On the formation of fibrous capsule and fluid space around machined and porous blood plasma clot coated titanium

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Machined and machined submicron porous titanium, with and without a thin blood plasma coating (100 nm), were implanted for 7 or 28 days in subcutaneous pockets on the back of the rat. After explantation the specimens were analyzed by light microscopy with respect to thickness of the fibrous capsule, the fluid space width between implants and fibrous capsule, and formation of blood vessels. The results at 7 days indicate a thinnest fluid space for the plasma clot coated porous titanium surface, and the spaces vanished at the light microscopic level after 28 days outside all the analyzed surfaces. The thickness of the fibrous capsule increased outside the different surfaces at 7–28 days, and in this respect no significant differences were observed between the different surfaces at any time. Analysis of neovascularization showed that the number of vessels and proportion of vessels in the fibrous capsule increased with time at all surfaces, except machined Ti where the number instead decreased from 7 to 28 days. The average distance between the blood vessels and the fluid space increased with time for all types of surfaces. The results in the present study indicate that the healing process around titanium can be modulated by porosity and thin pre-prepared plasma coatings.

1. Introduction

The surgical trauma caused by the implantation of titanium induces a series of inflammatory events, and an unwanted fibrous capsule may surround the implant after the wound healing period [1, 2]. One means to improve the healing process around the implants may be to change the surface topography [3, 4]. A surface enlargement facilitates mechanical interlocking and transport of nutrients and waste products at the interface [5]. It has, for example, been found that adhesion, migration area and the extracellular matrix production by osteoblasts and corneal cells were larger on rough than smooth surfaces (surfaces with pore sizes 0.1–10, 0.07–3.34 respectively 100–1000 μm) [6], although the behavior of implant close inflammatory cells during the very early phase is still poorly understood. In a previous work the recruitment of PMN and monocytes/macrophages was higher to plasma- than serum-coated surfaces, indicating that fibrin/fibrinogen was important for the inflammatory cell recruitment [7]. The fibrin clot network functions as a scaffold or framework for activated cells, and cellular mitosis and migration become stimulated [8–10]. The significance of the fibrin layer for the wound healing process at biomaterial interfaces is not well understood but believed to be important [11].

In the present work, approximately 100 nm thick plasma clot layers were prepared through a repeated clotting of rat plasma [12] on machined and submicron porous titanium. The implants were inserted subcutaneously in rats for 7 or 28 days and the width of the fluid space, the capsule thickness and the vessel formation in the capsule were analyzed by light microscopy.

2. Materials and methods

2.1. Implants

Machined circular c.p. titanium implant disks (Ti) (1 mm thick and ∅ 10 mm) were made (Meditech, Sweden). Half of the implants were etched porous to obtain a pore diameter of 200–300 nm, and with a porous layer thickness of about 700 nm (called PTi). The machined discs were then washed in acetone, ethanol and Milli-Q water in an ultrasonic bath and etched porous in 5 M NaOH aqueous solution at 60 °C for 24 h [13]. The disks

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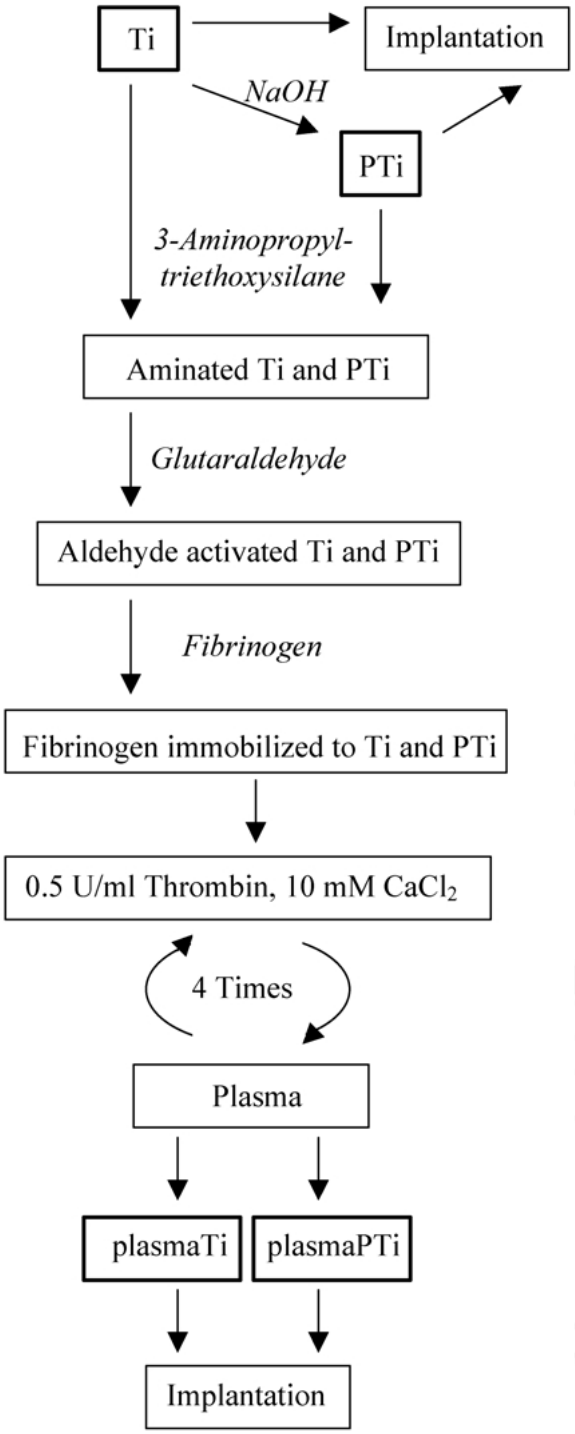

