The effect of titanium dental implant surfaces with varying microtopography on the temporal expression of the osteogenic phenotype *in vitro*

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Over the past 15 years, titanium implant surfaces, which exhibit two superimposed levels of surface roughness, have received increasing attention in implant dentistry in view of accelerating the osseous integration of dental implants. However, there are few studies, which compare the biological behavior of a range of these clinically used implant surfaces with varying mixed microtopography to each other. This study aimed at establishing the relationship between the surface properties of an array of clinically used titanium implant surfaces with varying mixed microtopography and their osteogenic potential. Type I collagen (Col I), alkaline phosphatase (ALP), osteopontin (OP), osteocalcin (OC), osteonectin (ON) and bone sialoprotein (BSP) are valuable markers for evaluating the osteogenic potential of endosseous implant materials. This study analyzed Col I, ALP, OP, OC, ON and BSP expression by SaOS-2 bone cells cultured on various implant surfaces with mixed microtopography for 3, 7, 14 and 21 days. Test materials were titanium disks with Cellplus® (CP), Promote® (Pro), SLA® (SLA), Titanium plasma-sprayed (TPS) and Osseotite® (Oss) surfaces. Machined Ti (MTi) was used as control. All surfaces supported cell proliferation, differentiation and extracellular matrix formation and mineralization. The SLA surface had the greatest stimulatory effect on osteoblast proliferation and differentiation; suggesting that this surface possesses the highest potency to enhance osteogenesis. This study shows that slight differences in surface microarchitectural features have a significant effect on the osteogenic phenotype expression *in vitro*. These differences are likely to influence the implant *in vivo* performance in patients.

Keywords: titanium dental implants, implant surfaces, microarchitecture, surface properties, SaOS-2, osteogenic phenotype, osteoblast, cell differentiation, bone

1. Introduction

The use of dental implants to replace missing teeth has become a common treatment modality in modern dentistry. Over the past two decades, numerous prospective long-term studies have documented excellent long-term success rates for osseointegrated implants [1–4]. Titanium (Ti) is widely used as dental implant material, because direct contact occurs between bone and the implant surface [5–10]. Preferably, roughened surfaces have been used for the endosseous portion of dental implants in order to increase the total surface area available for osseous apposition [11–15]. Over the past 15 years, the use of sandblasted and acid-etched, or dual-acid-etched, implant surfaces, which exhibit two superimposed levels of surface roughness, has received increasing attention in implant dentistry [1, 15–23]. This new generation of implant surfaces, which features a dual microtopographic level of roughness, a first level of 20–40 μm, or 5–8 μm roughness and a superimposed second level of 8–9 or 1–3 μm [16–22] has replaced the formerly widely used titanium plasma-sprayed (TPS) surfaces [2, 3, 8, 9, 13–15, 20, 24] with the goal of accelerating the osseous integration of dental implants and enhancing the bone-implant contact [1, 12–23], thereby, shortening healing times and improving anchorage of dental implants in areas with low bone quality such as areas with highly cancellous bone in the posterior maxilla.

Surface topography plays a critical role in the interaction of dental implants with adjacent tissues [6, 13, 15, 25, 26]. Many of the most important steps in the peri-implant healing cascade are profoundly influenced by implant surface micro-

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topography [6, 8, 23]. *In vitro* studies have shown that microroughened sandblasted and/or acid-etched surfaces enhance platelet activation and aggregation and fibrin retention [8, 21]. As a result a migratory pathway for the differentiating osteogenic cells to reach the implant surface is provided [8, 21]. Furthermore, these microroughened surfaces enhance osteoblastic attachment, differentiation and matrix production and as well as their growth factor and cytokine production [22–23, 25–30]. Animal studies demonstrated an enhanced bone-implant contact and removal torque values with microroughened surfaces compared to smooth and TPS surfaces [17, 20, 22, 31–35]. Clinical trials indicate high success rates after early loading [1, 16, 36–39]. In addition, improved success rates have been reported in low quality bone, such as the posterior maxilla [38, 39]. Obtaining greater bone to implant contact which forms in a shorter period of time, not only reduces the healing period of dental implants prior to functional loading but also protects them from a breakdown of their anchorage in bone and from developing marginal bone loss [1, 14].These are important aspects in view of preventing periimplantitis [1, 13]. Apart from surface roughness and topography, surface properties affecting the cellular response are chemical composition-bulk chemistry as well as surface chemistry, and surface energy and charge [13, 15, 23, 40–45].

In order to cause more abundant and more expeditious bone formation at the bone–implant interface, a dental implant surface has to possess the ability to enhance cell differentiation of osteogenic cells at its surface. The use of *in vitro* osteogenic cell cultures has proven valuable for studying the biological reactions to dental implant surfaces [6, 7, 23, 24, 26–29]. Quantitative evaluation of osteogenic markers expressed by osteoblasts grown on different implant surfaces facilitates assessing their osteogenic capacity by gaining insight into the effect of endosseous implant materials on osteoblastic cell differentiation [23, 24, 28, 44].

Few studies, however, have compared the surface properties and the biological behavior of a broader selection of these new generation commercially available dental implant surfaces with mixed microtopography to each other [13–15, 46–49], and existing data obtained in studies with varying experimental set-ups do not allow comparing these implant surfaces directly to each other [13–15]. Previously, we were able to show, with respect to bone grafting materials, that enhanced osteogenic marker expression and osteoblast differentiation *in vitro* led to greater bone bonding and bone formation *in vivo* [50–53]. Consequently, such *in vitro* data are valuable with respect to enhancing our understanding of *in vivo* results and clinical outcomes, and in view of optimizing implant surface design. Hence, the goal of the present study was to elucidate the effect of the surface characteristics of an ample selection of dental implant surfaces with mixed microtopography on osteoblast differentiation *in vitro* in terms of expression of an array of osteogenic markers under identical experimental conditions, thereby creating a comprehensive set of data, which may contribute to elucidating key factors affecting the performance of dental implants. To this end, the temporal expression of six osteogenic markers by human osteoblastic cells, i.e. SaOS-2 cells, cultured on an ample selection of new generation clinically used dental implant surfaces with varying mixed microtopography was characterized.

