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Biointerphases 7, 27 (2012)

<https://doi.org/10.1007/s13758-012-0027-9>



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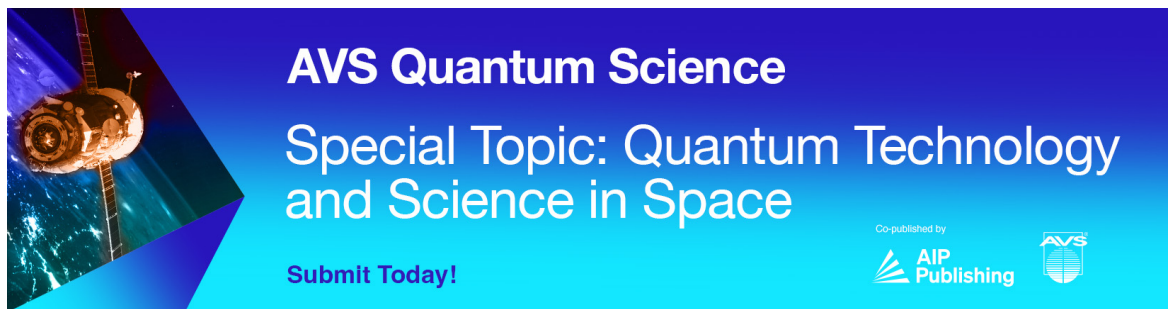
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Received: 7 December 2011 / Accepted: 5 March 2012 / Published online: 30 March 2012
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Abstract In the present study, we investigated the hydrothermal treatment of titanium with divalent cation solutions and its effect in promoting the adhesion of gingival epithelial cells and fibroblasts *in vitro*. Gingival keratinocyte-like Sa3 cells or fibroblastic NIH3T3 cells were cultured for 1 h on experimental titanium plates hydrothermally-treated with CaCl₂ (Ca) or MgCl₂ (Mg) solution, or distilled water (DW). The number and adhesive strengths of attached cells on the substrata were then analyzed. The number of Sa3 cells adhering to the Ca- and Mg-treated plates was significantly larger than in the DW group, but the strength of this adhesion did not differ significantly between groups. In contrast, NIH3T3 cell adhesion number and strength were increased in both the Ca and Mg groups compared to the DW group. Fluorescent microscopic observation indicated that, in all groups, Sa3 had identical expression levels of integrin β 4 and development of actin filaments, whereas NIH3T3 cells in the Ca and Mg groups displayed much stronger punctate cytoplasmic signals for vinculin and more bundle-shaped actin filaments than cells in the DW group. As a result, it was indicated that the hydrothermal treatment of titanium with Ca or Mg solution improved the integration of soft tissue cells with the substrata, which may facilitate the development of a soft tissue barrier around the implant.

1 Introduction

Dental implants are installed into alveolar bone and penetrate into the oral cavity, thus interfacing with at least three types of cells (epithelial cells in the peri-implant epithelium, fibroblasts in the connective tissue, and osteoblasts in the bone) [1]. In the oral cavity, it also establishes an interface with dental plaque, which can contribute to peri-implant inflammation [2]. Improvement in the adhesion of soft tissue cells to the implant surface is a key modality in preventing peri-implant soft tissue breakdown [3].

A laminin-5-containing basement membrane (internal basal lamina) intervenes between the tooth or implant and epithelial cells [4]. Cells are connected to this basement membrane by hemidesmosomes (HDs), which are multi-protein complexes that mediate stable anchoring by providing a tight link between the intracellular intermediate filament network system and the extracellular matrix of the underlying basement membrane [5–7]. This connective tissue link also acts as a key defensive element against peri-implant tissue breakdown [8], and the secure adhesion of fibroblasts to the substratum surface may play a key role in this protective property.

In some rodent oral implant studies, epithelial attachment structures such as HDs or basement membrane are of inferior quality and quantity [4, 9] to that observed around a natural tooth. It has been reported that peri-implant connective tissue attachments consisting of fibroblasts and collagen are similar to scar tissue, and have less cells than healthy periodontium [10]. These reports suggest that the enhancement of peri-implant soft tissue adhesion is an important stage in promoting implant acceptance into the oral cavity.

