ANGIOGENIC FUNCTIONALISATION OF TITANIUM SURFACES USING NANO-ANCHORED VEGF – AN *IN VITRO* STUDY

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Abstract

The aim of the present study was to test the hypothesis that sandblasted and acid etched titanium surfaces can be functionalised with vascular endothelial growth factor (VEGF) using oligonucleotides for anchorage and slow release. rhVEGF165 molecules were conjugated to strands of 30-mer non-coding DNA oligonucleotides (ODN) and hybridised to complementary ODN anchor strands which had been immobilised to the surface of sandblasted/acid etched (SAE) Ti specimens. Specimens with non-conjugated VEGF adsorbed to ODN anchor strands and to blank SAE surfaces served as controls. Specific binding of conjugated VEGF exhibited the highest percentage of immobilised VEGF (71.0 %), whereas non-conjugated VEGF only achieved 53.2 and 30.7 %, respectively. Cumulative release reached 54.0 % of the immobilised growth factor in the group of specifically bound VEGF after 4 weeks, whereas non-conjugated VEGF adsorbed to ODN strands released 78.9 % and VEGF adsorbed to SAE Ti surfaces released 97.4 %. Proliferation of human umbilical vein endothelial cells (HUVECs) was significantly increased on the surfaces with specifically bound VEGF compared to the control surfaces and SAE Ti surfaces without VEGF. Moreover, the released conjugated VEGF exhibited biological activity by induction of von Willebrand Factor (vWF) in mesenchymal stem cells. It is concluded that the angiogenic functionalisation of SAE titanium surfaces can be achieved by conjugation of VEGF to ODN strands and hybridisation to complementary ODN strands that are anchored to the titanium surface. The angiogenic effect is exerted both through the immobilised and the released portion of the growth factor.

Keywords: Vascular endothelial growth factor, nucleotides, titanium, controlled release, recombinant proteins, angiogenesis.

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Introduction

Periimplant bone formation constitutes the basis for the successful integration of endosseous titanium implants by establishing an intimate contact between bone tissue and the implant surface. This process is osteoconductive in nature and requires angiogenesis not only to provide blood supply for tissue formation but also to bring perivascular osteoprogenitor cells to the site of osteogenesis (Park and Davies, 2000). Excessive trauma, radiation therapy or systemic diseases can reduce the angiogenic capacity of periimplant bone tissue and thereby compromise the process of osseointegration (Nociti *et al*. 2002; Keller *et al*. 2004; Javed and Romanos, 2009; Javed *et al*., 2010). In these cases, a bioactive implant surface that improves the process of bone formation through enhancement of angiogenesis would be of considerable clinical benefit.

The biological performance of implant surfaces has been improved through increased surface roughness, which facilitates the adsorption of serum proteins and other biomolecules as well as the attachment of platelets (Park and Davies, 2000; Boukari *et al*., 2006). The increased release of growth factors due to surface microstructure and energy from cells growing on the surface (Raines *et al*., 2010) as well as subsequently enhanced platelet attachment to the implant surface has shown to enhance the process of tissue formation and of angiogenesis (Davies *et al*., 2003). A more direct approach to the improvement of bone formation through the enhancement of angiogenesis is the use of angiogenic growth factors (Kleinheinz *et al*., 2005; Kaigler *et al*., 2006; Poh *et al*., 2010; Wernike *et al*., 2010). Some authors have explored the potential of collagen coatings with and without heparin on titanium surfaces in order to bind and release vascular endothelial growth factor (VEGF) (Wolf-Brandstetter *et al*., 2006; Mueller *et al*. 2011). Others have used polysaccharides, polydopamine films or copolymeric hydrogel coating in conjunction with VEGF to enhance the biologic quality of titanium implant surfaces (De Giglio *et al*., 2010; Hu *et al*., 2010; Poh *et al*., 2010).

A critical aspect in bio-activation of implant surfaces is the mode of binding and release of angiogenic growth factors from the implant surface. Recent experimental results reporting on the use of VEGF to enhance angiogenesis suggest that a short-term release of higher amounts of VEGF after simple adsorption can impede formation of new vessels whereas a slow sustained release has been able to promote vascularisation (Wernike *et al*., 2010). Other reports describe enhanced vessel formation with VEGF release at early periods (Leach *et al*., 2006). However, a problem with growth factors integrated into

