Journal of Applied Biomaterials & Biomechanics 2004; 2: 169-176

The adhesion of Flow 2002 fibroblasts to titanium implant materials is influenced by different surface topographies and is related to the immunocytochemical expression of fibronectin

M.A. SANDRUCCI[1], V. NICOLIN[1], M. BIASOTTO[2], L. BRESCHI[2], L. CASAGRANDE[1*], R. DI LENARDA[2], V. GRILL[1]

[1]Department of Human Morphology, University of Trieste - Italy
[2]Department of Specialistic Surgery, Operative Unit of Odontology and Stomatology, University of Trieste - Italy
*At present Dental Hygienist

ABSTRACT: *Osteointegrated titanium dental implants are widely used biomaterials that have to integrate within the alveolar bone and interact with periodontal soft tissues. In this study, we investigated the immunocytochemical expression of the extracellular matrix (ECM) protein fibronectin (FN) and type I collagen (Coll I) in Flow 2002 fibroblast cultures spread on grade III-titanium samples with five different surface topographies and we correlated the immunocytochemical data to the adhesion capability of these cells to the above-mentioned substrates. Five different surfaces of grade III-titanium implants were at first characterized both by scanning electron microscopy (SEM) and by laser profilometry for surface roughness evaluation. After being spread on the biomaterial surfaces, the fibroblasts were left to proliferate for 72 hr and subsequently the cells underwent immunocytochemical procedures for detecting both FN and Coll I. The fibroblasts appeared more adherent to smoother titanium surfaces than to rougher ones; however, the highest cell density was detected on the roughest surface, even if it was unrelated to the highest FN expression. In the other biomaterial surfaces examined, as well as in controls, immunocytochemical FN expression correlated effectively to cell density on the examined substratum, whereas no determinant information was available regarding Coll I. It is reasonable to assume that surface roughness could be a relevant parameter influencing fibroblast adhesion to substrata; however, the evaluation of the cell density only is insufficient, and should be supported by the immunocytochemical FN expression, which could be confirmed as a useful tool in determining implant material biocompatibility. (Journal of Applied Biomaterials & Biomechanics 2004; 2: 169-76)*

KEY WORDS: *Titanium dental biomaterials, Surface topography, Fibroblasts, Cell adhesion, Fibronectin, Type I collagen*

Received 28/01/04; Revised 14/02/04; Accepted 13/05/04

INTRODUCTION

Osteointegrated dental biomaterials are currently widely used both to replace missing tooth elements and to anchor prostheses in the reconstruction of intra- and extraoral defects (1). The interface between dental implants and periodontal structures includes the attachment to periodontal soft tissues, as well as biomaterial osteointegration within the alveolar bone (2). Therefore, research on their biocompatibility has focused on both the bone-implant interface associated with the osteointegration process (1, 3-11) and on the implant-fibroblasts (12-16) and implant-epithelia interactions (17, 18). In addition, tissue responses in relation to different dental implant surface topographies have been largely investigated (2, 4, 5, 17, 19-23). Therefore, the evaluation of cell viability and cell proliferation

 1722-6899 / 169-08$15.00 / 0

rates (2, 6, 16, 17, 24-30), as well as cell adhesion capability on implant biomaterials (2, 9, 10, 12, 17), have been considered as indicators of *in vitro* biocompatibility. It has been already demonstrated that various extracellular matrix (ECM) molecules, and in particular the ECM protein fibronectin (FN), its integrin receptors and cytoskeletal molecules, are correlated to cell proliferation rates, as well as involved in cell differentiation, migration and adhesion (31). On this basis, various studies were performed on ECM molecule expression to evaluate dental biomaterial biocompatibility. In particular, our research group, by employing stabilized human embryonal fibroblast cultures in the presence of six single-phase dental metal alloys, evidenced that metal alloy composition influenced cell proliferation rates that correlated to the immunocytochemical FN expression (27). Further evaluation, extended to the expression of other ECM molecules, i.e. type I collagen (Coll I), chondroitin sulfate (CIS), as well as the FN receptor (the β_1 subunit of the $\alpha_5\beta_1$ integrin complex), also showed that the immunocytochemical feature of these ECM molecules could be useful tools in assessing the biocompatibility *in vitro* of biomaterials (28).