2. Materials and methods

2.1. Titanium implant test surfaces

The specimens used consisted of commercially pure grade 2 (CP G2, ASTM F67) Ti disks with dimensions of 10 mm in diameter by 2 mm in thickness. Ti disks were subjected to their respective surface treatments by various dental implant manufactures in order to produce the specific implant surfaces present with the respective commercially available implants. The implant surfaces tested were Cellplus® (CP; Dentsply, Germany), Promote® (Pro; Altatec, Germany), SLA® (SLA; Straumann, Germany), Osseotite® (Oss; Biomet 3i, Germany) and titanium plasma sprayed (TPS; Dentsply, Germany) surfaces. According to the dental implant manufacturers, CP, Pro and SLA are sandblasted and acid-etched surfaces; CP was grit-blasted using Al_2O_3 particles with a grain size 350–500 μm and acid-etched with hydrochloric acid/sulfuric acid/hydrofluoric acid/oxalic acid and neutralized with a sodium-based solution, i.e. NaOH or $NaHCO_3$, while SLA was grit-blasted using Al_2O_3 particles with a grain size of 250–500 μm and acid etched with HCl/H_2SO_4 [15, 18, 20]. Pro is manufactured by grit-blasting with Al_2O_3 particles followed by acid-etching with inorganic acids [15]. Oss is a dual-etched surface produced by a first etching with hydrofluoric acid, followed by a second etching with a combination of hydrochloric and sulfuric acid [22]. TPS is a rough surface created by plasma spraying of spherical Ti particles; Disks with a machined Ti surface (MTi, Dentsply, Germany) served as control. The various Ti dental implant test surfaces were packed, sealed, and sterilized by gamma irradiation by their respective provider

2.2. Surface characterization of dental implant surfaces

The surface roughness of the various test surfaces was determined by using an optical profilometer (MicroProf, Fries Research & Technologie GmbH, Germany) and specific software, i.e. Mark III V3.9.4.1 (FRT GmbH, Germany). A 600 μm sensor, with a precision of 200 nm and a vertical resolution of 20 nm, was used to perform the measurements. A total of 20 measurements per test surface were performed. Images were obtained from the center of the sample by using the sensor mentioned above in an area of 2000×2000 μm. The samples were placed horizontally under the sensor with a given distance so that the sensor showed a relative intensity medium value of 150 units. For the roughness an area of 4000×1200 μm was measured using 10 lines;

each line having 2000 dots/pixels. With one series 10 results (lines) were generated. Two series per sample were measured to obtain the following surface roughness parameters: R_a (the arithmetic mean of departures of the roughness profile from the mean line), R_z (the average of five consecutive values of roughness height which is defined as the distance between the top of the highest peak and the bottom of the deepest valley) and R_{max} (maximum peak to valley height of the profile in the assessment length) values.

The wettability of the surfaces was characterized by determining the contact angles utilizing a high-resolution drop shape analysis system (model DSA 10 Mk2, Krüss GmbH, Hamburg, Germany) and applying the sessile drop method. HPLC grade distilled water (Sigma Aldrich, Germany) was used as liquid to measure the contact angle. Briefly, 4 µl-droplets were placed automatically under software control on the surface of the samples. For each droplet 1825 frames were recorded during 50 s after droplet deposition by using a CCD-camera. The images were processed utilizing a special purpose software, i.e. Drop Shape Analysis 1.9 (Krüss, Germany) to determine the contact angles continuously. For all samples the picture/frame acquired after 15 s was used to determine the contact angle values. Ten contact angle measurements were performed for each surface group. Surface roughness and contact angles were measured at room temperature. The morphology of the surfaces was examined by scanning electron microscopy (SEM; Philips SEM 505, Phillips, the Netherlands) at an accelerating voltage of up to 20 kV in order to visualize the differences in surface morphology at two levels of magnification (500× and 1000×).

2.3. Cell culture

The human osteoblast-like osteogenic cell line SaOS-2, derived from a human osteosarcoma, was used for the *in vitro* experiments. SaOS-2 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). Cells were cultured in a modified McCoy's 5A medium (Sigma-Aldrich, Germany) supplemented with 10% FCS (Gibco, UK), 1% penicillin/streptomycin (Gibco, UK), 1% L-glutamine 200 mM (Gibco, UK) and 0.1M L-ascorbic acid phosphate Mg (Wako Pure Chemicals, Japan). Cells were maintained in an incubator in a 5% CO_2 atmosphere and at 37°C. Culture medium was renewed every 2-3 days. When cells reached confluence, a trypsin-EDTA (0.5 g/L trypsin and 0.2 g/L EDTA) solution (Gibco, UK) was used to detach cells from the bottom of the culture flasks; then, cells were reseeded into a new tissue culture flask or seeded onto the Ti surfaces.

