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ORIGINAL ARTICLE

Histological and immunohistochemical analysis of initial and early subepithelial connective tissue attachment at chemically modified and conventional SLA® titanium implants. A pilot study in dogs

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Abstract The aim of the present pilot study was to histologically/immunohistochemically investigate initial and early subepithelial connective tissue attachment at transmucosal parts of modified (mod) and conventional sandblasted, large grit and acid-etched (SLA) titanium implants. Implantation of modSLA and SLA implants was performed bilaterally in both the mandible and maxilla of four beagle dogs. The implants were submerged to prevent bacterial contamination. The animals were killed after 1, 4, 7 and 14 days. Peri-implant tissue reactions were assessed histologically (Masson Goldner Trichrome stain-MG) and immunohistochemically (IH) using monoclonal antibodies to fibronectin (FN) and proliferating cell nuclear antigen (PCNA). The surgical procedure of implant submerging resulted in the formation of an artificial gap in the transmucosal area of both types of implants. After 14 days of healing, MG stain revealed the formation of well-organized collagen fibres and numerous blood vessels in a newly formed loose connective tissue zone adjacent to modSLA. While some fibres were oriented in a parallel direction, others have started to extend and attach partially perpendicular to the implant surface. In contrast, SLA implants appeared to be clearly separated by a dense connective tissue zone with parallel-running collagen fibres and rare blood vessel formation. First signs of a positive FN and PCNA staining adjacent to both implant surfaces were observed at day 4. Within the limits of a pilot study, it might be concluded that modSLA titanium surfaces might possess the potential to promote subepithelial connective tissue attachment at the transmucosal part of the implant.

Keywords Titanium surface · Sandblasted and acid-etched surface · Animal study · Soft tissue integration · Histology · Immunohistochemistry

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Introduction

Today, there is considerable evidence supporting the view that marginal soft tissue integration plays a fundamental role in establishing an effective seal between the oral environment and the endosseous part of a titanium implant [11, 23, 35]. Indeed, the presence of bacteria on implant surfaces may lead to an inflammation of the peri-implant mucosa, and if left untreated, the inflammation spreads apically and results in bone resorption, which has been named peri-implantitis [5, 36]. As a consequence of the fact that rough surfaces accumulate and retain more plaque than smooth surfaces, nowadays, most implant systems use a highly polished titanium in the transmucosal part [6, 10, 32, 39, 40, 42]. However, as previous studies have also demonstrated that the surface texture significantly influences fibroblast and epithelial cell attachment, it was suggested that a certain surface roughness is needed for an optimal soft

tissue sealing [21, 22, 27]. Accordingly, in recent years, extensive research has been performed to investigate the biological soft tissue seal at different types, materials and roughness of dental implants [1, 3, 7, 8]. In general, the transmucosal attachment is comprised of a barrier epithelium and a zone of connective tissue attachment [6, 8]. Close attention has been paid to the implant/subepithelial connective tissue interface, as this zone of interaction is apparently not recognized as a wound and therefore does not call for an epithelial lining [7, 8, 18]. At both machined and rough surfaces, the subepithelial connective tissue is located between the apical part of the barrier epithelium and the implant supporting alveolar bone and can be divided into two different zones. In particular, the inner zone has been described to be poorly vascularised, consisting of numerous dense collagen fibres running close to the implant surface predominantly in a parallel direction [3, 9, 13, 15, 20]. The outer zone, however, appeared to be formed of fibres running in different directions, richer in cells and blood vessels [13]. In the past years, several modifications of specific surface properties such as topography, structure, chemistry, surface charge and wettability have been investigated to improve marginal soft tissue integration at different implants [4]. Although a direct connective tissue contact has been observed for smoothly polished, roughly sandblasted and plasma-sprayed implant surfaces, the collagen fibres were oriented parallel without showing any signs of perpendicular insertion to the respective surfaces [13, 24]. However, the lack of perpendicular insertion of collagen fibres at non-submerged or two-stage implants might also be influenced by uncontrollable plaque accumulation and, subsequently, bacterial contamination of the internal portion of implants, leading to an inflammatory cell infiltrate in the peri-implant mucosa [30, 31]. Most recently, a chemically modified sandblasted large grit and acid-etched (modSLA) titanium surface has been introduced to enhance bone apposition [12, 38, 45]. The specific production process used for modSLA surfaces (i.e. rinsing the titanium surface after the etching process under N_2 protection and continuous storage in an isotonic NaCl solution) has been reported to produce a chemically active surface with a small amount of hydrocarbons and carbonates. The resulting hydroxylated/hydrated modSLA surface was shown to have an initial advancing water contact angle of 0°, indicating its ultra hydrophilic character. Furthermore, an increased surface free energy (SFE) has been reported by Rupp et al. [34], resulting in an increased water/biomaterial contact area. Histological observations in miniature pigs have also shown that modSLA implants exhibited a significantly greater mean percentage of bone-to-implant contact as compared with conventional SLA surfaces at 2 and 4 weeks of healing [12, 37]. These preliminary results suggest that modSLA surfaces might also improve attachment and proliferation of fibroblasts, thus promoting a subepithelial connective tissue attachment at the transmucosal part of the implant. Therefore, the aim of the present pilot study was to histologically/immunohistochemically investigate initial and early subepithelial connective tissue attachment at transmucosal parts of modSLA and SLA titanium implants in a dog model. The implants were submerged to prevent bacterial contamination.

