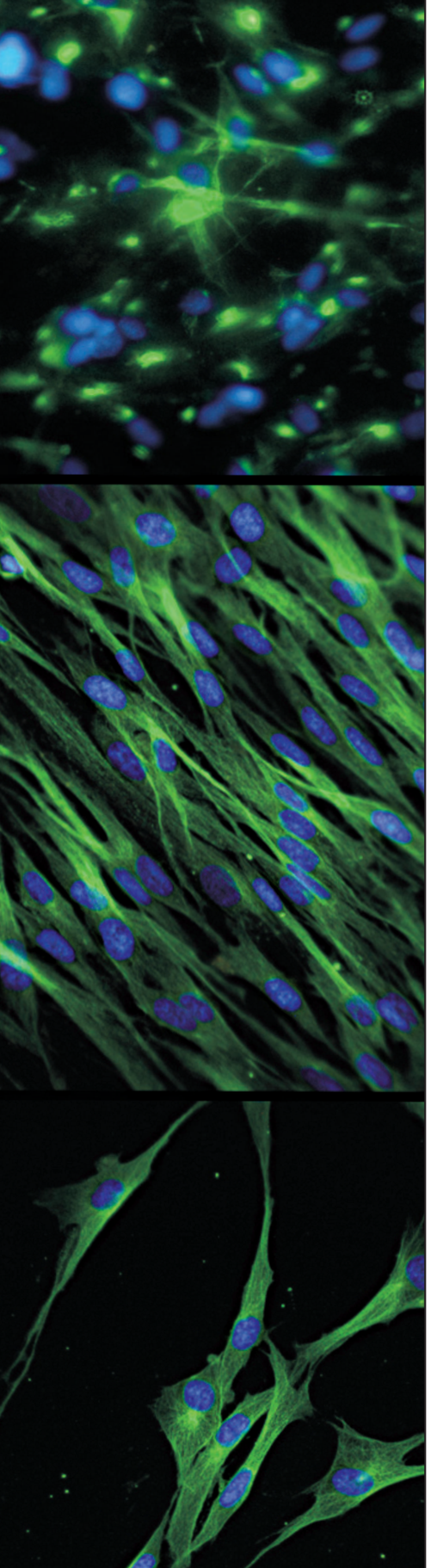

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Implant Surface Roughness Influences Osteoclast Proliferation and Differentiation

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#63

Implant Surface Roughness Influences Osteoclast Proliferation and Differentiation

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ABSTRACT

Introduction: The long term maintenance of a rigid implant-bone interface is related to a continuous remodeling of this interface. This remodeling replaces old bone, repairs fatigue damage foci and helps to maintain the interface structural integrity. Bone remodeling requires coordinated activities by osteoclasts and osteoblasts. In order to shed more light on this topic, we performed a multiparametric analysis of murine monocytes response to different bio-compatible Titanium surfaces.

Materials and Method: Murine RAW 264.7 type TIB-71, were cultured on the 3 different surfaces glass, machined titanium, rough titanium (Oralplant, Cordenons, Italy). For experiments, cells were treated with 10 ng/ml M-CSF and 25 ng/ml RANKL for 14 days and then processed for SEM and immunofluorescence microscopy.

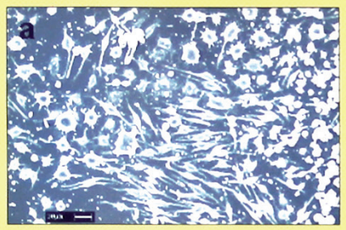
Results: The morphological analysis performed after 14 days of culture by means of immunofluorescence with the support of a specific anti β-tubulin antibody, revealed deep differences among the cells cultured on the different surfaces. On the glass surface, murine monocyte-macrophage RAW cells underwent strong morphological changes which were the hallmarks of differentiation, namely the migration of the precursor cells toward the formation of syncytii and aggregation into polynucleated cells which represented mature osteoclasts. On glass surfaces, round and uniformly distributed cells were present, generally without clusters formation, while cells cultured on machined titanium disks showed largely distributed clusters of cells with smaller cytoplasmic bodies than those observed on glass. On the rougher surface disks, cells were strictly aggregated into small isolated clusters. RAW cells, cultured onto the machined titanium surface, demonstrated a higher proliferation rate than cells cultured onto the rougher titanium surface, while the latter showed a higher capability to differentiate.