2.4. Cellular quantitative immunocytochemistry assay for osteogenic proteins

SaOS-2 cells of the 5th to 8th passage were seeded at a density of 8.5×10^4 cells/cm^2 on the different implant surface disks and cultured for 3, 7, 14, and 21 days. Eight disks (n = 8) per test surface and time point were used. A modified version of the cellular quantitative immunocytochemistry assay described by Wang et al. [54] was used to quantify the intracellular protein expression of an array of osteogenic proteins characteristic of the osteoblast phenotype [50–52]. Briefly, cells were harvested from the test surfaces by trypsinization using 0.02% trypsin/0.02% EDTA in phosphate-buffered saline solution (PBS) and counted with a hemocytometer as described previously. A defined number of cells, i.e. 1×10^4 cells, was placed into 96-well plates and centrifuged at 1000 rpm for 10 min. The supernatant from each well was removed and cells were then dried at the plates for 30 min at 37°C. Dried cells were fixed by immersing them in a methanol–acetone mixture, with a 9 : 1 ratio, for 1 min at room temperature. Subsequently, cells were rinsed twice with PBS. Then, incubation in 0.25% Triton 100-X (ICN, USA) and 0.25% Nonidet NP40 (Sigma-Aldrich, Germany) in PBS for 5 min was done to permeabilize the cell membrane. The incubation solution was removed and cells were rinsed twice in a 0.05% (v/v) Triton 100-X in PBS solution. To reduce nonspecific binding, 50 µl of a blocking solution of 2% (w/v) heat-inactivated bovine serum albumin (Sigma-Aldrich, Germany) in Hanks balanced salt solution (Sigma-Aldrich, Germany), was added to each well and the plates were incubated for 20 min at room temperature in a humidified chamber. The blocking solution was then removed from the wells before adding any monoclonal or polyclonal antibodies. Monoclonal antibodies for alkaline phosphatase (ALP; Sigma-Aldrich, Germany), osteocalcin (OC; Santa Cruz, USA), as well as polyclonal antibodies for type I collagen (Col I; LF-39, NIDCR, USA), osteopontin (OP; LF-124, NIDCR, USA), osteonectin (ON; BON-I, NIDCR, USA), and bone sialoprotein (BSP; LF-83, NIDCR, USA) were used to analyze the intracellular protein expression of these osteogenic markers [50–52, 55]. The presence of a biotin label on the $F(ab)_2$ fragment of the secondary antibody was quantitatively measured by using a one-step application of a soluble complex of streptavidin and biotinylated alkaline phosphatase (Dako Cytomation, Denmark) and by subsequently visualizing the para-nitrophenyl phosphate (p-NP) via a specific assay [52, 54]. A 5 mM (mmol/L) solution of levamizole was added to inhibit endogenous ALP activity. Quantification was performed by measuring the optical density of the yellow color of the p-NP in a plate reader (SPECTRA MAX 340PC, Molecular Devices, USA) at a wavelength of 405 nm. Finally, the results were normalized to the internal β-actin protein control. Furthermore, Alizarin red staining was applied after 21 days of culture using previously described protocols [56], in order to examine, whether mineralization of the extracellular matrix (ECM) produced by SaOS-2 cells on the various Ti surfaces had occurred. This was in addition to utilizing a Roentec® energy dispersive X-ray (EDX) microanalyzer attached to the Philips SEM 505 for EDX analysis to determine the presence

Table 1. Results of the roughness and contact angle measurements of the different dental implant surfaces

Implant surface	R_a, µm	R_z, µm	R_{max}, µm	Contac angle
CP	4.01 ± 0.04	21.22 ± 0.61	26.29 ± 0.90	91.3° ± 5.8°
Pro	1.97 ± 0.02	12.16 ± 0.21	16.35 ± 0.39	56.3° ± 9.8°
SLA	3.65 ± 0.16	18.72 ± 0.01	23.86 ± 0.23	90.9° ± 4.0°
TPS	5.58 ± 0.01	40.87 ± 0.98	52.45 ± 1.39	59.4° ± 7.5°
Oss	0.82 ± 0.04	5.01 ± 0.11	6.59 ± 0.58	70.5° ± 1.6°
MTi	0.20 ± 0.01	1.43 ± 0.04	1.98 ± 0.01	67.6° ± 2.7°

All values are mean ± standard deviation of 20 (surface roughness) or 10 (contact angle) measurements, respectively. CP = CellPlus; Pro = Promote; SLA = SLA; TPS = titanium plasma sprayed, Oss = Osseotite and MTi = machined titanium.

of calcium and phosphorus in the ECM produced by SaOS-2 cells after 21 days.

2.5. Statistical analysis

Two runs of experiment were performed and assays were run in quadruple. The measurements of the two experimental runs were pooled. To compare the different substrata to each other, the data were statistically analyzed using an analysis of variance (ANOVA; Origin Pro8; Originlab, USA) and a *post-hoc* Tukey test. Results were considered statistically significant at $p < 0.01$.

3. Results

3.1. Titanium implant surfaces characterization

The result of the surface roughness and contact angle measurement of the various test surfaces are summarized in Table I. The highest surface roughness values (R_a, R_z and R_{max}) were recorded for the TPS surfaces followed by CP, SLA, Pro, Oss and MTi. R_a, R_z and R_{max} values ranged from 0.2 to 5.58 µm, 1.43 to 40.87 µm and 1.98 to 52.45 µm, respectively. With respect to the wettability, Pro surfaces displayed the lowest contact angle that is the highest wettability, followed by TPS, MTi, Oss, SLA and CP, for which the highest contact angles and lowest wettability were noted. Contact angle values ranged from 56.3° to 91.3°. In Fig. 1 the topographic images obtained for the different implant surfaces utilizing the profilometer are depicted. The CP surface displayed the most rugged surface texture. In this it was followed by SLA, Pro and Oss. Furthermore, the highly rough texture of the TPS surfaces as well as the smooth morphology of the MTi control surfaces were visualized.