Despite some reports regarding the relationship between surface-modified substrata and epithelial cell or fibroblast adhesion, most describe topographical surface modification

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procedures and conclude that roughened substrata generate inferior soft tissue cell attachment [11–13]. Regarding chemical modification of titanium for the improvement of epithelial cell or fibroblast adhesion, coating of a micro-structured titanium surface with a laminin-derived peptide was reported to be effective for epithelial cell attachment [3]. In addition, a laser micro-grooved surface was effective for connective tissue attachment, and this attachment effectively resisted peri-implant bone resorption [8]. In the present study, we use hydrothermal modification of titanium with divalent calcium or magnesium cations [14, 15], which are known to be indispensable for cell-to-substratum contact [16, 17]. We hypothesized that this technique may promote the adhesion of epithelial cells or fibroblasts to titanium. It could also be performed with minimal change of surface topography, which may be an advantage given that a roughened surface has a negative effect on soft tissue cell adhesion [12] and is highly suited to dental plaque accumulation if the surface is exposed in the oral cavity. The aim of the present study was therefore to evaluate the effect of hydrothermal modification of titanium with calcium or magnesium ions on the attachment of oral epithelial cells and fibroblasts.

2 Materials and Methods

2.1 Titanium Plates

Commercially available, smooth, pure wrought titanium plates [diameter 15 mm, thickness 1 mm, roughness (Ra) 0.19 μm , Japan Industrial Standards Class 1 (equivalent for ASTM grade I)] (KS40, Kobelco, Kobe, Japan) were used in this study. These were divided into four groups. Non-process (NP) group specimens received no treatment. In the negative control (DW) group, specimens were hydrothermally treated in distilled water at 200 °C for 24 h. In the calcium (Ca) and magnesium (Mg) groups, plates were treated at 200 °C for 24 h with a 10 mmol/L solution of CaCl_2 or MgCl_2 , respectively, as reported previously [15, 18]. After the treatment, surface Ra was ranged 0.20–0.22 μm ; subsequently, all specimens were washed with DW and stored under a vacuum to prevent surface contamination. All groups were compared with NP as an additional control.

2.2 Surface Characterization

The surfaces of the specimens were analyzed by X-ray photoemission spectrometry (XPS) (ESCA 750, Shimadzu, Kyoto, Japan). All binding energies were referenced to the carbon 1s component set to 285.0 eV. Overlapping peaks in XPS spectra were separated by a computer-aided curve-deconvolution method.

2.3 Cell Culture

The human gingival squamous cell carcinoma cell line, Sa3, and the mouse embryonic fibroblast cell line, NIH3T3, were purchased from RIKEN Cell Bank (Tsukuba, Japan). Sa3 cells were cultured in basal medium Eagle (BME, Invitrogen, Carlsbad, CA, USA) containing 20 % fetal bovine serum (FBS) and antibiotics (100 U/mL medium of penicillin and 100 mg/mL streptomycin). NIH3T3 cells were cultured in alpha-minimum essential medium (α -MEM, Invitrogen, Carlsbad, CA, USA) containing 10 % FBS and antibiotics (as above). These cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2 in air. Cells were seeded in a 1 mL volume onto each titanium plate at a density of 1×10^5 cells per well of a 12-well plate (Falcon Labware, Oxford, UK).

2.4 Cell Number Measurement

The numbers of attached cells were measured using the water-soluble tetrazolium salt kit (WST-8, Dojindo, Kumamoto, Japan). Cells were plated and cultured for 1 h before plating medium was removed and replaced with 1.1 mL of serum-free medium containing 100 μL of WST-8 solution. After 2 h incubation at 37 °C under 5 % CO_2 , 100 μL of the supernatant was transferred to a 96-well plate and its absorbance measured at 450 nm wavelength using a spectrophotometer (NJ-2300, Biotec, Tokyo, Japan). In our pilot experiment, we failed to detach cells by the adhesion assay described below if we employed a culture time of more than 1 h. Thus, a single time point of 1 h was performed in the present study.

2.5 Adhesion Assay to Evaluate the Strength of Cell Adhesion to the Substratum

The adhesion strength of Sa3 and NIH3T3 cells was evaluated by adhesion assays as reported in a previous study [19]. In brief, after two washes with culture medium, any non-adherent or weakly attached cells were removed by shaking three times at 75 rpm for 5 min in culture medium using a rotary shaker (NX-20, Nissin, Tokyo, Japan). The remaining adherent cells were measured using the WST-8 kit (Dojindo) and calculated as a percentage of the initial count. Consequently, the results defined the adhesive strength of the cells.

