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**Keratinocytes protect soft-tissue integration of dental implant materials
against bacterial challenges in a 3D-tissue infection model**

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Short title: keratinocyte protection of tissue integration

ABSTRACT

The soft-tissue seal around dental implants protects the osseointegrated screw against bacterial challenges. Surface properties of the implant material are crucial for implant survival against bacterial challenges, but there is no adequate *in vitro* model mimicking the soft-tissue seal around dental implants. Here, we set up a 3D-tissue model of the soft-tissue seal, in order to establish the roles of oral keratinocytes, gingival fibroblasts and materials surface properties in the protective seal. To this end, keratinocytes were grown on membrane filters in a transwell system, while fibroblasts were adhering to TiO₂ surfaces underneath the membrane. In absence of keratinocytes on the membrane, fibroblasts growing on the TiO₂ surface could not withstand challenges by commensal streptococci or pathogenic staphylococci. Keratinocytes growing on the membrane filters could withstand bacterial challenges, but tight junctions widened to allow invasion of bacteria to the underlying fibroblast layer in lower numbers than in absence of keratinocytes. The challenge of this bacterial invasion to the fibroblast layer on the TiO₂ surface negatively affected tissue integration of the surface, demonstrating the protective barrier role of keratinocytes. Streptococci caused less damage to fibroblasts than staphylococci. Importantly, the protection offered by the soft-tissue seal appeared sensitive to surface properties of the implant material. Integration by fibroblasts of a hydrophobic silicone rubber surface was affected more upon bacterial challenges than integration of more hydrophilic hydroxyapatite or TiO₂ surfaces. This differential response to different surface-chemistries makes the 3D-tissue infection model presented a useful tool in the development of new infection-resistant dental implant

materials.

Keywords: co-culture, dental implants, infection, tight junctions, streptococci, staphylococci, soft-tissue seal.

1. Introduction

Despite reported failure rates between 0.0% and 1.1% [1,2], dental implants count as the most successful biomaterials implants in the human body. Failures are largely due to implant-associated infection [3]. Adopting the well-known concept of “the race for the surface between bacteria trying to colonize a biomaterial surface, and tissue cells attempting to integrate it” as predictive for the faith of a biomaterials implant [4], dental implants are at a disadvantage with respect to, for instance, total joint arthroplasties. Dental implants are inserted in the highly unsterile environment of the oral cavity, and consequently a protective soft-tissue seal has to develop around the implant surface, that can already be contaminated by bacteria during implantation. Once that hurdle has been overcome, and a soft-tissue seal has formed (see Fig. 1A), the soft-tissue seal should protect a dental implant, most notably its osseointegratable part, against oral bacterial invasion and subsequent “peri-implantitis” [5]. Therewith, the success of dental implants, functioning in a bacterially laden environment, presents a paradox with other biomaterial implants in the human body, usually functioning in a sterile environment, yet having similar failure rates due to infection as dental implants have [6].

The soft-tissue seal is a 3D-tissue structure consisting of keratinocytes and gingival fibroblasts on the abutment part of a dental implant [7–9] and protects the osseointegrated part of the implant against bacterial invasion (see also Fig. 1A). 3D-tissue models distinguish themselves by the prevalence of physiological cell-to-cell contact *versus* predominantly physical, cell-to-substratum contact and cell-to-cell edge-contact

in 2D-cultures [10,11]. Physiological cell-to-cell contact can either refer to cells of the same type or to different cell types such as keratinocytes and fibroblasts constituting the soft-tissue seal around dental implants. In the quest to develop new, infection-resistant dental implant materials, the 3D-tissue structure of the soft-tissue seal (see Fig. 1B) is mostly neglected and therewith the important physiological interplay between keratinocytes, fibroblasts, bacteria and implant surfaces. This is a severe shortcoming of current *in vitro* mono-culture models, especially in an era in which animal studies are regarded with more and more societal scrutiny and regulatory impediments are increasing [12]. Moreover, the outcome of animal experiments for human clinical outcome can be doubted [13], if only because the composition of the oral microflora in animals is highly different from the human oral microbial composition [14]. These developments, necessitate improvement of current *in vitro* models, including a better mimic of the 3D-model of the soft-tissue seal, for the evaluation of new, infection-resistant dental implant materials under development.

