Osteopontin Reduces Biofilm Formation in a Multi-Species Model of Dental Biofilm

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Abstract

Background: Combating dental biofilm formation is the most effective means for the prevention of caries, one of the most widespread human diseases. Among the chemical supplements to mechanical tooth cleaning procedures, non-bactericidal adjuncts that target the mechanisms of bacterial biofilm formation have gained increasing interest in recent years. Milk proteins, such as lactoferrin, have been shown to interfere with bacterial colonization of saliva-coated surfaces. We here study the effect of bovine milk osteopontin (OPN), a highly phosphorylated whey glycoprotein, on a multispecies in vitro model of dental biofilm. While considerable research effort focuses on the interaction of OPN with mammalian cells, there are no data investigating the influence of OPN on bacterial biofilms.

Methodology/Principal Findings: Biofilms consisting of Streptococcus oralis, Actinomyces naeslundii, Streptococcus mitis, Streptococcus downei and Streptococcus sanguinis were grown in a flow cell system that permitted in situ microscopic analysis. Crystal violet staining showed significantly less biofilm formation in the presence of OPN, as compared to biofilms grown without OPN or biofilms grown in the presence of caseinoligomacropeptide, another phosphorylated milk protein. Confocal microscopy revealed that OPN bound to the surface of bacterial cells and reduced mechanical stability of the biofilms without affecting cell viability. The bacterial composition of the biofilms, determined by fluorescence in situ hybridization, changed considerably in the presence of OPN. In particular, colonization of S. mitis, the best biofilm former in the model, was reduced dramatically.

Conclusions/Significance: OPN strongly reduces the amount of biofilm formed in a well-defined laboratory model of acidogenic dental biofilm. If a similar effect can be observed in vivo, OPN might serve as a valuable adjunct to mechanical tooth cleaning procedures.

Introduction

Bacteria in dental biofilms produce organic acids on account of fermentable dietary carbohydrates. Repeated pH drops at the biofilmtooth interface lead to slow demineralization of the dental hard tissues and the development of carious lesions. The most common and most effective means of caries prevention is the mechanical removal of dental biofilm. However, self-performed mechanical cleaning using both tooth brush and interdental floss does not result in full removal of the biofilm [1–3], and combating the high world-wide prevalence of caries is still one of the major challenges for dental research [4].

A large number of chemical adjuncts to support mechanical tooth cleaning have been developed and proven to contribute to caries control [5–7]. While most of these agents, such as chlorhexidine or essential oils, aim at killing bacteria in the oral cavity, non-bactericidal approaches that target bacterial adhesion and biofilm formation have gained increasing attention in recent years [8–9]. In particular, milk proteins, such as lactoferrin, α-lactalbumin and caseins have been shown to interfere with the adhesion and biofilm formation of oral organisms [10–14]. Since the development of a robust biofilm is a prerequisite for the establishment of highly acidic microenvironments at the tooth surface, these therapeutic approaches possess great potential for caries prevention.

In the present study, we investigate the effect of bovine milk osteopontin (OPN), a glycosylated and highly phosphorylated whey protein, on a well-described laboratory model of dental...
biofilm composed of Streptococcus oralis, Actinomyces naeslundii, Streptococcus mitis, Streptococcus downei and Streptococcus sanguinis [15].

OPN is also expressed in a variety of human tissues and involved in numerous biological processes, including bone and tooth mineralization, wound healing and leukocyte recruitment [16–18]. While considerable research effort has been spent on describing the interaction of OPN with mammalian cells [19–20], no data on the interaction with bacteria and a potential effect of OPN on bacterial biofilm formation have been published. We quantified biofilm formation in a flow cell system spectrophotometrically by crystal violet staining and investigated species composition, cell viability and structural stability of the biofilms by fluorescence labelling and confocal laser scanning microscopy.

Results and Discussion

Removal of dental biofilm and suppression of biofilm build-up are crucial means of caries prevention. In the five-species model of dental biofilm employed here, bovine milk osteopontin had a profound effect on biofilm growth. When 26.5 µmol/L of OPN were present in the flow medium, biofilm formation in the flow cells was affected considerably (Figure 1A). Quantification of the biofilm biomass by crystal violet staining showed a highly significant difference in OD585 between biofilms grown in the absence and presence of OPN ($OD_{585}^{} = 1.0 \pm 0.30$ SD without OPN and $0.26 \pm 0.06$ SD with OPN; $p < 0.001$). No such effect was observed when biofilms were grown in the presence of caseinoglycomacropeptide (CGMP), another highly phosphorylated milk glycoprotein ($0.89 \pm 0.27$ SD; $p = 0.26$) (Figure 1B).