Materials and methods

Animals

Four 3-year-old female foxhounds (mean weight, 29.3± 0.4 kg) were used in the study. All animals exhibited a fully erupted permanent dentition. During the experiment, the dogs were fed once per day with soft-food diet and water. Animal selection, management and surgery protocol were approved by the Animal Care and Use Committee of the Heinrich Heine University and the Bezirksregierung Düsseldorf. The experimental segment of the study started after an adaption period of 4 weeks.

Study design

The study was performed in two surgical phases. In the first phase, extraction of the mandibular and maxillary second, third, fourth premolar and first molar (P2-M1) was performed bilaterally. After 4 months of healing, surgical implantation of modSLA and conventional SLA screw-typed implants was performed in a submerged healing procedure during the second phase. Both test and control implants were randomly assigned to both jaws according to a split-mouth design, including five implants per group in the lower jaw and three implants per group in the upper jaw. Randomization was performed according to a computer-generated list (RandList®, DatInf GmbH, Tübingen, Germany). Accordingly, each animal received six implants bilaterally in the upper jaw (three modSLA and three SLA, respectively) and ten implants bilaterally in the lower jaw (five modSLA and five SLA, respectively; Fig. 1a,b). The animals were killed after a healing period of 1, 4, 7 and 14 days, including one animal each.

Surgical procedure

After intramuscular sedation with 0.17 mg/kg acepromazine (Vetranquil 1%, Ceva Tiergesundheit, Düsseldorf, Germany), anaesthesia was initiated using 21.5 mg/kg thiopental-sodium (Trapanal 2.5%, Altana GmbH, Konstanz, Germany). For all surgical procedures, inhalation anaesthesia was performed by use of oxygen and nitrous oxide and isoflurane. To maintain hydration, all animals received a constant rate infusion of

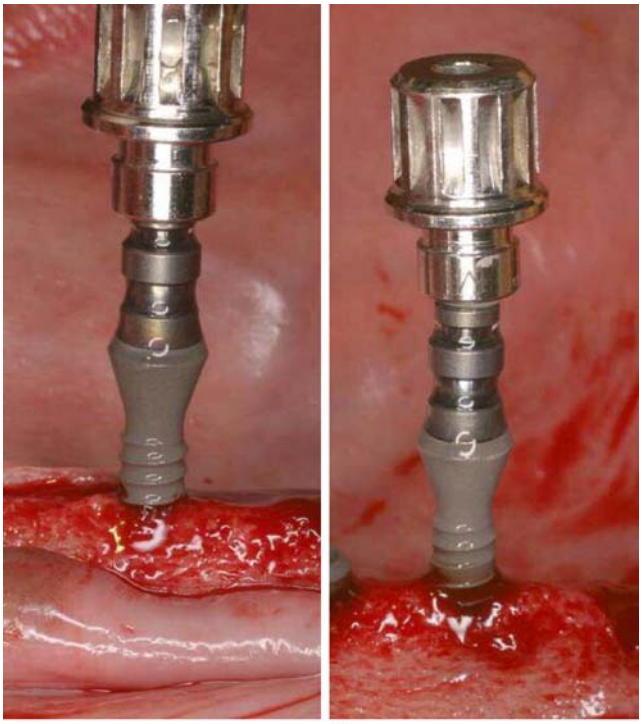

Fig. 1 Wettability of SLA (**a**) and modSLA (**b**) titanium surfaces as observed during implant installation. In case of SLA, the implants were thoroughly rinsed with sterile saline before insertion

lactated Ringer's solution while being anaesthetised. Intraoperative analgesia was performed by intravenous injection of 0.4 mg/kg piritramid (Dipidolor®, Janssen-Cilag GmbH, Neuss, Germany) and 4.5 mg/kg carprofene (Rimadyl®, Pfitzer Pharma GmbH, Karlsruhe, Germany). For postoperative treatment, piritramid and carprofene were applied subcutaneously for 3 days in the same dose as described before. In the first surgery, P2–M2 were carefully removed bilaterally in both jaws after reflection of mucoperiosteal flaps and tooth separation. After wound closure by means of mattress sutures, the sites were allowed to heal for 4 months. Prophylactic administration of clindamycine (11.0 mg/kg body weight; Cleorobe®, Pharmacia Tiergesundheit, Erlangen, Germany) was performed intra- and postoperatively for 10 days.

In the second surgery, bilateral vestibular incisions were made, and mucoperiosteal flaps were reflected to expose the respective sites for implant insertion in both jaws. Surgical implant sites were prepared bilaterally in both jaws, at a distance of 10 mm apart, using a low-trauma surgical technique under copious irrigation with sterile 0.9% physiological saline. Both test and control implants (regular neck, sandblasted, large grit and acid-etched transmucosal part, ∅ 3.3 mm, length 8 mm, Institut Straumann® AG, Basel, Switzerland; commercial name of modSLA is SLActive®) were inserted according to a low-trauma surgical technique with good primary stability. The modSLA implants were produced according to the SLActive® manufacturing process described by Rupp et al. [34]. The implants were inserted in a way so that the borderline between the bony and transmucosal part of the implant coincided with the bone crest. In the case of SLA, the implants were thoroughly rinsed with sterile saline before insertion. After irrigation, primary wound closure was achieved with consecutive resorbable 5.0 polyglygolic acid sutures (Resorba®, Nürnberg, Germany), and implants were left to heal in a submerged position (Fig. 2a–d).

Animal preparation and retrieval of specimens

The animals were killed (overdose of sodium pentobarbital 3%) after a healing period of 1, 4, 7 and 14 days, respectively, and the oral tissues were fixed by perfusion with 10% buffered formalin administered through the carotid arteries. The jaws were dissected, and blocks containing the experimental specimens were obtained. All specimens were fixed in 10% neutral-buffered formalin solution for 4–7 days.

