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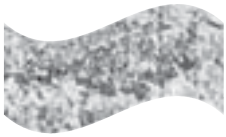

Different titanium surfaces influence the biological behavior of SaOS-2 human osteoblast-like cells: in vitro study

Differenti superfici in titanio condizionano il comportamento biologico di cellule umane simil-osteoblastiche SaOS-2: studio in vitro.

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Summary

Osseointegrated dental implants have been successfully used over the past several years, allowing functional replacement of missing teeth. Implant topography appears to modulate cell growth and differentiation of osteoblasts affecting the bone healing around the titanium implant. Optimal topography is still controversial. The aim of present study was to evaluate in vitro the biological behavior of Human osteoblast-like SaOS-2 cells, cultured on two different titanium surfaces.

Riassunto

Gli impianti dentali osteointegrati sono stati impiegati con successo nel corso degli ultimi decenni, permettendo il ripristino funzionale di denti mancanti. La topografia della superficie implantare appare modulare la crescita cellulare e la differenziazione verso la linea osteoblastica, condizionando la guarigione ossea peri-implantare. La topografia di superficie ottimale è ancora un aspetto molto controverso. Scopo della presente ricerca è stato valutare, in vitro, il comportamento biologico di cellule umane simil-osteoblastiche SaOS-2 coltivate su due differenti superfici in titanio.

Introduction

The biological phenomena at the bone/implant interface leading to implant osseoin-

tegration depends on several factors, including implant macrostructural, microstructural and ultrastructural properties 1,2. In order to increase the bone/implant contact as well as to accelerate bone healing processes, each of these aspects has been in-depth investigated in several studies. More than macrostructural features, the superficial layer of titanium-oxide and the surface microstructure seem to deeply affect bone/fixture interactions 3.After the initial implant/biological fluids contact, surface idratation occurs with absorption of ions, proteins, glycolipids, proteoglycans, polysaccharides and a stable fibrin-platelet network is established. Cell migration, adhesion, proliferation and differentiation with extra-cellular matrix (ECM) production and deposition at implant surface are the most important following events. Several studies 2-5 have shown that a rough surface, when compared to a relatively smooth one, grants a better biomulecular absorption, due to a wider surface, increases ECM production and promotes the differentiation of the pluripotent mesenchymal stem cells toward an osteoblastic phenotype. Different industrial treatments may modify chemical features and thickness of the oxide layer, as well as surface morphology and roughness. Optimal roughness and superficial morphology are still controversial and need to be clearly defined. SaOS-2 is a mature osteoblastic cell line, derived from a human osteosarcoma, that several studies have demonstrated to be a relevant cell model, due to its large analogies with immature osteoblast cells 6,7. The aim of the present study was to evaluate in vitro the biological behavior of SaOS-2 cells, cultured on two different titanium surfaces, smooth and sandblasted-acid-etched, by investigating proliferation, phenotypical bone expression and ECM deposition.

Materials and Methods

Titanium Disks

Commercially pure titanium disks, 1,5 cm Ø and 2 mm in thickness with two different surfaces were used: a relatively smooth, machined surface (S) and a sandblasted-acid-etched surface (SBA). Titanium samples were subjected to a routine plasma cleaning in order to minimize surface contamination. Disks were then subjected to several steps of conventional solvent cleaning and placed in a cold-plasma reactor. Furthermore, disks were treated by Air plasma, using a 100 W power, a flow rate of 20 scc/m (standard cubic cm/minute) for 15 min, rinsed with distilled water, and autoclaved prior to cell culture experiments.

Cell Culture

Human osteoblast-like SaOS-2 cells (ATCC85-HTB) were cultured in Dulbecco's modified Eagle's medium (DMEM), with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and 10 U/ml penicillin/streptomycin in a humidified 95% air/5% CO2 incubator, at 37°C. Medium was changed every 3-4 days. Cells were detached by 0.5 mmol/L ethylendiammintetraacetate in calcium and magne-

sium-free phosphate-buffered saline (PBS) with 0.05 % trypsin. For all experiments, 1.5x106 cells were plated and cultured on titanium disks in 60 mm-culture plates. Cells grown on a polyestere plastic surface were used as a control. All experiments were carried out in quadruplicate and repeated three times.

