Human-osteoblast proliferation and differentiation on grit-blasted and bioactive titanium for dental applications

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Physico-chemical and topographical surface quality of commercially pure titanium (c.p. Ti) dental implants is one of the most influencing factors in the improvement of their osseointegration. In this sense, previously, a two-step method (2S) for obtaining bioactive blasted-rough titanium surfaces was developed for improving short-term (due to its bioactivity) and long-term (due to its roughness) osseointegration. This 2S-method consists of: (1) Grit blasting on titanium surface in order to roughen it, and (2) thermo-chemical (TCh) treatment in order to obtain a bioactive surface with bone-bonding ability. The aim of the present work is to evaluate the *in vitro* human-osteoblast response (proliferation, differentiation – ALP activity- and cell morphology-studied by environmental scanning electron microscopy) of rough c.p. Ti (grit blasted), bioactive c.p. Ti (thermo-chemically treated) and rough-bioactive c.p. Ti (2S-treated). Different grit materials (Al_2O_3 and SiC) have been used in order to investigate their influence. The results showed that cell adhesion was statistically higher for the rough and bioactive surfaces, whatever the grit used. Cells proliferated very well on all the c.p. Ti surfaces. If comparing groups with and without TCh (all other treatments being equal) the ALP was always higher in the groups with TCh, indicating stimulation of osteoblast differentiation because of TCh, more significantlly in the groups that were first blasted. Those ALP results were accompanied by a decrease in the value of proliferation, which shows the good behavior of the cells. This results suggest that a rough and bioactive-titanium surface obtained by 2S-treatment enhances adhesion and differentiation activity of human osteoblasts cells.

1. Introduction

Physico-chemical and topographical surface quality of commercially pure titanium (c.p. Ti) dental implants is one of the most influencing factors in the improvement of their osseointegration [1].

In terms of topographical quality, it is known that an increased implant-surface roughness significantly influences the osteoblastic response *in vitro* [2, 3]. Moreover, a better long-term *in vivo* response is achieved when the surface roughness increases since the percentage of implant in direct contact with bone increases as well as loads and torques for extracting implant from bone [4, 5].

Grit blasting is one of the most frequently used treatments for obtaining a rough surface of a dental implant [6], which forces different surface-roughness values mainly depending on grit size. However, adhesion of the grits on the implant surface because of the high-velocity impact may change physico-chemical surface quality [7, 8]. In this sense, the chemical composition of the grits may play a significant role in the *in vitro* and *in vivo* behavior of the implant [9, 10].

In terms of physico-chemical surface quality, it is known that c.p. Ti is widely used as a dental implant mainly because of its excellent corrosion behavior in the physiological environment. This is because of the extremely passive titanium oxide that spontaneously covers the metal [11, 12]. However, c.p. Ti is a bioinert material with nonbone-bonding ability, i.e., the interaction between the metal and the tissue does not involve a chemical bond with bone [13]. The lack of ability to bond chemically and the lack of rapid favorable guided reactions lead to the well studied osteointegration

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process which takes place at a slow rate. In recent years, Kokubo *et al*. [14] have demonstrated that an alkali and heat treatment process becomes titanium bioactive, and that an *in vitro* chemically deposited bonelike apatite on cp Ti could be induced if the treated surfaces are soaked in a simulated body fluid (SBF). The bioactive c.p. Ti improves the short-term *in vivo* bone reactions [15].

Taking into consideration the benefits of a rough surface and of a bioactive surface, in previous studies [16], a two-step method (2S) for obtaining bioactive blasted-rough titanium surfaces was developed for improving short-term (due to its bioactivity) and long-term (due to its roughness) osseointegration. This 2S-method consists of: (1) Grit blasting on titanium surface in order to roughen it, and (2) thermo-chemical treatment, developed by Kokubo *et al*. [14], in order to obtain a bioactive surface with bone-bonding ability. It was also demonstrated that the material of the grits has a significant effect on the *in vitro* bioactivity of the rough-titanium surfaces [16]; and that a 600-μm grit-size gives a surface roughness that improves cell response [10].

The aim of the present work is to evaluate the *in vitro* human-osteoblast response (proliferation, differentiation, and cell morphology) of rough c.p. Ti, bioactive c.p. Ti and rough-bioactive c.p. Ti obtained with the 2S-treatment. Different grit materials have been used in order to investigate their influence in the *in vitro* osteoblastic behavior.

