Different titanium surfaces modulate the bone phenotype of SaOS-2 osteoblast-like cells

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Commercially pure titanium implants presenting a relatively smooth, machined surface or a roughened endosseous surface show a large percentage of clinical success. Surface properties of dental implants seem to affect bone cells response. Implant topography appears to modulate cell growth and differentiation of osteoblasts affecting the bone healing around the titanium implant. The aim of the present study was to examine the effects of 1cm diameter and 1mm thick titanium disks on cellular morphology, adhesion and bone phenotypic expression of human osteoblast-like cells, SaOS-2. SaOS-2 cells were cultured on commercially 1 cm pure titanium disks with three different surface roughness: smooth (S), sandblasted (SB) and titanium plasma sprayed (TPS). Differences in the cellular morphology were found when they were grown on the three different surfaces. An uniform monolayer of cells recovered the S surface, while clusters of multilayered irregularly shaped cells were distributed on the rough SB and TPS surfaces. The adhesion of SaOS-2 cells, as measured after 3h of culture, was not affected by surface roughness. ECM components such as Collagen I (CoI), Fibronectin (FN), Vitronectin (VN) and Tenascin (TN) were secreted and organized only on the SB and TPS surfaces while they remained into the cytoplasm on the S surfaces. Osteopontin and BSP-II were largely detected on the SB and TPS surfaces, while only minimal production was observed on the S ones. These data show that titanium surface roughness affects bone differentiation of osteoblast like-cells, SaOS-2, indicating that surface properties may be able to modulate the osteoblast phenotype. These observations also suggest that the bone healing response around dental implants can be affected by surface topography.

Keywords: titanium disks, implants, SaOS 2 cells, extra cellular matrix, bone tissue.

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Prosthetic restorations supported by dental implants represent a predictable treatment with long-term survival of osteointegrated titanium fixtures (Adell et al., 1981; Albrektsson et al., 1986; Buser et al., 1997).

Currently, commercially pure titanium implants, presenting a relatively smooth, machined surface or a roughened endosseous surface are clinically used, both types providing a large percentage of clinical success (Cochran, 1996; Cochran, 1999).

Nevertheless, it has been reported that the surface topography and composition of titanium implants seem to be important for the clinical outcome, since bone tissue shows a better interaction with a rough surface as compared to a relatively smooth one (Buser et al., 1991; Wong et al., 1995; Martin et al., 1995; Kieswetter et al., 1996).

Several *in vivo* (Sandberg et al., 1988; Nomura et al., 1988; Weinreb et al., 1990; Shalhoub et al., 1991) and *in vitro* (Aronow et al., 1990; Owen et al., 1990; Pockwinse et al., 1992) studies have shown that bone tissue differentiation around the bone-implant interface is a complex process involving sequential biological steps such as cellular migration, attachment and proliferation, followed by the expression of markers of the osteoblast phenotype.

In particular, differentiation toward an osteoblast phenotype is a multi-step process, characterized by a first proliferative phase, during which cell cycle progression genes (histones and cyclines), proto-oncogenes (myc and fos) and some Extra-Cellular Matrix (ECM) proteins, such as CoI, are expressed. A second phase follows, in which there is a decrease in cell proliferation and the induction of genes related to maturation and organization of bone extracellular matrix (alkaline phosphatase, matrix Gla-protein); finally, a third phase, consisting in ECM mineralization, with up-regulation of specific genes

correlated with this phenomenon (Osteopontin, BoneSialoprotein-II and Osteocalcin), concludes the process (Lian and Stein, 1993). Bone differentiation, including ECM production and deposition, is regulated by several factors, e.g. hormones and growth factors such as glucocorticoids, 1-25 (OH)2 D3, IGF-1, PDGF, TGF-β1 (Lian and Stein, 1993; Kaplan, 1995).

It is well known that CoI, FN, VN and TN are some of the components of the ECM produced by osteoblasts during differentiation and that CoI is the major component of the bone connective tissue (Owen et al., 1990). FN and VN play a pivotal role during the embryogenesis and in wound healing process, promoting cell proliferation and migration. In particular, FN represents one of the major proteins of the ECM that promotes cell migration during the tissues differentiation. Furthermore, it represents one of the major components of connective tissue, linking CoI and Proteoglycans and acting therefore as a connecting element between the cytoskeletal and the extra-cellular matrix (Alberts et al., 1983). TN is a glycoprotein characteristic of bone, cartilage and dentina during the embryonic development. The synthesis of TN decreases after tissue differentiation but it can newly increase during wound healing, in bone as well as in other tissues (Steffensen et al., 1992).

We have recently studied the in vitro behaviour of SaOS-2 cells, a well characterized osteosarcoma human cell line, showing clear osteoblast-like properties. These cells produce high levels of Alkaline Phosphatase and Osteonectin in culture and express high levels of 1-25(OH)2-D3 receptor (Rodan et al., 1987). Our results confirmed that SaOS-2 cells are well differentiated osteoblast-like cells, even in the absence of ascorbate and beta-glycerophosphate in the culture medium; we also showed that SaOS-2 cell differentiation is regulated by the Granulocyte-Macrophage-Colony Stimulating Factor (GM-CSF) (Postiglione et al., 2003).