Therefore, the aim of this study is to construct a 3D-tissue infection model of the soft-tissue seal around a dental implant using a transwell system (see Fig. 1C), in which bacterial challenges of the soft-tissue seal formed on different materials can be studied. The degree of physiological cell-to-cell contact will be regulated by employing membrane filters with different pore sizes. The model will be developed using titanium oxide as an implant surface, and *Streptococcus oralis* and *Staphylococcus aureus* as post-operative challenging organisms. Sensitivity to different surface-chemistries of substratum materials in the model will be demonstrated by using hydroxyapatite and

silicone rubber, in addition to titanium dioxide surfaces.

Fig. 1. Transwell 3D-tissue infection model to study soft-tissue integration of dental implant materials in presence of a bacterial challenge.

(A) Overview of the soft-tissue structure around a dental implant.

(B) Schematics of the 3D-tissue integration of a dental implant, providing the soft-tissue seal around an implant against bacterial invasion.

(C) Transwell 3D-tissue infection model of the soft-tissue seal around a dental implant for evaluating the role of keratinocytes in protecting implant integration by gingival fibroblasts against invading pathogens. Note, keratinocytes are seeded on a membrane filter to allow keratinocyte to fibroblast, physiological cell-to-cell contact.

2. Materials and methods

2.1. Substratum materials

Titanium oxide was provided by Salomon's Metalen (Groningen, The Netherlands)

and its surface was demonstrated by X-ray photoelectron spectroscopy to be composed mainly of TiO_2 (see below and Table S1). Hydroxyapatite (HAP) discs were commercially obtained from Himed (Old Bethpage, NY, USA). TiO_2 , cleaned in a 2% RBS 35 detergent solution (Omniclean, Breda, The Netherlands) under sonication and thoroughly rinsed in demineralized water, methanol, water again and finally washed with sterile water. TiO_2 (1.0 cm^2) and HAP discs (1.3 cm^2) were autoclaved at 121°C for 2 h before use. Medical grade silicone rubber discs (1.0 cm^2) were obtained from ATOS Medical B.V. (Zoetermeer, The Netherlands) and cleaned by immersing in 70% ethanol for 30 min. Discs had a thickness of 0.1 cm and their surface area was big enough to cover the membrane filter area of the transwell (0.3 cm^2 , see below).

The hydrophobicity of the different materials was determined using water contact angle measurements. Briefly, 1–1.5 μl water droplets were placed on the materials with a microsyringe and contact angles derived from the droplet contours after black-white thresholding using a customized, camera-equipped instrument.

The average surface roughness of the different materials was determined using atomic force microscopy (AFM, Nanoscope IIIa Dimension™3100, Bruker, Santa Barbara, CA, USA), equipped with a Si_3N_4 cantilever tip (DNP from Veeco, Woodbury, NY, USA), possessing a spring constant of 0.06 N/m. Images were taken in the contact mode. The roughness R_a was subsequently calculated using the NanoScope Analysis 1.8 software.

The elemental surface composition of the materials surfaces was measured using an S-probe X-ray photoelectron spectrometer (Surface Science Instruments, Mountain

View, CA, USA), equipped with an aluminum anode (10 kV, 22 mA) and a quartz monochromator. The direction of the photoelectron collection angle was 55 degrees with the sample surface and the electron flood gun was set at 14 eV.