Histological preparation

One part of the specimens (i.e. two implants from the upper jaw, and three implants from the lower jaw per group and time point) was dehydrated using ascending grades of alcohol and xylene, infiltrated and embedded in methylmethacrylate (MMA; Technovit 9100 NEU, Heraeus Kulzer, Wehrheim, Germany) for non-decalcified sectioning. During this procedure, any negative influence of polymerisation heat was avoided due to a controlled polymerization in a cold atmosphere (−4°C). After 20 h, the specimens were completely polymerized. Each implant site was cut in the bucco-lingual direction and along with the long axis of the implant using a diamond wire saw (Exakt®, Apparatebau, Norderstedt, Germany), resulting in four sections of approximately 500 μm in thickness [16]. Subsequently, all specimens were glued with acrylic cement (Technovit 7210 VLC, Heraeus Kulzer) to silanized glass slides (Super Frost, Menzel GmbH, Braunschweig, Germany) and ground to a final thickness of approximately 40 μm. The specimens were stained with Masson Goldner Trichrome (MG; quality and quantity of collagen fibres).

Another part of the specimens (i.e. one implant from the upper jaw and two implants from the lower jaw per group and time point) was processed according to the fracture technique [2] and decalcified in ethylenediamine tetraacetic acid under radiographic control of the decalcification process, dehydrated and fixed in paraffin. Bucco-lingual serial sections were cut along with the long axis of the implant, resulting in sections of approximately 5 μm. Sections representing the central part of the implant body were selected for immunohistochemical analysis.

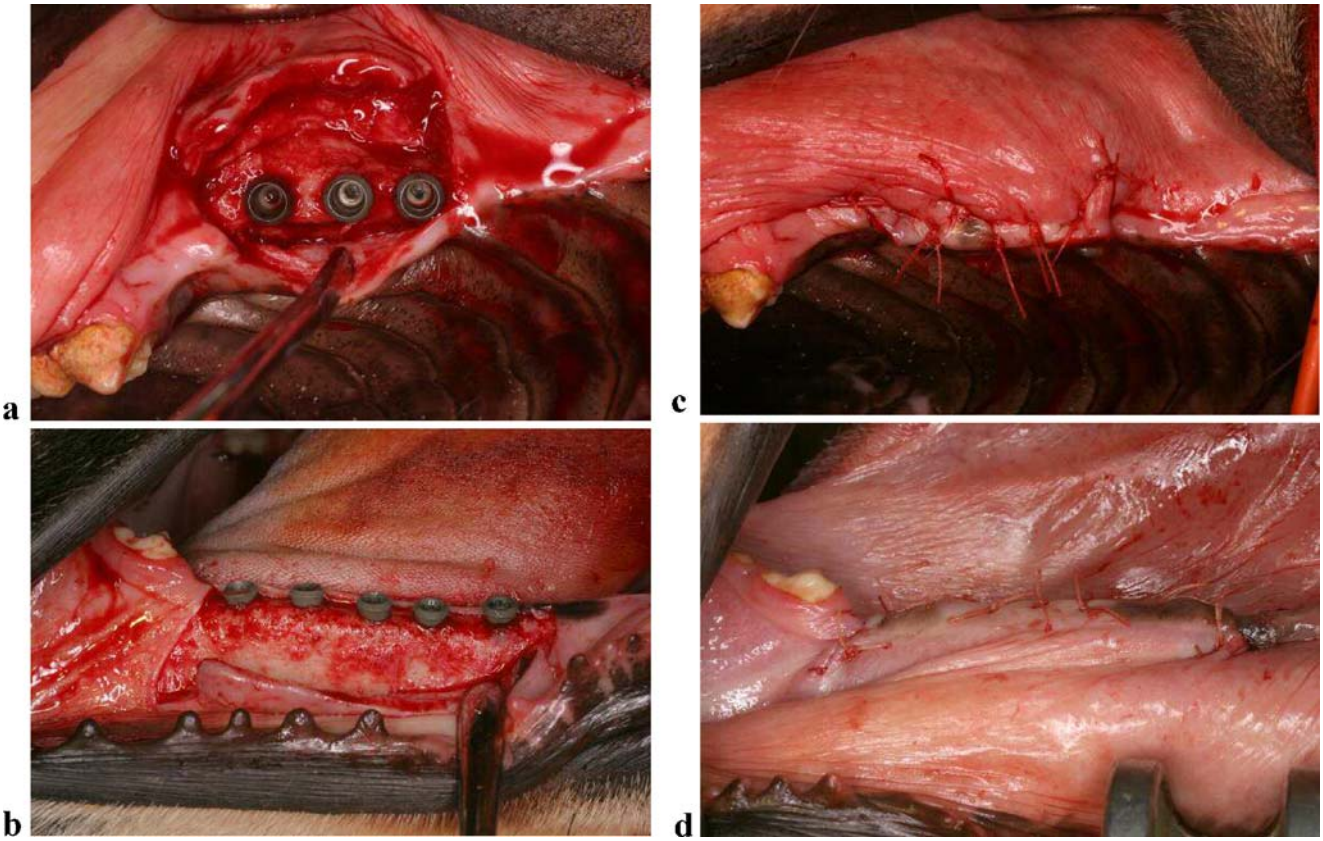

Fig. 2 In both upper (**a**) and lower (**b**) jaws, the implants were inserted in a way so that the borderline between the bony and structured transmucosal part of the implant coincided with the bone crest. Primary wound closure was achieved with consecutive resorbable polyglygolic acid sutures, and implants were left to heal in a submerged position (**c** and **d**)