3H-thymidine Incorporation and Cell Proliferation Assay

To determine the proliferation of SaOS-2 cells on titanium surfaces, we measured 3H-thymidine incorporation. SaOS-2 cells were plated on titanium disks and cultured up to 14 days in the presence of 100μCi of 3H-thymidine. Cells were harvested, at given times (24, 72, 96 hrs; 7th and 14th day) counted in a Neubauer cytometer, and absorbed onto nitrocellulose paper; radioactivity was then counted in a β-counter. Results were expressed as mean incorporation at the experimental point (counts *per* min)/104 cells. Titanium disks without cells and medium alone were used as negative controls.

Alkaline Phosphatase and Interleukine-6 measurement

Alkaline phosphatase (ALP) was determined with p-nitrophenylphosphate as a substrate. SaOS-2 cells, plated on titanium disks, were analysed at the same time intervals, as described above. Cells were scraped into 500-μl ice-cold harvest buffer (10mM Tris HCI, pH 7,4, 0,2% NP-40 and 2mM phenylmethylsulfonyl fluoride, PMSF). Enzymatic activity was measured by an automatic analyser.

The results were expressed as UI/(enzyme activity)/104 cells. A quantitative analysis to determine the production of IL-6 was performed by Enzyme-linked Immunoassay (ELISA). SaOS-2 cells were plated on titanium disks at 24, 72, 96 hrs and 7th, 14th days of culture. The results were expressed as IL-6 concentration (pg/mL) /104 cells.

ECM Deposition: in situ Enzyme-linked Immunoassay

A quantitative analysis of some ECM components (CoI, FN and TN) produced by SaOS-2 cells, cultured on titanium disks up to 14 days, was performed by in situ Enzyme-linked Immunoassay. SaOS-2 cells were plated on titanium surfaces and cultured for up to 14th days. At the same time intervals as above, cells were fixed on titanium surfaces by 50% (vol/vol) methanol-acetone for 10 min at room temperature and air-dried. Disks were incubated with calcium and magnesium-free phosphate-buffered saline (PBS)/0.5 % bovine serum albumin (BSA), for 2 hrs at 4°C, then filled with 50μL of one of the following rabbit anti-sera: anti-collagen I (Co I), anti-fibronectin (FN) and anti-tenascin (TN), in PBS/0.5 % BSA and 0.2 % Tween 20, and allowed to react for 1 hr at room temperature. Plates were then washed with PBS, filled with 50 L horseradish eroxidaseconjugated anti-rabbit IgG in PBS, 0.2% Tween 20, allowed to react for 1 hr, washed again with PBS and filled with 150μL of 1 mg/mL o-phenyl-enediamine, 0.006% hydrogen peroxide, 0.1 M citrate buffer, pH 5.0. After 30 min of incubation, the absorbance at 450

nm was measured by a spectrophotometer. Titanium disks without cells and disks coated with purified ECM components were used as controls. To evaluate the effect of serum on ECM deposition of SaOS-2 cells, serumstarved cells, cultured in DMEM 0.5% BSA, were used as controls.

Fibronectin and Tenascin Deposition by Western Blotting

The expression of fibronectin and tenascin by SaOS-2 cells was investigated by Western blotting.

Cell lysates were collected by scraping on titanium disks and centrifuged at 14.000 rpm for 5 min at 4°C. Supernatants were harvested and protein concentration in cell lysates was determined by a colorimetric assay. For each sample, 100µg of total proteins was incubated 5 min at 90°C in Laemmli sample buffer and separated by electrophoresis in 10% SDS-polyacrylamide gels. Gels were electroblotted on PVDF filters; membranes were blocked with 5% fat-free dry milk, 1% ovalbumin, 5% fetal calf serum (FCS) and 7.5% glycine for 30 min at room temperature. After three washes in washing solution (PBS/0.1% ovalbumin, 0.1% fat-free dry milk, 1% FCS), membranes were incubated overnight at 4° C with the specific primary monoclonal antibodies: anti- FN and anti-TN. After four 5 min washes, at room temperature, with washing solution, membranes were incubated for 30 min at room temperature with horseradish peroxidase-conjugated secondary antibody, diluted to 1:3000 in PBS. After three washes in washing solution and three final washes in T-TBS (50mM Tris, pH 7.5; 0.5M NaCl; 0.2% Tween 20), membranes were stained with an enhanced chemoluminescence (ECL) detection kit. A quantitative analysis was performed by Scanner Densitometry.

