Recouvrement luminal de greffons vasculaires en ePTFE à petit diamètre avec des matériaux organiques et non organiques: effets sur la neo endothelisation, hyperplasie de l'intima et la patence

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Cardiovascular Implants

Improved neo-endothelialization of small diameter ePTFE grafts with titanium coating

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ABSTRACT: Background: *Patency of small synthetic bypass grafts is inferior compared to autologous grafts for revascularization procedures. Titanium coating of foreign surfaces has shown to decrease thrombogenicity, enhance biocompatibility and promote adhesion of endothelial cells. The aim of this study was to test the effect of titanium coating of small diameter ePTFE grafts on short term patency, neo-endothelialization and neointimal proliferation.*

Methods: *Bilateral carotid graft interposition was performed in 5 pigs with uncoated (n=5) and titanium-coated (n=5) ePTFE grafts (internal diameter=4 mm, length=5 cm), thus each pig served as its own control. At the end of the study (30 ± 3 days), patency and stenosis severity was assessed by carotid angiography. Animals were sacrificed and grafts were excised for histology and scanning electron microscopy. Morphometry of histologic sections was carried out to determine neointimal proliferation and percentage of neo-endothelial coverage.*

Results: *Patency rate was 80% for uncoated and titanium-coated grafts. Quantitative angiography did not show any significant difference in lumen size between two groups. Morphometry revealed a significantly higher cellular coverage with CD^{31} positive endothelial cells for titanium-coated (84 ± 19%) than uncoated grafts (48 ± 26%, $p<0.001$). There was a non significant trend ($p=0.112$) towards increased neointimal proliferation in titanium-coated (94 ± 61 $\mu m^2/\mu m$) compared to uncoated grafts (60 ± 57 $\mu m^2/\mu m$).*

Conclusions: *Patency rate in uncoated and titanium-coated ePTFE grafts is similar at one month. However, titanium coated grafts show a significant improvement in neo-endothelialization compared to uncoated grafts. (Int J Artif Organs 2006; 29: 990-9)*

KEY WORDS: Experimental surgery, Prosthesis, Research, Revascularization, Vascular grafts, Prosthesis, Endothelium, Surface coating, Titanium, Experimental surgery

INTRODUCTION

Cardiovascular disease has been the leading cause of mortality in the world during the last ten years (1). Revascularization procedures, such as percutenous coronary intervention and coronary bypass grafting, are the methods of choice for the treatment of ischemic cardiovascular disease (2). Autologous graft material is the replacement material of choice for surgical revascularisation (3). However, because of a shortage of autologous bypass grafts due to disease and/or previous surgery, there is an increasing demand for synthetic vascular prosthesis (4). Furthermore, patency of small synthetic bypass grafts is inferior compared to autologous

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grafts for revascularization procedures (5, 6). There is increased early thrombogenicity and late neointimal proliferation associated with synthetic materials, especially due to the blood-surface interface (7, 8).

Titanium alloy coating has been used successfully in orthopedic implants and various medical devices. *In vitro* and *in vivo* studies have shown beneficial effects of titanium coating of foreign surfaces to decrease thrombogenicity, enhance biocompatibility and promote adhesion of endothelial cells (9-12). Thus, titanium coating of the luminal surface may be helpful to improve biocompatibility of small diameter synthetic vascular grafts.

The aim of this study was to test the feasibility of titanium coating of ePTFE grafts and to assess the effect on short term patency, endothelialization and intimal hyperplasia of titanium coated versus uncoated ePTFE grafts.

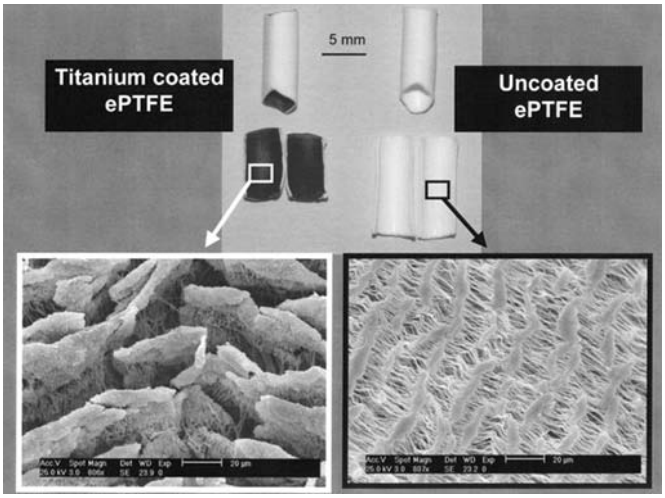

Fig. 1 - *Macroscopy and scanning electron microscopy (x800) pictures of non-perfused titanium-coated and uncoated ePTFE grafts.*

