Effects of Different Titanium Alloys and Nanosize Surface Patterning on Adhesion, Differentiation, and Orientation of Osteoblast-Like Cells

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Key Words

Adhesion · Alignment · Differentiation · Osteoblasts · Surface charge · Titanium alloy

Abstract

To test nanosize surface patterning for application as implant material, a suitable titanium composition has to be found first. Therefore we investigated the effect of surface chemistry on attachment and differentiation of osteoblast-like cells on pure titanium prepared by pulsed laser deposition (TiPLD) and different Ti alloys (Ti6Al4V, TiNb30 and TiNb13Zr13). Early attachment (30 min) and alkaline phosphatase (ALP) activity (day 5) was found to be fastest and highest, respectively, in cells grown on TiPLD and Ti6Al4V. Osteoblasts seeded on TiPLD produced most osteopontin (day 10), whereas expression of this extracellular matrix protein was an order of magnitude lower on the TiNb30 surface. In contrast, expression of the corresponding receptor, CD44, was not influenced by surface chemistry. Thus, TiPLD was used for further experiments to explore the influence of surface nanostructures on osteoblast adhesion, differentiation and orientation. By laser-induced oxidation, we produced patterns of parallel Ti oxide lines with different widths (0.2–10 μm) and distances (2–20 and 1,000 μm), but a common height of only 12 nm. These structures did not influence ALP activity (days 5–9), but had a positive effect on cell alignment. Two days after plating, the major-

ity of the focal contacts were placed on the oxide lines. The portion of larger focal adhesions bridging two lines was inversely related to the line distance (2–20 μm). In contrast, the portion of aligned cells did not depend on the line distance. On average, 43% of the cells orientated parallel towards the lines, whereas 34% orientated vertically. In the control pattern (1,000 μm line distance), cell distribution was completely at random. Because a significant surplus of the cells preferred a parallel alignment, the nanosize difference in height between Ti surface and oxide lines may be sufficient to orientate the cells by contact guiding. However, gradients in electrostatic potential and surface charge density at the Ti/Ti oxide interface may additionally influence focal contact formation and cell guidance.

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Abbreviations used in this paper

ALP	alkaline phosphatase
BSA	bovine serum albumin
ECM	extracellular matrix
PBS	phosphate-buffered saline
PLD	pulsed laser deposition
PMMA	polymethylmethacrylate
RT	room temperature
TiPLD	pure titanium prepared by pulsed laser deposition

Introduction

The surface as an interface between bulk material and biological tissue is crucial for a good acceptance of bone implants. Specific surface properties, such as composition, roughness, porosity, alignment of structures and surface charges, influence the interaction between cells and implants [Martin et al., 1995; Degasne et al., 1999; Soboyejo et al., 2002]. Thus, osteoblast adhesion depends on surface chemistry and structure. Cells can react to an artificial surface topography on the micrometer scale (2–10 μm depth), e.g. by aligning along groove- and ridge-patterned structures (contact guidance). These microgrooves also affect cell shape by elongating them along the grooves, which in turn can influence cell proliferation, differentiation and function [Brunette and Cheroudi, 1999; Soboyejo et al., 2002; Lu and Leng, 2003]. The influence of finer structures on cell guidance and functions was controversially discussed. Until recently, papers showing that nanostructures can influence cell behavior were rare. Clark et al. [1991] reported cell alignment on a 260-nm grated quartz surface with 100-nm-deep grooves. Now, Teixeira et al. [2003] found that corneal epithelial cells elongate and align along silicone ridges 70 nm wide and 150 nm deep. Reduced fibroblast adhesion was observed using nanosized pits on polymethylmethacrylate (PMMA, 35–120 nm in diameter, pitch 100–300 nm) [Curtis et al., 2004; Martinez et al., 2004]. On 13-nm-high islands produced by demixing of polymers, fibroblasts showed increased cell spreading and up-regulation of gene expression [Dalby et al., 2002]. Similar results were obtained on cylindrical PMMA columns (100 nm wide and 160 nm high) [Dalby et al., 2005]. However, to the best of our knowledge, we are the first to demonstrate osteoblast alignment on only 12-nm-high Ti structures.

A superior combination of toughness, density and corrosion resistance has made Ti and Ti alloys the materials of choice in many clinical applications. Metastable or stable $\alpha+\beta$ or β -Ti alloys exhibit high strength, good formability and high hardness. β -Alloys also offer the unique possibility of combined low elastic modulus and superior corrosion resistance [Long and Rack, 1998]. The $\alpha+\beta$ Ti6Al4V alloy is generally considered as a 'standard material' for orthopedic and dental implant applications. On the other hand, vanadium is cytotoxic and can initiate adverse tissue reactions. In response to these concerns, a number of new Ti alloys with Ta, Nb, and Zr were developed for biomedical applications. These elements produce essentially insoluble oxides, allowing the corre-

sponding alloys to be treated as 'completely biocompatible'. The $\alpha+\beta$ TiNb13Zr13 and the near- β TiNb30 alloys combine low elastic modulus and superior corrosion resistance in comparison to Ti6Al4V and are the materials of choice for long-term implants [Breme et al., 2000].

Bone consists of nanostructured hydroxyapatite and collagen fibers, whereas conventional metallic or ceramic materials used for orthopedic implants are structured in the micrometer range. Therefore, several nanophase materials, i.e. with grain or fiber size less than 100 nm, were recently developed. Compared to the conventional material, these nanosized fibers or grains increased osteoblast adhesion and functions. This was demonstrated for nanoscale carbon fibers, nanophase metals or alloys (Ti, Al, Ti6Al4V, CoCrMo) [Price et al., 2004; Webster and Eji-ofor, 2004; Webster et al., 2005]. In addition, greater bending moduli and increased osteoblast adhesion was observed on polymer composite formulations (either PMMA or poly-L-lactic acid) containing 30–50 weight% of nanophase alumina, Ti or hydroxyapatite [McManus et al., 2005]. Ceramics containing nanophase alumina also significantly enhanced osteoclast cell functions such as synthesis of tartrate-resistant acid phosphatase and formation of resorption pits [Webster et al., 2001].