Thus, previous studies clearly established that the SaOS-2 cells are a relevant cell model of the bone tissue which can be employed to investigate the *in vitro* behaviour of the osteoblast cells emulating, at the same time, the *in vivo* conditions.

The aim of the present study was to evaluate the effects of the increasing roughness of clinically employed titanium implant surfaces on in vitro cell morphology, adhesion and phenotypic bone expression of SaOS-2 cells.

Materials and Methods

Titanium disks

Commercially pure titanium disks of 1 cm in diameter and 1 mm in thickness were supplied by Sweden & Martina S.p.A. (Due Carrare, PD, Italy). Disks with three different surfaces of increasing roughness were used: a relatively smooth, machined surface (S), a sandblasted surface (SB) and a titanium plasma-sprayed surface (TPS). The 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 (Gambetti Kenologia, MI, Italy). Furthermore, disks were treated with Ar plasma, using a 100 W power, a flow rate of 20 scc/m (standard cubic cm/minute) for 15 minutes, rinsed with distilled water and autoclaved, before performing cell culture experiments.

Cell culture

Human osteoblast-like cells SaOS-2 (ATCC85-HTB) were cultured in Dulbecco' s modified Eagles medium (DMEM) (Sigma, St. Louis, MO, USA), with 10% heat-inactivated FCS (Gibco, Grand Island, NY, USA), 2 mM L-glutamine, 10 U/ml penicillin/streptomycin (Sigma), 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 magnesium-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 (Sarstedt, Newton, NC, USA). Cells, grown on a polyestere plastic surface, were used as a control. All experiments were carried out in quadruplicate and repeated three times.

Scanning electron microscopy (SEM)

Titanium disk surfaces were analysed by scanning electron microscopy (SEM). The disks did not require any special preparation for SEM examination. SaOS-2 cells were cultured on titanium disks up to 96 h. Disks were washed with PBS and fixed with 2.5% glutaraldehyde in 0.1 mol/L buffer phosphate at pH 7.3 for 30 min and 1% osmium tetraoxide in 0.1 mol/L buffer cacodylate, pH 7.4, for 1 h. Disks were washed with PBS three times, dehydrated in a graded series of ethanol (30%,

50%, 70% and 90%) and placed in a 100% ethanol bath and rinsed three times (30 min x 3). Disks were critical-point dried, sputter-coated with gold and the surface was then observed with a scanning electron microscope Philips 505 (Philips, Holland) and photographed with a Polaroid type 55 Positive-Negative 4x5 instant sheet film (Polaroid, Cambridge, MA, USA).

Cell attachment assay

SaOS-2 cells were grown on 100 mm plastic culture dishes (Sarstedt, Newton, NC,USA) until 50% confluence. Cells cultured for 2 days in presence of 3H-Thymidine 10 µCi/ml, were harvested and 3H-Thymidine labeled cells were counted with a beta-counter (Beckman LS 5000, CA, USA). Cells were plated on the titanium disks, and incubated for 3 h at 37°C, 5%CO_2. Disks were washed with PBS, transferred into counting tube with liquid scintillation (Wallac UK, Milton Keynes) and the radioactivity was measured as above.

Immunocytochemistry

SaOS-2 cells, after 96h *in vitro* culture on titanium disks or on glass coverslips, were fixed with 3% formaldehyde in PBS for 5 min at room temperature and gently washed with PBS. When it was needed, cells were permeabilized by Triton X100 buffer (20 mM HEPES, 300 mM sucrose, 50mM NaCl, 3 mM MgCl2, 0.5% Triton X100, pH 7.4). After several PBS washings, specimens were incubated with rabbit polyclonal antibodies against FN (Sigma) or with mouse monoclonal antibodies against CoI, TN, VN, Cadherin E (Chemicon, Temencula, CA), Osteopontin and Bone Sialoprotein II (BSP-II) (Iowa, Iowa City, IA, USA). After further washings with PBS, cells were stained with the secondary antibody, generally rhodamine labelled rabbit anti-mouse IgG and fluorescein-tagged goat anti-rabbit IgG. Both steps required 1 h incubation at room temperature in a moist chamber. Titanium disks as well as glass coverslips were then observed with an Axiophot microscope (Leitz, Germany) equipped for epifluorescence and microphotography. Each sample was evaluated by three independent observers, using a four points arbitrary scale ranging from 0 to 3.