2.6 Statistical Analysis

To eliminate inter-group error arising from differences in cell conditions, all data were standardized with the values from the NP group.

Data are expressed as the mean \pm SD. One-way analysis of variance (ANOVA) with Dunnett's post-test was

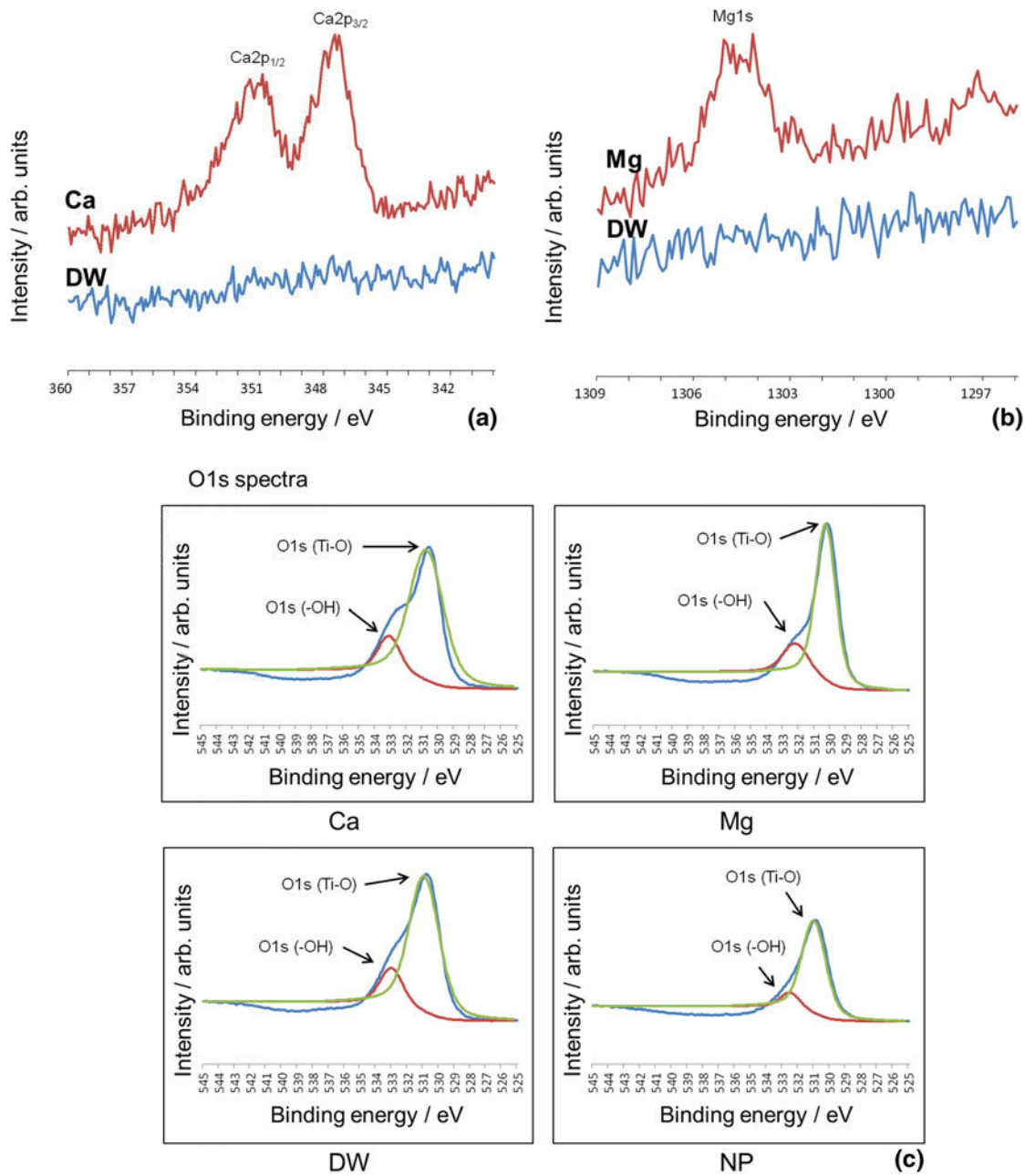


Fig. 1 XPS spectra of DW, Ca, Mg and non-process (NP) groups. **a** Ca2p peaks are observed between 340 and 360 eV (Ca). **b** Mg1s peak is observed between 1,297 and 1,309 eV (Mg). **c** O1s peaks comprise two peaks, namely Ti-O (approximately 530 eV) and Ti-

