Eklem Hastalkları ve Cerrahisi
Joint Diseases and Related Surgery

Joint Dis Rel Surg
2008; 19(2):84-90

Experimental Study / Deneysel *Çalışma*

Interaction of MC3T3-E1 cells with titanium implants

MC3T3-E1 hücrelerinin titanyum implantlarla etkileşimi

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Objectives

The aim of the present study was to evaluate in-vitro MC3T3-E1 preosteoblastic cell osseointegration on surfaces of polished, sand-blasted (smooth and rough) and sodium titanate coated titanium alloys.

Materials and methods

MC3T3-E1 cell proliferation and mineralization was assessed comparatively on polished, sand-blasted (smooth and rough) and sodium titanate coated titanium alloys. Cell morphology, attachment and proliferation were also comparatively evaluated using confocal (CM) and scanning electron microscopy (SEM).

Results

All implants used in this study were biocompatible. Cells started to attach on the surfaces of the implants following exposure to the in vitro medium for 3 days. The cells were viable and metabolically active as observed by CM. Cell population increased exponentially from day 3 to day 22. Proliferation rate was highest on polished surfaces and lowest on sodium titanate-coated surfaces. In contrast, mineralized nodules were numerous on sand-blasted and sodium titanate-coated surfaces when compared to the polished ones on day 30.

Conclusion

This study demonstrated that sand-blasting and sodium titanate coating provided by NaOH favored the attachment, mineralization and early differentiation of osteoblasts on titanium alloys.

Key words: Titanium, Implant, Bone, Osteoblast cell culture, MC3T3-E1, Scanning electron microscopy, Confocal microscopy.

Amaç

Çalışmanın amacı in vitro koşullarda MC3T3-E1 osteoblast öncülü hücre serisinin kemiğe integrasyonunu parlatılmış, ince veya kalın kumlama yapılmış ya da sodium titanat ile kaplanmış titanium implant yüzeylerinde karşılaştırmalı olarak değerlendirmektir.

Gereç ve yöntemler

Parlak, ince, kalın kumlama yapılmış ve sodium titanatla yüzey kaplaması uygulanmış titanium implantlar üzerine uygulanan MC3T3-E1 osteoblast öncülü hücrelerde karşılaştırmalı olarak yüzeye tutunma, çoğalma ve mineralizasyon hızları saptandı. Hücre morfolofisi, yapışma ve canlılık taramalı elektron mikroskobu ve konfokal mikroskop ile değerlendirildi.

Bulgular

Bu çalışmada kullanılan tüm implantlar doku ile uyumludur. Hücreler titanium yüzeylere deneyin 3. gününden itibaren tutundu. Bu hücrelerin konfokal mikroskopta canlı ve metabolik olarak aktif davranışa sahip oldukları gözlendi. Hücre sayısı 3. günden 22. güne belirgin olarak arttı. Çoğalma hızı parlak yüzeylerde en yüksek, sodium titanat kaplı olanlarda en düşük olarak saptandı. Diğer yandan 30. günde mineralize nodüller ince ve kalın kumlama yapılmış ve sodium titanat kaplı yüzeylerde daha çok sayıda izlendi.

Çıkarım

Bu çalışma ince ve kalın kumlama ile sodium titanat yüzey kaplamasının osteoblastların titanium yüzeyine tutunma, mineralizasyon ve erken farklanmalarını uyardığını göstermektedir.

Anahtar sözcükler: Titanyum, İmplant, Kemik, Osteoblast hücre kültürü, MC3T3-E1, Taramalı elektron mikrokopi, Konfokal mikroskopi.

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Biomaterials of various shapes and forms have been widely used in musculoskeletal reconstruction and repair. Steel, cobalt–chrome and titanium in pure and/or alloy form are currently used in bone and joint replacement. Although extensively used in daily practice, research to improve the biocompatibility of these metals is still going on as they will nevertheless cause tissue response when implanted into bone[1]. Biocompatibility of metals depends on their type, production technique, composition, mechanical properties, structure and surface geometry. Surface area modification of titanium implants may significantly enhance cell attachment and differentiation.[1]

In a recent study mirror-polished, alumina-blasted, sand-blasted, biphasic calcium phosphate grit-blasted and acid etched titanium surfaces exhibited similar osteoblastic cell attachment in vitro.[2] In another study, proliferation and matrix mineralization of MC3T3 osteoblastic cells reduced significantly on micro textured titanium, compared to polished titanium surfaces.[3] Initial cell attachment and early proliferation are affected by crystallographic texture of the substrate while late preosteoblast differentiation indicated less dependence on the texture of the material.[4] In a recent study, silica nanoparticles were functionalized by apropylsemicarbazide moiety by silanization prior to deposition onto titanium surfaces. MC3T3-E1 osteoblasts cultured on these surfaces revealed an excellent cytocompatibility as shown by the assessment of cell viability, vitality and morphology.[5] Although the effects of surface modification of titanium alloys are partially known, there is little information on the effects of novel surface modifications such as sodium titanate coating and sand-blasting. It is assumed that sodium titanate coating and sand-blasting might improve surface properties of titanium alloys.