Conclusions: The understanding of how osteoclasts respond to different implant surfaces and biomaterials may help to develop implants that improve osseointegration and the long-term function of dental implants.

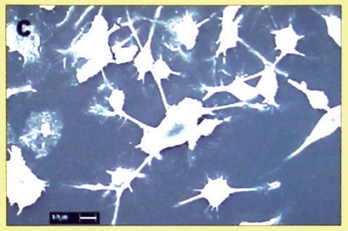
INTRODUCTION

Bone is a dynamic tissue and the long term maintenance of a rigid implant-bone interface is related to a continuous remodeling of this interface. This remodeling, occurring at the implant-bone interface, replaces old bone, repairs fatigue damage foci and helps to maintain the interface structural integrity. Bone remodeling requires coordinated activities by osteoclasts and osteoblasts. Bone replacement is initiated by osteoclast resorption followed soon after by osteoblastic formation. Implant surface roughness has been demonstrated to affect the characteristics and the behaviour of the osteoblasts at the bone-implant interface and a higher bone-implant contact percentage has been reported for implants with a rougher surface. Although osteoblasts play a pivotal role in implant integration in the bone context, osteoclast activity is also required for peri-implant bone remodeling. Osteoclasts are activated in specific focal sites by mechanisms that are still not well understood. Osteoclast-mediated bone resorption has been reported to occur around the implants, possibly playing a relevant role during peri-implant bone healing and in the long-term overall implant success. Osteoclasts are large, highly specialized multinucleated cells able to produce lacunar bone resorption: these cells are formed by fusion of marrow-derived mononuclear phagocyte osteoclast precursors which circulate in the CD14+ monocyte fraction of peripheral blood. The differentiation of the monocytes into mature osteoclasts is specifically induced by simultaneous stimulation of monocytes by two cytokines: Macrophage Colony Stimulating Factor (M-CSF) and Receptor Activator of NF-kB Ligand (RANKL). RANKL is a member of the TNF superfamily, that is essential for osteoclastogenesis and fundamental in mediating the osteoclastogenic action of TNF. The binding to its receptor, RANK, evokes the activation of a signalling cascade involving Jun N-Terminal kinase (JNK) that increases the *trans* activation activity and the production of AP-1 transcription factors. This would induce the migration activity of the cells and the formation of syncytii. It has been suggested that undifferentiated osteoclast progenitors bind to RANKL on stromal cells and differentiate into tartrate-resistant acid phosphatase (TRAP)-positive pre-osteoclasts which migrate towards the bone surface and multinucleate. RANKL-deficient mice showed a phenotype similar to severe osteopetrosis, in which osteoclastogenesis was completely inhibited. During normal bone remodeling, osteoclasts are thought to provide RANKL. However, progresses in the understanding of the biological events that osteoclasts undergo and, in turn, induce during bone remodeling, following the contact with the surface of dental implants, are difficult to get due to the lack of osteoclast cell lines. Therefore, in order to circumvent these problems, a variety of model systems have been developed, able to give rise osteoclast-like cells under certain stimulations and used with the aim to identify precursors, surface phenotype and characterize factors that affect osteoclast activity and formation. Among others, murine monocytes RAW 264.7 type TIB-71 obtained from murine ascitic fluids, represent a very useful cell system since they differentiate into osteoclasts following treatment of definite doses of the osteoclast-differentiation factor RANKL and macrophage colony-stimulating factor (M-CSF). Therefore, in this study we cultured and differentiated such cells on titanium disks whose surfaces displayed different roughness features in order to investigate their influence on cell differentiation into osteoclasts and on their morphological organization, distribution and adhesion capability.