2.2. Bacterial strains, growth conditions and harvesting

Streptococcus oralis J22 and *Staphylococcus aureus* ATCC 25923 were used. *S. oralis* is a well-known oral commensal strain [15] and *S. aureus* is recently recognized as an oral pathogen [16] frequently involved in peri-implantitis. Both strains have been demonstrated to form biofilms *in vitro* and have a diameter of around 1 μm [17]. Strains from a frozen stock were streaked onto blood agar plates and a fresh colony was transferred in 10 ml of Todd Hewitt Broth (THB; OXOID, Basingstoke, England) or Tryptone Soya Broth (TSB; OXOID) for *S. oralis* J22 and *S. aureus* ATCC 25923, respectively and cultured for 24 h at 37°C. Subsequently, these pre- cultures were added to 200 ml fresh medium and incubated for 20 h at 37°C. Then, bacteria were centrifuged at 6500 rpm for 5 min at 10°C and washed three times with buffer. Subsequently, bacteria were sonicated on ice (3×10 s) at 30 W (Vibra Cell model 375; Sonics and Materials, Danbury, CT, USA) in order to break bacterial chains and aggregates and suspended in their respective growth media, to concentrations desired in specific experiments as determined using a Bürker-Türk counting chamber.

2.3. Tissue cells, growth conditions and harvesting

Human gingival fibroblasts (HGF) and human oral keratinocytes (HOK) were employed in this study. HGFs were obtained from the American Type Culture Collection (HGF-1, ATCC-CRL-2014, Manassas, USA). HGF (passage between 20 and

25) were grown in 75 cm² tissue culture polystyrene flasks in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen, Breda, The Netherlands) and 0.2 mM ascorbic acid-2-phosphate at 37°C in 5% CO₂. At 80–90% confluency, cells were detached using a trypsin-EDTA solution (T/E, Invitrogen) and harvested by centrifugation at 1500 rpm for 5 min. Prior to each experiment, fibroblasts were diluted in culture medium to the density desired in specific experiments, as determined using a Bürker-Türk counting chamber.

HOK cells (passage 3 to 5) were purchased from ScienCell (Carlsbad, CA, USA). HOK cells were grown in Oral Keratinocyte Medium (OKM, ScienCell) supplemented with oral keratinocyte growth supplement at 37°C in 5% CO₂. Medium was changed every two days. The HOK cells were passaged after 80% confluency by adding 3 mL EDTA-Trypsin (2.5 g L⁻¹, Gibco, USA) for 4 min at 37°C to detach the cells. After detachment, 10% FBS in PBS was added for trypsin neutralization and cells were harvested by centrifugation at 1500 rpm for 5 min. Keratinocytes were diluted in culture medium to the density desired in specific experiments, as determined using a Bürker-Türk counting chamber.

2.4. 3D-tissue model

In order to create a 3D-tissue model with the typical structure of a soft-tissue seal, keratinocytes were grown on polyethylene terephthalate membrane filters (0.3 cm²; PET transparent, Greiner Bio-One, Frickenhausen, Germany) to allow close contact with fibroblasts, growing underneath the membrane on a substratum material.

Membrane filters with pore diameters of either 0.4 or 3 μm were used to vary the degree of physiological cell-to-cell contact, characteristic of a 3D-tissue model.

2.4.1. Bacterial challenges to cellular monolayers grown in polystyrene well plates

HOK cells were seeded on the bottom of tissue-culture polystyrene wells at a cell density of 5×10^4 cells per well (24 wells per plate) in 1 ml OKM medium and grown for 72 h. HGF cells were seeded to a density of 1×10^4 cells per well in 1 ml in DMEM medium with 10% FBS, also for 72 h. After 72 h, growth media were refreshed by 900 μl of the appropriate medium and 100 μl medium containing 10^2 , 10^3 , 10^4 and 10^5 *S. oralis* J22 or *S. aureus* ATCC 25923 per ml, added. Medium without bacteria were added as a control. After co-culturing for 24 h, cells were washed with phosphate buffered saline (PBS, 10 mM potassium phosphate, 0.15 M NaCl, pH 7). Then, cells were fixed with 3.7% paraformaldehyde and stained with green-fluorescent, cytoskeleton stain (phalloidin-TRITC) and blue-fluorescent nucleus stain (DAPI) for fluorescence microscopy (see section 2.5 for details). Images were quantitatively analyzed for cell surface coverage of the well surface and the number of cells using Image J.