The aim of the present study was to evaluate in-vitro MC3T3-E1 cell osseointegration on polished, sand-blasted (smooth and rough) and sodium titanate coated titanium alloys. MC3T3-E1 cell proliferation and mineralization was assessed. Cell morphology and viability were evaluated using confocal (CM) and scanning electron microscopy (SEM).

MATERIALS AND METHODS

Design

In this prospective randomized controlled experimental in vitro study, independent variables were groups (n=4) and time (n=3, 8, 11 and 15 days for the proliferation and n=30 days for the mineralization tests). Dependent variables were proliferation, mineralization, cell morphology and viability tests using CM and SEM.

Preparation and surface modification of titanium alloys. Commercially available titanium alloy Ti6Al4V (ISO 5832-3, ASTM F67) was cut into 1.0 mm thick discs of 10 mm diameter. These discs were divided into four experimental groups. The first group was directly used without surface modification. The second group was electro-polished. The third and fourth groups were sand-blasted using 100 μm and 300 μm size alumina (Al_2O_3) particles to develop smooth or rough surfaces were used. The surface of the fifth group was coated with sodium titanate. For this purpose, the Ti6Al4V alloy discs were immersed in 5N NaOH solution for 48 hours at 60°C and then treated thermally at 600°C for 2 hours. All discs were mechanically cleaned and gamma sterilized for tests.

Surface characterization. Implants were evaluated with a JSM-6400 electron microscope (JEOL) equipped with the NORAN 6 X-ray Microanalysis System and Semafore Digitizer. Surface properties were recorded and EDS analysis was conducted.

MC3T3-E1 cell culture. MC3T3-E1 (Subclone 4), an immortalized cell-line kindly provided by Dr. Martha J. Somerman from the University of Washington and Renny T. Franceschi from the University of Michigan that was derived from newborn mouse calvaria was used to evaluate osseointegration, cell proliferation, mineralization, cell morphology and viability.[6, 7] Cells were plated in 60 mm culture dishes. Implants and cells at 5x104 cells/cm2 were co-cultured. They were allowed to adhere for 3 days in α-MEM with 10% fetal bovine serum, 1 % penicillin-streptomycin and L-glutamine at 370C in a humidified atmosphere of 5% CO2 in air. The medium was replaced at every 2 days. Presence of cells on metal surfaces was examined with an inverted microscope at 3, 8, 13, 17 and 22 days.

Proliferation and mineralization tests. Cell proliferation tests were performed on 3, 8, 11 and 15 days. Cell number was determined after tripsinization of cells using a hemacytometer on a Neubbauer glass. For proliferation tests, cells and implants were transferred to 24 well plates and cultured. For each time point, three samples were investigated and the average number of cells was calculated.

Similar density of cells were seeded on the implant surfaces and placed in 24-well plates for the mineralization assay. The cells and implants were incubated within mineralization media consisting of 10 % fetal bovine serum, ascorbic acid (50 μg/ml) and α-glycerophosphate (10 mM) in α-MEM. Mineralized nodules were visualized by von Kossa staining on day 30.[8]

Evaluation of cell morphology.

Confocal microscopy (CM)

For CM evaluation, live cells on different titanium surfaces were incubated for 2 hours at 37oC with dialkylcarbocyanine probe DiL (Invitrogen 10 μg /ml in serum-free α-MEM culture medium). After

washing in 0.01 M phosphate buffered saline (PBS), cells and implants were fixed for 15 minutes in 2.5 % gluteraldehyde (in PBS). Than, they were washed with PBS, transferred onto glass bottom flasks, and examined. A confocal laser scanning microscope (LSM Pascal, Zeiss Germany) was used for fluorescent imaging. 543 nm laser-line was used for excitation and a 560 nm barrier filter was used for collecting the emitted fluorescence.