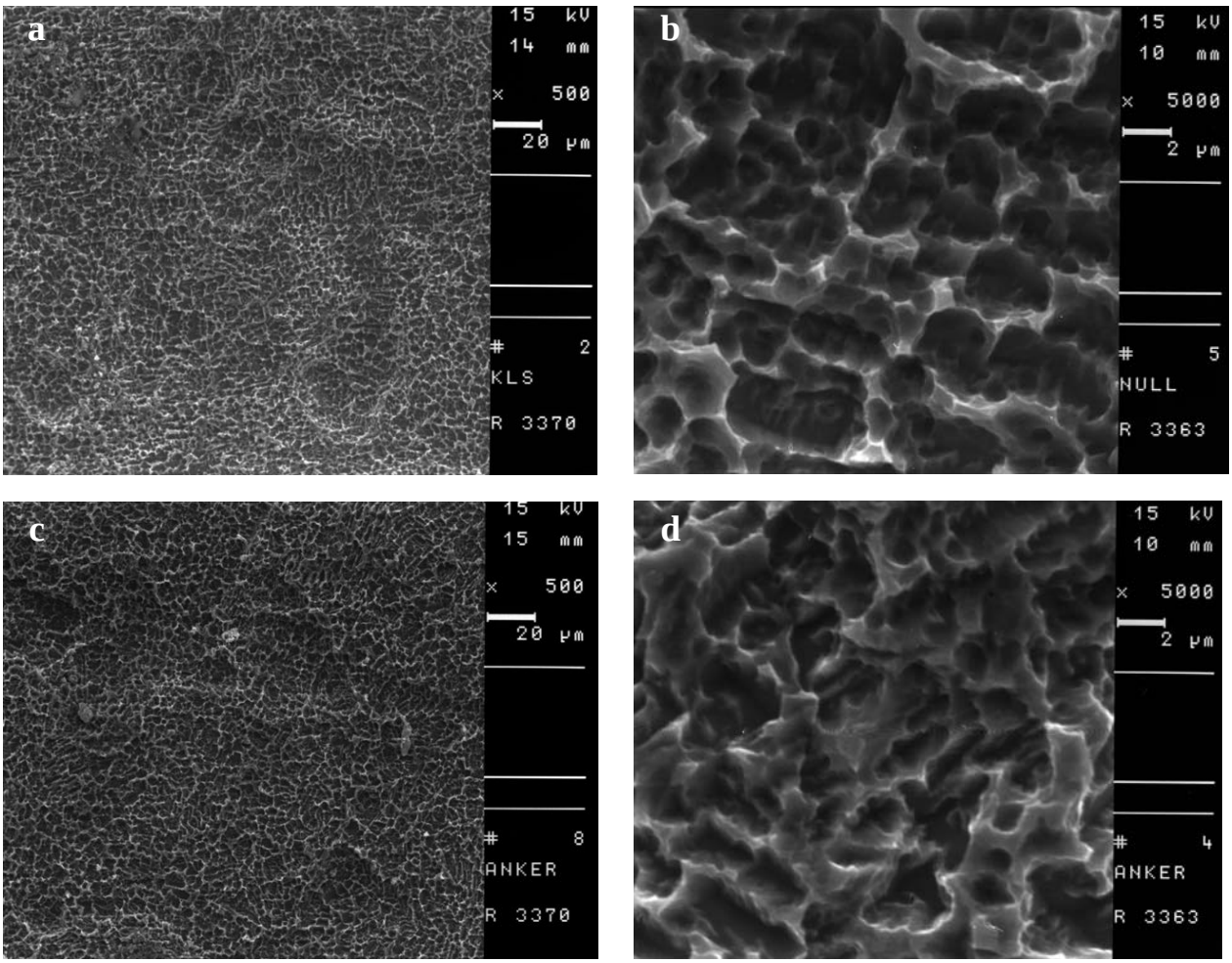

Fig. 1. SEM micrograph of **(a)** the sandblasted acid etched (SAE) surface, **(b)** the SAE surface with nano-anchored ODN strands. No change in surface morphology is visible.

a bioactive implant coating commonly arises when these implants undergo sterilisation in a commercial production process, because both gamma radiation and autoclaving can destroy the biological activity of the incorporated growth factors. Thus, a release system that allows for controlled binding and release of growth factors after sterilisation would be desirable to overcome this problem. Recently, a system of oligonucleotides nano-anchored to titanium surfaces through anodic polarisation has been developed which allows for specific binding of complementary oligonucleotides conjugated to bioactive molecules (Michael *et al*., 2007). Moreover, it has been previously shown by our group that bone morphogenic proteins (BMP2) can be immobilised on the surface of sandblasted acid etched titanium with subsequent release as active molecule (Schliephake *et al*., 2012). As the structure of oligonucleotides is likely to remain unaltered under certain conditions of γ-radiation (Reichert *et al*., 2011) they could be used as a system for release of peptide growth factors that are specifically bound to the oligonucleotides subsequent to gamma ray sterilisation immediately prior to insertion in the operation room.

The aim of the present *in vitro* study was, thus, to test the hypothesis that oligonucleotides can be used for anchorage and slow release of angiogenic growth factors such as VEGF from a titanium surface to enhance its angiogenic activity.

Materials and Methods

Specimen fabrication

Commercially pure titanium discs of 14.7 mm diameter were prepared by sandblasting and subsequent acid-etching (KLS Martin, Tuttlingen, Germany) (Fig. 1a,b). The samples were etched in 5.1 M hydrochloric acid and 4.6 M sulphuric acid solution for 300 s by 108 °C (Scharnweber *et al*., 2010). The 60-mer anchor strands were synthesised by Thermo Fisher Scientific (Ulm, Germany). The 31-mer Oligonucleotides (GS-ODN) to anchor strands were synthesised by Biomers (Ulm, Germany). For complementary immobilisation of anchor strands (AS) an electrochemical setup was used, which has already been described by Beutner *et al*. (2009). Briefly, the samples were mounted to the bottom of a conical cell made from polyetherimide. A conventional three-electrode setup was used with a spiral gold wire acting as auxiliary electrode and a saturated Ag/AgCl reference electrode connected to the cell via a salt bridge made from a tube filled with agarose gel (2 % w/v in 2 mol/L acetate buffer, pH 4.0). Initial adsorption inside the electrochemical cell was carried out in 300 µL of 0.5 mol/L acetate buffer at pH 4.0 with additional 5 mol/L ethanol for 15 min. The electrolyte contained 800 nmol/L AS. After adsorption time 6 mL buffer (with ethanol analogous to the adsorption electrolyte) were added to the cell and the anodic

