A Hydrophilic Dental Implant Surface Exhibit Thrombogenic Properties In Vitro

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ABSTRACT

Background: Surface modifications of dental implants have gained attention during several years and the thrombotic response from blood components with these materials has become more important during recent years.

Purpose: The aims of this study were to evaluate the thrombogenic response of whole blood, in contact with clinically used dental surfaces, Sandblasted Large grit Acid etched titanium (SLA) and Sandblasted Large grit Acid etched, and chemically modified titanium with hydrophilic properties (SLActive).

Methods: An in vitro slide chamber model, furnished with heparin, was used in which whole blood came in contact with slides of the test surfaces. After incubation (60-minute rotation at 22 rpm in a 37°C water bath), blood was mixed with ethylenediaminetetraacetic acid (EDTA) or citrate, further centrifuged at +4°C. Finally, plasma was collected pending analysis.

Results: Whole blood in contact with surfaces resulted in significantly higher binding of platelets to the hydrophilic surface, accompanied by a significant increase of contact activation of the coagulation cascade. In addition, the platelet activation showed a similar pattern with a significant elevated release of β-TG from platelet granule.

Conclusions: The conclusion that can be drawn from the results in our study is that the hydrophilic modification seems to augment the thrombogenic properties of titanium with implications for healing into bone of, that is titanium dental implants.

KEY WORDS: coagulation, complement, contact phase activation, dental implant, hydrophilicity, osseointegration, platelet activation, surface modification, titanium

INTRODUCTION

The importance of the design of an implant surface for improved osseointegration has gained much attention during recent years. Surface modifications of dental implants have resulted in accelerated healing and increased bone formation.[1,2]

In addition, the importance of the various constituents of blood attributed to wound healing of tissues has been elucidated in several studies for many years. Both purified blood products as fibrin sealant and fractions of cells, such as platelet-rich plasma or whole blood, have been discussed.[3,4]

Bone tissue is a very well-vascularized tissue, and the implantation of a biomaterial into this tissue causes bleeding and subsequent exposure of the material to blood. The importance of the blood components for bone regeneration, in particular around implants, has gained attention during several years.[5,6] The end-product of the coagulation cascade is the formation of fibrin fibers. Early bone healing around implants relies on the anchorage of these fibers to the implant surface in a sufficient manner. The fixation of the fibers to the surface restrains the possible sequence of shrinkage of the clot resulting in a gap formation between the new tissue and the implant surface. The first healing phase relies on the recruitment and migration of osteogenic cells through the blood clot to the implant surface where the bone formation may be initiated directly on the surface.[7] As a result of shorter healing periods with faster bone formation around dental implants, the possibility

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of early loading of the implants arises, which in turn is favorable for patients seeking oral rehabilitation.[1]

We have previously presented results using an in vitro model where a surface modification with hydrofluoric acid displayed enhanced thrombogenic properties and improved healing in vivo.[8]

A modified titanium surface (SLActive), that has recently become commercially available, is grit-blasted, acid-etched and chemically modified to introduce hydrophilic properties. Studies suggest that the bone response in vivo is stronger with this modification compared with control surfaces with hydrophobic properties (SLA).[9]

Therefore, this study was designed to explore if this hydrophilic modified implant surface has increased ability to promote the thrombogenic response in whole blood as well as to stimulate the release of growth factors from platelets. This study deals with a conceptually new hypothesis implying that thrombogenicity promotes osseointegration and tissue growth.

MATERIALS AND METHODS

Biomaterial Surfaces and Sample Preparation

For blood compatibility evaluations, surfaces of SLA and SLActive were prepared and provided by Institut Straumann AG (Basel, Switzerland). The exact preparation sequence of these surfaces is proprietary of the company and not known by the authors. The SLActive were stored in sealed glass vials in physiological salt solution without air contact until immediately prior to blood contact. The SLA surfaces were stored in a dry and sterile package until blood testing. Pieces of polyvinyl chloride (PVC) and smooth titanium (s-Ti) prepared by chemical vapor deposition were cleaned in 5% (w/v) ammonium persulphate at 60°C, for 1 hour and used as reference test surfaces for control of experimental setup.

