ADHESION, GROWTH AND DIFFERENTIATION MARKERS IN HUMAN OSTEOBLAST-LIKE CELLS CULTURED ON SURFACE-MODIFIED METALLIC MATERIALS DESIGNED FOR BONE IMPLANTS

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Abstract

A series of metallic materials with different surface treatments were prepared: pure machined titanium (T), titanium polished by diamond paste (TL), machined Ti6Al4V alloy (TS), Ti6Al4V alloy polished by diamond paste (TSL), Ti5Al2.5Fe alloy treated by electro-erosion (A) and Ti5Al2.5Fe plasma-sprayed with Ti (PL). The materials were seeded with human osteoblast-like cells MG 63. One day after seeding, the highest cell numbers were obtained on the samples of medium surface roughness (T and TS; R_a 0.63±0.30 um and 0.89±0.57 um, respectively). From day 1 to 4, the cell proliferation was the quickest on the samples with the lowest surface roughness (TL and TSL; R_a 0.17±0.13 for both materials). The cells on TL also contained the highest concentration of integrin adhesion molecules with alpha V chain, i.e. receptors for vitronectin and fibronectin. On day 8 after seeding, the cells on all metallic samples as well as tissue culture polystyrene reached similar population densities. The cells on electro-eroded Ti5Al2.5Fe (samples A; R_a 15.27±0.74 um) contained the highest concentration of osteocalcin and osteopontin, i.e. markers of osteoblastic differentiation. Thus, the latter newly developed material could be considered as promising for construction of bone implants well anchored in the surrounding bone tissue.

Key Words: *titanium, Ti6Al4V, Ti5Al2.5Fe, surface roughness, surface chemistry, bone cells, adhesion, integrins, proliferation, osteocalcin, osteopontin*

[Engineering of Biomaterials,58-60,(2006),1-3]

Introduction

Alloys of titanium, aluminium, vanadium, iron and other metals are traditional materials used in hard tissue surgery, e.g. for fabrication of screws, splints and other devices for bone fixation, or for construction of joint prostheses, mainly their bone-anchored stems (Boden *et al.* 2006, Cobb and Schmalzreid 2006, Levine *et al.* 2006). The anchorage of the metallic materials into the surrounding tissue depends of their mechanical and other physical and chemical properties. Unfortunately, the metals are generally very rigid and heavy in comparison with the bone tissue. For example, the modulus of elasticity and specific weight of metals commonly used for implantation into the bone (if they are in solid non-porous form) is higher by 1 to 2 orders than the values measured for the osseous tissue (Blazewizc *et al.* 1997, Levine *et al.* 2006). These disproportions often lead to damage and aseptic loosening of the bone adjacent to the implant. In addition, also the chemical composition of the metallic materials does not often support the viability and function of the surrounding as well as remote cells – several metals, for example cobalt, nickel or chromium, have been found to be cytotoxic or immunogenic, which is due to the release of metallic ions from the implant (Ibris and Rosca 2002, Cobb and Schmalzreid 2006; for a review see also Bacakova *et al.* 2001). On the other hand, the metallic prostheses are usually mechanically resistant and durable; their life span in the patient's organism can exceed 10 years (Boden *et al.* 2006, Reikeras and Gunderson 2006). In addition, the integration of metallic material with the surrounding tissue can be markedly improved by appropriate physicochemical surface properties of the material, such as roughness, topography, wettability or presence of certain chemical functional groups (Bacakova *et al.* 2000, 2001, 2004, Stary *et al.* 2003, Sul *et al.* 2005, Boden *et al.* 2006, Reikeras and Gunderson 2006).

Therefore, in the present study, the surface roughness of samples of pure Ti or Ti6Al4V alloy was modified by machining or subsequent polishing by diamond paste in order to influence the adhesion, growth and presence of bone differentiation markers (osteocalcin, osteopontin) in human osteoblast-like MG 63 cells. In addition, we investigated the interaction of these cells with a newly developed material for construction of bone-anchoring parts of hip joint replacements, i.e. Ti5Al2.5Fe alloy treated either with electro-erosion or plasma-spraying with Ti. Earlier, the Ti5Al2.5Fe alloy has been found to have high corrosion resistance, due to the presence of non-titanium atoms, particularly iron, which enhance the formation of the oxide film on the material surface (Ibris and Rosca 2002). Moreover, the Ti5Al2.5Fe alloy has been reported to carry low risk for patients of ferromagnetically induced secondary loosening caused by magnetic resonance scanning (Thomsen *et al.* 2001).

