Evaluation Study

HISTOLOGICAL AND IMMUNOHISTOCHEMICAL EVALUATION OF HUMAN GINGIVAL FIBROBLASTS ON DIFFERENT TITANIUM SURFACES

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ABSTRACT

The aim of the research is to evaluate the response of cells such as fibroblasts to titanium surfaces treated differently. The reason why this type of cells was chosen is related to their role in the bone healing process and in the abutment adhesion, forming a barrier that protects the underlying bone. Human gingival fibroblasts cells (HGF) were cultured for 18 and 72 hours on machined titanium grade 5 (Ti6Al4V) interstitial elements (ELI) titanium disks, coated disks with titanium nitride (TiN) and sandblasted disks with hydroxyapatite (HA) (OsseoGRIP). Cell morphology was analyzed by scanning electron microscopy (SEM), while focal adhesion kinase (FAK) protein was analyzed by confocal laser scanning microscopy (CLSM). From the results of this study, it is clear that SEM and CLSM showed great HGF cell adhesion and filopodium-like extensions on the isotropic nanorough surface (OsseoGRIP), especially after 72 hours. FAK protein was localized along cellular extensions on the OsseoGRIP disks. Within the limits of the study, we could observe that the micro-geometric differences of the various surfaces analyzed lead to a difference in cell growth in qualitative and quantitative terms. In fact, the less rough TiN surfaces are those that show less fibroblast growth. This consideration may be important in implant systems that require a long transmucosal canal where it would be desirable, in order to compose a coronal seal important for implant and bone, to achieve good growth and adhesion of the connective part. For this is useful the application of the machined and OsseoGRIP type surfaces which, being rougher, allow better seal. OsseoGRIP surfaces, thanks to their 0.5 µm surface nano-roughness, allow both to obtain a good seal from soft tissues and to control bacterial adhesion.

KEYWORDS: *OsseoGRIP, bone healing process, adhesion, titanium, endosseous implants*

INTRODUCTION

Titanium is a metal that, in its pure state, is shiny, white, and quite ductile. It has a high melting point (1668°C) and a rather low modulus of elasticity (similar to that of bone), which makes it particularly flexible and able to absorb the

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masticatory loads and then transmit them to the bone without developing tension, fundamental property to use it in dental implants together with its excellent biocompatibility.

The titanium used for the realization of endosseous implants is defined as "commercially pure" (cpTi) and has been classified by ASTM (American Society for Testing and Materials) in grades 1 to 4, depending on the oxygen content (increasing from 0, 18 to 0.40) and iron (increasing from 0.20 to 0.50). This increase corresponds to an improvement in the mechanical characteristics (1).

For the realization of dental implants, it is also possible to use titanium alloys such as Ti6Al4V (titanium grade 5) composed of 90% titanium, 6% of aluminum, 4% of vanadium, 0.25% of iron and 0.2% of oxygen (2). There is also a version of this alloy with low levels of interstitial elements (ELI), which is an excellent choice in those circumstances where a combination of high strength, lightness, toughness and corrosion resistance is required.

The titanium implant surfaces can be subjected to additive treatments (coating with hydroxyapatite or calcium phosphate, titanium plasma-spray, and ion deposition) or subtractive type (electropolishing, mechanical polishing, sandblasting, acid attack, and oxidation), which create irregularities in the surfaces (3).

Surfaces with a clear orientation of irregularities are defined as anisotropic; those that do not have an easily identifiable orientation are instead called isotropic.

Subtractive techniques

Turning/cutting/smoothing: the surfaces treated this way are defined as "machined". The cutting device used is a rotating carborundum disk, which creates a jagged and irregular design on the titanium surfaces with a low finish degree. Instead, the turning is made with a stainless-steel instrument, through which a highly anisotropic surface is obtained with microscale irregularities. To have a more delicate finish degree, the surfaces can be exposed to a smoothing process by using grit-papers or diamond clothes made of abrasive particles such as corundum (SiC) of various sizes or with alumina powder (4).