OH (approximately 532 eV). Note that hydrothermal treatment increased surface OH regardless of the presence of cations in the solution. *Red*, -OH group; *green*, Ti-O group; *blue*, total oxide

performed. Values of $P < 0.05$ were considered to be statistically significant.

2.7 (Immuno)-Fluorescent Staining for Adhesion Proteins

Sa3 and NIH3T3 cells on the experimental titanium were fixed with 4 % formaldehyde for 10 min, blocked with 10 % BSA for 30 min at 37 °C, and then incubated overnight at

4 °C with rabbit anti-human integrin $\beta 4$ polyclonal antibody (Chemicon International, Phillipsburg, NJ, USA; 1:200 dilution) and rabbit anti-mouse vinculin polyclonal antibody (Chemicon International; 1:200 dilution), respectively. After washing, both samples were labeled for 2 h at 37 °C with fluorescein isothiocyanate-conjugated anti-rabbit IgG secondary antibody (Sigma-Aldrich, St. Louis, MO, USA; 1:100 dilution). Actin filaments were stained with tetramethylrhodamine isothiocyanate-conjugated phalloidin

(Sigma-Aldrich; 1:200 dilution) for 1 h at 37 °C. Finally, the samples were mounted with anti-fade reagent containing 4',6-diamidino-2-phenylindole (ProLong[®] Gold with DAPI; Invitrogen) for nuclear staining. The stained cells were observed (and images obtained) with a fluorescence microscope apparatus (BZ-9000, Keyence, Osaka, Japan).

3 Results

3.1 Surface Characteristics of Specimens (Fig. 1)

Ca and Mg spectra on the surface of the Ca-treated and Mg-treated titanium plates are shown in Fig. 1a, b, respectively. Ca2p and Mg1s peaks can be seen in the spectra for these groups, neither of which are evident in samples from the DW group.

High-resolution XPS spectra of the O1s region indicated that this region is composed of two peaks, namely Ti–O (approximately 530 eV) and Ti–OH (approximately 532 eV) [20]. The results indicate that hydrothermal treatment increases surface hydroxyl groups, regardless of the presence of cations in the treatment solution (Fig. 1c).

3.2 Adhesion of Sa3 Cells (Figs. 2, 3)

The Ca and Mg groups had significantly larger numbers of attached Sa3 cells on the substrate after 1 h culture than in the DW group (Fig. 2). However, there was no significant

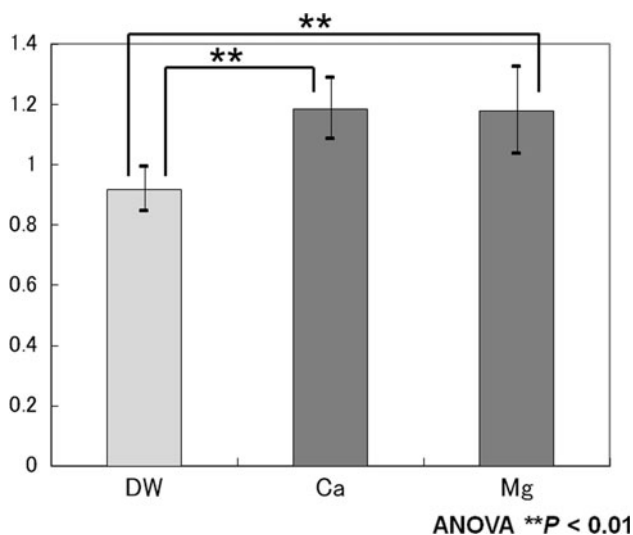


Fig. 2 Sa3 cell adherence. Sa3 cells were plated at 1×10^5 cells per well of a 12-well plate, each well containing a titanium plate treated with either distilled water (DW), 10 mM CaCl₂ (Ca) or 10 mM MgCl₂ (Mg). Cell numbers were determined using WST-8 reagent and measurement of absorbance at 450 nm. Data are mean \pm SD. Statistical analysis was by ANOVA with Dunnett's post-test. Asterisk denotes a statistically significant difference ($P < 0.01$)

difference in the adhesion strength among these groups (Fig. 3a). Fluorescent microscopic findings revealed punctate integrin $\beta 4$ signals (green) localized around the nucleus (blue), with actin filaments (red) forming a thick band at the intracellular margin of these cells in all groups. The distribution of these markers did not differ among groups (Fig. 3b).