Reverse Transcriptase - Polimerase Chain Reaction (RT-PCR)

Total RNA was isolated from cells cultured at 8 days using a total RNA isolation reagent, according to the manufacturer's instructions. Contaminating DNA was digested with DNase using a DNase Kit and 2µg of total RNA was reverse transcribed with 100 U of a DNA polymerase in a volume of 40µl, using 100 mM random hexamer primers according to the manufacturer's instructions. Primer sequences for RT-PCR were as follows: Collagen type I forward primer, 5'-GAG GAA GGC CAA GTC GAG G-3'; Collagen type I reverse primer, 5'-CCG AGT GAA GAT CCC CTT TTT A-3'; and generated an 86 base-pair fragment. The amplification was established using a DNA Thermal Cycler for 35 cycles as follows: denaturation: 95°C for 45s; annealing: 60° C for 30s; extension: 72°C for 30s. At the beginning of the reaction, a cycle at 95°C for 5 min was carried out to activate Taq Polimerase.

Real-time quantitative PCR

A quantitative assay for type I (α1) collagen mRNA expression was established using a Real-time PCR system. All measurements were normalized to an endogenous control using the 101 bp fragment at 3' of the beta-glucuronidase region.

PCR oligo-primers were: h-COL1A1 forward primer 5'-GAG GAA GGC CAA GTC GAG G-3' and h-COL1A1 reverse primer 5'-ACG TCT CGG TCA TGG TAC CTG-3' generating an 86 bp fragment; h-GUSB forward primer 5'-GAA AAT ATG TGG TTG GAG AGC TCA TT-3' and h-GUSB reverse primer 5'-CCG AGT GAA GAT CCC CTT TTT A-3'. Real-time PCR was performed using a Real-time PCR solution and 50 ng of cDNA in a total volume of 15µl. Each sample was run in triplicate, for both type I (α1) collagen and beta glucuronidase. The PCR cycling profile consisted of AmpErase UNG incubation for 2 min at 50°C and AmpliTaq Gold activation for 10 min at 95°C, as predenaturation steps, and in 50 two-step cycles at 95°C for 15 s, and at 60°C for 60 s. For type I (α1) collagen mRNA relative quantification, the comparative Ct method was used according to the manufacturer's instructions.

Statistical Analysis

The data obtained in the present study were calculated as mean +/- S.D. Statistical analysis for normally distributed parametric data was performed using the Students t-test, and the differences were considered statistically significant with "p" values less than 0.05. For non parametric data obtained by Real-time analysis the differences were analyzed using the REST program and the Pair Wise Fixed Reallocation Randomisation test. Randomisation tests with a pair-wise reallocation are considered the most appropriate approach for this application. They are more flexible than nonparametric tests based on ranks (Mann-Whitney, Kruskal-Wallis) and do not suffer a reduction in power relative to parametric tests (t-test, ANOVA). A p-value <0.05 was considered significant.

Results

Cell Proliferation

Cell proliferation was not influenced by the type of surface (data not shown). The observed differences in the growth curves were not statistically significant ($p>0.05$).