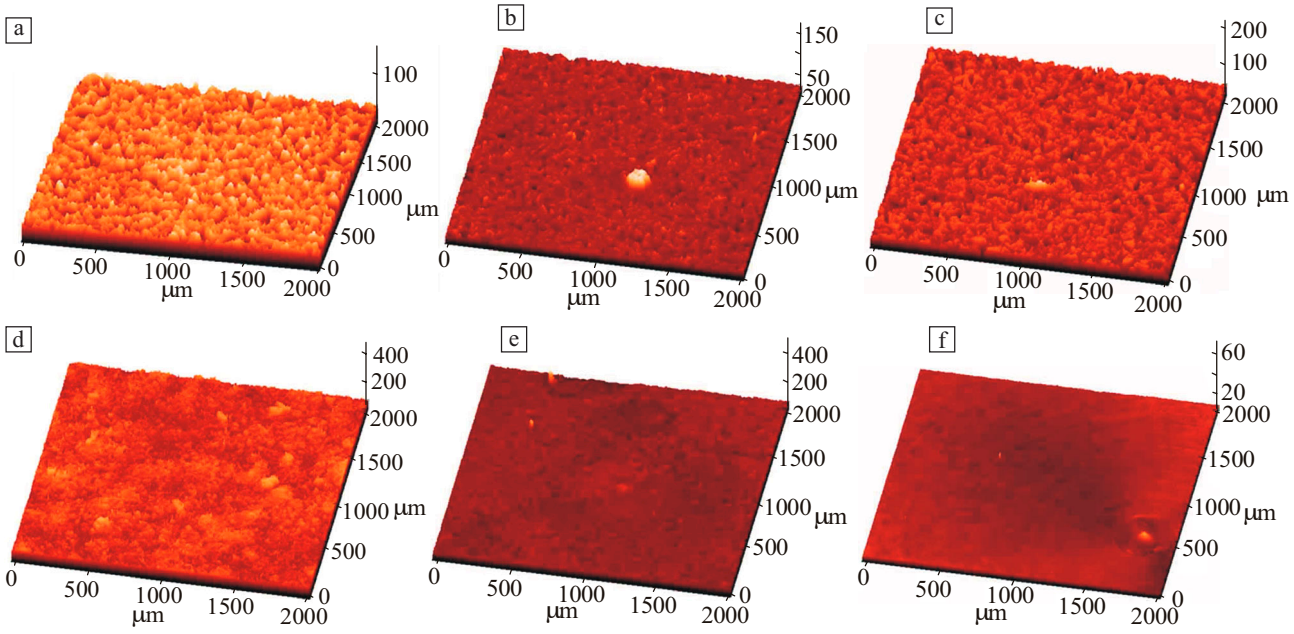

Fig. 1. Visualization of the surface microtopography of the various dental titanium implant surfaces used in this study by optical profilometry; CellPlus (CP) (a), Promote (Pro) (b), SLA (c), titanium plasma sprayed (TPS) (d), Osseotite (Oss) (e), and machined titanium (MTi)–control surface (f).

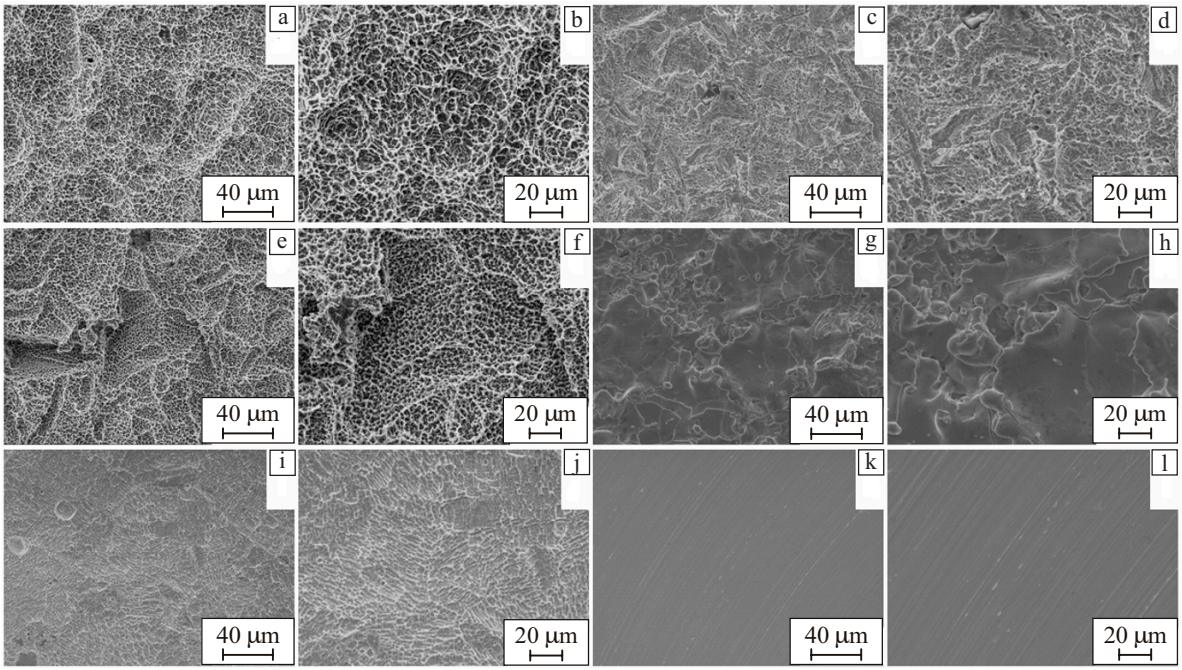

Fig. 2. Scanning electron micrographs of the various dental implant surfaces, acquired at two levels of magnification (500× and 1000×); CellPlus (CP) (a, b), Promote (Pro) (c, d), SLA (e, f), titanium plasma sprayed (TPS) (g, h), Osseotite (Oss) (i, j), and machined titanium (MTi)–control surface (k, l).

Figure 2 displays the scanning electron micrographs of the different Ti dental implant test surfaces supplied by the various dental implant manufacturers including the machined control surfaces (MTi). SEM analysis at a magnification of up to 1000 times showed that SLA and CP surfaces exhibited a surface texture of greater roughness compared to Pro and Oss. In addition, SLA and CP showed somewhat similar features at the 20–100 micrometer- and micrometer-scale. The SLA surface, however, displayed a more heterogeneously-structured surface featuring larger diameter crater-like cavities with greater concavity and more steeply sloped walls and undercuts (Figs. 2e and 2f) compared to the CP surface, which exhibited a less textured surface geometry with cavities of lesser diameter, concavity and less steeply sloped walls (Figs. 2a and 2b). At the micrometer-scale both implant surfaces featured superimposed microtopographic pits. TPS surfaces displayed a surface morphology which differed significantly from the sand-blasted and/or dual-acid-etched surfaces due to the molten titanium particles deposited on the Ti substrate. TPS surfaces had the highest surface roughness values but lacked the superimposed levels of surface roughness present with CP, SLA, Pro and Oss samples. All implant surfaces exhibited a rougher surface than the MTi control specimens, which had the smoothest surface (Table 1), and for which only the machining threads were visible.