2. Materials and methods

2.1. Surface preparation

Seven different groups with 28 c.p. Ti Grade II discs of 12 mm in diameter were prepared:

- Ct: polyethylene (negative control);
- Ti: as machined;
- Si: grit blasted with SiC;
- Al: grit blasted with Al_2O_3;
- Ti–2S: as machined + thermo-chemical treatment;
- Si–2S: grit blasted with SiC + thermo-chemical treatment;
- Al–2S: grit blasted with Al_2O_3 + thermo-chemical treatment.

The grit blasting was carried out with a laboratory grit-blasting machine at 2.5 MPa pressure during the time required for roughness saturation of the samples. The grits used for each sample series were those mentioned above with 600 μm in mean size in all cases.

After grit blasting the samples were ultrasonically cleaned in acetone and distilled water. Then the discs in Al and Si-groups were passivated with a 0.6 HCl solution for 30 s at room temperature, and immediately washed in distilled water.

The thermo-chemical treatment was an alkali and heat treatment described by Takadama *et al*. [17]. The plates were treated with 0.5 M NaOH aqueous solution at 60 °C for 24 h, washed gently with distilled water and dried at 40 °C for 24 h. Subsequently they were heated up to 600 °C at a rate of 5 °C/min in an electric furnace; kept at 600 °C for 1 h and then allowed to cool in the furnace. All the discs were sterilized with ethylene oxide.

2.2. Surface roughness

Surface roughness measurements were done with a 2D-profilometer with a diamond tip (Surftest SV-500©, Mitutoyo, Japan); and the profiles and R_a values were obtained and calculated using appropriate software (Surfpack v3.00©, Mitutoyo, Japan). R_a is the arithmetical mean deviation of the profile and is calculated as the arithmetical mean of the absolute values of the profile deviations from the mean line.

The main assay conditions were:

- Sampling length: 0.8 mm
- Number of sampling lengths: 3
- Evaluation length: 2.4 mm (0.8 mm × 3)
- Pre- and post-travel: 0.8 mm
- Filter type: Gaussian Standard
- Cut-off length: 0.8 mm
- Number of measurements per sample series: 9 (3 measurements × 3 samples)

2.3. Surface observation

The surfaces were studied by means of scanning electron microscopy (SEM) (6400©, Jeol, Japan) and environmental scanning electron microscopy (ESEM) (2020©, Electroscan, USA) in order to evaluate qualitatively the effect of the different surface treatments.

2.4. Thin film X-ray diffraction and X-ray dispersion energy

Thin film X-ray diffraction (TF-XRD) (X'Pert MPD©, Philips, The Netherlands) were carried out in order to evaluate the compositional changes in the surface of the c.p. Ti treated with the thermo-chemical treatment.

X-ray dispersion energy (EDS) was used for surface chemical composition measurements of the grit-blasted surfaces (Analytical LZ-5©, Link, UK).

2.5. Cell cultures

Cells were cultured as previously described [18]. Briefly, human bone cells were obtained from the surgical specimens of three patients, two women aged 69 and 74 years and one man aged 68 years, all undergoing surgery for degenerative joint disease. Osteoblasts were obtained from 1 to 2 mm explants of trabecular bone maintained between two glass rings and cultured in supplemented medium with 20% fetal calf serum. About a week later, osteoblasts started to grow out from the explants over the mesh. Meshes were completely covered by osteoblasts after 3–4 weeks. Cells were isolated using 0.25% trypsin and then subcultured together to make a pool from the three patients. Osteoblasts were characterized by alkaline phosphatase activity and osteocalcin synthesis in response to stimulation with $1,25(OH)_2D_3$. In all cases, cells stained intensely using a histochemical reaction for alkaline phosphatase and were found to secrete osteocalcin after stimulation with $1,25(OH)_2D_3$.

The cells were seeded in 24-well plates at a density of 1.2×10^4 cells/well. Incubation was carried out in a complete Iscove's modified Dulbecco's medium

(IMDM; Gibco, Grand Island, NY) supplemented with 10% FCS and 1% penicillin/streptomycine in a humidified atmosphere with 5% CO_2 at 37 °C. The medium was changed twice a week.

Cells were cultured on negative control samples and on the studied surfaces for 14 days and were characterized at defined time intervals.