The adhesion of osteoblasts to the Ti surface is a prerequisite for successful osseointegration of the implant in vivo. Cell adhesion can be divided into three sequential events: (a) cell attachment, (b) cell spreading, and (c) the formation of focal adhesions and actin stress fibers [Greenwood and Murphy-Ullrich, 1998]. Focal contacts are formed at the leading edge of a migrating cell and often mature into focal adhesions, being the closest contacts between cell and implant surface. These streak-like structures are associated with the cytoplasmic domain of transmembrane integrin receptors on the one side and with actin stress fibers on the other. Vinculin is one of several proteins involved in the formation of a focal contact [Burrige and Chrzanowska-Wodnicka, 1996]. Osteoblast adhesion and subsequent functional differentiation are important steps in the overgrowth of artificial implants by living cells up to ex vivo production of spare organs. Both processes are affected by the composition of the Ti alloy and the surface geometry.

In this paper, we first analyzed the effect of surface chemistry on attachment and differentiation of osteoblast-like cells on pure Ti prepared by pulsed laser deposition (TiPLD) and on different Ti alloys (Ti6Al4V, TiNb30 and TiNb13Zr13). The influence of surface structure on osteoblast adhesion, differentiation and orientation was investigated using TiPLD implants. Here, the

Table 1. Effect of the TiPLD surface structure on the alignment of human SaOS-2 osteoblast-like cells

Line distance	Line width μm	Vertical %	Parallel %	Not considered %	Microscopic fields
2 μm	0.2	$34.2 \pm 8.6^{\text{a,b}}$	$46.0 \pm 7.7^{\text{c}}$	19.6 ± 3.2	19
5 μm	0.7	$33.8 \pm 6.2^{\text{a,b}}$	$43.5 \pm 10.7^{\text{c}}$	22.5 ± 7.3	14
10 μm	1.2	$35.9 \pm 10.1^{\text{a,b}}$	$42.4 \pm 10.0^{\text{c}}$	21.5 ± 7.3	8
20 μm	1.5	$32.5 \pm 23.4^{\text{a,b}}$	$41.9 \pm 14.2^{\text{c}}$	25.6 ± 23.4	4
Control, 1,000 μm	10.0	34.2 ± 13.2	34.6 ± 11.5	32.5 ± 11.0	19
Average 2–20 μm		$34.1 \pm 12.0^{\text{d}}$	$43.4 \pm 10.6^{\text{d,*}}$	22.3 ± 10.3	45

Values are means $\pm$ SD. * $p < 0.05$: a vs. parallel for a specific line distance, b vs. vertical control, c vs. parallel control, d vs. control.

surface was patterned by parallel Ti oxide lines with different widths (0.2–10 μm) and distances (2–20, 1000 μm), but a common height of only 12 nm.

Materials and Methods

Titanium Alloys

Pure Ti and Ti alloys (TiNb30, TiNb13Zr13 and Ti6Al4V) were used as different substrates for the cell experiments. Thin films of technically pure Ti were deposited by high-vacuum PLD. PLD is an energetic, highly non-equilibrium method of thin-film synthesis, with hyperthermal species having energies in the range between several eV to above 1 keV [Gorbunov et al., 2002]. The PLD films possess a nanocrystalline or quasi-amorphous crystal structure and are very smooth [roughness (root mean square) $r_{\text{rms}} = 0.71$ nm]. They show excellent adhesion properties and can withstand a long-term sonification in water without any visible damage. The 50-nm-thick oxide specimens with a composition similar to the native surface oxides of the TiNb13Zr13 and TiNb30 alloys has been produced by means of the sol-gel process on glass substrates. Ti6Al4V films were deposited by magnetron sputtering.

Surface Modification of Titanium Films

The 15-nm TiPLD films were modified by laser-induced oxidation using a commercial laser scanning microscope. By scanning the laser beam line by line along the probe, a pattern of fully oxidized lines with a height of 12 nm above the surface of the initial Ti film and a width between 0.2 and 10 μm was produced (table 1). The thickness ratio of the initial Ti film and the resulting oxide structure was about 1: 1.8, which coincides with the molar volume ratios of Ti and TiO_2 [Gorbunov et al., 1996; 1997].

For cell culture experiments, the coated glass discs (diameter 16 mm) were sterilized with either 100% ethylene oxide at 42°C for 12 h or by ultraviolet radiation at room temperature (RT) for at least 30 min.

Cultures of Osteoblasts

Primary osteoblastic cells were isolated from fetal rat calvariae as described previously [Roehlecke et al., 2001]. In brief, calvaria bone samples were dissected in approximately 1-mm³ fragments

after removal of the periosteum. All samples were washed with Tyrode solution (Sigma, Taufkirchen, Germany), followed by Ham's F12 medium (Gibco BRL, Karlsruhe, Germany) and thereafter digested in 4 ml of collagenase-trypsin solution [137 mg of collagenase type I (Biochrom, Berlin, Germany) and 50 mg of trypsin type III (Sigma) in 10 ml of a buffer containing 8 g of NaCl, 0.2 g of KCl and 0.05 g of $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$ per 100 ml of deionized H_2O] at RT. Cells from the first 45-min digest were discarded. The second and third 30-min digests (each in 4 ml of fresh collagenase-trypsin solution) were centrifuged for 2 min at 580 g. The cell pellets were resuspended in 4 ml of Ham's F12 medium containing 20% fetal calf serum (Gibco BRL). After centrifugation (10 min at 500 g), the cells were transferred into 12 ml of Ham's F12 medium containing 12% fetal calf serum, 2.3 μM Mg^{2+} , 100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate (Gibco BRL). The cultures were maintained at 37°C in humidified air and 5% CO_2 . The medium was changed every 3 days. After 5 passages, the cells were used for the experiments. The osteoblast phenotype was confirmed by determination of alkaline phosphatase (ALP) activity, collagen type I synthesis and formation of calcium phosphate deposits according to published procedures [Bretaudier and Spillman, 1984; Scutt et al., 1992; Becker et al., 2002].