polarisation was started within 20 s. The potentiostat/galvanostat (Voltalab 4.0, Radiometer Analytical, Lyon, France) was operated in galvanostatic mode with 11 mA/cm^2 to ensure a polarisation time of approx. 3 s to reach a potential of 7.5 $V_{Ag/AgCl}$. Subsequently the cell was rinsed two times with 9 mL buffer, followed by dipping the samples in sterile deionised (SDI) H_2O (0.055 mS/cm) to remove the buffer. After that treatment the samples were dried for 2 h at room temperature in the dark and finally packed in aluminium peel bags (Früh Verpackungstechnik, Fehraltorf, Switzerland). The surface structure of the sandblasted acid etched (SAE) titanium surface with ODN strands is shown in Figs. 1c,d.

Conjugation of growth factors with complementary oligonucleotides (GS) was done by using disuccinimidyl suberate (DSS) as linker molecule. In a first step the aminomodified GS-ODN was activated with DSS in 1 mM acetate buffer pH 4.0. The excess DSS was separated using an Illustra NAP-10 column (GE Healthcare, Freiburg, Germany). The DSS-activated GS-ODN was mixed with rhVEGF165 (Reliatech, Braunschweig, Germany) in phosphate buffered saline (PBS) containing 2 M sodium chloride pH 7.4 and incubated at room temperature for at least 1 h.

Hybridisation of anchored oligonucleotides with growth factor conjugated with complementary oligonucleotides (VEGF-ODN)

For each disc, 100 µL of VEGF-ODN solutions were prepared as follows: 10 µL PBS, 80 µL pure water and 10 µL conjugated growth factor solution containing 1,800 ng of rhVEGF165 (Reliatech. Wolfenbüttel, Germany).

The volumes of 100 µL were pipetted onto the discs and left for hybridisation in a dark chamber at room temperature for 1 h. Care was taken to accommodate the complete volume on the disc surface. Subsequently, the specimens were washed four times in PBS. The amount of VEGF that was washed away was measured using a custom made commercially produced sandwich ELISA (Reliatech) according to the recommendation of the manufacturer.

Experimental design

The experiments comprised three series:

1. Evaluation of release of rhVEGF165 from the loaded titanium surfaces,
2. Specific activity related to growth factor loading and release was assessed using human umbilical vein endothelial cells (HUVECs),
3. Angiogenic activity of the released growth factor was measured through the induction of endothelial phenotype in human mesenchymal stem cells.

Release of growth factor rhVEGF165

For release experiments, Titanium discs with three different conditions were analysed in order to compare unspecific binding by adsorptive coating with specific binding through oligonucleotides:

- **Group R1**: Sandblasted acid-etched (SAE) (Fig. 1c,d) surface with nano-anchored oligonucleotides (ODN) that were hybridised with complementary oligonucleotides conjugated to rhVEGF165 as described above. Assessment of washed-out VEGF indicated that 1,277.6 ng (71.0 %) of VEGF remained attached to the disc surface.
- **Group R2**: SAE surface with nano-anchored oligonucleotides (ODN) and adsorbed non-conjugated rhVEGF165 to test for the effect of non-specific binding of VEGF to the ODN strands. Discs were loaded in an identical manner as described for Group R1. This yielded an adsorbed amount of 957 ng (53.2 %) of VEGF on the disc surface.
- **Group R3**: Blank SAE (Fig. 1a,b) surface without nano-anchored ODN and adsorbed non-conjugated rhVEGF165. Discs were loaded with VEGF by dripping 1,800 ng of VEGF in 100 µL onto the disc surface. The growth factors were allowed to adsorb for 1 h in a dark chamber at room temperature after which the discs were rinsed four times in PBS, leaving 553.3 ng (30.7 %) of VEGF attached to the disc surface.

The discs scheduled for the release experiments were placed in cell culture medium (DMEM, 1 g Glucose; Biochrom, Berlin, Germany) in bovine serum albumin (BSA) (Carl Roth, Karlsruhe, Germany) coated 24 well plates. The medium was collected and replaced after 24 h, 3 days and every 3-4 days thereafter until day 28. The amount of VEGF was assessed using the custom made commercially produced sandwich ELISA mentioned above. The minimum detectable amount was 100 pg/mL rhVEGF165. Three discs of each condition were evaluated and all measurements were performed in triplicate. The residual volume of the eluates was used for the induction experiments in mesenchymal stem cells.

Specific activity of the loaded Titanium surface

For the evaluation of the angiogenic activity of the loaded surface by assessment of HUVEC proliferation, the following groups were used:

- **Group C1**: Blank SAE surface.
- **Group C2**: SAE surface with adsorbed non-conjugated rhVEGF165.
- **Group C3**: SAE surface with nano-anchored oligonucleotides (ON) and loaded with non-conjugated rhVEGF165.
- **Group C4**: SAE surface with nano-anchored oligonucleotides (ON) and loaded with rhVEGF165 conjugated to complementary oliogonucleotides.