Sandblasting

Sandblasting consists of the realization of surface irregularities through the collision with microscopic particles of variable size (2). Sandblasting can be made with titanium dioxide particles (5), alumina, hydroxyapatite, or rutile, and the surface roughness is directly proportional to the size of the used particles. Surfaces treated with larger particles have a greater roughness and, therefore a greater contact area (4).

Regarding the sandblasting with hydroxyapatite (HA) particles (OsseoGRIP surfaces), a comparison was made with the machined surfaces. The impact of HA particles causes plastic deformation and induces an increase in the surface available for osseointegration. The surface does not have a clear orientation and contrarily to the machined ones, in which peaks and valleys are evident, appears homogeneous. Based on these characteristics, a 67% osseo-implant was considered after 12 weeks, greater than 62% of the machined surfaces (6).

Etching

The implant is immersed in acid substances that remove a small amount of metallic material, thus creating a surface micro-roughness. The most commonly used acids are HCL, H_2SO_4, and HF, increasing the available surface and transforming anisotropy into isotropy (7). These are the only acids capable of reacting with titanium surface oxide, which normally has a very low chemical reactivity. What occurs at the level of the implant surface are redox reactions, which are responsible for the metal dissolution (8). The dual acid-etching technique involves the immersion of titanium implants for some minutes in a mixture of concentrated HCl and H2SO4 heated above 100°C. This method increases osteoconductive by attaching osteogenic cells and fibrin, resulting in direct bone formation (9).

Sandblasted and acid-etched surface (SLA)

The combination of these two treatments is perhaps the most used. This technique involves first a sandblasting phase with 250-500 μm particles that create macrostructures and then a second phase in which micro-irregularities are created using acids (13). Through sandblasting, an optimal roughness is guaranteed for a mechanical fixation. Then, the etching smooths the sharp peaks and may add a high-frequency component on the implant surface with potential importance for protein adhesion, which is important for the early bone-healing process (10).

Oxidation

The titanium itself has a surface oxide film. Still, the oxidized implants are made by applying a thicker layer of oxide obtained by heat treatment or by placing the implant in a galvanic cell with an electrolyte [H_2SO_4, H_3PO_4,

CH_3COOH, NaOH, $Ca(OH)_2$]. Current (80 V) is then passed through the galvanic cell, and the oxide will grow from a thickness of 5 nm to 1 mm or more (10, 11). In addition to increasing the thickness of the TiO2 film, there is an increase in roughness and surface area (12). More precisely, a dissolution of the oxide layer along the convective current lines and its thickening in other areas occurs. Dissolution creates micro and nano-pores on the implant surface (13).

Additive techniques

Titanium Plasma-Spray (TPS): this technique requires a titanium powder to be introduced into a high-temperature plasma torch. The particles then collide with the implant surface on which they condense and fuse to form a film about 30 μm thick. The resulting TPS coating will have a roughness of about 7μm, increasing the implant's surface area (14).

Hydroxyapatite coating

This technique involves the addition of HA on the implant surface to increase the osteoconduction. Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is considered a bioactive material, as it allows the realization of calcium phosphate-rich layers on the surface (15) and is also capable of absorbing large amounts of fibronectin and vitronektin, which favor the adhesion of osteoblasts and therefore osseointegration (16). The release of calcium phosphate in the peri-implant region determines the precipitation of biological apatite on the implant surface, which acts as a matrix for the growth and adhesion of osteogenic cells. There are several methods for coating the titanium surface. Still, the one used in clinical practice is the plasma-spray technique, which involves a mixture of amorphous calcium phosphate with calcium phosphate crystals and hydroxyapatite (2). The procedure is analogous to coating with TPS and involves the deposition of a film with a thickness varying from a few micrometers to a few millimeters.

