Surface Chemistry Effects of Topographic Modification of Titanium Dental Implant Surfaces: 2. In Vitro Experiments

Clara Cassinelli, Dr Biol[1]/Marco Morra, Dr Chem[1]/Giuseppe Bruzzone, MD[1]/Angelo Carpi, MD[2]/Giuseppe Di Santi, MD[3]/Roberto Giardino, MD[4]/Milena Fini, MD[5]

Purpose: *To determine, in vitro, cytotoxicity and cell adhesion on 3 different implant surfaces.* ***Materials and Methods:*** *All samples had machined surfaces, but they were subjected to different cleaning procedures, which produced 3 different surface chemistries. One of the samples was "as-produced" from the machining tools. The other samples were subjected to partial and total cleaning routines. Cytotoxicity was evaluated using mouse fibroblast cultures, and cell adhesion was evaluated with osteoblast-like SaOS-2 cells.* ***Results:*** *The "as-produced" sample showed a pronounced surface contamination by lubricating oils. For partially and totally cleaned samples, an increasing amount of titanium and a decreasing carbon/titanium ratio was observed as cleaning became more complete.* ***Discussion:*** *Differences in surface chemistry such as those normally found on titanium implant surfaces (see part 1 of this series) can lead to those same effects which, in in vitro experiments, are normally accounted for in terms of surface topography alone.* ***Conclusion:*** *Effects related to surface chemistry can operate over and above surface topography, making it impossible, without proper characterization, to make definite statements about the role of topography alone.* (INT J ORAL MAXILLOFAC IMPLANTS 2003;18:46–52)

Key words: *cell adhesion, cytotoxicity, dental implants, surface properties, surface topography, titanium*

Cell culture experiments have become more attractive in the attempt to understand, control, and direct interfacial interactions at dental implant surfaces.[1–3] In particular, cultures of osteoblast cells, either primary or from tumor lines, are frequently used to evaluate the effect of surface roughness on cell behavior and metabolism. Cooper and coworkers[4] studied the use of osteoblast cultures on titanium surfaces having different roughness to evaluate the effect of topography on mineralization. Boyan and coworkers addressed the effect of surface topography on surface cell density, alkaline phosphatase and growth factor production, and protein synthesis.[5–8]

Previous papers have shown that the response of cells on titanium surfaces is different on surfaces having different roughness. The behavior of cells in culture (and the clinical performance of dental implants[9,10]) is very often accounted for in terms of surface topography only—that is, in terms of the effect of surface topography on cell behavior. This view contains an implicit assumption, ie, when comparing samples with different roughness, topography is the only variable and surface chemistry is constant.[11] This carries the connotation that surface topography is responsible for all measured differences of cell response. In the studies described above, this is a serious assumption, since it is well known that surface chemistry very definitely affects cell behavior.[12] Actually, modification of surface chemistry to direct cell behavior is a very intriguing area of the surface modification of medical materials.[12,13]

[1]Researcher, Nobil Bio Ricerche, Villafranca d'Asti, Italy.
[2]Professor, Department of Reproduction and Aging, University of Pisa, Italy.
[3]Private Practice, Porto Ercole, Italy.
[4]Professor and Chair of Surgical Pathophysiology, Faculty of Medicine, University of Bologna and Experimental Surgery Department, Research Institute Codivilla Putti, Rizzoli Orthopaedic Institute, Bologna, Italy.
[5]Researcher, Experimental Surgery Department, Research Institute Codivilla Putti, Rizzoli Orthopaedic Institute, Bologna, Italy.