Human umbilical vein endothelial cells (HUVECs, www.lonza.com; Lonza, Verviers, Belgium) were obtained and processed according to the instructions of the provider. Cells were cultured and expanded using the EGM-2-Bullet-Kit provided by Lonza (CC-3162 EGM-Bulletkit (CC3156 & CC-4176), containing medium, supplements and 2 % FCS). 2 ng/mL VEGF and gentamycin 2 % (PAN Biotech, Aidenbach, Germany) were added during expansion of HUVECs until the cells were harvested and seeded onto the titanium surfaces. Passaging was done using the ReagentPack Subculture provided by Lonza (CC-5034 ReagentPack Subculture containing HBBS buffer, trypsin and trypsin neutralising agent) when the cells reached 80 % confluence. After harvesting, 30,000 HUVECs were

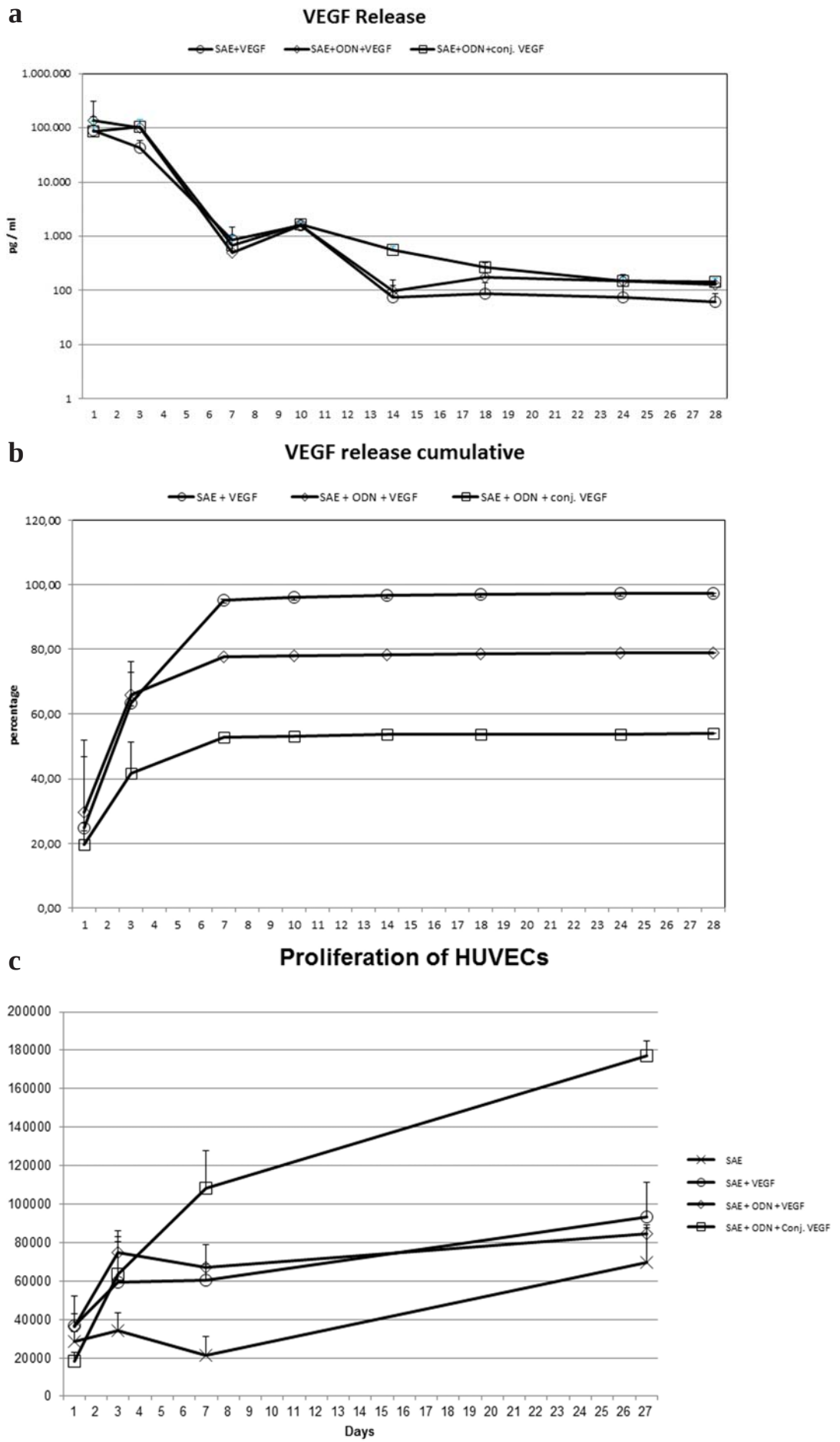

Fig. 2. **(a)** Release of VEGF (absolute amounts, y-axis logarithmic scale), **(b)** Cumulative release of VEGF (percentage of immobilised VEGF), **(c)** Proliferation of human umbilical venous endothelial cells (HUVECs).

seeded onto each titanium disc and fresh cell culture medium without VEGF was added. The ability to induce HUVEC cell proliferation was expressed as an increase in cell numbers compared to the blank controls. Cell numbers were assessed using automated cell counting (CASY®, Model TT; Roche Diagnostics, Mannheim, Germany) after 1, 3, 7 days and 4 weeks (Tillner *et al*., 1994). Three discs of each condition were evaluated at each interval and all measurements were performed in triplicate.