Scanning electron microscopy (SEM)

For SEM evaluation, cells and implants were fixed for 15 minutes with 2.5 % gluteraldehyde. After removal from their petri compartment, the samples were dried and sputter-coated with gold. The SEM study was conducted with a JSM-6400 Electron Microscope (JEOL), equipped with the NORAN 6 X-ray Microanalysis System and Semafore Digitizer.

Statistics. Multivariate analysis of variance (MANOVA) was carried out to define group and time interaction. When significant values were obtained, the Tukey HSD comparison test was used to define the source of difference. Proliferation tests were triplicate at each time point and the average was taken into consideration. At histological, CM and SEM analyses, two independent blind observers evaluated and scored cell morphology and viability. Significance was set at alpha equal to or smaller than 0.05.

RESULTS

Surface Characterization by SEM

The electronmicrographs shown in Figure 1 correspond to those of polished, smooth and rough sand-blasted and sodium titanate coated surfaces, respectively. The topography of the implants presented enhanced bioactivity. EDX analysis that was performed at 2000x magnification revealed the presence of the alkaline titanate layer on the surface from day 1. The EDX patterns of the different titanium surfaces are presented in Figure 2a-d.

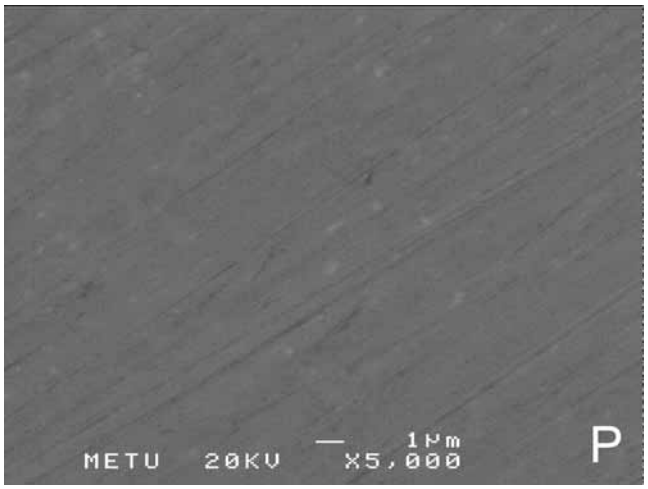


Figure 1 a.

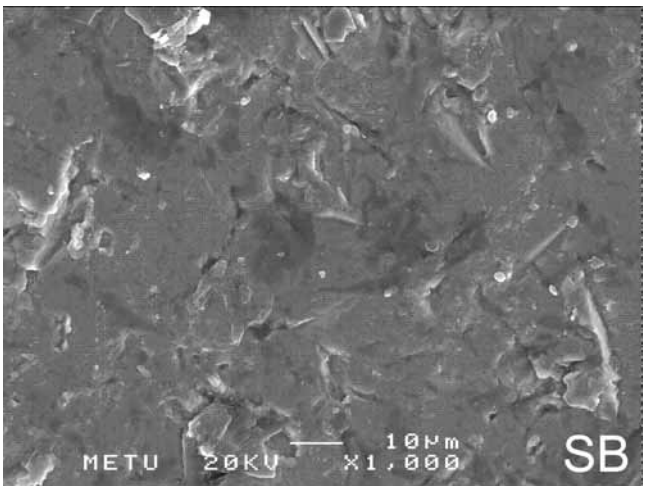


Figure 1 b.

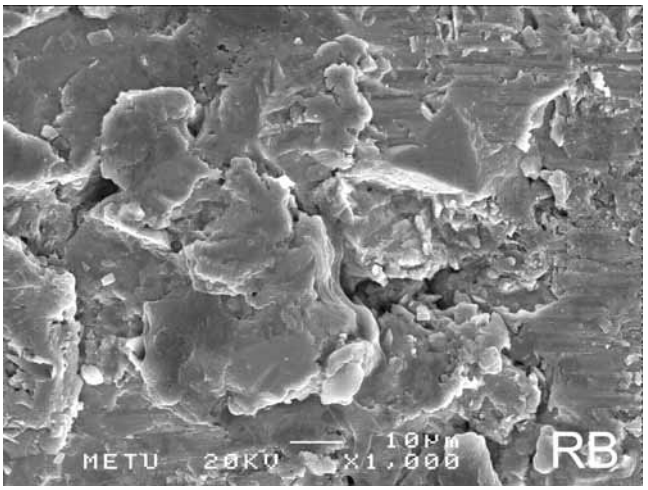


Figure 1 c.