MATERIALS AND METHODS

Grafts

Standard ePTFE grafts with 4 mm internal diameter, through porosity and inter-nodal distance of 30 micrometers (provided free of charge by Atrium, USA) were used for this study. Uncoated grafts and titanium-coated grafts, were prepared for surgery after ethylene oxide sterilization (Fig. 1). Bilateral carotid graft interposition was performed in 5 pigs (length of interposed ePTFE grafts 50 mm). Each pig received uncoated (n=5) and titanium-coated (n=5) grafts. Thus, each pig served as its own control.

Manufacturing of titanium-coated grafts

Magnetron sputtering was used for coating of ePTFE grafts (Institute Ginalmazzoloto, Moscow, Russia) (European Patent Office. 16.09.2002, Sedelnikov Nikolai. Patent number: W00158504; Non-thrombogenic implantable devices). It creates a thin (5-20 micrometer), preferably non-continuous, amorphous metal layer with high adhesiveness. This technique includes three steps: ionization of a plasma forming gas, electrical glow discharge in vacuum, and sputtering of the metal target's material by bombarding it with accelerated titanium ions.

The ePTFE grafts were immersed in ultrasonic bath containing a low residue detergent solution in distilled water for ultrasonic cleansing followed by distilled water rinsing and dessicator drying. Then the bare graft was placed in the substrate holder, and was inserted into the working chamber via the air-lock. The working chamber was de-aired by means of the evacuation system to a residual pressure of about 0.1 Pa and replaced by argon. The substrate holder (graft) was displaced by means of the conveyor system. The coating was performed by a sputtering method (titanium ion deposition).

Assessment of coating quality, stability and thrombogenicity

In order to evaluate the quality and homogenicity of the titanium coating, grafts were assessed by scanning electron microscopy (SEM) with three different magnifications (x25, x200, x800). The femoro-femoral *ex vivo* arterio-venous shunt (AVS) method was used to evaluate the coating stability, thrombogenicity and cellular deposition of the titanium coating and the results were compared with semi-quantitative assessment to the bare ePTFE grafts (13, 14). Cellular and thrombi deposition criteria were graded from 0 to 3 (0=no deposition, 3=severe deposition) for semi-quantitative assessment (15) and then expressed as % increase or decrease from the control ePTFE grafts for each AVS with the following equation: [(Score of coated graft / Score of uncoated PTFE) – 1] x 100.

Pig carotid artery interposition model

Animal model

The protocol was reviewed and approved by the Ethical Animal Committee of the University of Geneva and the Veterinary Office of the State of Geneva (Protocol No. 1081/2330/III). All animals received standard care according to Principles of Laboratory Animal Care and the Guidelines for the Care and Use of the Laboratory Animals (U.S. National Institutes of Health, 1996). Five Swiss house swine aged 3-4 months (30 ± 2 kg) were used in this study. They were fasted the night before surgery and received normal food throughout the post-operative period.

Anesthesia and monitoring

The following pre-medication was used: midazolam (Dormicum®, 0.5 mg/kg), azaperon (Stresnil®, 6 mg/kg) and atropin (Atropine sulfat®, 0.04 mg/kg). Animals were hydrated during the operation with balanced electrolyte solution via ear vein (Ringer Lactate®, 3-5 ml/kg/h). Anesthetic induction for intubation was performed with a face mask using isoflurane 5% (Forene®) and fentanyl injection (Sintenyl®, 50 mg/kg). After induction, pigs were intubated and ventilated with a volume control respirator (Servo 900D®, Siemens, Sweden). The maintenance of the anesthesia during the procedure was based on isoflurane inhalation (Forene®, 2-3%) and fentanyl perfusion (Sintenyl®, 50 µg/kg/h).