Material and methods

The metallic materials were provided the company Beznoska Ltd., Kladno, Czech Republic, a company producing and trading instruments and implants for orthopedics, traumatology and maxillofacial surgery (http://www.beznoska.cz). The materials were of cylindrical shape (diameter 2.4 or 2.9 cm, thickness 2 mm). The following groups of samples were prepared:
T: titanium, machined
TL: titanium, mechanically grinded and finally polished by diamond paste (grain size 2 μm),
TS: Ti6Al4V alloy, machined
TSL: Ti6Al4V alloy, mechanically grinded and finally polished by diamond paste (grain size 2 μm),
A: Ti5Al2.5Fe, electro-eroded
PL: Ti5Al2.5Fe, plasma-sprayed with Ti
PS: conventional tissue culture polystyrene dish (TPP, Trasadingen, Switzerland) as a control reference material.

The surface roughness was measured using a profilometer Hommel-Tester T 1000 with T1E and T3E scanners (Hommelwerke GmbH, VS-Schwenningen, Germany) and also by TALYSURF6 profilometer (Taylor-Hobson Ltd, Leicester, UK). The parameters R_a (arithmetic mean of the departures of the roughness profile from the mean line) and S_m (mean spacing of adjacent local peaks) were evaluated according the norm ISO 4287.

For cell culture, the samples were cleaned in ethanol, rinsed with distilled and deionized water, sterilized for 2 hours at 160°C in hot air sterilizer, inserted into 6-well plates (TPP, Trasadingen, Switzerland; diameter 3.4 cm). They were seeded with human osteoblast-like cells of the line MG 63, derived from osteosarcoma of a 13-year-old boy (European Collection of Cell Cultures, Salisbury, UK). Each dish contained 120 000 cells (i.e., 13 230 cells/cm^2 and 6 ml of Dulbecco Minimum Essential Medium supplemented with 10% of fetal bovine serum and 40 µg/ml of gentamicin). Cells were cultured for 1-8 days at 37°C in humidified air atmosphere containing 5% of CO_2. For evaluation of the morphology of cells and their distribution over the material surface, the cells were fixed with 70% cold ethanol and stained with propidium iodide (this compound stains nucleic acids, i.e. preferentially the nuclei, but the cytoplasmic part of the cell is, at least partly, also visible). For evaluation of cell number, the cells were detached with a trypsin-EDTA solution (Sigma, U.S.A, Cat. N° T4174) in phosphate buffered saline (PBS) for 10 min at 37°C, resuspended in cell culture medium (see above) and analyzed in a ViCell XR analyzer (Beckman Coulter, U.S.A).

The concentration of alpha V integrins, i.e. molecules participating in cell adhesion, as well as osteocalcin and osteopontin, i.e. extracellular matrix proteins typical for the bone and indicators of osteoblastic differentiation, was determined semi-quantitatively by the enzyme-linked immunosorbent assay (ELISA) in cell homogenates obtained by sonication (Bacakova et al. 2000). The following primary polyclonal rabbit anti-human antibodies were used: anti-integrin alpha V (Chemicon Int., Cat. N° AB1930), anti-osteocalcin (Chemicon Int., cat. N° Cat. No.ALX-210-309). Dilution of all antibodies was 1:500, incubation time 1 hour at room temperature. As the secondary antibody, goat anti-rabbit IgG (whole molecule) conjugated with peroxidase (Sigma, St. Louis, MO, U.S.A.; Cat. N° A0545) was applied (dilution 1:5000, incubation 1 hour at room temperature). Alpha V integrins, osteocalcin and osteopontin were also visualized by immunofluorescence staining using the above mentioned primary antibodies (similar dilution, incubation at 4°C overnight). The secondary antibody was represented by F(ab')2 fragment of Goat Anti-Rabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes, OR, U.S.A., Cat. N° A11070; dilution 1:500, incubation 1 hour at room temperature).

