Osteoblast Adhesion onto Titanium Dental Implants

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Abstract

One of the many characteristics of an implant that must be improved is its surface topography. Cells are able to respond differently to the degree of substratum roughness. In this work we investigated the adhesion of human primary osteoblast-like cells to differently roughened titanium dental implants. Using HOB cells pulsed with [^{3}H]-thymidine, we could infer that smooth surfaces show significant decrease in adhesion rates. Scanning electron microscopy of HOB cells adhered to titanium discs and implants showed that cells orient themselves along grooves of the substrate. Most cell cultures on smooth surfaces form continuous monolayers, while those grown on rougher ones are unable to. We conclude that cells grown on rough surfaces adhere better than those grown on their smooth counterparts. The biological reasons for such behavior are further discussed.

Keywords: Osteoblast, implants, surface roughness, cell adhesion, scanning electron microscopy

Introduction

Osteoblasts are cells of mesenchymal origin which produce and regulate mineralization of the bone matrix. Committed osteoprogenitor cells divide and differentiate into osteoblasts forming bone (5).

Cell-cell and cell-substrate interactions are both processes of prime importance related to many biological phenomena. As such, cell attachment influences the way by which cells proliferate and differentiate when found in contact with both metal and ceramic implants. Cell adhesion may be regulated by various membrane proteins, most notably integrins, which bind to extracellular matrix (ECM) proteins, such as fibronectin, laminin and collagens. As such integrins not only physically connects the external environment to the cell cytoskeleton but also start signaling cascades that control the way cells behave (22,26). There are some similar adhesions complexes involving ECM, integrins and cytoskeleton (1,28) and focal contact or focal adhesion (7,23) is one of these that are known to occur in osteoblast cell lines (14,21).

It has been shown that among the biomaterials currently used in dental implants those made of titanium are highly biocompatible (9). Biocompatibility is a concept often related to the ability of an inert surface to be a substrate for cell adhesion, growth and division. Additional advantages of titanium are its lightweight, excellent corrosion resistance and easy shaping by a number of mechanical and chemical processes (18). Although the material itself is of great importance, the cell-inert surface interaction depends on other factors as well, such as substrate topography, energy and chemistry (2).

Previous studies have pointed out the importance of the surface topography of implants on the regulation of the osteoblast physiology. For instance, on smooth surfaces osteoblasts seem to have decreased adhesion, but proliferate at a high extent. On rougher surfaces they seem to adhere further, proliferate at a low extent and show an increased protein secretion, resembling a more differentiated phenotype (19). However, if the peaks of the surface topography are too high or the distance between them are too great, the cells may be essentially dealing with smooth surfaces.

Also important to perceive is the ECM components adsorbed to the implant's surface. Some of the matrix proteins involved are not only structurally important for the tissue integrity but act as survival factors modulating cell proliferation, differentiation and mineralization (10,13,15,25). Iuliano et al. (1993) has highlighted the importance of the conformation and orientation of fibronectin adsorbed to the substratum,

which influences the affinity of the protein to its cell surface receptor.

The aim of the present study was to evaluate the ability of primary human osteoblast-like cells (HOB) to adhere to differently roughened titanium dental implants.

Materials and Methods

Titanium cylinders and implants

Commercially pure titanium grade 3 was machine cut into either cylinders (6mm in diameter) or dental implant screws (3.75mm x 15mm). Subsequently they were exposed to HNO_3 for 20, 60 or 90 seconds. These treatments are here named I, II and III, respectively (I for 20s of acid-etching, II for 60s and III for 90s). Surface II have also been blasted with Al_2O_3, to achieve a new degree of roughness. The last treatment is here referred as II-Al.

Titanium surface topography

Titanium surface topography was analyzed by scanning electron microscopy. Briefly, treated cylinders were treated with methanol, air-dried, thinly sputter coated with gold (10-15 nm thick) and visualized under a JSM 5310 scanning microscope (JEOL).