Activity of the released rhVEGF165

Human mesenchymal stem cells (hMSCs, http://www.lonza.com) were expanded according to the instructions of the provider in mesenchymal stem cell growth medium provided by the manufacturer in order to assess the angiogenic activity ability of the released VEGF by induction of von Willebrand factor (vWF) as an indication for induction of an endothelial phenotype in hMSCs (Poh *et al*., 2010). Cells from passage 2 of the expanded hMSCs were used for the analysis. The cells were exposed to the media containing VEGF released from the discs on days 1, 3, 7 and 28. After 6 days the expression of vWF was assessed using immunocytochemistry. The cells were fixed with 4 % paraformaldehyde (PFA, Carl Roth, for 7 min and permeabilised for 10 min. using Triton X-100 (0.1 %) (Sigma-Aldrich Chemie, Taufkirchen, Germany). For staining of the vWF, the primary antibody (ab6994, Abcam, www.abcam.com) (1:100) was added for 24 h with the secondary antibody Alexa Fluor (Invitrogen; www.invitrogen.com) (1:100) for 1 h. Phalloidin (1:100; 1 h) (Millipore, Merck Bioscience, Schwalbach, Germany) was employed to stain actin filaments and nuclei were stained with DAPI (1:1000; 20 min) (Millipore). Inductions were performed in duplicate.

Statistics

Repeated measures ANOVA (PASW Statistics 18.0, http://support.spss.com) were used to compare the release kinetics between the three different surfaces. Univariate ANOVA with Bonferroni correction was employed to compare proliferation of HUVECs. All tests were performed at a significance level of $p < 0.05$.

Results

Release of rhVEGF165

SAE surfaces that had been loaded with non-conjugated VEGF showed a burst release with a range of 90 ng/mL to 41 ng/mL within the first three days with a rapid decline in release until day 7. The release of non-conjugated VEGF from the surfaces equipped with ODN strands was even higher with 138.9 ng/mL and 101.8 ng/mL during the first two intervals. The release from surfaces loaded with conjugated VEGF hybridised to nano-anchored oligonucleotides exhibited a burst release kinetic, too with a high release during the first 5 days between 87.1 ng/mL and 104.9 ng/mL. The release after the first week showed that the surface loaded with conjugated VEGF hybridised to nano-anchored oligonucleotides had a significantly higher release from day 10 to the end of the observation period than the surface loaded with non-conjugated VEGF (Fig. 2a). Differences in release between the three groups were significant only at day 14 and at day 28 ($p < 0.001$ and $p = 0.008$, respectively).

Calculation of the cumulative release showed that the rapid desorption of non-conjugated rhVEGF165 from the SAE surfaces has led to an almost complete release after one week (95.3 %, SD: 0.2) which did hardly change until the end of the observation period (97.4 %, SD: 0.01) The release of non-conjugated rhVEGF165 from ODN equipped SAE surfaces was identical in the first three days where after it slowed down reaching a plateau at 77.6 % (SD: 0.07) after one week, too, with almost no change until day 28 (78.9 %, SD: 0.01). The SAE surface with ODN strands and conjugated rhVEGF165 exhibited the slowest increase with a plateau also after one week, however only at 52.8 % (SD: 0.03). After that no changes in percentage of release occurred over the following three weeks (54.0 %, SD 0.01) (Fig. 2b).

Proliferation of HUVECs

During the 4 weeks of culture HUVECs proliferated from 30,000 cells to 69,895 and 93,432 cells on average, respectively, both on the uncoated SAE surface as well as on the surface loaded with non-conjugated rhVEGF165. Surfaces with nano-anchored ODN strands and non-conjugated VEGF have shown increased proliferation to 74,073 cells on average during the first three days after which the number of cells decreased to a mean of 63,570 cells and slowly increased towards the end of the observation period to an average number of 83,353 cells. Only on the surface loaded with conjugated rhVEGF165 hybridised to complementary ODN strands HUVECS continued to proliferate to a number of 177,192 over the period of four weeks. Differences in cell numbers between this surface and the two latter ones were significant at day 7 and day 28, respectively ($p < 0.001$, $p < 0.001$) (Fig. 2c).

Induction of vWF in mesenchymal stem cells

Supernatants from discs with blank SAE surfaces had not shown any induction of vWF in the immunofluorescent images at any interval (Fig. 3a-c). Non-conjugated rhVEGF165 adsorbed to SAE surfaces without (Figs. 3d,e) and with nano-anchored ODN (Figs. 4a,b) and released into the supernatant clearly has shown to induce expression of vWF. The vWF was located perinuclear in the cytoplasm of the hMSCs. The same held true for the conjugated rhVEGF165 released from the discs with nano-anchored ODN. The vWF was clearly visible after exposure to supernatants also from the late intervals (Figs 4c,d).

Discussion

The present study has shown that rhVEGF165 can be effectively immobilised on titanium surfaces through conjugation to oligonucleotides and hybridisation to complementary nucleotide strands anchored to the surface by anodic polarisation induced oxide growth. The amount of VEGF that was washed away from the surface after loading was the smallest in the group of SAE surfaces with