In situ enzyme-linked immunoassay

A quantitative analysis of some ECM components (CoI, FN, VN and TN) produced by SaOS-2 cells cultured on titanium disks up to 96 h was performed by an *in situ* enzyme-linked immunoassay (ELISA). Cells were fixed on titanium disks by acetone/methanol (V/V), for 10 minutes at room temperature, incubated in PBS/BSA 0.5%, Tween20 0.2% for 2 hours at 4°C, to minimize aspecific binding sites, and washed in PBS. Disks were incubated then with primary antibodies anti-CoI from goat, anti-FN, anti-Laminin, anti-TN from rabbit (Chemicon, Temencula, CA, USA) for 1 hour at room temperature. After three washes in PBS, disks were incubated with horseradish peroxidase-conjugated anti-rabbit IgG and anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 minutes at room temperature. After further washes in PBS, the specific substrate was added (1mg/ml OFD/0.1 mol/L citrate buffer PH 5.0/ 0.006% H_2O_2) and disks were incubated for 30 minutes at 37°C, in the dark. The reaction was then stopped by 1N H_2SO_4 and the absorbance was read at 450nm by spectrophotometer.

In order to evaluate serum effects on SaOS-2 cell ECM deposition, serum starvation with DMEM 0.5% BSA was used as a control.

Statistics

The results are reported as mean values ± S.D. Student's t-test was performed in each experiment to evaluate the modifications observed in comparison to the unstimulated SaOS-2 cells. Differences at $p<0.05$ were considered statistically significant.

Results

Scanning electron microscopy (SEM)

The titanium surfaces of smooth (S), sandblasted (SB) and titanium plasma sprayed (TPS) disks were observed by scanning electron microscopy at various magnification (X500-X5000). As shown in Figure 1A, the surfaces of the S disks were very smooth. On the contrary, the surface of SB disks was characterized by pits and bumps; furthermore, grain boundaries were seldom observed (Figure 1B). The surfaces of TPS disks were highly irregular, with larger bumps, pits and frequent fissures and cracks (Figure 1C); grain boundaries were not observed.

After the sterilization of the disks by autoclave, the SEM analysis failed to individuate any change of the titanium surfaces, so suggesting that they

