SOFT TISSUE ATTACHMENT TO TITANIUM COATED WITH GROWTH FACTORS

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Abstract

Background: Peri-implant tissues form a crucial but fragile seal between the oral environment, the bone and the implant surface. Enhancing the seal formed by the peri-implant soft tissues at the titanium/connective tissue interface may be an important factor in implant survival. Additionally, enhancing soft tissue adherence to the implant surface when implants are placed in dehiscence type defects may mean that simultaneous osseous grafting procedures will not always be required. **Objective:** The aim of this study was to investigate the effect of implant surface modification with either platelet-derived growth factor (PDGF) or enamel matrix derivative (EMD) on the connective tissue attachment to moderately roughened titanium implants. **Material and Methods:** 18 moderately roughened titanium implants were subcutaneously implanted into 14 rats. 6 implants each were coated with PDGF and EMD immediately priorto implantation and 6 implants were left uncoated. The implants were retrieved with a sample of surrounding tissue at 4 and 8 weeks. The specimens were resin-embedded and sections viewed under confocal microscopy for collagen autofluorescence and prepared for qualitative and histomorphometric analysis under light microscopy. ANOVA and t-tests were used to compare the thickness of fibroblast encapsulation on the implant surface and the depth of connective tissue penetration onto the implant grooves. **Results:** Qualitative analysis under confocal and light microscopy showed encapsulation ofall implants by fibroblasts and good soft tissue integration at the end of 4 and 8 weeks. Coating of the implants with growth factors did not alter the orientation of fibroblasts and collagen fibres. Histomorphometric analysis demonstrated that the depth of connective tissue penetration into the implant grooves was significantly greater for the implants coated with PDGF at 4 weeks (ANOVA, P value 0.0014). The thickness of the fibroblast encapsulation onthe implant surface was significantly less for the implants coated with PDGF at 8 weeks (ANOVA, P value 0.0012). **Conclusion:** Good soft tissue integration can be achieved on a moderately roughenedtitanium implant surface. Coating the implant surface with rhPDGF-BB could increase the speed of soft tissue healing around an implant surface but this increased rate of healing with rhPDGF-BB coating could also result in a less robust titanium/connective tissue interface.

Keywords- rhPDGF-BB, rats, titanium, platelet-derived growth factor

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1. Introduction

Osseointegrated dental implants are transmucosal "masticatory devices" that penetrate the oral mucosa, with the peri-implant tissues expected to exercise a protective function (Weber & Cochran 1998). Research and clinical focus in dental implantology in the past two decades has primarily concentrated on the bone-to-implant interface and the peri-implant mucosa, with the soft tissue seal around implants investigated to a much lesser degree. Both bone and soft tissue integration to dental implants are wound healing processes involving several stages of tissue formation and degradation (Berglundh et al 2003, Abrahamsson et al 2004, Berglundh et al 2007). Osseointegration is the result of the modelling and remodelling of bone tissue thatoccurs after implant placement, whilst the wound healing that occurs following the closure of mucoperiosteal flaps during implant surgery results in the establishment of a mucosal attachment (transmucosal attachment) to the implant. The establishment of the mucosal barrier around the implant is characterised by a gradual shift from a coagulum to granulation tissue followed by the formation of a barrier epithelium and the formation of connective tissue(Berglundh et al 2007).

Several studies using animal and human models have investigated the structure and function of the peri-implant mucosa (Berglundh et al 1991, 1992, 1994, 2003, 2007; Buser et al 1992, Ericsson et al 1996, 1997; Abrahamsson et al 1996, 1997, 1998, 1999, 2002, 2004; Berglundh & Lindhe 1996, Cochran et al 1997, Moon et al 1999, Glauser et al 2005,Schüpbach & Glauser 2007, Welander et al 2007, 2008, Allegrini Jr et al 2008, Nevins et al 2008). In an early study in dogs, Berglundh et al (1991) compared the gingiva around teeth and the mucosa around two-stage implants (Branemark System®, Nobel Biocare, Götenburg, Sweden). It was found that the peri-implant mucosa consisted of a 2 mm long barrier epithelium and a zone 1-1.5 mm high where the connective tissue was in direct contact with the TiO_2 layer of the implant. This area was termed a zone of "connective tissue integration".