Reprint requests: Dr Clara Cassinelli, Nobil Bio Ricerche, Str. S. Rocco 36, 14018 Villafranca d'Asti, Italy. Fax: +390-141-941956. E-mail: cclara@tin.it

Results of a companion study, an x-ray photoelectron spectroscopic (XPS) analysis of 34 different commercially available dental implants,[11] have suggested that a statistically significant relationship exists between surface chemistry and treatments used to increase surface roughness, confirming that surface topography and surface chemistry are inextricably bound. The interest of the present investigation is to show that surface chemistry variations such as those observed previously[11] can lead to the same biologic effects in vitro that are generally interpreted in terms of surface topography only. To this end, cytotoxicity testing and cell adhesion experiments were conducted on implant surfaces having the same topography (machined surfaces) and different surface chemistries, which were obtained using different cleaning routines. The continuous line L-929 mouse fibroblast was used in the former experiments, and the tumor cell line SaOS-2, osteoblast-like cell,[14] was used for cell adhesion.

MATERIALS AND METHODS

Titanium dental implants were supplied by Dentaltech (Misinto, Milan, Italy). All implants were machined, with no roughening treatments applied. Samples were divided into 3 groups. The first group contained "as-machined" implants, ie, implants produced by machining tools without any cleaning. These samples were coded nc (not cleaned). To simulate different surface chemistries within the range of those commonly found on titanium dental implants, different cleaning routines were used. The second group was water washed only (pc, partially cleaned implants), and the third group of implants was completely cleaned following the complete proprietary cleaning protocol of the producer (which does not involve acid pickling). These samples were coded cc (completely cleaned).

XPS Analysis

XPS analysis was performed with a Perkin-Elmer PHI 5500 ESCA system (Shelton, CT). The instrument is equipped with a monochromatic x-ray source (Al Kα anode) operating at 14 kV and 250 W. The diameter of the analyzed spot was approximately 400 μm, the base pressure was 10^{-8} Pa, the angle between the electron analyzer and the sample surface was 45 degrees, and the pass energy was 187.8 eV. Quantification of elements was accomplished using the software and sensitivity factors supplied by the manufacturer. The correctness of the sensitivity factors used was checked independently by the evaluation of lightly sputtered titanium dioxide reference samples (Sigma, Milan, Italy).

Scanning Electron Microscopic Analysis

Sample morphology was observed using a LEO 420 scanning electron microscope (SEM) (LEO Electron Microscopy, Cambridge, United Kingdom). The accelerating voltage was maintained between 15 and 2 kV (low-voltage SEM [LV-SEM]), as discussed in the following.

Cytotoxicity Testing

Cytotoxicity was evaluated using the continuous mouse fibroblast L-929 cell line. This is an established cell line commonly used for this kind of testing. The experimental cell culture medium (Sigma) was minimum essential Eagle's medium without L-glutamine, 10% fetal bovine serum, streptomycin (100 μg/L), penicillin (100 U/mL), and 2 mmol/L L-glutamine in a 250-mL plastic culture flask (Falcon, Franklin Lakes, NJ). Cells were cultured at 37°C in a humidified incubator equilibrated with 5% CO_2. Fibroblasts were harvested from the culture flasks prior to confluence by means of a sterile trypsin-EDTA solution (0.05 trypsin, 0.02 EDTA in normal phosphate-buffered saline [PBS], pH 7.4), resuspended in the experimental cell culture medium, and diluted to 1×10^5 cells/mL. From this, 3.5 mL of the cell suspension were seeded into 6-well tissue culture polystyrene plates (9.6 cm^2 of growth area; Falcon) containing the samples.

A gold cylinder of the same size as the implants was used as a negative control, and a cylinder of a copper-nickel-aluminum dental alloy was used as the positive control.[15] After 3 days of growth at 37°C and 5% CO_2 in a humidified incubator, the following evaluations were performed:

1. Cell death and cell morphology. The cell monolayer around the samples was observed by an inverted microscope (DM IL, Leica, Wetzlar, Germany). The boundary between the samples and the cell monolayer was carefully controlled to check for cell death or number reduction. Also, the cell morphology was carefully controlled and compared with the results obtained with the negative control.
2. Biosynthetic activity. At a more in-depth level, a biochemical assay was performed to evaluate cell health through biosynthetic activity. In particular, the widely used Mosmann toxicity test (MTT) was performed. This test permits measurement of the succinate dehydrogenase (SDH) activity of cells after 72 hours contact with the alloy sample. SDH is a key enzyme of the Krebs