Results and discussion

On day 1 after seeding, the highest numbers of adhering cells were found on machined samples of Ti and Ti6Al4V (T and TS, respectively), i.e. surfaces of the medium surface roughness (R_a less than 1 µm, TAB.1, FIG.2A). On Ti6Al4V, this number vas even significantly higher in comparison with that on control tissue culture polystyrene. The cells were usually spindle-shaped, and their longitudinal axis was found on both polished Ti and Ti6Al4V, i.e. surfaces with the lowest roughness (R_a less than 200 nm; TAB.1, FIG.2A). On these smooth surfaces, the cells were homogeneously distributed like on the control tissue culture polystyrene (FIG.2B,D,G). However, from day 1 to day 2, the cells on the smoothest surfaces had the shortest population doubling

Sample / Parameter	T	TL	TS	TSL	A	PL	PS
R_a (µm)	0.63± 0.30	0.17± 0.13	0.89± 0.57	0.17± 0.13	15.27± 0.74	39.72± 9.58	0.02± 0.006
S_m (µm)	54.13± 23.09	31.52± 22.86	73.25± 53.62	33.25± 21.26	332.85± 22.62	519.5± 101.26	8.47± 1.33

R_a: arithmetic mean of the departures of the roughness profile from the mean line
S_m : mean spacing of crossing of the mean line, i.e between profile peaks
Means ± SD from 3 measurements on each of 14 samples of every experimental group.

TABLE 1. Parameters of the surface roughness in metallic materials with different surface treatments (see Materials and methods) designed for construction of bone implants.

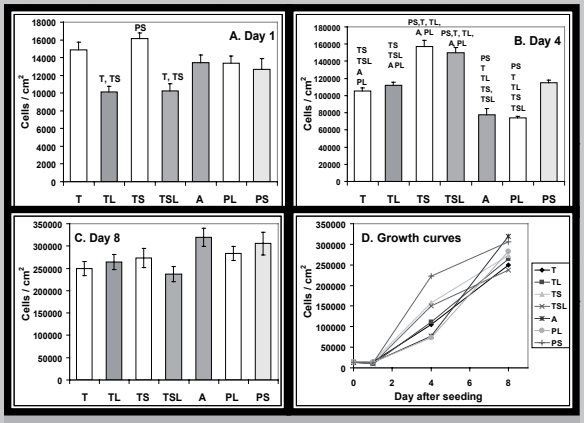

FIG.1. The population density of human osteoblast-like MG 63 cells on day 1, 4 and 8 (A, B and C, respectively) after seeding on metallic materials with different surface treatments (see Materials and methods), and their growth curves from day 0 to 8 (D).
Means ± SEM from 4 to 10 samples for each experimental group. One Way Analysis of Variance (ANOVA), Holm-Sidak test. T, TS, PS etc.: significantly different ($p \leq 0.05$) compared to samples the abbreviations of which are explained in the Material and methods).

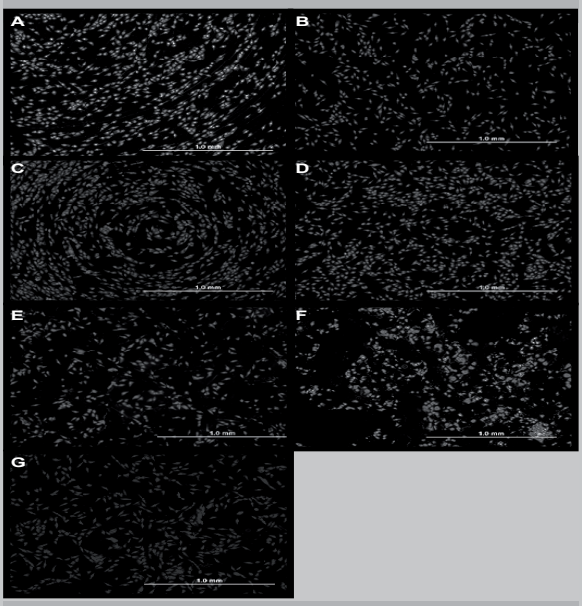

FIG.2. Morphology of human osteoblast-like cells MG 63 on machined pure titanium (A), titanium polished using diamond paste (B), machined Ti6Al4V alloy (C) Ti6Al4V alloy polished by diamond paste (D), Ti5Al2.5Fe alloy treated by electro-erosion (E) or plasma-spraying with Ti (F) and polystyrene tissue culture dish TPP (G). Day 1 after seeding, ethanol-fixed cells stained with propidium iodide, inverted fluorescence microscope Olympus IX 50.

time (TAB.2), so that on day 4, their population densities exceeded the values on the corresponding non-polished samples (FIG.1B). These results are in accordance with our previous findings of a more active cell proliferation on polished carbon-fibre reinforced carbon composites (Bacakova *et al.* 2001). On polished materials, the cells are usually better spread and forming multiple cell-material contacts, which could be positively correlated with their growth activity (for a review see Bacakova *et al.* 2000, 2001, 2004). More intensive cell-material contacts were suggested by a higher concentration of integrin adhesion molecules with