The observed reduction in biofilm formation could either be caused by a bactericidal effect of OPN, or by an impact on the mechanisms involved in biofilm formation. CGMP and OPN’s effect on cell growth was investigated for individual strains in planktonic culture, and none of the two proteins affected the effect on cell growth was investigated for individual strains in planktonic culture, and none of the two proteins affected the

While biofilm formation was strongly affected by introducing OPN in early stages of growth, the addition of OPN after 28 h did not remove the already established biofilms: No significant difference in biomass quantified by crystal violet staining could be observed ($OD_{585}^{} = 1.21 \pm 0.35$ with OPN, $p = 0.26$). Collectively, these results show that OPN affects biofilm development, but it does not disperse or disrupt already established biofilms.

To further investigate the changes induced by OPN, we subjected biofilms to FISH (fluorescence in situ hybridization) with specific probes targeting the five species in the model and determined the bacterial composition. Confocal microscopy and subsequent digital image analysis revealed considerable changes in the absolute and relative biovolumes of individual strains, as compared to biofilms grown without OPN. The abundance of S. mitis SK24, the predominant organism in biofilms grown in the absence of OPN, was reduced dramatically from 78% to 14% of the total biovolume ($p < 0.001$). The relative biovolumes of all other organisms increased when OPN was present in the medium ($p < 0.001$), and S. sanguinis SK150 became the most abundant organism in the biofilms, representing 48% of the bacterial volume (Figure 4). Detailed biofilm composition data are shown in Figure 5 and Figure S4. The total biovolume detected with EUB338 also decreased significantly ($p < 0.001$), confirming the results obtained by crystal violet staining.

A. naeslundii AK6 was inoculated before S. mitis SK024 and was predominantly found in the basal layers of the biofilms (Figure 4C, D). As both organisms form coaggregates in OPN-free THB, we hypothesized that attached cells of A. naeslundii facilitate the adhesion of S. mitis, and that OPN in the medium might interfere with this interaction. However, pairwise coaggregation of planktonic organisms in THB was not affected by the presence of OPN (Table S1). In two-species biofilms, grown with A. naeslundii and S. mitis, as well as in monospecies biofilms of S. mitis, biofilm formation was significantly lower in the presence of OPN (Two-species biofilms: $0.63 \pm 0.31$ SD without OPN; $0.27 \pm 0.11$ SD with OPN; $p < 0.05$). Monospecies biofilms: $1.06 \pm 0.49$ SD without OPN; $0.29 \pm 0.12$ SD with OPN; $p < 0.05$; Figure S5). While an effect of OPN on receptor-adhesin interactions mediating interspecies coaggregation in the model biofilms cannot be ruled out,
these data suggest that OPN also interfered with intra-species coaggregation and bacterium matrix interactions in the biofilms. Bovine milk osteopontin bound to the bacterial cell surfaces in the employed dental biofilm model. Without affecting cell viability, it reduced biofilm stability and had a highly significant impact on the amount of biofilm formed in the flow cells. If OPN has a similar effect on in vivo grown dental biofilms, the protein might be used as a supplement to mechanical tooth cleaning procedures. OPN, provided by, for example, a mouth rinse or a chewing gum during biofilm build-up might compromise dental biofilm stability and reduce the amount of biofilm formed on tooth surfaces. Thereby, the acid challenge would be reduced, and biofilm removal by mechanical debridement might be facilitated. Hence, OPN might be a valuable adjunct to professional and self-performed oral hygiene procedures and contribute to caries control. Further investigations should explore if the results presented here can be extrapolated to in vivo grown dental biofilms.

**Materials and Methods**

**Bacterial strains**

*Streptococcus oralis* SK248, *Streptococcus mitis* SK24, *Streptococcus sanguinis* SK150, *Streptococcus downei* HG594 and *Actinomyces naeslundii* AK6 were used in the experiments. All organisms were moderately acidogenic human oral isolates [15]. 16S rRNA gene sequences have been deposited in GenBank (accession numbers: HQ219654-HQ219658) [21]. All organisms were cultivated aerobically on blood agar (SSI, Copenhagen, Denmark) and transferred to THB (Roth, Karlsruhe, Germany) at 35°C until mid to late exponential phase prior to experimental use.