Histologically, the peri-implant epithelium and the surrounding connective tissue of dental implants have similar characteristics to those structures surrounding teeth (Abrahamsson & Soldini 2006) but differ in terms of the orientation of collagen fibres (Buser et al 1992), the composition of the connective tissue (Moon et al 1999, Abrahamsson et al 2002), and the distribution of the vascular system of the peri-implant mucosa (Berglundh et al 1994). The connective tissue in the zone of integration has a low density of blood vessels but a large number of fibroblasts and collagen fibres appearing to originate from the periosteum of the bone crest and extending towards the margin of the soft tissue in a direction parallel to thelong axis of the abutment. More detailed analyses of the soft tissue/implant interface using transmission electron microscopy found that the zone of connective tissue directly adjacent to the implant surface has a large number of round and flat-shaped fibroblasts with their long axes parallel with the implant surface but virtually no blood vessels. Further away from this zone the number of fibroblasts decreases but there are more collagen fibres and there is an increase in vascularity (Moon et al 1999, Abrahamsson et al 2002). Berglundh et al (1991) stated that the main difference between the mesenchymal tissues present at a tooth and at an implant site is the occurrence of cementum (acellular or cellular) on the root surface.

There is no doubt that the peri-implant soft-tissues form a crucial seal between the oral environment, the bone and the implant surface (Cochran et al 1994, 1997). The seal is fragile and due to the absence of periodontal ligament fibres when subjected to bacterial or mechanical challenge the

destruction of peri-implant tissues can be a faster and more devastating process than in periodontal tissues (Salcetti et al 1997, Maksoud 2003). Thus, enhancing the seal formed by the peri-implant soft tissues, especially that of thetitanium/connective tissue interface, may be an important factor in implant survival.

The titanium/connective tissue interface, certainly for smooth, machined surface dental implants, lacks a mechanical attachment of inserting collagen fibres, unlike that of periodontaltissues of teeth. Whether this lack of mechanical attachment differs for roughened surface implants has not been extensively investigated. A small number of recent in vivo studies have indicated that microtexturing of the implant can be used to control the soft tissue response (Glauser et al 2005, Schüpbach & Glauser 2007, Nevins et al 2008). Until recently most dental implants were designed such that the transmucosal portion of the implant was of a smooth or polished nature. Recently, these design concepts have changed, with several implant designs allowing crestal placement and incorporating roughened surfaces into the coronal portion of the body of the implant, up to the level of the implant-abutment platform (eg. Nobel Replace, Straumann Bone-level, Astra Osseospeed). Some clinicians have advocated that roughened surfaces may in fact be conducive to very good soft tissue adherence in dehiscence type defects and therefore placement of implants into these defects may not always require osseous grafting procedures to correct these defects (Dragoo, personal communication).

Surface modification of titanium implants may improve the ability of connective tissue components in the peri-implant mucosa to attach to the implants. Currently, most dental implant types incorporate a "roughened" surface as part of their macro-design. Many of these surfaces are able to absorb proteins and thus act as a reservoir or carrier for attachment proteins, growth factors and other biological agents which may be of assistance for soft or hard tissue integration. In vitro studies have shown that epithelial cell adhesion to titanium surfaces coated with biological agents such as fibronectin, laminin and collagen was enhanced in comparison with uncoated titanium (Dean et al 1995, Tamura et al 1998, Park et al 1998, Roessler et al 2001, Nagai et al 2002). However, in a recent study investigating soft tissue healing around implants in a canine model, it was found that the vertical dimensions of the epithelial and connective tissue components as well as the composition of the connective tissue zone directly adjacent to the implant were similar for collagen-coated and non-coated implants at 4 and 8 weeks of healing (Welander et al 2007).

