



Oral microbial biofilm stimulation of epithelial cell responses

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ARTICLE INFO

Article history:

Received 11 August 2011
Received in revised form 9 November 2011
Accepted 22 December 2011
Available online 21 January 2012

Keywords:

Oral bacteria
Biofilms
Epithelial cells
Cytokines
Chemokines

ABSTRACT

Oral bacterial biofilms trigger chronic inflammatory responses in the host that can result in the tissue destructive events of periodontitis. However, the characteristics of the capacity of specific host cell types to respond to these biofilms remain ill-defined. This report describes the use of a novel model of bacterial biofilms to stimulate oral epithelial cells and profile select cytokines and chemokines that contribute to the local inflammatory environment in the periodontium. Monoinfection biofilms were developed with *Streptococcus sanguinis*, *Streptococcus oralis*, *Streptococcus gordonii*, *Actinomyces naeslundii*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis* on rigid gas-permeable contact lenses. Biofilms, as well as planktonic cultures of these same bacterial species, were incubated under anaerobic conditions with a human oral epithelial cell line, OKF4, for up to 24 h. Gro-1 α , IL-1 α , IL-6, IL-8, TGF α , Fractalkine, MIP-1 α , and IP-10 were shown to be produced in response to a range of the planktonic or biofilm forms of these species. *P. gingivalis* biofilms significantly inhibited the production of all of these cytokines and chemokines, except MIP-1 α . Generally, the biofilms of all species inhibited Gro-1 α , TGF α , and Fractalkine production, while *F. nucleatum* biofilms stimulated significant increases in IL-1 α , IL-6, IL-8, and IP-10. *A. naeslundii* biofilms induced elevated levels of IL-6, IL-8 and IP-10. The oral streptococcal species in biofilms or planktonic forms were poor stimulants for any of these mediators from the epithelial cells. The results of these studies demonstrate that oral bacteria in biofilms elicit a substantially different profile of responses compared to planktonic bacteria of the same species. Moreover, certain oral species are highly stimulatory when in biofilms and interact with host cell receptors to trigger pathways of responses that appear quite divergent from individual bacteria.

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1. Introduction

Host gingival tissues respond to an array of microbial challenges in the oral cavity that are crucial for maintaining homeostasis within this constantly infected environment. These responses comprise a wide array of pro- and anti-inflammatory molecules produced by resident cells of the periodontium [1,2], as well as response molecules derived from infiltrating inflammatory and immune cells in the tissues [3,4]. While these innate and adaptive immune responses are generated to protect the host from the microbial burden juxtaposed to the tissues, magnitude of the burden and chronicity of the inflammatory response can result in both soft and hard tissue damage defined as periodontitis [5].

Many *in vitro* studies have reduced the complex *in situ* microbial infection into individual proposed pathogenic or commensal species as planktonic challenges of host cells [6–8]. These types

of studies have frequently focused on a particular host target molecule [9,10], emphasized molecular aspects of Toll-like receptor engagement [11,12] and/or developed data to identify intracellular signaling pathways that account for the response profile of the cells [13]. Moreover, it appears clear that the net result of these host–bacterial interactions as maintaining health or manifesting disease is reflected in the relative distribution and abundance of a wide range of biomolecules with competing and complementary activities in the tissue microenvironment [14,15]. Many of these conclusions have resulted from studies that attempt to sample the oral environment and compare the patterns of various responses in health and disease [16], during progression of the periodontal lesion [17], or following clinically successful therapy of disease [18]. While providing a robust snapshot of the characteristics of the compendium of responses that can occur in the oral cavity, these types of studies are not able to delineate details of the microorganisms that have a predilection to elicit the particular responses or the relative contribution of individual host cell types to these response profiles.

It is clear that the bacteria inhabiting the oral cavity in contact with oral tissues reside in complex multispecies biofilms [19]. These biofilm structures arise via interaction with host substrates [20,21] and accrue and mature based upon the oral environment

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and specific interactions among individual species of bacteria [22,23]. However, there are very few reports that have evaluated the response profiles of specific host cells to oral bacteria in biofilms. Recently, Guggenheim and colleagues used a hydroxyapatite disc model to prepare oral multispecies biofilms and used these to challenge epithelial cell cultures [24,25]. While numerous species were used to create the biofilms, of the nine species used, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* made up a rather small proportion of the overall microbial composite at a level approximating 1% of the total. Thus, while the architecture of these very complex biofilms was described using confocal scanning laser microscopy, it remains undetermined the density of the bacteria that interacted with the individual cells, nor the species that may have been primary participants in this process. Nevertheless, this report did document a range of host responses molecules produced in response to the biofilm challenge that occurred under aerobic conditions over 24 h. The primary findings were an apparent increase in apoptosis and degradation of IL-1 β , IL-6 and IL-8 cytokines that were elicited from the epithelial cells that was predicted to be related to the presence of proteases, such as those produced by *P. gingivalis* in these biofilms.

The present report describes our use of a novel biofilm model, created on rigid gas permeable contact lens (RGPL) material [26,27] that were used to challenge oral epithelial cell cultures in an anaerobic environment that may better reflect the subgingival sulcus. We evaluated patterns of cell responses to single species biofilms, compared with planktonic challenge with the same species, to elucidate unique features of the cellular responses to the biofilm challenge.