Biomaterials both in commercially pure titanium and in titanium alloy are currently widely used in dental implantology, since they express a high biocompatibility both *in vitro* and *in vivo*. This biocompatibility could be attributed to the thermodynamically and mechanically stable oxide layer that instantly forms on the metallic surface. However, various implant surface treatments, i.e surface machining, acid etching, sand blasting and plasma spraying require performing to improve titanium implant osteointegration (10). The interaction between cells and titanium implants is regulated by chemical, physical and biological factors: in particular, the implant surface topography seems to influence dental implant osteointegration (8, 11, 19-21). For this purpose, some authors concluded that implant osteointegration is more enhanced by rough surfaces than by smooth ones (5). Recently, by performing a study *in vivo*, it was evidenced that the interaction between the alveolar bone and dental implants with smooth surfaces was mediated by proteoglicans and collagen fibers interacting with osteoblasts, whereas regarding dental implants with rough surfaces, they closely interacted with a bone matrix presenting many osteocytes (20). However, since the development of new implant surfaces to achieve osteointegration is an aim in biomaterial research, new surface treatments have always been proposed (32). Concerning the interactions between dental implants and soft tissues, various studies were performed mainly on cell adhesion and proliferation in relation to implant surface topography (2, 12, 14, 17) and it was shown that soft tissue integration was better with smooth surfaces, and that it could be influenced more by implant surface morphology than the osteointegration process (12-18).

On this basis, this investigation *in vitro* aimed to evaluate the influence of five different surface topographies on titanium implant material biocompatibility, by considering the adhesion capability of stabilized human fibroblasts (cell line Flow 2002) to grade III-titanium biomaterial surfaces in correlation to the immunocytochemical expression of the ECM proteins, i.e. FN and Coll I, in 72-hr cell cultures.

MATERIALS AND METHODS

Biomaterials

Commercially grade III titanium discs (5 mm diameter, 1 mm thickness, 19.6 mm^2 area) with five different surfaces, obtained by mechanical, chemical or coating treatments were used (Plan 1 Health s.r.l., Villanova di S. Daniele del Friuli, UD, Italy). According to the different surface treatments, the discs were classified as follows.

A. Machined (MACH).
B. Sandblasted with Al_2O_3 (S-Al).
C. Sandblasted with glass microspheres (S-G).
D. Sandblasted with hydroxyapatite (S-HA).
E. Coated with titanium plasma spray (Ti-PS).

After surface treatment the discs were washed and sterilized by γ radiation.

Characterization of biomaterial surfaces

The surface features and the elemental analysis of the biomaterial composition of each sample were detected by a scanning electron microscopy (SEM) Stereoscan 430 Leica (Milano, Italy), at a magnification of 500x, equipped with an energy dispersive spectrometer (EDS). The roughness of the biomaterial samples surface was evaluated by a laser profilometer UBM (Sunnyvale, CA, USA) and measurements carried out on different regions of the sample surface.

Table I summarizes the following roughness parameters that were considered: the measure of the average roughness and profile depths (R_a), the profile length relationship (L_r), the profile symmetry (S_k) and the surface waviness (W_t). Roughness measurements were performed on five samples for each biomaterial surface.

TABLE I - ROUGHNESS PARAMETERS

Roughness parameters	Equation	Parameter description
R_a	$R_a = \frac{1}{n} \sum_{i=1}^{n} \lvert y_i \rvert$	Arithmetic mean of the departures of the roughness profile from the mean line, measured in µm. This parameter gives a good general description of height variations but is insensitive to wavelength and occasional high peaks and low valleys
L_r	$L_r = \frac{L_0}{L_m}$	Rate between the length obtained by strengthening a defined profile (L_0) and the real length of the measured profile (L_m)
S_k	$S_k = \frac{1}{n} \sum_{i=1}^{n} z^3_i / R^3_q$	The measure of the symmetry of the profile S_k=0: symmetric distribution; S_k>0: asymmetric distribution with more peaks than valleys; S_k<0 asymmetric distribution with more valleys than peaks
W_t		The greatest height between peaks and valleys of a low-pass filtered profile

Cell cultures

Human stabilized fibroblasts (cell line Flow 2002) were cultured in 75 cm^2 flasks in minimum essential medium (MEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS) (Sigma), 100 UI/ml penicillin and 100 µg/ml streptomycin sulfate, at 37 °C in a fully humidified air atmosphere containing 5% CO_2.
At confluence, cells were harvested using trypsin-EDTA and plated on the surface of each biomaterial sample at a cell density of 5 x 10^3 cells/cm^2. Subsequently, each sample was placed in a 24-well plate and 100 µl of fibroblast suspension carefully applied and the cells allowed to attach to the biomaterial surface for 2 hr. Finally, 1 ml of culture medium was added. Fibroblasts were cultured for 72 hr without renewing the culture medium. As controls, further series of cultures were performed in the same experimental conditions on glass coverslips. In each test, duplicate samples were run and each experiment was repeated five times, 10 replications in total.