3.2. Cellular proliferation

Figure 3 depicts the results of the cell proliferation on the different implant surfaces at the different time points. In general, all the implant surfaces supported increasing cell proliferation, compared to the initial cell density of 8.5×10^4 cells/cm^2, over the 21day incubation period. MTi and TPS, however, displayed significant lower cell numbers than SLA, CP, Pro and Oss, i.e. the new generation of titanium implant surfaces, which exhibit two superimposed levels of surface roughness, at 7, 14 and 21 days of incubation. At 21 days most cells were present on SLA followed by CP and Pro, while at 3 days cell numbers were highest on Pro and SLA closely followed by Oss.

3.3. Cellular differentiation–immunocytochemistry assay for osteogenic proteins

Figure 4 shows the results of the quantification of the different osteogenic proteins produced by the SaOS-2 cells

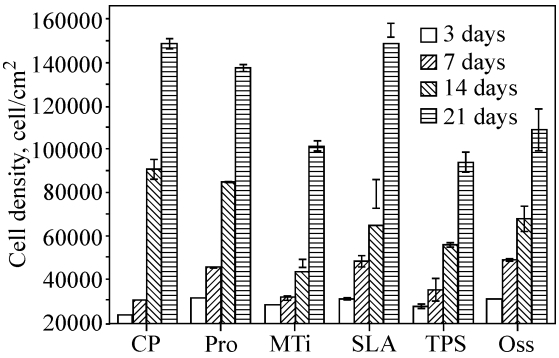

Fig. 3. Number of SaOs-2 human bone cells cultured over 21 days on different titanium dental implant surfaces. Values are mean ± standard deviation.

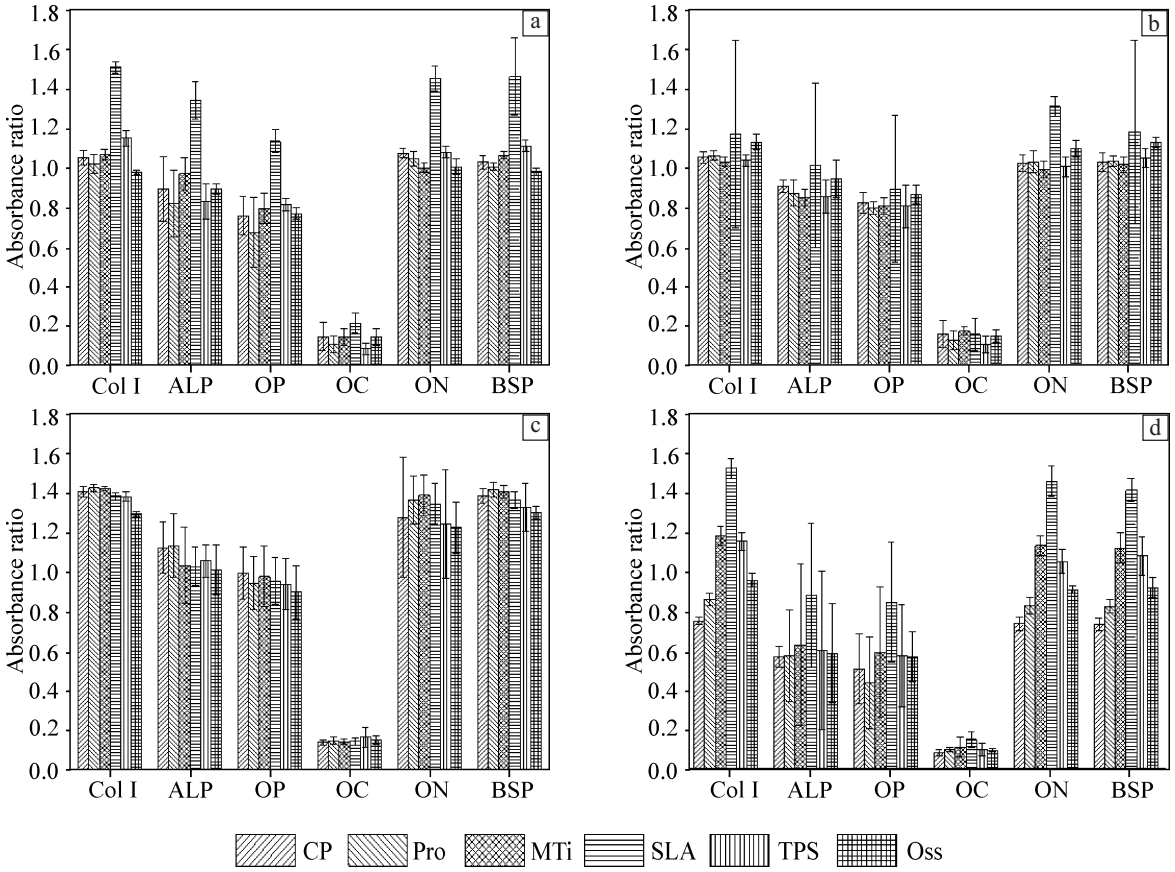

Fig. 4. Expression of bone-related proteins by SaOs-2 cells cultured on different titanium dental implant surfaces for 3 weeks. Results are normalized to the internal control, i.e. β-actin protein, for each time point and each substratum. All values are mean ± standard deviation of 8 measurements. Absorbance ratio (Y-axis) at 405 nm: day 3 (a), day 7 (b), day 14 (c), and day 21 (d). Col I, type I collagen; ALP, alkaline phosphatase; OP, osteopontin; OC, osteocalcin; ON, osteonectin; and BSP, bone sialoprotein. CP = CellPlus; Pro = Promote; SLA = SLA; TPS = titanium plasma sprayed, Oss = Osseotite, and MTi = machined titanium (control).

cultured on the various Ti samples at the different time points. At 3 days, cells cultured on SLA and TPS expressed significantly higher levels of Col I compared to cells grown on all other samples, while Col I expression by cells cultured on Oss was significantly lower compared to all the other samples except for Pro; ALP and OP expression levels in cells on SLA were also significantly higher compared to all other samples; OC expression was significantly higher in cells grown on SLA compared to osteoblasts cultured on Pro and TPS, and intracellular ON expression on SLA was significantly higher compared to all other surfaces. Furthermore, cells grown on TPS and CP expressed significantly higher ON levels than cells cultured on Oss and MTi. With respect to BSP expression, significant differences were only observed for cells on SLA, which expressed more BSP than cells grown on all other materials.

