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Gingival Keratinocyte Adhesion on Atomic Layer-Deposited Hydroxyapatite Coated Titanium

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Abstract

This study aimed to evaluate the effects of the atomic layer deposited hydroxyapatite (ALD-HA) coating of the titanium (Ti) surface on human gingival keratinocyte (HGK) cell adhesion, spreading, viability, and hemidesmosome (HD) formation. Grade 2 square-shaped Ti substrates were used (n=62). Half of the substrates were ALD-HA coated, while the other half were used as non-coated controls (NC). The ALD-HA surface was characterized with scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) analysis. The initial cell adhesion and HD formation of HGKs were evaluated after a 24-hour cultivation period. The cell proliferation was assessed by cultivating cells for 1, 3, and 7 days. The expression levels of the integrin mediating cell adhesion were detected with the Western Blot method. In addition, cell spreading and expression of the proteins mediating cell adhesion were imaged using a confocal microscope. SEM-EDS analysis demonstrated the formation of HA on the ALD-HA surfaces. The relative cell attachment was significantly higher ($p< 0.05$) on the ALD-HA compared to the NC surface after 1 and 3 d of cell culture. No significant difference was found in integrin $\alpha 6$ or $\beta 4$ expression. The microscope evaluation showed significantly increased cell spreading with peripheral HD expression on ALD-HA compared to the NC surfaces ($p= 0.0001$). Moreover, laminin $\gamma 2$ expression was significantly higher on the ALD-HA than on the NC surfaces ($p< 0.001$). Compared to the NC Ti surface, the ALD-HA coating has favorable effects on HGK proliferation, growth, and cell spreading. This indicates that the ALD-HA coating has good potential for improving mucosal attachment on implant surfaces.

Keywords: cell adhesion, epithelial cells, gingival tissue, hydroxyapatite, dental implant, noncrystalline surface, titanium

1. Introduction

Various titanium (Ti) implant surface modifications have been implemented to improve its surface characteristics, such as topography, chemical composition, and roughness, for better biological response at the implant-tissue interface.[1–3] Among these surface modifications, hydroxyapatite (HA; $Ca_{10}(PO_4)_6(OH)_2$) implant coating has demonstrated its ability to enhance direct bone growth and increase bone-to-implant contact compared to uncoated Ti.[4,5] Additionally, calcium-phosphate (Ca-P) coating has been shown to positively influence the attachment, cell spreading, and focal adhesion of human gingival fibroblasts (HGFs).[6] Furthermore, Zhao et al. illustrated in an in vivo study that implants coated with Ca-P exhibit promising chemical alterations at the implant-peri-implant tissue interface.[7] Their study involved implanting Ca-P-coated implants in dogs, revealing that the coating simulates better orientation and modulation of soft tissue in the gingival connection area compared to uncoated implants.

Several coating methods and techniques have been used to form calcium phosphate layers on Ti surfaces, such as the sol-gel, plasma spraying, and sputter coating. However, several problems are associated with these methods, such as flaking, brittleness, and delamination.[4,5] Atomic layer deposition (ALD) is a method that can overcome these issues and produce nanoscale coatings with controlled thickness on complex three-dimensional surfaces.[6] Nanocrystalline HA film 300 - 380 nm in thickness, deposited by ALD (ALD-HA), demonstrated a good adhesion strength (6.71 Mpa) to the Ti surface.[7] Therefore, this nano-coating can potentially reduce the risks associated with the HA layer produced by conventional methods. Previous studies have shown that ALD-HA on Ti surfaces can promote osteoblast cell adhesion and viability and support blood coagulation.[8,9] However, when human peripheral blood monocytes were cultured on ALD-HA, they could not differentiate into bone-resorbing osteoclasts but displayed a foreign body reaction, which resulted in their incapacity to resorb the coating.[10]

The soft tissue seal around the implant abutment plays a vital role in the prognosis of dental implant treatments. The gingival fibers in connective tissue cannot adhere to the Ti implant surface but run parallel and form a capsule-like structure. This structure makes it easier for bacteria to dive into peri-implant tissues and be exposed to peri-implant infections.[11,12] As peri-implantitis is challenging to treat, prevention is essential. The prevention is possible, for example, by enhancing the gingival tissue attachment to the implant surface with implant surface modifications.

