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In a Pig Model ePTFE Grafts will Sustain For 6 Weeks a Confluent Endothelial Cell Layer Formed *In Vitro* Under Shear Stress Conditions

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Objective: *to investigate in a pig model whether small diameter ePTFE grafts will sustain a confluent endothelial cell layer formed* in vitro *under shear stress conditions.*
Material and Methods: *thirteen ePTFE (4 mm) grafts were implanted end to end in the right femoral artery of; 8 grafts had been endothelialized* in vitro. *Grafts were left* in situ *for 6 weeks then evaluated with ultrasound and histology.*
Results: *seven endothelialized graft were patent with confluent endothelial cell lining. None of the control grafts were patent or showed evidence of an endothelial lining.*
Conclusion: *in this pig model ePTFE grafts sustained for 6 weeks a confluent endothelial cell layer formed* in vitro *under shear stress.*

Key Words: Tissue engineering; Vascular grafts; Shear stress.

Introduction

Autologous saphenous vein is the preferred conduit for femoro-distal bypass but is often unavailable.[1] The patency rates of small diameter ePTFE grafts are disappointing[2–10] partly because of the absence of an endothelial cell lining.[11–16] However, the artificial seeding of ePTFE grafts with autologous harvested endothelial cells failed to improve patency *in vivo*,[17–19] possibly because such cells fail to adhere on to the graft under normal blood flow conditions.[20,21] We have previously reported the endothelialization of ePTFE grafts under shear stress[22] and were able to demonstrate that such grafts sustained endothelial cell confluency when exposed to pulsed physiological flow conditions.[23] Therefore, with regard to the potential use of flow processed ePTFE grafts as a substitute to autologous bypass, a prospective animal study was carried out. The aim of this study was to extend these observations to a pig model.

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Materials and Methods

Thirteen 25–30 kg, 10–14 week female pigs (Deutsche Landrasse, Charles River Wiga, Ansbach, Germany) were housed under standard laboratory conditions, allowed food and water *ad libitum* and randomized into two groups. Group 1 ($n = 5$) received an uncoated 4 mm ePTFE graft and group 2 ($n = 8$) a shear stress endothelialized 4 mm ePTFE graft.

For endothelial cell harvest and cultivation the animals were anaesthetized with i.m. Stresnil (Azapiron, Janssen, Germany) 4 mg/kg, 0.05 mg Atropine and Ethomidat-Lipiro 0.15–0.3 mg/kg. The external jugular vein was dissected over 5 cm, repeatedly flushed with Dulbecco's PBS (Biochrom AG, Berlin, Germany), filled with 1 U/ml Collagenase P® (Boehringer, Mannheim, Germany) diluted in PBS and incubated at 37 °C (gazed 5% CO_2 at 100% humidity) for 15 min. Collagenase activity was stopped with 10% FCS supplemented standard RPMI medium (Biochrom AG, Berlin, Germany). After centrifugation at 1000 rpm for 5 min the supernatant was rejected and the pellet resuspended in PBS. This procedure was repeated twice before the pellet was suspended in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich, Steinheim, Germany) and Endothelial Cell Growth Medium (ECGM) (PromoCell GmbH, Germany), mixed 1:1.

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Cells were cultivated in an incubator at (37 °C, 5% CO_2 at 100% humidity) and grown to subconfluence (80% confluent) before passaging. Harvesting was performed by incubating the cell monolayer 5 min with PBS diluted Trypsin/EDTA solution (Biochrom AG, Germany). Trypsin was inactivated by adding 10% FCS supplemented standard RPMI medium. After centrifugation and washing in PBS the cells were seeded again at a ratio of 1:2 to 1:4 until final harvest. Cell number and viability were controlled with an electronic cell counting system. Cell identity was confirmed *in situ* with von Willebrand/Factor VIII staining (Sigma–Aldrich, Steinheim, Germany) using the EnVision™ AP detection system (Dako, Hamburg, Germany) and by observing the typical cobblestone morphology of endothelial cells in confluent monolayer. Contamination by Mycoplasma and other bacteria was tested routinely by a fluorescence DNA staining kit (Biochrom AG, Berlin, Germany). Photographic documentation of cell growth was performed under light microscopy daily and before new passages of the cells. Control cultures of endothelial cells were performed for each harvest to check growth characteristics and cell viability. Data were tested for normal distribution by Spearman's correlation test. Categorical data are displayed as mean and range.