Human osteogenic sarcoma cells (SaOS-2) were obtained from the American Type Culture Collection (ATCC HTB 85) and cultured in 85% McCoy's 5A medium (Gibco BRL) containing 15% fetal calf serum.

For the experiments, the different metal-coated glass discs were placed into 12- or 24-well Falcon culture plates (Becton Dickinson; Heidelberg, Germany) and carefully covered with the osteoblasts at a density of 10,000 cells/cm². The cells were allowed to settle for 2 h in the incubator at 37°C, after which 2 ml of complete medium was added.

Fluorescence Labeling of Actin and Vinculin

After the indicated time periods, the cells were fixed using formaldehyde (4% in phosphate-buffered saline, PBS) for 5 min at RT. The cells were permeabilized with Triton X-100 (0.5% in PBS) for 6 min and then incubated with bovine serum albumin (BSA, 1% in PBS) for 10 min to block nonspecific binding. To demonstrate focal contacts, the cells were incubated with a mouse monoclonal antibody against human vinculin (1:20; Serotec, Oxford, UK) for 1 h at RT. After three short washing steps with PBS, the cells were in-

cubated with a fluorescein-isothiocyanate-coupled goat anti-mouse antibody (1:100, Dianova, Hamburg, Germany) for 1 h at RT. To detect filamentous actin of the cytoskeleton, the cells were additionally incubated with tetramethylrhodamine-isothiocyanate-conjugated phalloidin (0.1 $\mu\text{mol/l}$; Sigma) for 1 h at RT. In some cases, nuclei were visualized by incubation for 5 min at RT with 4',6-diamidino-2-phenylindole (1:50, Sigma). After mounting, the cells were observed using a fluorescence microscope.

Fluorescence-Activated Cell Scanning of Osteopontin and CD44 Expression

The expression of osteopontin or CD44 in calvarial osteoblasts plated on the different Ti-alloy-coated discs was measured after 10 days of culture, when cells were fully confluent. After 3 days of culture, media were supplemented with 50 $\mu\text{g/ml}$ ascorbic acid and 10 mM β -glycerophosphate (Sigma) to induce osteogenic differentiation. The cells were washed twice with PBS and detached using trypsin/ethylenediaminetetraacetic acid (0.05%/0.02% in PBS). After washing, the cells were resuspended at a density of 5×10^6 cells/ml, immediately fixed in formaldehyde (2% in PBS) for 20 min and centrifuged shortly. Then the cells were resuspended in a washing buffer consisting of BSA (0.5% in PBS). Aliquots of 10^5 cells were used to measure the relative level of osteopontin expression by flow cytometry. The cells were permeabilized for 20 min using 0.5% saponin (Sigma) in washing buffer. They were then washed, centrifuged shortly, resuspended in washing buffer and incubated for 1 h at RT with specific monoclonal antibodies: 1:100 mouse anti-rat osteopontin monoclonal antibodies, clone MPIII b10₁ (Chemicon, Temecula, Calif., USA) and CD44 (R&D Systems, Abingdon, UK). Subsequently, the cells were washed twice in PBS containing 0.5% BSA and 0.5% saponin and incubated with fluorescein isothiocyanate-anti-mouse IgG (Sigma) for 1 h at RT. The cells were washed again, resuspended in 400 μl of PBS, and analyzed using a FACS-Calibur (Becton Dickinson).

ALP Activity and Histochemical Staining for ALP Activity

After 3 days of culture, media were supplemented with 50 $\mu\text{g/ml}$ ascorbic acid and 10 mM β -glycerophosphate. After 8 days of culture, histochemical detection of ALP activity was done using the fluorescence-based ELF-97 endogenous phosphatase detection kit (Molecular Probes, Göttingen, Germany) according to the manufacturer's instructions. ALP activity in cell layers was assessed by measuring the release of *p*-nitrophenolate from *p*-nitrophenylphosphate at pH 10.0 [Bretaudier and Spillman, 1984]. At the indicated time points, cells were washed twice with PBS and lysed in 10 mM Tris-HCl (pH 8.2), containing 2 mM MgCl_2 and 0.05% Triton X-100 at 4°C for 1 h. The lysates were cleared by centrifugation at 14,000 g for 30 min (4°C) and incubated with 10 mM *p*-nitrophenylphosphate in 0.1 M sodium carbonate buffer, pH 10.0, containing 1 mM MgCl_2 at 37°C for 30 min. After addition of 0.1 M NaOH, *p*-nitrophenolate release was determined spectrophotometrically at 405 nm. Protein concentration in the supernatant was analyzed using the BioRad protein assay (BioRad, Hercules, Calif., USA) with BSA as standard.

Influence of Surface Structure on Cell Orientation

Human SaOS-2 cells were seeded onto the patterned TiPLD discs placed in 24-well plates for 48 h. The cell concentration (10,000/disc) was very low to exclude possible influences on cellular orientation by contact with other cells. Cells were fixed and fluores-

cent stained for actin and vinculin as described before. After fluorescent imaging, the cells were additionally stained with hematoxylin and eosin and analyzed using phase contrast microscopy. The orientation of cells towards the Ti oxide lines was investigated. The proportion of cells with alignment of their longitudinal axes in the direction of the Ti oxide lines (orientation angle 0–30°, parallel) was considered to show contact guidance. Cells with an orientation angle of 60–90° were considered as vertically orientated. Randomly orientated cells showed angles around 45° [Lu and Leng, 2003].

Statistical Analysis

All experiments were carried out in duplicate or triplicate and repeated at least three times with different cell culture preparations. The results reported correspond to one representative experiment. Data are expressed as means $\pm$ SD, and differences between groups were analyzed by two-tailed Student's *t* test.