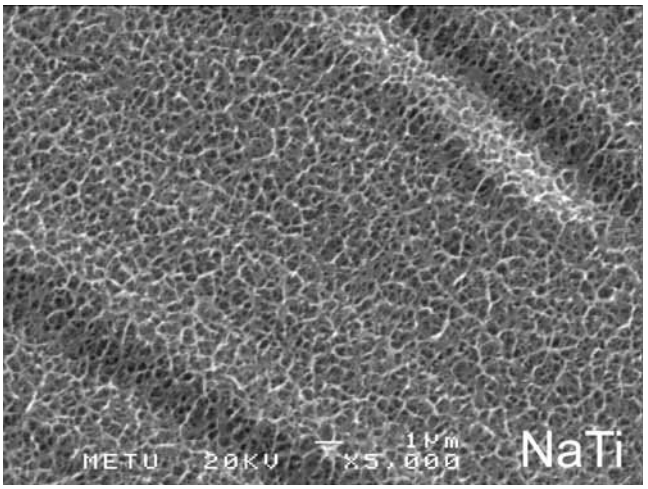


Figure 1 d.

Figure 1: Morphology of the different titanium surfaces at SEM. P: Polished, SB: Smooth sand-blasted, RB: Rough sand-blasted, and NaTi: Sodium titanate coated.

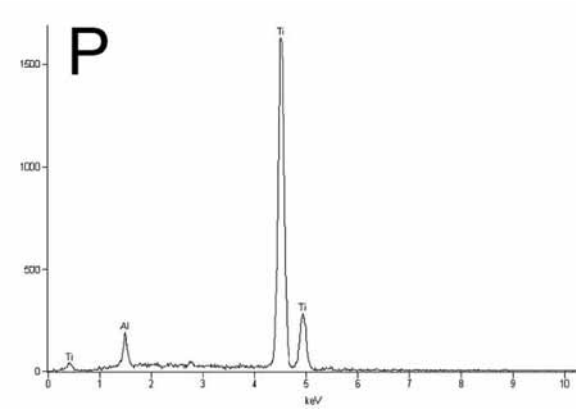
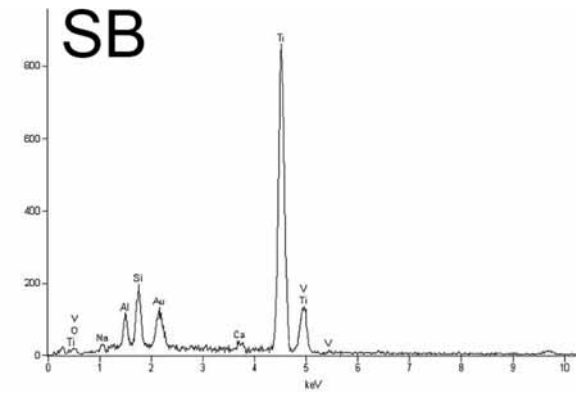


Figure 2 a.



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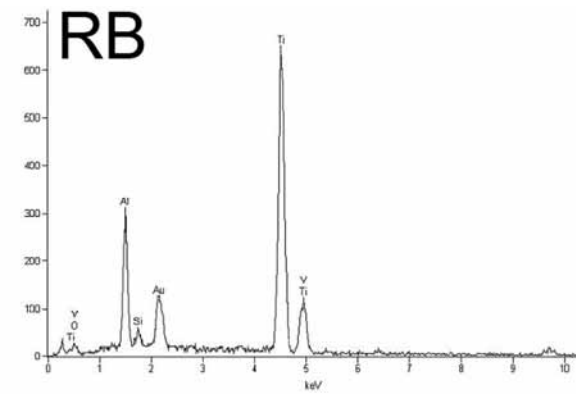

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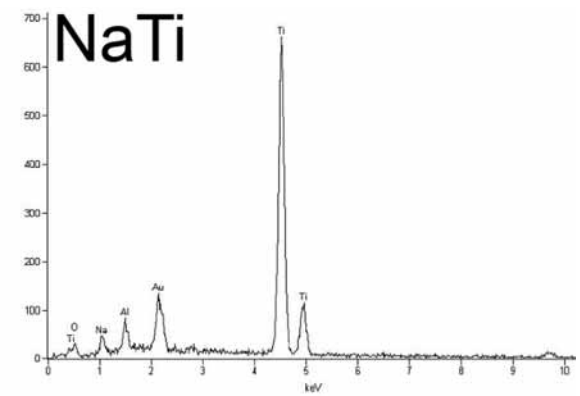


Figure 2 d.