The epithelial attachment forms the first barrier against oral microbes and mechanical forces around oral implants. The epithelial cells in the junctional epithelium, also called human gingival keratinocytes (HGKs), can attach to the implant surface via HDs and basal lamina as they do to natural teeth.[13–17] However, the HGK attachment is weaker around implants compared to natural teeth.[18,19] HDs are molecular

structures that consist of basement membrane proteins, cell membrane penetrating proteins called integrins, and cytosolic proteins. The internal basal lamina is rich in laminins, of which laminin 332 plays the most critical role in epithelial attachment. Integrins are a family of transmembrane proteins composed of α and β subunits. They function as adhesion receptors for extracellular matrix (ECM) proteins, including laminin, collagen, and fibronectin.[20] Binding to integrins facilitates processes such as gene expression, cell adhesion, spreading, migration, and alterations in cell shape.[21] Integrin $\alpha 6\beta 4$ is an important factor concerning hemidesmosomal adhesion in gingival keratinocytes as it binds to laminin 332.[22,23] The expression of these adhesion molecules has been shown to correlate with enhanced HGK adhesion and spreading in earlier studies.[24]

The risk of infections is significantly increased in all medical devices that penetrate the skin or mucosa, such as oral implants. Proper adhesion between the implant and surrounding tissue at the exit site can form an effective seal and thus control microbial penetration into the peri-implant area. This would significantly maintain the health of the peri-implant tissue and improve the success rate of oral implants. Previous studies by the authors[8-10] have demonstrated that the ALD-HA-coated surfaces promote blood coagulation and platelet adhesion, which may enhance early events of wound healing around implant and abutment surfaces. Moreover, The incapacity of human peripheral blood monocytes to resorb the ALD-HA coating prompted us to explore the potential of utilizing it for coating the trans-mucosal part of implants to create a soft tissue seal around the implant. Therefore, further investigations are needed to evaluate the compatibility of these surfaces with gingival cells and their interaction with adhesion proteins. This study aimed to evaluate the potential of a nanostructured ALD-HA-coated surface to enhance wound healing and enable bioadhesion between peri-implant tissues and implant surfaces. In this study, we wanted to study if the ALD-HA coating of Ti would affect the cell adhesion, spreading, growth, and HD formation of HGK. The study was carried out by measuring the relative cell attachment, cell spreading, and adhesion protein expression using confocal microscopy and Western blotting. The hypothesis was that the ALD-HA coating would enhance initial epithelial cell adhesion and proliferation, promote cell spreading, and increase the expression of adhesion molecules.

2. Materials and Methods

2.1 Preparation of Nanocrystalline hydroxyapatite coating on Ti discs

The process for preparing ALD-HA coatings has been explained in detail elsewhere.[25,24] In summary, a 1 mm thick Ti sheet (Grade 2, ASTM B265 specification, William Gregor Ltd., London, UK) was used as a substrate for the coating. The ALD-HA coating was made by first depositing a $CaCO_3$ thin film using $Ca(thd)_2$ and O_3 as precursors in an F-120 ALD reactor (ASM Microchemistry Ltd., Helsinki, Finland).

The Ca(thd)2 (Volatec Oy, Porvoo, Finland) was vaporized at 188 ◦C, and O_3 was generated from O_2 (99.9999%) using a Wedeco Ozomatic Modular 4 HC Lab ozone generator. Depositions were conducted at 250 ◦C with 2000 ALD cycles., The $CaCO_3$ film was then transformed into HA in a 0.2 M $(NH_4)_2HPO_4$ (Merck, 99%) solution at 95 ◦C. The HA coating had a thickness of about 300 nm. Finally, the samples were rinsed with deionized water, dried using compressed air, and cut into 0.7 x 0.7 cm discs using a manual plate cutter (Bernardo PTS 1050 S Manual disc cutter, Linz, Austria).