**Biofilm growth**

Biofilms were grown as described previously [15]. Briefly, bacterial cultures (OD = 0.4 at 550 nm) were injected sequentially into flow cells (ibiTreat, µ-slide VI, Ibidi, Munich, Germany) in the following order: 1. *S. oralis* SK248; 2. *A. naeslundii* AK6; 3. *S. mitis* SK24; 4. *S. downei* HG594; 5. *S. sanguinis* SK150. Each organism was allowed to settle for 30 min, then nonadherent cells were removed by 10 min of flow and the next organism was inoculated. After inoculation, biofilms were grown for 26 h at 35°C with a flow rate of 250 µL/min (28.3 mm/min), using 1/10 diluted THB (pH 7.0), 1/10 diluted THB containing 26.5 µmol/L (0.9 g/L) OPN (pH 7.0) or 1/10 diluted THB containing 26.5 µmol/L (0.18 g/L) CGMP as the flow medium. OPN and CGMP were added in approximately the same molar concentration. For practical reasons, calculation of the molar concentration of OPN assumed a molecular weight of 34 kDa, although part of the OPN is likely to have formed fractions with lower molecular weight, leading to a slight underestimation of the true molar concentration.

In additional experiments, single species biofilms with *S. mitis* SK24 and dual species biofilms with 1. *A. naeslundii* AK6 and 2. *S. mitis* SK24 were grown in the same way. At least five replicate biofilms were grown for each experimental setting.

**Quantification of biofilm formation**

After biofilm growth, THB was removed from the flow channels by aspiration with paper points. The channels were rinsed with distilled water, dried again and stained with 100 µL of 2% crystal
violet solution (in 19.2% ethanol containing 0.8% ammonium oxalate) for 1 h. Then channels were rinsed again with distilled water, dried, and filled with 120 μL of absolute ethanol (Sigma-Aldrich, Brøndby, Denmark) for 30 min to destain the biofilms. Thereafter, 100 μL of the stained ethanol solutions, diluted 1:8, were transferred to a 96 well plate (Sarstedt, Newton, NC, USA), and optical density at 585 nm was measured with a spectrophotometer (BioTek PowerWave XS2, Bad Friedrichshall, Germany). Empty flow channels were processed in the same way and used for background subtraction.

**Growth in planktonic culture**

Bacteria were transferred to THB, THB containing 26.5 μmol/L OPN or THB containing 26.5 μmol/L CGMP. Aliquots of 100 μL were transferred to a 96 well plate (Sarstedt, Newton, NC, USA) and OD at 550 nm was measured with a spectrophotometer (BioTek PowerWave XS2, Bad Friedrichshall, Germany). Experiments were carried out in triplicates and repeated once.

**Confocal microscopy**

An inverted confocal microscope (Zeiss LSM 510 META, Jena, Germany) equipped with a 63× oil immersion objective, 1.4 numerical aperture (Plan-Apochromat) was used for microscopic analysis unless otherwise stated.

Viability in the biofilms was assessed using BacLight (Invitrogen, Taastrup, Denmark) according to the manufacturer’s instructions.

Figure 4. Biofilms grown in the presence of OPN, hybridized with EUB338 and species-specific probes SMIT, SSAN, ANAES, SDOW or SORA2. EUB338 targets all organisms in the biofilms and was labelled with Atto633 (red). Species-specific probes were labelled with Cy3 (green).

A. S. mitis SK24, the dominant organism in biofilms grown without OPN, accounted for 14% of the bacterial biovolume. B–F. The relative biovolumes of all other organisms increased in biofilms grown with OPN, as compared to biofilms grown without OPN. S. sanguinis SK150 (B) was the most abundant organism in the biofilms (48% of the biovolume). A. naeslundii AK6 was a prominent colonizer in basal layers of the biofilms (C, 22% of the biovolume in the basal layer), but was detected less frequently in upper layers of the biofilm (D, 9% of the total biovolume). S. downei HG594 (E, 11% of the biovolume) and S. oralis SK248 (F, 3% of the biovolume) represented smaller fractions of the bacterial biofilm.

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Figure 5. Bacterial composition of biofilms grown in the presence and absence of OPN. In biofilms grown without OPN (−OPN), S. mitis SK24 was the predominant organism. When OPN was present in the medium (+OPN), the abundance of S. mitis was dramatically lower, and the relative abundance of all other organisms increased. S. sanguinis SK150 became the predominant organism.

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Biofilms were stained with SYTO9 (Invitrogen, Taastrup, Denmark). After growth phase, biofilms were incubated for 45 min with 100 μL of the labelled protein at 35°C and imaged using the 488 nm laser line and a 500–550 nm band pass filter. XY resolution was set to 0.1 μm/pixel and Z resolution corresponded to 1 Airy unit (0.8 μm optical slice thickness).

To investigate binding of OPN to the biofilms, the protein was labelled with fluorescein according to the manufacturer’s instructions (Invitrogen, Taastrup, Denmark). After growth phase, biofilms were imaged repeatedly for 1000 sec. Biofilm substratum interface (20 μm from biofilm substratum interface) was imaged for 1000 sec. The biofilm stability was documented by time lapse imaging. Biofilms were stained with SYTO9, and a single field of view (20 μm optical slice thickness).