Figure 2. EDX patterns of the titanium surfaces P: Polished, SB: Smooth sand-blasted, RB: Rough sand-blasted, NaTi: Sodium titanate coated.

They correspond to those of polished, smooth sand-blasted, rough sand-blasted and sodium titanate coated surfaces, respectively.

Proliferation and mineralization tests

The MC3T3 osteoblastic cells that were seeded on titanium discs with different surface modifications are presented in Figure 3. Implants were biocompatible and cells grew on their surfaces. Cells formed multiple layers on the surfaces of the implants however their detailed morphology could not be investigated as the

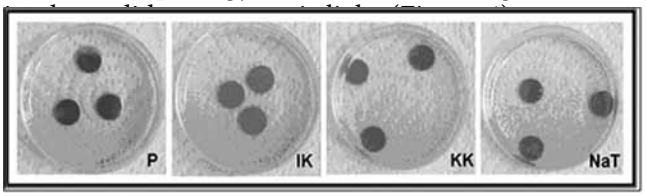


Figure 3: MC3T3 osteoblastic cell on the implants. P: Polished, SB: Smooth sand-blasted, RB: Rough sand-blasted, NaTi: Sodium titanate coated.

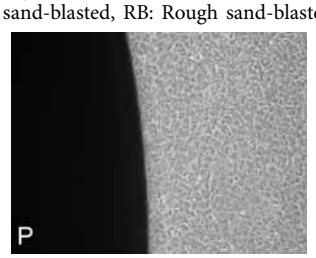
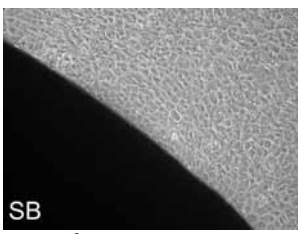


Figure 4 a.



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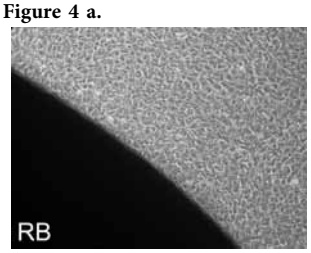

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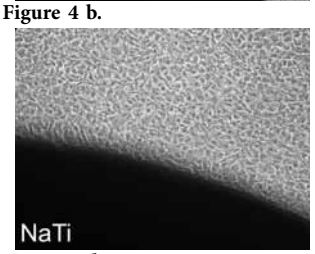


Figure 4 d.

Figure 4. The appearance of MC3T3 osteoblastic cells on different titanium surfaces under inverted microscope. P: Polished, SB: Smooth sand-blasted, RB: Rough sand-blasted, NaTi: Sodium titanate coated.

Proliferation assays revealed the highest cell population on polished surfaces on days 3, 8 and 15 when compared to other groups. The smooth and rough sand-blasted groups presented better proliferation when compared to the sodium titanate group in which the proliferation rate was minimal (Figure 5).

Mineralization assay revealed the presence of numerous more nodules on sodium the titanate coated and sand-blasted surfaces when compared to the polished surfaces (Figure 6).

Histology

Scanning Electron Microscopy

Cells attached to the metals beginning at day 3. Osteoblasts migrated into the grooves of the smooth sand-blasted, rough sand-blasted and sodium titanate coated surfaces of implants. They secreted their extracelluar matrix on the surfaces beginning at day

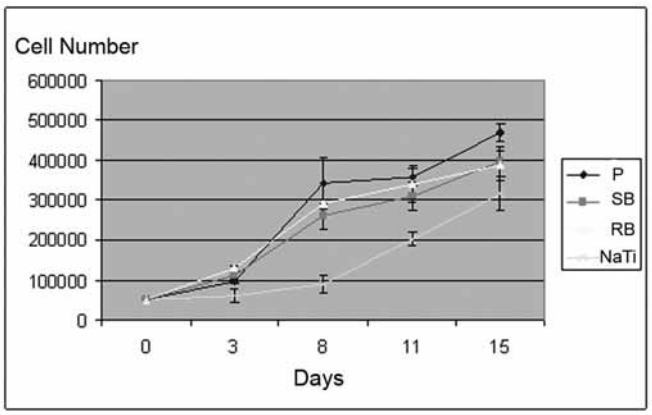


Figure 5. Proliferation tests of osteoblastic cells on the different titanium surfaces on days 3, 8, 11, and 15. P: Polished, SB: Smooth sand-blasted, RB: Rough sand-blasted, NaTi: Sodium titanate coated.