Immunocytochemistry of extracellular matrix antigens

After 72 hr of cell culture, the cells fixed with 4% paraformaldehyde in phosphate buffer saline (PBS) underwent the following immunocytochemical reactions.
- FN: anti-human FN rabbit antibody (Sigma, St. Louis, MO, USA) diluted 1:100 in 4% bovine serum albumine (BSA), 5% normal goat serum (NGS) in PBS, followed by anti-rabbit IgG FITC-conjugated antibody (fluorescein isothiocyanate, green fluorescence; Sigma, St. Louis, MO, USA) diluted 1:50 in 4% BSA, 5% NGS in PBS.
- Type I collagen (Coll I): anti-human Coll I monoclonal antibody developed in mouse (Sigma) diluted 1:10 in 4% BSA, 5% NGS in PBS, followed by anti-mouse IgG antibody Cy3-conjugated (cyanine 3, red fluorescence; Sigma, St. Louis, MO, USA) diluted 1:150 in 4% BSA, 5% NGS in PBS.

Both reactions with primary antibodies were performed after pre-incubation with 4% BSA, 5% NGS in PBS to block aspecific binding sites. Samples were then rinsed in PBS and incubated with 0.1% 4',6-diamidino-2-phenylindole, 1% Tween 20 in PBS (DAPI, blue fluorescence) for staining cell nuclei. Finally, the fibroblasts were washed in PBS, dehydrated and mounted using glycerol containing 2.3% 1.4-diazobicyclo (2.2.2.) octane. Samples were observed using a Zeiss Axiophot microscope under epifluorescence conditions. Photographs were taken on Agfachrome 100 film under constant microscope settings and at the same exposure times for all specimens regarding the expression of each considered ECM antigen.

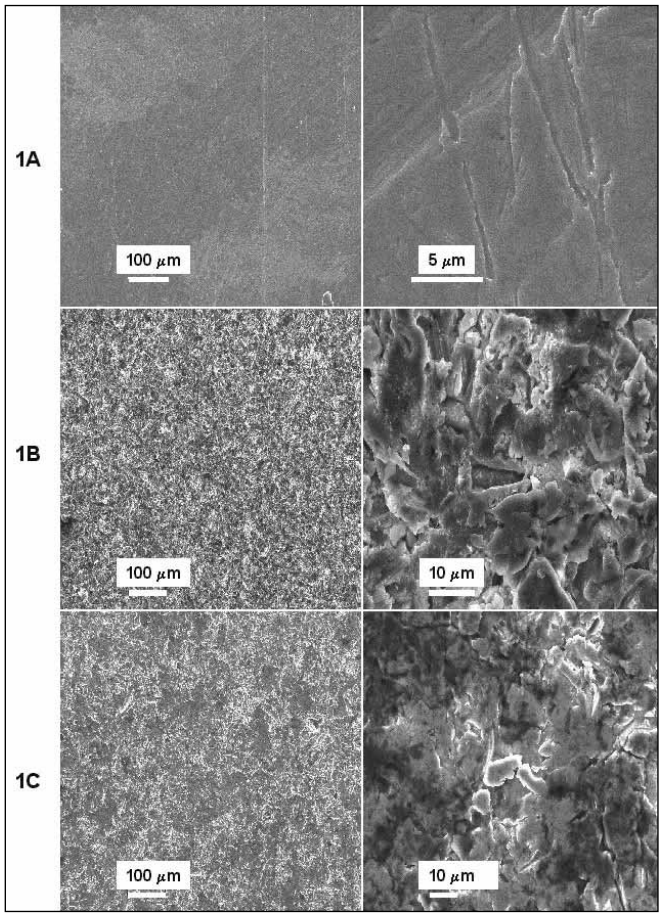

Fig. 1 - *SEM micrographs of biomaterial surfaces. 1A (machined surface), 1B (Al_2O_3 sandblasted surface), 1C (glass microspheres sandblasted surface).*

Immunofluorescence intensity was expressed as mean gray values in a scale of arbitrary units (AU) ranging from 0 (black, minimum fluorescence) to 255 (white, maximum fluorescence) related to pixels over a defined area in micrographs previously converted from RGB to 8 bit gray. Five areas for each micrograph were considered using the image analysis system Optimas 6 (Optimas Corporation, Bothell, WA, USA).