Cell culture

Primary human osteoblast-like cells (HOB) were obtained from the cell bank at Clementino Fraga Filho University Hospital (Federal University of Rio de Janeiro, Brazil), and maintained in Dulbecco's Modified Eagle Medium with low glucose (GIBCO) containing 10% fetal bovine serum (Seromed), 1% Minimum Essential Medium amino acid solution 100x (Sigma), ascorbic acid (0.15g/L) and buffered with Hepes (1M). Cell cultures were kept at 37°C in a humidified atmosphere with 5% CO_2.

Cell adhesion

Cell attachment was accessed by measurement of HOB pulsed with [^{3}H]-thymidine, attached to titanium cylinders. Cells were cultured for 24h in medium containing 1μCi [^{3}H]-thymidine. Afterwards, osteoblasts were harvested from culture bottles by the addition of 0.2% trypsin and 0,02% EDTA in a saline solution (0.8% NaCl, 0.01% KCl, 0.29% $NaHPO_4.7H_2O$ and 0.02% KH_2PO_4 in H_2O) for 5 minutes at 37°C. The reaction was stopped by addition of fetal calf serum. Harvested osteoblasts were counted in a haemacytometer and 10^5 cells were seeded to each cylinder found inside a 24-well plate. The plate was then incubated for 2h at 37°C in 5% CO_2, washed three times with 0.01M phosphate buffered 0.15M NaCl (PBS), followed by addition of 1ml of 0.1% SDS in 0.1% NaOH. Finally, 15ml of Aquasol-2 (DuPont) were added to the solution and cell attachment was evaluated by counting radioactivity in a beta counter (1214 Rackbeta Liquid Scintillation Counter, LKB Wallac). Negative controls followed the same procedures described above, except that cells were not seeded onto cylinders.

Scanning Electron Microscopy

Cell morphology was accessed by visualization of cultured HOB cells onto the titanium cylinders and dental implants. Cells were harvested and counted as described above. 10^5 cells were seeded to each implant, and 10^3 cells were seeded onto cylinders placed inside a 24-well plate. The plate was then incubated for 6 or 72 hours at 37°C in 5% CO_2. Just after culture time, the samples were washed three times with 0.01M PBS, and fixed with Karnovsky solution (0.25% glutaraldehyde grade I, 0.64% paraformaldehyde, 0.1g/L picric acid made in PHEM buffer pH 7,2. PHEM buffer: PIPES 60mM, HEPES 20mM, EGTA 10mM, KCl 70mM, $MgCl_2$ 5mM in H_2O) overnight on the refrigerator. Following fixation, implants or cylinders were washed with 0.1M Cacodylic Acid pH 7.2, post-fixed with 2% OsO_4 for 30 minutes in the dark, washed again in the same buffer and dehydrated in ethanol. Samples were then critical point dried (CPD020, Balzers Union) and thinly sputter coated with gold (10-15nm thick). Images were digitally acquired from a JSM 5310 scanning microscope (JEOL).

Results

Titanium surface topography

The four treatments originated highly different surface topographies. First of all there seems to be a roughness gradient: I, II, III and II-Al, from the smoothest to the roughest (Figure 1). Besides that, before being exposed to acid etching, these surfaces were machine cut, a process that leaves grooves on the titanium surface. Surfaces treated for shorter times retrieved its grooves (noticeably in I) while others did not (III and II-Al). Surface II had very subtle, almost negligible grooves (Figure 1b). Also, surfaces III and II-Al do not hold any apparent topographic pattern (Figures 1c and 1d), while surfaces I and II does, as represented by the parallel grooves done in the process of machine cutting (I have more evident grooves than II, thus a higher degree of organization). Finally, Al_2O_3 blasting in treatment II-Al resulted in particle incrustation (Figure 1d).

Cell attachment

As can be seen in figure 2, osteoblast attachment was affected by surface treatment. There is a slight tendency of increase in cell attachment as the surface becomes rougher. The smoothest surface, though, was the only one to have significant ($p<0,05$) decrease in adhesion when compared to suspension counting. While the treatment related to the surface II had more than 10% of

decrease in its mean value of cell adhesion against suspension counts, it was not statistically significant. No differences in osteoblast adhesion were evident between surface treatments III, II-Al and suspension counts.

