Influence of Titanium Surface Charge on Fibroblast Adhesion

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ABSTRACT

Background: Although dental implants have a high success rate, failure owing to the absence of adhesion between the gingival connective tissue and the implant surface is still being reported.

Purpose: This study was designed to evaluate the effect of a titanium surface charge on fibroblast adhesion.

Material and Methods: An electrical chamber was custom-made to generate negative and positive surface charges on commercially pure titanium cylinders with a potential difference of 4.5 V. Twenty-seven titanium cylinders were divided into three experimental groups. In each group, cell attachment to a positively charged titanium cylinder, a negatively charged titanium cylinder, and a titanium cylinder (control) was studied at three time intervals of 15, 30, and 60 minutes. NCTC clone 929 fibroblasts were used in these experiments. The effect of the potential difference in the pH of Dulbecco's Modified Eagle Medium (DMEM) was also evaluated using two new specimens at time intervals of 15, 30, 60, and 80 minutes.

Results: The fibroblast cell attachment was more statistically significant to the positively charged titanium cylinder than the negatively charged titanium cylinder ($p = .002$) and the control ($p = .000$), whereas the cell adhesion difference between the control and the negatively charged titanium cylinder was not statistically significant ($p = .808$). The range of pH difference of the DMEM in the negative and positive parts of the electrical chamber was 0.46 and 0.30, respectively.

Conclusion: Within the limitations of this in vitro study, the positive surface charge of the titanium cylinder results in significantly favorable cell adhesion.

KEY WORDS: cell attachment, fibroblast adhesion, implant surface, titanium surface charge

The control of cell adhesion and its consequences are fundamental in various biologic processes, such as embryonic development, immunologic responses, injury healing, and tissue maintenance. Moreover, cell adhesion is also important in the context of implants because it is considered to be the determinant of the success or failure of implantation. Anchorage-dependent cells such as fibroblasts and osteoblasts need the adhesion to survive.[1] The fibroblast cell has a negative surface charge[2,3] and participates in the production of various essential components of connective tissues, such as glucosaminoglycans and collagen in fibrous tissue.[4]

Generally, there are two types of cell adhesion: adhesion of cells to each other and the extracellular matrix and adhesion of cells to adsorbed proteins.[1,5] One of the primary mechanisms of cell adhesion to the surface of a substrate is the focal contact, also known as focal adhesion or adhesion plaque, where it adheres to adsorbed proteins or to the extracellular matrix.[6,7] Focal adhesions are distinct regions in the cell membrane where the membrane intimately associates with the substrate surface.[8–11]

Basically, dental implants are encircled by three different tissues: the epithelium, the connective tissue, and

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the bone. The absence of adhesion between the gingival connective tissue and the implant surface might be a major factor in implant failure.[12] Peri-implant mucosa is a scar tissue that has fewer cells and more fibers. Therefore, peri-implant mucosa will be less effective in encapsulating lesions associated with dental plaque. Thus, peri-implantitis would affect the osseous tissue and may result in implant failure.[13]

Collagen fiber orientation is an important difference between peri-implant mucosa and the gingiva around natural teeth. Collagen fibers are principally perpendicular or oblique to the tooth surface but are mainly parallel around dental implants.[14–21]

The dentogingival fibers perpendicularly attached to the cementum form part of an abutment to the sulcular epithelium.[22] They serve as a barrier to the epithelial migration and thus impede bacterial invasion. It has been suggested that the creation of a perpendicular insertion of collagen fibers to the implant surface would enhance the barrier capacity and reduce the implant failure rate.[12] However, in a previous study, parallel collagen fiber orientation to nonsubmerged titanium-coated implants was observed, with no apical migration of the junctional epithelium.[19] In another study, no inflammatory cell infiltrate was found around the dental implant in a canine model, and it was suggested that the parallel collagen fibers provide a cufflike barrier to bacterial invasion.[17]

From the above-mentioned studies, it might be concluded that enhancement of fibroblast adhesion and improvement in the insertion direction of collagen fibers to the titanium implant surface might improve the biologic seal around the dental implant and the resistance of peri-implant mucosa to inflammation and bacterial invasion. In addition, the improvement in cell adhesion to the implant surface, which is covered with a layer of titanium dioxide, would help integrate the implant to the connective tissue, improve the vascularity at the implant surface and decrease the opportunity for fibrous encapsulation and bacterial infection.[23–26]

Principally, there are different surface parameters that may influence cell adhesion to the substrate surface, such as hydrophilicity and hydrophobicity,[27] surface electrical charge,[28] surface roughness,[29] and surface free energy.[30,31]

