Suppression of neointimal hyperplasia by sirolimus-eluting expanded polytetrafluoroethylene (ePTFE) haemodialysis grafts in comparison with paclitaxel-coated grafts

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Abstract

Background. Haemodialysis vascular access dysfunction caused by aggressive venous neointimal hyperplasia is a major problem for haemodialysis patients with synthetic arteriovenous (AV) grafts. Several different strategies to prevent venous stenosis by inhibiting smooth muscle cell proliferation and migration using local delivery of potent antiproliferative agents are currently under investigation. We performed this study to evaluate the efficacy of sirolimus-eluting vascular grafts in preventing stenosis and to compare the effectiveness of sirolimus-coated grafts with that of paclitaxel-coated vascular grafts that we characterized in a previous study.

Methods. AV grafts were implanted laterally between the common carotid artery and external jugular vein of 14 female Landrace pigs. Three types of grafts were implanted: grafts coated with 1.08 μg/mm² sirolimus (low dose, n = 6), grafts coated with 2.41 μg/mm² sirolimus (high dose, n = 2) and uncoated control grafts (n = 6). Animals were sacrificed 6 weeks after surgery. Cross-sections of the venous anastomoses were analysed to determine the percentage of luminal stenosis in each group, and immunohistochemistry was performed to identify the cellular phenotypes of the neointimal hyperplasia and tissues adjacent to the implanted grafts.

Results. Compared with the control group, neointimal hyperplasia in the venous anastomoses of the groups implanted with sirolimus-coated vascular grafts was significantly suppressed without infection. The mean ± standard error values for the percentage of luminal stenosis were 75.7 ± 12.7% in the control group and 22.2 ± 1.41% in the low-dose sirolimus-coated group. Myofibroblasts and fibroblasts were the major cell types found in the neointimal hyperplasia.

Conclusions. Neointimal hyperplasia was effectively suppressed by sirolimus-eluting grafts. However, the inhibitory effects of sirolimus-eluting grafts were weaker than those observed for paclitaxel-coated grafts in our previous study.

Keywords: controlled drug release; drug delivery; haemodialysis; intimal hyperplasia; vascular graft

Introduction

Millions of end-stage renal disease patients depend on native arteriovenous (AV) fistulas or expanded polytetrafluoroethylene (ePTFE) grafts for permanent vascular access, but haemodialysis vascular access complications are a major clinical problem. Currently, the cost of vascular access care is approximately 1 billion US dollars [1]. AV fistula placements have increased as a result of the Fistula First Breakthrough Initiative, but decreased patency or failure of fistulas to mature has resulted in the increased use of central venous catheters [2]. Although more salvage procedures are required for AV grafts, it is recommended that ePTFE grafts should serve as the initial primary access in patients with poor vessels, such as elderly patients [3, 4]. The elderly haemodialysis population is the largest group of patients in the USA, and up to 40% of incident patients are >65 years old in many European countries [5].

The failure of haemodialysis vascular grafts is predominantly due to stenosis that results from progressive neointimal hyperplasia, and 50–70% of these stenoses occur within 3 cm of the venous anastomosis site [6]. The 1- and 2-year primary patency rates of vascular grafts are as low as 50 and 25%, respectively [7].

The proliferation and migration of vascular smooth muscle cells and myofibroblasts into the intima can result in a pathological narrowing of access [8]. Many efforts have been made to prevent venous stenosis in vascular grafts by inhibiting smooth muscle cell proliferation and migration using the local delivery of potent anti-proliferative agents such as paclitaxel or sirolimus [9–15]. Positive outcomes have been reported in animal experiments, but the efficacy and safety of these methods have not been proven in clinical trials.

Sirolimus, an anti-proliferative agent that blocks G1 to S cell cycle progression by interacting with the specific target protein mammalian target of rapamycin (mTOR), thereby
inhibiting its activation, can also prevent tissue hyperplasia following vascular injury [16–18]. In this study, we coated ePTFE grafts with sirolimus and evaluated the efficacy of sirolimus-eluting vascular grafts for preventing neointimal hyperplasia in a pig model. We compared the results to those we obtained in a previous study in which we investigated paclitaxel-eluting vascular grafts.