Topographical Characterization

The 3-D roughness parameters of the clinical titanium surfaces were measured by Straumann AG using confocal white light microscopy (OS04358). The micro roughness parameters were determined using a moving average gauss filter with a cut-off wavelength of 30 µm (x = 31 µm, y = 30 µm; 20 × 19 image points) (Table 1). The surface roughness of PVC and smooth Ti was measured using an Alpha-step 200 (Tencor Instruments, Mountain View, CA, USA). The hydrophobicity of the modified surfaces was estimated from contact angles against water measured using a Fibro DAT-1100 (Fibrosystem AB, Hägersten, Sweden).

TABLE 1 Results of Topographical and Wettability Measurement

Material	Sa (µm)	Sdr (%)	Contact Angle (deg)
SLActive	1.78	143	0
SLA	1.5	34	>90
Smooth- Ti	0.005	n.d.	41.3
PVC	0.033	n.d.	85.4

n.d. = not determined; PVC = polyvinyl chloride; Sa = average height deviation; Sdr = the developed interfacial area ratio; SLA = Sandblasted Large grit Acid etched titanium; SLActive = Sandblasted Large grit Acid etched chemically modified titanium.

Heparin Treatment

Whenever indicated, materials were heparin-coated using the Corline method (Corline Systems AB, Uppsala, Sweden) according to the manufacturer's recommendations. The surface concentration of heparin was 0.9 µg/cm^2, with an antithrombin (AT) binding capacity of 10–12 pmol/cm^2.[10] All surfaces except the test objects were coated with heparin to prevent activation by the chamber itself.

Experimental Setup

A modification of the slide chamber, previously described by Hong and colleagues,[11] containing two wells with an inner diameter of 14 mm and a volume of 1.45 mL was used.

Samples of 25 mL whole blood was collected from healthy volunteers in a heparinized 50 ml Falcon tube containing 125 µL heparin solution to a final concentration of 0.5 IU heparin/ml and used within 15 minutes. The wells of the slide chamber were filled with 1.2 mL of blood.

Test surfaces were attached with two clips as a lid creating two circular chambers.

After closure, the slide chamber was mounted on the outer rim of a plastic disc and rotated vertically in a 37°C water bath for 60 minute.

After incubation, the blood was mixed with ethylenediaminetetraacetic acid (EDTA) or citrate to give final concentrations of 4 or 13 mM, respectively. Prior to centrifugation, the EDTA samples were analyzed for leukocytes and platelets on a Coulter AcT diff™ hematology analyzer (Coulter Corporation, Miami, FL, USA). The EDTA-treated blood was centrifuged at 2,200 g for

10 minutes at +4°C, and the citrated blood was centrifuged twice (10 minutes at 1,000 g and 10 minutes at 10,000 g) at +4°C within 30 minutes. The plasma samples were collected and stored at −70°C. The EDTA plasma was used for measurements of coagulation and complement activation. The citrated plasma samples were used for detection of platelet granule release of thrombospondin.

In order to assess the blood compatibility in the model, macroscopic clotting was monitored and the blood analyzed for blood cell counts, platelet activation markers, coagulation activation, and complement activation.

Enzyme Immunoassays (EIAs)

Phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA) and 0.05% (v/v) Tween 20 was used as working buffer and PBS containing 0.05% (v/v) Tween 20 as washing buffer in all EIAs.

Detection of Thrombin-Antithrombin Complex (TAT)

Plasma samples from EDTA-treated blood were analyzed for TAT. Microtiter plates were coated with anti-human thrombin antibody (Enzyme Research Laboratories, South Bend, IN, USA). Horseradish peroxidase (HRP)-coupled anti-human antithrombin antibody (Enzyme Research Laboratories) was used for detection. Pooled human serum diluted in normal citrate-phosphate-dextrose plasma was used as standard. Values are given in μg/L.