Results

Effect of Surface Composition on Attachment and Spreading of Osteoblasts

The initial adhesion of rat calvarial osteoblasts on Ti-PLD, Ti6Al4V and the simulated surface of Ti13Nb13Zr and Ti30Nb alloys was compared using the parameters cell attachment and spreading, focal contact formation and actin cytoskeleton. Reorganization of actin stress fibers was visualized by incubation with tetramethylrhodamine-isothiocyanate-conjugated phalloidin, whereas focal contact formation was analyzed by immunohistochemistry using a monoclonal antibody against vinculin. Thirty minutes after plating, initially round osteoblasts began to attach to all of the Ti surfaces, with subsequent spreading of the cells (fig. 1). Only on TiPLD, some osteoblasts showed spreading already after 15 min. After 30 min, an approximately equal ratio between round osteoblasts and spread cells was observed on the surface of the alloys TiNb13Zr13 and TiNb30, whereas more stretched cells were seen on Ti6Al4V. In contrast, most of the osteoblasts showed already spreading on pure Ti (TiPLD, fig. 1). At this time point, actin staining was rather diffuse in round cells, whereas spread osteoblasts showed cortical actin filaments below the cell membrane

Fig. 1. I Effect of the surface composition on the initial adhesion of rat calvarial osteoblasts characterized by detection of focal contacts and actin stress fiber formation. Osteoblasts were plated for 15, 30 and 90 min, respectively, on TiPLD (**a**), TiNb13Zr13 (**b**), TiNb30 (**c**) or Ti6Al4V (**d**). **II** Osteoblasts plated on TiPLD after 6 or 24 h. Cells were fixed and double fluorescence stained for vinculin (green) and actin (red). Bar = 20 μm .

Fig. 2. Effect of surface composition on osteopontin expression in calvarial osteoblasts after 10 days of culture analyzed by flow-cytometric analysis. Cells were plated on TiPLD or different Ti alloys. After 3 days of culture, media were supplemented with 50 $\mu\text{g}/\text{ml}$ ascorbic acid and 10 mM β -glycerophosphate to induce osteoblastic differentiation. The vertical line indicates the maximum fluorescence intensity of osteopontin.

with some radially orientated filaments. Ninety minutes after plating, these differences in adhesion parameters between pure Ti and the Ti alloys investigated were less pronounced (fig. 1). Nearly all the cells were spread and displayed cellular extensions in different directions. The cytoskeleton began to show a pattern of parallel-orientated

actin filaments (especially on TiPLD), although most of the actin staining was still located circumferential near the cell membrane. Focal contacts were only sparsely detected at these early time points. Nearly all of the vinculin was located throughout the cytoplasm. Later (6 and 24 h), osteoblasts grown on TiPLD (fig. 1II) were polygonal with well-formed actin filaments that were orientated parallel to one another and to the long axis of the cell. Discrete, streak-like focal contacts were found at the termini of stress fibers near the cellular plasma membrane. Cells grown on the Ti alloys displayed identical patterns of actin and vinculin staining (not shown).

Effect of Surface Composition on Osteopontin and CD44 Expression

Expression of differentiation-associated markers of osteoblasts, osteopontin and CD44 was analyzed by fluorescence-activated cell scanning using specific antibodies. Osteopontin production was measured after 10 days of culture. As shown in figure 2, osteoblasts adhering to pure Ti surfaces produced the most osteopontin (~ 200 fluorescence units, TiPLD) compared to cells grown on the other Ti alloys tested. Cells seeded on Ti6Al4V or TiNb13Zr13 showed similar levels of osteopontin expression (~ 100 units), whereas osteoblasts grown on TiNb30 displayed by far the lowest levels (~ 30 units). In contrast, no differences in CD44 protein expression were observed by flow-cytometric analysis after 10 days of culture (not shown).

Effect of Surface Composition on ALP Activity

The specific activity of ALP was measured 5, 7, and 9 days after plating on the different Ti alloys. Osteoblasts cultured for 5 days on TiPLD or Ti6Al4V showed similar ALP activities, which are significantly higher ($p < 0.05$) than those plated on TiNb13Zr13 or TiNb30 (fig. 3). However, no major changes in the ALP activity could be observed at later time points.

Effect of the Surface Structure on ALP Activity

Figure 4 shows the time course of ALP activity in calvaria osteoblasts grown on structured TiPLD (parallel Ti oxide lines, width 0.2 μm , distance 2 μm , height 12 nm) during the first 9 days after plating. Specific ALP activity increased with time on both surfaces, but at the indicated time points no significant differences were observed. Also SaOS-2 cells grown for 8 days on the structured TiPLD [parallel Ti oxide lines with 2 μm distance (fig. 5b) or grids of 2 $\times$ 2 μm (fig. 5c), width 0.2 μm , height 12 nm) showed no major differences in ALP activity compared to smooth

Fig. 3. Effect of surface composition on the specific activity of ALP in calvarial osteoblasts. Cells were seeded on TiPLD or different Ti alloys. At the indicated time points, ALP activity was measured by determining *p*-nitrophenolate (pNPP) released from *p*-nitrophenylphosphate at 405 nm. Results are presented as means $\pm$ SD ($n = 3$). * $p < 0.05$ vs. the ALP value on TiPLD.

Fig. 4. Effect of the TiPLD surface structure on the time course of the specific activity of ALP in calvarial osteoblasts. Cells were seeded on smooth or structured (parallel TiO₂ lines, width 0.2 μ m, distance 2 μ m, height 12 nm) TiPLD. ALP activity was measured by determining *p*-nitrophenolate (pNPP) released from *p*-nitrophenylphosphate at 405 nm. Results are presented as means $\pm$ SD ($n = 3$).

