PEG-hirudin/iloprost Coating of Small Diameter ePTFE Grafts Effectively Prevents Pseudointima and Intimal Hyperplasia Development

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Objectives. Small diameter PTFE grafts are prone to thrombosis and intimal hyperplasia development. Heparin graft coating has beneficial effects but also potential drawbacks. The purpose of this study was to evaluate the experimental efficacy of PEG-hirudin/iloprost coated small caliber PTFE grafts.

Methods. Thirty-six femoro-popliteal ePTFE grafts (expanded polytetrafluoroethylene, diameter 4 mm) were inserted into 18 pigs. Grafts were randomised individually for each leg and grouped for 3 groups. Group I consisted of native ePTFE grafts, group II were grafts coated with a polylactide polymer (PLA) without drugs and group III grafts were coated with PLA containing a polyethylene glycol (PEG)-hirudin/iloprost combination. The follow-up period was 6 weeks. Patency rates were calculated and development of pseudointima inside the grafts was noted. Thickness of intimal hyperplasia at the distal anastomoses was measured using light microscopy.

Results. Patency rates for group I were 6/9 (67%), for group II 9/10 (90%) and 12/12 (100%) for group III. In groups I and II there was a significant reduction of blood flow proximal to the graft at graft harvest, to 29±12 and 28±20 ml/min respectively (both p<0.01 versus preoperative value), whilst in group III blood flow, 99±21 ml/min, remained at the preoperative level. Subtotal stenosis due to development of pseudointima was noted in each of the native and PLA coated grafts but not in group III grafts. Intimal hyperplasia at the distal anastomosis was lowest in group III.

Conclusions. The PEG-hirudin/iloprost coating of ePTFE prostheses effectively reduced pseudointima and intimal hyperplasia development and led to superior graft patency.

Keywords: Intimal hyperplasia; Peripheral grafts; Antithrombotic coating; Hirudin; Iloprost.

Introduction

It is generally accepted, that the primary choice for infrainguinal reconstructions is the autologous vein.1,2 However, in the absence of a suitable vein, a prosthetic graft has to be inserted. Small caliber vascular prostheses are associated with high rates of graft failure due to thrombosis and development of intimal hyperplasia.3,4 Particularly the prognosis of crural PTFE grafts remains poor. In order to prevent early graft failure, systemic anticoagulation using vitamin K antagonists is commonly administered.5,6 Since the PTFE surface is highly thrombogenic, especially small prostheses providing marginal flow rates are prone to early graft thrombosis.7,8 Several approaches have been developed in order to reduce the thrombogenicity of graft surfaces.9–11 In the past, various efforts were undertaken to facilitate endothelial cell seeding to enhance the thromboresistance.11 However the endoethelial cell seeding procedure is cumbersome and time consuming since the cells have to be harvested from a patient’s vein, several weeks ahead of the definite peripheral reconstruction.11,12 Alternatively prosthetic grafts can be bonded with antithrombotic drugs like heparin or dipyridamole.10,13 However the biggest drawback of the use of heparin remains the risk of heparin-induced thrombocytopenia.14–16

A promising and safe alternative to heparin with even higher anticoagulative properties represents the combination of hirudin and iloprost.17 Hirudin is
a direct thrombin antagonist and inhibits both free and clot-bound thrombin. It is not dependent, unlike heparin, on antithrombin III. Iloprost acts as a strong inhibitor of thrombocyte activation and aggregation.\textsuperscript{17,18} In addition it has a strong vasodilator effect which could be particularly important for peripheral grafts with poor runoff.\textsuperscript{19,20} The use of a polylactide polymer coating as a drug carrier allows a safe delivery of the anticoagulant drugs from the graft surface to reduce the thrombogenicity of the prosthesis during several weeks and months.