After 7 days of incubation, Col I, ALP, OP, OC, and BSP expression levels by cells grown on the various test surfaces did not show and statistically significant differences. Only intracellular ON production on SLA was significantly higher compared to all other surfaces; the same was true when comparing intracellular ON expression on Oss to TPS and MTi.

At day 14, cells grown on Oss expressed significantly lower Col I levels than cells on all other substrata; the same was true when comparing Col I levels in cells on SLA and TPS to those in cells on Pro and MTi. ALP, OP, OC, and ON expression levels did not show any statistically significant differences when comparing the various implant surfaces, while with respect to BSP significantly lower expression levels were noted in cells on Oss compared to those on Pro and MTi.

After 21 days of incubation, SLA surfaces displayed significantly higher levels of Col I and BSP compared to any other test surfaces and significantly greater OC levels than cells on CP and Oss in combination with significantly higher ON levels than MTi and TPS. Cells cultured on Oss exhibited significantly lower Col I production compared to cells grown on MTi and TPS. Col I expression levels by cells on MTi and TPS did not display any statistically significant differences. Cells cultured on CP and Pro showed significantly lower ON production than cells on all other

surfaces. The same was true when comparing ON production by cells on Oss to that by cells grown on MTi and TPS. Cells cultured on Oss expressed significantly higher BSP levels than cells cultured on CP and significantly lower BSP levels than cells cultured on MTi and TPS. Also cells grown on CP and Pro showed significantly lower BSP expression than cells cultured on MTi and TPS. When comparing BSP expression by cells on Pro to that by cells on CP or BSP levels in cells on Oss to those in cells on MTi, differences were not statistically different. With respect to ALP and OP production no statistically significant differences were noted for cells grown on any of the test surfaces. Alizarin red staining and EDX analysis, which yielded peaks for phosphorus and calcium, confirmed mineralization of the extracellular matrix produced by SaOS-2 cells on all titanium test surfaces after 21 days of culture.

Regarding the effect of the different implant surfaces on osteogenic marker expression over time, already at day 3, SLA induced greater expression of all osteogenic markers than all other surfaces, and expression of all osteogenic markers already peaked at day 3 and then again at day 21, while with the other surfaces Col I, ALP, ON and BSP production only peaked at day 21 (Fig. 4). Cells grown on Oss expressed significantly greater OP levels at 21 days than at 3 and 14 days, while the difference in OC levels at 21 days was not statistically significant. On Pro, TPS and MTi OC levels also peaked at 21 days, but were significantly lower compared to those recorded for cells grown on SLA. Differences in OP levels observed in cells cultured on CP, Pro, TPS and MTi did not show any statistically significant differences at the various time points.

4. Discussion

In implant dentistry there has been an ongoing effort to enhance and accelerate osseointegration of dental implants by optimizing their implant surface design [12–15], which led to the development of implant surfaces with mixed microtopography. This is related to the fact that treatment outcomes in dental implantology are critically dependent on implant surface designs that optimize the biological response during the different mechanisms by which bone becomes juxtaposed to an endosseous implant surface [6–8, 12–15]. The mechanisms by which endosseous implants become integrated in bone can be subdivided into three distinct phases [6]. The first, osteoconduction relies on the migration of differentiating osteogenic cells to the implant surface through a temporary connective tissue scaffold. Anchorage of this scaffold is a function of implant surface design. The second phase, *de novo* bone formation results in a mineralized interfacial matrix, being laid down on the implant surface. Implant surface topography will determine, if the interfacial bone formed is bonded to the implant. A third tissue response is that of bone remodeling, also resulting in *de novo* bone formation at discrete sites [6]. In this context, an important aspect is the influence of the implant surface on osteoblastic cell differentiation. To enhance osseous integration dental implant surfaces should possess the ability to stimulate differentiation of osteogenic cells and matrix formation at their surface.

Consequently, the current study aimed at elucidating the effect of varying surface properties and microtopography of new generation clinically used dental implant surfaces, which exhibit two superimposed levels of surface roughness, on osteoblast differentiation. To this end, first the surface characteristics of these commercially available implant surfaces were determined in the same experimental set-up. Profilometry and contact angle measurements were utilized to characterize the surface roughness and wettability. This was in addition to visualizing surface microtopography and architecture by scanning electron microscopy as well optical profilometry. SEM analysis and optical profilometry imaging allowed visually assessing morphological differences between the test surfaces and comparing the morphology of the various implant surfaces to each other in addition to correlating the morphological findings with the surface roughness and contact angle values as well as osteogenic marker expression by bone cells. Second, a human osteoblast-like osteogenic cell line, i.e. SaOS-2, was used to analyze the effect of the different Ti implant surfaces and their surface properties on osteogenic marker expression by these cells with the purpose to establish the relationship between surface characterization data and the cellular response in terms of osteoblast differentiation.

To our knowledge, there are very few studies which compare the effect of a broader selection of new generation dental implant surfaces with mixed microtopography on the temporal expression of a range of osteogenic proteins as a measure of phenotypic differentiation in the same experimental set-up. Furthermore, we were able to show previously that with respect to calcium phosphate bone grafting materials evaluation of osteogenic marker expression *in vitro* was an excellent predictive indicator for *in vivo* performance [50–53]. Consequently, the hypothesis tested in our study presented here was that elucidating the effect of the surface characteristics of various dental implant surfaces on osteoblast differentiation *in vitro* in terms of temporal expression of an array of osteogenic markers under identical experimental conditions may enhance our understanding of the bone tissue response to these surfaces at the molecular level and may contribute to identifying key factors affecting the performance of dental implants.