Fig. 5. Effect of TiPLD surface structures on the activity of ALP in SaOS-2 osteoblast-like cells. ALP activity was detected by immunohistochemical staining and fluorescence microscopy. Cells were seeded at a density of 10,000 cells/cm² onto either smooth (**a**) or structured (**b**; parallel TiO₂ lines with 2 μ m distance) or grids of 2 $\times$ 2 μ m (**c**; width 0.2 μ m, height 12 nm) TiPLD discs. After 8 days of culture, cells were fixed and fluorescence stained for actin (phalloidin-tetramethylrhodamine isothiocyanate, red) and ALP activity (ELF-97 immunohistochemistry kit, Molecular Probes, green). Bar = 50 μ m.

TiPLD (fig. 5a). The fluorescence staining intensity and the number of positive cells were similar in cells seeded on smooth or nanosize-structured surfaces.

Effect of Surface Structure on Cellular Orientation

Human SaOS-2 cells grown on surfaces structured with parallel Ti oxide lines of different widths and distances (table 1) but the same height (12 nm) often orientated along the parallel lines (fig. 6, 7). The cells stretched their cytoskeleton to align along the parallel structures and formed small filopodia to make contact with the oxide lines (fig. 6b). Cells grown on the smooth TiPLD surface did not show any preferences in cell alignment or form (fig. 6c). The majority of the focal contacts were placed on the oxide lines (white arrows in fig. 7), whereas only a few were located between them (yellow arrows). Occasionally, larger focal contacts or focal adhesions formed bridges between two oxide lines (red arrows). The portion of focal contacts bridging two oxide lines depends on the distance between the lines. Most of them were observed on structures with 2 μm distance, some on those with a distance of 5 μm , but they were rare on structured surfaces with 10 μm distance of lines (fig. 7). The shape of the focal contacts seems to be altered, when SaOS-2 cells grew on the structured surface. On smooth TiPLD, most focal contacts were like streaks (fig. 7d), but they appeared much rounder on the oxide lines fig. 7a). Thus, form and alignment of focal contacts were influenced by the topographic structures. In contrast, cells cultured on smooth TiPLD formed focal contacts and filopodia with no orientation being preferred (fig. 7d). In the range of 2–20 μm , the proportion of aligned cells did not significantly depend on the distance of the Ti oxide lines (table 1), although, there was a trend to more orientated cells with the narrowest line distance. On average, 43% of the SaOS-2 cells orientated parallel towards the oxide lines, whereas 34% orientated vertical (table 1) This difference was significant ($p = 0.03$) compared to the control. 22% of the cells were not considered for alignment due to their cell shape (round or triangular) or due to their angle (approximately 45°) towards a line. As a control, we used a pattern of oxide lines with distances being much greater than the

Fig. 6. Effect of TiPLD surface structure on the alignment of osteoblast-like cells. SaOS-2 cells were analyzed 2 days after plating on a smooth (**c**) or structured TiPLD surface (**a**, **b**): parallel Ti oxide lines, height 12 nm, width 0.7 μm , distance 5 μm . **a** One representative microscopic field out of 14 showing cells stained with hematoxylin/eosin and analyzed using phase contrast. Arrows indicate

cells orientated parallel to Ti oxide lines; arrowheads indicate cells orientated vertically, and asterisks cells not considered for alignment. Bar = 100 μm . **b** Cells were fluorescence stained for actin. Ti oxide lines were visualized by bright-field microscopy and appeared in white. Bar = 20 μm . **c** Cells grown on smooth TiPLD were fluorescence stained for actin and vinculin. Bar = 20 μm .

Fig. 7. Effect of the TiPLD surface structure on alignment and focal contact formation of SaOS-2 cells. Cells were plated for 2 days on smooth (**d**) or differently structured TiPLD surfaces (parallel Ti oxide lines, height 12 nm and various widths (w) and distances (d): (w; d): **a** (0.2; 2 μm), **b** (0.7; 5 μm), **c** (1.2; 10 μm) and then fixed and double fluorescence stained for vinculin (green) and actin (red); nuclei were stained with 4',6-diamidino-2-phenylindole (blue). Ti oxide lines were visualized by bright-field microscopy and appeared in white (**a**) or black (**b**, **c**; negative image). Arrows mark focal contacts located directly on Ti oxide lines (white), between oxide lines (yellow), or bridging two oxide lines (red). Bar = 40 μm (**a**) or 20 μm (**b–d**).

size of the cells (distance 1,000 μm , width 10 μm). Here the cells were randomly distributed (table 1). In summary, we found that a significant surplus of 9.3% of the SaOS-2 cells prefer a more parallel than a vertical alignment on Ti oxide lines with a distance of 2–20 μm .

Discussion

Surface Composition and Cell Adhesion

We found that within 30 min after plating, primary osteoblasts adhere fastest on pure titanium (TiPLD) followed by Ti6Al4V, whereas cell spread was decreased and cells were more frequently round and hence poorly attached cells on TiNb13Zr13 and TiNb30 alloys. Thereafter, no significant differences in terms of cell spreading, focal contact formation and cytoskeleton reorganization could be observed. With time, osteoblastic cells began to secrete several extracellular matrix (ECM) proteins. They will also attach on the implant surface and are necessary for adhesion due to their specific binding to cell surface receptors. This may explain the lack of a difference in the adhesion quality among the different surfaces at later time points. The formation of cell attachment to the alloys seems to be slower than on pure Ti. A reasonable explanation for this observation is that the formation of cell-implant contacts may be hampered on rough surfaces. As measured by atomic force microscopy, the surface roughness of TiPLD ($r_{\text{rms}} = 0.71 \text{ nm}$) is an order of magnitude lower than that of the sputtered and sol-gel coatings. As a consequence, the formation of stable cell adhesion on the latter may be delayed resulting in initially reduced attachment rates. Similar results were observed by Walboomers et al. [1998], who showed a decreased early attachment (30 and 45 min) but a similar, or even exceeding, later attachment of fibroblasts on micro-grooved surfaces (depth 0.5 μm , width 1–10 μm) compared to smooth polystyrene. Increased attachment, spreading and formation of focal contacts on a smoother surface are also observed in a number of different studies. Greater spreading of fibroblasts occurred on smooth electropolished Ti, rather than on the rougher etched or sand-blasted Ti surfaces [Könönen et al., 1992]. Fibroblasts exhibited larger cell areas on smoother surfaces when compared to implants with grooves [Oakley and Brunette, 1993]. Osteoblasts also showed greater spreading on Ti compared to the much rougher hydroxyapatite surface [Okumura et al., 2001]. Better attachment and spreading of osteoblasts was observed on the smoothest Ti samples [Mustafa et al., 2000] and on polished Ti sur-