The PEG-hirudin/iloprost coating technology has previously been shown to effectively reduce thrombogenicity of clinically used vascular prostheses \textit{in vitro}.\textsuperscript{21} We therefore intended to compare a PEG-hirudin/iloprost coated prosthesis against an uncoated and a graft with polylactide sealing in a randomized fashion in an animal experimental setting. We used a porcine model, since pigs produce a reliable intimal hyperplastic response and because the coagulation system of the pig resembles the human system.\textsuperscript{22–24}

\textbf{Material and Methods}

\textit{Graft coating}

Eight centimeter long segments of 4 mm diameter ePTFE grafts were coated under sterile conditions with a polylactide polymer (PLA, MW 30 kDa, Resomer R203, Boehringer Ingelheim, Ingelheim, Germany) as described previously.\textsuperscript{17} An 8\% PLA solution was used, containing 5\% polyethylene glycol (PEG) hirudin (lepirudin, Aventis Pharma GmbH, Germany) and 1\% iloprost (Ilomedin\textsuperscript{®}, Schering AG, Berlin, Germany). The grafts were dip-coated twice into the solution to achieve a homogenous coating and then dried and sterile packed. The detailed release characteristics of PEG-hirudin and iloprost from the coating have been described elsewhere.\textsuperscript{21} After an initial accelerated release of both drugs during the first 48 h, a slower and continuous release follows over a period greater than 3 months. After 90 days approximately 60\% of the PEG-hirudin and 10\% of the iloprost were released.

\textit{Animals, surgical and randomization procedures}

Thirty-six 4-mm ePTFE grafts were implanted in eighteen female domestic pigs (25-30 kg, age 10 to 16 weeks, German Landrace). Since each animal received two grafts, each side was randomised using closed envelopes. Two animals died during the course of the experiments, one due to an infected port system and one due to malignant hyperthermia (each ePTFE/PLA combination). One infected graft (ePTFE) was excluded from the study. Eventually, group I consisted of native ePTFE grafts (n = 9), group II comprised PLA coated ePTFE grafts (n = 10) and group III PLA containing PEG-hirudin/iloprost (n = 12).

After intramuscular sedation with 4 mg/kg azapiron (Stresnil, Janssen, Germany), 10 mg/kg ketamine (Ursotamin, Serumwerk Bernburg, Germany) and 0.05 mg/kg atropine (B.Braun AG, Melsungen, Germany), a 20-gauge needle was placed in an ear vein. General anaesthesia was maintained after endotracheal intubation with isoflurane (Baxter AG, München, Germany) and oxygen. Antibiotic prophylaxis was provided with 1.2 g amoxicillin (Augmentan, GlaxoSmithKline GmbH, München, Germany) intravenously during surgery and for two doses after surgery. A port system for intravenous administration of drugs and blood sampling was placed into the first 5 animals. This procedure was discontinued after one animal died due to an infection of the port system.

Longitudinal incisions (12 cm) were made in both hind limb groins. The femoral artery was dissected free from the inguinal ligament to the first segment of the popliteal artery. The deep femoral artery was ligated and dissected out. The baseline flow was measured by means of an ultrasonic flow meter (T206, Transonic Systems, Ithaca, USA). Following systemic heparinisation with 300 IE/kg heparin the common femoral artery was clamped. The proximal anastomosis was created end-to-end using a 7-0 polypropylene (Prolene, Ethicon, Germany) running suture. The distal anastomosis was sutured to the distal superficial femoral artery in an end-to-side fashion using a 7-0 Prolene running suture. After restoring blood flow, the flow rate was measured again to confirm patency. The wounds were closed subcutaneously with a running 4-0 poliglecaprone 25 (Monocryl, Ethicon, Norderstedt, Germany) suture for the skin. All animals received 100 mg aspirin/day (ASS 100, Ratiopharm, Germany). The study protocol was approved by the local ethical committee. Animal care complied with the “Principles of Laboratory Animal Care” and the Guide for the Care and Use of Laboratory Animals” (NIH Publication No. 80-23, revised 1985).

\textit{Follow-up and termination}

Graft patency was confirmed once a week and on the day of the harvest using color-coded duplex sonography under sedation, as described previously.