Immunohistochemical labelling

After deparaffinization and rehydration of 5-µm-thin tissue sections, antigen unmasking was performed by heating for 15 min in target retrieval solution (DakoCytomation, Hamburg, Germany). After quenching the activity of endogenous peroxidase with 0.9% hydrogen peroxide in phosphate-buffered saline (PBS) for 15 min at room temperature, non-specific binding sites will be blocked with blocking solution for 30 min (Dako). After a 5-min wash with PBS, the primary mouse monoclonal antibody to FN and PCNA (1:40 dilution, Labvision, Fremont, CA, USA) or unspecific antibodies, respectively, as negative control were applied to tissue sections in a humidified chamber at room temperature. The slides were washed in PBS and incubated with secondary biotinylated anti-mouse antibody (Dako) for 1 h. The presence of antibody–antigen complexes was visualized using a streptavidin-peroxidase solution with analog aminoethylcystine as the chromogen (Dako).

Histological analysis

Histomorphometrical analyses as well as microscopic observations were performed by one experienced investigator masked to the specific experimental conditions. For image acquisition, a color CCD camera (Color View III, Olympus, Hamburg, Germany) was mounted on a binocular light microscope (Olympus BX50, Olympus). Digital images (original magnification ×200) were evaluated using a software program (analySIS FIVE docu®, Soft Imaging System, Münster, Germany).

Results

The postoperative healing was uneventful in all dogs. No complications such as allergic reactions, abscesses or infections were observed throughout the whole study period. Furthermore, there were no signs of any wound dehiscence or exposure of the transmucosal part of the implant body in both groups.

Histological analysis

Day 1

The conditions of the soft tissues adjacent to the transmucosal part of modSLA and SLA implants are illustrated in Fig. 3a and b. In particular, the surgical procedure of implant submerging resulted in the formation of an artificial

