiplicity of infection (MOI) of 50. This concentration was found to be optimal.

Vitamin D3 (1a,25$[OH]_2D_3$) (VD) obtained from Delifab was dissolved in 0.1% of absolute ethyl alcohol at a concentration of 10^{-10} mol/L, 10^{-9} mol/L and 10^{-8} mol/L. Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), glutamine, penicillin, streptomycin, amphotericin B and trypsin were obtained from Gibco (Invitrogen S.r.l., Milan, Italy).

2.2. Isolation, cell culture and treatments

Primary cultures of HGE cells used in this study were obtained after informed consent from a healthy young individual (male; aged 23 years) in need of premolar extraction for orthodontic reasons. Gingival tissue was isolated at the cement-enamel junction of the extracted tooth by means of a surgical blade. The harvested tissue was rinsed several times in DMEM containing antibiotics (penicillin 100 U/ml; streptomycin 125 μg/ml and amphotericin B 5 μg/ml). The tissue was cut into small pieces and cultured with a complete medium (CM) containing 10% fetal bovine serum, L-glutamine (600 μg/ml), penicillin (100 U/ml), and streptomycin (125 μg/ml) in a humidified atmosphere of 5%CO_2 and 95% air at 37 °C. The cells that grew from the explanted tissues were sub-cultured. Cell cultures used in all experiments were between passages 2 and 4.

Primary cultures of HPL cells were isolated from the middle part of the root of one periodontally healthy molar extracted from an orthodontic patient (male; aged 26 years) after informed consent was signed. Briefly, the tooth was washed twice in sterile PBS supplemented with antibiotics (100 U/ml penicillin, 125 μg/ml streptomycin and 5 μg/ml amphotericin B). The periodontal ligament was scraped out from the middle portion of the tooth root with a sterile surgical scalpel and put in a tube with 1 ml of PBS containing 1 mg/ml collagenase P (Worthington Biochemical, USA) and 0.25% trypsin, and rotated for 10 min. Cells from this initial digestion were discarded. A fresh collagenase/trypsin solution was added and tissue fragments were rotated for 2 h at 37 °C. The solution was then centrifuged at 1000*g* for 5 min. The cell pellet, consisting primarily of HPL cells, was collected and washed three times with DMEM. Cells were seeded into 6-well culture plates with CM; the cells were incubated in a humidified atmosphere, 95% air, 5%CO_2 at 37 °C. The medium was changed twice/week. When the cultures were preconfluent (10–14 days in culture), the cells were passed with trypsin-EDTA and placed onto 24-well culture plates; HPL cells of the second to fifth passages were used for the experiments described below. Cell viability was assessed by means of the trypan blue dye exclusion method (Sigma, Aldrich). Total cells treated with 0.1% absolute ethyl alcohol were used as the control.

The total cell lines were confirmed to be free of *Mycoplasma* infections by using 4, 6-diaminodino-2-phenylindole (DAPI) fluorescent staining (Sigma-Aldrich S.r.l. Milan, Italy). For some experiments, HGE and HPL cells were treated with 20 ng/ml concentration of human recombinant HBD-3 (rHBD-3) (Sigma-Aldrich S.r.l. MI,USA) or