Cell density determination on biomaterials

DAPI-stained fibroblast nuclei were counted over each sample under epifluorescence conditions to calculate the cell density on each sample, expressed as cell number / mm^2 (mean values and standard deviations, SD) on five duplicate cultures. Statistical evaluations were performed with one-way ANOVA and Bonferroni Post-hoc tests (statistical significance $p \leq 0.05$).

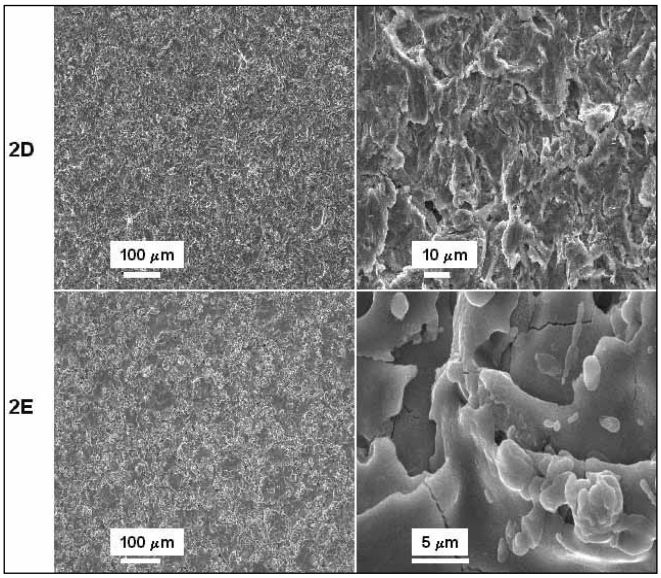

Fig. 2 - *SEM micrographs of biomaterial surfaces. 2D (hydroxyapatite sandblasted surface), 2E (titanium plasma spray coated surface).*

RESULTS

Characterization of biomaterial surface

The profilometric analysis followed by roughness parameter evaluation revealed that the five surfaces presented an increasing roughness in the following sequence regarding, in particular, the parameter R_a (range from 0.2-1.7 μm): A (Mach), B (S-Al), C (S-G), D (S-HA) and E (Ti-PS).

By SEM observation, surface A appeared the smoothest of those considered and presented some scratches with irregular disposition (Fig. 1A). Surface B presented many indentations that formed peaks and valleys (Fig. 1B). Surface C was also very rough (Fig. 1C), as was surface D (Fig. 2D). Surface E presented deep indentations and was the roughest among the samples examined (Fig. 2E). Table II reports the quantitative results related to roughness parameters. In particular, by considering the most frequently used roughness parameters, i.e. R_a and W_t, surface A could be considered the smoothest, since it presented the lowest values of both R_a (0.1845 ± 0.0606) and W_t (1.4432 ± 1.1166). Sample E exhibited the roughest surface (R_a 1.7036 ± 0.1851; W_t 11.9953 ± 2.9147).

Cell density determination on biomaterials

Tables III and IV, and Figure 3 report the results related to cell density on the implant surfaces. The highest cell density (expressed in mean values) was

TABLE II - ROUGHNESS PARAMETER VALUES OF FIVE TITANIUM SURFACES

	A machined	B sandblasted with Al_2O_3	C sandblasted with glass microspheres	D sandblasted with HA	E titanium plasma sprayed
R_a (μm)	0.1845 ± 0.0606	0.5108 ± 0.0506	0.6895 ± 0.0885	0.8190 ± 0.1076	1.7036 ± 0.1851
L_r (μm)	1.0009 ± 0.0003	1.0060 ± 0.0014	1.0033 ± 0.0011	1.0068 ± 0.0015	1.0106 ± 0.0023
S_k (μm)	-0.0660 ± 0.6784	-0.0370 ± 0.3089	-0.0381 ± 0.2682	-0.0855 ± 0.1801	-0.1340 ± 0.2204
W_t (μm)	1.4432 ± 1.1166	2.2794 ± 0.6958	4.2533 ± 0.5171	4.3647 ± 1.3086	11.9953 ± 2.9147

Mean values ± SD

TABLE III - FIBROBLASTS CULTURED ON VARIOUS TITANIUM SURFACES: CELL DENSITY

	A machined	B sandblasted with Al_2O_3	C sandblasted with glass microspheres	D sandblasted with HA	E titanium plasma sprayed	Control glass coverslips
Cell density (cells/mm^2)	16.87 ± 3.82	10.29 ± 2.34	15.89 ± 3.56	15.02 ± 4.41	17.73 ± 3.14	21.08 ± 4.71