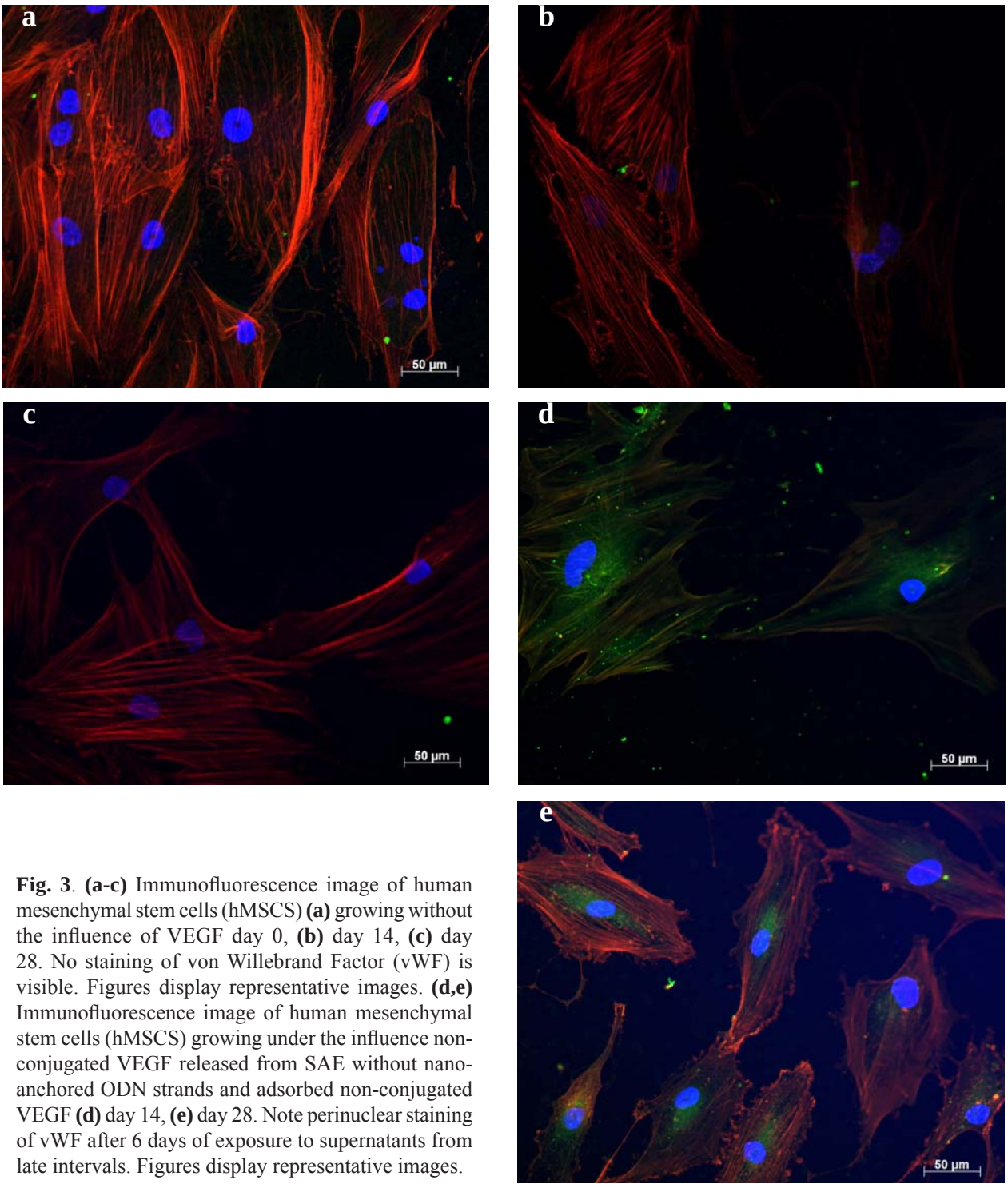

Fig. 3. **(a-c)** Immunofluorescence image of human mesenchymal stem cells (hMSCS) **(a)** growing without the influence of VEGF day 0, **(b)** day 14, **(c)** day 28. No staining of von Willebrand Factor (vWF) is visible. Figures display representative images. **(d,e)** Immunofluorescence image of human mesenchymal stem cells (hMSCS) growing under the influence non-conjugated VEGF released from SAE without nano-anchored ODN strands and adsorbed non-conjugated VEGF **(d)** day 14, **(e)** day 28. Note perinuclear staining of vWF after 6 days of exposure to supernatants from late intervals. Figures display representative images.

ODN strands combined with conjugated VEGF indicating that binding of VEGF to the titanium surface through this modular system is the most effective. This supports previous reports showing that binding of organic molecules to titanium surfaces through hybridisation of ODN strands is specific (Michael *et al*., 2009) and that other growth factors like rhBMP2 can be effectively immobilised on the surface using this system (Schliephake *et al*., 2012). The portion of growth factor that has been retained is lower than what has been reported for carboxymethylchitosan (94 %) and hydroxylapatite coatings (85 %) (Hu *et al*., 2010), but it was considerably higher than that reported for polydopamine grafting (52 %, Poh *et al*., 2010) or coatings with collagen and heparin (38-42 % (Wulf-Brandstetter *et al*., 2006). The second best group in terms of growth factor retention have been the SAE surfaces with ODN strands combined with non-conjugated VEGF whereas the non-conjugated VEGF adsorbed to the blank SAE titanium surface had shown the lowest degree of growth factor retention on the surface. This has been different for rhBMP2, where titanium surfaces with ODN strands had bound the non-conjugated growth factor to an even smaller extend than blank SAE titanium surfaces (Schliephake *et al*., 2012). The different behaviour of the two non-conjugated growth factors under these conditions may be explained by differences in both structure and size of