2.4.2. Bacterial challenges to HOK layers grown on membrane filters in a transwell system

HOK cells in growth medium (100 μl) were seeded in a transwell system with PET membrane filters with pore sizes of 0.4 μm and 3 μm to a density of 5×10^4 per transwell

in 24 well plates for 72 h. The growth medium was refreshed every 24 h. Then, 100 μ l bacterial suspension was added (1×10^3 bacteria per ml) for 24 h of co-culturing, after which HOK cells were quantitatively analyzed as described above and in section 2.5.

In addition, HOK layers were examined using transepithelial electrical resistance (TEER) measurements, using a Millicell-ERS Volt-Ohmmeter (Millipore, MA, USA) to determine the integrity of HOK layers. Also, to count the number of bacteria invading through HOK layers, aliquots of the fluid underneath the membrane filter were taken and the number of colony forming units (CFU) present counted after serial dilution and plating on tryptone soy agar. Enumeration was done after incubation at 37°C for 48 h and data expressed as (log CFUs)/ml.

2.4.3. Bacterial challenges to HGF layers grown on substratum materials in a 3D-tissue model

Next, discs of the different substratum materials were placed in 24 well plates, one material in each well. HGF were seeded from 600 μ l suspensions in growth medium (2×10^4 cells per ml) and grown for 72 h. After 72 h, the growth medium was refreshed and a transwell insert with membrane filter pore sizes of 0.4 μ m or 3 μ m was placed in the wells plate. When required, 100 μ l suspensions of HOK (5×10^5 cells per ml) were seeded on the membrane filters and grown till confluency, after which transwell inserts were placed in wells with substratum materials covered by HGF. The distance between the bottom of the transwell insert and the bottom of the well amounted ≥ 0.1 cm to leave a narrow gap for physiological cell-to-cell contact and bridging by extracellular matrix

and collagen, as produced by fibroblasts [18].

Bacterial challenges were applied by adding 100 μ l of a bacterial suspension (1×10^3 bacteria per ml of medium) to the transwell, placed in a well. Tri-cultures were incubated for 24 h.

At the end of the experiments, HGF and HOK cells were fixed with 3.7% paraformaldehyde, stained with phalloidin-TRITC/DAPI and analyzed as described above and in section 2.5. All experiments were performed in triplicate.

2.5. Immuno-fluorescence staining and fluorescence microscopy of cellular layers

Tissue integration of the membrane filters and the substratum materials by cells was quantitated in terms of the percentage of membrane or substratum surface covered by cells, and the number of cells per unit surface area jointly achieving this coverage. Spreading of individual cells simply follows by dividing the surface coverage by the number of cells, but as it can be easily derived from surface coverage and number of cells, was not separately presented in this article. To visualize cell spreading, cells were fixed with 3.7% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS for 3 min, and stained with phalloidin-TRITC/DAPI for fluorescence microscopy (Leica DM4000, Leica Microsystems Ltd., Wetzlar, Germany). Surface coverage and number of cells per unit area were subsequently derived from the images using image J software.

For visualization of focal adhesions, HGF cells on the TiO₂ surfaces after fixing and permeabilization, were first exposed to 5% BSA in PBS for 30 min to block non-specific binding followed by exposure to anti-vinculin mouse anti-human (clone h-

VIN-1, Sigma, 1:100) primary antibodies for 1 h at room temperature. After washing three times for 10 min with 1% BSA in PBS, cells were incubated with a secondary antibody FITC-labeled goat-anti-mouse IgG (Jackson Immunolab, 1:100) for another 1 h in the dark. Vinculin staining was applied in combination with phalloidin-TRITC/DAPI. For visualization of focal adhesions, images were acquired using a confocal laser scanning microscope (CLSM, Leica SP8 Confocal microscope, Leica Microsystems Ltd, Germany). The number of focal adhesions per cell and the surface area of an individual cell covered by these focal adhesions were quantified through Focal Adhesion Analysis Server [19], and ImageJ software. Note that number of focal adhesions and cell coverage by focal adhesions are related through the size of the focal adhesions.

2.6. Statistical analyses

All data were analyzed and plotted in Graphpad Prism version 7.0. $p < 0.05$ was considered statistically significant after a One-way ANOVA test with Sidak's multiple comparisons test or a pair-wise Student t-test with Bonferroni correction for evaluating the effect of absence or presence of an HOK layer on HGF response in different experiments.