Before surgical incision, 750 mg intravenous cefuroxime (Zinacef®) was applied for infection prophylaxis. During surgery, animals were monitored for ECG, invasive blood pressure, temperature as well as oxygen saturation and end-tidal carbon dioxide. To prevent hypothermia during surgery, a heating blanket was used and rectal temperature was monitored. In addition, hematologic, blood glucose, blood gas and activated clotting time (ACT) tests were performed at baseline during and after surgery. During surgery heparin was given intravenously in order to prolong the ACT values at least two-fold. In addition, a single dose of 0.5 g acetyl salicylic acid (Aspegic®) was given intravenously before carotid artery clamping.

Surgery

Bi-lateral carotid artery graft interposition was performed through a cervical mid-line incision with preparation lateral to the trachea and medial to the sternocleidomastoid muscle on each side. The carotid arteries were prepared and freed on a length of about 8-10cm by taking care to protect the vagus nerve. To prevent vascular spasm papaverin solution was applied externally (40 mg papaverin / 50 mL saline).

Before starting surgery a baseline intra-operative carotid artery flow measurement was carried out using a transit time flow meter (Cardiomed, MediStim, Norway) under standard, stable hemodynamic conditions.

After proximal and distal clamping of the carotid artery a segment of approximately 3 cm was excised in a bevelled way. The prostheses were trimmed to a length of 5cm in a bevelled way. Proximal then distal anastomoses were performed with 7-0 polypropylene interrupted sutures using surgical loupes (x 3.5 optical magnification). Before finishing the anastomosis the vessels were rinsed with heparinized saline. The distal clamp was removed for de-airing and then the proximal clamp was removed. If a bleeding persisted after 2-3 minutes of compression, this was fixed by additional single stitch.

Before wound closure, intra-operative flow measurements were performed proximal and distal to the graft on each side.

Follow-up

After wake up, animals were extubated and returned to their cage where they were followed, assessed and medicated as required, during seven days. Standard postoperative medication included morphine and cefuroxime during the first three days. Throughout the survival period, animals were administered orally 0.5 g of aspirin daily until sacrifice at one month. After one week, duplex examination was repeated after premedication and induction as mentioned earlier, and maintenance anesthesia with a face mask using isoflurane narcosis (4%). Thereafter, animals were allowed to return to the farm until sacrifice.

Sacrifice

At the end of the study animals were brought back to our laboratory and the same pre-medication, anesthesia, monitoring and laboratory samples as described above, were carried out. First, a control carotid angiography was performed (General Electric, Cardiac Series 9800, Salt Lake, USA) (Fig. 2), followed by surgical exposure of the

grafts. After administration of 10,000 units of heparin IV, grafts were excised with about 2 cm of native proximal (marked by suture) and distal carotid artery. They were then rinsed with saline followed by formaldehyde (4%) immersion for fixation.

Morphologic evaluations of explanted grafts

After formalin (4%) fixation, the grafts were cut in half (proximal and distal plus a ring segment in the middle of the prosthesis). Each half was then opened longitudinally making two halves of the vessel, one of which was used for SEM and the other for histology and morphometry. Qualitative assessment was made by normal histologic stainings with hematoxilin and eosin followed by special CD^{31} endothelium specific immuno-histochemical staining.

Inner surface morphology of the graft was examined by scanning electron microscopy (Philips XL20 SEM, Netherland) at predefined locations, settings and magnifications (x20, x200 and x800 magnification).

Quantitative morphometric evaluation of the neointimal proliferation and neo-endothelial coverage were performed using a planimetric computerised microscope (Leica DMRB, Germany). The neointimal proliferation was calculated as "area per graft length (micrometer^2/micrometer)" and neo-endothelial coverage was expressed as "percent of the total graft length".

Statistics

Results are expressed as mean values ± standard deviations. The following statistical tests were performed using SPSS (Version 11.5): Parametric values compared with Chi-square test. Mann Whitney U test was used to compare non-parametric values. The level of significance was set at $p < 0.05$.

