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ORIGINAL ARTICLE

Interactions between endothelial progenitor cells (EPC) and titanium implant surfaces

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Abstract
Objectives Endothelial cells play an important role in peri-implant angiogenesis during early bone formation. Therefore, interactions between endothelial progenitor cells (EPCs) and titanium dental implant surfaces are of crucial interest. The aim of our in vitro study was to investigate the reactions of EPCs in contact with different commercially available implant surfaces.
Materials and methods EPCs from buffy coats were isolated by Ficoll density gradient separation. After cell differentiation, EPC were cultured for a period of 7 days on different titanium surfaces. The test surfaces varied in roughness and hydrophilicity: acid-etched (A), sand-blasted-blasted and acid-etched (SLA), hydrophilic A (modA), and hydrophilic SLA (modSLA). Plastic and fibronectin-coated plastic surfaces served as controls. Cell numbers and morphology were analyzed by confocal laser scanning microscopy. Secretion of vascular endothelial growth factor (VEGF)-A was measured by enzyme-linked immunosorbent assay and expressions of iNOS and eNOS were investigated by real-time polymerase chain reaction.
Results Cell numbers were higher in the control groups compared to the cells of titanium surfaces. Initially, hydrophilic titanium surfaces (modA and modSLA) showed lower cell numbers than hydrophobic surfaces (A and SLA). After 7 days smoother surfaces (A and modA) showed increased cell numbers compared to rougher surfaces (SLA and modSLA). Cell morphology of A, modA, and control surfaces was characterized by a multitude of pseudopodia and planar cell soma architecture. SLA and modSLA promoted small and plump cell soma with little quantity of pseudopodia. The lowest VEGF level was measured on A, the highest on modSLA. The highest eNOS and iNOS expressions were found on modA surfaces.
Conclusions The results of this study demonstrate that biological behaviors of EPCs can be influenced by different surfaces. The modSLA surface promotes an undifferentiated phenotype of EPCs that has the ability to secrete growth factors in great quantities.
Clinical relevance In correlation with recent clinical studies these results underline the hypothesis that EPC could promote and increase neovascularization by secreting paracrine factors which support osseointegration of dental implants.

Keywords Endothelial progenitor cell · EPC · Stem cell · Titanium implant surface · SLA · SLActive · Hydrophilicity

Thomas Ziebart and Anne Schnell contributed equally to this work.

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Introduction

In the last years, various researchers focused on modification of surface characteristics of dental implants with the objective of optimizing and precipitating the dental implant healing cascade. The main goals are immediate implantation, immediate prosthetic treatment, and immediate exposure

for the future. There is a demand for bioactive surfaces that are able to enhance requested properties such as cell adhesion, cell proliferation, and secretion of paracrine growth factors. Hydrophilic surfaces were developed, which showed encouraging results regarding optimized implant stability and shortened healing time [1–3]. The exact biological mechanisms on cellular level are still unclear. Despite the knowledge that implant healing is exceedingly dependent on the successful establishment of a vessel network, most of the in vitro studies focus on the osteogenic potential of implant surfaces [4–7]. Increased endothelial stimulation may accelerate new bone formation with increased healing implant osseointegration [8]. Only a few studies investigate the effect of implant surfaces on matured endothelial cells [9].

Endothelial progenitor cells (EPC) were first described by Ashara et al. in 1997 [10]. Different research fields (e.g., tumor biology or regenerative medicine) have delved into characterization of EPCs, which display new hope, e.g., for curing cardiovascular diseases. EPCs are able to promote neovascularization under hypoxic conditions in adult humans [11–17] through a process called vasculogenesis. Vasculogenesis is a de novo blood vessel development compared to angiogenesis, where new blood vessels originate from pre-existing vessels by migration and proliferation of endothelial cells [18]. EPC effects neovascularization by secreting paracrine factors (cytokines, growth factors) like vascular endothelial growth factor (VEGF) and by building a primary cell network through differentiation in endothelial cells or muscle cells [14, 19, 20].