Detection of β-Thromboglobulin (β-TG)

For detection of β-TG citrated plasma was analyzed using Asserachom assay (Diagnostica Stago, Asnie'res-sur-seine, France). Samples were prepared according to the recommendations for Quantikine™ (R & D Systems Inc., Minneapolis, MN, USA). The values are expressed as units/mL.

Detection of Contact Activation Complexes

For determination of FXIIa–AT, FXIIa–C1INH, FXIa–AT, and FXIa–C1INH complexes in plasma, EIAs described by Sanchez J and colleagues[12] was used. The values are expressed as nmol/L.

Detection of Thrombospondin-1 (TSP-1)

Citrate samples were analyzed for thrombospondin-1. Samples were added to microtiter plates coated with capture antibody, clone B7 (AbCam, Cambridge, UK) diluted 1/1,000. Biotinylated anti-human TSP-1 clone P10 (Immunotech, Marseilles, France) diluted 1/400 was used as detection antibody, followed by HRP-conjugated streptavidin (GE Healthcare, Buckinghamshire, UK) diluted 1/500. Human serum diluted in working buffer was used as a standard. Values are given in ng/mL.

Detection of C3a and sC5b-9

C3a levels were measured using the method described by Nilsson Ekdahl and colleagues.[13] Zymosan-activated serum, calibrated against a solution of purified C3a, served as a standard. Values are given in ng/mL.

sC5b-9 was assessed using a modification of the method described by Mollnes and colleagues.[14] Plasma samples were diluted and added to microtiter plates coated with antineoC9 monoclonal antibody. sC5b-9 was detected by polyclonal anti-C5 antibodies diluted 1/500, followed by HRP-conjugated anti-rabbit immunoglobulin diluted 1/500. Zymosan activated serum containing 40,000 AU/mL served as a standard.

Statistical Analyses

Statistical evaluation was calculated with Prism 5 for Mac OS X, GraphPad Software Inc. The results are expressed as mean ± standard error. Statistical significance was calculated using one factor ANOVA, followed by Bonnferroni's post hoc test. The final amount of test surfaces used for statistical analyses were SLActive $n = 16$ and SLA $n = 15$ (one excluded due to technical error), and for control surfaces Smooth-Ti $n = 6$ and PVC $n = 4$ which were tested with blood from three different donors.

RESULTS

After incubation in the slide chamber model, the difference between biomaterial surfaces was readily visible for the eye; the SLActive surface showed extensive clotting compared with the SLA surface (Figure 1). The smooth Ti surface showed reduced clotting and the PVC test surface induced clotting at a low level.

Platelet Reduction in Incubated Blood

The platelet binding to test surfaces reflected by platelet consumption in the fluid phase was most prominent with SLActive (Figure 2). The platelet count decreased 99% with SLActive compared with 90% with SLA. The control surfaces induced less consumption; the

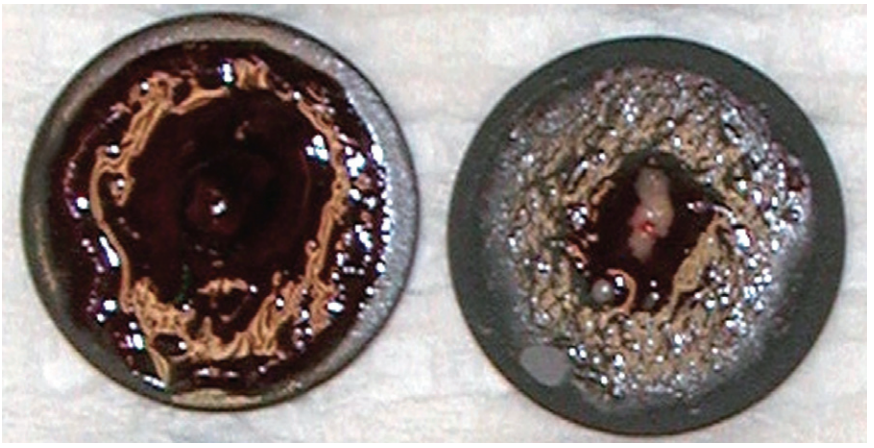

Figure 1 Example of SLActive left and SLA right, after blood contact 1 hour in the slide chamber model.

reduction was 36 % with s-Ti and 31% with PVC. The platelet reduction with SLActive was significantly higher compared with the SLA surface ($p < .05$).