RESULTS

Assessment of coating quality, stability and thrombogenicity

Comparing the handling properties of uncoated and titanium-coated grafts, the latter was found softer and more flexible. The coating quality was uniform and regular by SEM evaluation (Fig. 1). *Ex vivo* shunt test indicated that titanium-coated surfaces were unaltered after arteriovenous perfusion. The semi-quantitative analysis with SEM showed less micro-thrombus formation and cellular deposition on the titanium-coated grafts (Tab. I, Fig. 3). None of the shunted grafts occluded during this procedure.

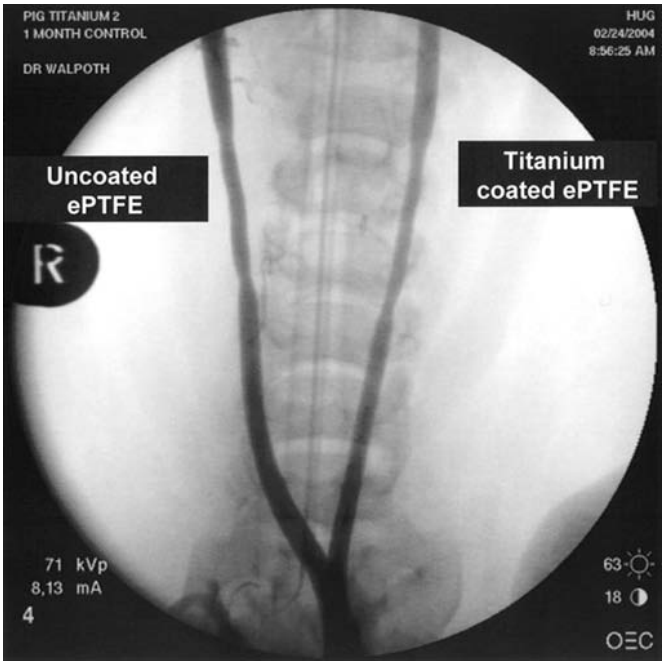

Fig. 2 - *Selective carotid angiography before sacrifice (1 month) shows patent uncoated (right side) and titanium-coated ePTFE grafts.*

Pig carotid artery interposition

All the 5 pigs reported in this paper have survived 30 ± 3 days as planned without any complications. Patency control was performed with transit time flow measurement

TABLE I - RESULTS OF THROMBOGENICITY TEST AFTER ARTERIOVENOUS SHUNT PERFUSION FOR 9 MINUTES*

	% of uncoated ePTFE	
	Thrombogenicity score	Cell deposition score
Titanium-coated grafts	-53 ± 23	-50 ± 28

* Cellular and thrombi deposition criteria is graded from 0 to 3 (0=none or few deposition, 3=severe deposition) for the semi-quantitative assessment and then expressed as % increase or decrease from the control ePTFE grafts for each AVS with the following equation: [(Score of coated graft / Score of uncoated PTFE) – 1] x 100.