Materials and methods

Materials

Sirolimus was purchased from LC Laboratories. The ePTFE vascular grafts (IMPRA, F4006C) were purchased from Bard Peripheral Vascular Inc. High-performance liquid chromatography (HPLC)-grade acetone and acetonitrile were obtained from Fisher Scientific. Tween-20 was purchased from Sigma Chemical Co, and phosphate-buffered saline (PBS) was obtained from Cambrex Corporation. All other reagents were of analytical grade and used without further purification.

Preparation of sirolimus-coated ePTFE grafts

The ePTFE vascular grafts were used to test the efficacy of a sirolimus coating. Sirolimus-coated vascular grafts were made using a single dipping method. Briefly, sirolimus was dissolved in acetone to a concentration of 2.0 or 4.0 mg/mL in polypropylene tubes. Each of the ePTFE vascular grafts, 6 mm in diameter and 20 cm long, was dipped into one of these solutions and incubated for 50 min at 37°C in a roller incubator (Combi-H; FinePCR, Korea). The sirolimus-coated ePTFE grafts were then air-dried and kept under vacuum overnight to remove the solvent completely. These grafts were sterilized in ethylene oxide gas before use. The corresponding amounts of sirolimus on the grafts for the coating solution concentrations of 2.0 or 4.0 mg/mL were 1.08 and 2.41 μg/mm², respectively.

In vitro release test

For the in vitro release test, PBS (pH 7.4) containing 0.5% (w/vol) Tween-20 was used as the drug release medium. Sirolimus-coated vascular grafts with a length of 3 cm were soaked in polypropylene tubes with 10 mL of release medium and shaken at 37°C in a roller incubator. At designated time points over a period of 20 days, release medium was removed completely and replaced with fresh PBS. The release medium was collected at each time point, and the amount of sirolimus was determined by HPLC (Agilent 1200 Series) using a 4.6 × 150 mm C18 reverse phase column and a UV detector set at 278 nm. The mobile phase used was water:acetonitrile (30:70 vol/vol) under isocratic conditions. The sirolimus was eluted at 18 min.

Experimental animals and operative technique

Eight female Landrace pigs in good health, weighing 50 ± 7 kg on average, received a single sirolimus-coated ePTFE graft between the common carotid artery and the external jugular vein. Of the eight pigs, six received either 2.0 or 4.0 mg/mL in polypropylene tubes. Each of the ePTFE vascular grafts was sterilized in ethylene oxide gas before use. The corresponding amount of sirolimus on the grafts for the coating solution concentrations of 2.0 or 4.0 mg/mL were 1.08 and 2.41 μg/mm², respectively.

Animals were euthanized 6 weeks after the operation described above. Until then, the animals were maintained in standard animal care facilities at Samsung Biomedical Research Institute. All operating procedures conformed to the Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

The pigs were anesthetized with intramuscular ketamine HCl (20 mg/kg) and xylazine HCl (2 mg/kg). Then, they were intubated and ventilated with a mixture of O₂ and air (1:2) containing enflurane (2%) for the maintenance of anaesthesia. Vecuronium bromide (0.1 mg/kg) was administered continuously through an ear vein.

As in previous studies, we adopted the animal experiment model proposed by Rotmans et al. [19]. After standard surgical cleansing, a longitudinal incision was made in the right lateral side of the neck along the sternocleidomastoid muscle. The common carotid artery and the external jugular vein were exposed and heparin was given intravenously at 100 IU/kg before vessel manipulation. The common carotid artery was clamped using vessel loops and an 8-mm arteriotomy was made. An end-to-side anastomosis was made at ~45° using a 6-0 polypropylene suture. Venous anastomoses were created in a similar manner. When the animals were euthanized at 6 weeks post-surgery, the implanted ePTFE grafts were excised along with the adjacent vessels and immersion fixed in 10% neutral-buffered formalin (NBF) for at least 24 h. All pigs were administered aspirin (100 mg/day) and clopidogrel (PLAVIX®; Sanofi Aventis, France; 75 mg/day) from day 0 to the day of euthanasia.