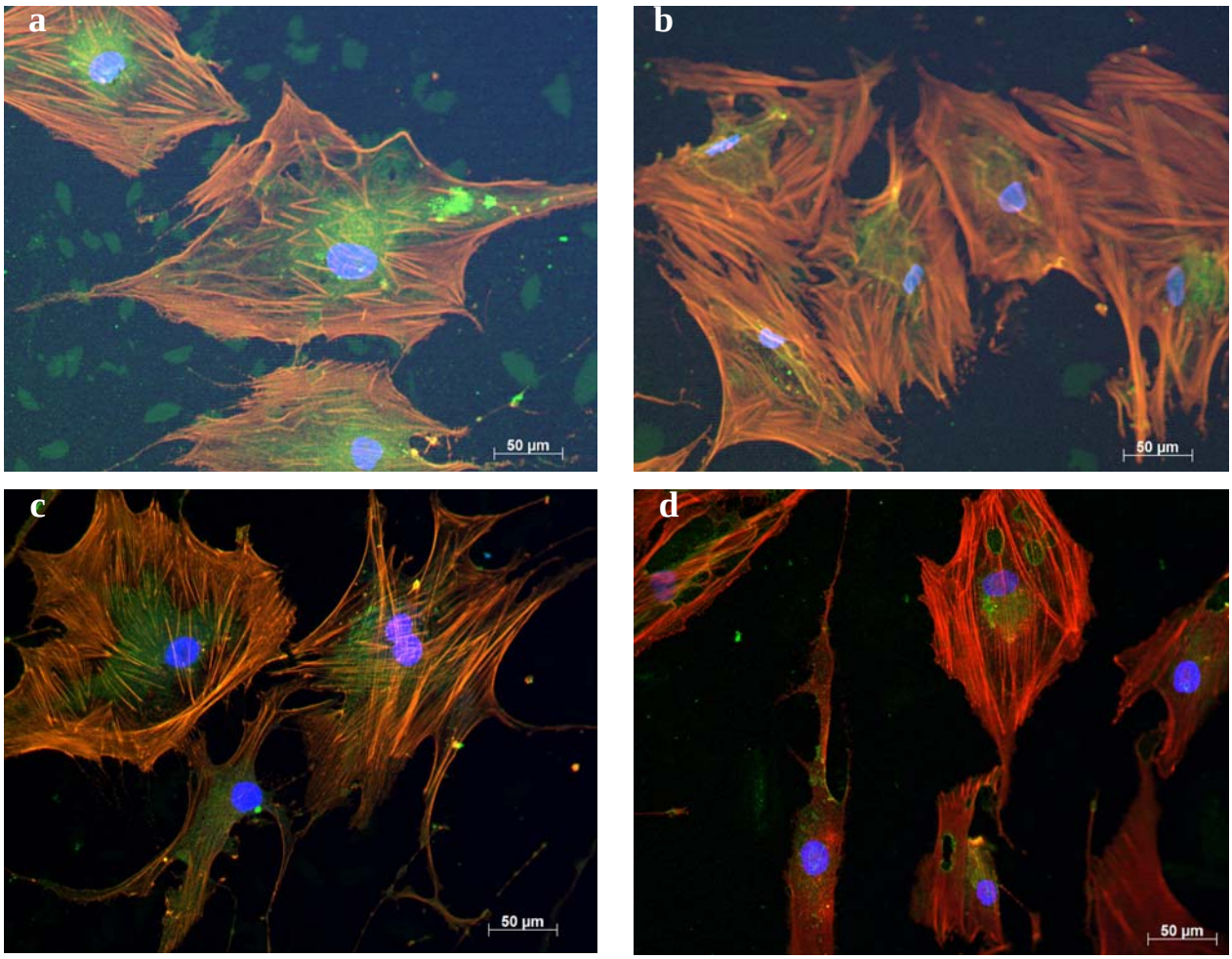

Fig. 4. **(a,b)** Immunofluorescence image of human mesenchymal stem cells (hMSCS) growing under the influence of non-conjugated VEGF released from SAE surfaces with nano-anchored ODN strands and adsorbed non-conjugated VEGF **(a)** day 14, **(b)** day 28. **(c,d)** Immunofluorescence image of human mesenchymal stem cells (hMSCS) growing on SAE surfaces under the influence of conjugated VEGF released from SAE surface with nano-anchored ODN strands and hybridised conjugated VEGF: **(c)** day 14; **(d)** day 28. Note perinuclear staining of vWF after exposure to supernatants from late intervals.

the two different growth factors, which may account for non-specific interaction between the ODN strands and the growth factor molecules.

However, despite the higher potential of immobilisation for conjugated rhVEGF165 bound to ODN strands, the release characteristic for this specifically bound VEGF has been very similar to non-conjugated VEGF particularly during early periods of release and is in contrast to the previously reported results for BMP2 (Schliephake *et al*., 2012). The burst release of much higher amounts of non-conjugated growth factor in the present study compared to BMP may be explained by the increased amount of conjugated VEGF, which has been used for immobilisation on the titanium surface, indicating that the amount of growth factor that can be immobilised on the titanium surface though hybridisation on ODN strands is limited and excess growth factor such as higher amounts of VEGF in the present study will be rapidly released. The reason for this limitation may be that the process of hybridisation between the ODN strands immobilised on the titanium surface and those conjugated to the growth factor is impeded by the size of the growth factor molecule. The portion of conjugated growth factor that would be left on the surface without specific binding would then be released in the burst like fashion that has been observed in the present study. A possible solution for this problem could be the use of smaller peptide fragments that carry the receptor binding domain.