The relationship between surface charge and fibroblast cell adhesion was investigated in previous studies by charging surfaces through chemical or physical means.[28,32–34] However, variables inherent in chemically charged surfaces used in previous studies make it difficult to separate the electrical charge and chemical effects.[28] Qiu and colleagues generated charged surfaces of indium tin oxide (ITO) electrodes without affecting their chemical composition and morphology. These electrodes were fabricated using photolithography and charged through the application of electrical surface potential.[28] Generally, these studies indicate that a positive surface charge seems to enhance cell adhesion.[28,32,33,35] In the present study, a potential difference to titanium cylinders was applied to generate positive and negative surface charges. Then the effect of the titanium surface charge on fibroblast cell adhesion was investigated. Furthermore, the effect of the potential difference in the pH of Dulbecco's Modified Eagle Medium (DMEM) was evaluated at different time intervals.

MATERIALS AND METHODS

A six-well culture plate (Gibco, Madrid, Spain) was used to prepare the electrical chamber in this study. The center of three wells, which would receive the titanium cylinders, was marked at the top cover. Then, with a 5 mm–diameter trephine bur, holes were made to receive their respective titanium cylinders of 5 mm diameter. Two additional holes of 1 mm diameter with an internal distance of 50 mm were made to receive the salt bridge, which connected two wells electrically.

The titanium cylinders were sterilized in autoclave, whereas the salt bridge and modified top cover of the culture plate were sterilized using ultraviolet sterilization.

Salt Bridge Preparation

Three grams of agar-agar, 14 g of potassium nitrate, and 100 mL of distilled water were used. First, the potassium nitrate was dissolved in distilled water, and then the agar-agar was added. The solution was heated until the agar-agar became easy to aspirate. With a vacuum pump, a U-shaped glass tube was carefully filled with the salt solution in order not to introduce air bubbles. Then it was disconnected from the vacuum pump and immediately placed under running tap water to solidify the agar-agar. The salt bridge was kept in distilled water.

Fibroblast Culture

The mouse fibroblast cell line NCTC (clone 929) was purchased from the American Type Culture Collection

(Virginia, USA). Cells were cultured in Minimal Essential Medium (Gibco, Madrid, Spain) containing 10% horse serum (Gibco), supplemented with 100 IU/mL penicillin G (sodium salt), 100 µg/mL streptomycin (Gibco) with 2 mm L-glutamine, and Earle's Balanced Salt Solution (Gibco) adjusted to contain 1.5 g/L sodium bicarbonate.

The culture medium was renewed to maintain the cells on every other day. Fibroblast cells were first inspected under an inverted light microscope (Leica, Tokyo, Japan) to verify their condition. Then old culture medium was carefully aspirated using a sterile Pasteur pipette connected to a vacuum pump. After that, 10 mL of complete culture medium was added to the cell culture flask. Finally, the cell culture flask was stored in an incubator (INCO-2, Memmert, Germany) at 95% humidity, 5% CO_2, and 37°C.

The cell suspension used in this study was prepared from a confluent cell layer that was washed three times with phosphate buffer salt (PBS) solution. The cells were then incubated with 2 mL of trypsin–ethylenediaminetetraacetic acid (EDTA) at 95% humidity, 5% CO_2, and 37°C for 2 minutes. The trypsinization of the cells was stopped by the addition of an excessive amount of complete culture medium (at least three times the trypsin quantity).[34] The cells were then counted to determine the suspension cell concentration [S]. The volume of the cell suspension (V) needed to make 30 mL of a cell suspension with a density of 14×10^4 cells/mL was calculated from the following equation:

$$V = (30\,\text{mL} \times 14 \times 10^4\,\text{cells/mL})/[S]$$

where [S] equals cell suspension concentration.

The calculated volume was transferred to a 50 mL sterile test tube, and the volume was completed to 30 mL. All handling of the fibroblast cells was done in a sterilized fume hood (Telsatr, Terrasa, Spain).

Cell Attachment Assay

Twenty-seven titanium cylinders used to study the cell attachment were divided into three groups of nine cylinders each. For each time interval, three titanium cylinders were placed in the electrical chamber. Two cylinders were connected to a 500 mA universal pulse width modulated, regulated AC/DC adaptor. A chargefree third cylinder served as a control. Three milliliters of cell suspension with a density of 14×10^4 cells/mL was added to each titanium cylinder, and a potential difference of 4.5 V was applied for 15, 30, and 60 minutes.

After each time interval, the cell suspension was aspirated from around the titanium cylinders and the cylinders were washed three times with PBS solution. Three milliliters of trypsin-EDTA was then added, and the cell culture plate was incubated for 2 minutes at 95% humidity, 5% CO_2, and 37°C. The detachment of the cells was ensured by an inverted light microscope. The trypsin action was neutralized with the addition of an excessive amount of complete culture medium. After that, the cells were counted using a Neubauer counting chamber (Brand, Wertheim, Germany) and an inverted light microscope. The cell attachment assay was repeated three times.