The characterization of EPCs is very difficult since different subtypes with various origins and stages of development are summed up to this group of cells. In the last few years, hundreds of research groups concentrated on role, identity, and characterization of EPCs. In September 2011, a pubmed-search for "endothelial progenitor cell" resulted in 10,173 articles including 1,760 reviews.

Several researchers divide EPCs in two main groups: CD-14+ (so called "early" EPCs) and CD-34+ EPCs (so called "late" EPCs) [21, 22]. Confusingly several other research groups call "late" EPCs "ECFCs" (endothelial colony-forming cells) and still others "OECs" (outgrowth endothelial cells) [23, 24]. Diverse authors actually talked about four instead of two subpopulations [25].

In vitro diverse subpopulations of EPCs show functional differences, but in vivo it seems that they all contribute to neovascularization [26, 27] for which the exact mechanisms are unclear.

eNOS and iNOS expression is essential for the functional activity of EPC and reflects the differentiation level and angiogenetic potency of these cells [28, 29].

The aim of this in vitro study is to investigate the interaction and biological behavior of EPC with different commercially available implant surfaces. Surface modification could lead to increased angiogenesis and vasculogenesis which influence implants integration and osseointegration.

Materials and methods

Titanium disks

Instead of inapplicable three-dimensional origin implants, we used titanium disks (diameter 15 mm, thickness 1 mm) produced and supplied by the Straumann Group (Basel, Switzerland). Four different titanium surfaces were applied: A, SLA, modSLA, and modA. A stands for "acid-etched" and SLA is the abbreviation for "sand-blasted, large grit, acid-etched". In addition to this modA and modSLA are rinsed under nitrogenic atmosphere and stored in isotonic NaCl to avoid contact with atmospheric carbonates after SLA-treatment and have a hydrophilic surface [30]. The commercial name for modSLA is SLActive. In 2007, Qu et al. reported on the fabrication and surface characterization of the four used titanium surfaces [7]. A and modA constitute a smoother titanium surface (mean roughness 0.6 μm) compared to SLA and modSLA (mean roughness 1.2 μm) [7]. Cell culture compatible plastic and fibronectin-coated plastic served as control surface (coated by 30 min incubation with 10 μl fibronectin per milliliter PBS).

Isolation and culture of EPCs

Mononuclear cells were isolated from buffy coats from healthy blood donors by ficoll density gradient separation as previous described [27, 31]. After that, mononuclear cells were plated on fibronectin-coated culture bottles at a concentration of 8×10^6 cells/ml medium and stored under following culture conditions: 37°C, 5% CO_2, and 95% humidity. The culture medium was endothelial basal medium (Lonza Group AG, Basel, Switzerland) combined with endothelial growth medium SingleQuots (0.1% human epidermal growth factor, 0.1% hydrocortisone, 0.1% gentamicin+amphotericin-b, and 0.4% bovine brain extract; Lonza Group AG, Basel, Switzerland) and 20% fetal calf serum. On the fourth day of culturing mononuclear cells, nonadherent cells were washed out with phosphate-buffered saline (Gibco, Cleveland, USA), while EPCs adhering to the fibronectin-coated plastic surface were detached by 0.5% trypsin/EDTA (Gibco, Cleveland, USA).

Confocal laser scanning microscopy

To detect the number of living EPCs, EPCs were cultured at a concentration of 300,000 cells/ml medium, stained, visualized via fluorescence excitation, photographed, and counted afterwards. EPCs were stained with CellTracker green CMFDA (Invitrogen, Karlsruhe, Germany) and visualized by confocal laser scanning microscopy (CLSM; Leica Group AG, Basel, Switzerland). We investigated nine samples each and took five pictures at a time of 24, 72, and 168 h of each surface (SLA, modSLA, A, modA, plastic, and fibronectin-coated plastic). In parallel, cell morphology was analyzed on CLSM images using a 40-fold immersion lens.

Cell count

We investigated the mean cell number of each surface by counting the cells number on 45 randomized CLSM pictures.

