A Novel Perfusion System for the Endothelialisation of PTFE Grafts Under Defined Flow

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Objectives: to develop a perfusion system for culturing human endothelial cells on small-diameter PTFE grafts under defined pulsatile shear stress.

Methods: to benefit from a stronger adhesion of endothelial cells to the substrate, we developed a perfusion system which enables culture of endothelial cells on PTFE grafts to confluence under a wide range of shear stress. We also developed an in situ staining method for the determination of the endothelialisation stage by upper light microscopy.

Results: the application of pulsatile flow with high shear stress (6.6 dyn/cm², 5 min) to a graft endothelialised under perfusion did not lead to a disruption of the confluent cell layer. In contrast, a shear stress of 3 dyn/cm² applied for 5 min was sufficient to wash more than 50% of endothelial cells off the PTFE graft when cultured to confluence under static conditions.

Conclusions: this technique induces a stronger cell adherence of endothelial cells to a PTFE graft in comparison with grafts endothelialised under static conditions. Endothelialised vascular grafts can be pre-conditioned to defined shear stress values.

Key Words: Prosthesis; Endothelialisation; Shear stress.

Introduction

The prevention of postoperative thrombotic occlusion of small diameter vascular PTFE grafts is one of the greatest challenges in bypass surgery. If no autologous saphenous vein or internal mammary artery are available, PTFE is the vascular prosthesis of choice. However, PTFE grafts with a diameter of about 4 mm or less show a high incidence of occlusion because of their thrombogenic surface.

To prevent this early graft obstruction, the biomaterial may be modified by altering the physico-chemical properties of the inner polymeric surface,¹ or by endothelialising seeding.²,³ Therefore, in the so-called “two-stage in vitro lining”, enriched autologous endothelial cells are seeded preoperatively onto the lumen of the vascular graft, establishing a confluent non-thrombogenic cellular monolayer. The use of autologous cells avoids an inflammatory immune response directed against the implant. Autologous cells used in this technique are mainly of venous origin. In a significant number of cases, due to previous bypass surgery or systemic vascular diseases, patients may lack an appropriate vein to obtain a sufficient number of cells for the endothelialisation process. In such a case, the isolation of microvascular endothelial cells from adipose tissue by means of a magnetic separation technique⁵ may offer an alternative source of cells for the endothelialisation process.

Recently, it has been demonstrated that shear stress is responsible for changes in structural and functional properties of endothelial cells both in vivo and in vitro.⁶ For example, the shear stress influences significantly the expression of several surface receptors like ICAM-1⁷ and VCAM-1,⁸ which account for thrombogenicity in a time- and shear stress-dependent manner. Furthermore, endothelial cell adherence is influenced by shear stress, causing an increased autocrine laminin production.⁹,¹⁰ Therefore, growing endothelial cells to confluence under shear stress should improve adherence, stability and integrity of the endothelial cell monolayer. Since the adherence and retention of endothelial cells on a PTFE surface is negligible under flow, fibrin has been used as a mediating attachment
We developed a perfusion circuit which enables us both to pre-coat the PTFE graft with fibrin, and to culture endothelial cells seeded on this fibrin matrix during the whole proliferative phase under defined shear stress. The design of the perfusion chamber allows the sampling of graft material for histological investigations within the culture period.

**Material and Methods**

*Endothelial cell harvest and culture*

Human umbilical vein endothelial cells (HUVEC) were isolated according to the method described by Jaffe *et al.* Briefly, a 7–10 cm long umbilical vein was cannulated and gently washed with PBS followed by 15 min of collagenase digestion. Afterwards, cells were flushed out and gently washed in PBS. The cells were cultured in a medium mixture containing one part of Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, Steinheim, Germany) supplemented with 20% fetal calf serum (Seromed, Berlin, Germany), 4 mM L-glutamine (Seromed, Berlin, Germany), 2.5 μg/ml amphotericin B (Seromed, Berlin, Germany), 28 μg/ml gentamicin (Sigma-Aldrich, Steinheim, Germany) and 50 U/ml heparin (Seromed, Berlin, Germany), and one part of endothelial cell growth medium (PromoCell, Heidelberg, Germany). Microvascular endothelial cells (MVEC) from adipose tissue were isolated using UEA-1-lectin-coated dynabeads (Deutsche Dynal, Hamburg, Germany) and cultured in an identical medium. Seeding of both cell types was performed in 1% gelatin-coated flasks. Before reaching confluence, cells were trypsinised, washed and diluted in a 1:2 ratio. This procedure was repeated until a sufficient number of endothelial cells could be harvested for graft-coating. Cell identity was confirmed by *in situ* 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 monolayers. Contamination by mycoplasm and other bacteria was tested routinely by means of a fluorescence kit (Seromed, Berlin, Germany). Cells used for the experiments were freshly isolated and not older than three passages.