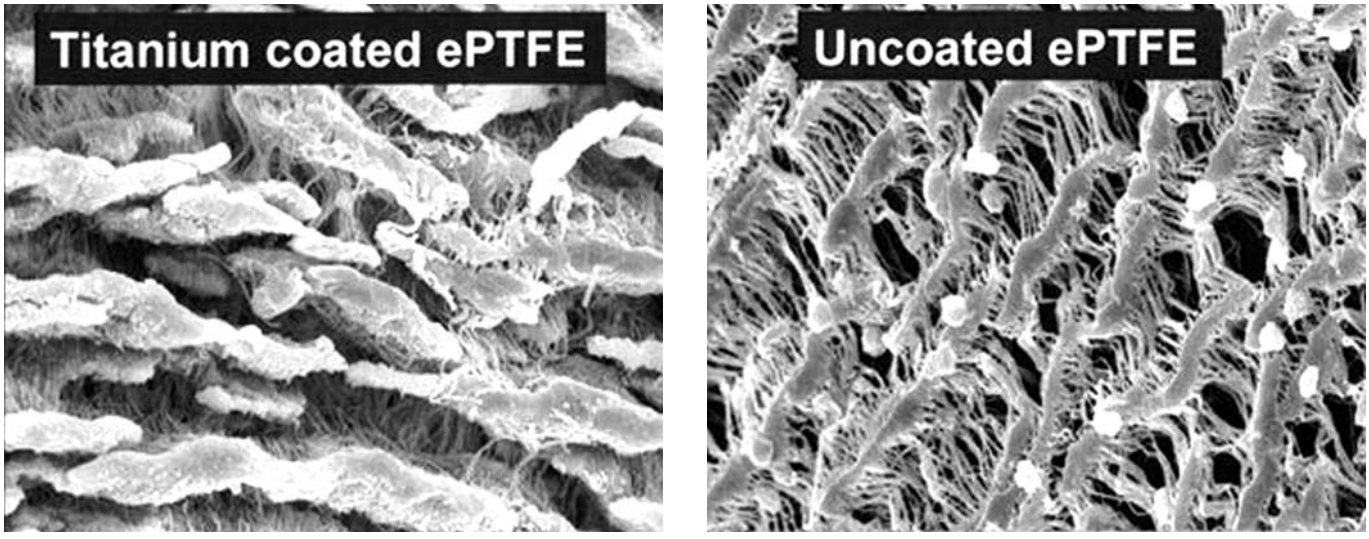

Fig. 3 - *The SEM pictures of the luminal side of the grafts after 9 minutes perfusion in an arteriovenous shunt in the pig model (x800). Significantly less cellular deposition is found in titanium-coated grafts.*

at the end of surgery. All grafts showed an adequate flow without significant differences between two groups (Tab. II).

All grafts were patent at one week by ultrasonography. After one month one graft of each group was occluded (in the same animal). Thus, patency rate was 80% for uncoated and titanium-coated grafts. Pre-sacrifice quantitative carotid angiography did not show any significant difference between the groups (Tab. III).

Morphologic evaluation

Titanium-coated grafts had deeper inner surface cryptae, more fibrin deposition, larger cells in the cryptae and better neo-endothelialization especially in the middle of the grafts than uncoated grafts (Fig. 4). On longitudinal sections across the anastomosis, we found in both groups

TABLE II - SURGICAL QUALITY CONTROL WAS EVALUATED WITH INTRAOPERATIVE BLOOD FLOW MEASUREMENT AFTER COMPLETION OF CAROTID GRAFT INTERPOSITION

	Intraoperative flow measurement (ml/min)
Uncoated grafts (n=5)	200 ± 73
Titanium-coated grafts (n=5)	170 ± 108
p	NS

NS = non significant.

TABLE III - PATENCY AND QUANTITATIVE ANGIOGRAPHY AT ONE MOTH FOLLOW-UP AFTER CAROTID ARTERY GRAFT INTERPOSITION

		Percental narrowing (stenosis) compared to native proximal carotid artery diameter *		
	Patency	Proximal anastomosis (B_1)	Middle Graft (B_2)	Distal Anastomosis (B_3)
Uncoated grafts	4/5	28 ± 13	14 ± 11	28 ± 13
Titanium-coated grafts	4/5	33 ± 9	7 ± 8	30 ± 6
P	NS	NS	NS	NS

NS = non significant; * Modified NASCET formula = (1-(Bx / A)) x 100; B_1 = Diameter of the proximal anastomosis, B_2 = Diameter of the middle part of the ePTFE graft, B_3 = Diameter of the distal anastomosis; A = Diameter of native carotid artery (5 cm before the proximal anastomosis).