Titanium nitride coating

Titanium alloys are known for their high strength, low density, and ability to resist corrosion. Despite this, they are not very resistant to abrasion, which can dissolve the surface oxide layer. Titanium nitride (TiN) is instead known for its particular hardness and mechanical strength, with a low dissolution of titanium ions, and this type of coating allows to solve the problem of poor resistance to abrasion of the titanium alloy. These conclusions were observed in the study by Tamura et al. (12) after analyzing a nitride titanium surface. Regarding the abrasion resistance, the surface treated with nitride showed much better characteristics; for this reason, the use of this surface is recommended in those circumstances where both a good biocompatibility and a good resistance to abrasion is required, as in the case of 'abutment (17).
Surface topography can be divided into macro, micro, and nano roughness (13, 14):

- Macro roughness: this feature can range from millimeters to microns and, if appropriate, can directly improve the initial implant stability and long-term fixation by mechanical interlocking the rough surface irregularities and the bone (13). However, a major risk with high surface roughness may be an increase in peri-implantitis (14).
- Micro roughness: it ranges from 1-10 microns and determines superior growth and interlocking of bone at the implant interface (13).
- Nano roughness: it is widely used in implant dentistry and ranges from 1-100 nm. The roughness is believe to promote absorption of proteins and adhesion of osteoblasts, improving osteointegration (13). Nanoscale topography can be obtained by various methods, such as sandblasting, ionization and etching.

The aim of the research is to evaluate the response of cells such as fibroblasts to titanium surfaces treated differently. This type of cells was chosen due to their role in the bone healing process and in the abutment adhesion.

MATERIALS AND METHODS

Sample preparation

The study was carried out using:

- machined Ti6Al4V ELI titanium disks;
- coated disks with TiN;
- sandblasted disks with HA (OsseoGRIP).

All the samples had a diameter of 15 mm and a thickness of 1.5 mm and were previously sterilized in an autoclave at 160 ° for 2 hours. Acrylic resin discs (Sintodent and Jet Lang) of the same size as the test samples, also sterilized in an autoclave, were used as the control group.

Cells and cell culture

The study used human gingival fibroblasts HGF obtained from ATCC (ATCC® PCS-201- 018 ™). Cells were seeded at a density of 1.5 x 10^5 cells/cm^2 in tissue culture plastic dishes containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics as penicillin and streptomycin in a fully humidified atmosphere consisting of 95% air, 5% CO_2 at 37°C. For SEM the cells were seeded on the titanium disks (1.5 x 10^4 cells/disk) and incubated for 18 or 72 hours.

To analyze the expression of FAK protein was used a confocal laser scanning microscopy (CLSM). The cells were seeded on the titanium disks with a density of 1.5 x 10^5 cells/disk as well as on the tissue culture plastic wells, which were used as control. The cells were incubated for 18 or 72 hours.

SEM analysis of cell morphology

HGF cell morphology was analyzed by SEM (Zeiss EVO, Germany) after a culture of 18 or 72 hours. The samples were prepared as follows: all the disks with cultured cells were washed twice in PBS, phosphate-buffered saline, to remove nonattached cells and then twice with 0.1 M SCB, sodium cacodylate buffer, pH 7.4, at 37°C for 5 min. The cells on the disks were fixed for 3 hours at 4°C according to Karnovsky's method (4% paraformaldehyde, 3% glutaraldehyde, 0.1 M SCB, pH 7.4). After fixation, the disks with HGF were washed twice in PBS and then dehydrated using a graded ethanol series. The procedure ended with critical point drying and ion-sputter coating.

CLSM analysis of immunofluorescent staining

The expression of FAK protein and actin was analyzed by immunofluorescent staining with CLSM (confocal laser scanning microscopy) after HGF cell culture for 18 or 72 hours. The samples were prepared as follows:

All the disks with cultured cells were washed twice in PBS to remove nonattached cells. Then the cells were fixed in 4% paraformaldehyde in PBS for 10 mint 37°C and permeabilized with 0.1 Triton X-100 in PBS for 5 min at 37°C. The cells were labeled with rabbit anti-FAK antibody for 1h at 37°C and then overnight at 4°C. After washing the cell layer with 0.2% Triton X-100 in PBS, anti-rabbit IgG were added for 30 min at 37°C. Finally, the cellular actin was stained using rhodamine-phalloidin.

RESULTS

The samples were analyzed by SEM after 18 and 72 from the positioning of the HGFs. Following we can appreciate the images of the various samples (Fig. 1-4).