faces [Bagambisa et al., 1994]. On the other hand, some studies showed that attachment, proliferation or differentiation of osteoblasts is best on the rougher surfaces [Degasne et al., 1999; Schwartz et al., 1999]. Grössner-Schreiber et al. [1991] found that osteoblasts cultured on porous and rough surfaces synthesized much more bone specific-collagen and had a better mineralization than on smooth Ti. In addition, Ti with a certain roughness is superior in the production of growth factors (prostaglandin E_2 , TGF- β_1) compared to smooth Ti surfaces [Kieswetter et al., 1996a, b].

Some recent studies showed that compared to conventional (μm -sized) material, osteoblast adhesion and function are increased on nanophase materials (grain or fiber size <100 nm). Osteoblasts showed increased adhesion when grown on nanoscale carbon fibers, nanophase metals or alloys (Ti, Al, Ti6Al4V, CoCrMo) [Price et al., 2004; Webster and Ejiófor, 2004; Webster et al., 2005]. Interestingly, carbon nanofibers exclusively promote osteoblast adhesion but not adhesion of chondrocytes, fibroblasts or smooth muscle cells [Price et al., 2004]. Advanced adhesion was also observed on polymer composite formulations (either PMMA or poly-L-lactic acid) containing 30–50 weight% of nanophase alumina, Ti or hydroxyapatite [McManus et al., 2005]. Since osteoblast adhesion occurred preferentially at surface particle boundaries and more of them are present on the nanophase compared to conventional material, this may explain the observed increase in osteoblast adhesion [Webster and Ejiófor, 2004]. However, this phenomenon may be cell specific, since reduced fibroblast adhesion was observed on nanosized pits (PMMA, 35–120 nm in diameter) [Curtis et al., 2004; Martinez et al., 2004].

We observed well-formed focal contacts by fluorescence labeling for vinculin only at later time points (6 and 24 h), but not during the first 90 min of cell adhesion. This corresponds to results of Könönen et al. [1992], who demonstrated that vinculin, playing a part in focal adhesion, appeared within 3 h of cell attachment. Our results that rat calvarial osteoblasts effectively adhere to all the different implants used within 90 min in vitro are in agreement with those of others [Puleo et al. 1991; Geißler et al., 2000; Roehlecke et al., 2001], who observed that approximately 90% of the rat osteoblasts attached to Ti6Al4V within 2 h. Baxter et al. [2002] showed that rat calvarial osteoblasts and 3T3 fibroblasts grown for 24 h on anodized Ti or polyethylene terephthalate produced a greater amount of cell spreading and focal adhesion than on hydroxyapatite. Variations in the surface topography may be responsible for the observed differences: anodized Ti or polyethylene

terephthalate displayed a smoother surface than hydroxyapatite with its high density of fine roughness discontinuities. Increased spreading of fibroblasts and osteoblasts on smoother substrates has been observed in a number of different studies [Könönen et al., 1992; Oakley and Brunette, 1993; Okumura et al., 2001].

Regarding surface composition, Rosa and Beloti [2003] found no major differences in initial rat bone marrow cell attachment (2 h) on pure Ti or Ti6Al4V. In contrast, cell proliferation was significantly enhanced on pure Ti. Significantly lower cell numbers and delayed proliferation of human fetal osteoblasts on cobalt-chrome-molybdenum and stainless steel compared with Ti and polystyrene have also been reported [Hendrich et al., 2002].

Surface Composition and Cell Differentiation

To investigate the influence of the surface chemistry on the differentiation of rat calvaria osteoblasts, we analyzed the expression of osteopontin, CD44 and ALP. To induce osteogenic differentiation, the medium was supplemented with 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate after 3 days of culture [Bellows et al., 1986]. ALP activity is an important marker of the phenotype of osteoblasts. We discovered significantly enhanced ALP activities in calvarial osteoblasts plated for 5 days on TiPLD or Ti6Al4V compared to cells grown on TiNb13Zr13 or TiNb30. However, these differences were not observed at later time points. This time course was similar to data of Hendrich et al., 2002. They observed significantly higher ALP activities in human fetal osteoblasts grown on pure Ti than on a cobalt-chrome-molybdenum alloy only on day 4 and 6 but not thereafter. Similar ALP activities on smooth surfaces of pure Ti and Ti6Al4V alloy were also reported in rat bone marrow cells [Rosa and Beloti, 2003]. However, these authors observed a significant difference in ALP activity between rough Ti and the Ti alloy. These surfaces were sandblasted using Al₂O₃ particles, which may affect the Ti6Al4V alloy to release more Al and V ions compared to the unmodified smooth surfaces. Osteoblastic differentiation or ALP activity may be hampered here due to mild toxic effects by the release of aluminum and vanadium ions from Ti6Al4V [Rae, 1986; Thompson and Puleo, 1996; Bellows et al., 1999].