2.2 Scanning Electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) analysis.

SEM images and EDS analysis of the ALD-HA coated and uncoated Ti surfaces were performed using scanning electron microscopy (LEO Gemini 1530, Carl Zeiss, Germany) and an X-ray detector (Thermo Scientific, Wisconsin, USA). The other surface characterization of the ALD-HA coating, including surface roughness, contact angle (CA), and surface free energy (SFE), was performed in our previous study [25].

2.3 Cell Cultures

Before the cell cultures, the discs were sterilized in an autoclave. In all experiments of this study, spontaneously immortalized human gingival keratinocytes (HGKs) with a passage of 24 were cultured on Ti discs at a density of 25.000 cells/cm^2. The cells were collected from a human gingival biopsy sample and mixed in a keratinocyte-serum-free medium (SFM, Gibco®, Thermo Fisher, USA). For relative cell attachment, 12 samples were analyzed per group (n=12/group) using four technical replicates per group, and the experiment was conducted three times. The HGKs were incubated on discs for 1, 3, and 7 days. After each time point, the discs were covered with Alamar Blue reagent (Thermo Fischer, USA) diluted in SFM (1:10) for 3 h at 37 °C. After incubation, the absorbance of each solution (200 µl on the 96-well plate) was measured with wavelengths of 569 and 594 nm (Multiskan FC, Thermo Scientific). The relative cell attachment was calculated by comparing the values of the test group to the values of the control group. HD formation of HGKs was studied with a confocal microscope; cells were cultured on Ti discs (n=4/group) for 24 h, washed once with PBS, and fixed in 4 % paraformaldehyde (15 min). The fixative was washed away with PBS, and the discs were stored at +4 °C.

2.4 Adhesion protein analyses

For Western blotting, the HGKs were cultured on discs for 3 days, and each group consisted of 12 samples, representing three biological replicates. The discs were washed with PBS and lysed with prewarmed (95 °C) TXLB-buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% Triton-X, 0.5% glycerol, 1% SDS, Complete protease inhibitor (Sigma- Aldrich) and phos-stop tablet (Sigma-Aldrich)]. The solution was collected into Eppendorf tubes, reheated at 95 °C (10 min), and stored at -20 °C. Protein contents were measured with Protein Assay Reagent (Bio-Rad) following the

manufacturer's instructions using a wavelength of 595 nm (EnSight, PerkinElmer, Kaleido 3.0 software). Equal amounts of protein solution were mixed in 6x sample buffer and pipetted on Mini Protean TGX Precast SDS-PAGE Gels (Bio-Rad). The protein bands were transferred to gels (Trans-Blot Turbo Transfer System, Bio-Rad), washed twice with Milli-Q and once with TBST (Tris-buffered saline with tween) before treating the gels in diluted milk in TBST (5 %, 1 h) in a shaker (Orbital Shaker SSL1, Stuart). The gels were stained with primary antibodies [integrin α6 (1:500, HPA012696) integrin β4 (1:100, ab182120, Abcam), GAPDH (1:20000, 5G4MaB6C5, Hytest)] diluted in milk overnight (+4 °C). The next day, the gels were washed three times with TBST and treated with secondary antibodies [IRDye 680 RD Donkey Anti-Rabbit (926-68073), LI-COR Biosciences), IRDye 680 RD Donkey Anti-Mouse (926-68072)] for 1 h at room temperature and washed three times with TBST. The stained gels were imaged with Li-Cor, Infrared Imager, Odyssey. Three biological replicates were used in Western blotting.