Enzyme-linked immunosorbent assay

EPCs were cultured at a concentration of 400,000 cells/ml medium on five different surfaces (SLA, modSLA, A, modA, and fibronectin-coated plastic) in 24-wells. An additional medium without cells was analyzed as control. The experiments were repeated six times. Supernatants were investigated by Quantikine® Human VEGF Immunoassay (R&D Systems, Inc. Minneapolis, USA) after 24, 72, and 168 h using a microplate reader (Metertech, Inc., Taipei, Taiwan).

Real-time PCR

EPCs were cultivated in 400,000 cells/ml medium on five surfaces (SLA, modSLA, A, modA, and fibronectin-coated plastic) in 24 wells. mRNA was isolated after 24, 72, and 168 h and the iNOS and eNOS expression was measured. $N=3$ examples of each surface were investigated and summed up to one PCR run. mRNA was isolated by using a commercial Qiagen-Kit and QIAshredder (both from Qiagen, Hilden, Germany) according to the manufacturer's information; 1.5 μl of each mRNA was used for an analysis of concentration and purity of mRNA with NanoDrop-Spectrophotometer ND-100 (peqLab Biotechnologie GmbH, Erlangen, Germany). cDNA was synthesized by iScript cDNA synthesis kit (BioRad, Hercules, USA) and expressions were measured with Gene Amp PCR system 2400 (PerkinElmer, Massachusetts, USA). Thereby, 500 ng RNA were applied and oligodT primers were used. All steps were done as specified by the manufacturer. β-Actin served as a housekeeping gene. The following primer sequences (Eurofins MWG Operon, Ebersberg, Germany) were used: β-actin: sense 5′-GGAGCAATGATCTTGATCTT-3′, antisense 5′-CCTTCCTGGGCATGGAGTCCT-3′; eNOS: sense 5′-GCAAGTCCACGAGGGCCACC-3′ antisense 5′-GCTCAGCAGCGCCTCCACAA-3′; iNOS: sense 5′-TTTCCTTACGAGGCGAAGAA, antisense 5′-GGCCCTGTGACCTCAGATAA-3′. Quantitative real-time PCR was run with I-Cycler IQ5 and IQ5 Optical System software version 2.0 (both from BioRad, Hercules, USA). Each sample for RT-PCR consisted of 25 μl 2× SYBR Green Supermix (BioRad, Hercules, USA), 9.5 μl DEPC water, 2 μl cDNA, and 1 μl primer, which had a concentration of 20 pmol/μl. For the RT-PCR the following protocol was used: Initial denaturation at 95°C for 5 min, then 40-fold rerun of this cycle: 95°C 30 s; 58°C 30 s; and 72°C 30 s. At the end of the run temperature increased from 60°C to 95°C, while fluorescence intensity was being measured gradually. Relative quantification of each gene between its control gene was realized through ΔΔCT method.

Statistical analysis

SPSS 16.0 was used for the statistical analysis. To detect a difference between the groups in the cell count experiments and VEGF enzyme-linked immunosorbent assay (ELISA) data one-way ANOVA was used with the post hoc Tukey test. A p value of 0.05 was considered statistically significant. Cell morphology on different surfaces was described descriptively. For RT-PCR, differences were detected by the ΔΔCT method.

Results

Cell numbers

We found significant deviations between different surfaces in terms of EPC numbers (Fig. 2). At all analyzed time points, the cell number measured on titanium surfaces were significantly decreased from days 1 to 7 compared the control surfaces ($p \leq 0.001$). After 24 and 72 h hydrophilic titanium surfaces (modA and modSLA) had smaller cell numbers than hydrophobic titanium surfaces (A and SLA). After 7 days, there were no significant differences detectable. After 7 days, higher cell numbers on smoother titanium surfaces (A respectively modA) than on rougher surfaces (SLA respectively modSLA) were detected ($p \leq 0.001$)

Cell morphology

Figure 1 shows representative photos. EPC showed the following characteristics on all surfaces: EPCs developed increasing pseudopodia from days 1 to 7; displaying