Platelet Release of α-Granule Content upon Activation (Figure 3)

The platelet release of β-TG increased from 243 ± 56.8 to 7,029 ± 570 IU/mL with SLActive, to 5,504 ± 282 with SLA, to 2,539 ± 326 with s-Ti and to 2,494 ± 657 with PVC.

SLActive surface showed a significantly higher release of β-TG compared with the SLA surface ($p < .05$). The release was less extensive with s-Ti and PVC.

The release of thrombospondin also stored in alfa-granules showed a different pattern (Figure 3).

The concentration in plasma increased from 918 ± 172 to 16,939 ± 1,182 ng/mL with SLActive, to 18,119 ± 860 with SLA, to 10,738 ± 1,128 with s-Ti and to 8,867 ± 3,344 with PVC.

The SLA surface showed a slightly higher release of Thrombospondin compared with the SLActive surface, but the difference was not significant. The release was less extensive with s-Ti and PVC.

TAT

Activation of the coagulation, as reflected by the generation of TAT showed the highest increase with SLActive. Values of TAT for SLActive increased most but were however not significantly higher than SLA. TAT increased from 4.0 ± 0.4 to 22,183 ± 3,755 µg/L with SLActive: in comparison, it increased to 19,084 ± 4,928 µg/L and 2,379 ± 9,622 µg/L with SLA and s-Ti, respectively. The control surface PVC generated 1,838 ± 771 µg/L (Figure 2).

Contact Activation of Coagulation (Figure 4)

Activation of the intrinsic coagulation system, as reflected by the generation of FXIIa–AT, FXIIa–C1INH,

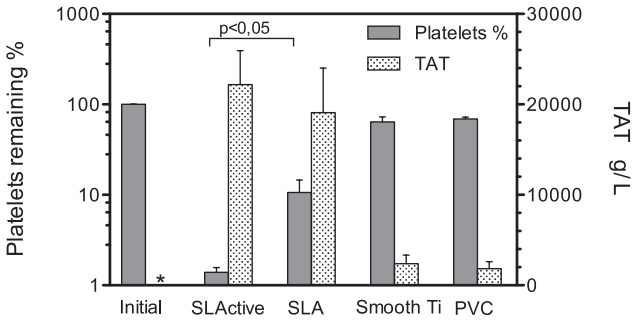

Figure 2 Thrombogenic properties of test surfaces in contact with whole blood, in the slide chamber model and results are presented, as reflected by the amount of platelets and the generation of TAT in blood and plasma samples, respectively. *Initial TAT value not visible. TAT = thrombin-antithrombin complex.

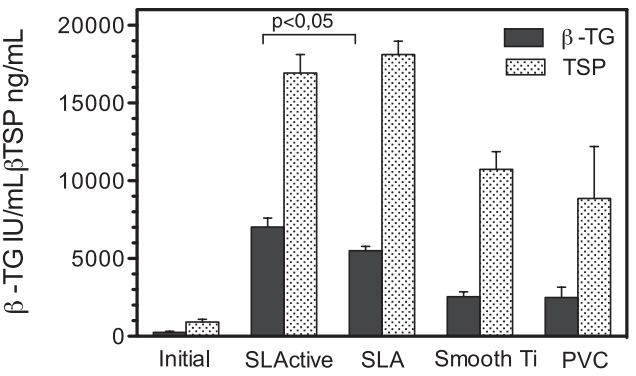

Figure 3 Platelet release of granula content following contact with test surfaces and whole blood in the slide chamber model and results are presented, as reflected by release of β-TG and thrombospondin of in plasma samples.