2.5 Immunofluorescence stainings for confocal microscopy

After fixation, the discs were treated with Triton-X-100 in PBS (0.5 %, 300 µl, 15 min) followed by overnight incubation at horse serum (30 %) mixed with primary antibodies [laminin γ2 (1:100, sc-7652, Santa-Cruz Biotechnology), integrin β4 (1:200, ab110167, Abcam)]. The next day, the discs were washed three times in PBS. Secondary antibodies (Anti-Rabbit 488, Anti-Goat 555, both from ThermoFisher Scientific), DAPI (nucleus staining, 1:200), and Phalloidin Atto (1:400, Sigma-Aldrich)] were mixed with horse-serum in PBS (30%) and pipetted on discs for 1 h. Again, the discs were washed with PBS and glued to microscope glass (Mowiol, Sigma-Aldrich). A high-resolution spinning disc confocal microscope (63x Zeiss Plan-Apochromat, 3i CSU-W1 Spinning Disk) was used in cell imaging. An area of 30 cells per group was measured to represent cell spreading. Integrin β4 and laminin γ2 signals were analyzed from each staining and normalized to the cell number. The ImageJ Fiji program measured cell areas and adhesion protein signals from the cell bottom layer. For cell spreading, the area of 30 cells per group was measured from each image to evaluate spreading, and the experiment was performed in three independent trials.

2.6 Statistical analyses

Western blot and confocal microscope images were analyzed using the ImageJ Fiji program. The graphs and analyses were accomplished with the GraphPad Prism program. An unpaired T-test was used in cell spreading and western blot analyses. Mann-Whitney test was used to analyze the results from cell proliferation assay and adhesion protein signals.

3. Results

3.1 Scanning Electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) analysis.

SEM images demonstrate that the ALD-HA coating is continuous as the HA crystals cover the substrate surface, as demonstrated by the EDS analysis (Fig. 1). Table 1, taken from a previous publication,[8] shows the surface roughness, contact angle, and free energy.

3.2 Cell proliferation

Relative cell attachment was measured to evaluate whether the ALD-HA surface would enhance cell proliferation on a Ti surface. The relative cell attachment was significantly higher ($p < 0.05$) on the ALD-HA surface compared to NC Ti after 1 and 3 days of cell culture (Fig. 2).

3.3 Western blotting

Western blotting was accomplished to analyze whether improved relative attachment would signify higher adhesion protein expression. After 3 d of cell culture, no significant difference was found in integrin $\alpha 6$ or $\beta 4$ expression between ALD-HA-coated and the NC Ti surfaces (Fig. 3).

3.4 Cell spreading

High-resolution confocal microscope imaging and analyses were performed to determine if increased cell viability would indicate improved cell adhesion complex formation and cell spreading on the ALD-HA coating. Cell spreading (cell area) was significantly increased on ALD-HA surfaces compared to the NC (Fig. 4). Well-spread cells with peripheral HD protein expression were found on confocal images. The signal of laminin $\gamma 2$ on the cell bottom layer was significantly higher on ALD-HA-coated surfaces compared to the NC surfaces (Fig. 5).

4. Discussion

Atomic layer deposition is a method that has been used to produce nanoscale HA coatings with controlled thickness on complex three-dimensional Ti surfaces.[6,25] This study aimed to evaluate the effects of ALD-HA coating on HGK cell adhesion, spreading, growth, and HD formation on the Ti surface. The results of this study demonstrated improved cell viability during the first 3 days on ALD-HA, as indicated by significantly higher proliferation levels of HGKs compared to NC Ti. However, the cell count became relatively similar in both investigated groups after one week.

Our previous work has shown that ALD-HA coatings on Ti surfaces have the potential to enhance the adhesion and viability of osteoblast cells, as well as support blood coagulation and platelet response.[8,9]