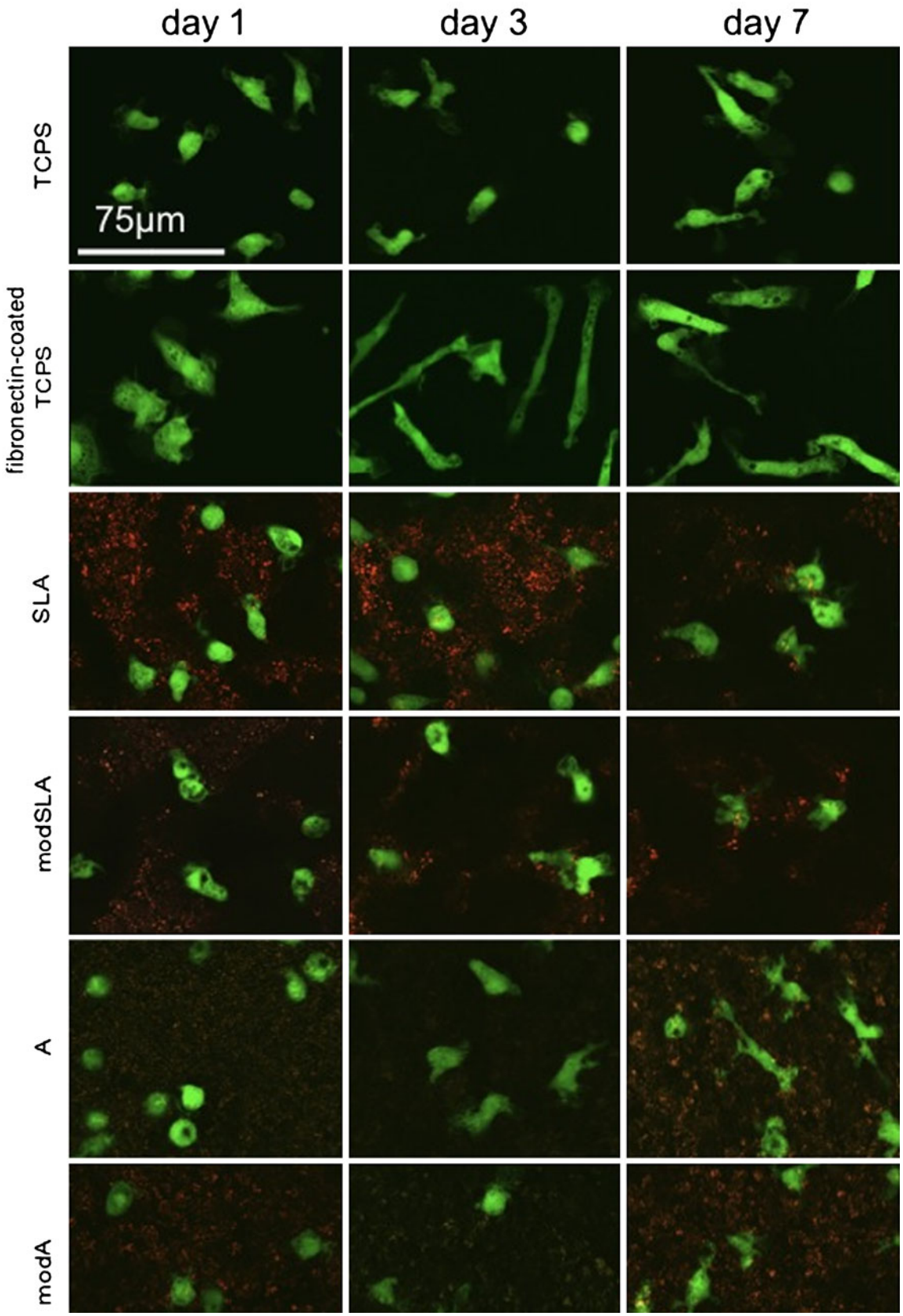

Fig. 1 Morphology of vital EPCs on six surfaces (tissue culture polystyrene (*TCPS*), fibronectin-coated TCPS, SLA, modSLA, A, and modA) after 24, 72, and 168 h; detected by confocal laser scanning microscopy with a 40-fold immersion lens. Green EPCs were stained with CellTracker green CMFDA (Invitrogen, Karlsruhe, Germany). *Red color* displays implant surface

adherence on the surface. Neither formation of cell clusters nor formation of tubular structures could be determined. The fibronectin-coated plastic surface showed biggest cell soma on the first point of examination and constituted spindle-shaped, elongated cell soma on the following days. The grade of roughness played a role for morphology: EPCs on control surfaces (plastic and fibronectin-coated plastic) together with A and modA displayed more extensive cell soma compared with rougher SLA and modSLA which showed smaller and plump cell soma. Hydrophilic properties had no influence on cell morphology (Fig. 2).