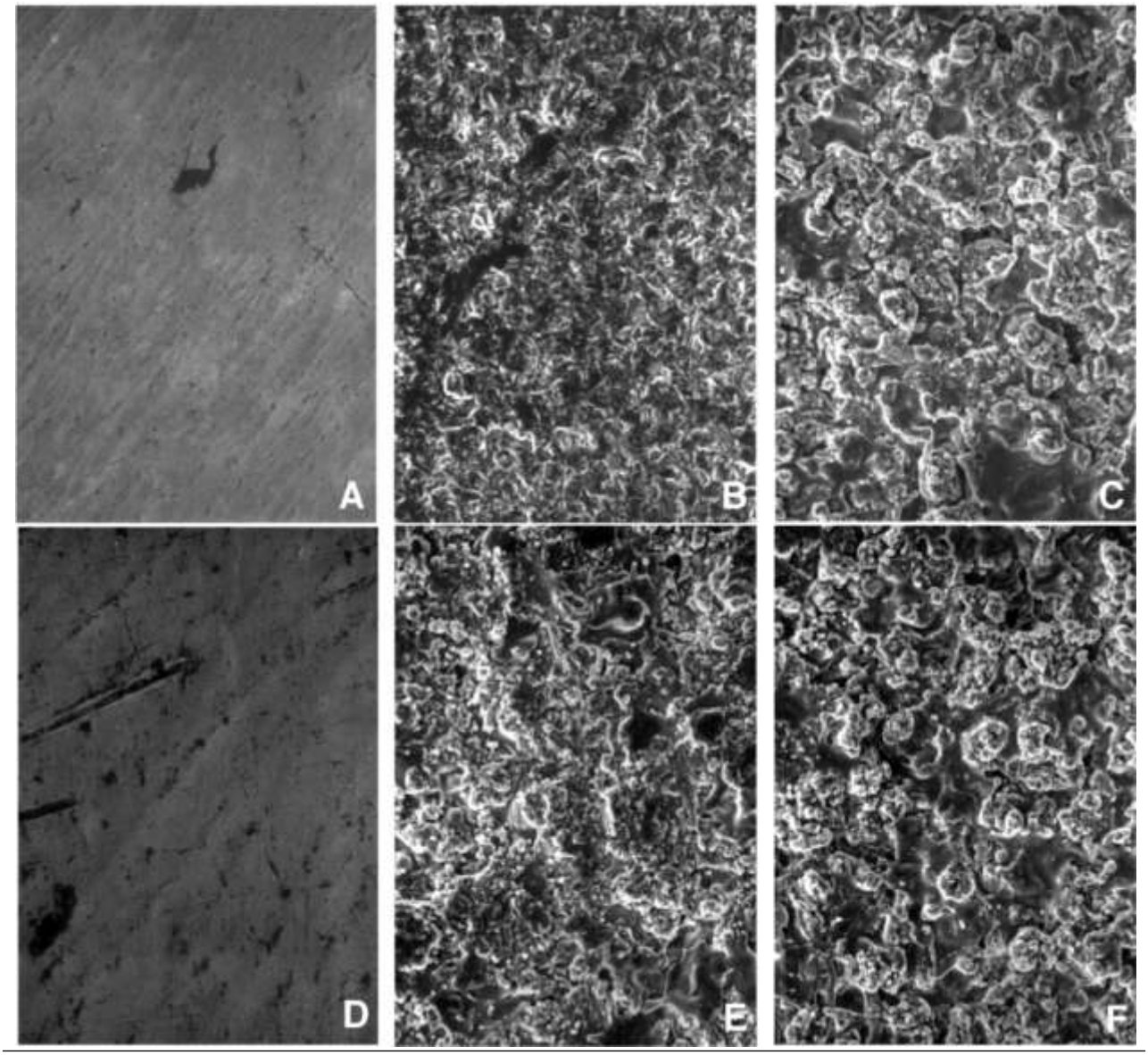

Figure 1 Scanning electron micrographs of disks surfaces (A. Smooth surface; B. Sandblasted surface; C. Ti-Plasmasprayed surface). After the treatment with autoclave, no significant difference was evident (D. Smooth surface; E. Sandblasted surface; F. Ti-Plasmasprayed surface) (Magnification, 274×).

were unaffected by this treatment (Figures 1 D,E, F). SaOS-2 cells plated on titanium surfaces were examined after 96h by SEM (Figure 2). Differences in cellular morphology on the various surfaces were observed. S disks were uniformly covered by a monolayer of flat and spreaded cells (Figure 2A). On the contrary, cell distribution on the SB and TPS surfaces was discontinuous, showing clusters of multilayered cells and cell shape was irregular, with filopodia crossing the disk surface (Figures 2B and 2C). Furthermore, cells never reached the confluence on the surfaces of the SB and TPS titanium disks but they were distributed in multilayered aggregates.

Cell attachment assay

In order to evaluate whether cell attachment was affected by different titanium surfaces, SaOS-2 cells were labelled with ^{3}H-Thymidine, plated on titanium disks and allowed to attach for 3 h. The radioactivity retained by the disks after extensive wash was 2861±435, 1985±495 and 2228±452 (mean c.p.m. ± SD) for S, SB and TPS disks respectively. Differences were not statistically significant

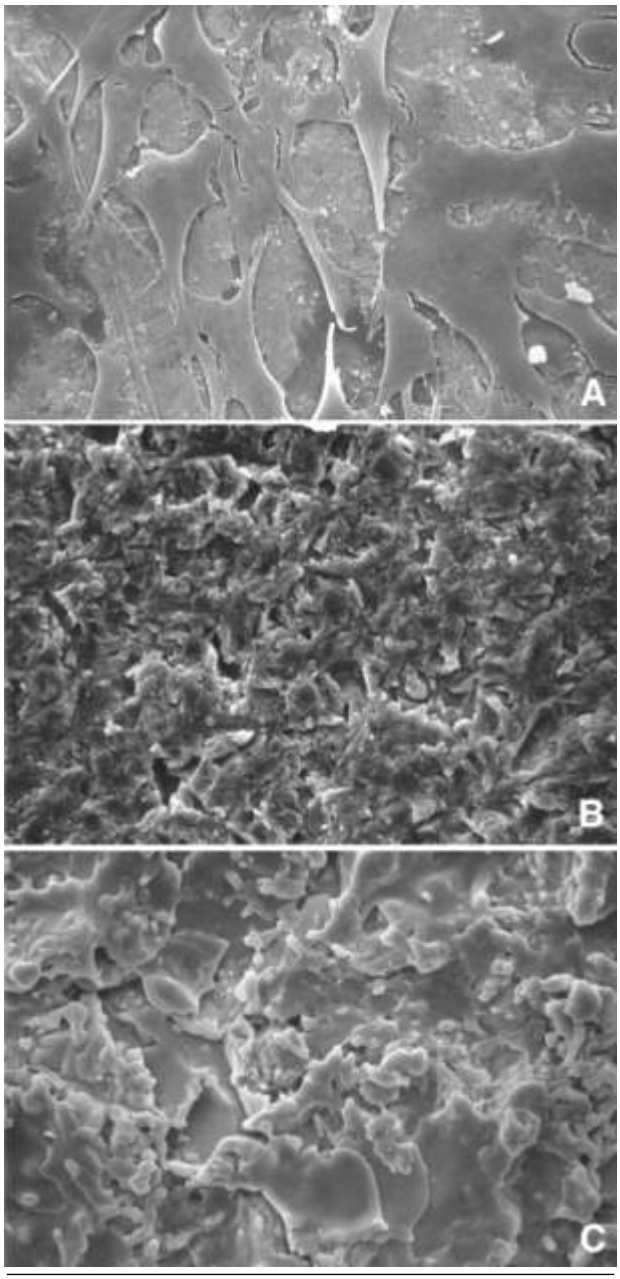

Figure 2 Scanning electron micrographs of cultured SaOS-2 cells adhering to different titanium surfaces. A. Smooth surface; B. Sandblasted surface; C. Ti-Plasmasprayed surface. Clusters of multilayered and irregularly shaped cells with filopodia crossing the disk surface are evident on SB and TPS surfaces (Figures 2B and 2C). (Magnification, 600×)

(p>0,05), demonstrating that the attachment of SaOS-2 cells was only slightly affected by the different titanium surfaces (Figure 3).