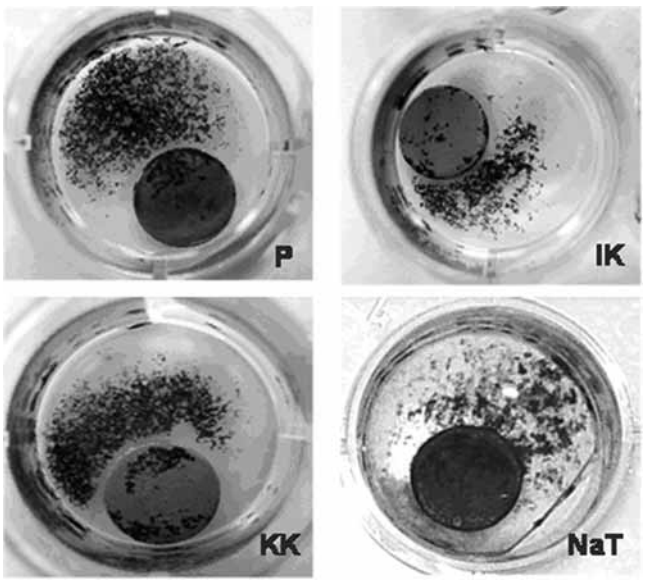


Figure 6. Mineralized nodules of osteoblastic cells on day 30. Note the presence of nodules on the sand-blasted and the sodium titanate-coated surfaces. P: Polished, SB: Smooth sand-blasted, RB: Rough sand-blasted, NaTi: Sodium titanate coated.

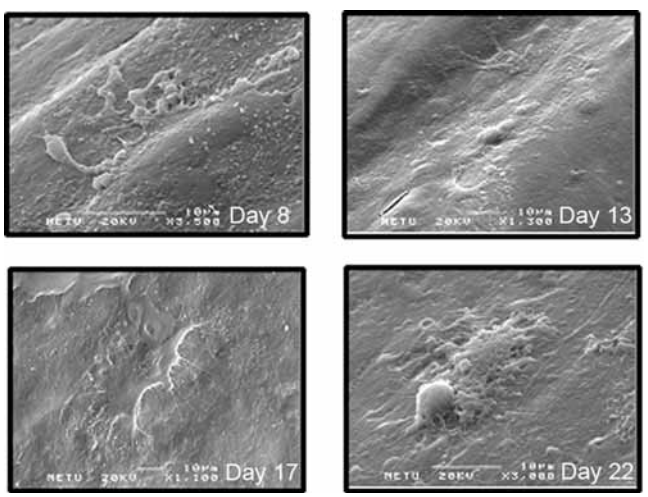
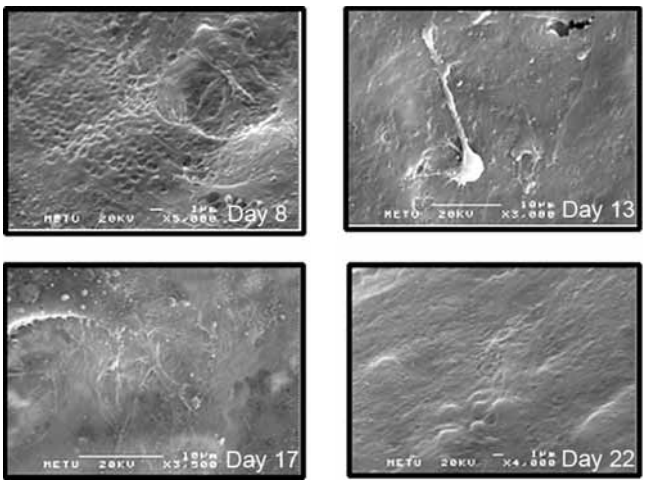


Figure 7 a.



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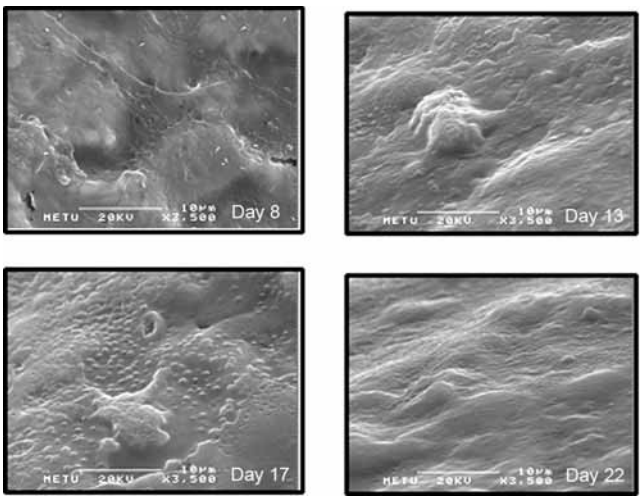