3. Results

In order to determine the influence of streptococcal and staphylococcal challenges on HOK layers, a bi-culture was set up in tissue-culture polystyrene wells. First, a layer

of HOK was grown for 72 h on the bottom of a well, and subsequently exposed to a bacterial suspension for another 24 h (Fig. 2A). In absence of a bacterial challenge and after phalloidin-TRITC/DAPI staining of HOK, CLSM imaging (Fig. 2B) showed blue-fluorescent nuclei and red-fluorescent cytoskeleton staining. Quantitative analysis indicated near full surface coverage of the bottom of a well by HOK (99% surface coverage, see also Fig. 2C), as established by 4.1×10^4 cells cm^{-2} (see Fig. 2D). Challenging of this HOK layer with streptococci or staphylococci had a minor decreasing effect on cell surface coverage (Fig. 2C) and number of adhering HOK (Fig. 2D), but in general these differences were not statistically significant. Fluorescence imaging of human gingival fibroblasts after bacterial challenges (see Fig. S1), demonstrated that streptococci mainly inhibited spreading but did not cause massive detachment of HGF. Staphylococcal challenges on the other hand, maintained a low degree of spreading of HGF up to a bacterial challenge concentration of $10^4 - 10^5$ CFU/ml, above which the majority of adhering HGF detached. Thus, it can be concluded that HOK maintained better integration of the polystyrene surface upon bacterial challenges than HGF.

Fig. 2. Bacterial challenge by streptococci or staphylococci in suspension for 24 h of human oral keratinocytes (HOK) grown for 72 h in tissue-culture polystyrene wells. (A) Bi-culture model with bacteria challenging an HOK layer on the bottom of a tissue-culture grade, polystyrene well. (B) Fluorescence image of phalloidin-TRITC/DAPI stained HOK grown in absence of a bacterial challenge. Scale bar represents 100 μ m. (C) Surface coverage by adhering HOK challenged by different concentrations of bacteria in suspension for 24 h. The dashed line indicates the cell surface coverage in absence of bacteria. Error bars denote SEM over three experiments with separately cultured bacteria and cells. * denotes a significant ($p < 0.05$, Student t-test) difference with respect to absence of bacteria. (D) As in panel C, now for the number of adhering HOK per unit area. (A log-scale of this graph is presented in Fig. S2.)

Also challenging HOK layers formed on membrane filters in a transwell by either streptococci or staphylococci, showed little microscopic damage to the HOK layers (compare Figs. 3A and 3B). However, exposure to bacterial suspensions yielded lower TEER values of the HOK layers indicative of tight junction widening (Fig. 3C and 3D).

TEER values were more strongly reduced on membrane filters with the larger pore size (Fig. 3D), regardless whether challenged with streptococci or staphylococci. Yet, despite the tight junction damage done by the bacterial challenges, the HOK layer remained microscopically intact.

Fig. 3. Bacterial challenge by streptococci or staphylococci in suspension of human oral keratinocytes (HOK) grown for 72 h on membrane filters with different pore size in a transwell.

(A) Fluorescence images of phalloidin-TRITC/DAPI stained HOK layers in absence of a bacterial challenge. Scale bar represents 100 µm and applies to all images.

(B) Fluorescence images of HOK layers challenged by bacteria in suspension (10^3 CFU/ml) for 24 h.

(C) TEER values of the HOK layers grown on membrane filters with 0.4 µm diameter pore size in absence and presence of bacterial challenges. Error bars denote SEM over three experiments with separately cultured bacteria and cells.

(D) As in panel C, now for HOK layers grown on membrane filters with 3 µm diameter

pore size.

* denotes a significant ($p < 0.05$, One-way ANOVA) difference with respect to absence of bacteria.