VEGF-A secretion

No VEGF-A was found in the medium without cells. VEGF-A could be observed at all time points in supernatant medium of all tested surfaces cultured with EPC (Fig. 3). VEGF-A was statistically significant increased in SLA, modSLA, and modA from days 1 to 7 ($p \leq 0.008$). On day 7, the smallest VEGF-A quantity was measured on A surface, all other surfaces did not show significant differences. The highest VEGF-A level were measured on modSLA surface.

eNOS and iNOS expressions

Both eNOS and iNOS expressions showed similar properties regarding different surfaces (Figs. 4 and 5). In summary, the highest values of eNOS and iNOS were measured on hydrophilic smooth modA. The exception was the primary high level of eNOS and iNOS in the SLA group. This effect was no longer visible on days 3 and 7. On day 7, the expression of eNOS and iNOS was higher on smooth titanium surfaces (A and modA) than rough titanium surfaces (SLA and modSLA). Due to the number of cases, only a descriptive analysis of the results was possible.

Discussion

Previously, most studies have concentrated on observing interactions between dental titanium surfaces and solitary cell lines. Most research groups focused on osteogenic cells [4, 32, 33], soft tissue cells like fibroblasts [34, 35] and endothelial cells (e.g., HUVEC) [36]. Based on the knowledge that sufficient implant healing depends on the establishment of efficient vascular plexus, implant surfaces should be tested for their vascular potential and not for their osteogenic potential alone. Ideal implant surfaces should promote adhesion and the stimulation of endothelial progenitor cells in addition stimulation of osteoblasts. To our knowledge, we investigated the first time the interaction of EPCs with different titanium surfaces in the context of implant healing. In the past, several research groups including us have shown the influence of microstructure and surface wettability on the adhesion, proliferation, and differentiation of osteoblasts in vitro [5–7, 37–39]. An explanation of the good clinical results of SLA and modSLA might be the microstructure of SLA/modSLA which is similar to natural osteoclasts lacuna [40, 41]. Wettability seems to influence direct protein adsorption on titanium surfaces. While hydrophobic surfaces trigger an unspecific and irreversible protein adsorption,