The hypothesis for this study is that surface modification of roughened surface (TiUnite) titanium implants with PDGF or EMD results in improved bioactivity of the implant surface, thereby promoting cell attachment and CT formation, which is expected to result in an improved attachment. The aim of this study is to investigate if surface modification of roughened surface (TiUnite) titanium implants with PDGF or EMD has the potential to enhance connective tissue attachment to titanium implants.

2. Methodology

Fourteen female Dark Agouti (DA) rats, each about 6 to 8 weeks old were used. These were acquired through the Animal Services Division, Indian Veterinary Research Institute Bareilly (IVRI), Adelaide. The research protocol related to the use of animals in this study was approved by the animal ethics committees of both the University of Adelaide and the IMVS. Eighteen Branemark System® Mk III Groovy NP (3.3 mmØ x 10 mm) (Nobel BiocareAB, Göteborg, Sweden) implants were used. Six test implants were coated with enamel matrix protein derivative

(Emdogain®, Biora AB, Straumann, Malmö, Sweden) and 6 test implants were coated with reconstituted recombinant human platelet-derived growth factor- BB (rhPDGF-BB, Pepro Tech, Rocky Hill, New Jersey, USA). The 6 control implants were uncoated. Two of these control implants were used for an initial pilot study to verify the feasibility of the experimental protocol.This experiment was conducted to check on the viability of the study, i.e. the ability to get a meaningful sample by ensuring that the animals tolerated the implants and thatthere were no ill effects over the time course planned for this study. For the major experimental study, there were three groups:

Group 1 – Group 1 – Animals with uncoated implants

In this group, two rats each had two uncoated implants surgically implanted, making a totalof four uncoated implants for the group.

Group 2 – Animals with enamel matrix protein derivative (Emdogain®) coated implants

Six implants coated with Emdogain® were placed into four animals. Two rats received twocoated implants and the other two rats had only one coated implant placed into their backs.

Group 3 – Animals with platelet-derived growth factor (rhPDGF-BB) coated implants

This group also had four animals with two rats receiving two rhPDGF-BB coated implants while the other two had only one coated implant surgically implanted, making a total of six rhPDGF-BB coated implants.

Animals in each group were sacrificed at 4 weeks and 8 weeks. At the time of sacrifice, theimplants and surrounding tissues were surgically retrieved, processed and analysed. Two implants were retrieved from Group 1 at each time point while 3 implants were retrieved from each of the other groups at each time point. A total of 16 implants were used for the major study.

Surgical Procedures

All surgical procedures were performed using inhalation anaesthesia induced with 2% v/v isofluorane with O_2 flow rates set at 2L/min. A modification of the implantation model used by Bartold et al (1989) was employed. Following the administration anaesthesia, a subcutaneous incision measuring approximately 20 mm was made along the ventral midline between the left and right shoulders (Figure 1).

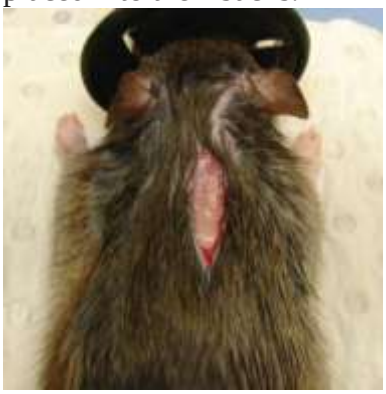


Figure 1. Initial subcutaneous incision in anaesthetised animal prior to implantation.

Figure 2. Implantation into subcutaneous pouch.

A subcutaneous pouch above either the right or left shoulder was created for the placement of the implant (Figure 2). If two implants were to be placed, then pouches were created below the left and right shoulder. The control implants were placed uncoated and the test implants were either coated with Emdogain® or rhPDGF –BB by submerging them for 30 seconds in the freshly prepared growth factor contained within an Ependorf tube (Figure 3) before immediate placement into the subcutaneous pouches.