3.3 Adhesion of NIH3T3 Cells (Figs. 4, 5)

The numbers of attached NIH3T3 cells (Fig. 4), and the strength of this attachment (Fig. 5a), were significantly greater in the Ca and Mg groups than in the DW group. Fluorescent microscopic findings revealed that cells in the Ca and Mg groups contained punctate signals for vinculin (green) throughout the cytoplasm, whereas those in the DW group exhibited only a low signal around the nucleus (blue). However, cells in the Ca and Mg groups had more highly developed actin filaments (red) than those in the DW group, and had many lamellipodia from actin bundles that seemed to shape the cytoskeleton (Fig. 5b).

4 Discussion

In this study, hydrothermal treatment was employed to introduce Mg or Ca onto the surface of titanium. With this treatment, a divalent cation-rich surface to the outermost portion of titanium substrata is easily generated, with minimal surface topographical alteration [18]. Because a rougher surface impairs the adhesion of both epithelial cells and fibroblasts of oral origin [12], this treatment may be advantageous in improving oral soft tissue cell adhesion to titanium implants.

The state of Ca and Mg on the surface of titanium (i.e., whether they are present as ions, crystals, etc.) remains unclear. In our pilot experiment, we tried to remove surface Ca or Mg using EDTA solutions, but we detected Ca and Mg peaks on the titanium surface even after the EDTA treatment (data not shown). In addition, despite the difference in experimental conditions (concentration, temperature, etc.), a possible calcium titanate peak was reportedly detected on the surface of titanium hydrothermally treated with CaCl₂ solution [21]. The resolution capability of our XPS apparatus was relatively low so that we could not obtain similar results; in our case, however, some Ca on the surface of titanium may also be present as calcium titanate. In addition, the surface of calcium titanate reportedly releases some Ca ions [22]. In this context, the state of surface Ca on the surface of titanium hydrothermally treated with CaCl₂ could be presumed to comprise both ion and stable compounds.