Scanning Electron Microscopy

The morphology of cells grown onto titanium cylinders and implants varied with the surface roughness. Those grown on surfaces that retrieved their grooves from the machine cut oriented themselves in a manner that was parallel to it (Figures 3a and 3b). After six hours of cell culture on implants, osteoblasts from all surfaces exhibited either a rounded shape or an elongated morphology reaching 100µm in length, with phyllopodial contact between them (Figure 4). However, after 72 hours of culture, cells grown on implants with treatment I were well spread forming almost a continuous monolayer, where cells in rectangular shapes could be observed (Figure 5a). On the other hand, cells grown on rougher surfaces that did not show grooves, such as II-Al,, had a dendritic shape, being loosely organized and no evidence of a monolayer was seen (Figure 5d). Cells in surface treatments II and III showed intermediate morphologies, some exhibiting a multilayered conformation (figures 5b and 5d).

Discussion

Osteoblast adhesion on biomaterials is a subject of great interest since it is closely related to both orthopedics and orthodontics. Several factors may influence inert materials integration with the surrounding tissue and the degree of surface roughness is certainly one of them.

Curtis and Wilkinson (1997) have pointed out the difficulties to define and quantify surface roughness. Nonetheless, there seems to be a relationship between cell response and surface roughness or even to its spatial organization. Anselme et al. (2000b) has, in fact, observed statistical correlation between surface roughness organization, cell adhesion and proliferation. Therefore it is possible that two surfaces with equal degree of roughness may differ in terms of spatial organization, triggering, as a consequence, different cell signaling.

The results here reported on the implants surface topography analyzed by scanning electron microscopy demonstrated a roughness gradient as expected by the prolonged exposure to acid etching. Such technique, as well as the Al_2O_3 blasting, generated random patterns in the titanium surfaces. This coarse arrangement was also expected since we may not control the way corrosion takes place. Surface types I and II, though, showed some degree of organization as the parallel grooves left by the machine cut were evident.

The Al_2O_3 particle incrustation observed in the II-Al surface treatment would represent risk of Al dissolution in the surrounding tissues constituting a threat to both the implant success and patient health (6). Thus this implant is not recommended in any way. Apart from the prolonged exposure problem, its surface characteristics might be of interest and it may only be a technical challenge to remove these incrustations from the implant surface.

Data concerning cell attachment reveal a tendency of smooth surfaces to be a poorer substrate for osteoblast attachment when compared to rougher ones. Although surface type I was the only one to show statistical divergence from suspension counts, surface type II mean count rate was 10% lower than suspension. This non-statistical discrepancy may be due to low sample number in the experiment. Eriksson et al. (2001) demonstrated that leukocyte adhesion on rough titanium surfaces is higher as well as its expression of adhesion receptors when compared to smooth counterparts. This could also occur with osteoblasts onto titanium surfaces.

We still do not know an explanation for the relationship of roughness and adhesion, but certainly surface energy is involved. Indeed, it has been shown that increases in the surface charge significantly influence osteoblast behavior, most probably through signals via focal contacts (20,27). Lampin et al. (1997) observed an increase in the apolar component of the surface as surface becomes rougher. A relation between cell adhesion enhancement, degree of roughness and hydrophobicity was then established.

Also, cells in contact with deep and narrow grooves (when compared to the cell dimensions) show major actin accumulations in the cell-surface contact areas (8). Actin stress fibers are formed in focal contacts mediated by integrins. Thus, if cells are allowed to contact various peaks and valleys on the inert surface, it may have more focal contacts resulting in greater attachment strength.

Morphological studies have raised even more intriguing issues. In all implant surfaces here studied by the time of 6 hours of culture cells were not yet well attached to the substrata. They seemed to have few contact points with the surface and, as such, had either a round or an elongated shape. By this time of culture it is possible that cells are still secreting ECM proteins that will adsorb to the substratum, and only then cell adhesion will be more effective. Our data related to attachment experiments, however, revealed that more than 80% of cells adhere within only 2 hours in any kind of implant here studied. Therefore, even though the adhesion strength may not be the strongest at the 6-hour point, certainly all treatments are excellent in regard to cell attachment. It should be noted that, prior to experiments, we use trypsin to release cells from tissue culture flasks. This enzyme treatment cause proteolysis of many membrane bound proteins crucial in the adhesion process. Thus, *in vivo*,

adhesion should take place even faster than periods mentioned above.