Figure 3. Coating of test implant with growth factor

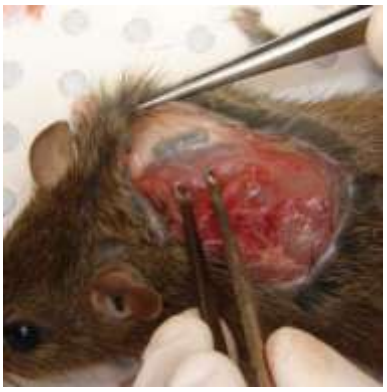


Figure 4. Implant in euthanized animal prior to retrieval.

After the implants were secured in their positions, the incision was closed using staples and swabbed with Betadine. Post-operatively the rats were administered 22.7 mg/ml enrofloxacin orally for 1 week. The staples were removed 2 weeks after implant placement and the rats were monitored daily and weighed weekly during the healing period.

The rats were euthanized by CO_2 asphyxiation and the implants were located through implantation records and palpation. For implant retrieval, a similar but larger ventral incision was made and the implant retrieved with a sample of surrounding tissue (Figure 4). The retrieved samples were placed in a fixative (10% PBS buffered formalin) for 48 hours prior to processing into resin blocks. The retrieved implant/tissue biopsies were transferred from the fixative and dehydrated in serial steps of alcohol concentrations and subsequently embedded in a methyl-methacrylate resin.

Confocal laser scanning microscopy of the sectioned implant/tissue resin embedded blocks was carried out using a Leica TCS SP5 Confocal Microscope System (Leica Microsystems, Heidelberg, Germany). The implant/tissue block sections were viewed using a 20x IMM objective lens (magnification of x200) on the Leica DMI6000B inverted microscope (Leica Microsystems, Heidelberg, Germany), using the argon-neon laser set at a power setting of 40% and emitting at a wavelength of 458 nm, allowing confocal laser scanning microscopic analyses of collagen autofluorescence. Mean values for each variable were calculated for each implant unit. The differenceswithin the 4 week and 8 week groups of control and coated implants were analysed using a one-way ANOVA and Bonferroni's Multiple Comparison Test was used as a post test. The null hypothesis was rejected at $P<0.05$. The differences between the control implants at 4 weeks and 8 weeks and the coated implants at 4 weeks and 8 weeks were analysed using Student's t-tests. Once again the null hypothesis was rejected at $P<0.05$. Statistical analysis was

carried out using a SPSS Version 22.

3. Results

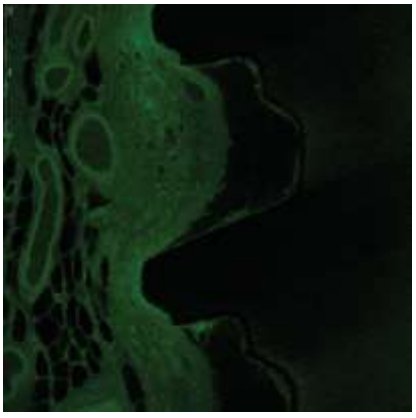
Healing following implant placement was uneventful for all animals involved in the study. The incision wounds appeared to have healed by 4 weeks. Although 18 implants were placed in the rats (including the pilot study), 16 were retrieved. Two implants were not recovered from two animals at the 8 week time point. One of these lost implants had been coated with Emdogain® and the other with rhPDGF-BB. This reduced the number for analysis to 5 for each coated groups at the 8 week time point.