Next, it was studied whether a layer of HOK could effectively protect a layer of HGF growing on a TiO_2 substratum against bacterial challenges. To this end, a tri-culture was set up in a transwell, comprised of a layer of HOK on the membrane filter and HGF growing on a TiO_2 substratum, placed on the bottom of the well. In absence of bacterial challenges, HGF spread well on the TiO_2 surface regardless of an HOK layer on the membrane filter (see Fig. 4) and pore size (data not separately shown for both pore sizes). Also, coverage of the membrane filter by a layer of HOK had no influence on HGF interaction with the TiO_2 surface (see also Fig. 4). However, in presence of bacterial challenges, both pore sizes significantly impacted HGF interaction with the TiO_2 surface. In absence of an HOK layer, bacterial challenges above the membrane filter negatively impacted substratum coverage by HGF (Fig. 4A) and their cell number (Fig. 4B). Larger pore size yielded a stronger negative impact on HGF interaction with TiO_2 surfaces, while the impact of *S. aureus* was more detrimental than of commensal *S. oralis*. Detrimental effects of bacterial challenges were significantly weakened when the membrane filter was covered by a layer of HOK, especially for membranes with 3 μm pores. However, also in presence of a protective HOK layer, detrimental effects of *S. aureus* were generally greater than of *S. oralis*. Clearly, 0.4 μm pores will only allow passage of secreted bacterial toxins towards the HGF layers, as streptococci or staphylococci are physically unable to pass 0.4 μm , but do pass through 3 μm diameter pores (Fig. S3). Importantly, a layer of HGF on the membrane filter, did

not offer protection to the HGF growing on the TiO₂ surface against bacterial challenges (Fig. S4).

Fig. 4. Bacterial challenges by streptococci or staphylococci in suspension (24 h at 10³ CFU/ml) of HGF grown for 72 h on a TiO₂ surface in absence and presence of an HOK layer on the membrane filter in a 3D-tissue infection model.

(A) Surface coverage of a TiO₂ surface by HGF in absence and presence of an HOK layer on membranes with different pore size challenged by *S. oralis* J22 or *S. aureus* ATCC 25923 in a 3D-tissue model. Error bars denote SEM over three experiments with separately cultured bacteria and cells.

(B) As in panel A, now for the number of HGF cells per unit area. (A log-scale of this graph is presented in Fig. S5.)

*indicates significant ($p < 0.05$, One-way ANOVA) differences between effects of bacterial challenges in absence and presence of a HOK layer (per strain) and #indicates significant ($p < 0.05$, One-way ANOVA) difference between the absence or presence of a bacterial challenge.

To further investigate the effect of bacterial challenges and the presence of an HOK layer on integration of TiO₂ surfaces by HGF, the formation of HGF focal adhesions was analyzed, using additional vinculin staining. In absence of a bacterial challenge,

adhering HGF demonstrated well-defined dash-like vinculin spots typical for mature focal adhesions [20], regardless of pore size or the absence (Fig. 5A) or presence (Fig. 5B) of an HOK layer on the membrane filter. Absence or presence of an HOK layer on the membrane filter yielded no significant difference in the focal adhesion area (Fig. 5C) or number (Fig. 5D) per cell. In absence of an HOK layer, streptococcal challenges of HGF on the TiO₂ substratum surface did not affect focal adhesion formation in case of 0.4 µm membrane filters, but in case of 3 µm membrane filters focal adhesion formation decreased significantly ($p < 0.05$, One-way ANOVA). Staphylococcal challenges decreased focal adhesion area per cell in case of both 0.4 µm and 3 µm membrane filters. Upon growing an HOK layer on the membrane filter, bacterial challenges yielded smaller decreases in focal adhesion formation (Figs. 5C and 5D).

Fig. 5. Focal adhesion (FA) expression by human gingival fibroblasts (HGF) adhering to TiO₂ surfaces and grown for 72 h, after exposure to streptococci or staphylococci in suspensions (24 h at 10³ CFU/ml) in a transwell.

(A) Confocal images of phalloidin-TRITC/DAPI/vinculin-stained HGF in absence of a bacterial challenge and without an HOK layer. Scale bar represents 30 μm and applies to all images.

(B) As in panel A, now for a membrane covered with an HOK layer.