2.6. Human-osteoblast proliferation

Cell count (CC) was carried out at days 1, 3, 7, and 14 of incubation. Each specimen was washed with phosphate buffered saline solution (PBS) and the cells were detached with the addition of 250 µl of 0.25% Trypsin–EDTA (Gibco, Grand Island, NY). CC was carried out in a Newbauer chamber.

2.7. Human-osteoblast differentiation

On day 14, medium was removed and fresh with FCS-free IMDM addition. After 24 h media was replaced and cells were incubated for further 72 h with IMDM + 3% charcoal stripped FCS (Gibco, Grand Island, NY) and each well supplemented with vitamin K (10^{-8} M) and vitamin C (50 µg/ml). 1,25$(OH)_2D_3$ (10^{-8} M) (Roche, Basel, Switzerland) was added to half of the samples (groups + D) and the other half were not supplemented with this vitamin (groups – D).

After 72 h of incubation, cell differentiation was determined by means of measurement of alkaline phosphatase activity (ALP). The ALP was determined in cell lysates obtained by treatment of the cultures with 25% of Triton-100 according to the method described by Bessey *et al*. [19]. This method consists of hydrolysis of p-nitrophenyl phosphate in alkaline buffer solution and colorimetric (yellow) determination of the product (p-nitrophenol) at $\lambda = 410$ nm.

Enzymatic activity was measured as equivalent units of the release of 1-µmol p-nitrophenol per minute. ALP has been determined as activity units per mg of protein. Protein was measured using a fluorimeter (Bio-Rad Laboratories, San Jose, CA).

2.8. Human-osteoblast morphology

The observation of cell morphology on the different substrates was studied by means of environmental scanning electron microscopy (ESEM). For that reason, the observations were carried out using the Peltier stage at a chamber presssure of 5 Torr (water vapor) and at 4 °C. This method enabled the cells to remain practically hydrated. The potential used in the ESEM was 5–10 keV to keep any damage caused by the electron beam to a minimum. The results were only partially satisfactory, since the cells were damaged during prolonged exposure times. For this reason it was decided to fix the cells with 4% paraformaldehyde for 5 min, with no other type of preparation.

2.9. Statistical analysis and figures

All statistical measurements and tests were made with appropriate software (Minitab Release 11©, Minitab Inc., USA). *t*-Student tests, ANOVA- tables and Tuckey's multiple-comparison tests were carried out in order to evaluate statistically significant differences between sample groups.

Figures summarizing cellular response were made with appropriate software (Origin© 6.1, OriginLab, USA).

3. Results

3.1. Surfaces description

The surface roughness (R_a-values) results are summarized in Table I. As expected, all the grit-blasted surfaces had significantly higher values than the nonblasted surfaces. Besides, surface roughness for all the blasted surfaces, whatever the material of the grit, were very similar. The c.p. Ti surfaces with grit-blasting + thermo-chemical treatment had very similar surface roughness compared with those without the additional thermo-chemical treatment.

An example of a SEM-image for a Al_2O_3-grit blasted c.p. Ti surface is shown in Fig. 1. The particles adhered on the surface are clearly seen. The nature of the grits was determined according to the existence of a high aluminum peak in the EDS-spectrum of the surface. Similarly, the SiC-grit blasted c.p. Ti surfaces had adhered SiC particles and a high silicon peak was obtained in their EDS-spectrum.

The ESEM-image of a Ti–2S sample is shown in Fig. 2. The topographical effect of the thermo-chemical treatment can be seen. The fine skeletal configuration of the topography and the TF-XRD pattern (Fig. 3) confirm the existence of a sodium titanate gel on the surface, which is essential in order to give to the c.p. Ti the bioactive behavior. Similar ESEM-images and TF-XRD

TABLE I Mean R_a-values and standard deviation (S.D.) for all the sample-groups

Group	Ti	Al	Si	Ti–2S	Al–2S	Si–2S
R_a [µm]	0.07	3.99	3.66	0.07	3.93	3.59
S.D. [µm]	0.02	0.31	0.27	0.03	0.21	0.21

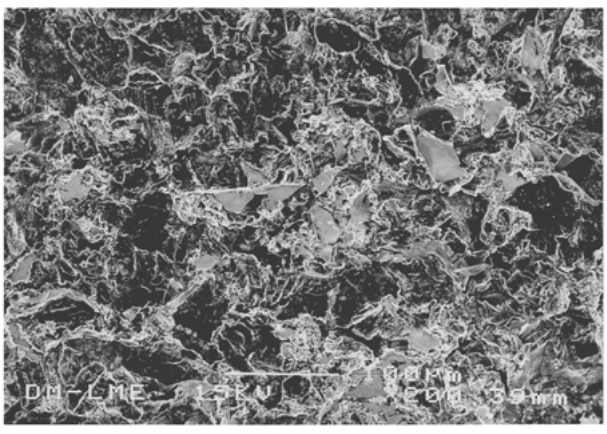

Figure 1 SEM-image of a rough Al-sample. The adhered Al_2O_3-grits can be clearly seen.