Table 1 Surface Composition as Detected by XPS Analysis of the Implants Tested

Sample	C	O	Ti	N	Si	Na	Mg	Ca	Cl
nc	79.8	15.6			4.3				0.3
pc	61.7	30.2	4.6	1.1	1.5		0.2	0.4	0.3
cc	36.4	42.9	17.0	1.5	1.3	0.2	0.2	0.3	0.2

Measurements were performed on 3 different spots. Data shown are means, typical variation was ± 1%.

cycle (that is, the citric acid cycle), and its evaluation by biochemical means is commonly used to check cell health.

Briefly, at the end of 72 hours contact, cells were washed with sterile PBS, and then the PBS was replaced with 2 mL/well of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) sodium succinate solution. The cells and MTT solution were incubated at 37°C for 3 hours in the incubator. During this time, yellow MTT solution is transformed by the cell mitochondrial dehydrogenase into insoluble blue formazan. By measuring the amount of formazan produced, it is possible to measure mitochondrial activity, and, as a consequence, cell viability. At the end of the incubation period, the MTT solution was removed and replaced with 2 mL/well of a 6.25% v/v 0.1 mol/L sodium hydroxide in dimethylsulfoxide to dissolve any formazan present. The wells were swirled for 5 minutes until the purple color was uniform and the adsorbance was evaluated at 560 nm. The adsorbance values obtained were averaged, and the average was expressed as a percentage of the negative control (which, by definition, is 100%).

Cell Adhesion

Osteoblast-like SaOS-2 cells were used in the cell adhesion experiments.[14] Experimental cell culture medium (Biochrom KG, Berlin, Germany) consisted of minimum essential Eagle's medium without L-glutamine, 10% fetal bovine serum, streptomycin (100 µg/L), penicillin (100 U/mL), and 2 mmol/L L-glutamine in a 250-mL plastic culture flask (Corning, Milan, Italy). Cells were cultured at 37°C in a humidified incubator equilibrated with 5% CO_2. Cells were harvested prior to confluence by means of a sterile trypsin-EDTA solution (0.5 trypsin g/L, 0.2 g/L EDTA in normal PBS, pH 7.4), resuspended in the experimental cell culture medium, and diluted to 1×10^5 cells/mL. For experiments, 5 mL of the cell suspension were seeded into 6-well tissue culture polystyrene plates (9.6 cm^2 of growth area; Falcon) containing the samples. Experiments were performed in triplicate. After 3 days, samples were carefully rinsed with PBS and fixed in a 5% glutaraldehyde-PBS. Samples were dehydrated using increasing concentrations of ethanol in water ethanol solutions up to 100% ethanol. The final dehydration step was performed with hexamethyldisilazane (HMDS, Aldrich, Milan, Italy). Dehydrated samples were gold sputter coated (AGAR Auto Sputter Coater, Stansted, UK) and observed with a LEO 420 SEM.

RESULTS

XPS Analysis

Results of the surface analysis of the 3 titanium dental implants tested in this study are reported in Table 1. In the case of nc implants, no titanium was detected. Clearly, contaminating hydrocarbons completely masked the underlying material, an observation that underlines the importance of cleaning procedures in the production of dental implants. For pc and cc samples, an increasing amount of titanium and a decreasing carbon/titanium ratio was observed as cleaning became more complete. While the nc sample was outside the range of surface chemistries commonly found on commercially available dental implants (see Table 1 of Morra and coworkers[11]), the surface compositions detected on pc and cc were well within the values reported. As to the quantitative aspects, cc could be defined as a "good" surface, from the standpoint of cleanliness (always making reference to the previous work[11]), while pc is not that satisfactory.