Yet, the most remarkable result was obtained by osteoblasts cultured onto titanium by 72 hours. As stated earlier, cells on smoother implants were well spread forming almost a continuous monolayer, containing cells in rectangular shapes. On the other hand cells grown on rougher implants in which grooves were not evident, had a dendritic shape and were loosely organized with no apparent formation of a monolayer.

The fact that osteoblasts on smoother surfaces exhibited a rectangular shape may be reflecting the grooves on the surface formed by the machine cut. As shown in figure 4, cells tend to grow along the grooves where those are seen. In consequence, if the grooves of the surface are arranged parallel to each other, cells become arranged in the same way. Osteoblasts also tend to spread over large areas in smooth surfaces. Thus, a rectangular shape, and an arrangement in a continuous monolayer are expected to occur.

In contrast, growth on rough surfaces tends to be different. Lincks et al. (1998) have shown that rough surfaces are related to increased expression of differentiation markers (alkaline phosphatase and osteocalcin) and local secretion (prostaglandin E_2 and latent transforming growth factor β), as well as decreased proliferation in osteoblast-like cells. Considering this, our results with the rough surfaces, particularly with surface type II-Al, could be explained as follows: the dendritic shape with no apparent cell arrangement is a consequence of the lack of an organized topography in which the cells would not have a pattern to follow, and the coarse layout of cells, which do not form any noticeable monolayer, could derive from a decreased proliferation rate and, possibly, increased differentiation.

One speculation may be addressed regarding the increase in adhesiveness on rough surfaces. One of the most intriguing features of focal contacts is their sensitivity to mechanical loading. For example, when a mechanical force is applied from the outside of a cell, increase of focal contacts is still achieved even though crucial signal molecules are inhibited (24). It was also demonstrated that there is a correlation between the local force applied and the individual adhesion site size, so that there is a constant for this phenomena of about $5nN.\mu m^{-2}$ (4). Although some possible explanations are at hand (12), an interesting matter is that growth of focal contacts is a tension-induced process. Thus, one possible explanation for the increase in adhesiveness on rough surfaces is that if a single cell is allowed to experience various peaks on the surface it lies upon, it may have several tension points in which focal contacts may grow due to mechanical force resulting in greater attachment strength. On the other hand, on smooth substrates most of the cell is in contact with the surface. Consequently, the mechanical loading on focal contacts becomes distributed between the many others, which, in turn, would not grow as much.

Finally, one should be aware of the ability of the implant surface to adsorb certain ECM proteins, as well as the way these proteins bind to it. Adsorption of proteins such as fibronectin, collagen, bone sialoprotein, osteonectin, and many others should take place if an implant is to become successfully integrated with the surrounding tissue. It should also expose the binding motifs of these proteins, for concealment would prevent the cells from perceiving them.

We conclude that smooth implant surfaces are poorer substrates for osteoblast attachment than rough ones. Also, differentiation of the cells surrounding the implant should be the primary concern as long as a calcified bone around the implant is wanted. Therefore, bone implants with treatments that generate rough surfaces, so long as these are thought to promote differentiation, are recommended the most for better and faster integration with the surrounding tissue.

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References

1. Adams, J.C. (2001). Cell-matrix contact structures. Cellular and Molecular Life Sciences 58: 371-392.

2. Anselme, K. (2000). Osteoblast adhesion on biomaterials. Biomaterials 21:667-681.

3. Anselme, K., Bigerelle, M., Noel, B., Dufresne, E., Judas, D., Iost, A. and Hardouin, P. (2000). Qualitative and quantitative study of human osteoblast adhesion on materials with various surface roughnesses. Journal of Biomedical Materials Research 49:155-166.

4. Balaban, N., Schwarz, U., Riveline, D., Goichberg, P., Tzur, G., Sabanay, I., Mahalu, D., Safran, S., Bershadsky, A., Addadi, L. and Geiger, B. (2001). Force and focal adhesion assembly: a close relationship studied using elastic micropatterned substrates. Nature Cell Biology 3: 466-472.