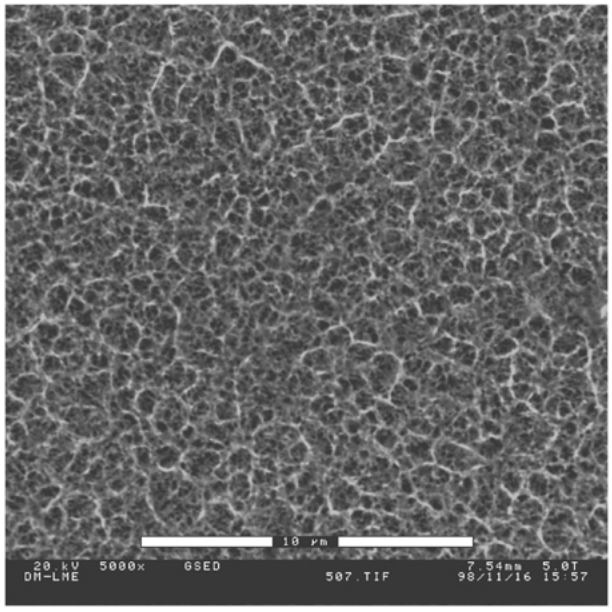

Figure 2 ESEM-image of a Ti–2S sample showing the fine skeletal topography obtained after thermo-chemical treatment.

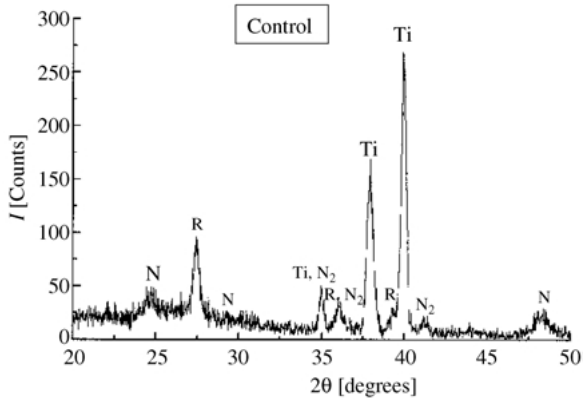

Figure 3 X-ray diffraction pattern of a Ti–2S sample. Ti, -titanium; N, N_2; sodium titanate ($Na_2Ti_5O_{11}$); *R*-rutile (TiO_2).

results were obtained in the samples of Al–2S and Si–2S groups.

3.2. Cell proliferation

The proliferation results of the cultures on sample-groups with and without the thermo-chemical treatment are summarized in Figs. 4 and 5 respectively. Mean and standard deviation of CC at each studied time are plotted.

The cultures on the negative control samples (Ct) proliferated adequately all over the studied time.

3.3. Cell differentiation

ALP activity results of all the studied sample-groups are summarized in Fig. 6. Mean and standard deviation of the osteoblastic differentiation values are represented. In all cases, the ALP activity in the same sample group was higher for (+ D) groups than for (− D) groups.

3.4. Cell morphology

ESEM-images of cultured cells on all the studied surfaces were not obtained probably because of sample preparation, which is discussed later on. Examples of the ESEM-images obtained are Figs. 7 to 9, which show osteoblasts on Al, Si, and Al–2S samples, respectively.

4. Discussion

The results of surface roughness (Table I) showed that statistically significant differences in R_a-values (p-value > 0.05) were not found comparing surfaces with or without the thermo-chemical treatment (all other treatments being equal). Moreover, the samples grit blasted with Al_2O_3 particles (Al and Al–2S) have a slightly higher surface roughness than those grit blasted with SiC particles (Si and Si–2S) but the differences were not statistically significant in any case (p-value > 0.05). All these results suggest that the main differences between all the grit-blasted surfaces were due to the different chemical compositions. The adhered grits on the surfaces, confirmed by the SEM and EDS results (Fig. 1), and bioactive sodium titanate on the c.p. Ti–2S surfaces (Fig. 3) were the major factors influencing cell performance on the blasted surfaces. However, the fine skeletal topography obtained with the thermo-chemical treatment must also be taken into account (Fig. 2) despite differences in surfaces roughness not being found. This is probably because the technique used for measuring surface roughness was not accurate enough.