To examine the effect of the potential difference in pH on DMEM, two new specimens of titanium cylinders were placed in the electrical chamber and 5 mL of DMEM was added. Finally, the salt bridge was placed to connect the two titanium cylinders electrically. A potential difference of 4.5 V was applied, and the pH values were measured in 15, 30, 60, and 80 minutes. Before reading the pH value, the potential difference and salt bridge were removed.

Statistical Analysis

It was hypothesized that the surface charge of the titanium dioxide layer would not affect fibroblast cell adhesion. The data for cell attachment assay were analyzed with two-way analysis of variance (ANOVA) ($\alpha = 0.05$). Accordingly, all pairwise multiple comparison procedures were performed using the Tukey test ($p < .05$) for the comparisons among individual means of the test groups.

RESULTS

Table 1 displays the calculated data for the adhesion assay results. The positively charged titanium surfaces had the highest mean in cell adhesion at all time intervals. On the other hand, the control group had the lowest mean in cell adhesion at all time intervals.

The results of two-way ANOVA, summarized in Table 2, demonstrated the presence of a statistically significant difference between electrical charge categories and between time intervals ($p = .000$ for both variables). This primary analysis resulted in the rejection of the null hypothesis because a significant effect was found between electrical charge categories and between time intervals. The results of a post hoc test (Table 3) indicated that the positively charged titanium surface had a

TABLE 1 Adhesion Assay Results Represented by Means of Cell Counts (Cells/mL) for the Experimental Groups (±SD)

Group	Cell Count T15*	Cell Count T30*	Cell Count T60*
Control	0.46×10^4 (±0.19)	1.08×10^4 (±0.38)	1.71×10^4 (±0.19)
Positive	1.00×10^4 (±0.00)	1.75×10^4 (±0.43)	2.42×10^4 (±0.38)
Negative	0.50×10^4 (±0.00)	1.00×10^4 (±0.29)	2.00×10^4 (±0.29)

*Cell count after 15, 30, and 60 minutes.

statistically significant difference in cell adhesion from both the control ($p = 0.000$) and the negatively charged titanium surface ($p = .002$). Conversely, no statistical significance was found when the control was compared with the negatively charged titanium surface ($p = .808$).

The results for the DMEM pH change are shown in Table 4. The initial pH of the DMEM around positively charged and negatively charged titanium cylinders was 7.73 and 7.69, respectively. At the negatively charged titanium surface, the initial pH value (at baseline) was 7.69 and the highest (at 80 minutes) was 8.15, whereas at the positively charged titanium surface, the initial pH value was 7.73 and the highest was 8.03.

DISCUSSION

It was observed from these results that in relation to the electrical charge, the positively charged titanium surface had the highest cell adhesion than both the control and the negatively charged titanium surface at all time intervals, whereas in relation to time interval, the 60-minute time interval had the highest cell adhesion and the 15-minute time interval had the lowest.

When the titanium is exposed to air, a titanium dioxide layer forms spontaneously (1 nm in <1 ms) and normally has a thickness of 5 to 10 nm. There is evidence that this layer grows firmly in vivo. The titanium dioxide layer is not electrically conductive, but electrons can tunnel through the layer. This tunneling of electrons results in conformational changes and denaturing of proteins. In titanium, layers more than 50 nm, which result during machining, are sufficient to prevent significant denaturing of proteins by electron tunneling.[24] Titanium dioxide has an isoelectrical point of 4.5; therefore, in pH values >4.5, it will have a negative electrical charge.[25]

The adhesion assay and the statistical analysis revealed that fibroblast cell adhesion was more statistically significant for the positively charged titanium cylinder than both the negatively charged titanium surface and the control, whereas the difference in cell adhesion between the control and the negatively charged titanium cylinder was not statistically significant (Figure 1). Qiu and colleagues demonstrated a new design to study the effect of the electrical charge on the adhesion, morphology, and expression of proteins in rat marrow stromal cells. In their study, transparent and electrically conductive ITO was used. The ITO electrodes were charged positively and negatively using an electrical current supplier. When it was exposed to a potential difference of 0.8 V for 24 hours in serum-supplemented culture medium, the cells adhered to the anode with a positive electrical charge more than the cathode with a negative electrical charge and the gap between the electrodes.

When the titanium surface was exposed to proteins from the culture medium supplied by horse serum, these proteins adhered passively to the titanium dioxide

TABLE 2 Two-Way Analysis of Variance ($p < .05$) for Cell Attachment Results

Source of Variation	SS	*df*	MS	F Ratio	Probability
Charge	2.171	2	1.086	13.594	.000
Time	8.001	2	4.001	50.094	.000
Charge* time	0.023	4	0.006	0.072	.990
Error	1.438	18	0.080		

df = degree of freedom; MS = mean square; SS = sum of squares.