Various methods have been employed to deposit CaP layers on Ti surfaces, including laser deposition, sputter coating, electrophoretic deposition, sol-gel deposition, and plasma spraying. Among these, plasma spraying has been the most extensively studied and can produce coatings on Ti with thicknesses ranging from 30 to 200 μm.[26] Different issues, such as flaking, brittleness, and delamination, have been reported[4] due to the low cohesive strength and high processing temperatures, which can cause inhomogeneities in coating chemical composition.[27] ALD presents a promising alternative capable of addressing these drawbacks by enabling the production of nanoscale coatings with precise thickness control, even on complex three-dimensional surfaces.[6] We previously demonstrated that human bone marrow-derived cells could be cultured on ALD HA-coated Ti surfaces as they fuse into multinuclear cells on the coating, similar to their behavior on natural bone slices[25], and support the adhesion and proliferation of osteoblast cells.[9]
There are, however, few studies have investigated the cellular interactions on surfaces created using the ALD technique. Among these, recent research utilized ALD to fabricate TiO_2 films, showing osteoblast adhesion, proliferation, and differentiation improvements while impeding fibroblasts' activities.[28] Another study revealed that applying an additional ALD TiO_2 coating to Ti and Ti nanotube surfaces enhances fibroblasts and osteoblast growth.[29] A study by Liang et al. [30] also demonstrated enhanced HA formation in simulated body fluid on ALD alumina (Al_2O_3) and TiO_2 coatings and observed fibroblast attachment to these coated surfaces. Furthermore, Radtke et al.[31] illustrated fibroblasts' adherence to Ti6Al4V samples coated with ALD-HA and showed higher cell proliferation levels than NC surfaces. However, this study is the first to fully characterize HGK cell attachment on HA coatings fabricated with the ALD method. HGKs are vital components of the oral epithelium and play a significant role in maintaining the health and functionality of the gingiva. They serve as a protective barrier against injury and microbial invasion, essential in preventing peri-implant diseases while supporting wound healing and tissue regeneration.[32] They regulate immune responses and maintain the integrity of peri-implant tissue.[33] Their key role in preventing and managing peri-implant diseases highlights their importance in clinical and research contexts.

Improved cell attachment on the Ti surface is considered highly significant, as it facilitates more consistent cell adhesion, creating a more substantial barrier against oral microbes. Subsequently, this could decrease the risk of peri-implant infections, enhance soft tissue attachment, and maintain the integrity of the alveolar ridge. Implant abutment surfaces interact with soft tissue primarily through their surface wettability, roughness, and topography.[34] These factors are crucial in determining initial cell responses at the interface between cells and materials, ultimately promoting a connection with surrounding tissues.[35] Research has shown that nanostructured abutment-coated surfaces, exhibiting favorable surface wettability, may enhance epithelial and fibroblast cell reactions compared to machined surfaces. This can potentially impact the rate and quality of new tissue formation.[36,37]

The ALD-HA coating improved the surface wettability and changed the surface topography by reducing the surface roughness and producing a nanocrystalline structure on the Ti surface, which could enhance the epithelial cell attachment and growth on Ti surfaces. In addition, cell spreading was wider on ALD-HA-coated surfaces, indicating well-elongated cells with better adhesion properties on coated surfaces. Western blot analyses, however, showed no significant differences in the expression of integrin α6 or β4. Nevertheless, in confocal microscope analyses, the signal of laminin γ2 was significantly higher on ALD-HA-coated Ti compared to the NC surfaces. The induced expression of laminin γ2 indicates enhanced cell adhesion, as laminin γ2 is one of the most essential adhesion markers in HGKs.[23] In comparison, Riivari et al.[24] showed that TiO_2-coating on Ti produced by sol-gel enhances cell adhesion, adhesion molecule expression, and cell spreading of integrin α6 and β4, while the coating did not significantly affect the expression of laminin y2. Previous studies have also indicated that altering the surface of Ti implants at the nanoscale level can change surface chemistry and topography.[38,39] These modifications influence interactions between the implant surface, ions, proteins, and cells. Such interactions can potentially improve molecular and cellular activities and encourage tissue healing at the Ti and tissue interface.[40,41] The favorable cell attachment might also be explained by Ca and P elements on the ALD-HA-coated surfaces. The Ca and P have been demonstrated to improve biocompatibility and promote protein adsorption, which, in turn, facilitates better soft tissue integration.[42] This property, together with its nanotopography and hydrophilicity, is the apparent reason for the favorable cell response of the ALD-HA-coated surface.