Statistical analysis (mean values ± SD)

TABLE IV - FIBROBLASTS CULTURED ON VARIOUS TITANIUM SURFACES: CELL DENSITY STATISTICAL ANALYSIS (COMPARISONS BETWEEN GROUPS USING BONFERRONI *t* TEST)

	Cont	A	B	C	D	E
Cont	–	N.S	p<0.000	p<0.05	p<0.05	N.S
A	N.S	–	p<0.005	N.S	N.S	N.S
B	p<0.000	p<0.005	–	p<0.05	N.S	p<0.005
C	p<0.05	N.S	p<0.05	–	N.S	N.S
D	p<0.05	N.S	N.S	N.S	–	N.S
E	N.S	N.S	p<0.005	N.S	N.S	–

Statistical significance p≤0.05. N.S: not significant

detected on sample E (17.73 ± 3.14), and the lowest one on sample B (10.29 ± 2.34). In the controls, i.e. fibroblasts cultured on glass coverslips, cell density (21.08 ± 4.71) was higher in comparison to the other biomaterial surfaces. By considering the statistical analysis, a significant difference in cell density (p<0.05) was detected by comparing the cell density on surface C to that on surface D. Surface B differed significantly (p<0.000) in comparison to control cultures on glass coverslips. On the contrary, no significant differences in fibroblast density were evidenced on surfaces A and E. As regards the comparison between titanium samples, significant differences were detected between samples B and A (p<0.005), samples B and C (p<0.05), as well as between samples B and E (p<0.005).

Immunocytochemistry of extracellular matrix antigens

Regarding immunocytochemical FN expression, it appeared both localized within the cytoplasm and organized in fibrils in the ECM in the fibroblast cultures on samples A, B, C and D (Figs. 4A-FN, 4B-FN, 4C-FN, 4D-FN); whereas cells cultured on sample E expressed FN not organized in fibrils (Fig. 4E-FN). The quantitative analysis of immunofluorescence

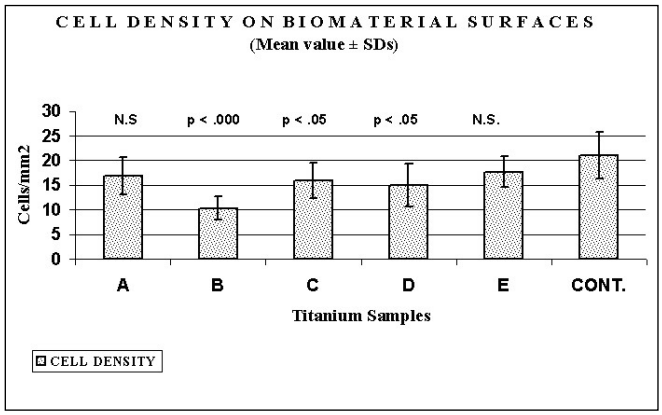

Fig. 3 - *Graphic presentation of cell density on biomaterial surfaces.*

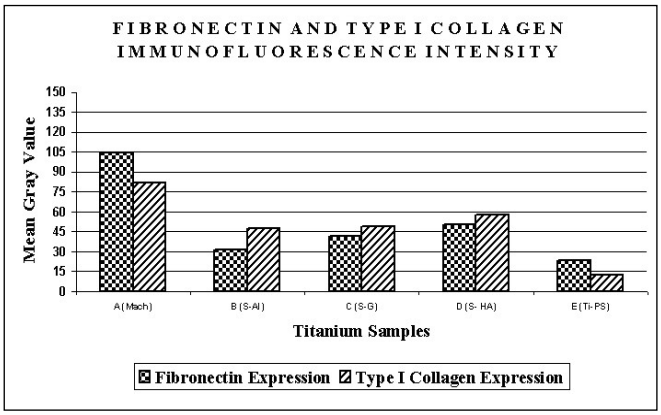

Fig. 5 - *Graphic presentation of the quantitative evaluation of FN and Coll I immunofluorescence intensity.*