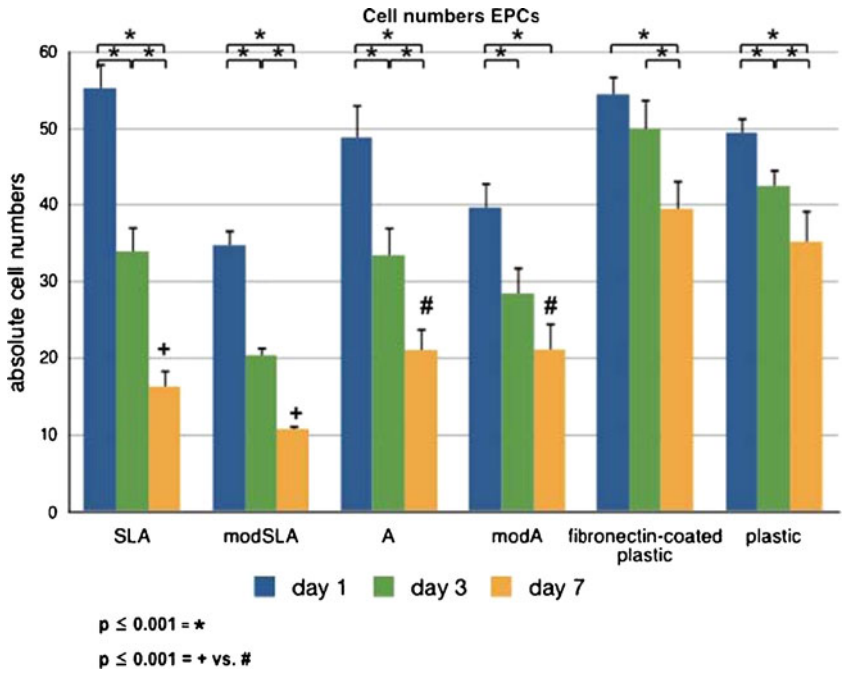

Fig. 2 Mean absolute numbers of EPCs counted out of pictures of the size 375×375 μm on six different surfaces (SLA, modSLA, A, modA, fibronectin-coated plastic, and plastic) at the point of 24, 72, and 168 h. Mean values; *error bars* show standard error of the mean. $n=9$

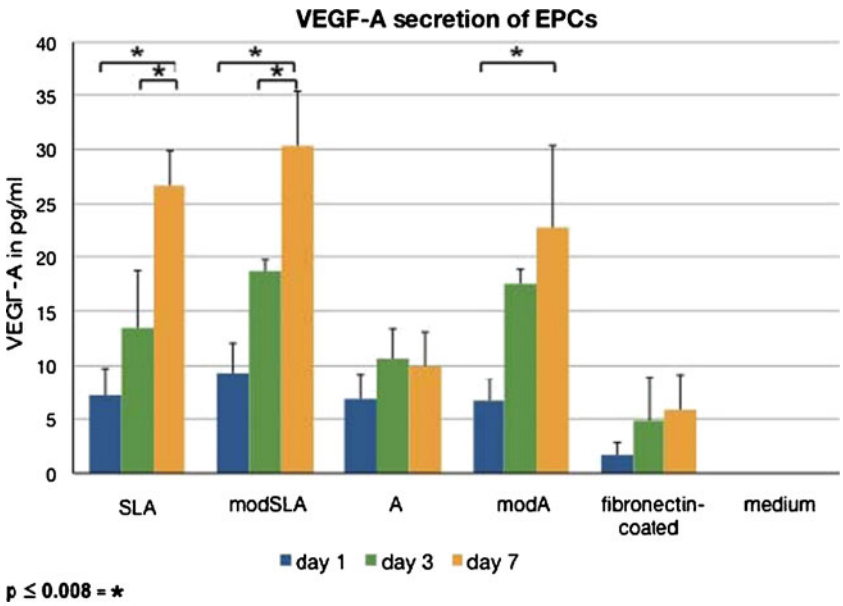

Fig. 3 Mean VEGF-A secretion in supernatant liquor of five surfaces colonized with EPCs (SLA, modSLA, A, modA, and fibronectin-coated plastic) and pure medium as control after 24, 72, and 168 h measured by ELISA. Data given in picograms per milliliter. $n=6$. *Error bars* show standard error of the mean

hydrophilic surfaces are supposed to cause an increased induction of osteogenesis through controlled adsorption of plasma proteins [7]. Current clinical studies often compared SLA with modSLA. The results of these studies attest that modSLA has better early implant stability when compared with SLA. This could be a hint that the healing time of hydrophilic titanium implants could be shortened compared with hydrophobic surface modifications [1–3, 42, 43]. In parallel to experiments with osteoblasts [4–7], we found that proliferation of EPCs was higher on hydrophobic than hydrophilic titanium surfaces at the beginning. In accordance with other research groups, we found lowest cell numbers on the hydrophilic rough modSLA at all time points. In a similar experimental setting, An et al. investigated human endothelial vein cells (HUVECs) on SLA, modSLA, A, and modA. They found lowest proliferation rates of HUVECs on A and highest on modA. These results are not coincident with ours and underline the differences between EPCs and mature endothelial cells (e.g., HUVECs). Osteoblasts mostly showed an increase of cluster formation on hydrophilic surfaces. Some researchers did not find morphological differences between hydrophilic and hydrophobic titanium surfaces [6, 7]. HUVECs displayed elongated and more quantity of cell clusters on modA in comparison to SLA and modSLA, where no cluster formations had been