Differentiating osteoblasts are known to synthesize and secrete type I collagen, alkaline phosphatase and other non-collagenous extracellular bone matrix proteins such as osteonectin, osteocalcin, osteopontin and bone sialoprotein [55, 57–60]. These bone-matrix proteins have been recognized to be particularly useful osteogenic markers characterizing the various stages of osteoblast differentiation. Differential bone-related marker expression of osteogenic cells can be defined by several principal biological periods: cellular pro-

liferation, cellular maturation and focal mineralization. It has been demonstrated that Col I is expressed during the initial period of proliferation and extracellular-matrix biosynthesis, whereas ALP is expressed during the post proliferative period of extracellular-matrix maturation, and the expression of OP, OC, ON and BSP occurs later during the third period of extracellular-matrix mineralization [55, 57–60]. Thus, the present study quantitatively records the response of human bone cells to the various dental implant test surfaces at four time points in terms of Col I, ALP, OP, OC, ON and BSP osteogenic protein expression as a measure of phenotypic differentiation [24, 44, 50–53].

The choice of the human osteoblast-like osteogenic cell line, i.e. SaOS-2 for examining the effect of the different dental implant test surfaces on osteoblast differentiation was based on previous experience of our group with this cell line. Previously, we obtained comparable results when using human primary osteoblasts and SaOS-2 cells for studying the effect of identical bone grafting materials on osteoblast differentiation [50–52]. Saldana et al. studied the use of SaOS-2 cells and compared them to other cell lines. In these studies the authors were able to prove that SaOS-2 cells are representative of human osteoblasts in biomaterial and cytocompatibility studies [61]. In addition, studies performed by others have also demonstrated the suitability of using SaOS cells for osteogenic assessment [62–64]. It also is important to note that an osteogenic medium was used which contained ascorbic acid, which is important for collagen production and osteoblast differentiation. In addition, osteogenic media need to contain phosphate in order to facilitate ECM mineralization. To this end, a phosphate containing ascorbic acid compound was used in the present study rendering additional use of β-glycerophosphate unnecessary [50–52, 54]. The choice of Ti grade 2 was based on the fact that this Ti grade material is the main commercially pure Ti used for fabrication of commercially available dental implants [65].

Meta-analysis of the existing literature emphasizes that, in order to establish the relationship between surface properties and biological performance of implant surfaces, a detailed characterization of their surface properties is required [13–15].

All surface modifications examined in the present study were provided by dental implant manufacturers. Hence, the surface modifications and properties of the Ti disks used in the present study are those present with the respective implants, which are used clinically and are commercially available. In general, all implant surfaces studied supported osteoblast proliferation, differentiation and matrix mineralization over the 21-day period without any adverse effects on cell function. In the current study, the new generation sandblasted and acid-etched or dual-acid-etched surfaces, i.e. SLA, CP, Pro and Oss, induced significantly greater cell proliferation compared to the TPS and MTi surfaces with SLA displaying greatest cell numbers after 21 days. SLA had the greatest effect on osteoblast differentiation already after 3 days in culture, as indicated by the OP, OC, ON and BSP protein expression, which are markers characteristic for the later stages of differentiated osteoblast function and are tightly linked to osteoid production and matrix mineralization. The significant higher osteogenic marker levels observed in cells on SLA after 3 days of incubation may be related to the initial cell–substratum interaction. Osteogenic protein expression peaked another time at 21 days. In this context, it should be noted that both surface chemistry as wells as surface geometry affect intracellular signaling which modulates cellular differentiation and survival, since there is a common link in the focal adhesions which are sites where integrin mediated adhesion links to the actin cytoskeleton. Thus, focal adhesions lie at the convergence of integrin adhesion, signaling and the actin cytoskeleton [66]. Focal adhesion kinase, FAK, has emerged as a key signaling component at focal adhesions. FAK can be activated, i.e. tyrosine phosphorylated both via integrin receptor substratum interaction [23, 67] leading to integrin receptor activation and outside-in signaling or via cytoskeletal orientation and activation [23, 68–71], which can be favorably influenced by the curvature design of pores and cavities [69]. At the same time integrin signaling and local factor production lead to transduction and perpetuation of the signal to subsequent cell layers. In addition, with increasing time of culture there also is an increase of extracellular matrix and type I collagen formation. Thus, it may be hypothesized that the cytoskeletal control exercised by the crater-like concave cavities may lead to enhanced osteoblast differentiation at day 3. This may be followed by a maturation phase at the intermediary time points and then a peak in osteogenic protein production after 21 days, which may result from the additive effects of first the perpetuation of the effect of the original activation of signaling cascades by the substrate geometry, and secondly the interaction of subsequently formed cells with the increasingly maturing extracellular matrix and components thereof such as type I collagen. Similar patterns of osteogenic marker expression were observed in previous studies on calcium alkali orthophosphate-based bioactive bone grafting materials, which induced enhanced osteoblast differentiation [51–53]. This was associated with enhanced fibronectin and collagen type I protein adsorption, subsequent $\alpha_5\beta_1$ and $\alpha_2\beta_1$integrin receptor binding and enhanced simultaneous activation of the ERK differentiation and PI3K/Akt cell survival pathways as well as of the alternate p38 pathway, all of which upregulate cellular differentiation and survival [72, 73]. It furthermore has been shown that (i) surface micron-scale topography of titanium substrates modulates $\alpha_5\beta_1$ integrin binding and FAK activation [70], that (ii) osteoblast differentiation on microtextured titanium substrates is dependent on $\alpha_2\beta_1$ integrin signaling [71] and that (iii) activation of the alternate p38 pathway is mediated via the $\alpha_2\beta_1$ integrin receptor, the so-called collagen receptor [73].

Consequently, the underlying mechanisms leading to the pattern of noticeably higher osteogenic protein expression recorded for cells cultured on SLA surfaces at day 3 and then again at day 21 warrant further investigation, in order to test the hypotheses outlined above.

The finding that SLA had the greatest stimulatory effect on osteogenic marker expression and osteoblast differentiation may, however, suggest that SLA possesses a higher potency to promote osteogenesis and matrix mineralization than the other titanium surfaces tested here.