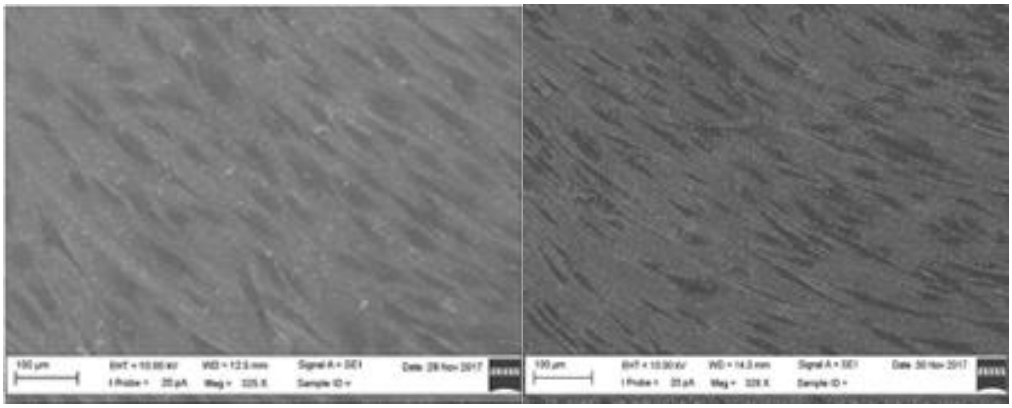

Fig. 1. *Ti6AL4V ELI alloy coated with TiN after 18 hours of fibroblast growth (left) and after 72 hours (right).*

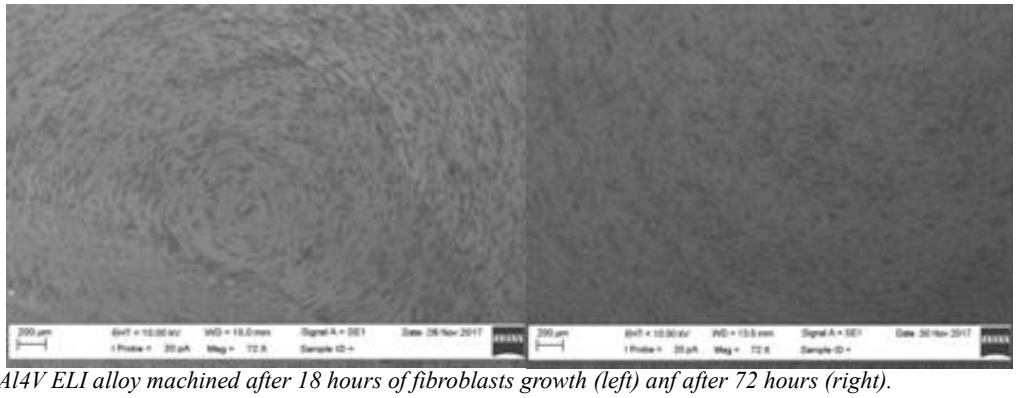

Fig 2. *Ti6Al4V ELI alloy machined after 18 hours of fibroblasts growth (left) anf after 72 hours (right).*