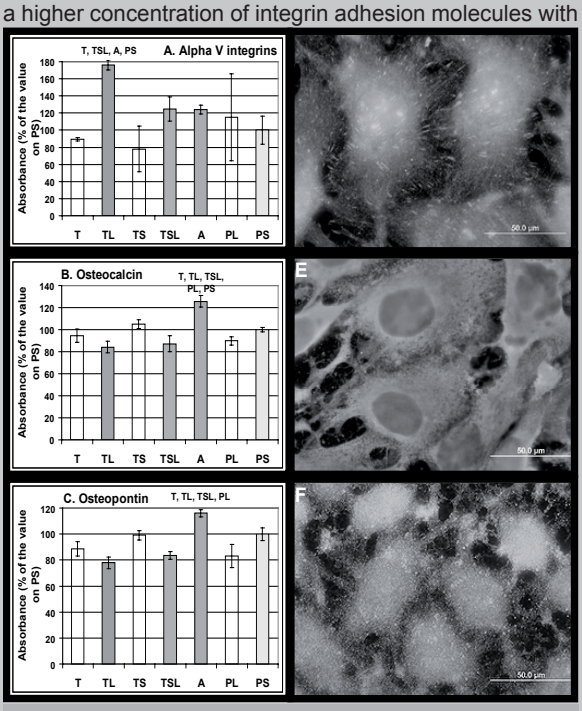

FIG.3. The concentration of alpha V integrins (A), osteocalcin (B) and osteopontin (C) in human osteoblast-like MG 63 cells grown for 8 days on metallic materials with different surface treatments (see Materials and methods). Measured by ELISA per mg of protein. D, E, F: Immunofluorescence staining of alpha V integrins, osteocalcin and osteopontin, respectively, in MG 63 cells on tissue culture polystyrene. Arrows indicate alpha V integrin-containing focal adhesion plaques, i.e. sites on the cell membrane with close cell-material contact.
Absorbance expressed in % of the value obtained from cells grown on tissue culture polystyrene. Means ± SEM from 4 measurements for each experimental group. Means ± SEM from 4 to 10 samples for each experimental group. One Way Analysis of Variance (ANOVA), Holm-Sidak test. T, TSL, A, PS etc.: significantly different ($p \leq 0.05$) compared to samples the abbreviations of which are explained in the Material and methods.

alpha V chain in cells grown on polished Ti in comparison with cells on the corresponding machined sample as well as polystyrene dish (FIG.3A). The alpha V integrins bind mainly vitronectin and fibronectin, which can be adsorbed on the materials surface from the serum of the culture medium.

On day 8 after seeding, the cells on all tested samples reached similar population densities (FIG.1A), although the density on both Ti5Al2.5Fe samples, especially those electro-eroded (referred as “A”), tended to be the highest. Moreover, these cells contained the highest concentration

Sample Parameter	T	TL	TS	TSL	A	PL	PS
DT (h)	25.5	20.8	21.9	18.6	28.5	29.2	17.4

DT = $(t\text{-}t_o)\log 2/(\log Nt\text{-}\log Nt_o)$, where Nt_o and Nt were the numbers of cells in 1- and 4-day-old cultures, respectively.

TABLE 2. The population doubling time (DT) in human osteoblast-like MG 63 cells grown on metallic materials with different surface treatments (see Materials and methods).

of osteocalcin and osteopontin, which suggested the highest differentiation potential of MG 63 cells on these materials towards the osteoblastic phenotype. The relatively good performance of MG 63 cells on the Ti5Al2.5Fe alloy could be due to a well-developed oxygen film on the surface of this material (Ibris and Rosca 2002). Oxygen-containing groups have been reported to support colonization of various artificial materials with cells (Bacakova *et al.* 2000, 2001, 2004, Stary *et al.* 2003). Therefore, it can be concluded that the Ti5Al2.5Fe alloy treated by electro-erosion might be promising material for construction of bone implants well-integrating with the surrounding bone tissue and permissive for bone tissue formation at the bone-material interface.

Acknowledgements

Supported by the Grant Agency of the Czech Republic (grant No. 101/06/0226). We also thank to Mrs. Ivana Zajanova for her excellent technical assistance. Mr. Robin Healey (Czech Technical University, Prague) gratefully is acknowledged for the language revision of the manuscript.

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