Immunocytochemistry

Immunofluorescence was performed to study the production and the organization of Osteopontin and BoneSialoprotein-II (BSP-II) and to determine if

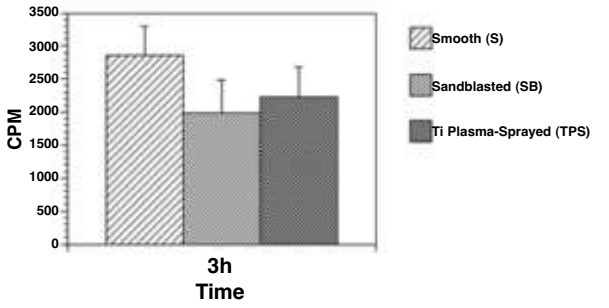

Figure 3. The attachment of SaOS-2 cells was only slightly affected by the different titanium surfaces.

the different titanium surfaces could affect the osteoblastic phenotype. Both Osteopontin and BSP-II were barely expressed in the cytoplasm by SaOS-2 cells cultured on S surfaces. Cells grown on rough surfaces showed an higher expression of these markers which seemed distributed on the cell membrane as well as in the extra-cellular environment (Figures 4 and 5). There is a slight production of E-Cadherin in S disks and an even lower production of this marker on SB and on TPS surfaces (*data not shown*).

CoI, FN and TN synthesized by SaOS-2 cells and retained in cytoplasm or deposited onto titanium surfaces were demonstrated by immunofluorescence. Their distribution was observed to investigate both production and morphological organization of ECM proteins. They were detectable on all the surfaces after 24 h and increased on all of them until 96 h of culture, with some differences among the three surfaces. In fact, a significant increase in production and organization of ECM proteins was detected in cells cultured on SB and TPS disks, as compared with ones cultured on smooth surfaces, where ECM components were present only in the cytoplasm (Figure 6).

The synthesis of VN evidentiates a similar pattern of intracellular production on S disks and an extracellular arrangement for SB and TPS ones (data not shown). This protein is however less abundant when compared with the previous ones.

In situ enzyme-linked immunoassay

The total amount of CoI, FN, VN and TN produced by SaOS-2 cells and localized in the cytoplasm or deposited on the titanium surfaces was evaluated by indirect immunofluorescence. ECM

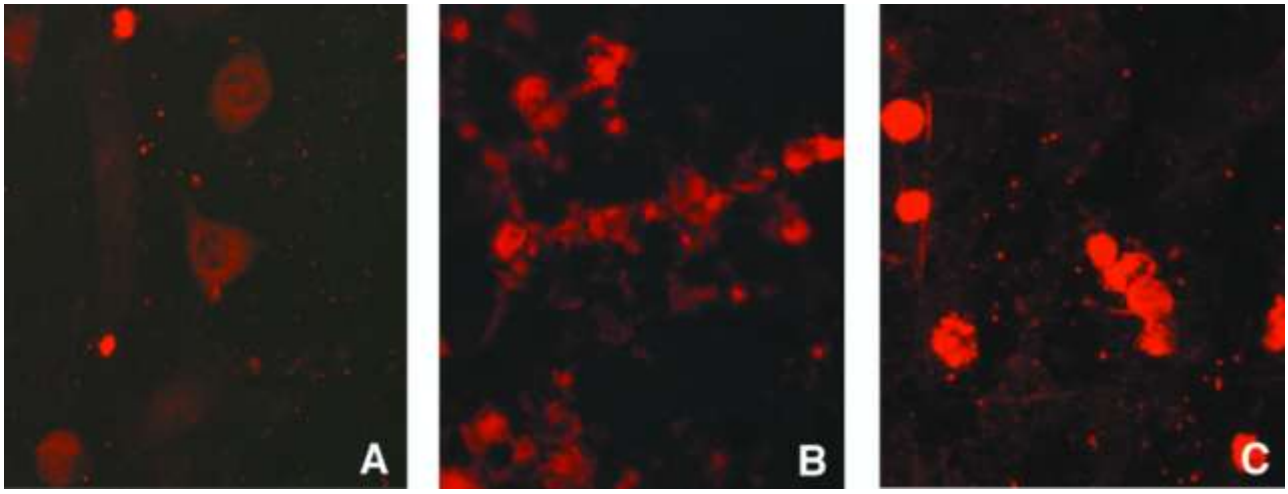

Figure 4. Production of markers of cell differentiation: BoneSialoProtein II is located in the cytoplasm on S disks (A), while it is distributed also on the cell membrane and appear in the extra-cellular spaces on SB and on TPS (B and C, respectively).