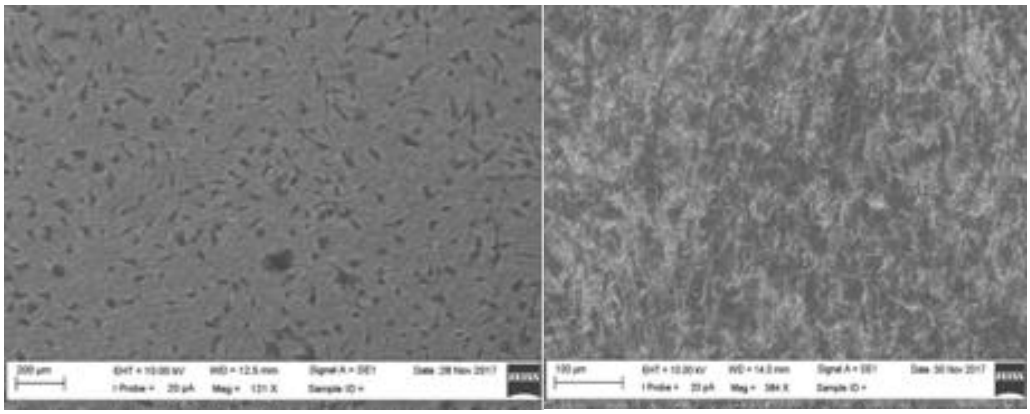

Fig. 3. *Ti6Al4V ELI alloy coated with HA after 18 hours of fibroblasts growth (left) and after 72 hours (right).*

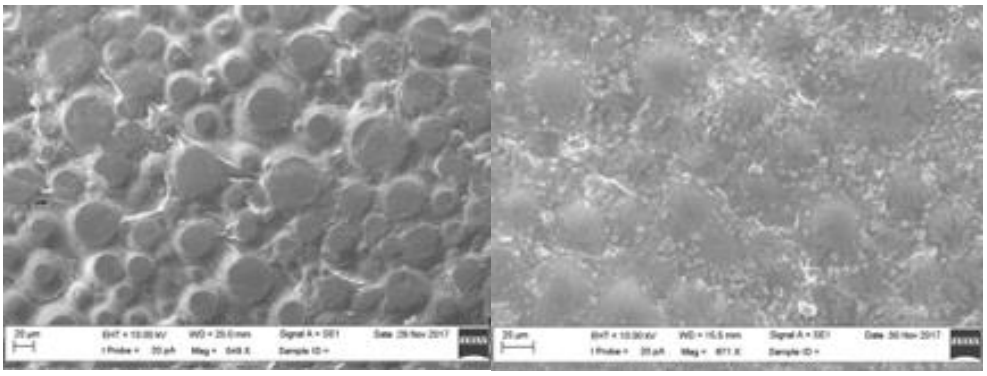

Fig. 4. *Resin Jet Lang after cell placement. The presence of an isolate fibroblast is highlighted.*

DISCUSSION

After an implant is inserted, a biological bone healing process is triggered. At the level of the bone, in the first few hours, a clot is formed that contains growth factors that influence the mesenchymal cells and intensify the activity of inflammatory cells. Over the next four days, the clot is gradually reabsorbed and replaced by granulation tissue containing blood vessels, leukocytes, macrophages, and mesenchymal-like fibroblast cells. Macrophages will take care of engulfing damaged tissue and cells and releasing growth factors and cytokines that further promote migration and differentiation of mesenchymal cells. The latter provides for the deposition of a new extracellular matrix, and together with an intense

angiogenesis mediated by endothelial cells, a temporary connective tissue is generated. Subsequently, the osteoprogenitor cells migrate and differentiate into osteoblasts. The formation of osteoid tissue is observed, followed by hydroxyapatite deposition around the 7th day with the consequent formation of immature bone or braided fibers bone (14th day) and then of mature lamellar bone (3 months) after a remodeling phase (4, 5).

Fibroblasts are the most numerous cellular elements within the connective tissue (about 65% of the total cell population), responsible for the synthesis of glycoproteins and proteoglycans of the amorphous matrix as well as the tropocollagen, the main fibrous component of the extracellular matrix (15). When an implant is introduced into the maxilla, it must interface correctly with 3 types of cells: osteoblasts, epithelial cells, and fibroblasts. The latter two adhere to the transmucosal component of the implant, forming a barrier that protects the underlying bone (13). The attachment of connective tissue to the implant blocks the apical migration of the junctional epithelium, preventing the loss of crestal bone (16). The topographic and physico-chemical properties of the implant surface affect hard and soft peri-implant tissues. As explained above, different treatments are used to increase the roughness of the implant and favor osseointegration. On the contrary, the transmucosal portion of the implant itself is generally made of smooth-turned titanium to minimize bacterial colonization (14). Regarding the type of surfaces that favor the attachment of fibroblasts, it has been observed that the fibroblasts grown on surfaces with micro-topography treated with double acid attack showed a greater production of type I collagen and fibronectin compared to those grown on the machined or smooth surfaces (17). According to the study by Miao et al. (13), after evaluating a sample with a polished surface, one with micro-roughness, and one with nano-sparse, the surfaces with nano-roughness are the ones that most stimulate the adhesion of epithelial cells and fibroblasts, increasing the percentage of survival of the implant. On this surface, the cells express more vinculin, a protein that achieves focal adhesion. Greater vinculin expression is associated with greater adhesion strength.