Cells proliferated very well on all the c.p. Ti surfaces (Figs. 4 and 5). Significant differences at days 3 and 7 between the different studied groups were not found. However, CC at day 1, which is a measure of the initial osteoblastic adhesion on the substrates, is statistically higher (Tukey's test) for the groups Si–2S and Al–2S compared with all the studied surfaces. This result may suggest that a rough and bioactive surface stimulates the adhesion of the cells due to an accumulative effect between the increase in surface roughness and the highly reactive surface of a thermo-chemically treated c.p. Ti. In fact, it is known that surface energy may influence protein adsorption and the structural rearrangement of the proteins on the surface [20], and the protein adsorption and conformation directly influence the initial steps in attachment and adhesion of the cells [21]. Both surface roughness and the bioactive-c.p. Ti influence surface energy significantly. The fine skeletal morphology of the bioactive surfaces may also play an important role in protein adhesion because roughness

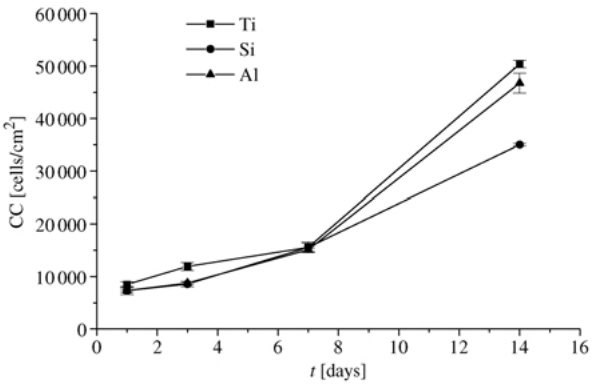

Figure 4 Cell proliferation curves for the surfaces that were not treated with the thermo-chemical treatment.

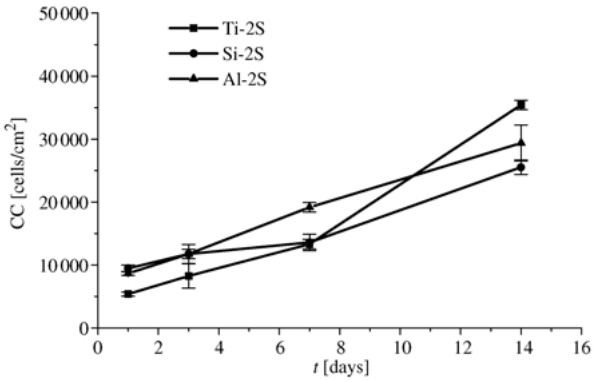

Figure 5 Cell proliferation curves for the surfaces that were treated with the thermo-chemical treatment.

features are in a low scale. Others [22] suggested that a hydrophobic surface enhances cell attachment because of the hydrophobic character of osteoblasts, but our results do not confirm this hypothesis because the rough and bioactive-c.p. Ti is highly hydrophilic, with contact angles near zero (nonpublished results).

Proliferation between day 7 and 14 was statistically lower for rough surfaces compared to the smooth surfaces and for thermo-chemically treated surfaces compared to the nonthermo-chemically treated. Controversial proliferation results between studies carried out by different authors on similar substrates have been found, as was clearly and extensively reviewed by Anselme [2]. As a consequence, correlation between surface quality and osteoblast proliferation is difficult. Probably, proliferation is a direct consequence of the differentiation response of the cells because a reciprocal relationship between proliferation and differentiation has been described in the development of the osteoblast phenotype [23]. The cell-response results (Figs. 4–6) revealed that the lower the osteoblastic proliferation, the higher the osteoblastic differentiation, which shows the good behavior of the cultured cells.

If comparing groups with and without the thermo-chemical treatment, i.e. bioactive or nonbioactive, with all other treatments being equal, then the ALP-activity (Fig. 6) was always higher (statistically significant, Tuckey's test) in the thermo-chemical treated surfaces, indicating stimulation of human-osteoblast differentiation because of the bioactive surfaces. This result confirms the conclusions of other authors [24].