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Six weeks after implantation the grafts were removed. Following induction of general anesthesia, the abdomen was opened using a midline incision and the distal aorta was dissected free. Longitudinal incisions in each hind limb groin were made and the grafts were identified. Both the proximal as well as the distal anastomoses were dissected free. In patent grafts, blood flow was measured in the common femoral artery just above the proximal anastomosis. After systemic heparinisation the distal aorta was ligated and a perfusion cannula inserted. For fixation of the femoral artery and the ePTFE graft a 4% formalin solution was injected into the distal aorta applying a perfusion pressure of 100 mmHg. The graft and the adjacent anastomoses were excised and stored in 4% formalin solution.

Specimen processing and morphometric analysis

Only patent grafts were included in the histological analyses. The specimens were dehydrated to 100% ethanol and were paraffin embedded. Serial longitudinal 5 μm sections were analyzed until the central anastomotic area containing the maximum graft diameter was identified. The representative sections were deparaffinized in xylene and rehydrated. After hydration, the slides were stained with the Verhoeff’s elastic stain and van Gieson’s counterstain technique to highlight the internal elastic lamina. In addition, haematoxylin and eosin stained slides were produced in a standard manner. Sections were analysed with digital planimetry (ImageJ 1.3, NIH, USA). The neointima of the heel and hood portions was measured as the distance between the internal elastic lamina and the lumen at four evenly spaced locations (distance 0.3 mm) starting at the prostheses level (location #1) and ending on the regular endothelial cell layer without significant neointimal thickening (location #4) (Fig. 1).

Clinical chemistry as well as hematologic and hemostatic laboratory values were measured before the operation, on each follow-up examination and before graft removal. The activated partial thromboplastin time (aPTT) was of particular interest, since this parameter would reveal a systemic release of PEG-hirudin.

Statistical analysis

Data were expressed as mean ± SD. The patency was analysed by means of a chi-square analysis. Differences between flow rates within the same groups were calculated using the Wilcoxon test, between the groups using the Mann-Whitney U test and the Kruskal-Wallis test. Differences in laboratory values were calculated after stratification of groups into antithrombotic and non-antithrombotic coating. P-Values <0.05 were considered statistically significant.

Results

Patency and flowrate measurements

In the native ePTFE graft group I (n = 9) three grafts occluded during the six weeks follow up period (patency rate 67%) while in group II (PLA, n = 10) one graft was closed, corresponding to a patency rate of 90%. However in group III (PEG-hirudin/iloprost, n = 12) no graft occlusion was seen ($\chi^2 p = 0.07$). While the baseline flow rates before graft implantation were comparable for all groups, a significant difference was seen between groups I-II and III at the time of removal (Table 1). Flow rates in the ePTFE and PLA group

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Baseline flowrates</th>
<th>Removal flowrates</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (uncoated ePTFE)</td>
<td>6</td>
<td>104 ± 9</td>
<td>29 ± 12</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Group II (PLA coated)</td>
<td>9</td>
<td>105 ± 10</td>
<td>28 ± 20</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Group III (PEG-hirudin/iloprost coated)</td>
<td>12</td>
<td>106 ± 8</td>
<td>99 ± 21</td>
<td>n.s.</td>
</tr>
</tbody>
</table>
were significantly lower than their pre-implantation rates, while the flow rates of the PEG-hirudin/iloprost group showed no differences between implantation and removal. The significant decline of the flow rates of groups I and II corresponded to a marked development of pseudointima leading to a circumferential stenosis of the graft lumen (Fig. 2A). Conversely only a thin and transparent layer of pseudointima was seen in group III grafts (Figs. 2B, 3).

**Histology and morphometric analysis**

Intimal hyperplasia (IH) was defined as the thickness of the layer between vessel lumen and internal elastic lamina. The results are depicted in Table 2. In all graft types the intimal hyperplasia decreased gradually with increasing distance from the anastomoses. IH development was more prominent in the anastomotic hood areas than in the heel regions. In all cases IH development was stronger in grafts without antithrombotic coating, compared to the PEG-hirudin/iloprost coating group. However, statistical significant differences between the groups were limited to the most centrally located IH areas of the anastomotic hood (#1 in Fig. 1).