(C) Focal adhesion area per cell of HGF growing on TiO₂ surfaces in absence and presence of an HOK layer on membranes with different pore size challenged by *S. oralis* J22 or *S. aureus* ATCC 25923 in a 3D-tissue model. Error bars denote SEM over three experiments with separately cultured bacteria and cells.

(D) As in panel C, now for the number of focal adhesions per HGF cell.

*indicates significant ($p < 0.05$, One-way ANOVA) differences in absence and presence of an HOK layer and #indicates significant ($p < 0.05$, One-way ANOVA) difference of the absence or presence of a bacterial challenge in presence of an HOK layer.

Finally, it was established whether the 3D-tissue infection model of the soft-tissue seal was sensitive to the surface-chemistry of implant material it formed upon, when

bacterially challenged. To this end, HGF layers were grown on TiO₂, hydroxyapatite and silicone rubber surfaces. All three substratum surfaces had a similar surface roughness in the sub-micron range, but differed greatly in hydrophobicity (see Table S2 for surface roughnesses and water contact angles). Elemental surface compositions obtained using XPS are given in Table S1 for the three materials used. In absence of a bacterial challenge, integration by HGF of the different materials (Fig. 6) was lowest on silicone rubber (Fig. 6A), the most hydrophobic material. Considering the minor differences in cell numbers (Fig. 6, right panels), this indicates that individual HGF round up more on silicone rubber than on TiO₂ and HAP. Streptococcal and staphylococcal challenges in presence of an HOK layer on 0.4 µm membrane filters have little influence on HGF integration of the different implant materials (compare Figs. 4A and 4B and see Fig. 6). HGF integration of the implant materials was most negatively affected by bacterial challenges on silicone rubber. Here too, *S. aureus* had a more detrimental effect than commensal *S. oralis*.

Fig. 6. Bacterial challenge by streptococci or staphylococci in suspension (24 h at 10^3 CFU/ml) of human gingival fibroblasts (HGF) adhering to different implant materials and grown for 72 h, in absence and presence of an HOK layer on the membrane in a 3D-tissue infection model.

(A) Surface coverage and the cell number on silicone rubber by HGF in absence and presence of an HOK layer on membranes with different pore size challenged by *S. oralis* J22 or *S. aureus* ATCC 25923 in a 3D-tissue model. Error bars denote SEM over three experiments with separately cultured bacteria and cells.

(B) As in panel A, now on HAP.

(C) As in panel A, now on TiO₂.

(For a log-scale of the graphs containing cell numbers, see Fig. S6.)

indicates significant ($p < 0.05$, One-way ANOVA) differences from corresponding data on silicone rubber.

4. Discussion

The 3D-structure of the soft-tissue seal, protecting the osseointegrated part of a dental implant is crucial to prevent bacterial invasion and peri-implantitis. Studies into new implant materials are often done in mono-culture, examining bacterial adhesion or tissue interactions separately and neglecting the 3D-structure of the tissue seal [13] in which physiological cell-to-cell contact between keratinocytes and fibroblasts may play a crucial role. Also, large differences exist between the human oral microflora and animal ones, invalidating extrapolation of *in vivo* results from animal experiments to the human clinical situation [14]. Human clinical trials are frequently under-powered and hard to obtain permission for in absence of convincing demonstration of potential benefits [21]. We here describe the development of a 3D-tissue infection model of the soft-tissue seal around a dental implant that can be used to evaluate new, infection-resistant implant materials and to study the behavior of the soft-tissue seal during bacterial invasion. To our knowledge, this is the first study to build a 3D-tissue infection model mimicking the 3D-structure of soft-tissue seal in the oral cavity and therewith the physiological interplay between bacteria, keratinocytes, fibroblasts and the implant material, while being sensitive to the surface-chemistry of the substratum material.