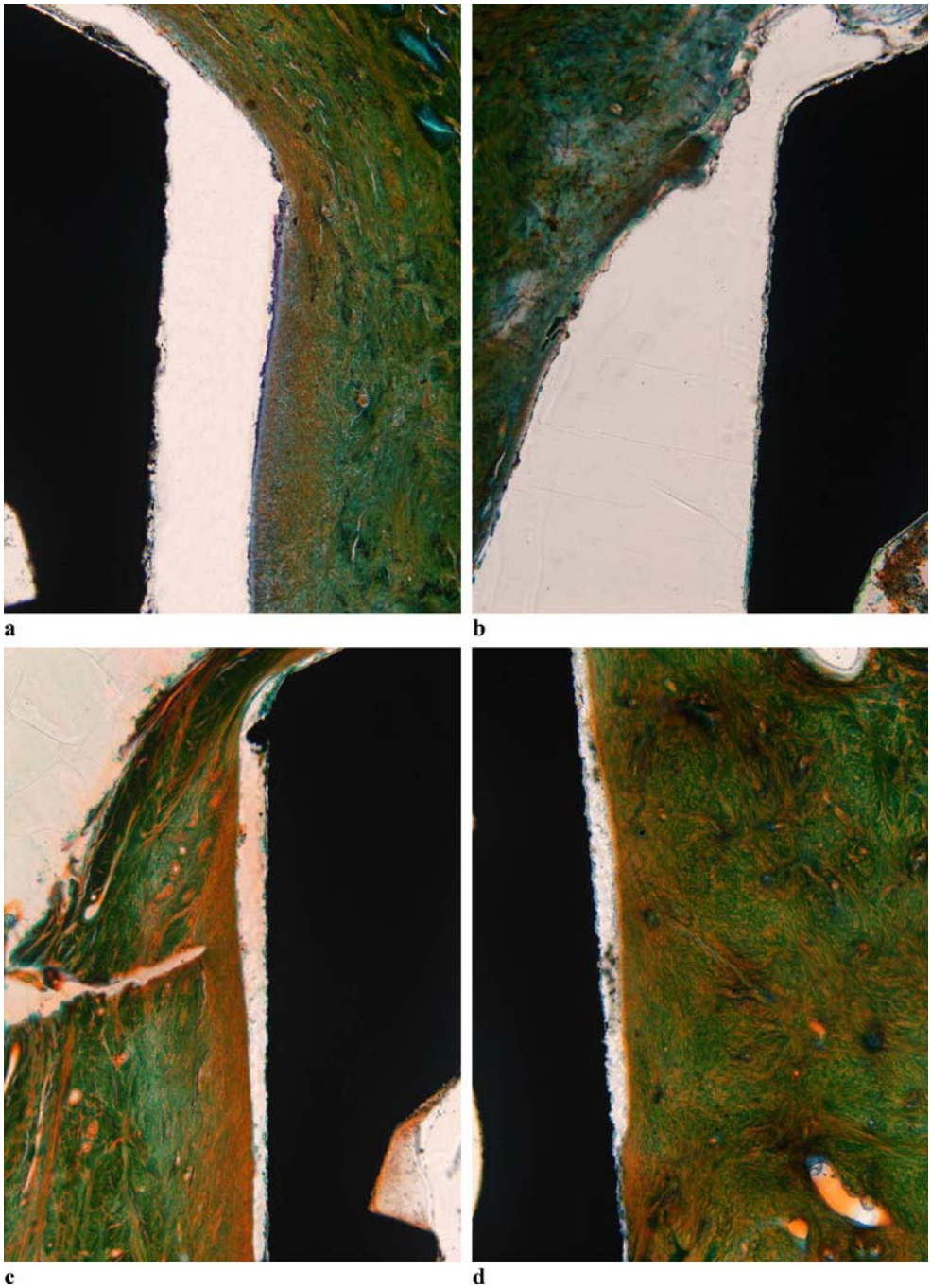

Fig. 3 Histological views of connective tissue reactions adjacent to the transmucosal part of modSLA (**a**, **c**, **e**, **g**, **i**, **k**) and SLA (**b**, **d f**, **h**, **j**, **l**) implant surfaces. **a**, **b** Implant submerging resulted in the formation of an artificial gap in the transmucosal area of both types of implants at day 1. **c**, **d** The artificial gaps were similarly minimised in the transmucosal area of both modSLA and SLA implants at day 4. **c** Collagen fibres adjacent to modSLA implants appeared to be replaced by a newly formed orange-stained loose connective tissue zone at day 4. **e** The newly formed loose connective tissue adjacent to modSLA has completely spanned the artificial gap and tended to be in close contact with the implant surface at day 7. **f** The transmucosal part of SLA implants at day 7 was characterized by the formation of a dense connective tissue zone with collagen fibres running parallel to the respective surfaces. **g** Ongoing and well-organized formation of collagen fibres and numerous blood vessels in the loose connective tissue zone adjacent to the transmucosal part of modSLA surfaces at day 14. **h** SLA implants appeared to be clearly separated by a dense connective tissue zone with parallel-running collagen fibres and rare blood vessel formation at day 14. **i**, **k** Collagen fibres have started to extend and attach perpendicularly to modSLA implant surfaces at day 14. **j**, **l** The newly formed dense connective tissue tended to be in close contact to SLA implant surfaces at day 14. Masson Goldner Trichrome Stain (**a**–**h** original magnification ×100; **i**–**l** original magnification ×500). *B* Alveolar bone crest

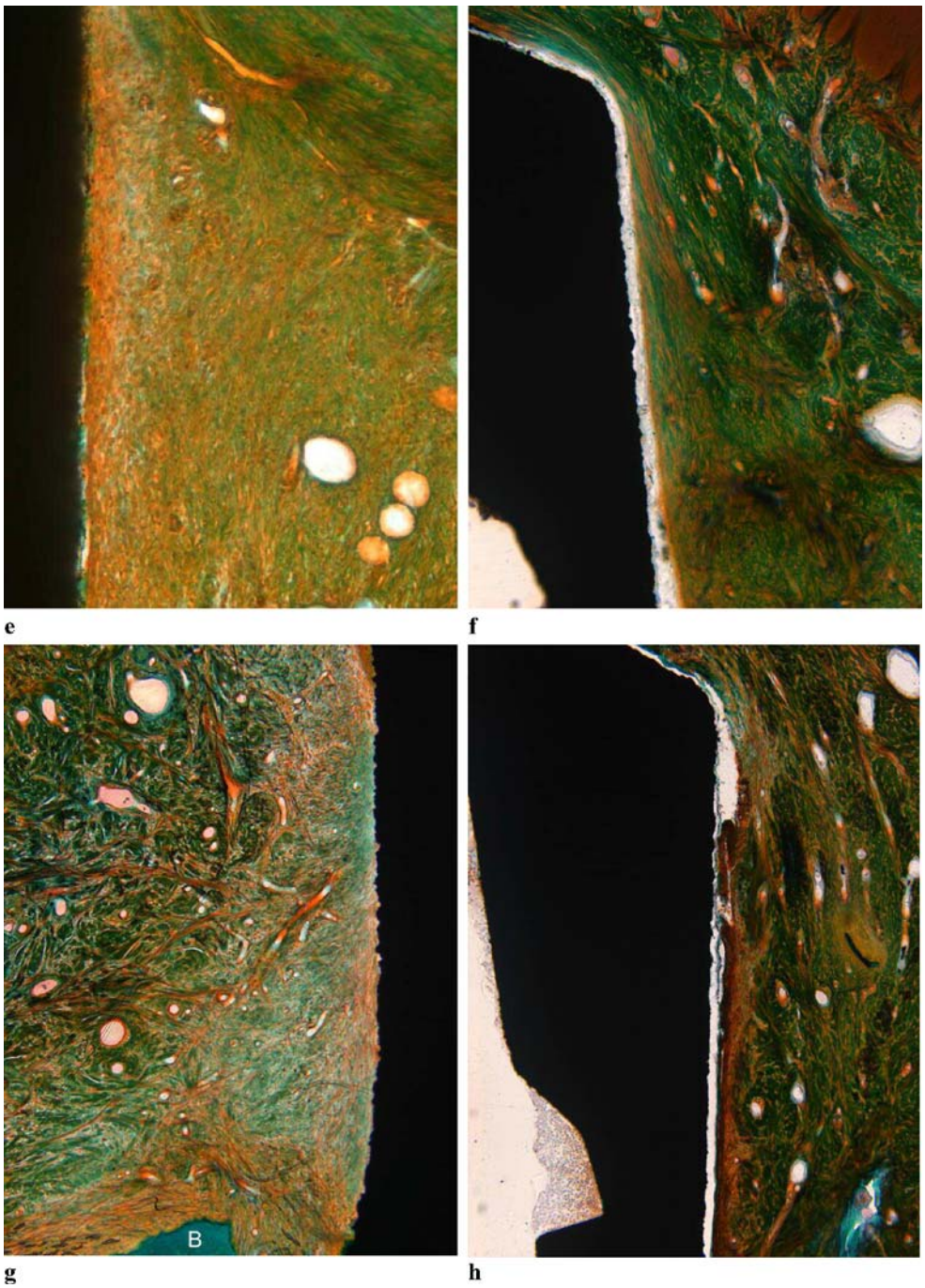

Fig. 3 (continued)

gap in the transmucosal area of both types of implants caused by the mechanical tension of the mucoperiosteal flaps over the respective implant shoulders (width approximately 300 μm). MG stain revealed that the collagen fibres in the adjacent subepithelial connective tissue appeared to follow different vertical and horizontal directions. Histological analysis failed to demonstrate any blood clot formation between both implant surfaces and the adjacent connective tissue (Fig. 3a,b).