Figure 1 Preparation schedule for the implanted surfaces: machined titanium (Ti) and machined porous titanium (PTi) with and without a thin (100 nm) blood plasma clot film.

were washed in acetone, ethanol and Milli-Q water in an ultrasonic bath, and dried for one week. The non-etched disks were treated similar to the porous but without the NaOH treatment. All disks were oxidized for 15 min on each side in an UVozone-cleaner (Model 42-220, Jelight Company Inc.). The preparation steps are shown in Fig. 1.

In order to prepare disks with a thin film of immobilized coagulated blood plasma (called plasmaTi and plasmaPTi) half of the porous disks and half of the non-porous were put (standing on the edge) in a chamber together with 0.2 ml 3-aminopropyltriethoxysilane, $H_2N(CH_2)_3Si(OC_2H_5)_3$, (APTES) [14–17], and reacted at 60 °C at 6 mbar pressure for 30 min. The temperature was then increased to 150 °C for 1 h. The disks were rinsed for 2 min in xylene in an ultrasonic bath, rinsed with ethanol (99%) and stored in ethanol. The so aminated disks were rinsed in distilled water (aqua dest) and incubated for 30 min in 6% glutaraldehyde, $OHC(CH_2)_3CHO$, in 0.05 M TRIS-buffer pH 9, at 21 °C [14, 15], rinsed three times in a 20 mM HEPES-buffer pH 7.4 with 0.15 M NaCl added. The aldehyde activated implants were incubated for 30 min at room temperature in 0.11 mg/ml rat fibrinogen (Sigma-Aldrich) dissolved in 20 mM HEPES-buffer pH 7.4, with 0.15 M NaCl and 0.06 mM sodium citrate added, and rinsed three times in HEPES-buffer. The fibrinogen coated surfaces were alternatingly incubated in two baths for 5 min (room temperature), four times in each. The first bath contained 0.5 U/ml rat thrombin (Sigma-Aldrich), 0.01 M Ca^{2+}, 20 mM HEPES-buffer pH 7.4, 0.15 M NaCl and 0.05 mg/ml albumin from chicken egg. The second bath 500 ml citrated rat plasma (platelet poor freshly frozen at −80 °C and thawed just before use, from Bacteriological laboratory of Sweden). By this procedure an approximately 100 nm thick plasma clot layer was created on the disks (plasmaTi and plasmaPTi). The implants were considered as sterilized after storage in 99% ethanol for more than 24 h. During the plasma clot preparation, sterilized equipment was used and the solutions were sterile filtered, except for the plasma itself. No traces of endotoxin were found on implants without plasma coatings (Limulus Amebocyte Lysate test). The plasma clot coated implants showed endotoxin levels between 6.6 and 16 and in one case 24 EU/ml.

2.2. Animal model

Female Sprague–Dawley rats (250 g) were anesthetized with intraperitoneal injections of 1 : 1 : 2 solution of sodium pentobarbital (60 g/l, Apoteksbolaget, Sweden), 0.9% saline and diazepam (5 g/l, Apozepam®, Apothekarnes Laboratorium AS, Norway), and their backs shaved and cleaned with chlorohexidine (in 70% ethanol). Each rat received one implant of each kind (Ti, PTi, plasmaTi and plasmaPTi) in subcutaneously created pockets in the back. In order to evaluate the cellular response due to the surgery, 3–4 sham sites were done for each implantation time (surgery without implant insertion). The wound edges were closed with 2 sutures (Suturamide® 5-0). Implants with surrounding tissue were retrieved after 7 and 28 days, respectively. The rats were fixed by perfusion (via the left heart ventricle) with 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) after pre-perfusion (1 min) with 0.05 M cacodylate buffer. The implant and surrounding tissue were excised *en bloc* and immersed in 2.5% glutaraldehyde over night. Skin and excessive tissue were removed carefully by dissection. The specimens were dehydrated in a graded series of ethanol and embedded in LR White (The London Resin Co. Ltd, Hampshire, UK). The experiments were approved by the Local Ethical Committee for Laboratory Animals (116/99).