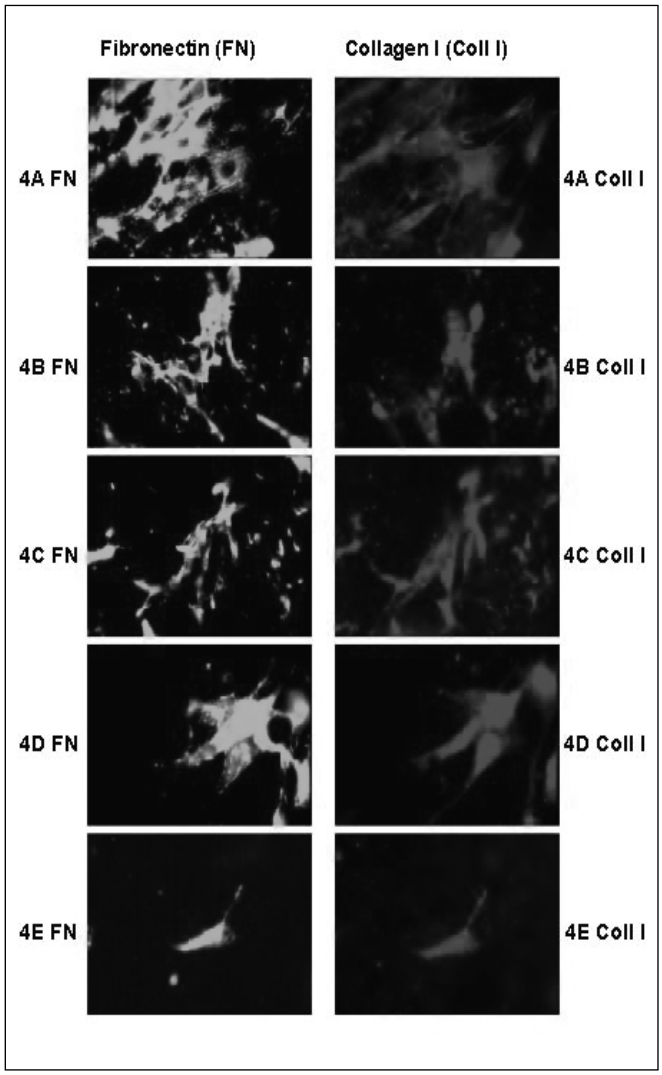

Fig. 4 - *Immunocytochemical expression of FN and Coll I in fibroblasts spread on different implant surfaces. 4A (machined surface), 4B (Al_2O_3 sandblasted surface), 4C (glass microspheres and blasted surface), 4D (hydroxyapatite sandblasted surface) and 4E (titanium plasma spray coated surface).*

by the gray values expressed in AU revealed the most intense FN immunofluorescence expression in fibroblast cultures on sample A (104 AU) and the lowest one in cultures on sample E (24 AU). FN expression intensities in the other cell cultures were 32 AU for sample B, 42 AU for sample C and 50 AU for sample D (Fig. 5).

Immunocytochemical Coll I expression was similar in all fibroblast cultures examined (Figs. 4A-Coll I, 4B-Coll I, 4C-Coll I, 4D-Coll I, 4E-Coll I). The highest Coll I immunofluorescence intensity was evidenced in fibroblast cultures on sample A (82 AU), the lowest one in cell cultures on sample E (24 AU). Intermediate intensity values were shown in cultures on samples B (48 AU), C (49 AU) and D (58 AU) (Fig. 5).

DISCUSSION

Since dental implants are biomedical devices applied in direct contact with hard and soft periodontal tissues, both experimental investigations in laboratory animals and clinical studies have focused on understanding the tissutal response mechanisms and, in particular, those involved in tissue integration with titanium implant biomaterials (1, 3-6, 7-16). In addition, experiments *in vivo* have to be accomplished by tests *in vitro* that give more detailed insights into biological mechanisms involved in cell-biomaterial interactions. However, few studies are currently available to correlate the evaluation of cell adhesion to implant biomaterials to an extensive description of the topographic and the physico-chemical aspects of implant surfaces (4). Indeed, many investigations were performed on the biological behavior of osteoblasts, as well as of osteoblast-like cells, which are widely involved