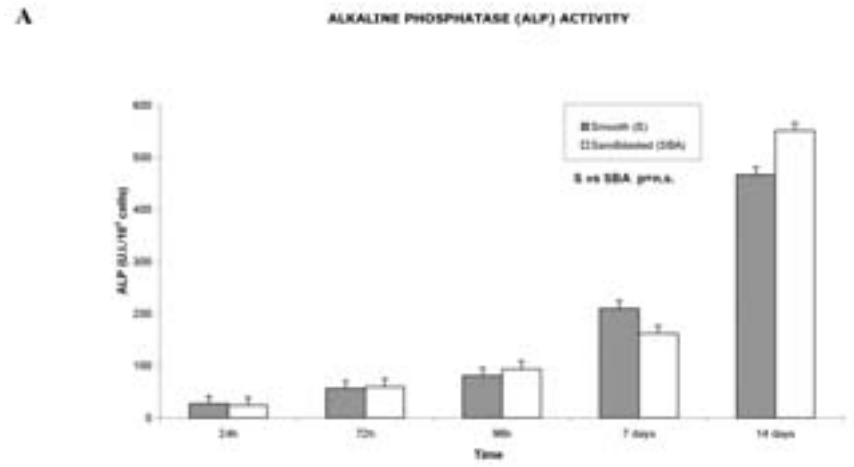
Production of Bone Differentiation Markers

SaOS-2 cells were analysed for Alkaline Phosphatase (ALP) production. The results (Figure 1, A) demonstrated a significant increase in ALP activity after 14 days of culture ($p<0.05$), but there was no statistically significant difference among different surfaces ($p>0.05$). Also IL-6 production increased significantly after 14 days, as shown in figure 1, B. Once again, there was no statistically significant difference between the two titanium surfaces ($p>0.05$).

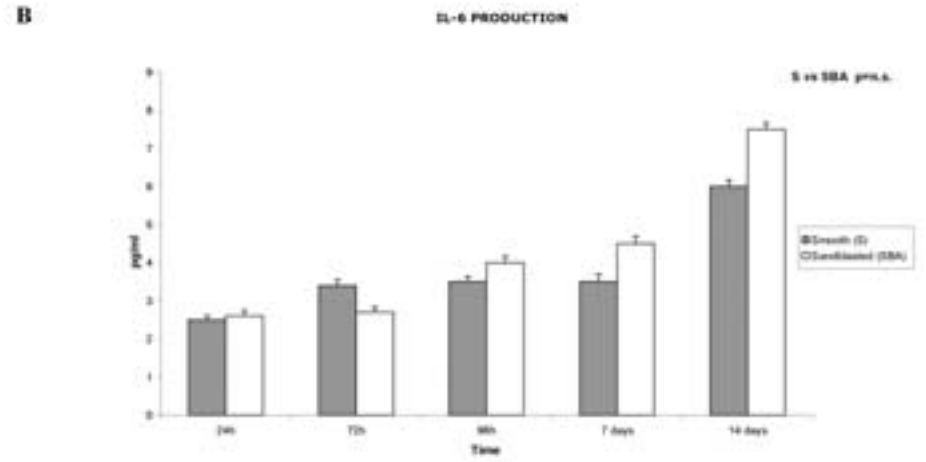
Production of Extracellular Matrix (ECM)

To investigate ECM production, we estimated the total amount of fibronectin (FN), tenascin (TN) and collagen I (Co I) synthesized by SaOS-2 cells by ELISA in situ. The difference between S and SBA surfaces was not significant for FN and TN dep-

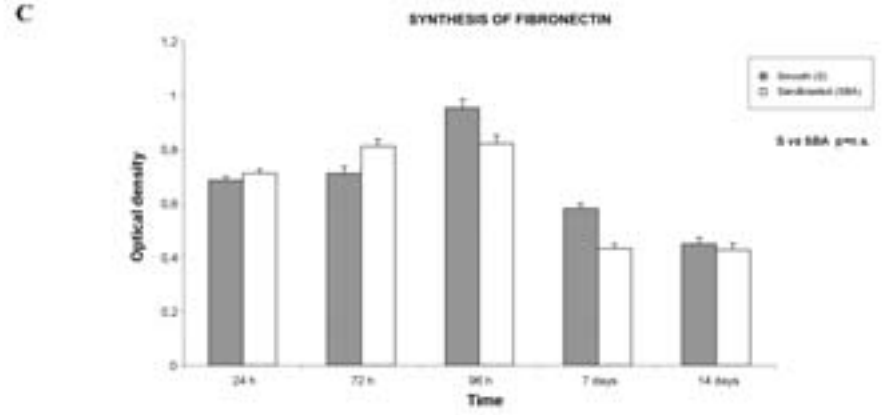
Figure 1



Alkaline phosphatase (ALP) activity of SaOS-2 cells on titanium surfaces determined by spectrophotometric analysis. S vs SBA p=n.s. (not significant).