Day 4

Histological analysis revealed that the artificial gaps were similarly minimized to a width of approximately 80 μm in the transmucosal area of both modSLA and SLA implants (Fig. 3c,d). However, MG stain demonstrated obvious differences with respect to both density and orientation of the collagen fibres in the subepithelial connective tissue adjacent to both types of implants.

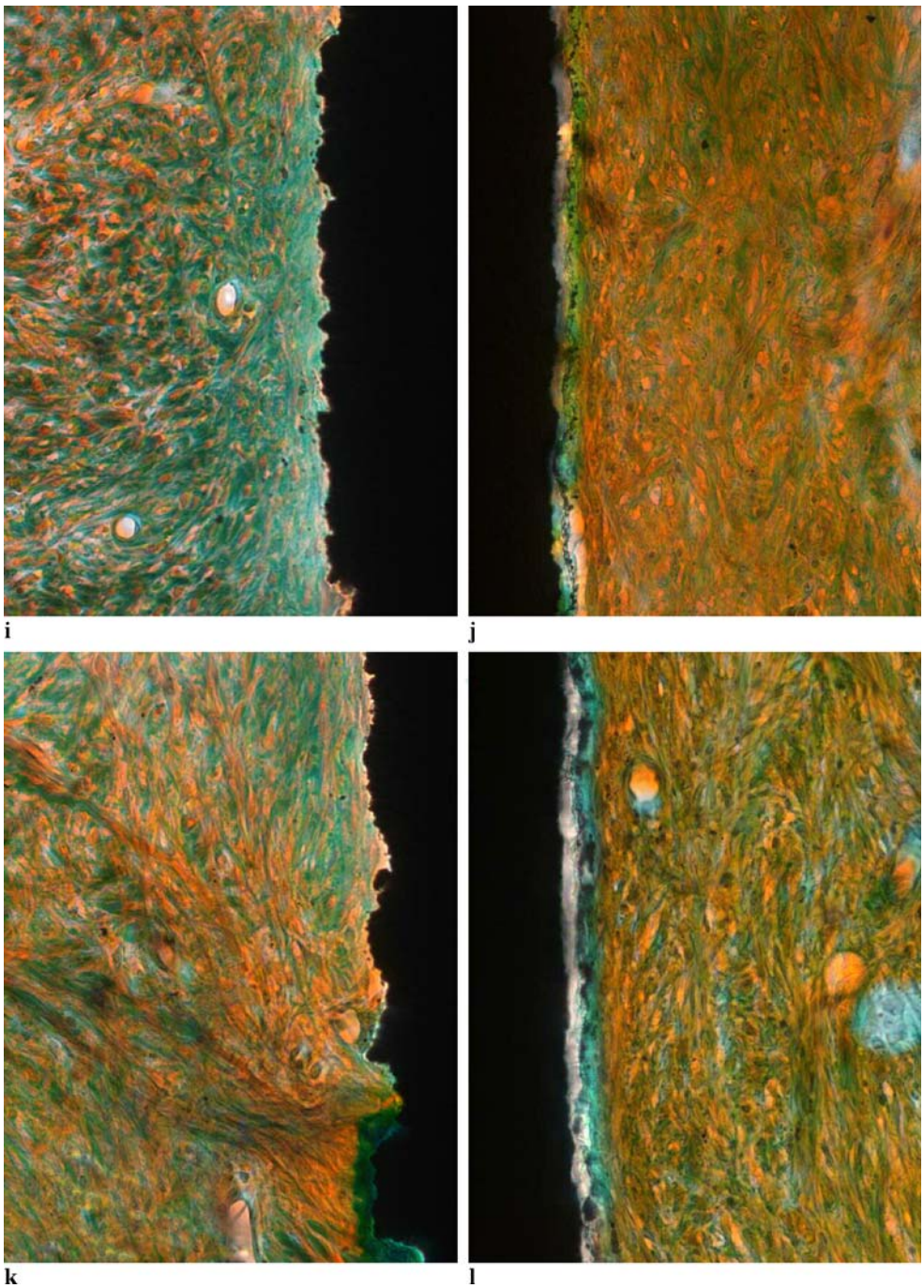

Fig. 3 (continued)

In particular, the collagen fibres adjacent to modSLA implants appeared to be replaced by a newly formed orange stained loose connective tissue zone, exhibiting a thickness of approximately 200 μm. In contrast, the density of the collagen fibres appeared to increase adjacent to the transmucosal part of SLA implants.

While fibre orientation adjacent to modSLA appeared to be dissolved due to their replacement by the loose connective tissue, collagen fibres have started to capture a parallel direction to the surfaces of respective SLA implants (Fig. 3c,d).

Day 7

MG stain revealed that the newly formed loose connective tissue adjacent to modSLA has completely spanned the artificial gap and tended to be in close contact with the

implant surface (Fig. 3e). The loose connective tissue appeared to become organized and replaced by newly formed collagen fibres, originating from its outer zone. These fibres tended to be partially organized in a perpendicular way towards the implant surface.