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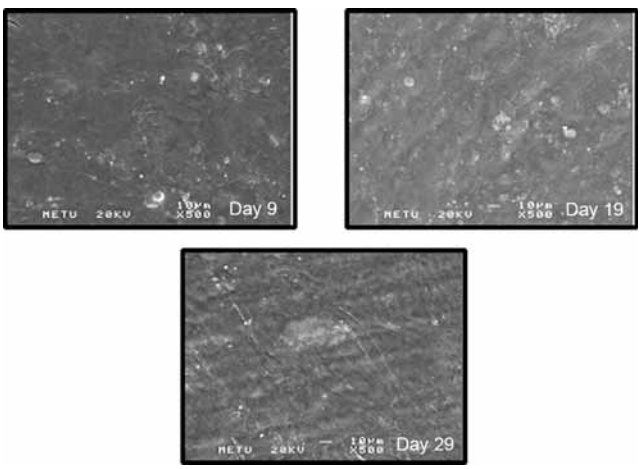

Figure 7 d.

Figure 7: SEM micrographs of MC3T3 cell-titanium composites P: Polished, SB: Smooth sand-blasted, RB: Rough sand-blasted, NaTi: Sodium titanate coated.

Cell population on the polished surfaces was more intense than the sodium titanate modified surfaces. The cellular cytoplasmic extensions and extracellular matrix production was higher on the sand-blasted surfaces with no significant difference in between the smooth and rough sand-blasted groups.

Confocal Microscopy Analysis

Fluorescent labeled cells dispersed as groups on titanium surfaces on day 10 (Figure 8a). Cell population with cytoplasmic processes increased and formed a multilayer with high amount of extracellular matrix on implants on day 20 (Figure 8b).

Osteoblastic cells connected to each other and formed a stronger network on sodium titanate-coated surface comparing to other groups on day 10. Both the smooth sand-blasted and sodium titanate cooted surfaces were covered by a stronger multilayered osseous tissue comparing to that of the polished surface group.

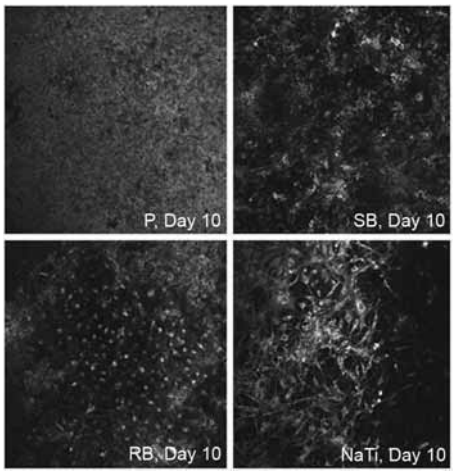

Figure 8 a.

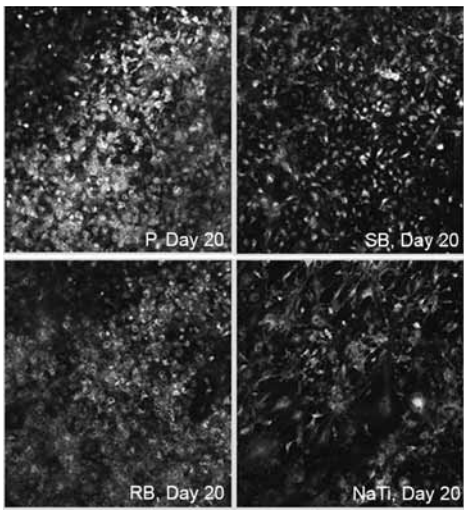

Figure 8 b.

Figure 8. CM micrographs (200x magnification) of osteoblasts on titanium surfaces A. Day 10, B. Day 20. P: Polished, SB: Smooth sand-blasted, RB: Rough sand-blasted, NaTi: Sodium titanate coated.

DISCUSSION

In vitro MC3T3-E1 cell osseointegration on polished, sand-blasted (smooth and rough) and sodium titanate coated titanium alloys were assessed in this study. Proliferation and mineralization was assessed. Cell morphology and viability were evaluated using confocal (CM) and scanning electron microscopy (SEM).