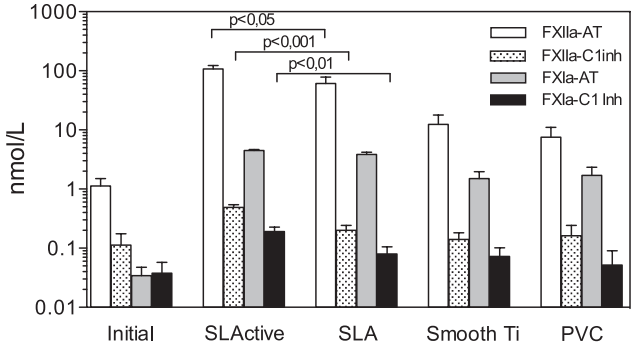

Figure 4 Activation of the contact system in whole blood, in contact with test surfaces were studied in the slide chamber model and results, as reflected in the generation of factor XIIa–C1INH, XIIa–AT, XIa–C1INH and XIa–AT complexes in plasma samples, are illustrated.

FXIa–AT and FXIa–C1INH complexes, showed that the activation was most pronounced with the SLActive surface compared with the SLA surface.

The FXIIa–AT generation was extensive with both SLActive and SLA. However, SLActive was significantly higher than SLA ($p < .05$). The s-Ti and PVC surfaces showed the lowest properties for contact activation. The complex generation increased from 1.12 ± 0.37 to 107 ± 16.0 with SLActive, to 60.8 ± 17.6 with SLA, to 12.3 ± 5.5 with s-Ti and to 7.5 ± 3.6 nmol/L with PVC.

The generation of FXIIa–C1INH increased from 0.11 ± 0.06 to 0.49 ± 0.06 with SLActive, to 0.20 ± 0.04 with SLA, to 0.14 ± 0.04 with s-Ti and to 0.16 ± 0.08 nmol/L with PVC. FXIIa- C1INH increased most with SLActive and was significantly higher than SLA ($p < .001$).

FXIa–AT generation showed a pattern similar with SLActive and SLA surfaces. FXIa–AT increased from 0.03 ± 0.01 to 4.5 ± 0.19 with SLActive, to 3.8 ± 0.34 with SLA, to 1.5 ± 0.46 with s-Ti and to 1.7 ± 0.65 nmol/L with PVC. The value of FXIa–AT was not significantly higher whith SLActive than for SLA.

FXIa- C1INH complex increased from 0.04 ± 0.02 to 0.19 ± 0.04 with SLActive, to 0.08 ± 0.03 with SLA, to 0.7 ± 0.03 with s- i and to 0.05 ± 0.04 nmol/L with PVC. The SLActive increased most. The values of FXIa–C1INH for SLActive was significantly higher than SLA ($p < .01$).

Complement Activation in Whole Blood, in Contact with Clinical Surfaces (Figure 5)

Complement activation, monitored with C3a and sC5b-9 showed a pattern similar for all surfaces. The generation of C3a increased from 39.3 ± 12.3 ng/mL to 827.1 ± 88.3 ng/mL with SLActive, to 776.9 ± 80.7 ng/mL with SLA, to 778.9 ± 118.9 ng/mL with s-Ti and to 924.9 ± 200.7 ng/mL with PVC. The most enhanced generation was obtained for SLActive, but was not significantly higher than SLA.

Generation of sC5b-9 increased from 28.6 ± 10.6 AU/mL to 73.1 ± 7.9 AU/mL with SLActive, to 72.8 ± 4.6 AU/mL with SLA, to 78.1 ± 12.6 AU/mL with s-Ti and to 87.6 ± 19.5 AU/mL with PVC. The highest generation was obtained with PVC. SLActive-values were not significantly higher than for SLA.