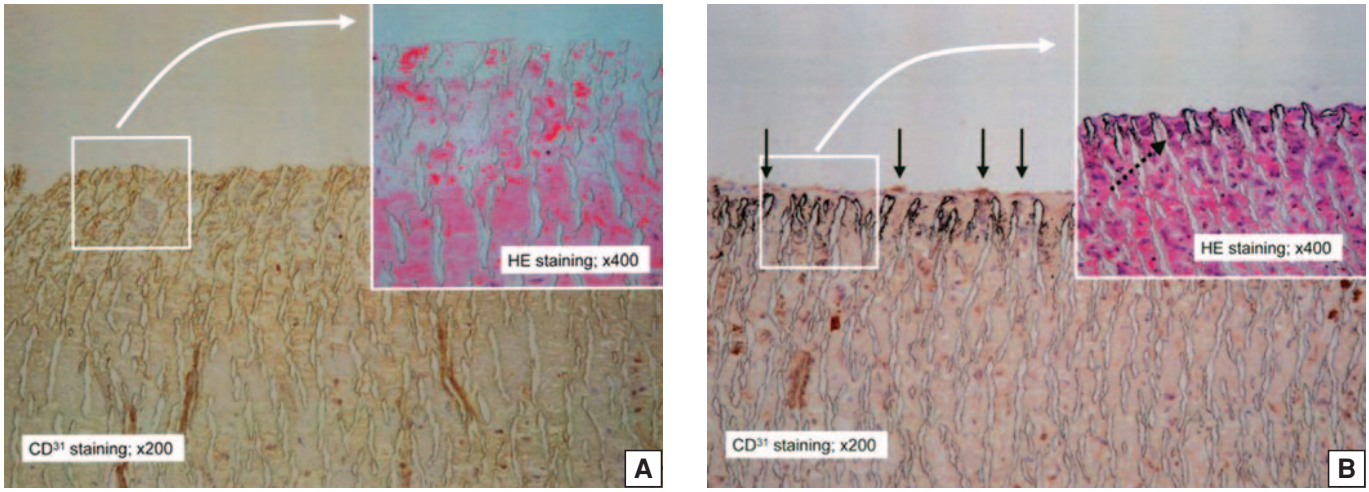

Fig. 4 - *Histologic evaluation of explanted (1 month) uncoated* **(A)** *and titanium-coated ePTFE grafts* **(B)**. *Cellular infiltration and neo-endothelialization with CD31 positive cells were less in uncoated ePTFE grafts compared to titanium-coated grafts* **(A)**. *The titanium-coated graft has deeper cryptae, these are filled with fibrin matrix and stem-like cells (dashed arrow) in the depth of cryptae (black arrows show CD31 positive endothelial cells)* **(B)**.

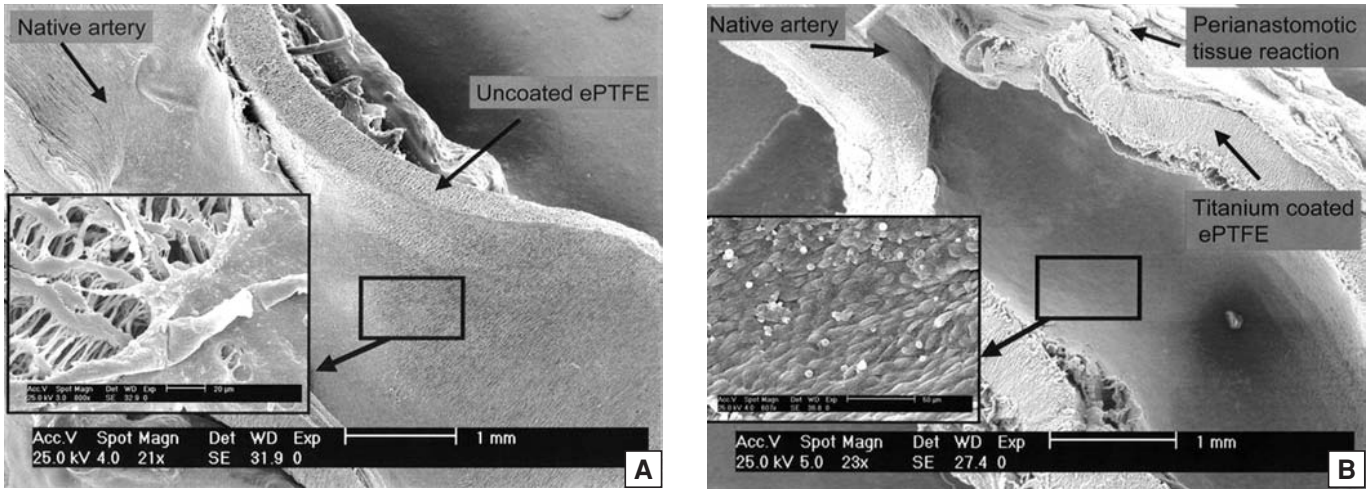

Fig. 5 - *Scanning electron microscopy of explanted (1 month) uncoated* **(A)** *and titanium-coated ePTFE grafts* **(B)**. *Uncoated ePTFE graft shows more non-endothelialized inner surface of the middle graft segment* **(A)**. *The middle segment of titanium-coated ePTFE graft shows a confluent layer of aligned endothelial cells* **(B)**.

a marked tissue reaction with inflammatory cells and an accompanying neointimal proliferation at the site of the proximal and distal anastomosis. The thickness of the neointima decreases towards the middle of the graft and it was more prominent at the proximal anastomoses. The neo-endothelialization was more extended towards the centre of the prosthesis in the titanium-coated group (Fig. 5). The scanning electron microscopic pictures were supporting the histology results.