In contrast, adjacent to the transmucosal part of SLA implants, histological analysis revealed the formation of a dense connective tissue zone with collagen fibres running parallel to the respective surfaces. However, in comparison to day 4, the newly formed tissue appeared to be in closer contact to the implant surface, although a direct adhesion was not observed (Fig. 3f).

Day 14

In comparison to day 7, MG stain revealed an ongoing and well-organized formation of collagen fibres and numerous blood vessels in the loose connective tissue zone adjacent to the transmucosal part of modSLA surfaces (Fig. 3g). In particular, this tissue zone appeared to be composed of fibres running in different directions. While some fibres were oriented in a parallel direction, others have started to extend and attach partially perpendicular to the implant surface (Fig. 3i,k). From a histological point of view, the subepithelial connective tissue adjacent to modSLA implants could not be separated in different zones.

In contrast, SLA implants appeared to be clearly separated by a dense connective tissue capsule with parallel-running collagen fibres and rare blood vessel formation (thickness of approximately 40 μm; Fig. 3h). In comparison to day 7, however, the fibres tended to be in closer contact to the implant surface. Towards the periphery, this inner zone was surrounded by a well-vascularised, loose orange-stained connective tissue formed of collagen fibres running in different directions similarly to that observed adjacent to modSLA implants (Fig. 3j,l).

Immunohistochemical analysis

First signs of a positive immunochistochemical staining for both FN and PCNA at the soft tissue interface adjacent to SLA and modSLA implant surfaces were observed at day 4. Although the intensity of both FN and PCNA antigen reactivity increased over time in both groups, PCNA appeared to be more pronounced at modSLA implants (Fig. 4).

Discussion

The present pilot study was designed to investigate initial and early subepithelial connective tissue integration at the transmucosal parts of submerged modSLA and SLA titanium implants by means of histological and immunohistochemical analysis. Within its limits, it was observed that after 14 days of healing, the artificial gap in the transmucosal area of modSLA surfaces appeared to be spanned by a newly formed and well-vascularised loose connective tissue exhibiting collagen fibres that have started to extend and attach partially perpendicular to the implant surface. In contrast, SLA implants appeared to be clearly separated by a dense connective tissue zone with parallel running collagen fibres and rare blood vessel formation. However, during the entire healing period of 14 days, both modSLA and SLA implants revealed an ongoing remodelling process within the newly formed adjacent connective tissue, identifiable as orange -stained components of the extracellular matrix. This observation may also be supported by a positive antigen reactivity of both FN and PCNA over time. Although the intensity of the immunohistochemical staining could not be quantified, the PCNA signal appeared to be more intense adjacent to modSLA implant surfaces. In this context, it must be emphasized that FN is involved in many cellular processes, including tissue repair, blood clotting and cell adhesion by anchoring cells to collagen or proteoglycan substrates [28, 43]. Immunohistochemical localization of PCNA can be used as a reliable marker of cells undergoing active proliferation [26, 29]. When interpreting these findings, however, it must also be emphasized that the present study has indeed some drawbacks. One key limitation was the small number of animals, and therefore, further experimental studies of higher power are needed to confirm the present results. Moreover, the submerged healing procedure is not appropriate to evaluate the epithelial component of the soft tissue interface at the implant surface. As described above, the submerged healing procedure was chosen to preserve subepithelial connective tissue healing from bacterial contamination and subsequently inflammatory reactions. In this context, however, it must be pointed out that the formation of peri-implant tissues, including the barrier epithelium, connective tissue integration and the establishment of bone-to-implant contact have been observed to be not dependent on submerged or non-submerged surgical approaches [3, 17, 44]. Indeed, Brånemark et al. [11] demonstrated that a 50–100 μm wide zone of circular collagen fibres was also present close to rough sandblasted, fine sandblasted or polished transmucosal surfaces of non-submerged implants and that this zone was free of vascular structures similarly to an inflammation-free scar tissue formation. This zone was surrounded by a looser connective tissue with a three-dimensional network of collagen fibres running in different directions [13]. This observation is in agreement with the histological healing pattern noted at the transmucosal part of SLA implants. The formation of a dense connective tissue zone with parallel running collagen fibres and rare blood vessel formation around