The soft-tissue seal consists of HOK and HGF that are brought in close contact in our transwell-based 3D-tissue model. HOK contact HGF through a porous membrane filter and HGF directly integrate the implant material, while also allowing to bridge an initial narrow gap between both cell types through production of extracellular matrix and collagen. Both cell types serve different physiological functions in the human body. HOK constitute the outer, keratinocytized epithelial layer of the gingiva as the first line

of defence against bacterial invasion [22]. HOK use beta-defensin-3 [23,24] and secretion of antimicrobial peptides [25] against bacterial attacks, explaining why neither surface coverage (Fig. 2C) nor (Fig. 2D) numbers of adhering HOK were affected by bacterial challenges. Evidently, HGF lack this defence against bacterial attacks (Fig. S1).

In the development of our 3D-tissue infection model, HOK and HGF were physiologically contacted through 0.4 μm and 3 μm diameter porous membranes. An HOK layer on 0.4 μm pores provided protection of the HGF against bacterial attack (Fig. 4) by secretion of antimicrobial peptides that may easily pass the membrane. However, an 0.4 μm diameter pore size only allows limited physiological contact between HOK and does not allow bacterial passage (Fig. S3). Thus with the use of an 0.4 μm diameter pore size the physical barrier function of an HOK layer cannot be studied. Therefore, we considered 0.4 μm pore membranes unsuitable for a good 3D-tissue infection model and in the forthcoming parts of the discussion we will focus solely on data pertaining to 3 μm pore size membranes. The extended physiological contact between HOK and HGF provided through 3 μm pores, allowed to distinguish an apical side, to which bacteria adhere and basolateral side, involved in cell-to-cell contact. Bacterial adhesion did not microscopically affect the integrity of HOK layers, but in line with literature [26], TEER (Fig. 3) indicated widening of the junctions between HOK through which bacteria could invade down to attack the HGF layer.

Several types of cell layers are known to survive bacterial attack through the production of adhesions, in order to more firmly attach themselves to a substratum

surface. *Shigella* virulence factor OspE can bind with integrin linked kinase from host cells to stabilize focal adhesion sites [27,28] and inhibited infected host cells from detaching from the basal membrane [27]. Also, LPS has been shown to stimulate focal adhesion formation in enterocytes via TLR4 and a similar reaction was also found when *Streptococcus mutans* colonizes oral soft-tissue [29]. In our 3D-tissue infection model, we do neither observe increased tissue integration by HGF upon bacterial challenges (Fig. 4) nor increased focal adhesion formation (Fig. 5). This suggests that in the 3D-tissue infection model, other factors than only bacterial challenges may stimulate focal adhesion formation. Keratinocytes for instance, can also stimulate fibroblasts to synthesize growth factors [30] and excrete wound healing promoting factors such as TGF- β 1 [31], that may increase the expression of focal adhesions in HGF [32,33], as shown for TiO₂ surfaces in our 3D-tissue model (Figs. 7A and 7B). Also, keratinocytes secrete antimicrobial peptides [25]. Therewith in a soft-tissue seal, keratinocytes do not merely serve as a physical barrier against bacterial invasion towards HGF, but also enhance their adhesion and spreading on the underlying implant material.

Fig. 7. HGF surface coverage of TiO_2 surfaces as a function of focal adhesion (FA) area (A) and FA number per cell (B) with and without bacterial challenges by streptococci or staphylococci in absence and presence of an HOK layer in a 3D-tissue infection model (data taken from experiments using membrane filters with 3 μm pore diameters). Arrows indicate an increase in surface coverage in presence of an HOK layer compared with absence of an HOK layer.

In conclusion, through our 3D-tissue infection model, important physiological interactions between keratinocytes, gingival fibroblast, bacteria and materials surfaces can be accounted for in the *in vitro* evaluation of new implant materials that are not revealed in mono-culture models. A commensal streptococcal strain was demonstrated to be less detrimental to tissue integration than *S. aureus* strain, known to be highly pathogenic through the secretion of a plethora of toxins [17][34]. The model is not only sensitive to bacterial virulence, but also to different surface-chemistries of the substratum material, which makes it suitable for screening of new implant materials in their race for the surface between tissue cells and invading bacteria. Therewith an alternative has become available for animal experiments and large scale clinical trials.

As a possible extension our 3D-tissue infection model, macrophages could be inserted in the bottom compartment of the transwell (the “tissue-side”) to account for HGF modulation of macrophage responses[35].

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