SEM Analysis

Figure 1 shows a 100× SEM image of the nc implant. This photograph clearly documents the XPS results, as the implant surface appears to be covered by a greasy layer of contaminants. This is apparently the result of lubricating fluids used in machining tools. It is important to realize that this dirty surface—and not an ideal titanium surface—could be the starting point of every sample intended for dental implant applications and experiments, in vitro, in vivo, or clinically. Thus, from this condition,

surface cleaning and chemical effects related to treatments aimed at the modification of the surface topography will determine the final surface chemistry of the device.

Figures 2a and 2b show 300× images of a pc sample. In particular, the surface depicted in Fig 2a was obtained using an accelerating voltage of 15 kV, which is the common practice in SEM (the accelerating voltage is normally maintained between 10 and 30 kV). Washing by water eliminated most of the greasy layer shown in Fig 1. Figure 2b shows the very same field of view as that shown in Fig 2a, but this time the image was obtained using an accelerating voltage of only 2 kV (LV-SEM).[16] A significant portion of the surface was covered by black areas, which were not captured using the conventional high-voltage mode (Fig 2a). LV-SEM has been used as a diagnostic tool to check organic contamination of titanium dental implants.[16] The black areas of Fig 2b, which go unnoticed in conventional SEM, are the result of organic contaminants not removed by the partial cleaning procedure. In agreement with the XPS findings, a significant amount of organic contamination remained on the pc surfaces.

As to cc surfaces, few or only very small black spots were detected in the LV-SEM mode, confirming previously reported quantitative relationships between XPS and LV-SEM analysis.[16] In summary, the different cleaning routines used (or the lack of cleaning) resulted in similar dental implants (from a topographic standpoint) with different amounts of organic contaminants.

Cytotoxicity Testing

The continuous cell line L-929 was used in the evaluation of cytotoxicity by the direct contact test. Titanium is obviously not toxic, but, as shown by Figs 1 and 2, it may not be the only element contained in this type of sampling. Figures 3a to 3c show inverted microscope images of results of direct contact test involving nc, pc, and cc samples. The portion of the implant in direct contact with the cells can be seen as an opaque (ie, not light-transmitting) body in the lower part of the figures. While the cell layer was well developed and came into contact with pc and cc samples, significant cell death was observed in the case of the nc sample (Fig 3a). Clearly, the lubricant shown in Fig 1, which was released from the implant surface to the culture medium, exerted a significant toxic effect. These findings were confirmed by results obtained from the MTT test (Table 2); no reduction of the cellular metabolism was observed in the case of cells cultured in contact with pc and cc samples, while a very significant effect was caused by nc samples.

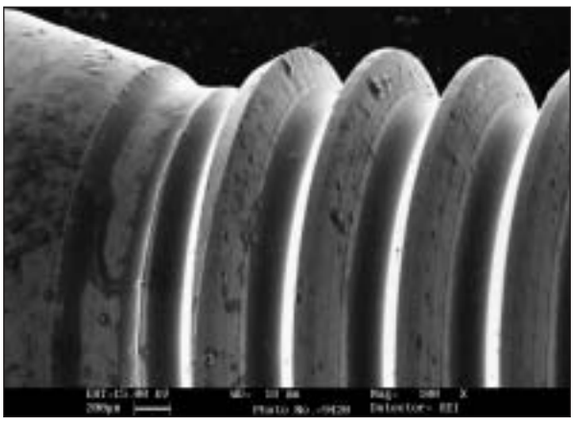
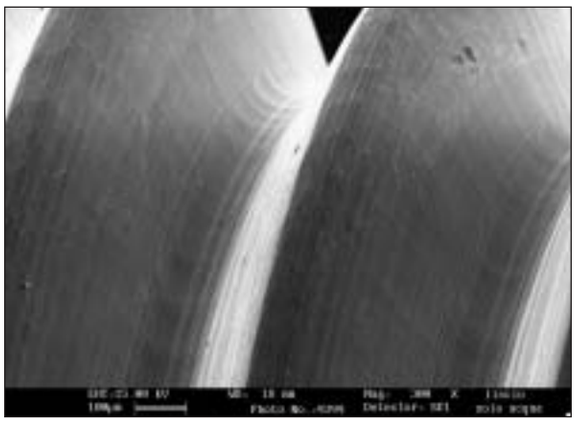