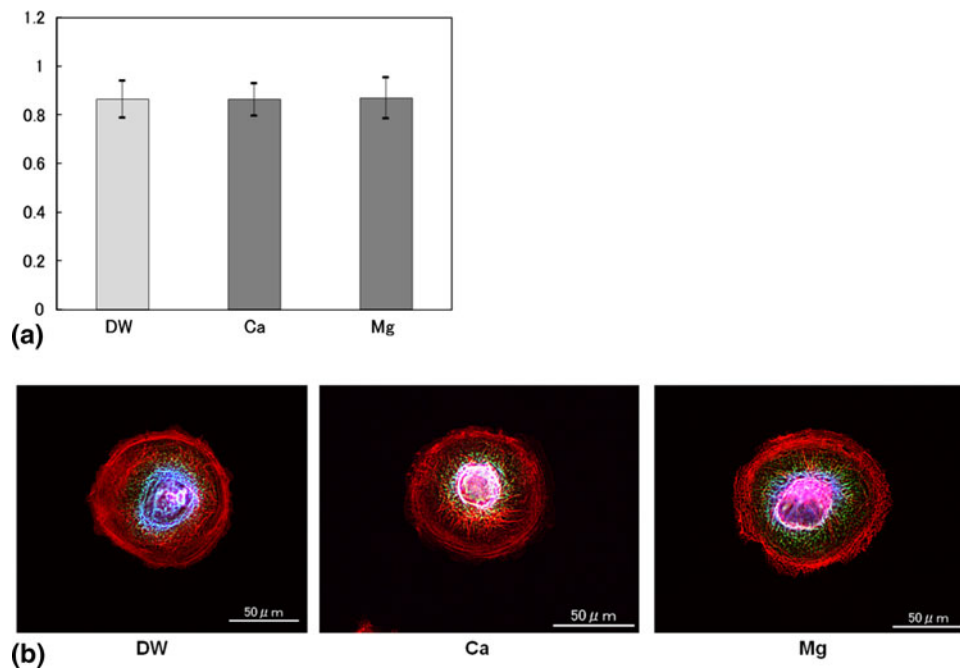


Fig. 3 a Adhesion assay of Sa3 cells. Sa3 cells were plated at 1×10^5 cells per well of a 12-well plate, each well containing a titanium plate treated with either distilled water (DW), 10 mM CaCl₂ (Ca) or 10 mM MgCl₂ (Mg). Plates were then shaken three times for 5 min at 75 rpm to remove non-/weakly-adherent cells. Remaining cell numbers were determined using WST-8 reagent and measurement of absorbance at 450 nm. Data are mean \pm SD. **b** Localization

of adhesive protein in Sa3 cells. Adherent cells were incubated firstly with rabbit anti-human integrin $\beta 4$ polyclonal antibody followed by fluorescein isothiocyanate-conjugated anti-rabbit IgG secondary antibody (green). Actin filaments were labeled with tetramethylrhodamine isothiocyanate-conjugated phalloidin (red). Blue staining represents DAPI visualization of the nuclei

After the hydrothermal treatment, the hydroxyl content was greater in the hydrothermal treatment group than in the non-treatment group regardless of the presence of cations in the treatment solution. The adsorption of protein (bovine serum albumin) on the titanium surfaces was reportedly positively related to the amounts of their surface hydroxyl groups [23]. In addition, it was reported that calcium titanate releases Ca ions, thereby forming a surface richer in TiO₂, which would in turn transform TiOH groups. Both surface Ca and TiOH sites then adsorb phosphate ions, which may cause apatite nucleation on its surface [22]. In this context, the surface Ca layer may contribute to protein/cell adhesion.

A large volume of literature describes bone cell adhesion to titanium substrata. It is hypothesized that titanium acquires, through interaction with the atmosphere, a passive surface layer of TiO₂ that adsorbs Ca²⁺ ions, which then react with Ca-binding proteins or glycosaminoglycans, such as osteocalcin, osteopontin or heparan sulfate proteoglycan (HSPG) [24]. Indeed, some reports suggest that osteocalcin and osteopontin are key factors in achieving osteoblastic cell adhesion to dental implants [25, 26].

As well as bone cell-titanium interaction, divalent cations have also been linked in part with the promotion of adhesion of gingival epithelial cells and connective tissue

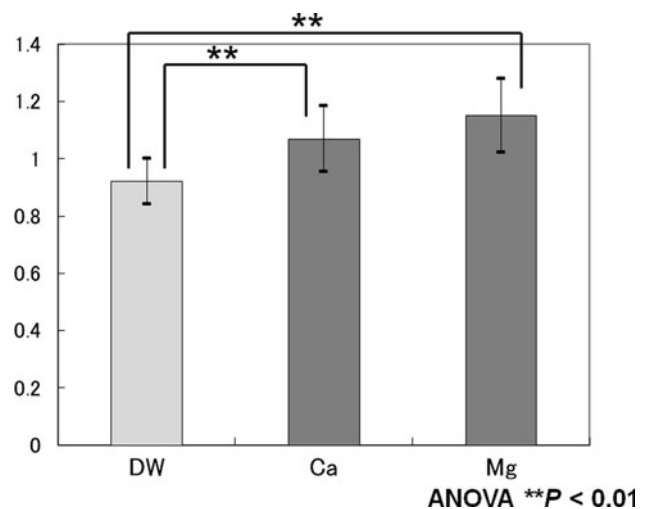


Fig. 4 NIH3T3 cell adhesion. NIH3T3 cells were plated at 1×10^5 cells per well of a 12-well plate, each well containing a titanium plate treated with either distilled water (DW), 10 mM CaCl₂ (Ca) or 10 mM MgCl₂ (Mg). Cell numbers were determined using WST-8 reagent and measurement of absorbance at 450 nm. Data are mean \pm SD. Statistical analysis was by ANOVA with Dunnett's post-test. Asterisk denotes a statistically significant difference ($P < 0.01$)

fibroblasts to titanium. Previous studies have discovered a basement membrane-like, laminin-5-rich layer interposed between epithelial cells and titanium [4, 9]. In addition,