Guida et al. (14) also observed that a greater surface area and better tissue-titanium contact are obtained on nanostructured surfaces. This type of surface can be obtained through anodization and is characterized by irregularities with a uniform and isotropic distribution over the entire surface.

It has been suggested that a certain roughness is necessary to obtain an excellent seal of the soft tissues and to counteract the migration of the epithelium, always bearing in mind that the same roughness favors bacterial adhesion and plaque formation and, therefore, it must not exceed the value (Ra) of 0.2 μm. The nanostructured surfaces obtained with anodic oxidation, which have such a low roughness to fall into the "smooth" category according to Alberktsson and Wennerberg (6), on the one hand, favor the formation of a soft tissue seal and, on the other appear to be little contaminated by the oral bacteria (14).

From the results of this study, in agreement with the literature, it is clear that with regard to machined surfaces, fibroblasts orientate themselves along the anisotropic irregularities of the surface, resulting in lower adhesion and reduced type I collagen production compared to other surfaces.

The OsseoGRIP surface is characterized by a greater nanorugosity than the TiN (0.5 μm) and does not present a clear orientation of the irregularities, thus falling into the category of isotropic surfaces. This surface, unlike the machined one in which peaks and valleys are evident, appears homogeneous, favoring a clearly greater cell adhesion as well as adequate plaque control thanks to its moderate roughness.

The surface coated with TiN is characterized by the presence of a nitride layer with a thickness of 2 μm, composed of TiN and Ti_2N, and an irregular roughness with a size of 0.32 μm, which determines a modest cell growth.

In general, surfaces with nano-roughness are those that most stimulate the adhesion of fibroblasts, as on this surface the cells express a greater amount of vinculin, a protein that achieves focal adhesion. Greater vinculin expression is associated with greater adhesion strength.

CONCLUSIONS

Within the limits of the study, we could observe that the micro-geometric differences of the various surfaces analyzed lead to a difference in cell growth in qualitative and quantitative terms. In fact, the less rough TiN surfaces are those that show less fibroblast growth. This consideration may be important in implant systems that require a long transmucosal canal where it would be desirable, in order to compose a coronal seal important for implant and bone, to achieve good growth and adhesion of the connective part. For this is useful the application of the machined and OsseoGRIP type surfaces which, being rougher, allow better seal. Those OsseoGRIP in particular, thanks to their 0.5 μm surface nano-roughness, allow both to obtain a good seal from soft tissues and to control bacterial adhesion.

With regard to osseointegration, OsseoGRIP appears to be clearly advisable due to its ability to grow precursors of bone cells while maintaining a moderate surface roughness that allows to counter bacterial colonization. During the process of osseointegration in the first few hours we observe the formation of a clot containing growth factors which

influence the mesenchymal cells and intensify the activity of inflammatory cells. Over the next four days the clot is gradually reabsorbed and replaced by granulation tissue, containing blood vessels, leukocytes, macrophages and mesenchymal-like fibroblast cells. Macrophages will take care of phagocytizing the damaged tissue and cells, as well as releasing growth factors and cytokines that further promote migration and differentiation of mesenchymal cells. The latter provide for the deposition of a new extracellular matrix and together with an intense angiogenesis mediated by endothelial cells, a temporary connective tissue originates. Subsequently the osteoprogenitor cells migrate and differentiate into osteoblasts and the formation of osteoid tissue is observed, followed by hydroxyapatite deposition around the 7th day with consequent formation of immature bone (14th day) and then of mature lamellar bone (3 months) after a remodeling phase. The surface of the OssGRIP is the one that most stimulates this process.

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