Osteopontin is one of the major non-collagenous ECM proteins and belongs to the super family of cell adhesion molecules. It has been shown to interact both with cell surface integrin and CD44v receptors through its RGD motif and carboxyl terminal portion, respectively [Ross et al., 1993; Weber et al., 1996]. The temporal and spatial

expression of the osteopontin (*opn*) gene during embryogenesis and skeletal tissue development underlines the function of osteopontin in bone metabolism. Here, osteopontin mediates cell adhesion, attachment and migration of osteoblasts, osteoclasts and fibroblasts [Carvalho et al., 1998]. Osteopontin is expressed early during osteogenesis and at different levels during maturation of osteoblastic cells [Zohar et al., 1998]. Using fluorescence-activated cell scanning, we clearly showed that osteoblasts adhering to pure Ti surfaces (TiPLD) produced most osteopontin, followed by cells seeded on Ti6Al4V or TiNb13Zr13. Osteoblasts grown on TiNb30 expressed significantly lower levels of osteopontin. Zreiqat et al. [2003] reported that in human osteoblastic cells, levels of non-collagenous proteins, including osteopontin, were significantly affected by Ti alloy and cobalt-chrome particles. However, the corresponding gene expression was not influenced by the different alloys. Calvarial osteoblasts grown on collagen-coated Ti6Al4V expressed more osteopontin protein when compared to the uncoated surface [Roehlecke et al., 2001].

Proteins of the CD44 transmembrane glycoprotein family are involved in the regulation of cell growth, survival, differentiation and motility. Interaction of CD44 with ECM proteins such as osteopontin, collagen and fibronectin seems to promote matrix-dependent migration. CD44 is also a major receptor for hyaluronan and glycosaminoglycans present in the bone matrix [Ponta et al., 2003]. However, we did not notice any differences in CD44 protein expression in osteoblasts grown for 10 days on TiPLD or different Ti alloys. Thus, surface chemistry influences secretion of the ECM protein osteopontin but not the expression of its corresponding receptor CD44.

The differences in adhesion properties and differential regulation of proteins like osteopontin and ALP occurring between cells grown on Ti and Ti alloys imply that rat calvarial osteoblasts respond to small differences in the surface chemistry, topography or charge. To further address this topic, we investigated the influence of different surface structures on osteoblast adhesion and differentiation. Osteoblasts grown on pure Ti adhered and spread fastest and produced most of the differentiation marker osteopontin. Therefore, to exclude the effects of differences in surface chemistry of the several alloys, we used only TiPLD implants for further experiments.

Surface Structure and Cell Differentiation

We found no significant differences in specific ALP activities in calvarial osteoblasts or SaOS-2 cells grown on structured Ti implants compared to those grown on

smooth TiPLD. Variations in ALP gene expression assessed by RT-PCR were also not noticed (not shown). A structured implant has a rough surface. Our finding of unchanged ALP activity on a structured surface is in line with results from Rosa and Beloti [2003]. They also failed to demonstrate significant effects of the surface roughness of Ti or Ti6Al4V (R_a 0.24–1.98 μm) on ALP activity in rat bone marrow cells. In contrast, Boyan et al. [1998] observed increasing ALP activities in human MG63 osteoblast-like cells with increasing Ti surface roughness (R_a 0.6–6.8 μm). De Santis et al. [1996] demonstrated a higher ALP activity in human bone marrow cells cultivated on rough Ti6Al4V surfaces. Hatano et al. [1999] showed increased ALP activity in rat calvarial cells grown on a rough polystyrene surface. A different study even showed enhanced enzyme activity in MG63 cells on smooth surfaces [Martin et al., 1995]. Differences in cell origin, osteoblastic differentiation and especially in the design of the structured surfaces may explain these discrepancies.

Surface Structure and Cell Orientation: Mechanical Guidance?

When adherent growing cells are seeded on a surface with a micrometer-sized groove and ridge pattern, they often migrate and align in the direction of the surface structure. This process is known as contact guidance [Brunette, 1986]. Depending on the surface topography, the cells can finally form a well-organized structure. In contrast, random cell orientations are often found on smooth surfaces [Soboyejo et al., 2002]. It was shown, that such random cell orientation may lead to increased scar tissue formation during wound healing [Wang et al., 2000b].

After seeding, a cell exposes thin filopodia protruding in all directions. In the leading edge, new cell adhesions are formed, and the actin meshwork contracts, which causes the cell to move in the direction of the new adhesion [Walboomers and Jansen, 2001]. In our study, the surface structures consist of Ti oxide lines with varied width and distance but a common height of only 12 nm. Experiments on cell orientation were performed with human SaOS-2 osteoblast-like cells for the following reasons: (i) SaOS-2 cells behave similar to calvarial osteoblasts regarding adhesion on Ti implants, (ii) they have a more stretched cell form, making it easier to detect the orientation angle and alignment and (iii) they have been previously used in contact-guiding experiments [Lu and Leng, 2003]. Two days after plating, we found significantly more (43%) SaOS-2 cells orientated parallel to the

Ti oxide structures, whereas only 34% orientated vertical. In the control pattern, however, with a line distance far beyond cell size or on the smooth TiPLD surface, cells distributed completely at random. The percentage of aligned cells did not significantly change with the distance of the Ti oxide lines within the range of 2–20 μm . This was similar to data of Teixeira et al. [2003], who found the percentage of aligned corneal epithelial cells to be constant on silicone patterns with pitches (sum of ridge + groove widths) ranging from 0.4 to 2 μm . The portion of cells aligning to a structured surface depends on the peak-to-valley height, with more orientated cells on the higher structures or the deeper grooves. The oxide lines used in our experiments were only 12 nm in height, which is very low compared to the micrometer-sized groove-and-ridge pattern used in other studies [Eisenbarth et al., 2002: 0.08–1.4 μm ; Soboyejo et al., 2002: 8–12 μm ; Lu and Leng, 2003: 2–10 μm]. Because in the majority of the cells, alignment was parallel to the lines, cells may recognize the nanosize difference in height between Ti bulk surface and Ti oxide lines. Our results are supported by recent studies showing that mammalian cells indeed react to nanosize order and symmetry. Contact guidance on nanometer-deep grooves (150 and 100 nm) was observed using silicone and quartz as substrates, respectively [Clark et al., 1991; Teixeira et al., 2003]. Recently, Dalby et al. [2004] demonstrated that fibroblasts can ‘sense’ structures being only 10 nm high. Here, cells grown on polystyrene islands produced more filopodia at the leading edge compared to the smooth control surface.