The ePTFE graft (Model: T04030C30, Baxter, Vascular Systems Division, Illinois, U.S.A.) was placed in the sterile perfusion chamber TCS[2c] (Cell-Lining GmbH, Berlin, Germany), which was configured with two separate, an inner and outer, circulation. The inner circulation was adjustable to different graft extents. The outer perfusion circuit was run as static (no pump connected, medium reservoirs were on the same level as perfusion chamber and outer circulation). Inner and outer perfusion circuit's medium reservoirs were fitted with a sterile membrane filter, guaranteeing gas exchange between medium and well defined atmosphere within the incubator, in order to keep the pressure inside the chamber constant. For further chamber specifications see Figure 1. For initial fibrin coating the graft was filled air bubble free with Beriplast® HS fibrin glue (Aventis Behring, Liederbach, Germany). The inlet was then pressurized using a simple 20 ml syringe until small fibrinogen drops could be observed on the graft's outside, indicating total "sweating" of the porous material hence displacing the air. Surplus fibrinogen was removed by blowing the graft with air. It was then filled up with diluted thrombin solution, air bubble free, which was immediately removed again by blow out. This procedure was repeated until the blown-out thrombin stayed liquid. The inner perfusion circuit was then filled with the prepared endothelial cell suspension and, after a seeding period of 4 h immediately connected to a peristaltic pump. The perfusion experiments were all conducted with Dulbecco's modified Eagle's medium (DMEM) supplemented with: 25 µg Amphotericin B (0.05 µg/ml), 5 ml Heparin (1000 U/ml) diluted in cell-culture tested water (50 U/ml) 50 ml FCS (10%), 10 ml L-Glutamin 200 mM (4 mM), 10 ml Hepes 1 M (0.02 M), 5 ml Pen/Strep (100 U/ml) (Sigma–Aldrich, Steinheim, Germany) containing 10% fetal calf serum and ECGM. This was supplemented with: ECGS/H (0.4%), FCS (2%), Epidermal Growth Factor 0.1 ng/ml, Hydrocortison (1 µg/ml), Basic Fibroblast Factor (1 ng/ml), Amphotericin B (50 ng/ml), Gentamicin (50 µg/ml)) (PromoCell GmbH, Germany), mixed 1:1. A constant flow was applied and slowly increased over the first 24 h with a maximum flow rate of 12.25 ml/s corresponding to a maximum shear stress rate of 15 dyn/cm^2. After 24 h the flow was pulsed with shear rate peaks of 15 dyn/cm^2 to simulate human blood flow circumstances. Shear rate values were calculated with the Hagen–Poiseuille equation: $\tau = 4\eta * Q/(\pi * r^3)$. Viscosity of DMEM was determined at 37 °C with Low-Shear-Viscosimeter LS 40 (Mettler Toledo, Zurich, Switzerland). The time point of endothelial cell confluence was determined via the established method based on the Bernouilli effect.[23]

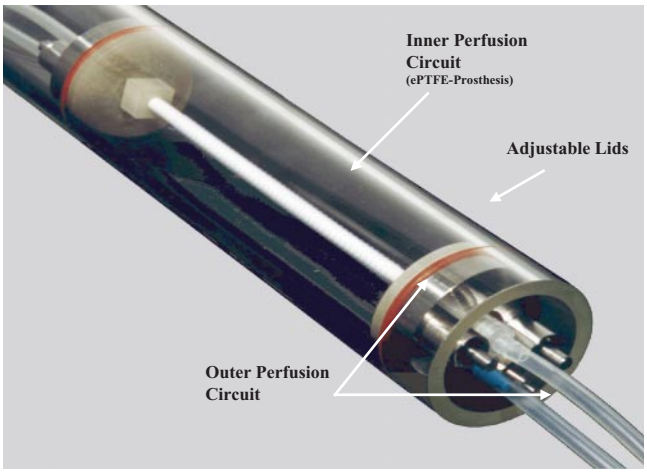

Fig. 1. Perfusion Chamber TCS[2c].

Fourteen days later, endothelialized ePTFE grafts were implanted end-to-end into the right femoral artery under general anaesthesia. A 7 cm right groin incision was performed and the right femoral artery dissected over 6 cm distal to the inguinal ligament. The flow at the artery was determined by Doppler ultrasound (Transonic Systems Inc., Ithaca, U.S.A.). The deep femoral artery was ligated to prevent early collateralization. The superficial femoral artery was

then clamped and incised over 4 mm and flushed distally and proximally direction heparin (2500 IU heparin in 250 ml saline). No systemic heparin or anti-fibrinolytic agents were administered. A 5 cm length of the superficial femoral artery was excised just below the inguinal ligament. A non-endothelialized ($n = 5$) or an under shear stress endothelialized ($n = 8$) 4 mm ePTFE graft was interposed in an end-to-end fashion. The arterial blood flow was remeasured. The incision was closed by running subcutaneous stitches and skin stapling.