There were no significant differences in the clinical chemistry and hematological or hemostatic values during the course of the experiments (data not shown). Especially the aPTT values in animals with PEG-hirudin/iloprost coated grafts (31 ± 6 s) and without antithrombogenic coating (30 ± 5 s) showed no significant difference.

**Discussion**

Small calibre PTFE grafts with low mean flow rates generally have a high risk of graft failure due to technical problems and the high thrombogenicity of PTFE.1,7 In order to reduce the thrombogenicity of the PTFE/graft interface several approaches exist.25 One potentially promising approach to reduce the thrombogenicity of PTFE grafts represents the pharmacological modification of the blood/graft interface. Anticoagulant drugs to be considered in this regard comprise heparin, dipyridamole and hirudin.10,17,26 The first clinical trials using heparin bonded PTFE grafts were promising, although the difference

![Fig. 2. Comparison of the distal anastomoses of an uncoated (A) versus a PEG-hirudin/iloprost coated (B) ePTFE graft. A marked pseudointima, resulting in a subtotal stenosis, developed in all uncoated/PLA coated prostheses (n = 19), while only a thin layer was seen in antithrombotic coated grafts (n = 12). * denotes distal femoral/popliteal artery. ePTFE - expanded polytetrafluoroethylene. (Verhoeff van Giesson stain, original magnification ×1.6).](image-url)
between heparin coated and uncoated grafts was statistically not significant after 5 years.\textsuperscript{13,27,28} Use of a heparinized graft has some drawbacks since the antithrombotic action of heparin is dependent on circulating cofactors (Antithrombin III) and is not effective against clot-bound thrombin.\textsuperscript{26} Another real concern is the occurrence of heparin-induced thrombocytopenia. Heparin, released from the coated graft, could sensitise patients with this disease resulting in bleeding and thrombo-embolic complications. The incidence of this potential harmful disease among vascular surgical patients ranges between 2\% and 21\%.\textsuperscript{14-16} The high incidence is probably due to the frequent exposure of these patients to heparin. There is a report of a patient, who developed a diffuse thrombosis following placement of a heparin-coated coronary stent.\textsuperscript{29} Since the immunogenic surface is much greater in long segments of 6 or 8 mm PTFE grafts, compared to a small coronary stent, the potential risk would appear to be higher in patients requiring peripheral reconstructions. In affected cases an immediate graft removal with potential limb loss would be mandatory.

A promising and safe alternative for the heparin coating represents the combination of hirudin and iloprost, used with a coating technique that gradually releases the drugs into the graft lumen. We therefore selected a polylactide polymer coating technology, that has been used for several other projects in cardiovascular and orthopaedic medicine.\textsuperscript{17,21,30} The hydrolytic step-by-step degradation of the polymer allows a continuous release of drugs over weeks and several months, depending on the thickness of the coating layer. Although the observation time of this study was limited to 6 weeks, the beneficial effect of the drug coating could be expected to last at least for 3 months.\textsuperscript{21} The rationale of using PEG-hirudin together with iloprost was the combination of a thrombin inhibitor with a strong antiplatelet drug. The PEG-hirudin/iloprost combination has been used successfully for coating of coronary stents in animal experiments.\textsuperscript{31} Hirudin is a specific and highly potent inhibitor of thrombin. It is able to inhibit both clot-bound and soluble thrombin. The recombinant form was found to be very effective for prevention of arterial thrombosis in animal models.\textsuperscript{32} When conjugated with polyethylene glycol (PEG) the duration of action is prolonged significantly.\textsuperscript{33} In a recent study the antithrombotic superiority of hirudin compared to heparin was clearly demonstrated.\textsuperscript{34} Since thrombin acts as a potent mitogen