Biofilm stability was documented by time lapse imaging. Biofilms were stained with SYTO9 (Invitrogen, Taastrup, Denmark), and different microscopic fields of view (20 μm from biofilm substratum interface) were imaged repeatedly for 1000 sec. 488 nm laser line was used for excitation, and emission was detected with the META detector set to 500–554 nm. XY resolution was 0.4 μm/pixel and Z resolution corresponded to 2 Airy units (1.6 μm optical slice thickness).

Supporting Information

Figure S1 Effect of OPN and CGMP on bacterial growth in planktonic culture. A. S. oralis SK248. B. A. naeslundii AK6. C. S. mitis SK24. D. S. dentis HG594. E. S. sanguinis SK150. Bacterial strains were grown aerobically at 35°C in THB alone (black lines), THB containing OPN (red lines) or THB containing CGMP (green lines). Neither OPN nor CGMP affected planktonic bacterial growth in THB. (TIF)

Figure S2 Quantification of biofilm formation by crystal violet staining. – OPN: Biofilms were grown for 30 h on 1/10 diluted THB without OPN. OPN≥12 h: Biofilm growth was initiated without OPN, and the protein was added to the medium after 12 h. Quantification of the biofilm biomass by crystal violet staining showed a significant effect of OPN on biofilm growth. OD585 was significantly lower when OPN was added after 12 h. Error bars indicate standard deviations. (TIF)

Figure S3 Biofilm formation after 12 h without OPN. 12 h old biofilms were stained with BacLight and examined with a wide field microscope (Zeiss Axiovert 200 M, Jena, Germany) equipped with a 100 W high-pressure mercury lamp (HB103, Osram, Winterthur, Switzerland). 12 h after biofilm initiation the bottom of the flow cell was covered with a monolayer of bacteria, and multilayered areas had started to develop. Bar = 20 μm. (TIF)

Figure S4 Detailed biovolume fractions for each organism in biofilms grown with and without OPN. Each circle represents one microscopic field of view. – OPN: Biofilms grown in the absence of OPN. +OPN: Biofilms grown in the presence of OPN. Bars indicate means. A. S. oralis SK248. B. A. naeslundii AK6. C. S. mitis SK24. D. S. dentis HG594. E. S. sanguinis SK150. (TIF)

Figure S5 Quantification of biofilm formation by crystal violet staining. Biofilms were grown for 30 h on 1/10 diluted THB without OPN (∼OPN) or with OPN (+OPN). A. Two-species biofilms were grown with A. naeslundii and S. mitis. B. Monospecies biofilms were grown with S. mitis alone. For both two-species and monospecies biofilms, OD585 was significantly lower when OPN was present in the medium. Error bars indicate standard deviations. (TIF)

Video S1 Time-lapse imaging of a biofilm grown without OPN. After growth phase, the biofilm was stained with SYTO9, and a single field of view (20 μm from the biofilm substratum interface) was imaged for 1000 sec. The biofilm appeared stable and cell mobility was very low. Field of view size: 143×143 μm. (WMV)

Video S2 Time-lapse imaging of a biofilm grown in the presence of OPN. After growth phase, the biofilm was stained with SYTO9, and a single field of view (20 μm from the biofilm substratum interface) was imaged for 1000 sec. Biofilm stability was compromised, and cell mobility was considerably higher than in biofilms grown without OPN. Field of view size: 143×143 μm. (WMV)

Table S1 Pairwise bacterial coaggregation. Pairwise coaggregation was determined 30 min, 2 h and 24 h after mixing in 1/5 diluted THB without OPN (∼OPN) and with 26.5 μmol/L OPN (+OPN). t1 = 30 min; t2 = 2 h; t3 = 24 h. Grade 0: No
visible aggregates in cell suspension. Grade 1: Small uniform aggregates in suspension. Grade 2: Definite coaggregates easily seen, but suspension remained turbid without immediate settling of coaggregates. Grade 3: Large coaggregates which settled rapidly leaving some turbidity in the supernatant fluid. Grade 4: Clear supernatant fluid and large coaggregates which settled immediately. No difference in coaggregation patterns was observed between OPN-free THB and THB containing OPN.

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Author Contributions

Conceived and designed the experiments: SS RLM BMS BN DSS. Performed the experiments: SS MKR. Analyzed the data: SS RLM. Contributed reagents/materials/analysis tools: SS PLW MKR RLM. Performed the experiments: SS MKR. Analyzed the data: SS RLM. Conceived and designed the experiments: SS RLM BMS BN DSS. Author Contributions

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