Quantitative morphometric analysis with computed planimetry

Mean results of all grafts (uncoated and titanium coated), showed more pronounced hyperplasia reaction at the anastomosis compared to middle part of the grafts (215 ± 90 micrometer^2/micrometer versus 49 ± 49 micrometer^2/micrometer; p=0.001). The proximal anastomosis showed more neointimal proliferation than

the distal anastomosis (261 ± 80 *versus* 165 ± 74 micrometer^2/micrometer; p=0.013). Endothelial coverage was less pronounced in the middle of the grafts (60 ± 29 %) when compared to the anastomoses (99 ± 4%; P = 0.001).

A significant difference for neo-endothelial coverage was found between the uncoated and titanium-coated grafts (48 ± 26 % *vs* 84 ± 19 %, p=0.001). There was a trend of more neointimal proliferation in titanium-coated compared to uncoated grafts (94 ± 61 vs 60 ± 57 micrometer^2/ micrometer, n.s.) (Tab. IV).

TABLE IV - NEOINTIMAL PROLIFERATION AND NEO-ENDOTHELIAL COVERAGE ASSESSED BY PLANIMETRY (ONLY PATENT GRAFTS WERE ANALYZED)

	Uncoated grafts (n=4)	Titanium-coated grafts (n=4)	p
Neointimal proliferation (μm^2/(μ)			
Whole graft	60 ± 57	94 ± 61	0.112
Anastomoses (proximal + distal)	172 ± 95	254 ± 68	0.051
Middle of the graft	27 ± 23	68 ± 58	0.175
Neo-endothelial coverage (%)			
Whole graft	48 ± 26	84 ± 19	0.001
Anastomoses (proximal + distal)	98 ± 5	100 ± 0	0.705
Middle of the graft	39 ± 16	77 ± 25	0.002

DISCUSSIONS

Titanium and medical implants

Titanium is a strong, silver gray, metallic element which is resistant to corrosion. It is the most biocompatible of all metals due to its inertness, high strength and high corrosion resistance. Titanium is often used as material or coating for implanted devices such as mechanical heart valves, artificial hearts, ventricular assist devices, coronary stents, vena cava filters as well as orthopedic prostheses, some used for more than 30 years (16-18).

Surface coating with titanium

Although great progress has been achieved in the field of synthetic vascular graft manufacturing, foreign-thrombogenic- surface of the grafts is the key issue for occlusive and stenotic complications. The inner surface of the grafts should be blood compatible in order to remain patent until neo-endothelial coverage. Several studies have shown improved blood-surface interaction by organic and/or biologic coating (i.e. carbon, heparin) as well as endothelial cell seeding (19-22). Although the effects of titanium surface coating on the hemocompatibility, tissue compatibility and cellular interactions has been shown in previous studies, this metal coating has not been used previously *in vivo* for coating of ePTFE vascular grafts.

The aim of the present study was to improve surface characteristics of small diameter ePTFE grafts to create a biologically inert barrier towards the circulating blood and to promote endothelial coverage by titanium coating. This may be important because thrombus formation triggers smooth muscle cell proliferation (23).

Yang et al have tested the hemocompatibility of titanium coating compared to other surface coatings. They implanted in sheep (superior and inferior vena cava) coated discs with different alloys including titanium. After two hours of exposure to flowing blood, the discs were explanted and evaluated with scanning electron microscopy for thrombogenicity. Titanium-coated surfaces showed significantly less thrombus formation (24).

Before performing our carotid implantation study, we tested the titanium-coated and uncoated grafts in an arteriovenous shunt circuit of the pig to assess hemocompatibility. Titanium-coated grafts showed 50% less microthombus formation.