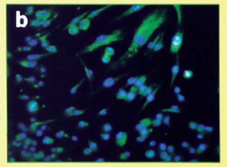
RESULTS



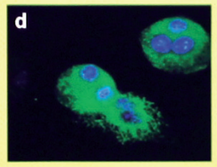
SEM
Morphological analysis of RAW cells on glass surface; note the migration of the precursor cells toward the formation of syncytii and aggregation into polynucleated cells which represents mature osteoclasts



β Tubulin was uniformly distributed in the monocytes, while its expression appeared to increase and to be reorganized around nuclei in the colony of the long and narrow cells going to confluence



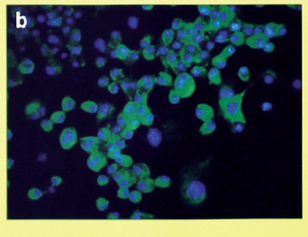
Immunofluorescence analysis of β-tubulin distribution (green) during raw cell differentiation. The nuclear counterstaining (blue) was obtained by means of dapi.

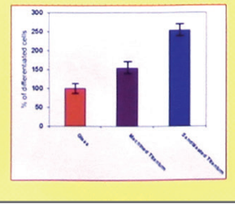


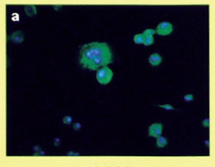
During the occurrence of cell fusion it could be noticed a slight green network, peripherally in the cytoplasm, while, in contrast, an evident protein expression giving rise to a green matrix around nuclei was observed

IMMUNOFLUORESCENCE ANALYSIS OF β-TUBULIN DISTRIBUTION IN RAW CELLS CULTURED ON DIFFERENT SURFACES:

MACHINED TITANIUM
Monocytes cultured onto machined surface were randomly located

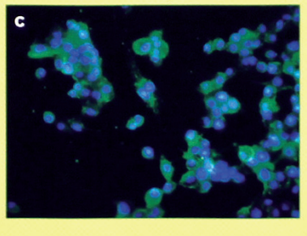




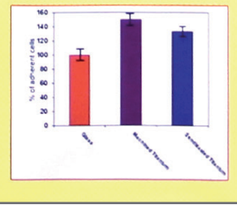


GLASS

SANDBLASTED TITANIUM
It is possible to observe isolated clusters of cells with a well defined cytoplasmic polarity, as indicated by the re-organization of β Tubulin. In fact, in the most of the clusters, cells showed not cytoplasmic extensions, but a rounded organized cytoplasm located inside the grooves while the nuclei were almost always oriented toward the peaks



Effect of machined and sandblasted Titanium surfaces on RAW cells viability and differentiation
P<0.005
The analysis of the effect of machined and TPSS surfaces on the cell adhesion and differentiation capabilities evidenced that cells cultured onto the machined titanium surface displayed a higher adhesion capability than cells cultured onto TPSS surface, while the latter surface induced the highest capability to differentiate with respect to the other surfaces.



MATERIALS AND METHODS

Materials: Sterile glass slides were used as controls; a set of sterile titanium disks grade 3 machined and a set of newly developed titanium disks (titanium pull spray superficial-TPSS surface, Oralplant, Cordenons, PN, Italy), the latter showing different roughness surface features (i.e. a rougher surface) were used for experiments. All disk types were of the same diameter (3 cm). The new surface (TPSS) was produced through a micro-mechanical removal of parts of the superficial oxide layer by means of the use of aluminum oxide 0.5 µm micropoints. Ten specimens were used for each material, and each experiment was repeated 3 times. The Ra values, obtained with the use of a Mitutoyo Surftest 211 Profilometer were 0.12 µm for the glass, 0.34 µm for the machined surface and 2.89 µm for the TPSS surface. The Rt values, obtained with an optical profilometer WYKO NT-2000, were, respectively, 0.92 µm, 2.89 µm, and 9.74 µm. An average of 5 readings was performed for each surface.

Cell Culture and treatments: Murine RAW 264.7 type TIB-71 monocytes, were cultured onto glass (control), machined surface and TPSS surface in RPMI 1640 containing 10% di FCS and 1% penicillin-streptomycin (50 IU/ml and 50 mg/ml respectively). Cell counts were performed by fixing the cells in 4% paraformaldehyde /PBS for 10 min at room temperature and counterstaining the nuclei with 4',6-diamidino-2-phenylindole (DAPI) a DNA specific dye. According to studies of phenotypic characterization, only multinucleated cells were considered as differentiated osteoclasts.