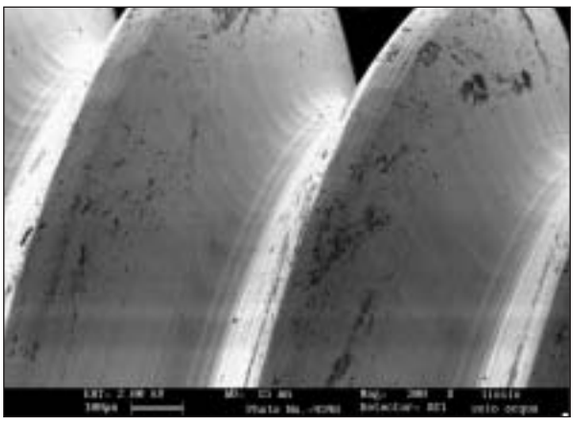
Fig 1 SEM image (×100) showing a dental implant "as produced" by the machining tool (not cleaned sample).

Cell adhesion studies, and the evaluation of surface density as a function of surface topography, are often reported in the literature. Osteoblast-like SaOS- 2 cells were used in the present case to evaluate cell density on surfaces with identical topography and different surface chemistry (Table 1). Results are depicted in Figs 4a to 4c. Confirming previous findings, no cells were detected on the surface of nc implants (Fig 4a), since the toxic effects described did not allow cells to adhere and grow on nc surfaces. On the other hand, cells adhered, spread, and grew readily on pc (Fig 4b) and cc (Fig 4c) surfaces. However, as clearly seen in the figures, the surface density of cells was definitely different, with a much higher density being detected on the cc surface. No attempts were made to give quantitative values of the differing cell densities, since the geometry of these samples could give rise to some artifacts (flat samples, rather than shaped implants, are more suitable for quantitative studies). However, the previous photographs showed clearly that the number of cells per unit area is different on pc and cc surfaces.

DISCUSSION

Tests involving the evaluation of cell response to titanium surfaces play an important role in the development of knowledge about biologic interactions at dental implant surfaces.[1,3] As basic knowledge on the biochemistry of tissue formation and healing increases, more sophisticated approaches can be used to capture the response of cells to the surface characteristics of implants. In the first article of this series, it was shown that surface topography and surface chemistry are inextricably bound, in