DISCUSSION

In the present study, we have investigated the thrombogenicity of a new surface treatment of a clinically available dental implant in contact with whole blood.

We were able to show a rather clear difference between the two test materials regarding the thrombogenic properties. The new hydrophilic surface modification shows enhanced activation of blood coagulation in our in vitro model. It has been well established that other hydrophilic surfaces, such as glass, augments the initial contact activation of coagulation.[12] It is previously shown that the thrombogenic effect of titanium in contact with whole blood is triggered by factor XIIa.[15] A possible explanation to the enhanced thombogenicity may be increased binding of intrinsic coagulation factors to the material surface. However, this paradigm have recently been questioned by Vogler.[16] One can speculate that the hydrophilic surface adsorbs proteins such as fibrinogen in a misfolded way, which in turn promotes both contact activation and platelet binding with subsequent activation.[17,18] Results by Van De Keere and colleagues supports this theory, as they could show larger perturbations to the native structure of fibrinogen

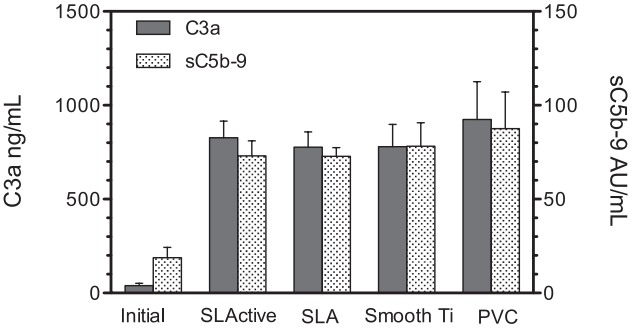

Figure 5 Complement activation in whole blood in contact with clinical surfaces tested in the slide chamber model as reflected by detection of C3a and sC5b-9.

when it adsorbed to titanium.[19] In addition, it is well established that fibrinogen is important for the binding of platelets by GPIIb/IIIa to the material surface.[20]

In our model, we could show increased amounts of C1INH complexes of contact activation factors, implying that the material initiates the contact activation and the initial thrombin formation, which in turn causes platelet aggregation. Indeed there was a strong reduction of the platelet count with both test materials, but the hydrophilic material caused a significant decrease of platelets compared with the hydrophobic one. As a consequence, this new surface with activated platelets serves as an amplifier of contact activation and the coagulation cascade revealed by the relatively high amounts of AT complexes.[21,22] We could also show an increased formation of thrombin reflected as TAT generation with the tested, exceptionally hydrophilic, SLActive surface (not significant). The hydrophilicity is reported to have a profound importance on the activation of blood coagulation. In a study by Yahyapour, it was shown that significantly higher amounts of thrombin were found on hydrophilic titanium surfaces compared with hydrophobic ones.[23] On the other hand, in a recent study by Sperling and colleagues reduced platelet binding and intermediate thrombin formation on a hydrophilic surface was described. The most reactive material consisted of mostly hydrophilic moieties with approximately twenty percent hydrophobic components. Therefore, the increased thrombin formation with SLActive can not solely be explained by the hydrophilic property of the material. The deduction may be that contact activation and platelet adhesion have a strong synergistic effect on coagulation.

Several studies have addressed the importance of blood coagulation and platelet factors for improvement of bone healing. Previously, we have shown that there seems to be a correlation between an implant thrombogenic behavior and enhanced osseointegration.[8]

The experiments in the present study showed increased activation of the intrinsic pathway of coagulation with the hydrophilic surface. It is established that activation of FXII causes augmented fibrinolytic activity via the kallikrein plasminogen pathway. Tanaka and colleagues showed that the inactivation of Plasminogen activator inhibitor-1 (PAI-1) by FXIIa was found to be responsible at least in part for the enhancement of fibrinolysis by activated contact factors.[24] The structure of the generated fibrin clot is proposed to be dependent of the in situ thrombin generation and thereby regulates the wound healing.[25] Additionally, mesenchymal cells may, in the bony compartment, contribute to the lysis of fibrin clots adjacent to the implant surface and facilitate stem cell invasion.[26]