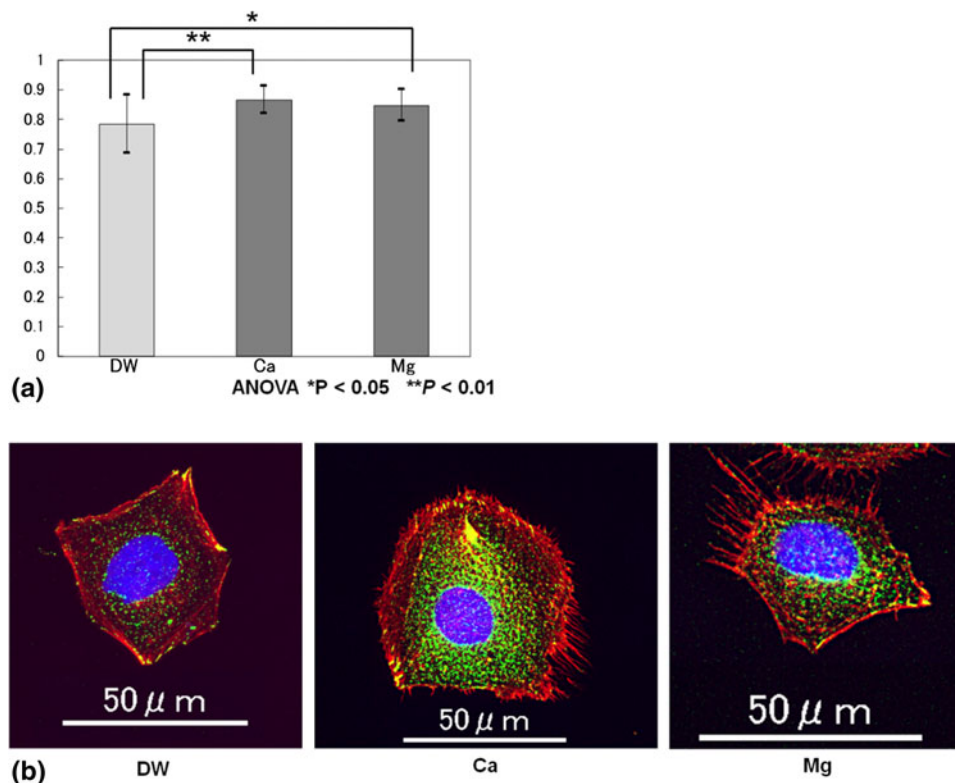


Fig. 5 **a** Adhesion assay of NIH3T3 cells. NIH3T3 cells were plated at 1×10^5 cells per well of a 12-well plate, each well containing a titanium plate treated with either distilled water (DW), 10 mM CaCl₂ (Ca) or 10 mM MgCl₂ (Mg). Plates were then shaken three times for 5 min at 75 rpm to remove non-/weakly-adherent cells. Remaining cell numbers were determined using WST-8 reagent and measurement of absorbance at 450 nm. Data are mean \pm SD. Statistical analysis was by ANOVA with Dunnett's post-test. Asterisk denotes a