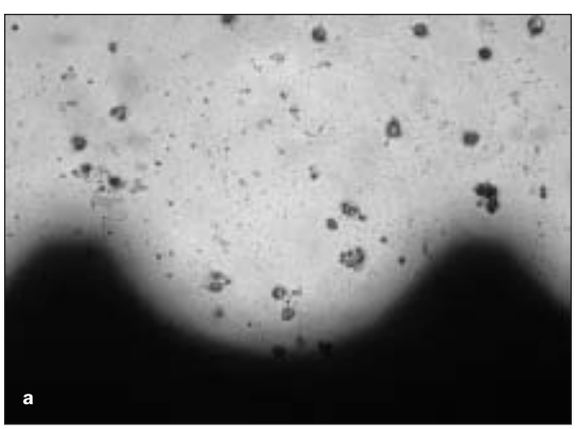


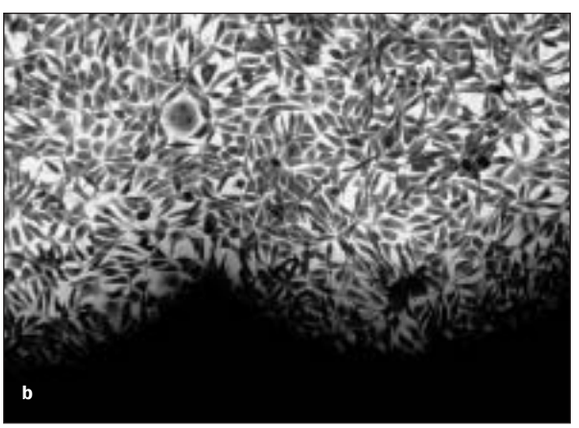


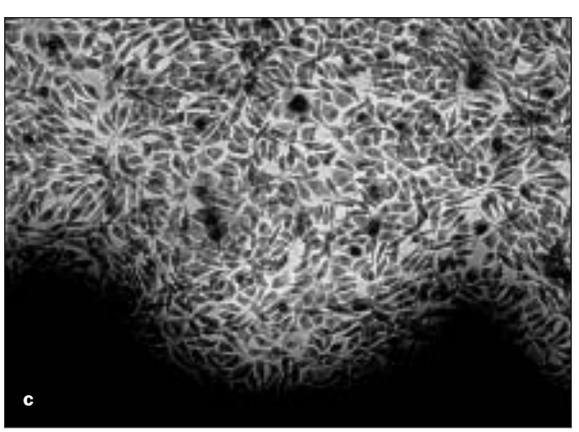
Figs 2a and 2b SEM images (×300) showing the partially cleaned sample. *(Left)* conventional (15 kV accelerating voltage) image; *(right)* same field of view obtained in LV-SEM mode (2 kV accelerating voltage).

Table 2 Results of MTT Test of Cytotoxicity (Mean ± SD)

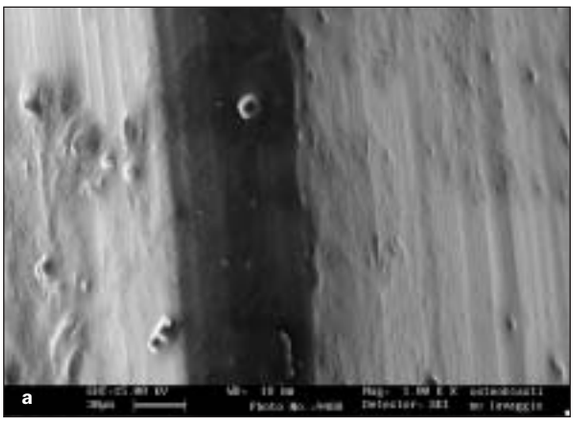
Sample	SDH activity (% negative control)
nc	34 ± 6
pc	99 ± 7
cc	98 ± 6
Positive control	27 ± 8

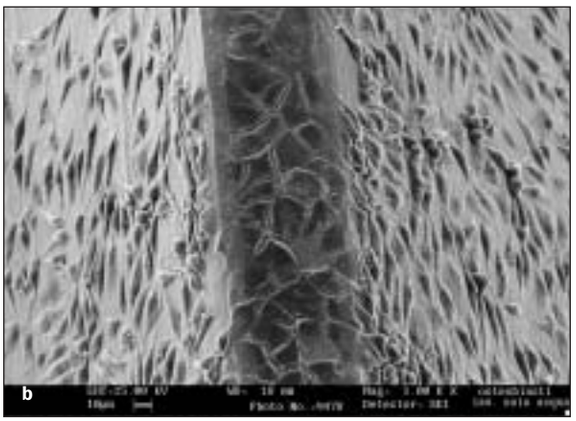


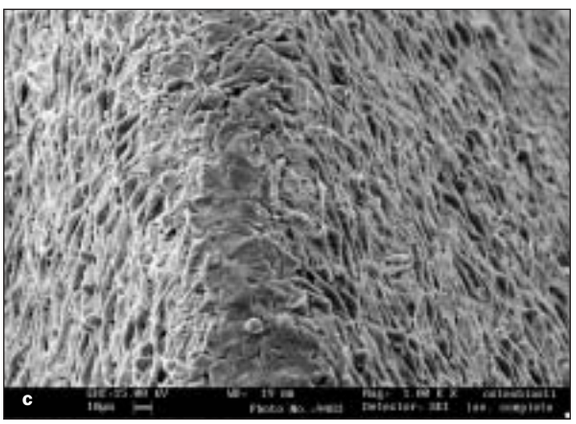




Figs 3a to 3c Optical microscope images (inverted microscope) showing the results of direct contact cytotoxicity testing. *(Above left)* Not cleaned sample; *(above right)* partially cleaned sample; *(left)* completely cleaned sample.