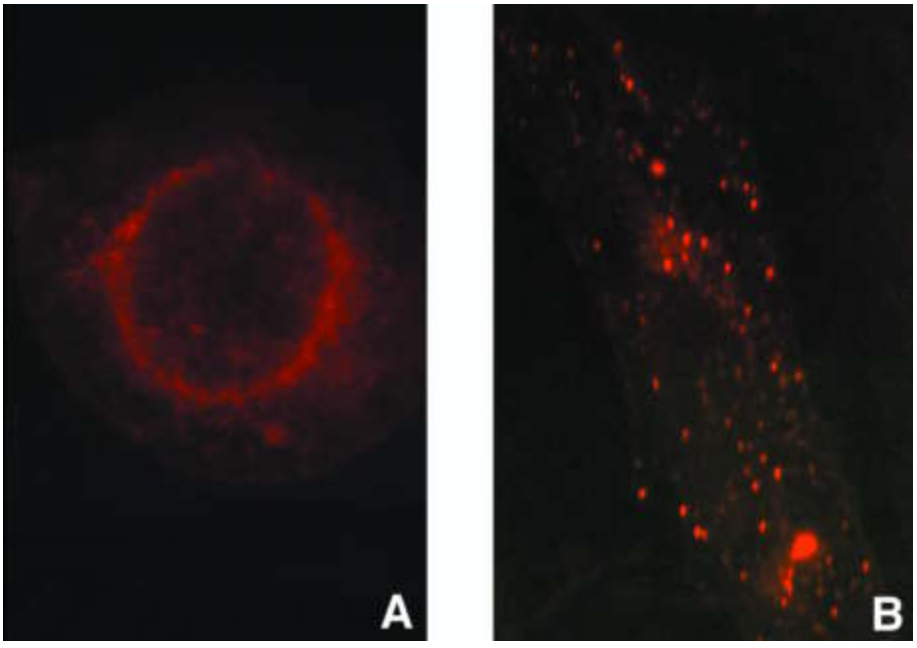

Figure 5 Production of markers of cell differentiation: Osteopontin. is in the cytoplasm on S disks (A), where it shows an interesting circular distribution, while on SB and on TPS (B), it is clearly located on the cell membrane with a punctate pattern of distribution.

proteins were detectable on all the surfaces after 24 h and increased until 96 h of culture. Some differences were revealed among the various surfaces. Significant increase in production and organization of ECM proteins was detected in SaOS-2 cells cultured on SB and TPS as compared with S disks, so confirming the immunocytochemical data.

The total amount of CoI, FN, VN and TN present in the SaOS-2 cells or deposited onto titanium surfaces was quantified by Enzyme-linked immunoassay *in situ* to better investigate the ECM production. After 96h of culture all the matrix proteins were detectable and were significantly more abundant on the TPS than on the SB and S surfaces ($p<0,05$). VN production was higher on the TPS surfaces than on the SB and S surfaces ($p<0,05$) (Figure 7). The results obtained in Serum starvation condition (DMEM 0.5%BSA) did not influence ECM production for the whole period of culture, showing a lower synthesis of ECM but an equal trend (*data not shown*).