IL-6 production in SaOS-2 cells on titanium surfaces determined by enzyme-linked immunoassay in situ. S vs SBA p=n.s. (not significant).



Synthesis of Fibronectin in SaOS-2 cells on titanium surfaces determined by enzyme-linked immunoassay in situ. S vs SBA p=n.s. (not significant).

osition (Figure1, C and Figure 2, A); on the contrary, the Co I production was significantly higher in SaOS-2 cells cultured on SBA titanium surface as compared to S surfaces, starting from day 7 (Figure 2, B) ($p<0.05$). Serum-starved cells, cultured in DMEM/0.5% BSA, still produced ECM components, although to a lower extent, thus showing that matrix production was not due to the deposition of serum components (data not shown). Production of FN and TN was confirmed by Western blotting analysis of SaOS-2 cells lysates, after incubation overnight at 4° C with the specific primary monoclonal antibodies, that showed bands, of variable intensities, with the expected molecular weights of 220 kDa for FN and 200 kDa for TN. A quantitative analysis was also performed; again no statistically significant difference in the expression of FN and TN (data not shown) was observed between the two surfaces. Due to though molecular weight of Co I, Western blotting analysis for this protein was not performed.

RT-PCR and Real-time quantitative PCR

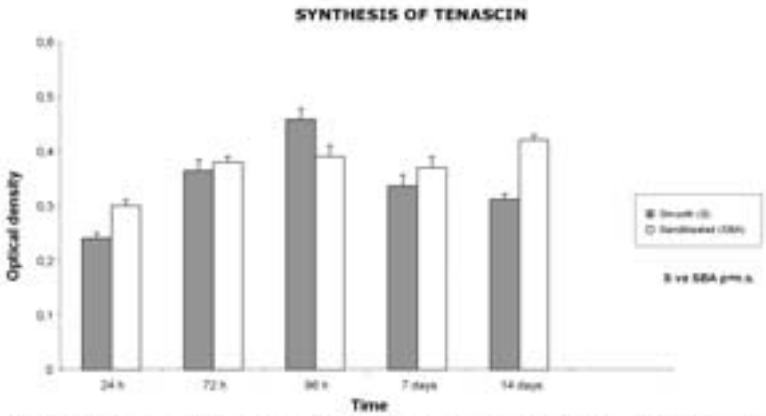
Type I collagen deposition was investigated also by RT-PCR and Real-time quantitative PCR. The results show that the expression of type I collagen mRNA is 2-fold higher ($p<0.05$) in SaOS-2 cells cultured on SBA titanium surface as compared to S surfaces (Figure 2, C), in agreement with the data obtained by enzyme-linked immunoassay in situ.

Discussion

Bone healing process at implant surfaces involves a complex sequence of events 8. Several studies suggest that surface properties of titanium dental implants are able to affect bone healing processes 2-4. In our study, we investigated in vitro proliferation and differentiation of human osteoblast-like cells SaOS-2 cultured on two different titanium surfaces, smooth (S) and sandblasted-acid-etched (SBA). Literature data 9 suggest that differentiation toward an osteoblastic phenotype correlates with a decrease in cell proliferation and an increase in production of the alkaline phospatase (ALP), a fundamental osteogenic activity marker 6. Cell proliferation increased significantly up to 7 days of culture, both on S and SBA surfaces ($p<0.05$), according to the observations of Martin et al. 2.With regard to ALP production, the data showed a significant increase in production on both surfaces, after 14 days of culture. Also the production of IL-6, the other bone differentiation marker examinated, increased significantly after 14 days, but there was no statistically significant difference between the two surfaces ($p>0.05$). This result seems to indicate a marked tendency of the SaOS-2 cells cultured on both surfaces to differentiate towards an osteoblastic phenotype. The differentiation towards an osteoblastic phenotypye was confirmed by the study of matrix deposition.The analysis of ECM production in SaOS-2 cells demonstrated that the synthesis of Co I, FN and TN increases with time and a significant difference ($p<0.05$) between SBA