*Perfusion chamber*

The perfusion chamber (Fig. 1) was manufactured using autoclavable plastics. It consists of a hollow cylinder (A) closed at both ends with a sterile lid (C) and piston (L), respectively. The latter may be moved into the chamber, maintaining sterility within the chamber because of two washers on the piston that allows the installation of PTFE grafts (B) of various lengths. The lid and piston are equipped with two sets of adapters. Peripheral adapters link the tubing of the outer circuit to the perfusion chamber, which will supply the outer surface of the prosthesis with medium at a lower flow rate compared to the inner circuit. The second set of adapters placed in the centre of lid and piston connect the inner lumen of the prosthesis with the inner medium circuit. The special geometry of this adapter guarantees a smooth change in diameter, preventing both turbulence flow and areas of dead volume where fibrin could accumulate and obstruct the lumen. Luer-lock connectors at the outer side of lid and piston permit the installation of the PTFE graft in the inner perfusion circuit. This new technique enables a gas and substance exchange between the perfused graft lumen and the outer chamber volume which is simulating the interstitial tissue liquid. By loosening the lid (C) and shifting the piston (L) into the chamber volume, it is possible to take a sample of the PTFE graft (max. 2 cm). After reconnecting the graft to the central adapter of the lid and retracting the piston, the perfusion of the PTFE graft may be continued. The cylindrical shape of the perfusion chamber allows the axial rotation of the chamber during the phase of initial cell seeding on a roller (O). Such a design enables an equal distribution of the endothelial cells on the PTFE graft surface.

*The perfusion system*

The perfusion system (Fig. 2) consists of an inner (M) and outer circuit (N). The inner circuit connects the perfusion chamber (A) to a pressure sensor (D), the rolling pump (E; IPC-4, Ismatec, Zurich, Switzerland) and the media bag (F; Stedim, Nancy, France). The media bag serves not only as a medium reservoir, but also as the compliance element in the circuit.
Vascular Graft Endothelialised Under Flow

107

rather different from that of other research groups, being faster and easier to handle.

**Endothelial cell seeding and perfusion**

Endothelial cells were trypsinised and washed in medium. Cell number and suspension volume were adjusted to seeding density, graft surface area and graft volume. The cell suspension was filled into the PTFE graft, avoiding air bubbles. In the next step, the perfusion chamber was constantly rotated on a roller 20 times per hour over a period of 4 h followed by 14 to 16 h of static incubation. The static incubation was necessary to give the cells time to adhere strongly enough to the underlying fibrin matrix. Additionally, the surface is gas-permeable, guaranteeing the gas exchange between medium and well-defined atmosphere within the incubator. Connecting the media bag in parallel to the inner circuit via two 4-way stopcocks (G & H), it is possible to regulate the medium exchange rate between circuit and media bag via clamp (I). Furthermore, it is possible to exchange the media bag without interrupting the medium circulation in the inner circuit. The perfusion chamber is coupled to the inner and outer circuit via four multi-adapters (J & K; Sarstedt, Nümbrecht, Germany). This offers the possibility of removing the chamber from the perfusion circuit without losing sterility.