2.3. Sectioning

The polymerized specimen block was divided into one half and two quarters by sawing. One quarter of the tissue block was processed using an electrochemical technique [18]. After electrochemical dissolution of the bulk Ti the specimens were reembedded in Agar 100 (Agar Aids, Stanstead, England) and about 0.5 μm thick sections were cut and stained [19] for light microscopy (Microm HM 350, Zeiss, Germany).

2.4. Morphometry

Light microscopy (LM, magnification × 160) was used to evaluate the tissue morphology (Leitz microscope equipped with a Microvid Image Analysis System). Two quantitative tissue parameters were analyzed at five equally distributed locations on both sides of the implant disk: the width of the fluid space (separating the implants from the fibrous capsule) and the thickness of the fibrous capsule surrounding the implants. In addition, the number of vessels, the vessel surface area and the distance of blood vessels to the fluid space were determined in a defined total area (100 μm times the fibrous capsule thickness) in three equally distributed sites in the fibrous capsule on both sides of the implant disk.

2.5. Statistics

Mean values (based on 6–8 observations) were calculated for each implant ± standard error of the mean (SEM). In the non-parametric Wilcoxon Signed Rank test [20], matched pairs were made within the same rat, (i.e. each rat was its own control). By this procedure inter-individual differences were excluded. The Mann–Whitney (Wilcoxon two sample) test was applied for the analysis between the time periods [20]. The statistics were performed without adjusting for multiple testing.

3. Results and discussion

The aim of the present study was to evaluate the influence of two separate implant parameters on the morphology of the tissue around the implants: the widths of the fluid space and the fibrous capsule and the proportion and distribution of blood vessels in the capsule. One parameter was the surface topography, comparing machined and porous structures (pore size of 200 nm). The porous surface with sub-surface channels and many holes could hypothetically reduce the contact area to adhering cells, thereby lowering the cellular stress that may be caused by the cell to implant contact. The second variable was the presence or absence of a thin plasma clot coating. Experimental studies using titanium in soft tissues reveal a transient inflammatory response, followed by the formation of a fibrous capsule. Studies comparing titanium with other materials indicate that the leukocyte recruitment differs between titanium and other materials. However, very little is known about how other parts of inflammation and tissue repair are influenced by material properties and whether titanium has special characteristics.

3.1. Fluid space

After seven days the implant was separated from the fibrous tissue by a fluid space. The narrowest space was found outside the plasma coated porous titanium surface (Fig. 2). Our observations after 28 days showed that the fluid space was largely absent around all surface modifications, in agreement with previous observations after six weeks using machined titanium [21, 22]. In parallel with the recruitment of the inflammatory cells, extracellular fluid and proteins accumulate in the tissue, creating a fluid space close to the implant surface [21, 22]. Previous immunohistochemical studies around machined titanium one week after implantation demonstrated albumin, complement factor C3c, immunoglobulins, fibrinogen and fibronectin [23]. The fluid space contained plasma proteins at an apparently low concentration, indicating that the fluid has a composition similar to the extracellular fluid in general and less than plasma. After six and 12 weeks, the expression and distribution pattern of proteins had changed [24]. Fibronectin, but not fibrinogen, was detected in the surrounding tissue around machined titanium. Collagen type I had a distribution similar to that of fibronectin, reaching close to the titanium oxide, but always separated from it by intervening layers of macrophages.

3.2. Fibrous capsule

The organization of the wound around titanium leads to the formation of a fibrous capsule. The fibrous capsule was defined by the presence of collagen bundles, elongated fibroblasts and inflammatory cells arranged in parallel with the implant surface. The fibrous capsule thickness was about 80 μm for all types of implants after seven days and around 200 μm after 28 days of implantation (Fig. 3).