Temperature, body weight, activity status and food intake were monitored daily. Eight days after implantation, graft flow was measured with a hand-held Doppler ultrasound twice weekly. Six weeks after implantation animals were re-anaesthetised, the incision reopened, the bypass was dissected, and arterial blood flow measured (Transonic® Flowprobe). The grafts were excised and placed in 4% formalin for histology.

Grafts were embedded in paraffin and sectioned at in the middle and at both end. Haematoxylin and eosin slides were prodcued in a standard manner. Endothelial cell verification was performed by peroxidase–antiperoxidase immunhistochemistry. Using antibodies against endothelial cell typical clusters of differentiation 31 as well as von Willebrand/Factor VIII.

Results

Endothelial cell harvest and cultivation was conducted without complications or contamination. Overall mean cell gain was 1 590 475 (range: 1 123 800–3 360 000) cells/ml per vein after a mean cultivation time of 11.25 (range: 9–13) days. Mean cell vitality was 92.5% (range: 89–96%). For graft cultivation 75 000 cells/cm^2 graft lumen were seeded. After the initial roller period of 4 h, a cell number of 71–96% was calculated as being adherent to the grafts surface. Histologically, this corresponds to a confluence of 35–50% on the porous surface depending on the varying cell diameter of the endothelial cells used in our experiments.[22] After 4 h, perfusion of the inner circuit was started and steadily increased to a shear stress rate of 15 dyn/cm^2 (12.25 ml/s) after 24 h. The perfusion scheme was then altered to pulsation, with shear stress peaks of 15 dyn/cm^2 (Fig. 2). Over the whole perfusion period the perfusion chamber weight was closely monitored four times an hour in order to determine cell confluency based on the established method following the Bernouilli principle. Mean perfusion time for each graft was 86.5 h (range: 65.5–161.5 h). No signs of infection or bacterial

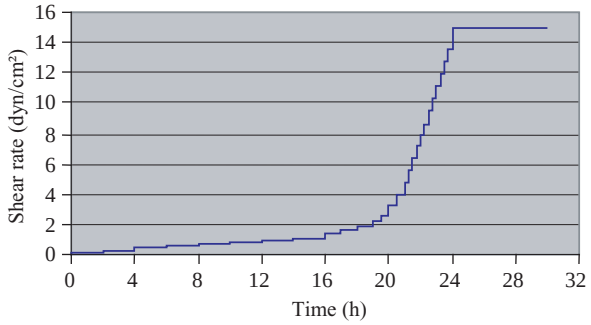

Fig. 2. Shear stress rates under perfusion culture.

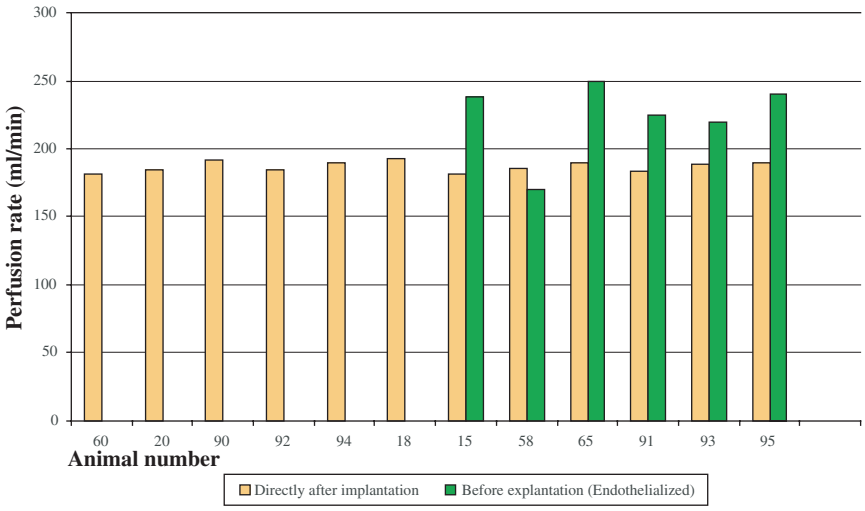

Fig. 3. Perfusion rates directly after implantation vs before explantation.

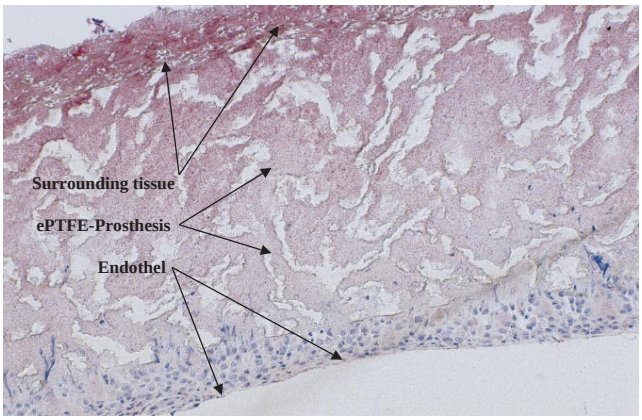

Fig. 4. Endothelialized graft after 6 weeks *in vivo*, CD 31 staining.