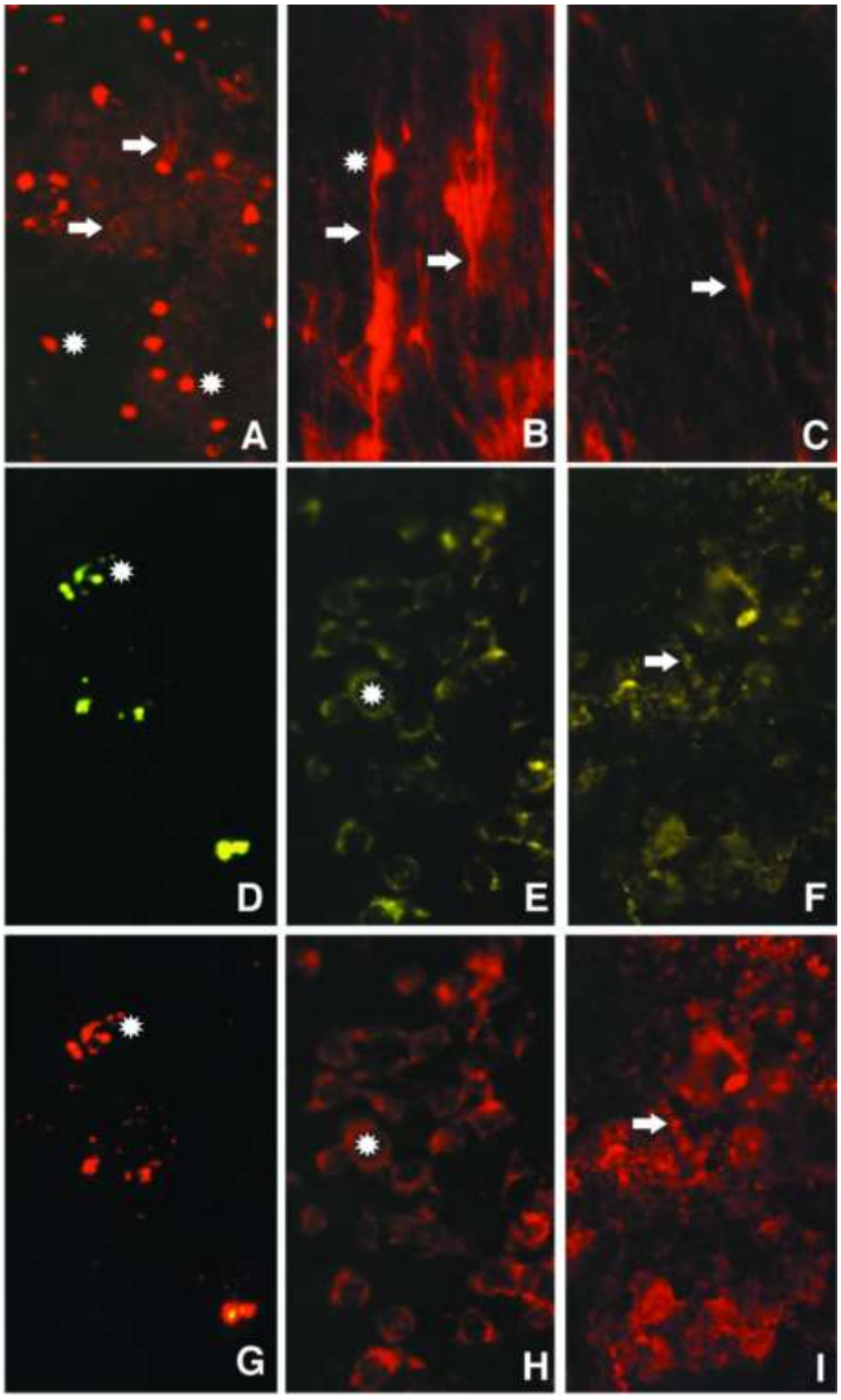

Figure 6. Extra Cellular Matrix production by SaOS-2 cells on titanium disks: the left column shows cells plated on smooth disks (S), the central column on sandblasted (SB) disks and the right one on titanium plasma sprayed (TPS). Arrows indicate the Extra Cellular Matrix and stars are positioned strictly near the cells. Col (A, B and C) is intracellular on S, strictly resembling in vitro basal condition, while it is well organized outside the cells on SB (more abundant) and TPS. TN (D, E and F) and FN (G, H and I) synthesis and assembly present pattern similar to those of Col. Cells which adhere on S disks are prevalently involved in intracellular production and storage while those plated on SB disks present a pericellular arrangement of these matrix components. On TPS disks both TN and FN are distributed on the titanium surface. The double staining of TN (green fluorescence) and FN (red fluorescence) demonstrates that these proteins largely co-distribute in our experimental system.

Discussion

The process of the bone formation around the titanium implants *in vivo* is a very complex process and we know that many factors are implied in this phenomenon. The published data on the *in vivo* study of titanium dental implants are sometimes contradictory and do not clarify the real biological differences between the various types of surface. In addition, the clinical success of titanium dental

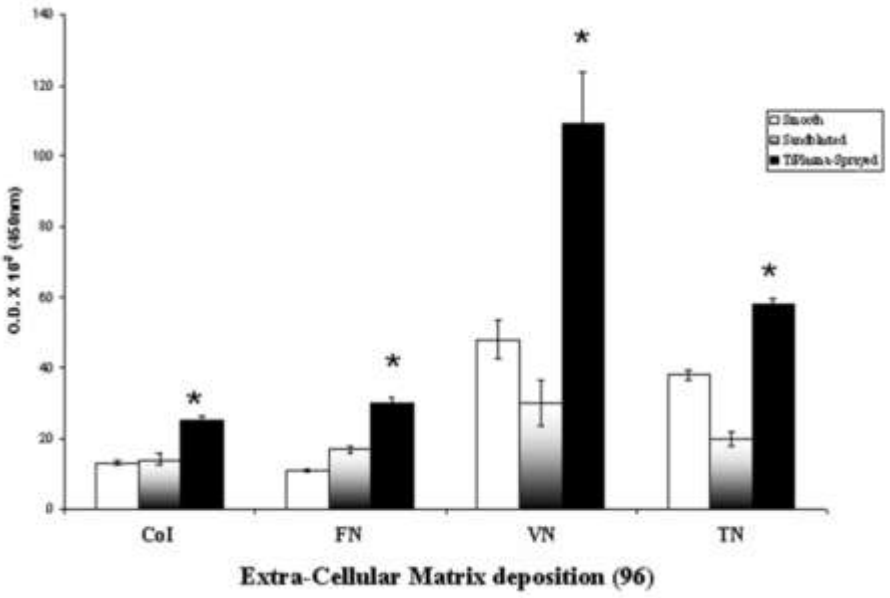

Figure 7 Effects of titanium surfaces on Extra-Cellular Matrix synthesis. Cells were cultured on titanium disks up to 96 h and analysed by an in situ enzyme-linked immunoassay (ELISA), for the quantification of a) CoI; b) FN; c) VN d) TN. The expression of all components was significantly increased on TPS disks.