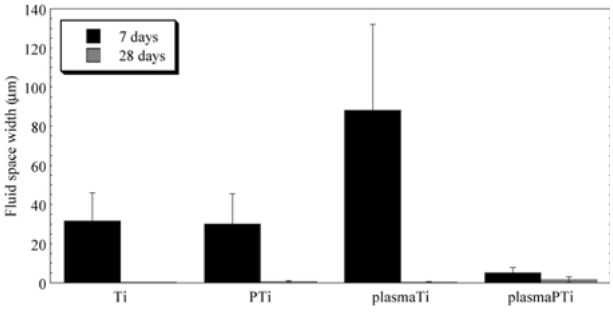

Figure 2 Width of the fluid space at seven or 28 days of implantation at the different surfaces, mean ± SEM, $n = 6$–8.

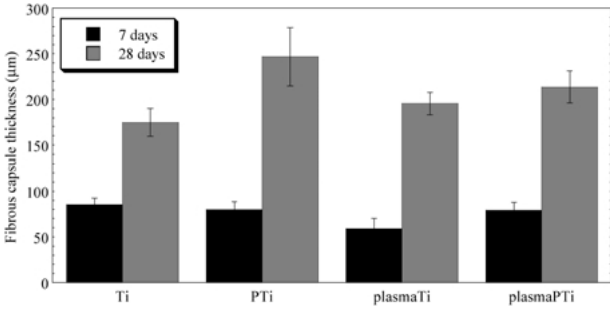

Figure 3 Thickness of the fibrous capsule at seven or 28 days of implantation at the different surfaces, mean ± SEM, $n = 6$–8.

No significant differences between the surfaces were observed at any time period. On the other hand, the present results showed a significant increase in fibrous capsule widths between seven and 28 days. The latter observation is in contradiction to findings in a previous study showing that the width of the fibrous capsule decreases with time (up to 30 days after implantation in rats) [2]. One possible explanation for this discrepancy could be that the extent of the fibrous capsule is difficult to define, in particular the outer boundary, therefore leading to large variations between studies.

Our observations on the similarity of the fibrous capsule width between different titanium surface modifications are in agreement with long-term studies on the fibrous capsule around implants showing no significant differences in fibrous capsule widths when comparing titanium, TiO_2-coated titanium, Ti6Al4V, TiO_2-coated Ti6Al4V, TiN-coated Ti6Al4V, Ti5Al2.5Fe and 316 L stainless steel [25]. Similar results in another rat model were found using machined titanium and Ti6Al4V (up to 12 weeks) [21]. In the rat abdominal wall model, PTFE implants were surrounded by a significantly thicker fibrous capsule than machined titanium, coinciding with larger cellular numbers, after 12 weeks but not earlier [26].

The surface topography of polymers modulates the foreign body reaction in soft tissues [27–32]. Several mechanisms have been suggested, including effects of surface roughness on protein adsorption [33], cell shape [34, 35], and mechanical stability [36–38]. In contrast to polymers there are relatively few studies on the relationship between metal surface topography and fibrous repair in soft tissues. Smooth titanium implants caused a thicker fibrous capsule than blasted and anodized titanium with rough surfaces [39]. However, the present results do not indicate any major effect of titanium submicron porosities on capsule thickness. Studies on the tissue ingrowth (after 4–12 weeks of s.c. implantation) in a porous titanium fiber mesh showed that macrophages and connective tissue were mainly concentrated on the inside of the mesh [40]. Different fiber mesh materials had no effect on the tissue response. In contrast, the thickness of the mesh had an influence, causing less inflammation and thinner fibrous capsules at thinner titanium mesh materials, indicating that implant material flexibility could influence the local soft tissue response and possibly reducing focal stresses at the tissue–material interface. Porous Ti6Al4V pacemaker tips, in comparison with solid ones, were better anchored than solid ones in the heart muscle wall due to tissue ingrowth into the pores [41].

3.3. Neovascularization

The neovascularization in the tissue around implants is generally considered to be important but basic information related to the degree of vascularization and distribution of blood vessels is yet incomplete. Concomitant with the growth of the fibrous capsule an angiogenic response was evident after seven days. At this early time point the capsule around Ti was most vascularized (Ti vs. PTi, $P < 0.05$) (Fig. 4), having a relatively higher number of vessels and a larger vessel luminal surface (Table I). Very few blood vessels were observed in the capsule around plasma Ti after seven days (Fig. 4, Table I). In contrast, after 28 days both porosity and the coating of Ti with plasma were associated with a significant increase in vascularity (PlasmaTi vs Ti, $P < 0.02$ and plasmaPTi vs Ti, $P < 0.05$). The proportion of vessels in the capsule increased significantly with time for PTi ($P < 0.01$) and plasmaTi ($P < 0.001$). The majority of blood vessels were distributed in the external part of the fibrous capsule at both early (seven days) and late (28 days) observation times. The distance between the vessels and the fluid space increased significantly with time for all types of surface modifications (Fig. 5).