**Fibrin coating of hybrid-PTFE grafts**

In our experiments, we used 2 × 8 cm long thin-walled hybrid-PTFE grafts (Atrium, Hudson, U.S.A.) with a diameter of 4 and 6 mm, respectively. The grafts were a gift from B. Braun, Aesculap (Tuttlingen, Germany). The graft was mounted into the chamber and fixed at the adapters of lid and piston. The fibrin coating starts with the pressurised filling of Tissucol® (70 ± 110 mg/ml human fibrinogen, 2 ± 9 mg/ml plasma fibronectin, 10 ± 50 E coagulation Factor XIII, 0.02 ± 0.08 mg/ml plasminogen, NaCl, Na-citrate, aprotinin, glycine, heparin, triton, human albumin; Immuno Heidelberg, Germany) using a syringe. Pressure was maintained until small Tissucol® drops appeared at the outer graft surface. Excessive fibrinogen was flushed out of the graft lumen. To start the polymerisation cascade, 10 I.E. of human thrombin were injected over a period of about 5 s and flushed out immediately with air. After an incubation time of 3 min, the graft was rinsed twice with PBS and stored filled with PBS for a maximum of 2 h. It should be stated that this technique is

**Endothelial cell seeding and static culture**

In the case of static culture, the seeding procedure was performed under identical conditions compared to the perfusion experiments. After finishing the initial seeding phase of 4 h, the graft seeded with endothelial cells was cautiously rinsed with fresh medium to remove non-adherent cells and then closed at either end with a stopcock. To avoid poor nutrition of the seeded cells, the medium was replaced every 10 h. After the static culture period of 48 h, the graft was mounted into the circuit and perfused with medium at a shear stress of 3 dyn/cm², respectively, over a period of 5 min.

**Histological examination**

The endothelialised grafts fixed in 4% formalin were cut transversely with a microtome blade to obtain 4 to 6-mm long ring segments from the distal and central segments of the graft. These segments were examined by standard paraffin histology and standard immunohistochemistry for CD31 and Factor VIII staining. Sections were made in transverse orientation.
Fig. 3. Paraffin section of a fibrin-coated PTFE-graft (eosin-stained, 200×). Notice the thin and uniform fibrin layer attached to the PTFE.

To examine the stage of endothelialisation, some ring segments were fixed in a solution containing 4% formalin and 0.5% glutaraldehyde in PBS for at least 8 h, then washed in PBS and stained with haematoxylin. After staining, the ring was cut longitudinally into four segments. The segments were flattened on a slide with the outer graft surface directed towards the slide surface, and observed with a modified light microscope.

Results

In contrast to other research groups using electron microscopy and Indium111-labelling to determine seeding efficiency, we were able to examine the distribution of freshly seeded and cultured cells on the whole surface of the endothelialised PTFE graft by upper light microscopy as well as paraffin histology. The critical point of cell-staining for upper light microscopy consists in the intensive background signal of the fibrin matrix which stains very strongly with methylene blue, eosin, May–Gruenwald and Giemsa. Summarising, haematoxylin staining gave the best result because of the pronounced contrast between fibrin- and cell-staining. The paraffin histology of cross-sections of fibrin-coated PTFE grafts revealed an 8-μm thin fibrin layer of even distribution and thickness. Furthermore, the distal ends of the PTFE graft displayed this characteristic fibrin film despite being in contact with the graft holders (Fig. 3).

After seeding HUVEC onto the graft with a density of 1.5 × 10⁵ cells/cm² at a rotation velocity of 0.3 r.p.m., a uniform cell distribution could be observed along the whole graft. Lower rotation velocity led to an unequal distribution of cells, whereas higher rotation velocities inhibited cellular attachment. The formation of multicellular aggregates on the graft surface as described by other research groups was not observed. After 4 h of rotation, the cells were attached to the PTFE-surface, but revealed a rounded shape. Only after static incubation did the cells develop their typical flattened morphology. In contrast, cells seeded in the culture flask attached faster and reached their typical morphology after only 2 h. Therefore, grafts were only perfused after finishing the static incubation of 14 to 16 h. The perfusion lasted 24 h, including a stepwise increase in applied shear stress of up to 6.6 dynes/cm² for the first 14 h. Perfusion of the cell-seeded PTFE graft did not lead to a reduction in the total endothelialised area. The opposite took place: the cells proliferated rapidly to a confluent monolayer after 24 h of perfusion (Figs 4 and 5). The endothelial cell layer also grew to confluence under static conditions.