implants is nevertheless related to a follow-up often executed for a short and insufficient time. It is well known that the interaction between dental implants and bone tissue depends on several conditions, including host and implant factors. Among implant factors, type of material, configuration and surface characteristics are important (Cochran, 1996; Cochran, 1999; Wong et al., 1995; Martin et al., 1995). The surface of titanium implants is one of the most important factors in the modulation of osteoblastic activity (Buser et al., 1991).

In the present study, we investigated the relevance of three different titanium surfaces on cellular morphology, adhesion, and *in vitro* phenotypic expression of the osteoblast-like cells SaOS-2.

The scanning electron microscopy (SEM) analysis showed a smooth surfaces on the S disks whereas SB and TPS disks were characterized by relatively rough surfaces with pits and bumps. Some studies demonstrated that the treatment of the titanium disks by autoclave sterilization does not change the surface characteristics of the titanium materials (Kelier et al. 1990). In our study, we confirmed that the surface features of titanium disks before and after sterilization were comparable, leading us to hypothesize that the autoclave treatment does not exert any subsequent influence.

The SEM analysis of the SaOS-2 cells morphology on the three different surfaces demonstrated some differences in cell number and cell shape. In fact, S disks were uniformly covered by a monolayer of flat cells, whereas the cell distribution on the SB and TPS surfaces was lower and discontinuous, showing clusters of multilayered cells which never reached the confluence. Cellular shape was irregular, with filopodia crossing the disk surface. These data seem to indicate that SaOS-2 cells are able to respond to the differences of the surface topography and can modify their morphology to ensure a better outcome with the surface of the titanium disks.

Cellular adhesion on titanium, as evaluated in the early hours of culture, was not affected by the type of the surface. Our data are in agreement with the observation of other Authors reporting that the type of surface does not influence *in vitro* adhesion and spreading of osteoblasts (Lowenberg et al. 1987). On the other hand, several studies have demonstrated that *in vitro* osteoblast adhesion to titanium surfaces can be affected by various factors including components of the ECM and the Bone Morphogenetic Protein-2 (BMP-2), a member of the super-family of the TGF-β (Degasne et al., 1999; Shah et al., 1999).

As regards CoI, FN, VN and TN, some of the components of the ECM produced by osteoblasts during differentiation, the analysis of the ECM production in SaOS-2 cells demonstrated that the synthesis of CoI, FN, VN and TN was influenced by the kind of the surface.

Immunofluorescence revealed that all the ECM proteins are synthesized but not yet secreted on S disks, whereas the ECM proteins were synthesized, secreted and organized as insoluble matrix on the

SB and TPS titanium surface after 4 days *in vitro*. As it has been reported that some components of the foetal calf serum (FCS) can mediate cellular attachment and spreading on artificial substrates by the involvement of the cytoskeleton of the cells (Grinell F, 1978), we must puntualize that the above described effects were evident after 24-48 h of culture and increased after 72-96 h, so suggesting that they were not affected by the FCS component, which was present in the same concentration during this whole period. The same experiments were repeated in serum starvation (0.5% BSA), but the results of ECM detection were unvaried in respect to the ones in 10% FCS, so we conclude that the presence of serum FCS does not influence the deposition of ECM after 6-96 hours.

The quantitative analysis of CoI, FN, VN and TN levels demonstrated a high production of matrix by SaOS-2 cells, with specific differences among the studied surfaces; in particular, the TPS surface presented the highest production of VN when compared with the other ones.

Osteopontin (OPN) and BoneSyaloprotein-II (BSP-II) are the major non-collageneous bone proteins incorporated into the mineralized tissue matrix (Kasugai et al., 1992). Morphological analysis of OPN and BSP-II production demonstrated that both of them were produced early and in large abundance in culture on the SB and TPS surfaces, whereas the S surfaces appear to induce only a slight intracellular production of OPN and BSP-II during the entire period of culture.

Indeed, our results suggest that rough surfaces promote osteoblast differentiation in vitro better than smooth surfaces. Therefore, it seems that they promote a better integration of dental implants *in vivo*.

Other studies *in vivo* are however required in order to confirm the *in vitro* effects of surface topography on the biological behaviour of bone cells. In conclusion, our data demonstrate that surface roughness affects *in vitro* morphology and differentiation of osteoblast-like cells, suggesting that the implant surface properties may modulate the biological behaviour of osteoblasts in bone tissue response.

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