Tissue compatibility is another aspect to consider for synthetic vascular grafts. Titanium is an inert material and may cause faster healing with less tissue reaction (25, 26). Kraft et al have shown that titanium implants had less inflammation, leucocyte extravasation and tissue toxicity using the hamster dorsal skinfold chamber model (27). Therefore, titanium coating may limit tissue reaction (e.g. neointimal proliferation) on the luminal side of our vascular graft. Neointimal proliferation includes mainly two components: smooth muscle cells and extracellular matrix. Furthermore Balasubramanian et al have shown that titanium has a limiting effect on the growth of smooth muscle cells (28).

In vivo *results with titanium-coated vascular implants*

The effect of titanium coating on coronary stents was assessed by Windecker et al in the porcine overstretch model (12). These stents showed less platelet adhesion,

fibrinogen binding and decreased neointimal proliferation. These experimental data were confirmed in a prospective, randomized clinical study by the same group (29). Ninety-two patients were randomized into a study group receiving titanium-coated stents (n=45) and a control group receiving bare metal stents (n=47). Six months after implantation, in-stent restenosis was evaluated with re-angiography and intravascular ultrasonography. Titanium-coated stents showed a significantly reduced restenosis compared to non-coated bare metal stents (29).

Ueberrueck et al coated polyester (Dacron) vascular prostheses with titanium (n=7) and implanted these into pig infrarenal aorta (30). At the conclusion of the study (3 months) all titanium-coated grafts were found occluded. Differently than the previous study, we used ePTFE grafts and they were interposed into the pig carotid artery. Our patency rate was 80% and equal in both (titanium-coated and uncoated) groups. On the other hand the observation period is longer in Ueberrueck's article. Difference between the two studies (Ueberrueck's study *vs* our study) may be explained by the graft materials (Dacron *vs* ePTFE), coating techniques (luminal and external coating *vs* only luminal coating) and observation period (3 months *vs* 1 month).

Effect of titanium coating on the endothelial cell response

ePTFE grafts have hydrophobic properties which may play a role for the high failure rate of small diameter grafts (31). Thus, rapid neo-endothelialization of the internal surface of ePTFE grafts may prevent formation of neointimal proliferation and thus improves thrombogenic and stenotic complications (32).

Yeh et al has performed an *in vitro* study to assess the effect of coating material on the behavior of human umbilical vein endothelial cells (HUVEC) grown on stent materials (33). They used bare stainless steal or nitinol, and titanium oxide (TiO) or titanium nitride (TiN) coated metallic sheets. The results showed that the levels of cellularity of HUVEC on the TiO and TiN coated sheets were significantly higher compared to the other materials.

In the present study, titanium-coated ePTFE grafts show a better covering with CD^{31} positive endothelial cells, compared to the bare ePTFE grafts. It may be partly related to changes of surface properties of ePTFE graft with titanium. Lehle et al showed that titanium coating of ePTFE did not influence the roughness of the surface, but the wettability/ charge conditions and possibly also the cytotoxic effects of ePTFE grafts (9). They have shown *in vitro* a significant increase of proliferation for endothelial cells on titanium coating of ePTFE patches compared to bare surface. They also found that titanium coated ePTFE surfaces are more hydrophilic and have better attachment by endothelial cells compared to the uncoated surfaces.

Surface evaluation of our titanium-coated ePTFE grafts with light microscopy and SEM showed deeper cryptae and more fibrin deposition compared to uncoated grafts. Fibrin has a wound healing function and triggers cellular ingrowth. Deep cryptae and fibrin matrix combination theoretically may trap for blood born circulating cells like endothelial progenitor cells (EPCs) (34-36). Recent publications have shown the positive effects of EPC's on endothelialization of injured or foreign surfaces (37, 38).

CONCLUSION

Our study shows that coating of small diameter ePTFE vascular grafts with titanium alloy is technically feasible. The coating quality of the grafts is uniform and surgical handling properties are good. Titanium coating decreases thrombogenicity in the arteriovenous shunt perfusion test in the pig model. After carotid artery replacement, patency rate at one month is 80% for both (coated and uncoated) groups. Endothelial coverage is better for titanium-coated grafts but with a trend for higher neointimal proliferation compared to noncoated grafts. Thus, we believe that the use of titanium coating of small diameter vascular prosthesis might have a positive effect on graft neo-endothelialization, but simulation of the clinical applications and long term implantation studies are necessary.

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