Avila et al.[43] have characterized the coating used in this study and demonstrated that the coating method allows for creating a highly conformal and uniform nanolayer that can be effectively applied to complex three-dimensional structures with precision. The thickness of the nanolayer coating can be controlled by the number of ALD cycles applied. Additionally, the coating has demonstrated good adhesion strength to the coated Ti substrate. However, some aspects that might affect the stability of the coating at the soft tissue-implant interface deserve further investigation. Therefore, studies evaluating the long-term stability of the coating in a physiologic environment are required, as it may undergo resorption over a relatively short period. Despite this potential limitation, a sufficiently proper interface could form between the Ti and adjacent peri-implant tissues during the resorption period.

The results of this study support the hypothesis that the ALD-HA coating can promote epithelial cell proliferation, spreading, and the expression of adhesion molecules on the Ti surfaces. These findings are significant as they could pave the way for developing more effective implant surfaces that promote better soft tissue integration. However, further in vivo studies are needed to understand and prove the clinical benefits of this coating.

5. Conclusion

In conclusion, our in vitro study demonstrates that the nanoscale crystalline hydroxyapatite coating obtained through the atomic layer deposition method has favorable effects on epithelial cell proliferation, viability, and spreading compared to the non-coated titanium surfaces. This suggests a potential enhancement in attaching soft tissue to the implant or abutment surface, thereby improving the success rate of dental implant treatment.

Author contributions

Faleh Abushahba: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Sini Riivari:** Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Nagat Areid:** Investigation, Methodology, Visualization, Writing – original draft. **Elisa Närvä:** Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – review and editing. **Elina Kylmäoja:** Writing – review and editing. **Mikko Ritala:** Conceptualization, Methodology, Writing – review and editing. **Juha Tuukkanen:** Conceptualization, Methodology, Writing – review and editing. **Pekka K. Vallittu:** Resources, Writing – review and editing. **Timo O. Närhi:** Conceptualization, Methodology, Supervision, Writing – review and editing.

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Figure legends:

Figure 1. SEM images for the non-coated (A) and the ALD-HA coated (B) surfaces at 2.5 Kx magnification. Region of interest (ROI; red boxes) shows the same area at 25 Kx magnification. The lower panel shows the EDS analysis of the ALD-HA surface.

Figure 2. Relative attachment of HGKs on non-coated (NC) and ALD-HA coated (HA) Ti after A.) 1, B.) 3, and C.) 7 d of cell culture based on Alamar Blue reagent. Mean ± standard deviation (SD) + individual values, Mann-Whitney test.

Figure 3. Expression of focal adhesion proteins. A.) Results from western blotting and B.) Quantification of of integrin α6 and β4 signal levels were balanced to GAPDH on HA-coated and non-coated surfaces after 3 days of cell culture. Mean ± standard deviation (SD) + individual values, T-test.

Figure 4. A.) Representative images of the cell area on non-coated and ALD-HA coated (HA) Ti.B.) Quantification of the cell area measurements after 24 h of cell culture. Mean ± standard deviation (SD), unpaired T-test An area of 30 cells per group was measured to represent cell spreading (biological replicates n = 3).

Figure 5. A.) Representative confocal microscope images on non-coated Ti and ALD-HA-coated Ti imaged with 63x Zeiss Plan-Apochromat, 3i CSU-W1 Spinning Disk. B.) Analysis of signal intensities of laminin γ2 and integrin β4 on the cell bottom. Mean ± standard deviation (SD), Mann-Whitney test.

Table Heading:

Table 1. Mean and standard deviation of surface roughness (Ra; arithmetical mean deviation, μm), water contact angle (°), and surface free energy (TOT; total, D; dispersive, and P; polar) determination by Owens and Wendt (OW) approach on non-coated and ALD-HA-coated surfaces. * $p<0.001$. Data were extracted from a previous publication [25].