Figs 4a to 4c SEM images showing results of cell adhesion and growth (SaOS-2 osteoblast-like cells). *(Above left)* Not cleaned sample; *(above right)* partially cleaned sample; *(right)* completely cleaned sample.

the sense that the treatment used to obtain a given surface topography is often reflected in the surface chemistry of the implants. Thus, while topography can be assumed to be the only variable, this is often not the case. The question is, are differences in chemistry such as those previously shown[11] enough to affect cell behavior? Literature suggests that they could, as demonstrated, for instance, by Keller and coworkers in in vitro studies concerning the effect of sterilization procedures on cell behavior.[17]

Two simple in vitro experiments, cytotoxicity testing and cell adhesion, were conducted in this investigation. The nc sample was explicitly included to stress that even if one mentions a titanium device, its surface composition is far from being predictable. Whenever a titanium sample is produced, its surface can look like that shown in Fig 1, its composition as shown in Table 1, and its effect on cell behavior as depicted in Figs 3a and 4a. The condition can be improved by cleaning procedures; but the final surface that will be used in sophisticated tests assaying cell biochemistry will result from the convolution of the accuracy of cleaning, of the effect of the treatment(s) used to impart a given surface topography, and eventually of the effects related to packaging materials. The results of the tests reported in Figs 3 and 4 and Table 2 show that, if topography is kept constant and only surface chemistry is changed, it is possible to span the whole range from cytotoxicity to full cytocompatibility (Figs 3a to 3c, Table 2), from cell death to complete surface colonization (Figs 4a to 4c). Of course, all markers and biochemicals related to cell metabolism are more than likely to show the same wide range of variation and to give rise to an erratic behavior if interpreted only in terms of surface topography. When surfaces with different roughness have been compared, differences in cell density such as those shown in Figs 4b and 4c have often been explained in terms of surface topography only. It is suggested here that surface chemistry effects can operate over and above surface topography, making it impossible, without proper characterization,[18] to make definite statements about the role of topography alone.

The topography assumption is further exacerbated by recent suggestions regarding the role of early blood-implant interactions in bone healing.[19,20] The marked difference in the outcome of interaction between blood and an organic (as opposed to an inorganic) surface has been known since 1885, when it was observed that blood clotting time is increased if

glass test tubes used to collect blood are covered by petroleum jelly, an observation later (1940) encoded by the Lampert rule of blood clotting time.[18] The variable amount of organic presence on the supposedly inorganic titanium surface (see Fig 2b, for example) can give rise to conflicting results, if only surface topography is taken into account[20] or if the actual surface chemistry is overlooked. Problems arising from artifacts induced by organic contamination on metal surface preparation are not new. In 1970, Baier and coworkers[21] explained that the enhanced blood compatibility of Stellite (a cobalt-chromium alloy then used in heart valve production) was actually the result of the polishing routine. Because the alloy hardness allowed polishing to a high luster, the permanent embedding of waxes in the metal surface was prompted, making it organic-like and more blood compatible.[21] Abnormally high contact angles, typical of organic rather than metal surfaces, are often found even in the case of titanium surfaces.[22]

CONCLUSIONS

The present results of this limited investigation show that cell response can be affected by the surface composition of dental implants, confirming that, at least in vitro, chemical effects operate over and above the commonly invoked topographic effects. As a consequence, accurate analysis of the surface chemistry of titanium implants (or of the titanium samples used in in vitro studies) should be an integral part of every study on the biologic response to roughened titanium implant surfaces.

In vitro studies dealing with the basic mechanisms of cell-material interactions should temper the implicit assumption of constant titanium surface chemistry. As biochemical characterization of the cell response to titanium surfaces becomes more sophisticated,[4–8,19,20] the risks involved in such an approach are magnified, and proper characterization of all the variables involved is the only solution for minimizing risks.

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