Histological Assessment – Qualitative Analysis at Four weeks

Fibrous encapsulation of the control and growth factors coated implants was evident after 4weeks. The fibroblast layer adherent to the implants and the surrounding connective tissues appeared well-organised with little indication of any residual inflammation. The images seen under confocal microscopy were well correlated with the images seen for the H&E stained thin sections viewed under light microscopy For the control uncoated implant viewed under confocal implant at 4 weeks.

microscopy, collagen autofluoresecence indicated that the collagen fibres were aligned parallel with the long axis of the implant, with a high concentration of collagen. The same thin sections, when stained with H&E, showed a dense distinct layer of fibroblasts over the implant threads and suspended over the implant grooves, surrounded by less dense connective tissue (Figure 8b). The fibroblasts also appeared to be aligned parallel to the long axis of the implant. A thin (1-3 cells thick) but distinct cellular layer, in intimate contact with the surface of the implant grooves, that autofluoresced for collagen was evident . The collagen fibre orientation and fibroblast alignment observed in the coated implants at 4weeks was no different to that reported for the uncoated implants when viewed under confocal and light microscopy. However, from this qualitative histological analysis, there appeared to be a greater depth of connective tissue penetration into the implant grooves with the Emdogain® and PDGF coated implants and a thicker dense cellular/fibroblast layer with the Emdogain® coated implants. The presence of an adipose-like cell layer almost devoid of other cell types surrounding the dense fibroblast layer was a distinctive featu of the rh-PDGF-BB coated

Figure 5. Control (uncoated) implant at 4 weeksof healing. Thin section (16μm/unstained), confocal microscopy, thread 4 (LHS), original magnification x200.



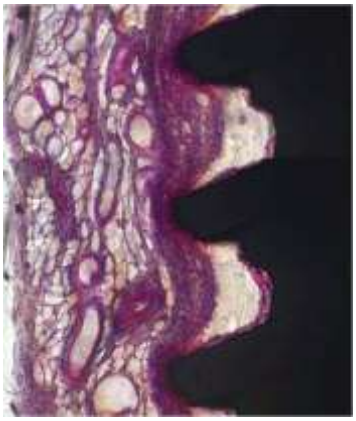


Figure 6. Control (uncoated) implant at 4 weeksof healing. Thin sections (16μm/H&E stained), light microscopy, threads 3-5 (LHS), original magnification x 200 (composite image).