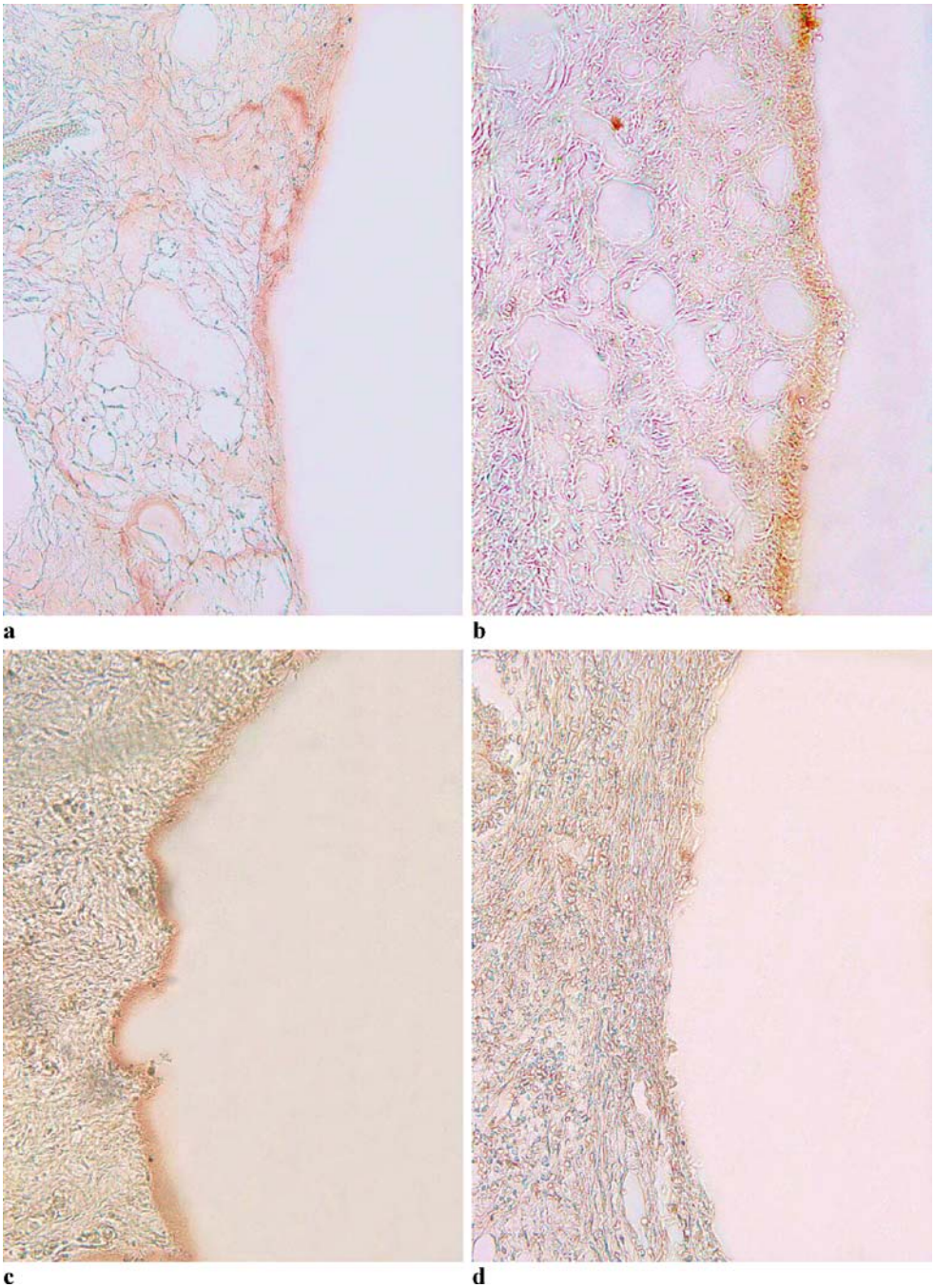

Fig. 4 Immunohistochemical analysis of FN (**a**, **b**) and PCNA (**c**, **d**) adjacent to the transmucosal part of modSLA (**a**, **c**) and SLA (**b**, **d**) implant surfaces (original magnification×500) after 14 days. A positive FN and PCNA staining was observed adjacent to both implant surfaces. However, the intensity appeared to be more pronounced at modSLA

differently structured titanium implants was also confirmed in several experimental studies using various animal models [3, 9, 15, 19, 33]. Accordingly, it might be hypothesised that surface roughening itself might not be the crucial step to promote a soft tissue attachment at the transmucosal part of the implant. However, this hypothesis seems to be inconsistent with previous studies, which have indeed demonstrated that the surface texture significantly influences fibroblast and epithelial cell attachment [21, 22, 27]. One possible explanation for this discrepancy might be due to the fact that results obtained by using an in vitro experimental model cannot recreate the complex interactions of cells in vivo. In contrast, as mentioned above, it was also observed that modSLA implants were surrounded by a well-vascularised loose connective tissue exhibiting collagen fibres that have started to extend and attach partially perpendicular to the implant surface. There might be several aspects to explain the present findings. First of all, it must be emphasized that the hydrophilic surface properties noted for hydroxylated/hydrated modSLA resulted in a higher wettability when compared to conventional SLA surfaces [34]. In particular, the main outcome of dynamic wettability measurements was that modSLA implants revealed increased SFE and hydrophilicity with

initial water contact angles of 0° compared to 139.9° for SLA implants. Accordingly, it might be hypothesised that the wettability of modSLA surfaces also ameliorated the adsorption of plasma proteins providing ligand sites for the interaction with cellular receptors. Indeed, immediately after wound closure, the artificial gap adjacent to the transmucosal part of both types of implants was partially filled by a blood clot, although histological observation revealed no coagulum formation at day 1. Furthermore, the results obtained from a recent in vitro study have indicated that osteoblasts grown on modSLA surfaces exhibited a more differentiated phenotype characterised by increased alkaline phosphatase activity (ALP) and osteocalcin and generated an osteogenic microenvironment through higher production of prostanglandine E2 (PGE2) and transforming growth factor (TGF)-beta 1 [45]. Moreover, Masaki et al. [25] observed significant increases in ALP gene expression in osteoblasts grown on modSLA when compared to SLA implant surfaces. All these data, taken together with the results from the present pilot study, seem to indicate that SFE and hydroxylation/hydration of modSLA implant surfaces might also have a beneficial effect on differentiation, attachment and proliferation of regenerative potential cells derived from the subepithelial connective tissue zone. When interpreting the present results, it must also be emphasised that there are currently no histological data available supporting the clinical relevance of collagen fibre attachment and orientation. However, the role of the connective tissue in preventing a down-growth of the junctional epithelium and thus increasing soft tissue stability has been elucidated in several experimental animal studies [14, 41]. Therefore, further studies using a higher number of animals are necessary to verify the present findings particularly at non-submerged implants. Within the limits of a pilot study, it might be concluded that modSLA titanium surfaces might possess the potential to promote subepithelial connective tissue attachment at the transmucosal part of the implant.

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