in the osteointegration process (3-6, 7-11), vs. titanium implant biomaterials, whereas minor attention has focused on the interaction with cells of soft periodontal tissues, i.e. gingival epithelial keratinocytes and fibroblasts (12-18). Based on previous reports demonstrating that the evaluation of the immunocytochemical ECM molecule expression involved in cell adhesion processes was a useful tool for evaluating dental biomaterial biocompatibility *in vitro* (16, 27-30), in this study we investigated how both FN and Coll I expression could be influenced in human fibroblast cultures spread on different implant surfaces of grade III-titanium biomaterials. Concerning the surface topographies, by considering the most used roughness parameters, i.e. R_a and W_t, it was observed that surface A was the smoothest, whereas surface E the roughest of those considered. Based on a previous study reporting the best fibroblast adhesion to smooth biomaterial surfaces (2), a better fibroblast adhesion was expected to the smoothest surface A and a worse one to the roughest surface E. In effect, data obtained by cell density evaluation revealed a higher number of adhered fibroblasts on surface A than on surfaces B, C and D; however, in contradiction with the previously mentioned hypothesis, more cells adhered to the roughest surface E than to the smoothest surface A. Regarding immunocytochemical FN expression, detected by a quantitative evaluation of gray levels, which did not yield absolute quantitative data but showed relative changes in protein expression in different culture samples (33), it was higher in cultures on sample A, according to cell density data on the same biomaterial surface. On the contrary, as expected, the lowest immunocytochemical FN intensity was detected in cultures on the roughest sample E, although it revealed the higher cell density. Concerning immunocytochemical Coll I expression, no significant differences were detected among the different culture specimens, which confirmed our previous observations (28). Results of both cell adhesion capability and immunocytochemical FN expression regarding surfaces A, B, C and D confirmed that fibroblasts should adhere better to smoother surfaces than to rougher ones, in accordance with previous reports (2, 12, 13). However, the contradictory results regarding the highest fibroblast density on the roughest surface E require further investigation, although these data correlated with low immunocytochemical FN expression; and therefore, this evidence could suggest a labile cell adhesion capability.

CONCLUSION

The results of this investigation confirmed that immunocytochemical FN expression evaluation could be a useful tool in the assessment of implant material biocompatibility. Moreover, each peculiar surface topography could be relevant in influencing cell adhesion to biomaterial surfaces, even if together with other factors such as the physical and chemical biomaterial composition it could be evaluated in further investigations.

ACKNOWLEDGEMENTS

This work was supported by grants from the Regione Autonoma Friuli Venezia Giulia, Italy (grants 2000) and FIRB (Italian MURST, 2001).

Address for correspondence:
Vittorio Grill, PhD
Dipartimento di Morfologia Umana Normale
Università degli Studi di Trieste
Via Manzoni, 16
34138 Trieste - Italy
grill@units.it

REFERENCES

1. Albrektsson T, Meredith N, Wennerberg A. Osseointegration of craniofacial implants. In Brånemark PI, Toleman DE eds. Osseointegration in craniofacial reconstruction. Chicago, Berlin, London, Tokyo, Paris, Barcelona, Sao Paulo, Moscow, Prague, Warsaw Quintessence Publishing Inc, 1998.
2. Könönen M, Hormia J, Kivilahti J, Hautaniemi J, Thesleff I. Effect of surface processing on the attachment, orientation and proliferation of human gingival fibroblasts on titanium. J Biomed Mater Res 1992; 26: 1325-41.
3. Ahmad M, McCarthy MB, Gronowicz G. An *in vitro*