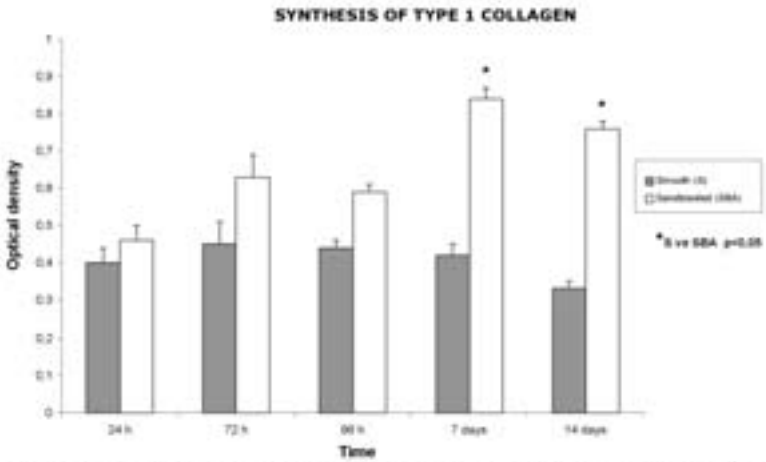
Figure 2

A



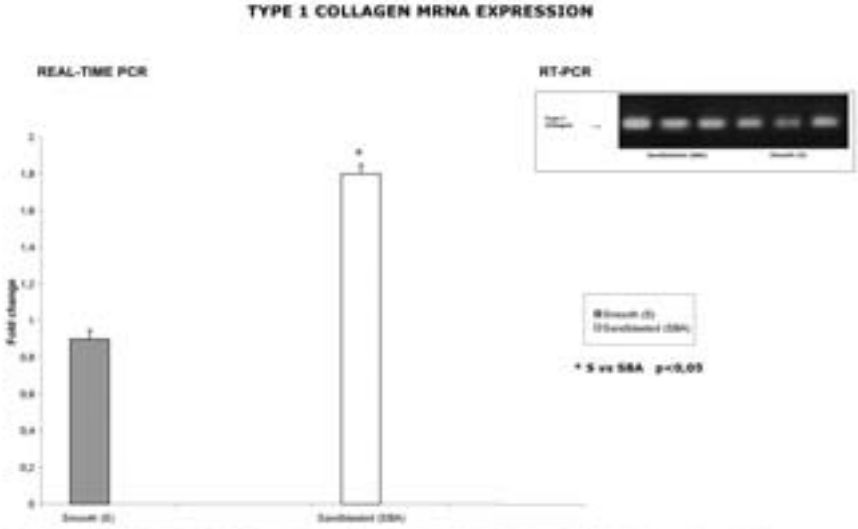
Synthesis of Tenascin in SaOS-2 cells on titanium surfaces determined by enzyme-linked immunoassay in situ. S vs SBA p=n.s. (not significant).

B



Synthesis of type I Collagen in SaOS-2 cells determined by ELISA. Co I deposition is significantly higher in SBA vs S p<0.05.

C



Expression of type I collagen mRNA, in SaOS-2 cells cultured, as shown by Real-time PCR and RT-PCR. SBA vs S p<0.05.

and S was observed for Co I, indicating a more efficient differentiation of cells cultured on SBA surfaces. Our data demonstrate that a SBA surface topography characterized by pore dimension less than 1µm, may affect in vitro the differentiation of osteoblast–like cells, as analyzed by ALP production, ECM deposition and integrin expression. In this regard, the SBA surface seems to grant a better differentiation towards an osteoblastic phenotype, as compared to the S surface. According to our previous study of smooth, sandblasted and titanium plasma sprayed surfaces, it seems that the sandblasted-acid-etched titanium surface is able to support in vitro proliferation and differentiation of osteoblast–like cells. It is therefore likely that implant surface properties can modulate in vivo the biological behavior of osteoblasts during bone tissue healing.

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