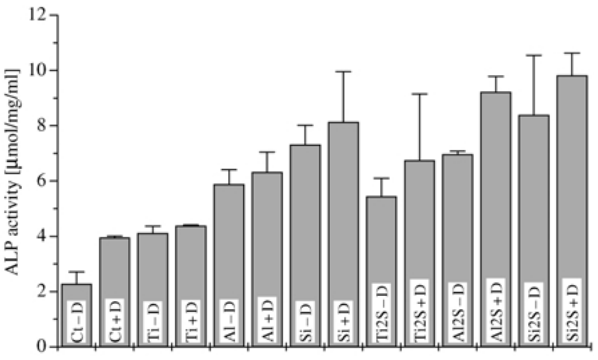

Figure 6 ALP activity results for all the studied groups, including the different groups containing (+ D) and noncontaining (− D) vitamin D.

Additionally, in this study, specially significant differences have been found between the groups of samples that were grit blasted + thermo-chemical treated (Al–2S and Si–2S) compared to those that were only thermo-chemical treated (Ti–2S). This observation demonstrates again that an accumulative effect on the osteoblast response is produced between the roughness and the bioactive surface.

The formation of the extracellular matrix is the last event of the osteoblastic differentiation and essential to the osseointegration of the implant, that is, the bone formation at the bone/biomaterial interface [25]. The enhanced osteoblast differentiation in the Al–2S and Si–2S surfaces on day 14 of culture may be an indicator of the more rapid formation of a mineralized extracellular matrix [25]. As a result, the short-term osseointegration of the material could be accelerated because of the grit blasting and thermo-chemical treatment.

Statistically significant differences (ANOVA-table, $p < 0.05$) have been found between the samples grit-blasted (Al and Si) and the as-machined c.p. Ti (Ti). This result revealed influence of surface roughness on osteoblast differentiation, which has been evidenced by others [26].

The material of the grit did not negatively influence on cell reponse whatever the grit material used. Others concluded the same for Al_2O_3-grit blasted c.p. Ti surfaces [25]. Besides, the material of the grit did not significantly influence (*t*-Student test, $p > 0.05$) the ALP-activity, neither in the grit blasted surfaces (Al vs. Si) nor in the grit blasted + thermo-chemically treated surfaces (Al–2S vs. Si–2S). However, the surfaces grit blasted with SiC particles had mean ALP-activity values higher than those blasted with Al_2O_3-particles. This suggests that, although nonstatistically significant, there is a different influence in cell response depending on the material of the grit, which could be related to differences in the surface energy because of differences in the chemical surface heterogeneities [2], differences in the texture of the surface (nondetermined by the roughness measurements) [26] or even differences in the residual compression stresses induced by the blasting treatments [27]. Moreover, this is an interesting result because the presence of SiC-particles on grit blasted and thermo-chemically treated c.p. Ti surfaces inhibited *in vitro* bioactivity of the metal [16]. Consequently, a nonbioactive behavior *in vivo* for Si–2S samples could be expected. However, in the present work the human-osteoblast showed an excellent behavior cultured on Si–2S samples. These are facts that can be taken into account when deciding the material of the grit.

The value of ALP-activity in + D groups is higher than in − D groups, with all the surface treatment being equal. However, these differences are not statistically significant (*t*-Student test, $p > 0.05$). The higher values for + D groups suggest an appropriate cell behavior because D vitamin stimulate cell differentiation, but the synergistic effect between the vitamin and surface roughness [28] was not clearly shown.

The complete study (all sample-groups and all studied times) of cell morphology was not carried out because difficulties in the preparation of the ESEM-samples were found. The observation of cells on substrates in special

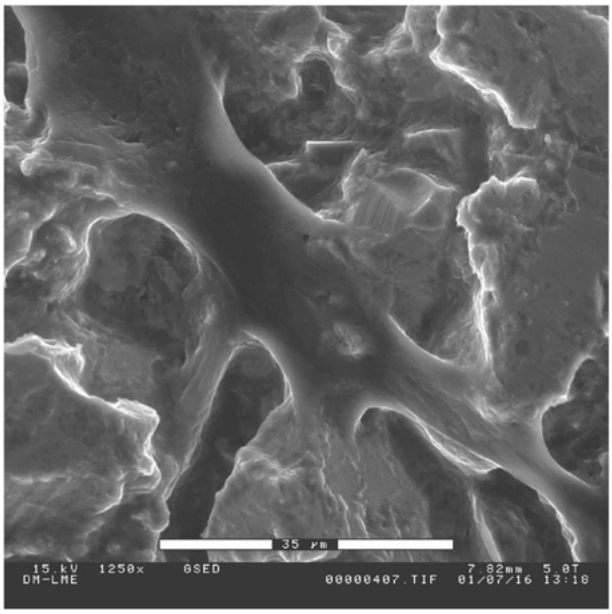

Figure 7 ESEM-image showing an osteoblast on an Al-surface after 7 days of culture.