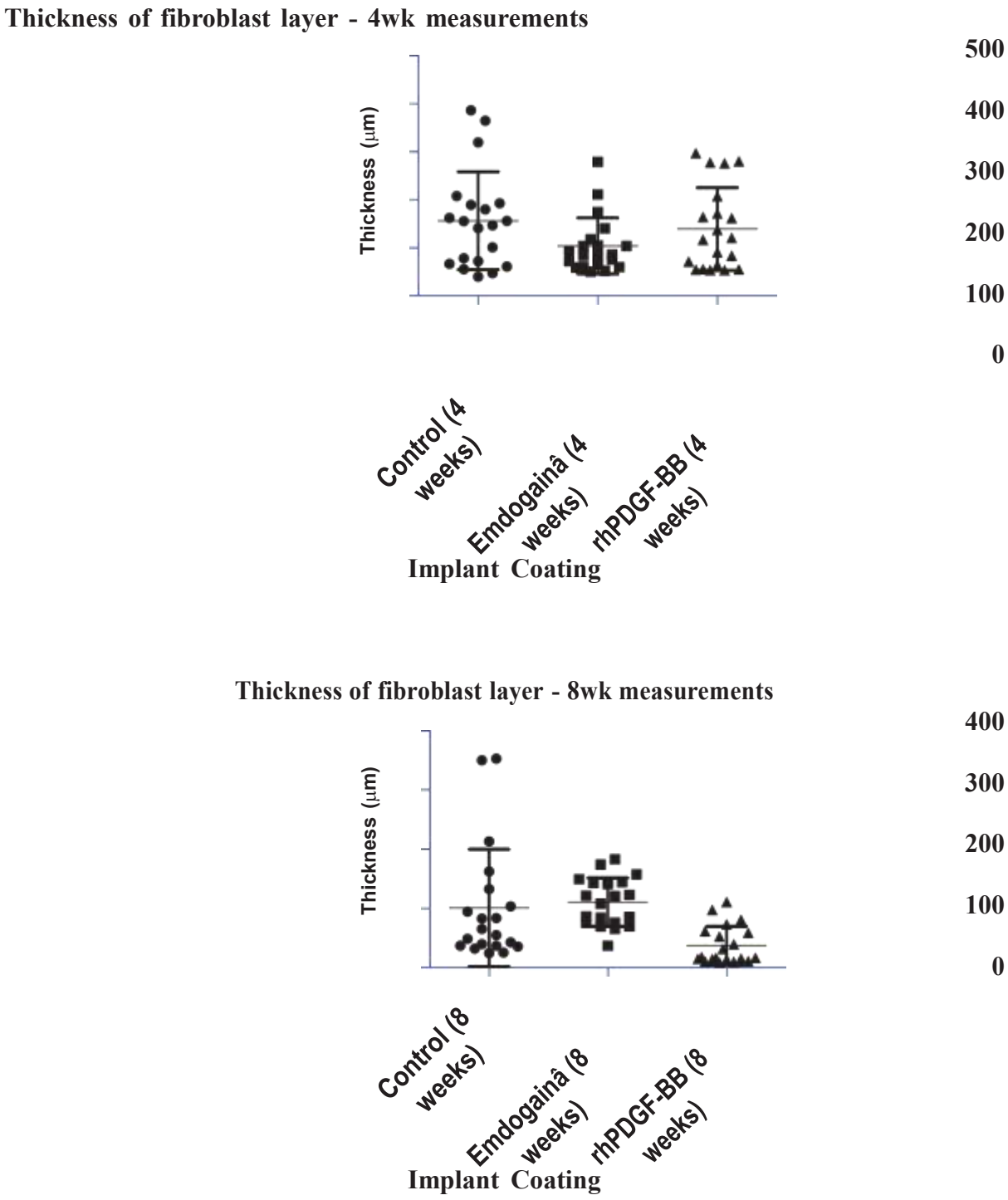


Figure 7. Vertical scatter plots illustrating the thickness of the fibroblast layer at implant threads 1 -10 (LHS and RHS) of uncoated and coated implants at (a) 4 weeks and (b) 8 week.

Control (uncoated)		Emdogain□	rhPDGF-BB
Number of values	20	20	20
4 Weeks			
Minimum	39.86	50.17	52.82
Maximum	386.0	278.4	296.3
Mean	155.9	103.7	139.2
Std. Deviation	102.2	58.21	86.33
One-way ANOVA P value 0.1431			
(ns)			
Number of values	20	20	20
8 Weeks			
Minimum	24.25	36.88	8.310
Maximum	352.5	183.1	110.6
Mean	100.9	110.6	37.54
Std. Deviation	98.72	40.74	32.24
One-way ANOVA P value 0.0012			
(s)			
t-test (4 wks. Versus 8 wks.) P value	0.0912 (ns)	0.06638 (ns)	<0.0001 (s)

Table 1. Thickness of fibroblast layer at the implant thread – 4 and 8 week measurements (μm).

	Control (uncoated)	**Emdogain□**	**rhPDGF-BB**
Number of values	20	20	19
4 Weeks			
Minimum	80.40	95.35	112.6
Maximum	182.4	170.4	194.7
Mean	127.2	131.5	156.9
Std. Deviation	24.52	22.46	30.53
One-way ANOVA P value 0.0014			
(s)			
Number of values	20	20	20
8 Weeks			
Minimum	131.2	118.3	120.6
Maximum	214.6	198.3	205.0
Mean	165.4	174.0	170.1
Std. Deviation	25.44	17.89	25.51
One-way ANOVA P value 0.5092			
(ns)			
t-test (4 wks. versus 8 wks.) P value	<0.0001 (s)	<0.0001 (s)	0.1515 (ns)

Table 2. Depth of connective tissue penetration into the implant grooves – 4 and 8 week measurements (μm).