Immunocytochemical Detection: Cells growing onto glass, machined surface and TPSS surface were fixed in 4% paraformaldehyde/PBS for 10 min at room temperature, washed twice with PBS, incubated with PBS for additional 15 min to quench the remaining paraformaldehyde, and saturated/permeabilized using NET gel solution (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.4), 0.05% NP-40, 0.25% Carrageenan Lambda gelatin, and 0.02% Na-N3) for 30 min at room temperature. Later on, cells were treated with anti-β-Tubulin monoclonal antibody (diluted 1:50) for 3h at room temperature. After two washes with NET gel solution, goat anti-mouse IgG-FITC (diluted 1:150) was added to the cells and incubated for 45 min at room temperature. After three additional washes, the nuclei were counterstained with DAPI and mounted in DABCO-glycerol-PBS. The immunostaining specificity for the monitored proteins was confirmed by the absence of any reactivity when the secondary antibody Fitc conjugate (diluted 1:150) were used. The influence of the different materials on cell adhesion capability was assessed through fluorescence microscopy by counting the number of the cells onto the surface area identified by a 20X objective. The same method was applied to asses the number of RAW cells differentiated into osteoclasts (number of cells disclosing three or more nuclei).

SEM: Samples belonging to the 3 different groups were mounted onto aluminium stubs and sputter gold coated in Emitech K 550 for SEM examination. The samples were rinsed with saline solution and fixed in a buffered solution at pH 7.2 of 2.5% of glutaraldehyde and 2.5% of paraformaldehyde. Samples were washed again with buffer and dehydrated in an ascending alcohol series of 50%, 70%, 90%, 95% and 100%. All the specimens underwent critical point drying in Emitech K 850 and treated for SEM examination as described above.

Data Analysis: Differences between mean values of groups were tested for statistical significance either with Student's t or, where applicable, with one-way Analysis-of-Variance (ANOVA).

DISCUSSION AND CONCLUSIONS

The osteoclasts formation appears to be strongly dependent on the tissue environment therefore influencing bone integration with biomaterials. It has been observed that, in rat femur, rough-surfaced plasma-coated implants induces, when compared to smooth implants, a layer of multinucleated giant cells. Studies on cell and matrix reactions at titanium implants, in rat tibiae, have evidenced a rapid osteoclastic activity at implant surfaces. In addition, a steady formation of osteoclast-like giant cells was observed as response to titanium implants in cortical bone of the rat femur, around the implant. Further, different chemical treatments of implant surfaces produce differential bone responses, including induction of osteoclast formation. The differential response of β Tubulin arrangement as detected by immunocytochemical analysis, strongly suggests that different cytoskeletal reorganization occur during the interaction with the different biomaterials. β Tubulin is not the only structural protein involved in the organization of the cytoskeletal matrix; fluorescence analysis shows, however, that its different rearrangements might be considered as marker of cytoskeleton adaptation evidently produced, in absence of other differences, by the surface features. In fact, machined surfaces disclose concentric ridges and grooves produced during the turning procedure that could account for the modulation of the cell orientation and adhesion. On the other hand, TPSS surfaces, did not display any well-oriented ridges or grooves but peaks and valleys. Such a surface organization was likely responsible for the organization in clusters, which, in turn, might explain the high rate of differentiation observed on this surface. It is possible, in fact, that the tight contacts produced by the cell clustering would inhibit the proliferation by influencing the cell cycle progression, switching on, as a consequence, the differentiation machinery. Titanium particles have been already demonstrated to stimulate in vitro bone resorption by inducing osteoclast differentiaton. Therefore the manufacturing of implant surfaces might be a crucial step able to influence the marrow microenvironment and, in turn, osteoclast formation and activity. This could obviously affect the biological response of the tissue environment in the period immediately after implant placement, during which osteoclasts may play a critical role in priming or at least preparing the tissue environment for the osteoblast activity. Since osteoclasts apparently initiate the remodelling of interfacial bone which occurs as a consequence of the continuous loading after implant placement, the understanding of the molecular machinery controlling the biological response of osteoclasts to differently organized biomaterial surfaces may help to develop implants that improve osteo-integration and long-term function of dental implants. Preliminary work in our laboratories would indicate that, among other molecules suggested to play some role in the signalling regulating osteoclast activity, phosphoinositide cycle- related enzymes are likely to play a pivotal role.