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Interleukin-1β is internalised by viable *Aggregatibacter actinomycetemcomitans* biofilm and locates to the outer edges of nucleoids

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ABSTRACT

The opportunistic pathogen *Aggregatibacter actinomycetemcomitans* causes periodontitis, which is a biofilm infection that destroys tooth-supportive tissues. Interleukin (IL)-1β, a central proinflammatory cytokine of periodontitis, is an essential first line cytokine for local inflammation that modulates the cell proliferation and anti-pathogen response of human gingival keratinocytes. Previously, we demonstrated that *A. actinomycetemcomitans* biofilms bind IL-1β; however, whether this binding is an active process is not known. In this study, we showed for the first time with immuno-electron microscopy that viable bacterial biofilm cells internalised IL-1β when co-cultured with an organotypic mucosa. Decreased biofilm viability hindered the ability of biofilm to sequester IL-1β and caused IL-1β leakage into the culture medium. In some *A. actinomycetemcomitans* cells, intracellular IL-1β localized to the outer edges of the nucleoids. We identified the DNA-binding protein HU as an IL-1β interacting protein with mass spectroscopy and showed the interaction of recombinant HU and IL-1β *in vitro* using enzyme-linked immunosorbent assay (ELISA). Close contact with a viable *A. actinomycetemcomitans* biofilm decreased the proliferation and apoptosis of human gingival keratinocytes as demonstrated using Ki-67 and the terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) staining, respectively. Our results suggest that viable *A. actinomycetemcomitans* biofilms may disturb the critical first steps of local inflammation in periodontitis by binding and internalising IL-1β. The interaction of IL-1β with conserved HU provides a potential mechanism for shaping bacterial gene expression.

1. Introduction

The inflammation and destruction of tooth-supporting tissues develops when a polymicrobial biofilm spreads subgingivally into the gingival sulcus. Periodontitis and the periodontal pocket is formed when the balance between the biofilm and the host defence system is disrupted. The opportunistic periodontal pathogen *Aggregatibacter actinomycetemcomitans* is linked to aggressive forms of periodontitis [1,2] and may systemically contribute to cardiovascular diseases [3,4]. Active tissue destruction sites of periodontitis contain high concentrations of the proinflammatory cytokine interleukin (IL)-1β [5,6], and the secretion of IL-1β from macrophages is stimulated by the *A. actinomycetemcomitans* leukotoxin [7]. Although macrophages are the main cell type that produces IL-1β in periodontal tissues [8], human gingival epithelial cells [9] and fibroblasts (HGF) [10] produce IL-1β after experiencing a bacterial challenge. In general, IL-1β modulates human cell differentiation, proliferation and apoptosis [11–13] and regulates the release of other proinflammatory cytokines such as IL-6 and IL-8 [14]. Significantly higher IL-6 levels have been detected in the gingival crevicular fluid from the inflamed sites than in healthy sites [6,15] and also levels of IL-8 have been increased in inflamed sites [6]. A number of *in vitro* studies have shown that the synthesis and release of these cytokines increases from human gingival

Abbreviations: BSA, bovine serum albumin; Cdt, cytolethal-distending toxin; CLSM, confocal laser scanning microscope; DAB, 3,3′ diaminobenzidine tetrahydrochloride; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; FBS, foetal bovine serum; GMC, gingival mucosa culture; HGF, human gingival fibroblast; HGK, human gingival keratinocyte; HRP, horseradish peroxidase; IL, interleukin; IL-1Ra, interleukin-1 receptor antagonist; LC–MS/MS, liquid chromatography–mass spectroscopy; LDH, lactate dehydrogenase; PBS, phosphate buffered saline; PI, propidium iodide; STI, soybean trypsin inhibitor; TBS, tris buffered saline; TSA, trypticase soy agar; TSB, trypticase soy broth; TUNEL, termal deoxynucleotidyl transferase dUTP nick-end labelling.

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cells and human blood cells after *A. actinomycetemcomitans* infection or induction with components released from the species [9,16–21]. IL-6 stimulates the growth and differentiation of B-lymphocytes whereas IL-8 activates and attracts leukocytes to the infection site. In the periodontal area, IL-1β causes alveolar bone loss by inducing osteoclast formation and activation (reviewed in [22]). During periodontitis, IL-1β may enhance host resistance to inflammation by increasing the expression of cell-stress and DNA-repair-related genes in human gingival keratinocytes (HGKs) [23].

Certain opportunistic pathogens respond to IL-1β by increasing growth [24] and modifying their virulence gene expression [25]. For example, the gram-positive bacterium *Staphylococcus aureus* decreases the expression of virulence genes, such as leukotoxin, after stimulation with IL-1β [25] when the levels of IL-1β are comparable to the levels in active periodontal inflammation sites [5]. Our recent findings suggest that periodontal pathogens can also sense and respond to IL-1β. We have demonstrated that biofilms and single cells of *A. actinomycetemcomitans* bind human IL-1β, which is presumably transported inside the bacterium and decreases the metabolic activity of *A. actinomycetemcomitans* by binding to a conserved ATP synthase subunit β [26]. These studies imply that bacteria can become more persistent after sensing the presence of IL-1β.