The mean thicknesses of the fibroblast layer at implant threads 1 – 10 (LHS & RHS) for the uncoated, Emdogain® and rhPDGF-BB coated implants were 155.9 ± 102.0μm, 103.7 ± 58.2μm and 139.2 ± 86.3μm respectively at 4 weeks and 100.9 ± 98.7μm, 110.6 ± 40.7μm and 37.5 ± 32.2μm at 8-weeks. One-way ANOVA found that there were no significant differences between the thicknesses of the fibroblast layer at the implant threads between the uncoated and coated implants at 4- weeks (P = 0.1431) (Table 1). However at 8 weeks, a significant difference in the thicknesses of the fibroblast layer (P = 0.0012) (Table 1) was detected. Using Bonferroni's Multiple Comparison Test as a post-test, the rhPDGF-BB coated implant was found to have a significantly thinner fibroblast layer at the implant threads than the uncoated (t = 3.112) and Emdogain® coated (t = 3.589) implants (Appendix 2.12). There was no significant difference in the thickness of the fibroblast layer between the Emdogain® coated and uncoated implants at 8 weeks, confirmed by the post-test (t = 0.4763).

Student's t-tests indicate that there was no significant change in the thicknesses of the fibroblast layer for the uncoated and Emdogain® coated implants between 4 and 8 weeks (Table 1). This was not the case for the rhPDGF-BB coated implants where the thickness of the fibroblast layer significantly decreased between 4 and 8 weeks (P < 0.0001) (Table 1).

The mean depth of connective tissue penetration into implant grooves 1 – 10 (LHS & RHS) for the uncoated, Emdogain® and rhPDGF-BB coated implants were 127.2 ± 24.5μm, 131.5 ± 22.4μm and 156.9 ± 30.5μm respectively at 4-weeks and 165.4 ± 25.4μm, 174.0 ± 17.9μm and 170.1 ± 25.51μm at 8-weeks.

4. Discussion

The adherence of the peri-implant tissues to the implant/abutment surface is crucial to its function as a barrier between the oral environment and the bone and implant surfaces. Enhancing this adherence by surface modification with biological agents can serve to improve implant survival, as well as potentially contribute to an improvement in implant success rates by preventing recession and improving aesthetic outcomes.

The aim of this study was to investigate the connective tissue attachment to the roughened surface of (TiUnite) titanium implants and roughened surfaces modified with rhPDGF-BB or EMD. Although there are distinct differences between gingival and subcutaneous connective tissues in terms of remodelling, turnover rates and architecture, both of these connective tissue types contain principally type I and III collagen as the most abundant biochemical component. As this study investigates connective tissue attachment by examining the collagen fibreorientation to the implant surface, the use of a subcutaneous murine model, whilst not mimicking the conditions in the oral cavity as accurately as would a buccal dehiscence model in a larger animal, does provide an appropriate cost-effective means to test the hypothesis of this study.

To our knowledge, this is the first study to utilize confocal laser scanning microscopy (CLSM) and collagen autofluorescence to image connective tissue attachment to titanium implants. The basic rationale behind CLSM is that illumination of tissues with a short wavelength light from a monochromatic punctiform laser source leads to excitation of endogenous substances, resulting in the emission of fluorescence light of longer wavelengths. The resulting emission energy is detected by a spatially filtered optical system, the pinhole, which filters out light signals from out of focus planes (Lucchese et al 2008). Amongst the molecules, called fluorophores, responsible for this tissue autofluorescnce include collagen (DaCosta et al 2002, 2003). Recent studies investigating the collagen fibre orientation in the peri-implant mucosa have used a variety of methods including: decalcified

ground sections stained with toluidine blue (Abrahamsson & Cardaropoli 2007, Berglundh et al 2007, Welander et al 2007, 2008; Nevins et al 2008), decalcified ground sections stained with methylene blue/Azure II (Schüpbach et al 2007), "fracture technique" sections stained in PAS and toluidine blue (Welander et al 2007), scanning electron microscopy (Schüpbach et al 2007, Welander et al 2007, Nevins et al 2008, Tete et al 2009), transmission electron microscopy (Schüpbach et al 2007) and circular polarised light microscopy (Allegrini Jr et al 2008, Tete et al 2009). Collagen fibre orientation in bone around osseointegrated implants have also been investigated using circular polarised light microscopy with tissue incorporated fluorescent dyes in human peri-implant bone (Traini et al 2005) and peri-implant bone from minipigs (Neugebauer et al 2006). These methods, whilst effective and allowing for high quality imaging of collagen, require complex and time-consuming sample preparation techniques.