5. Bilezikian, J.P., Raisz, L.G. and Rodan, G.A. (1996) Principles of Bone Biology. Academic Press, San Diego, 1398p.

6. Bushinsky, D.A., Sprague, S.M., Hallegot, P., Girod, C., Chabala, J.M. and Levi-Setti, R. (1995). Effects of aluminum on bone surface ion composition. Journal of Bone Mineral Research 10: 1988-1997.

7. Critchley, D.R. (2000). Focal adhesions – the cytoskeletal connection. Current Opinions in Cell Biology 12:133-139.

8. Curtis, A. and Wilkinson, C. (1997). Topographical control of cells. Biomaterials 18:1573-1583.

9. Davies, J.E., Lowenberg, B. and Shiga, A. (1990). The bone-titanium interface *in vitro*. Journal of Biomedical Materials Research 24: 1289-1306.

10. Degasne, I., Baslé, M.F., Demais, V., Huré, G., Lesourd, M., Grolleau, B., Mercier, L. and Chappard, D. (1999). Effects of roughness, fibronectin and vitronectin on attachment, spreading, and proliferation of human osteoblast-like cells (Saos-2) on titanium surfaces. Calcified Tissue International 64: 499-507.

11. Eriksson, C., Lausmaa, J. and Nygren, H. (2001). Interactions between human whole blood and modified Ti-O_2-surfaces: Influence of surface topography and oxyde thickness on leukocyte adhesion and activation. Biomaterials 22:1987-1996.

12. Geiger, B. and Bershadsky, A. (2001). Assembly and mechanosensory function of focal contacts. Current Opinion in Cell Biology 13:584-592.

13. Globus, R.K. (1998). Fibronectin is a survival factor for differentiated osteoblasts. Journal of Cell Science 111: 1385-1393.

14. Gronowicz, G. and McCarthy, M.B. (1996). Response of human osteoblasts to implant materials: integrin-mediated adhesion. J. Orthop. Res. 14: 878-887.

15. Gronthos, S., Simmons, P.J., Graves, S.E. and Robey, P.G. (2001). Integrin-mediated interactions between human bone marrow stromal precursor cells and the extracellular matrix. Bone 28: 174-181.

16. Iuliano, D.J., Saavedra, S.S and Truskey, G.A. (1993). Effect of the confrontation and orientation of adsorbed fibronectin on endothelial cell spreading and the strength of adhesion. Journal of Biomedical Materials Research 27: 1103-1113.

17. Lampin, M., Warocquier-Clérout, R., Legris, C., Degrange, M. and Sigot-Luizard, M.F. (1997). Correlation between substratum roughness and wettability, cell adhesion, and cell migration. Journal of Biomedical Materials Research 36: 99-108.

18. Lautenschlager, E.P. and Monaghan, P. (1993).Titanium and titanium alloys as dental materials. International Dental Journal 43: 245-253.

19. Lincks, J., Boyan, B.D., Blanchard, C.R., Lohmann, C.H., Liu, Y., Cochran, D.L., Dean, D.D. and Schwartz, Z. (1998). Response of MG63 osteoblast-like cells to titanium and titanium alloy is dependent on surface roughness and composition. Biomaterials 19:2219-2232.

20. Ohgaki, M., Kizuki, T., Katsura, M. and Yamashita, K. (2001). Manipulation of selective cell adhesion and growth by surface charges of electrically polarized hydroxyapatite. Journal of Biomedical Materials Research 57: 366-373.

21. Okumura. A., Goto, M., Goto, T., Yoshinari, M., Masuko, S., Katsiki, T. and Tanaka, T. (2001). Substrate affects the initial attachment and subsequent behavior of human osteoblastic cells (Saos-2). Biomaterials 22: 2263-2271.

22. Parsons, J.T., Martin, K.H., Slack, J.K., Taylor, J.M. and Weed, S.A. (2000). Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. Oncogene 19: 5606-5613.