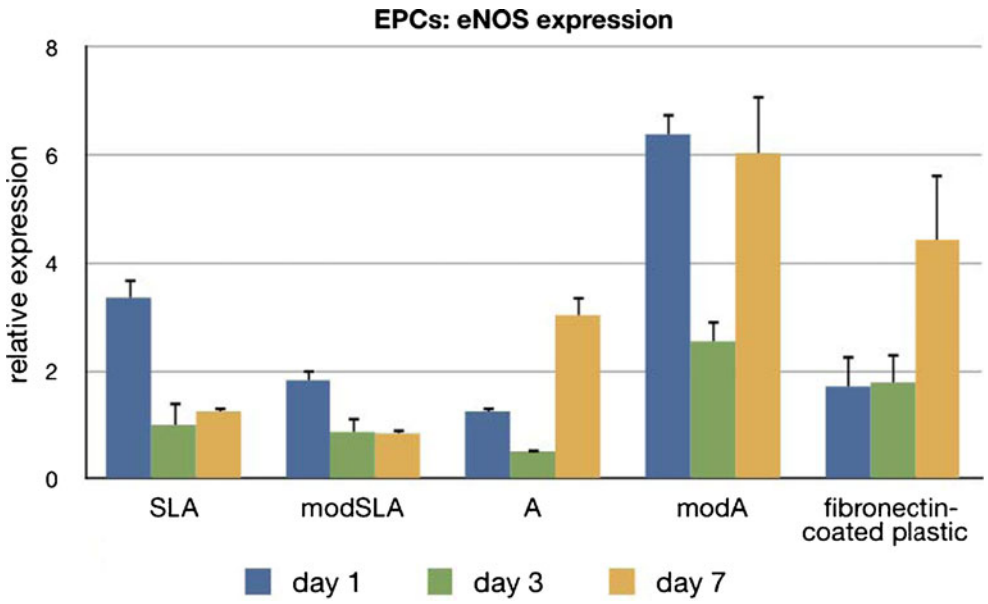

Fig. 4 Mean relative eNOS expression (normalized to thehousekeeping gene β-actin) of EPCs after 24, 72, and 168 h on five surfaces (SLA, modSLA, A, modA, and fibronectin-coated plastic) measured by RT-PCR. $n=3$. *Error bars* show standard error of the mean

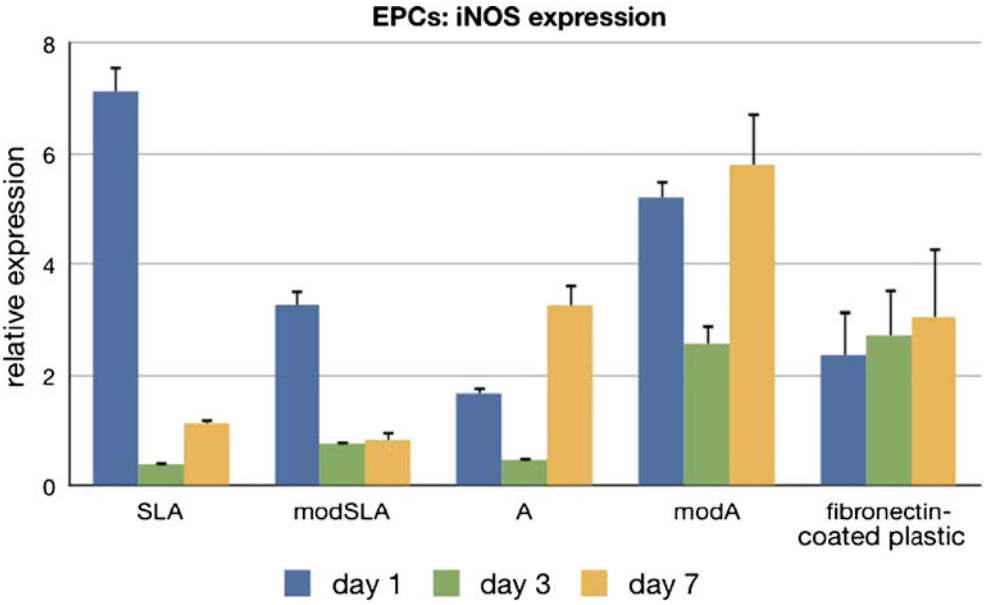

Fig. 5 Mean relative iNOS expression (normalized to the housekeeping gene β-actin) of EPCs after 24, 72, and 168 h on five surfaces (SLA, modSLA, A, modA, and fibronectin-coated plastic) measured by RT-PCR. *n*=3. *Error bars* show standard error of the mean