In contrast to the perfused graft, the medium contained a number of dead cells. The confluence was not stable enough to withstand a medium flow at 3 dyn/cm² for 5 min. The shedding of cells did not take place at the moment of flow onset, but was continuous over the whole observation period. After 5 min, more than 50% of initially adherent cells were washed off.

The immunohistological studies of pre-cultured cells performed in chamber slides™ (Nunc, Naperville, U.S.A.) revealed that approximately 90 per cent of
in vitro with a closed endothelial cell monolayer. However, most of these studies have not taken into account that shear stress may modify important physiological cell functions, like endothelial cell adherence. Static endothelialisation of PTFE grafts could not solve the two problems: the shedding of endothelial cells after the onset of perfusion, and the massive use of fibrinolysis inhibitors, like aprotinin or tranexamic acid, to prevent matrix degradation by the fibrinolytic activity of endothelial cells themselves. About 20% of all endothelial cells will be washed off the PTFE graft during the initial phase of perfusion as demonstrated in vitro. Unfortunately, little is known about the stability of the cell layer after implantation since no direct examination is yet possible. One could expect that a depletion of fibrinolysis inhibitors occurs after implantation, i.e. fibrinolysis takes place followed by shedding of endothelial cells from the graft. As a consequence, the thrombotic fibrin and/or PTFE-surface will be exposed to the coagulation and immune system.

Our experiments suggest that culturing endothelial cells from a sub-confluent to a confluent monolayer under increasing shear stress should provide a solution for both problems. We were able to culture the endothelial cells on fibrin-coated PTFE grafts without using any fibrinolysis inhibitor and did not observe any massive shedding of cells. Using upper light microscopy, the distribution and density of cells attached to the PTFE surface was semiquantified. The cells proliferated to a close monolayer while perfused. Even the application of high shear stress of 6.6 dyn/cm² over a period of 14 h did not influence the cell adherence. Culturing endothelial cells to confluence under flow seems to improve their adherence to the graft surface significantly. Possibly, the production of laminin and other factors mediating cell adherence while proliferating is responsible for this effect. Furthermore, cellular fibrinolytical activity might be suppressed.

In addition, this study revealed no advantage of a discontinuous rotation in endothelial cell seeding onto the PTFE graft surface compared to a continuous rotation technique. We favour the continuous rotation rather than a discontinuous rotation technique often described in literature, since the latter should lead to a polarisation of cell distribution on the graft surface. The proliferation rate was higher for perfused cell cultures compared to static monolayer cultures in flasks. This may result from the special design of this perfusion system, including two different perfusion circuits supplying both the inner and outer side of the PTFE graft with nutrients.

Discussion

In the past, a number of research groups have demonstrated the ability to coat small-calibre PTFE grafts with a closed endothelial cell monolayer. However, most of these studies have not taken into account that shear stress may modify important physiological cell functions, like endothelial cell adherence. Static endothelialisation of PTFE grafts could not solve the two problems: the shedding of endothelial cells after the onset of perfusion, and the massive use of fibrinolysis inhibitors, like aprotinin or tranexamic acid, to prevent matrix degradation by the fibrinolytic activity of endothelial cells themselves. About 20% of all endothelial cells will be washed off the PTFE graft during the initial phase of perfusion as demonstrated in vitro. Unfortunately, little is known about the stability of the cell layer after implantation since no direct examination is yet possible. One could expect that a depletion of fibrinolysis inhibitors occurs after implantation, i.e. fibrinolysis takes place followed by shedding of endothelial cells from the graft. As a consequence, the thrombotic fibrin and/or PTFE-surface will be exposed to the coagulation and immune system.

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We used routinely an upper light microscopy technique for the evaluation of the endothelialised PTFE-surface. Scanning as well as transmission electron microscopy are not suitable for analysing large surfaces because of the small sample size and its extensive preparation. On the other hand, paraffin histology is only suitable for transverse examination of the graft, because of the circular sample geometry which makes it difficult to obtain longitudinal cuts of a single-cell layer. The transverse examination is not suitable for the characterisation of the graft surface coated with endothelial cells but is important for immunohistological studies. Thus, upper light microscopy combined with haematoxylin staining is sufficient to visualise the cells in situ on the PTFE graft surface which enables us to characterise the whole graft surface.

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References


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