23. Petit, V. and Thiery, J.P. (2000). Focal adhesions: structure and dynamics. Biology of the Cell 92:477-494.

24. Riveline, D., Zamir, E., Balaban, N.Q., Schwarz, U., Ishizaki, T., Narumiya, S., Kam, Z., Geiger, B. and Bershadsky, A.D. (2001). Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by a mDia 1-dependent and ROCK-independent mechanism. The Journal of Cell Biology 153: 1175-1186.

25. Robey, P.G. (1989). The biochemistry of bone. Endocrinology and Metabolism Clinics in North America 18: 859-902.

26. Sastry, S.K. and Burridge, K. (2000).Focal adhesions: a nexus for intracellular signaling and cytoskeletal dynamics. Experimental Cell Research 261: 25-36.

27. Sommerfeldt, D.W., Kenneth, J.M., Clinton, T.R. and Hadjiargyrou, M. (2001). Differential phophorylation of paxillin in response to surface-bound serum proteins during early osteoblast adhesion. Biochemical and Biophysical Research Communications 285: 355-363.

28. Zamir, E. and Geiger. B. (2000). Molecular complexity and dynamics of cell-matrix adhesions. Journal of Cell Science 114: 3583-3590.

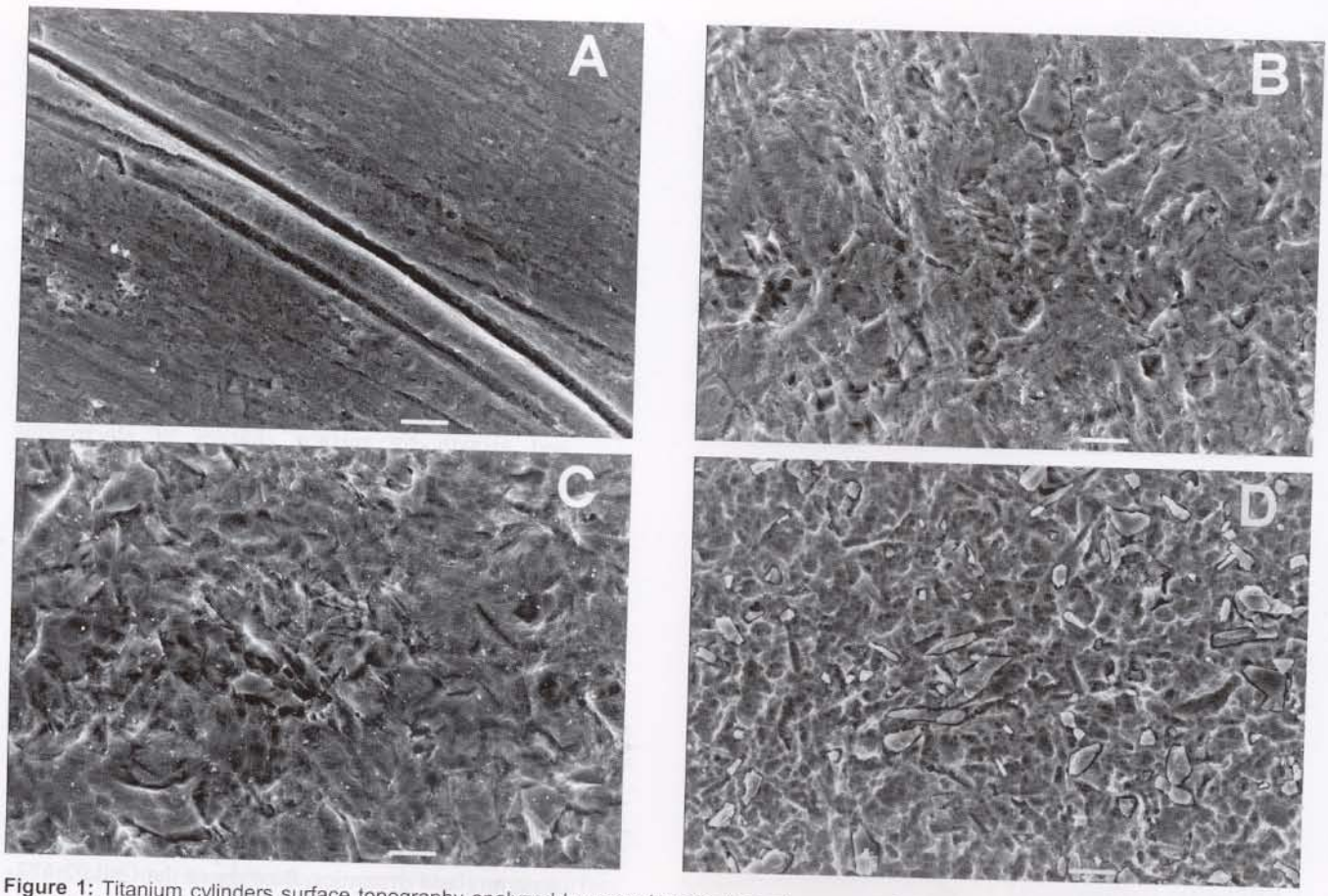

Figure 1: Titanium cylinders surface topography analyzed by scanning electron microscopy. A shows treatment type I ; B – type II ; C – type III ; and D – type II-Al. Bars=10μm

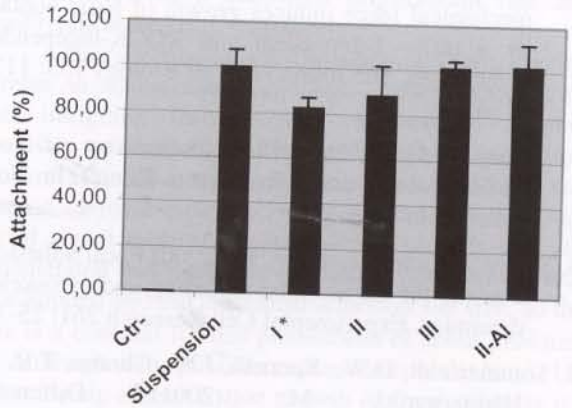

Figure 2: Cell attachment on titanium surfaces accessed by measurement of HOB cells pulsed with [^{3}H]-thymidine.

* Significantly different ($p<0,05$) from cell counts in suspension.

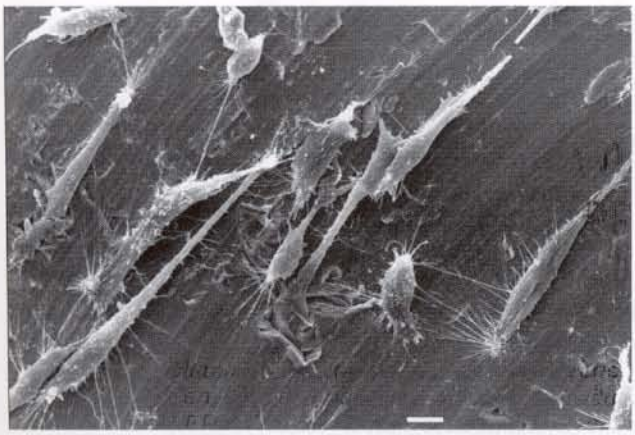

Figure 3: Cell morphology accessed by scanning electron microscopy of HOB cells cultured for 6h onto titanium cylinder with treatment type I. Bar=10μm.

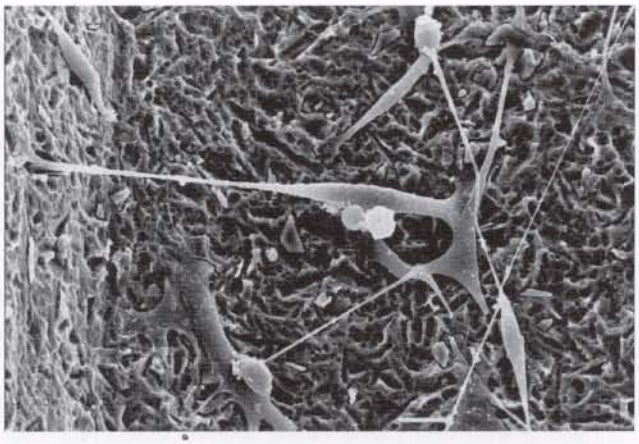

Figure 4: Cell morphology accessed by scanning electron microscopy of HOB cells cultured for 6h onto titanium dental implant with treatment type II-Al. Bar=10μm.