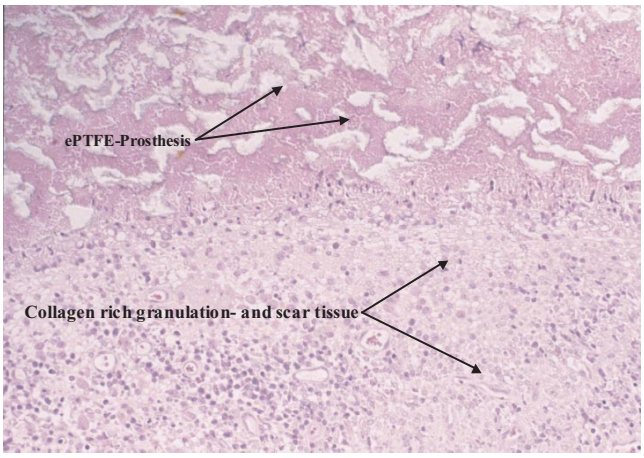

Fig. 6. Non-endothelialized graft after 6 weeks *in vivo*, Animal 60, HE staining.

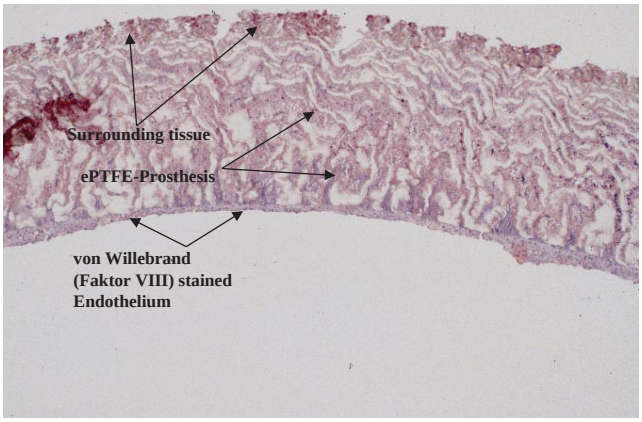

Fig. 5. Endothelialized graft after 6 weeks *in vivo*, VWF (factor VIII) staining.

contamination were seen throughout the repeated testing in the whole series and in control cultures.

As one pig was lost after implantation from malignant hyperthermia, only 12 grafts were evaluable. Otherwise, surgery and follow up were uneventful. In the endothelialized group limb development was normal (average diameter 51 cm) and there was no loss of function. In the control group one hind limb was underdeveloped (diameter 41 cm) in association with a graft infection. The other 11 prosthetic grafts were surrounded by typical fibrous tissue. At 6 weeks all but one of the endothelialized grafts showed normal pulsation in contrast to the control grafts which were all non-pulsatile.

Mean flow directly after implantation of the processed graft group was 187 (range: 182–193) ml/min, and prior to explantation was 225 (range: 220–250) ml/min (Fig. 3). No perfusion could be measured in the grafts implanted not endothelialized. Flow was demonstrated in all but one endothelialized graft up to explantation time by ultrasound control. In the unprocessed grafts, flow could only be verified up to the beginning of the fourth week after implantation. An endothelial cell lining was found in seven endothelialized grafts removed from living animals at 6 weeks (Figs 4 and 5). Only one graft showed thrombotic occlusion and endothelial cell detection was weak. The endothelialized graft in the animal deceased of malignant hyperthermia also demonstrated a confluent endothelial cell lining. In control grafts no endothelial cell-lining could be verified. All grafts were partly or completely filled with either organized thrombotic material and/or granulation and collagen rich scar tissue (Fig. 6).

Discussion

We have previously established *in vitro* that endothelial cells grown under shear stress conditions do not only show a higher proliferation rate, but also better ePFTE anchorage.[22–25] We chose a pig model to extend these observations because pigs, like humans, do not spontaneously form an endothelial monolayer.[26] After 6 weeks *in vivo* all but one endothelialized graft showed good blood flow. By comparison, all unprocessed grafts were occluded. Histology of the endothelialized grafts verified an endothelial cell layer integrity. Further studies are required to optimize the seeding process, to determine the long term durability of the endothelial layer *in vivo*, and to adapt the process for use in patients.

Acknowledgments

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