Moreover, there is increasing evidence of the importance of the regulatory role of platelets for early blood vessel development. Platelet granular mediators as growth factors and platelet concentrate have been explored.[27,28] In this work, we were able to show significantly increased release of platelet granule content reflected by β-TG when blood was incubated with SLActive. In a study by Brill it was shown that the highest amount of new vessels were achieved when platelet were activated with thrombin. They attributed this finding to the fact that the full effect only can be achieved by the concerted action of all growth factors within platelets.[27] Platelets have also been shown to contribute to mesenchymal cell proliferation with increased alkaline phosphate activity and bone formation.[29] In an in vitro study, where mesenchymal cells cultured in human blood serum displayed enhanced osteogenic potential, this could be attributed to factors released by platelets.[30] Moreover, the mesenchymal cells have ability to promote angiogenesis in dense fibrin tissues.[31] It has also been shown that platelet-rich plasma activated with thrombin causes enhanced release of growth factors.[32] Further on, in a histological dog study of SLActive by Schwarz, a positive relationship between de novo angiogenesis and new bone formation was indicated.[9] Taken together, these results support our findings that the thrombogenic properties of dental implants seem to correlate with improved healing in bone. The lack of significant difference for thrombospondin in tested plasma samples of SLActive and SLA can be explained by the binding of the molecule to platelets bound on the test surface. The amount of soluble thombospondin in plasma was therefore subsequently decreased as almost all platelets where found consumed by the clot on the test SLActive surface. Binding of thrombospondin to platelets is previously described in the literature by Wolff who could show that there is a specific binding site on thrombin-stimulated platelets.[33] Our results indicate that thrombospondin is not a versatile tool for detection of alfa-granule release from platelets when testing highly thrombogenic materials.

The success of titanium as a material for dental implants may also be explained by TGF-beta released,

for instance, from platelets, which have the ability to promote expansion of peripheral regulatory T-cells suppressing the inflammatory response at a wound site.[34,35] In a study by Suska and colleagues in 2003, it was shown in an animal model that the inflammatory response to titanium discs exhibit a transient initial pro inflammatory cytokine release and sustained IL-10 levels in the tissue.[36] This may partly be explained by the time-dependent degradation of fibrin into peptides. These fragments have been shown to exhibit lymphocyte-suppressive properties.[37] In addition, the complement system has been shown to be involved in induction of peripheral regulatory T-cells.[38] In this study, we could not show any significant difference in complement activation between the two tested dental surfaces. One could maybe expect lower complement activation on the hydrophilic surface while it is known that hydrophobic materials causes denaturation and activation of the key molecule C3.[39] A possible explanation is that most of complement activation occurs on the coagulum as it rapidly grows on the materials. In a study by Hamad and colleagues, it is shown that complement is activated by chondroitin sulfate released by thrombin receptor activated platelets.[40]

Our findings of enhanced deposition of fibrin as well as platelets on SLActive and in the clinic markedly shortened healing time are in contradiction to apatite-generation properties in simulated body fluid (SBF). In a study by Wang and colleagues, it is shown that delayed apatite formation occurs when serum proteins as Albumin are added to SBF.[41] One might speculate that the generation of a thrombus with subsequent release of cell-attracting molecules on the implant surface creates an improved milieu for new bone formation, while the in vitro test with SBF reveals chemical interactions important for the deposition rate of apatite by bone producing cells. Further studies are warranted to elucidate if thrombogenic properties affect apatite formation on dental implants.

CONCLUSION

In summary, the conclusion that can be drawn from the results in our study is that the surface modification of SLActive augments the thrombogenic properties of the material, which would be a favorable feature according to our hypothesis that enhanced thrombogenicity promotes osseointegration and tissue growth.

ACKNOWLEDGMENTS

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