TABLE 3 Tukey Post Hoc Analysis of Different Charge Categories ($p < .05$)

Group	Mean Difference	*p*	$p < .05$
Positive vs negative	0.55556	.002	Yes
Positive vs control	0.63889	.000	Yes
Negative vs control	0.08333	.808	No

TABLE 4 Evaluation of pH Change in Dulbecco's Modified Eagle Medium with 4.5 V Applied Potential Difference

Time (min)	pH Negative*	pH Positive†
0	7.69	7.73
15	7.84	7.73
30	8.06	7.86
60	8.10	7.97
80	8.15	8.03

*Negatively charged titanium cylinder.
†Positively charged titanium cylinder.

surface, forming a protein layer. One explanation of this behavior of cell adhesion was that the titanium surface electrical charge affects the adsorbed protein layer composition, thus favoring the fibroblast adhesion to the positively charged titanium surface.[33]

Kapur and colleagues evaluated the passive adsorption of proteins in cell free culture medium to fluorinated ethylene propylene (FEP) and bacteriologicgrade polystyrene (PS).[5] In FEP and PS surfaces with a negative charge, it was observed that protein adsorption was initially strong and increased with the increase in the negative charge until reaching a specific value, after which the protein adsorption started to decrease, whereas in FEP and PS with a positive charge, the protein adsorption was initially weak and increased with an increase in the positive electrical charge.

Fibroblasts have a negative surface electrical charge because their extracellular matrix is rich in negatively charged glucosaminoglycans.[2] It was demonstrated that the distribution of cationized ferritin in nonirradiated fibroblast cells was in a polar manner (the cationized ferritin particles were mainly localized at cell apical surfaces with cluster formation).[3] Therefore, the other possibility to explain the observed cell adhesion behavior is the electrostatic forces, that is, attraction and repulsion.

In a previous study, it was concluded that the oxidized thiol surface (has a negative electrical charge) had the lowest cell adhesion.[32] Therefore, it was hypothesized that this negative electrical charge could inhibit electrostatically the interaction with the cell surface proteoglycans or prevent the adsorption of adhesive proteins. Another study concluded that the enhancement of cell attachment was not controlled by proteins in the culture medium.[28] Based on the fact that the cell membrane has a negative electrical charge in normal physiologic conditions, this improvement in cell adhesion could be the result of the attraction forces between the positively charged anode and the negative cell membrane, but the effect of electrostatic forces alone could not explain why the cell adhesion to the negatively charged cathode did not decrease significantly.

Surface energy could be affected by several surface characteristics, such as chemical composition, surface electrical charge, and microstructural topography.[31] Hallab and colleagues demonstrated that cell adhesion is proportional to the surface energy.[31] In this study, an electrical charge was applied to titanium surfaces that could modify the surface energy of the titanium surfaces in a manner that favors cell adhesion to the positively charged titanium cylinder.

In the present study, the application of a potential difference of 4.5 V did not significantly affect the pH of the DMEM. The results demonstrated a small range of difference between the pH values, along with the passed time of the experiment intervals (see Table 4).

Other biologic processes, such as cell shape, extension, proliferation, and biosynthesis profile, need to be investigated, not only to specify the effect of an electrical surface charge of the titanium surface on fibroblast culture but also to reveal the mechanisms by which an electrical charge can affect these cellular processes.

CONCLUSIONS

Within the limitations of this in vitro study, it was concluded that the application of a potential difference of 4.5 V did not significantly affect the pH of DMEM. Cell adhesion was more statistically significant for the positively charged titanium surfaces at all studied time intervals.

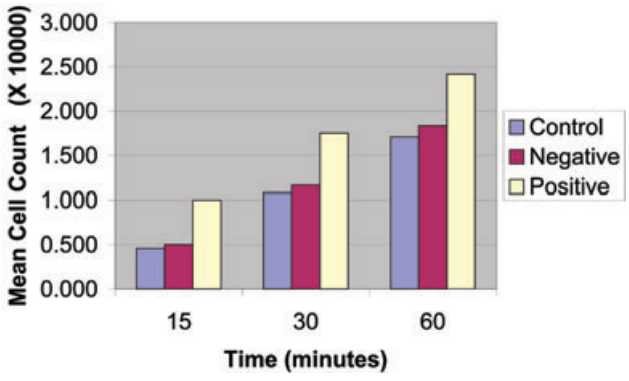

Figure 1 Fibroblast cell adhesion. Control = uncharged titanium cylinder; Negative = negatively charged titanium cylinder; Positive = positively charged titanium cylinder.

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