All implants used in this study were biocompatible. Contamination was not observed throughout the experiments. Cells attached on the surfaces of the implants on day 3. These cells were viable and metabolically active as observed by CM. Cell number increased exponentially from day 3 to day 22. Proliferation rate was highest on polished surfaces and lowest on sodium titanate-coated surfaces. In contrast, mineralized nodules were numerous on sand-blasted and sodium titanate-coated surfaces when compared to the polished ones on day 30. In a recent study polished and sand-blasted titanium surfaces exhibited similar osteoblastic cell attachment in vitro.[2] Hacking et al created a rough irregular surface texture by blasting with small hard 24 grit particles of Al_2O_3 or polished the titanium surfaces. In that study, proliferation and matrix mineralization of MC3T3 osteoblastic cells significantly reduced on microtextured compared to polished titanium surfaces.[3] Our findings were in agreement with Hacking et al., in means of osteoblastic cell attachment onto the metal surface which revealed that cell attachment was dependent on the metals surface texture. This study and the study of Hacking et al[3] revealed that polished surfaces present a favorable area for initial cell attachment and proliferation. In this study, cells migrated through the grooves of both smooth and rough sand-blasted titanium discs on day 10. Osteoblastic cells connected each other by their cytoplasmic extensions. They formed their extracellular matrix expansively on metals between days 10 to 20 in CM. MC3T3-E1 cells form a well-structured extracellular matrix on sodium titanate-coated and sand-blasted surfaces when compared to polished surfaces. This indicates that the osteoblastic cells need to grow and expand in a three-dimensional environment simulating in vivo conditions. Cell networks covered all the grooves created on sand-blasted titanium surfaces on day 20. Both smooth and rough sand blasted groups were equally covered with cells indicating that both methods of surface modification were suitable for cell attachment. Saint Pierre et als' data is almost consistent with ours. They reported that three-dimensional porous titanium scaffolds with different pore sizes ranging from 336 to 557 mm, equally induced the proliferation (DNA content) and differentiation of MC3T3 cells using polished titanium as reference material.[9] In our study,

osteoblastic differentiation and cell maturation was observed on the grooves. This finding opposes that of Faghihi et al.[4] Same group reported in their more recently published article that surface nanostructured titanium with ultra-fine crystals (<50 nm) and a surface oxide layer produced by the high pressure torsion favors degree of osteoblast attachment, rate of growth and, fibronectin expression. This data means that surface modification mediates also further osteoblast differentiation including the interaction with extracellular matrix protein fibronectin.[10] Roux et al reported that the silanized titanium surfaces presented excellent cytocompatibility for MC3T3-E1 osteoblast-like cells as shown by the assessment of cell viability, vitality and morphology.[5] Our data is consistent with theirs. Surface coating of polished commercially pure titanium substrates coated by different oxides: TiO_2, SiO_2, Nb_2O_5 and SiO_2–TiO_2 revealed higher cell proliferation rates in SiO_2–TiO_2 and TiO_2, and lower in Nb_2O_5 and SiO_2.[11] In contrast, cytochemical assays showed that all substrates induced a normal cytoskeleton and well-developed focal adhesion contacts. Thus both Roux et als' and our results need to be confirmed by immunocytochemical and RNA analyses revealing the further osteblastic maturation and the synthesis of bone extra cellular matrix proteins.

An in vivo study on bone growth around commercially pure titanium dental implants under masticatory loading did not demonstrate significant difference among the different surface roughness in the range of Ra 0.4–1.9 μm, Rz 2.8–11.2 μm, Rmax 3.6–28.1 μm and Sm 2.9–41.0 μm, which was estimated by measuring the bone contacts, bone occupancies and bone bonding strengths at the implant/bone marrow interface.[12] Lack of biomechanical tests to evaluate cell detachment and in vivo testing of the modified surfaces were the limitations of this study. However, a well-defined biomechanical cell detachment test is not defined in the literature. For in vivo testing, ethical board application is prepared and after allowance tests will be conducted.

In this study, cytocompatibility and osseointegration of different titanium surfaces with MC3T3 cells are presented by CM and SEM in vitro. Further molecular analysis for differentiation and maturation steps of osteoblastic cells on different titanium surfaces was not carried out. In conclusion, the results of this study demonstrated that sand-blasting and, sodium titanate coating provided by NaOH, favored the attachment, mineralization, and early differentiation of osteoblasts, which may have significant effects on the ultimate biomaterial related bone healing in vivo.

Acknowledgment:

Technical and Research Council of Turkey (Grant No: 105-S-097 (SBAG 3130)) supported this study. Cell culture facilites in Research Center of Faculty of Dentistry, Selçuk University was used.

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