2. Materials and methods

2.1. Bacteria and culture conditions

P. gingivalis (FDC381), *F. nucleatum* ATCC 25586, *Actinomyces naeslundii* ATCC 49840, and *Streptococcus gordonii* ATCC 10558 were cultured in Brain Heart Infusion (Becton Dickinson and Company, Sparks, MD) medium supplemented with 5 μ g hemin ml⁻¹ and 1 μ g menadione ml⁻¹ under anaerobic conditions (85% N₂, 10% H₂, 5% CO₂) at 37 °C. *Streptococcus sanguinis* ATCC 10556 and *Streptococcus oralis* ATCC 10557 were grown in Trypticase yeast extract salts (TYS) medium under anaerobic conditions. All bacterial strains used in this study have been described previously and were obtained from the ATCC, except *P. gingivalis* (FDC381) [26].

2.2. Biofilm growth conditions

Biofilms were grown on rigid gas permeable lenses (RGPLs) (Advanced Vision Technologies, Golden, CO) as previously described [26]. Briefly, prior to biofilm formation, RGPLs were coated with 1% fetal bovine serum (FBS; Invitrogen) to support the adherence of bacteria and incubated at room temperature until dry. Each RGPL was inoculated with a 5 ml of monospecies planktonic culture at 0.3 OD₆₀₀ in a single well of a 6-well polystyrene tissue culture plate (BD Falcon, Franklin Lakes, NJ) and incubated in an anaerobic chamber for 3 days for development of biofilms under static conditions. At each 24-h interval spent media was replenished by fresh medium. After incubation, RGPLs with adherent biofilms were washed in 1X PBS twice to remove loosely adherent cells and used in subsequent epithelial cell challenge. Biofilms grown on three additional RGPLs were used for bacterial enumeration by qPCR analysis as described previously [26].

2.3. OKF4 cell growth and bacterial challenge

An immortalized epithelial cell line OKF4 (Rheinwald 2002) was cultured in keratinocyte serum free medium (Invitrogen, Carlsbad,

CA) and seeded into 48-well tissue culture plates (Costar, Cambridge, MA) at a density of 10⁵ cells per well in a 1 ml volume and allowed to adhere for 24 h in a 5% CO₂ chamber at 37 °C to form a confluent monolayer. Planktonic, biofilm and control treatments were each carried out in six wells in 1 ml/well fresh media and continuously incubated for 6 h under anaerobic conditions (85% N₂, 5% CO₂, and 10% H₂). For planktonic challenge, overnight cultures were harvested by centrifugation and resuspended in keratinocyte medium. A 10⁸, 10⁷ and 10⁶ cells/well challenge corresponding to a multiplicity of infection (MOI) at 1000:1, 100:1 and 10:1 was used to stimulate the OKF4 cells. The estimated MOI for the planktonic challenge represents the numbers of bacteria that could be predicted to be in association with the OKF4 cells interacting with the biofilms. Three day old biofilms grown on contact lens were overlaid with biofilm-side facing the epithelial cells. OKF4 cells with or without overlaid RGPL were used as controls for the biofilm or planktonic bacterial challenges, respectively. OKF4 cell supernatants from each of two wells were pooled and stored at –80 °C for cytokine determination. Previous studies with this model system demonstrated that these biofilm challenges did not result in any obvious toxicity or cell death, as determined by both metabolic activity and housekeeping gene expression [27].

2.4. Inhibition of biofilm growth during the course of challenge

In order to constrain the biofilms from replicating in the keratinocyte media during the 24 h challenge with OKF4 cells, biofilms were treated with green fluorescent nucleic acid stain SYTO 24. SYTO 24 was chosen as it yielded lowest optical density with highest fluorescence intensity values for *S. gordonii*, *A. naeslundii* and *P. gingivalis* indicating that this stain inhibited replication while not affecting viability [26]. *S. oralis* and *S. sanguinis* biofilms were also treated with SYTO 24 to inhibit replication. Prior to challenging OKF4 cells, biofilms were immersed in 10 μ g/ml SYTO 24 stain in keratinocyte media for 5 min. after which they were immersed in 1X PBS twice to remove excess stain. *F. nucleatum* was not treated with any SYTO stains as it did not replicate in keratinocyte media.

2.5. Detection of cytokines/chemokines

The level of cells by 24 h was determined using a multiplexed beadlyte kit (R & D systems, Minneapolis, MN, USA) and a Luminex IS100 (Luminex, Inc., Austin, TX) instrument. The mean $\pm$ standard error of the mean of the planktonic and biofilm stimulation of OKF4 cells was compared with unchallenged and RGPL overlaid OKF4 cells, respectively. Statistical comparison of the data was accomplished using an ANOVA on ranks test with Dunn's test for multiple comparisons for multiple comparisons (SigmaStat 3.5; Systat Software, Inc., Chicago, IL).

3. Results

3.1. Characteristics of cytokine responses

An array of cytokines and cell communication factors produced by epithelial cells and having some potential role in responses to oral bacterial challenge was evaluated and included, IL-1 α , IL-6, and TGF α . The results in Fig. 1A–C demonstrate the responses of the epithelial cells to challenge with the range of oral bacteria. Biofilms of *A. naeslundii* and all three streptococci provided minimal stimulus of IL-1 α , while the planktonic challenge with these microorganisms appeared to inhibit production of this cytokine. The *F. nucleatum* biofilms significantly upregulated production of IL-1 α , while *P. gingivalis* biofilms significantly inhibited this

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