In contrast to a similar release of absolute amounts of growth factor during the first week, calculations of the cumulative figures have shown that the percentage of growth factor released during this period is only 54.0 % in the group of specifically bound VEGF, whereas significantly greater portions between 78.9 and 97.4 % of the non-conjugated VEGF have been released from the titanium surfaces with and without ODN strands. Because the absolute amounts of VEGF released in the following 3 weeks is very similar in all three groups but almost 50 % of the conjugated rhVEGF165 have remained immobilised on the surfaces in the group with specifically bound growth factor this may indicate that the large portion of immobilised growth factor in this group has accounted

for the significant increase in proliferation of HUVECs on these surfaces compared to those with adsorbed non-conjugated growth factor.

The question may arise, whether surface topography would have an effect on the results of HUVEC proliferation. A comparison with smooth machined or polished titanium surfaces would have quite likely resulted in a difference in terms of attachment and proliferation of HUVECs. However, the current standard of surface characteristics of oral implants are microrough sandblasted and/or acid etched surfaces. Moreover, previous studies have shown that the use of biological signals is more challenging in microrough surfaces than on smooth Ti surfaces with respect to *in vivo* effects (Schliephake *et al*., 2005; Schliephake *et al*., 2009). Thus, developments aiming for clinical applications would preferably focus on this type of surfaces.

The released amounts of VEGF itself have shown to be angiogenic also for the conjugated VEGF as could be seen from the induction of vWF in mesenchymal stem cells, where no difference has been found between the conjugated and the non-conjugated VEGF with respect to staining intensity. This indicates that the ODN complementary strand that had been conjugated to the VEGF molecule has obviously not reduced the ability of the growth factor to bind to its receptor and activate the expression of vWF. The induction of vWF in mesenchymal stem cells during differentiation into endothelial phenotype has been shown previously by Oswald *et al*. (2004).

The percentage of release of conjugated VEGF from the different surfaces has shown an interesting similarity of the release curves of VEGF from calcium phosphate ceramics with a high release kinetic for adsorbed VEGF with release of approx. 85 % and a low release of co-precipitated VEGF of approx. 45 % within the first 7 to 10 days of release (Wernike *et al*., 2010). *In vivo* the low release characteristic has shown increased vessel formation and biomaterial incorporation after implantation into cranial bone. Although the *in vivo* behaviour of the functionalised titanium surfaces in the present *in vitro* study is difficult to predict, the release characteristic provided by the ODN mediated immobilisation appears to be favourable with respect to bone tissue response.

Conclusions

The present study has shown that VEGF molecules can be effectively immobilised by conjugation to ODN strands and hybridisation to complementary ODN strands that have been anchored to titanium surfaces. The released VEGF molecules have exhibited angiogenic activity by induction of VWF and in conjunction with the molecules immobilised to the surface have significantly enhanced proliferation of human umbilical venous endothelial cells.

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Discussion with Reviewers

Reviewer I: Do you think that the present technique to bind VEGF to titanium could be applied to other artificial surfaces ?
Authors: The presented technique relies on the nano-anchorage of oligonucleotide strands by oxide growth of titanium. Thus, in the present form it will be limited to the application to titanium surfaces. However, other means of controlled binding of oligonucleotide strands to biomaterial surfaces can make this technique more universally applicable.

Reviewer I: Why does gamma-ray sterilisation not affect VEGF conjugated to ODN ?
Authors: VEGF will be added after sterilisation of the ODN loaded surface. This would allow specific loading and release from the sterilised surface.

Reviewer I: If VEGF release induced an endothelial phenotype of MSC, I wonder how stable is the osteogenic differentiation of MSC for bone formation? Are these conditions useful for MSC (osteogenic differentiation)? Are any data on *in vivo* experiments available?
Authors: The induction of an endothelial phenotype in MSCs has only been used as an *in vitro* essay. *In vivo*, MSCs are not the primary target for VEGF. Here, VEGF is only one factor among the large number of signals and growth factors that are involved in the differentiation of MSCs and its primary function will be to enhance angiogenesis. *In vivo* experiments are underway.

Reviewer II: The proliferation study was based on cells detachment and subsequent counting in suspension using CASY method. I was wondering if the SAE Ti surface coated with ODN, and conjugated or non-conjugated VEGF potentially affected cell adhesion differently. In such situations, HUVEC may separate easier from affected surfaces. This therefore, may erroneously results in a higher cell count in suspension.
Authors: The surfaces were checked after trypsinisation to make sure that no relevant or even varying numbers of cells were left on the surface.

Reviewer II: The main aim of this study was to test if the ODN coating of the Ti-surface can be used as an anchorage for the slow release of conjugated or non-conjugated VEGF. I was wondering why authors complicated the experiment by using a convoluted Ti surface topography such as SAE. I believe a smooth polished Ti surface should have been used instead to test the hypothesis. This way the potential additional effects of a complex Ti surface topography could be eliminated.
Authors: SAE surfaces have been chosen for two reasons: (i) Today, microstructured surfaces are standard in clinical oral implantology. A possible clinical application would therefore use this type of surfaces; (ii) Previous studies have shown that the use of biological signals is more challenging in microrough surfaces than on smooth Ti surfaces with respect to periimplant bone formation (Schliephake *et al*., 2005; Schliephake *et al*., 2009, text references). Thus, it has been concluded that the more appropriate test surface for a clinically useful development would be an SAE surface.