Recently, Lucchese et al (2008) analyzed collagen fibre distribution in human crown dentine using CLSM and found an intense autofluorescence that was ascribed to collagen fibres in all their samples. In our study, we were able to correlate the collagen autofluorescence seen in the CLSM images to the fibroblasts observed in the same thin sections when stained with H&E and viewed under light microscopy. The use of CLSM thus appears to provide a less time-consuming method of preparing tissue samples and therefore is a useful auxiliary tool for investigating the presence, distribution and collagen fibre orientation in the peri-implant soft tissues.

In this study, two of the coated implants, one coated with Emdogain® and the other coated with rhPDGF-BB, were not recovered from two animals at the 8 week time point. The reasonsfor this exfoliation are unknown. We suspect that this exfoliation would have occurred early on in the experiment as healing following implant placement was uneventful for all animals involved in this study. A distinct encapsulation by a layer of fibroblasts occurred around allthe retrieved implants, regardless of whether the implants were coated or uncoated. This is similar to what occurs with an osseointegrated implant intraorally, whereby the connective tissue forms a non-vascularised, circular, scar-like structure around the transmucosal portion of the implant. The qualitative analysis and histomorphometric measurements of the uncoated implants indicate that resolution of inflammation and connective tissue formation appeared to be completed by 4 weeks. However the healing process, which included tissue maturation and organisation continued between the 4 and 8 week period, as evidenced by the significant change in depth of connective tissue penetration into the implant grooves. Our observations ina murine model appear to be consistent with the conclusions made recently by Berglundh et al (2007). In their investigation of the morphogenesis of the peri-implant mucosa in a canine model, they concluded that the peri-implant mucosa exhibited minor signs of inflammation during the first 2 weeks of healing but from 4 weeks, the mucosa was stable and well attached to the bone. Berglundh et al (2007) further concluded that the soft tissue barrier adjacent to titanium implants placed in a non-submerged protocol takes about 6 to 8 weeks to establish a soft tissue barrier with proper dimensions and tissue organization.

However, in this pilot study, with and without the application of EMD, an implant-connective tissue interface morphologically consistent with a periodontal connective tissue attachment was not observed in sections from any of the implant or autogenous cell grafts (Craig et al 2006). In this study, surface modification of the TiUnite surface of titanium implants with Emdogain® or rhPDGF coating was not found to change the orientation of the fibroblasts or collagen

fibres in the encapsulating fibroblast layer. The orientation of the fibroblasts and collagen fibres when viewed under light microscopy and confocal laser scanning microscopy respectively appeared parallel to the long axis of the implant. Although reorientation of the fibroblasts and collagen fibres did not occur, there was good adaptation of the fibroblast layer onto the TiUnite surface and implant grooves for both the uncoated and growth factor coated implants at the end of the study period. This could indicate a degree of soft tissue integration onto the TiUnite surface that is more adherent than previously thought.

5. Conclusion

In conclusion, this study shows that good soft tissue integration can be achieved on a moderately roughened TiUnite surface. Surface modification of the TiUnite surface by coating with rhPDGF-BB could increase the speed of soft tissue healing around the implant surface. However, the increased speed of healing with rhPDGF-BB coating could result in a less robust titanium/connective tissue interface. The positive influence of implant surface modification with Emdogain® on soft tissue attachment and maturation around the implant surface should not be discounted and more research into this area is warranted.

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