Bacterial DNA is condensed by various DNA-binding proteins including HU, which is one of the most abundant of these proteins. In Enterobacteriaceae, such as *Escherichia coli*, HU consists of two subunits, HUα and HUβ. However, in the majority of gram-negative species, including *A. actinomycetemcomitans*, HU forms a homodimer [27]. Because the abundant HU can bind to DNA regardless of its sequence, similar to eukaryotic histones, it has been thought to function mainly as DNA compacting protein [28,29]. However, recent studies with mutated HU proteins have proved that the changes in DNA packing and supercoiling can result in changes in the gene-expression profile, which may alter the cellular metabolism and morphology of *E. coli* [27]. *E. coli* HU regulates the expression of 8% of the *E. coli* genome, targeting genes encoding proteins related to energy metabolism and stress response [30]. HU controls the allocation of RNA polymerase in the nucleoid, which could serve as a mechanism driving the changes in gene-expression [31]. In one major opportunistic periodontal pathogen, *Porphyromonas gingivalis*, HU is essential for efficient expression of K-antigen polysaccharide capsule synthesis genes [32], a capsule that prevents the attachment of the bacterium to the surface and inhibits biofilm formation *in vitro* [33].

The mechanism by which pathogens sense and internalise IL-1β is unknown. Because the extracellular matrix, which comprises proteins, DNA and polysaccharides, forms a substantial part of the total biofilm mass, it needs to be confirmed that the intense binding of IL-1β to biofilm [26] is not due to the nonspecific binding of IL-1β to the inert extracellular matrix. Whether the binding of the central proinflammatory cytokine IL-1β by bacteria could affect host cells is also unknown. In this study, we investigated how the presence of antibiotics affected the ability of the bacteria in a biofilm to bind IL-1β. In addition, we aimed to identify IL-1β-binding proteins that localise in the bacterial nucleoid, as IL-1β was detected in the outer edges of the *A. actinomycetemcomitans* nucleoid. Furthermore, we explored specific indicators, such as changes in epithelial cell proliferation and apoptosis, that suggest that biofilm affects epithelial cells by sequestering IL-1β. We infected an organotypic gingival mucosa culture (GMC) with *A. actinomycetemcomitans* biofilms in the presence and absence of antibiotics that were proven to kill biofilm cells. Only the viable biofilm bound and internalised IL-1β and decreased the proliferation and apoptosis of gingival keratinocytes. In addition, the conserved DNA-binding protein HU interacted with IL-1β, which might explain the localisation of IL-1β in the nucleoid. To our knowledge, the present study is the first to demonstrate that viable biofilm cells bind and internalise substantial amounts of IL-1β, which might subsequently interact with a DNA-binding protein.

2. Materials and methods

2.1. A. actinomycetemcomitans biofilm culture

A clinical *A. actinomycetemcomitans* strain, D7S, [34] was selected as test strain because it is a clinical isolate with a sequenced genome [35], allowing the identification of potential IL-1β-binding proteins using a recombinant DNA technique. The strain was revived from a frozen milk stock through growth on trypticase soy agar (TSA) blood plates for 3 days. Additional suspensions [36] of the plate-grown cells were produced in trypticase soy broth medium (TSB; composed of 30 g/l of trypticase soy broth, 6 g/l of yeast extract, 8 g/l of separately autoclaved glucose and 5% defibrinated sheep blood). The bacterial suspension (5×10^7 cells/well) was placed on sterile hydrophilic polyethersulfone membranes (Supor®-200; diameter of 5 mm; 0.2 μm pore size; Pall Corporation, Ann Arbour, MI) in a 48-well culture plate. Control membranes were incubated in TSB without bacteria with identical conditions in a candle jar at 37 °C for 24 h. On the following day, the membranes were briefly washed twice with 0.85% NaCl prior to a 24 h incubation in RPMI-1640 medium (Sigma) supplemented with 0.6 g/l L-glutamine (Sigma).

2.2. Biofilm viability assay and mass quantification

The biofilms (1.5×10^8 cells/well) were grown on microscope cover glasses (18 mm × 18 mm) in 6-well cell culturing plates as described above with the modification that after 24 h of incubation in RPMI-1640, the incubations were continued in Green's medium [37] in a candle jar for 6 h or 25 h. Half of the cultures were grown with a combination of penicillin (63.4 IU/ml) and streptomycin (63.4 μg/ml). The biofilms were rinsed with sterile water before they were stained using a FilmTracer™ LIVE/DEAD® biofilm viability kit according to the manufacturer's instructions (Invitrogen, Molecular Probes Inc., Eugene, OR). This viability assay is based on the different permeabilities of the dyes; propidium iodide (PI) penetrates only the bacterial cells with a damaged membrane whereas SYTO9 stains all bacterial cells; the SYTO9 stain is gradually displaced by the PI stain over time in dead cells. The samples were examined using a confocal laser scanning microscope (CLSM) as described below.

The cover glass with the stained biofilm was placed upside down on a microscope slide with a drop of nail polish on each corner. A water immersion objective, HCX PL APO 63x/1.20 W CORR, was used to avoid a substantial refractive index mismatch. The CLSM images were acquired using a Leica TCS SP5 confocal microscope with the LAS AF scanning software (Leica Microsystems CMS, Mannheim, Germany). Each collected image covered an area that was 246 μm × 246 μm. The fresh specimens were scanned using an argon laser with excitations of 488 nm for SYTO9 and 514 nm for PI. A sequential scanning mode was used to optimize the emission detection for both of the channels separately in the 500–550 nm and 600–650 nm bandwidths. Three independent specimens were prepared, and three images of each specimen were acquired. The scan settings for comparable specimens were adjusted according to the antibiotic treatment, which caused a pronounced brightness saturation in the untreated specimen images. An averaging of three frames for the green images and six frames for the red images were used to improve the signal-to-noise ratios. The dynamic range for the digital images was determined using the