Tissue preparation and morphometry

Excised specimens were fixed in 10% NBF and embedded in paraffin. Sections (5-μm thick) of veins 1 cm proximal and distal to the anastomosis were prepared by taking slices perpendicular to the blood flow. Serial sections were taken to obtain cross-sections around the centre of the graft–venous anastomosis and three sections were analysed in order to determine the percentage of luminal stenosis, as shown in Figure 1. All sections were stained with haematoxylin and eosin (H&E) to roughly confirm the presence of inflammation around the implanted ePTFE graft. The percentage of luminal stenosis was calculated from the area of the neointima divided by the total luminal area inside the ePTFE graft; the sections were then further stained with Masson’s trichrome. The areas of the intima and media were traced manually on the captured images using an Aperio ImageScope (Aperio). A microscope (BX50F; Olympus Digital Camera System, Japan) was used to identify the cellular phenotypes in the adjacent tissue outside the ePTFE graft. The percentage of luminal stenosis was calculated from the area of the neointima divided by the total luminal area inside the grafts and vascular tissues. We measured the percentages of luminal stenoses in three sections from each graft-venous anastomosis site, and the mean values for these three sections were analysed to compare between each groups. We compared the results we obtained in this study with those we obtained for paclitaxel-coated grafts in a previous study.

Fig. 1. Diagram of a graft-venous anastomosis. (A) Serial sections were taken to obtain cross-sections around the centre of the graft-venue anastomosis and three of these sections were analysed to find the mean percentage of luminal stenosis. One section was obtained at the centre of the anastomosis; the others were obtained 2 mm to the proximal side and 2 mm to the distal side from the centre of the anastomosis. (B) A cross-section was taken in the centre of the anastomosis site. The grey area indicates neointimal hyperplasia in the lumen of the graft-venous anastomosis.
Immunohistochemistry and cellular phenotyping

Five-micrometre sections from each paraffin-embedded tissue block were immunostained with antibodies against alpha-smooth muscle actin (α-SMA; Abcam PLC, UK), vimentin (AbD Serotec, UK) and desmin (GeneTex) using Vectastain ABC kits (Vector Laboratories) according to the manufacturer’s instructions. Briefly, sections were deparaffinized and hydrated through a xylene and graded alcohol series. After antigen retrieval in antigen-unmasking solution (Vector Laboratories) for 15 min, the slides were incubated in 0.3% H₂O₂ for 30 min, in normal blocking serum for 20 min, in primary antibody overnight at 4°C, in biotinylated secondary antibody for 30 min, in Vectastain ABC reagent for 30 min and finally in peroxidase substrate solution. The sections were then counterstained with haematoxylin, dehydrated through a graded alcohol series and scanned using an Aperio ImageScope.

To analyse the cellular phenotypes for graft neointima and adjacent tissue outside the graft wall, semiquantitative scoring of all sections was performed on a scale from 0 to 4, which corresponded to the percentage of cells positive for the specific marker in several different areas (0, 0–10% positive; 1, 11–25% positive; 2, 26–50% positive; 3, 51–75% positive and 4, 76–100% positive) [20].

Statistics

All data are presented as means ± standard errors. The significance of differences in variables between the control and sirolimus-coated groups was evaluated using the Mann–Whitney test (SPSS 18.0 for Windows; SPSS Inc.). P-values <0.05 were considered statistically significant.

Results

In vitro sirolimus release profiles

Because sirolimus is not soluble in PBS alone, Tween-20 is typically added to the medium for in vitro release tests of drug-coated medical devices. Tween-20, as a surfactant, forces the insoluble drug to dissolve in the release medium. Figure 2 shows the in vitro release profiles of the sirolimus-coated ePTFE grafts. Coating the grafts with 2 mg/mL sirolimus dissolved in acetone resulted in a sirolimus-loading amount of 1.08 μg/mm² on the ePTFE grafts. A burst release of sirolimus was followed by a slower sustained release, but <10% of the initial amount of the loaded drug was released after 20 days. Due to technical difficulties in obtaining an in vitro release curve using a solvent containing more Tween-20, we show an in vitro release profile using only 0.5% Tween-20. Therefore, it is difficult to effectively mimic in vivo conditions using in vitro drug release profiles for sirolimus-coated ePTFE grafts. This is partially due to the characteristics of the ePTFE material, in that sirolimus is strongly adsorbed on the fibrous structure within the ePTFE graft wall via hydrophobic interaction.