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|}
\hline
Group & n & Hood #1 & Hood #2 & Hood #3 & Hood #4 & Heel #1 & Heel #2 & Heel #3 & Heel #4 \\
\hline
ePTFE & 7 & 0.48 ± 0.1 & 0.25 ± 0.1 & 0.19 ± 0.1 & 0.15 ± 0.1 & 0.37 ± 0.1 & 0.28 ± 0.1 & 0.21 ± 0.1 & 0.15 ± 0.1 \\
PLA coated ePTFE & 7 & 0.47 ± 0.1 & 0.36 ± 0.1 & 0.30 ± 0.1 & 0.22 ± 0.1 & 0.34 ± 0.1 & 0.29 ± 0.1 & 0.20 ± 0.2 & 0.14 ± 0.1 \\
PEG-hirudin/iloprost coating & 11 & 0.20 ± 0.1 & 0.16 ± 0.1 & 0.13 ± 0.1 & 0.11 ± 0.1 & 0.18 ± 0.1 & 0.14 ± 0.1 & 0.12 ± 0.1 & 0.10 ± 0.1 \\
p-value & <0.05 & n.s. & n.s. & n.s. & n.s. & n.s. & n.s. & n.s. & n.s. \\
\hline
\end{tabular}
\caption{Effect of antithrombotic coating of ePTFE prostheses on the development of intimal hyperplasia (mm) at the distal anastomosis of femoro-popliteal grafts in pigs. ePTFE = expanded polytetrafluoroethylene}
\end{table}
and chemotactic agent, hirudin also could reduce neointimal thickening.\textsuperscript{35}

The copartner of hirudin, iloprost is a prostaglandin \(\text{I}_2\) analogue which as been shown to effectively inhibit platelet activation and aggregation.\textsuperscript{18} It additionally has vasodilator effects, which are particularly important for peripheral reconstructions with poor run-off vessels. After arterial application iloprost induces a peripheral vasodilatation, thus reducing the peripheral resistance and impedance, leading to an enhanced blood flow through the grafts.\textsuperscript{19,20} Both drugs bear antiproliferative capabilities and it has previously been shown that they had beneficial effects regarding neointimal development.\textsuperscript{31,36}

The present study clearly demonstrated the benefit of a PTFE coating with a PEG-hirudin/iloprost bonding. In order to create realistic and disadvantageous conditions, we used small ePTFE grafts, implanted into a relatively low flow area of the porcine hind limb. The measured blood flow of 100 ml/min resembles that of crural arteries.\textsuperscript{19} The use of a distal end-side anastomosis was chosen to induce a flow pattern, which is prone to neointimal hyperplasia development.\textsuperscript{37,38}

All of the implanted antithrombotic coated grafts were patent and showed only very small amounts of pseudointima development. The remaining grafts developed a marked pseudointima leading to a significant graft stenosis which corresponded to a significant decrease of the flow rates, which eventually contributed to graft failure. The small layer which was seen in antithrombotic coated grafts was covering the entire graft surface. We hypothesized that the small neocoating was responsible for a decreased surface thrombogenesis of the ePTFE prostheses.

Despite differences in patency and development of pseudointima, which were ascribed to a reduced thrombogenicity of the grafts, we additionally observed a considerable reduction of intimal hyperplasia development inside the distal anastomoses. In addition to enhanced flow characteristics, this was attributed to the antiproliferative properties of hirudin and iloprost.\textsuperscript{31,35}

We did not observe local or systemic effects of the graft coating. The coating did not influence the graft healing into the subcutaneous tissue and we did not observe peri-graft reaction. The locally released drugs did not impair the systemic coagulation. This supports the hypothesis, that the anti-thrombotic effect of the eluted drugs is essentially limited to the prosthesis surface. The available data suggest that the coating of ePTFE grafts with a PEG-hirudin/iloprost eluting polymer meets the safety requirements for peripheral reconstructions.

In conclusion the PEG-hirudin/iloprost coating of PTFE prostheses represents a promising approach for improvement of small PTFE grafts and should be further investigated in a randomised clinical trial.

References


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