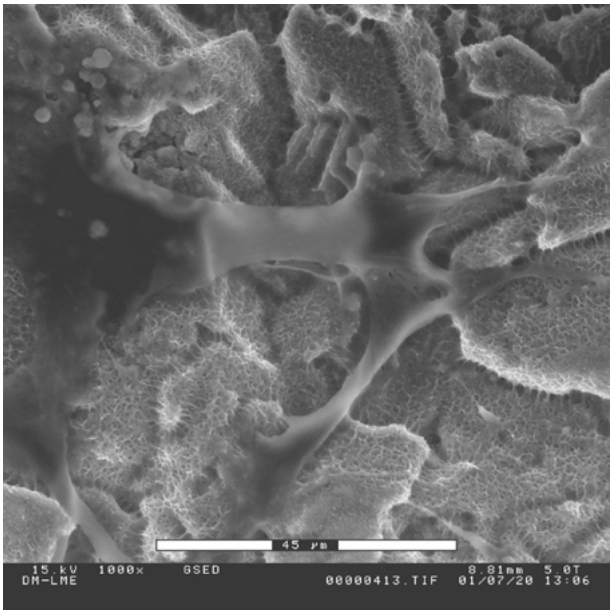

Figure 9 ESEM-image showing an osteoblast on an Al–2S surface after 3 days of culture.

conditions is allowed with ESEM technique compared to those needed with SEM. The special conditions consist on that dehydration, critical point and gold coating are not required. In fact, in a previous study with osteoclasts [29], fixation with paraformaldehyde for 5 min was enough in order to obtain high-resolution and clear ESEM-images. These conditions were reproduced in the present study, but the results were not as satisfactory as previously, which implies that the technique for sample preparation must be improved if the cultured cells are osteoblast. However, Figs. 7 to 9 are the examples that demonstrate the ESEM-images of osteoblasts with a rapid sample preparation could be obtained. The observation of cells on rough surfaces, including rough and bioactive surfaces (Fig. 9), was easier because the lack of adherence of the cells on the substrates after ESEM-sample preparation is reduced. This is because cells adhered on rough surfaces adapted to the irregularities with filopodia extensions (Figs. 7 to 9), which makes a tight adhesion for the cells. All the cells shown in Figs. 7 to 9 exhibit cell activity.

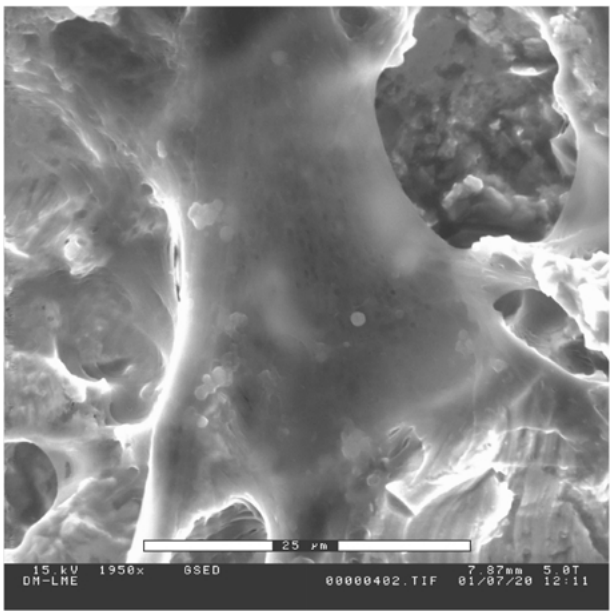

Figure 8 ESEM-image showing an osteoblast on a Si-surface after 7 days of culture.

5. Conclusions

A rough and bioactive-c.p. Ti surface obtained by a grit blasting + thermo-chemical treatment, provided enhanced adhesion and differentiation of human osteoblast cells. This fact may play an important role in a rapid formation of the extracellular matrix and, consequently, in an accelerated short-term osseointegration.

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