We found the majority of the focal contacts placed on the Ti oxide lines, whereas only a few were located in between or formed bridges between two lines. Focal contacts of SaOS-2 cells grown on the smooth surface did not show any preferred orientation. In addition, the shape of the focal contacts seems to be altered on the structured surface. On smooth TiPLD, most focal contacts resembled streaks, but they appeared much rounder on the oxide lines. Thus, form and alignment of focal contacts were influenced by the topographic structure. Our results correspond to data of Teixeira et al. [2003], who spotted the focal contacts on top of the ridges, with only occasionally spanning the groove. Here, fibroblasts cultured on a smooth silicone surface formed focal contacts with no preferred orientation, whereas on the nano-patterned substrates focal contacts aligned along the topographic structures. Moreover, the width of the focal contacts was dictated by the width of the underlying ridges. Dalby et al. [2004] also found focal contacts on top of the nano-structures to be smaller than those on the flat control.

However, Waalboomers et al. [1998] noted an orientation behavior of focal adhesions and actin filaments of fibroblasts on microgrooves (1 μm deep and 1–10 μm in distance), but no obvious preference for focal adhesion complexes to locate along edges of the ridges. In summary, topographic features in the nanometer range may influence orientation, alignment and shape of cells and focal contacts by contact guidance.

Surface Structure and Cell Orientation: Guidance by Surface Charges or Electrical Fields?

The Ti oxide lines used in this study slightly differ in their physicochemical properties compared to the main Ti surface. Ti is naturally covered by a thin (about 5 nm) oxide layer primarily consisting of an amorphous anatase modification of Ti oxide. However, the laser beam produced Ti oxide lines with a total thickness of 27 nm by heat-induced (about 1,100°C) oxidation of the Ti surface. Here, a highly crystalline (rutile) form of Ti oxide developed. Transmission profile analysis indicated that the maximum transparency of the line is 61%, whereas the initial transparency of the Ti film was only 6.3% [Gorbunov et al., 1997]. This optical 1:10 contrast clearly indicates differences in physicochemical properties between Ti surface and Ti oxide lines. Due to the different physicochemical properties of the Ti/Ti oxide surfaces used in this study, mechanism other than mechanical guidance may also be responsible for the alignment of the osteoblast-like cells. Chemotaxis, i.e. diffusion of molecules from the interface of the different materials, seems to be unlikely, but other effects like surface charges or electrical fields produced at the interface of the materials are more probable. Weak direct-current electrical fields are known to cause movement and guidance of various cell types, including bone cells, in vivo and in vitro [Ferrier et al., 1986]. Endogenous electric field gradients causing an electrotaxis response were shown in healing wounds, developing embryos and lens epithelial cells [Robinson, 1985; Wang et al., 2000a]. Similarly, coatings that alter the charge or the spatial arrangement of charges of an implant surface are known to induce cellular orientation and proliferation. Chondroitin sulfate proteoglycans and glycosaminoglycans are components of the embryonic ECM. They contain charged sulfate and carboxyl groups in a specific three-dimensional orientation and markedly enhance the orientation of nerve cells in a direct-current electric field by interacting with charged membrane proteins [Erskine and McCaig, 1997]. Hydroxyapatite ceramics with modified surface charges affected adhesion, growth and ori-

entation of different cell types, including MC3T3-E1 osteoblast-like cells [Ohgaki et al., 2001].

A direct measurement of potential differences at the interface semiconductor Ti oxide/metal Ti is difficult. Therefore, we used a model surface consisting of a regular pattern of Ti and Au sputtered on glass. Here, preliminary experiments using a raster Kelvin probe indeed demonstrated differences in the electrical potential at the Ti/Au interface.

We found the majority of the focal contacts placed on the Ti oxide lines. According to Walboomers and Jansen [2001], precursor contacts precede the formation of a focal contact and are made in two steps: (i) a nonspecific approach of the cell membrane to the substrate surface based on electrostatic or much weaker van der Waals forces, and (ii) the specific binding via membrane integrin receptors to ECM proteins absorbed on the implant surface. At the interface between Ti ground and the Ti oxide lines, the surface charge density may differ due to varying physicochemical properties of the thin naturally formed oxide film and the thicker thermal-induced oxide lines. The ξ potential is the electric potential at the interface between a solid surface and a liquid. In case of the laser-induced oxide lines, it is dependent on the thickness of the electric double layer formed at the interface semiconductor Ti oxide/cell culture medium. In case of the very thin natural oxide layer, the ξ potential is additionally dependent on a second phase, i.e. the interface Ti metal/semiconductor Ti oxide [Gerischer, 1989]. Reported ξ potentials for the natural oxide layer (~ 5 nm) and a thicker, anodically formed amorphous oxide (~ 150 nm) are around -40 and -55 mV (pH 7, 1 mM KCl), respectively [Roessler et al., 2002]. The negative ξ values are caused by the preferential adsorption of chloride and hydroxyl anions in the amorphous oxide layer. Consequently, the surface of a Ti or Ti6Al4V implant is negatively charged at physiologic pH. The observed differences in the ξ potential reflect variations in the surface charge density of the natural and the anodically formed oxide. Thus, surface charge density depends on the formation of the oxide. Therefore, similar differences in surface potential and resulting charge should also occur at the interface Ti natural oxide/laser-induced oxide lines. These gradients also seem to attract the osteoblast-like cells to migrate, to align towards these lines and to make new focal contacts on the lines.

Conclusion

Adhesion and differentiation of osteoblast-like cells occurred earlier on pure Ti (TiPLD) and Ti6Al4V implants compared to TiNb13Zr13 or TiNb30. Structuring of the TiPLD surface with parallel Ti oxide lines being only 12 nm in height did not affect ALP activity as a marker of cell differentiation, but markedly influenced cell and focal contact alignment. Thus, apart from surface composition and structure, other factors such as surface potential and charge density may also affect cell adhesion and alignment.

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