model for mineralization of human osteoblast-like cells on implant materials. Biomaterials 1999; 20: 211-20.
4. Anselme K, Linez P, Bigerelle M et al. The relative influence of the topography and chemistry of TiAl6V4 surfaces on osteoblastic cell behaviour. Biomaterials 2000; 21: 1567-77.
5. Buser D, Schenk RK, Steinemann S, Fiorellini JP, Fox CH, Stitch H. Influence of surface characteristics on bone integration of titanium implants, histomorphometric study in miniature pigs. J Biomed Mater Res 1991; 25: 809-902.
6. Ramires PA, Giuffrida A, Milella E. Three-dimensional reconstruction of confocal laser microscopic images to study the behaviour of osteoblastic cells grown on biomaterials. Biomaterials 2002; 23: 397-406.
7. Ramires PA, Romito A, Casentino F, Micella E. The influence of titania/hydroxyapatite composite coatings on *in vitro* osteoblasts behaviour. Biomaterials 2001; 22: 1467-74.
8. Schwartz Z, Lohmann CH, Oefinger J, Bonewald LF, Dean DD, Boyan BD. Implant surface characteristics modulate differentation behavior of cells in the osteoblastic lineage. Adv Dent Res 1999; 13: 38-48.
9. Swart KM, Keller JC, Wightman JP, Draughn RA, Stanford CM, Michaels CM. Short-term plasma-cleaning treatments enhance *in vitro* osteoblasts attachment to titanium. J Oral Implantol 1992; 18: 130-7.
10. Anselme K. Osteoblast adhesion on biomaterials. Biomaterials 2000; 21: 667-81.
11. Ku CH, Pioletti DP, Browne M, Gregson PJ. Effect of different Ti-6Al-4V surface treatments on osteoblasts behaviour. Biomaterials 2002; 23: 1447-54.
12. Guy SC, McQuade MJ, Scheidt MJ, McPherson JC III, Rossman JA, Van Dyke TE. *In vitro* attachment of human gingival fibroblasts to endosseous implant materials. J Periodontol 1993; 64: 542-6.
13. Goto T, Brunette DM. Surface topography and serum concentration affect the appearance of tenascin in human gingival fibroblasts *in vitro*. Exp Cell Res 1998; 244: 474-80.
14. Hormia M, Könönen M. Immunolocalization of fibronectin and vitronectin receptors in human gingival fibroblasts spreading on titanium surfaces. J Periodontal Res 1994; 29: 146-52.
15. Eisenbarth E, Meyle J, Nachtigall W, Breme J. Influence of teeth surface structure of titanium materials on the adhesion of fibroblasts. Biomaterials 1996; 17: 1399-403.
16. Grill V, Sandrucci MA, Rizzo R, Narducci P, Bareggi R, Dorigo E. Biocompatibility *in vitro* of titanium dental implants. Minerva Stomatol 2000; 49: 77-85.
17. Lauer G, Wiedmann-Al-Ahmad M, Otten JL, Hübner U, Schmelzeisen R, Schilli W. The titanium surface texture effects adherence and growth of human gingival keratinocytes and human maxillar osteoblast-like cells *in vitro*. Biomaterials 2001; 22: 2799-809.
18. Jansen JA, Den Braber ET, Walboomers XF, De Ruijter JE. Soft tissue and epithelial models. Adv Dent Res 1999; 13: 57-66.
19. Kasemo B, Gold J. Implant Surfaces and Interface Processes. Adv Dent Res 1999; 13: 8-20.
20. Steflik DE, Corpe RS, Young TR, Sisk AL, Parr GR. The biologic tissue responses to uncoated and coated implanted biomaterials. Adv Dent Res 1999; 13: 27-33.
21. Chehroudi B, Gould TR, Brunette DM. A light and electron microscopic study of the effects of surface topography on the behavior of cells attached to titanium-coated percutaneous implants. J Biomed Mater Res 1991; 25: 387-405.
22. Wennerberg A, Albrektsson T, Andersson B. Design and surface characteristics of 13 commercially available oral implant systems. Int J Oral Maxillofac Implants 1993; 8: 622-33.
23. Wennerberg A, Albrektsson T, Ulrich H, Krol JJ. An optical three-dimensional technique for topographical descriptions of surgical implants. Biomed Eng 1992; 14: 412-18.
24. Craig RG, Hanks CT. Cytotoxicity of experimental casting alloys evaluated by cell culture tests. J Dent Res 1990; 69: 1539-42.
25. Wataha JC, Hanks CT. Biological effects of palladium and risk of using palladium in dental casting alloys. J Oral Rehabil 1996; 23: 309-20.
26. Grill V, Sandrucci MA, Basa M, et al. The presence of implant materials influences fibronectin arrangement and cell growth in fibroblast cultures.Boll Soc Ital Biol Sper 1996; 72: 87-94.
27. Grill V, Sandrucci MA, Basa M, et al. Dental metal alloys influence cell proliferation and fibronectin arrangement in human fibroblast cultures. Arch Oral Biol 1997;42: 641-47.
28. Grill V, Sandrucci MA, Di Lenarda R. Biocompatibility evaluation of dental metal alloys *in vitro*: expression of extracellular matrix molecules and its relationship to cell proliferation rates. J Biomed Mater Res 2000; 52: 479-87.
29. Grill V, Sandrucci MA, Di Lenarda R, et al. Cell proliferation rates and fibronectin arrangement as parameters for biocompatibility evaluation of dental metal alloys *in vitro*. J Oral Sci 2000; 42: 1-7.
30. Grill V, Sandrucci MA, Di Lenarda R, et al. *In vitro* evaluation of the biocompatibility of dental alloys: fibronectin expression patterns and relationships to cellular proliferation rates. Quintessence Int 2000; 31: 741-7.
31. Burridge K, Fath K, Kelly T, et al. Focal adhesion: transmembrane junctions between the extracellular matrix and the cytoskeleton. Annu Rev Cell Biol 1988; 4: 487-525.
32. Sandrini E, Chiesa R, Rondelli G, et al. A novel biomimetic treatment for an improved osteointegration of titanium. Journal of Applied Biomaterials & Biomechanics 2003; 1: 33-42.
33. Ermert L, Hocke AC, Duncker HR, et al. Comparison of different detection methods in quantitative microdensitometry. Am J Pathol 2001; 158: 407-17.