observed and cell soma seemed more nodular [36]. These findings coincide with our results: Rough titanium surfaces (SLA/modSLA) promoted nodular cell soma with only wispy pseudopodia, smoother surfaces promoted planar cell soma with lots of pseudopodia. Cytokine release plays an important role in implant osseointegration. Researchers found highest secretion of osteoblast-specific factors (e.g., TGF-β1 and PGE2) on modSLA [5, 6]; HUVECs showed highest expressions of angiogenic factors (e.g., von Willebrand factor) on modSLA [36]. Our experiments also indicate a trend that EPCs secrete the most amount of VEGF on modSLA. VEGF plays a central role in neovascularization and bone healing: VEGF stimulates mature endothelial cells for migration and differentiation [44]. Furthermore, it promotes mobilization, recruitment, extravasation, and differentiation of EPCs [45–47]. In addition to that, it promotes recruitment, activation, and differentiation of osteoblast cells [48]. Compared to fibronectin-coated plastic surface, which is usually used as culture surface for EPCs, we found significant higher VEGF values on titanium surfaces. Researchers look for techniques to multiply secretion of paracrine factors by EPCs for cytokine therapies [25]. Our study could be a stimulus to investigate different culture surfaces to find out how to increase secretion of paracrine factors by EPCs. The majority of research groups determined that the differentiation grade of osteoblasts on different titanium surfaces is in inverse proportion to proliferation grade [4–7, 49], i.e., that rough and hydrophilic modSLA showed highest differentiation grade. This osteoblast type is often called "post-mitotic mature osteoblast" and is attributed to a high potential to build new bone [4, 50]. In our study, we investigated eNOS and iNOS expressions of EPCs as differentiation markers. eNOS is a commonly used marker of differentiation but correlation of iNOS and the grade of differentiation is still unclear [51–55]. We found that iNOS and eNOS behaved in a very similar way on different surfaces. This supports the hypothesis that iNOS is also a marker for advanced differentiation. eNOS as well as iNOS expressions were highest on smooth hydrophilic surfaces.

In summary, the results of this study show that different surfaces influenced interactions with EPCs significantly. The grade of roughness seems to be more important than grade of wettability. Smoother surfaces promoted a more differentiated polymorphic phenotype with high differentiation grade and permanent stable proliferation rate. Rougher titanium surfaces promoted an undifferentiated plump phenotype with low proliferation rates and eNOS/iNOS rates. They tended to show higher VEGF-A secretion rates. Taken together with clinical trials, which demonstrate modSLA very good implant stability and shortened healing period [1–3, 42, 43], our study supports the hypothesis that cytokine secretion by EPCs is more important than their direct vascular incorporation [20, 56]. EPCs could be an interesting key in implantology in the future. Novel implant surfaces could stimulate EPCs cytokine secretion. Osseointegration of implants could be increased in patients with reduced neovascularization capacity, e.g., after radiation therapy or bisphosphonate treatment. Local stem cell therapy with EPCs during implantation could be helpful in this condition, too. EPCs or their ex vivo extracted cytokines as cell-free alternative could be added to the local bone wound together with implant. This could result in a quicker and more effective

neovascularization with higher success implantation rates even in disadvantageous bone situations. Before the use of EPCs in dental medicine can take place, a lot of fundamental questions must be clarified first. A risk–benefit analysis should be done before stem cell takes place in dental implantology. As a next step, the results of this study should be transfer to an in vivo model to underline the findings and to exclude negative effects of stem cell therapy [57].

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