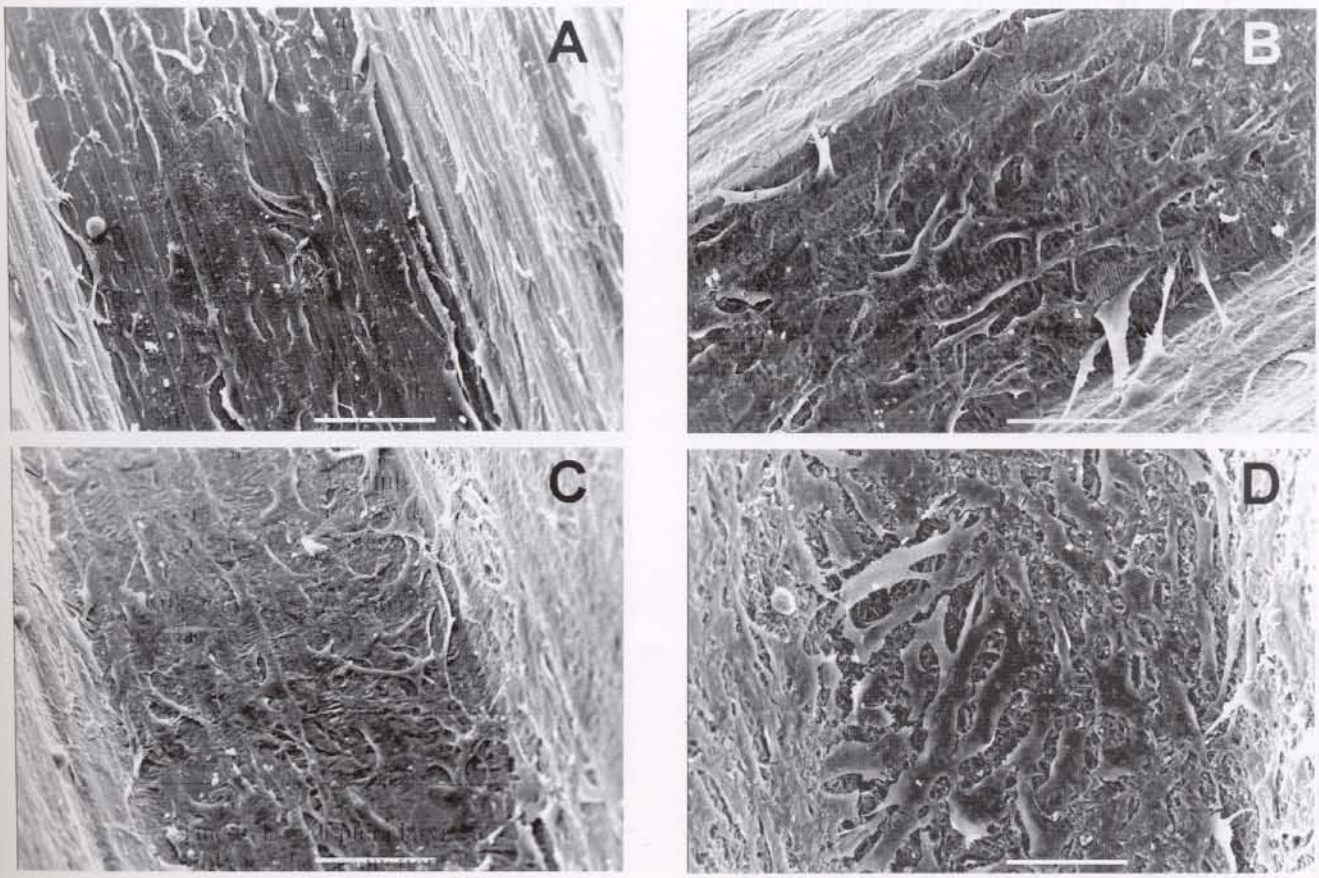

Figure 5: Cell morphology accessed by scanning electron microscopy of HOB cells cultured for 6h onto titanium dental implants. Panel A shows treatment type I ; B treatment type II ; C treatment type III ; and D treatment type II-Al. Bars=50μm