Our data are not completely in line with other studies on neovascularization at porous or non-porous polymer interfaces, largely due to differences in experimental set ups and implantation times. After three to four months of subcutaneous implantation in rat of polyvinyl alcohol (PVA) or polytetrafluorethylene (PTFE) with 0–700 μm porosity, it was concluded that maximal neovascularization was incited outside surfaces with pore sizes on the order of cellular dimensions [42]. In our study, the submicron porosity induced low neovascularization at seven days which increased at 28 days, and vice versa for

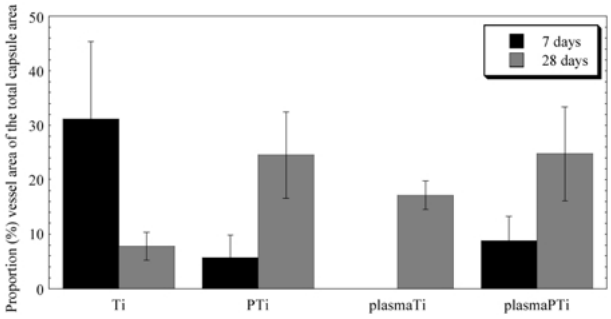

Figure 4 Proportion (%) of blood vessels in the fibrous capsule (lumen area/total area × 100) at seven or 28 days. Mean ± SEM, $n = 7$–8.

TABLE I The number of blood vessels per unit area and the mean blood vessel area (μm²) in the fibrous capsule after seven and 28 days

	Number of vessels per implant		Mean vessel area (μm²)	
	7 days	28 days	7 days	28 days
Ti	6 ± 2	9 ± 4	493 ± 70	160 ± 40
Pti	3 ± 2	21 ± 8	207 ± 52	365 ± 63
PlasmaTi	0 ± 0	11 ± 4	0 ± 0	315 ± 64
PlasmaPTi	3 ± 2	25 ± 7	349 ± 123	277 ± 64

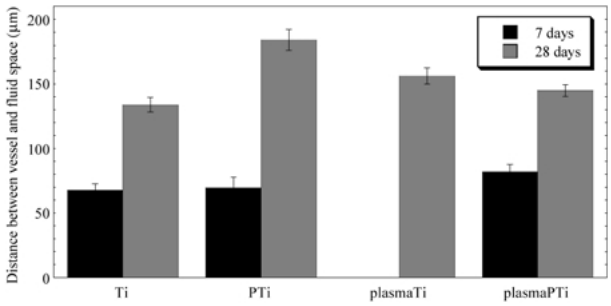

Figure 5 Mean distance (μm) between vessels and the fluid space, mean ± SEM, seven or eight implants per type of surface.

the non-porous Ti implant. Similar effect was observed when porous or non-porous surfaces were coated with the thin plasma clot. Thus, no clear-cut relationship existed between submicron porosity on Ti and the superimposed thin plasma clot layer for neovascularization (Fig. 4). Salzmann *et al*. implanted subcutaneously or in the adipose tissue different polymer fabrics for five weeks, and concluded upon the concomitant *ex vivo* tissue analysis that there seemed to exist an inverse relation between the degree of inflammation and neovascularization [43]. In the present study, elevated fibrin degradation products were believed to be released from the clot-coated surfaces, and again this could not override the effect of the submicron porosity with regard to vessel formation. When different thin polymer membranes with pore sizes 0–15 μm were implanted subcutaneously in the rat for three weeks, the larger pores induced a greater implant-close (1 cell layer to 15 μm from surface) vascularization compared to submicron pore sizes, and this difference in vascularization was maintained after one year [37]. The studies [37, 42, 43] indicate to us that increased neovascularization around implants can be obtained by pores with size one order of magnitude larger than in the present study, but also that neovascularization most likely is modulated by a superimposed submicron porosity and fibrin-like structures.

4. Conclusions

The fluid space outside the differently treated Ti-surfaces disappeared and the fibrous capsule thickness increased between seven and 28 days of implantation. The number of vessels per implant increased significantly during the time period for all types of surfaces, except Ti. At 28 days of implantation, porous surfaces or surfaces coated with a thin plasma clot had a larger proportion of blood vessels in the fibrous capsule. A general observation was that the blood vessels were distributed in the outer part of the fibrous capsule and the average distance between the fluid space and the vessels increased between seven and 28 days. The results in the present study indicate that the healing process around titanium can be modulated by porosity and thin pre-prepared plasma coatings.

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