Our findings are in correspondence with observations made by Orsini et al., who noted an enhanced osteoconductive behaviour for SLA surfaces *in vivo* [74]. The present results are also in agreement with the findings obtained by Donos et al., who reported upregulation of a large number of genes which are functionally relevant in the context of skeletogenesis, mesenchymal cell differentiation, angiogenesis and neurogenesis when comparing SLA surfaces to smooth titanium surfaces after implantation *in vivo* for 14 days [75]. Furthermore, our results are corroborated by numerous clinical studies, which demonstrated excellent clinical outcomes for SLA dental implants [1, 16, 20, 36]. Thus, the findings of the current study regarding the SLA surface are in agreement with previous studies, in which we were able to demonstrate that, when using the same *in vitro* osteogenic assay as applied in the current study, enhanced osteoblast differentiation *in vitro* by cells cultured on calcium alkali orthophosphate bone grafting materials correlated with enhanced and more expeditious bone formation and osteogenic marker expression *in vivo* [52, 53]. In this context, it needs to be noted that in an *in vivo* clinical setting additional factors may influence the final outcome such as patient age, health status, smoking, diabetes [14, 15].

In the current study, SLA samples displayed a rough and hydrophobic surface, whose roughness and contact angle values were similar to those of CP (Table 1). SEM analysis, however, revealed a difference in surface topography. While the SLA surfaces displayed a more highly textured surface architecture featuring distinct concave crater-like cavities with more steeply sloped walls and undercuts (Figs. 2e and 2f). CP surfaces exhibited a less textured surface geometry with cavities of lesser diameter and concavity and less steeply sloped walls (Figs. 2a and 2b). The walls of these cavities, however, contained microtopographic pits with both implant surfaces. In this context, it is noteworthy that various authors have demonstrated that repetitive concavities on the surface of bone implants enhance osteoblast differentiation *in vitro* as well as osteogenesis *in vivo*. Moreover, these repetitive concavities induce bone formation when implanted in ectopic locations *in vivo* [76–78], which has led to the development of osteoinductive biomaterials with smart concavities displaying geometric cues by Ripamonti et al. [77]. In fact, this principle, namely that concave surface features are capable of inducing bone formation, was first formulated by Marshall Urist in 1965 [79, 80]. It may be hypothesized that the specific crater-like cavities displaying steeply sloped walls found on the SLA surfaces may have a similarly beneficial effect resulting in enhanced osteoblast maturation and bone formation. In addition, micron and submicron surface characteristics of titanium implant surfaces have been shown to have a considerable effect on osteoblastic differentiation and maturation, and to also affect the vascular endothelial growth factor production [12, 23, 26, 74, 75, 81, 82]. In this context studies performed by Zhao et al. and Zinger et al. demonstrated that both micron and submicron scale surface topographical structures of titanium surfaces were required for enhanced osteoblast differentiation and local factor production [83, 84]. Taken together, these findings are in agreement with our results and suggest that the greater stimulatory effect on osteoblast differentiation of the SLA surfaces when compared to CP (as well as Pro and Oss) may be related to the defined mixed microtopography of the SLA surfaces with its specific distribution of repetitive crater-like steeply sloped concave cavities at the 100 μm scale in combination with micron and submicron scale surface topographical structures in the form of pits. Furthermore, the results of our present study suggest that the differences in microarchitectural features had a greater effect on the cellular response than the wettability, as the SLA surface displayed relatively high contact angle values. In spite of the higher wettability of the Oss and Pro surfaces compared to the SLA surfaces, and although similar contact angle values were recorded for SLA and CP, the SLA surfaces induced more enhanced osteoblast differentiation than the CP, Oss and Pro surfaces. This in agreement with observations by Pivodova et al. [85], who concluded that surface topography had a greater impact on osteoblastic cell differentiation than chemical surface modifications.

More recently, the SLActive® surface was developed by Straumann, exhibiting a chemically modified SLA surface with the same surface roughness but high hydrophilicity and hence wettability. In clinical studies, an enhanced bone tissue response was reported for the SLActive® surface during the early healing phase when compared with the SLA surface. However, the later biological response was quite similar for the two surfaces and both demonstrated very good clinical results [86]. Our results are in accordance with those of Khan et al. who noted that the expression of osteogenic markers was higher on both hydrophilic and hydrophobic SLA surfaces when compared to tissue culture plastic and smooth Ti disks [87]. Wall et al. reported that this difference was linked to the activation of the osteogenic promoter WNT5A [88].

Moreover, the findings of our current study demonstrate that obtaining long-term data regarding the success and survival rate of these various commonly clinically used new generation dental implants in the context of comparative prospective multi-center studies would be highly desirable

[13, 14]. This would allow correlating the distinct differences, which were observed with respect to the initial cellular response by osteoblasts in terms of osteoblast differentiation, to the long-term clinical performance of these implants, as their surfaces displayed considerable differences with regard to their surface properties, microtopography and microarchitectural features.

5. Conclusions

This study established the relationship between the surface properties of an array of clinically used titanium implant surfaces with varying mixed microtopography and their effect on osteoblast proliferation and differentiation. All test surfaces supported osteoblast growth and differentiation. Hence, their osteoconductive properties were confirmed at the molecular level. The new generation titanium surfaces, which exhibit two superimposed levels of surface roughness induced greater cell proliferation after at 7, 14 and 21 days of incubation than the smooth and plasma-sprayed surfaces. SLA surfaces had the greatest stimulatory effect on cell proliferation and osteoblast differentiation, which seemed to be related to their defined mixed surface microtopography with its specific combination of repetitive steeply sloped concave crater-like cavities with microtopographical pits. This study shows that differences in surface microarchitectural features at the 100micron and micron scale have a significant effect on the osteogenic phenotype expression *in vitro*. Similar to previous studies, in which the same *in vitro* osteogenic assay was used for evaluating the osteogenic potential of novel bone grafting materials, and in which we were able to demonstrate an excellent correlation between *in vitro* and *in vivo* results, the findings of the present *in vitro* study are in good agreement with results reported by various *in vivo* studies. Even if there may be limitations to directly extrapolating the results of *in vitro* studies to the *in vivo* clinical environment [13, 14], the findings of our present study with respect to the bone cell-implant surface interaction suggest that differences in surface properties and microtopography of dental implants are likely to influence their *in vivo* performance in patients.

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Author declaration

The authors declare to have no conflict of interests.

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