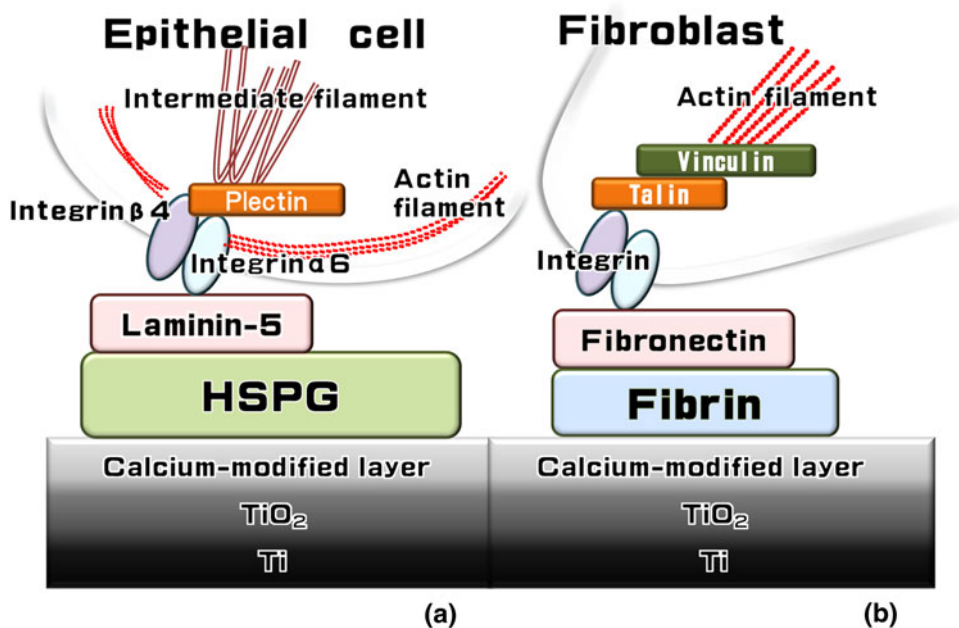
statistically significant difference. **b** Localization of adhesive protein in NIH3T3 cells. Adherent cells were incubated firstly with rabbit anti-mouse vinculin polyclonal antibody followed by fluorescein isothiocyanate-conjugated anti-rabbit IgG secondary antibody (*green*). Actin filaments were labeled with tetramethylrhodamine isothiocyanate-conjugated phalloidin (*red*). *Blue* staining represents DAPI visualization of the nuclei

HSPG, a proteoglycan found at high levels in blood plasma, is known to bind to both Ca²⁺ ions and laminin-5 [27–30]. Although there are other mechanisms of cell adhesion onto titanium substrata, the interposition of laminin-5, HSPG and Ca between epithelial cells and a titanium substratum could be one hypothetical model for epithelial cell adhesion to titanium (Fig. 6a). Similarly, the Ca-binding property of fibrin [31] could enable it to play a bridging role between the substratum and fibroblasts. The present study sought to clarify the role of the divalent cations, Ca²⁺ and Mg²⁺, in the adhesion of oral soft tissue cells to titanium substratum. Although the calcium ion is a more recognized mediator of cell adhesion to substrata or other cells, the magnesium ion is reported to be much more effective than calcium in promoting cell attachment to substrata (although not in cell-to-cell adhesion) [32], hence our investigation of both cations.

We believe that our adhesion assays are a direct measure of cell-to-substratum adhesion strength, because their short (1 h) time course eliminates any effect of cell-to-cell adhesion. According to these assays, Ca and Mg

significantly enhanced the adhesive properties of epithelial cells and fibroblasts, consistent with previous reports in which divalent cations promote the initial attachment of epithelial and fibroblastic cells to a negatively charged plastic dish [32, 33]. These treatments also enhanced adhesive strength in fibroblasts, although they were unable to act similarly in epithelial cells. These results were reinforced by microscopic observations showing strong expression of adhesion proteins in fibroblasts in both the Ca and Mg groups, whereas no significant differences in expression were found between epithelial cell groups. The reason for this discrepancy is currently unclear, but may indicate the presence of another mechanism of epithelium–titanium adhesion that does not involve divalent cations. An alternative explanation could be a lack of laminin-5 in the culture medium, which may impair initial adhesion of epithelial cells by requiring them to produce their own laminin. Fibroblasts have no such drawbacks, because fibronectin and fibrin are both contained at high levels in serum and are adsorbed onto the titanium substratum to generate rapid and secure fibroblast adhesion.

Fig. 6 Schematic showing the hypothesized mechanism of cell adhesion to the surface of Ca-modified titanium substratum. We speculate that heparan sulfate proteoglycan (HSPG) and laminin-5 intervene between titanium and epithelial cells (*left*), whereas fibrin and fibronectin intervene between titanium and fibroblasts (*right*)



5 Conclusion

Hydrothermal treatment of titanium with CaCl₂ or MgCl₂ enhanced initial attachment of both Sa3 epithelial cells and NIH3T3 fibroblasts, and improved the adhesion strength of the fibroblasts. We conclude that Ca and Mg in the titanium surface layer has an affirmative effect on epithelial and fibroblastic cell adhesion to the titanium surface, and may contribute to the quality of the soft tissue seal around the implant.

Acknowledgments We appreciate the contributions of Prof. Kunio Ishikawa and Associate Prof. Kanji Tsuru, Department of Biomaterials, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University in making the hydrothermally treated titanium plates.

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