Animal experiments and histomorphometry

Infection was defined as the presence of pus and cloudy exudate around the surgical site in the pig experiments. No signs of infection or bacterial contamination were seen in the implantation sites of either the control or the sirolimus-coated groups for 6 weeks.

At euthanasia, little of the drug remained in the explanted ePTFE grafts as measured by HPLC. The implanted ePTFE grafts in the control and low-dose sirolimus-coated groups were firmly attached to the adjacent tissue, but the high-dose grafts did not adhere strongly to the surrounding tissue. The high concentrations of sirolimus predisposed the grafts to slipping out.

Cross-sections of the venous anastomoses from the six uncoated control grafts and the six low-dose sirolimus-coated grafts 6 weeks after surgery are shown in Figure 3.

![Fig. 2. In vitro release profiles of sirolimus from ePTFE grafts containing 1.08 μg/mm² sirolimus. Data are the means of five experiments and bars represent standard deviations.](https://academic.oup.com/ndt/article-abstract/27/5/1997/1838330)

![Fig. 3. Cross-sections of Masson’s Trichrome-stained venous anastomoses 6 weeks after implantation of the control grafts (upper panel, A–F) and low-dose sirolimus-coated grafts (lower panel, G–L).](https://academic.oup.com/ndt/article-abstract/27/5/1997/1838330)
Only one of the six control grafts was patent; the neo-intima was identified as a pale blue area inside the vascular media, which appeared as a layer of thick red fibres that were more evident at high-power magnification. Compared with the control group, all of the sirolimus-coated grafts were patent. However, a small area of neointima that seemed to be growing into the luminal surface of the graft wall was observed in several drug-coated samples. Therefore, we conducted additional experiments by doubling the amount of drug with which the graft was coated, as shown in Figure 4.

Although neointimal hyperplasia decreased markedly in pigs in which high-dose sirolimus-coated vascular grafts were implanted, neo-intima was still observed inside the graft wall.

To evaluate quantitatively the aggressiveness of neointimal hyperplasia in the three groups, we measured the percentages of luminal stenosis in the venous anastomosis sites (Figure 5). The mean ± standard error values for the percentage of luminal stenosis were 75.7 ± 12.7% in the control group, 22.2 ± 1.41% in the low-dose and 14.2 ± 4.2% in the high-dose sirolimus-coated group. The percentages of luminal stenosis were significantly lower in the sirolimus-coated groups than in the control group (P < 0.05, Mann–Whitney test), but dose effect was not statistically significant due to the small sample size of the high-dose group.

**Immunohistochemistry and cellular phenotyping**

In addition to Masson’s trichrome stain, samples were stained with antibodies against α-SMA, vimentin and desmin to determine the cellular phenotypes of the graft neointima and adjacent tissue outside the graft wall, the site of which is indicated in Figure 1B. These markers were used to identify the presence of myofibroblasts, fibroblasts and contractile smooth muscle cells (see Table 1 [20–22]).

Because slight neointimal hyperplasia was observed near the suturing sites in the sirolimus-coated groups, we analysed all of the cellular phenotypes of the neo-intima adjacent to the suturing area. As shown in Figure 6, which depicts neointimal hyperplasia within the graft luminal surface just beside the suturing site of the venous anastomosis, the majority of cells in the neo-intima were α-SMA-positive, vimentin positive and desmin negative, suggesting that they were myofibroblasts. Furthermore, a number of cells were α-SMA-negative, vimentin-positive and desmin-negative, suggesting the presence of fibroblasts. There were few desmin-positive cells, suggesting that contractile smooth muscle cells were rare in the neo-intima. The graft neo-intimas in the control (A–C) and low-dose sirolimus-coated group (D–F) showed very similar cellular phenotypes, which included a very large number of myofibroblasts and a considerable amount of fibroblasts. However, the high-dose sirolimus-coated group (G–I) had different cellular phenotypes in that the number of myofibroblasts was drastically reduced just at the boundaries of the graft wall. In addition, the number of fibroblasts was much higher than in the other groups (H). To determine whether the implanted sirolimus-eluting ePTFE graft influenced the cellular phenotypes in the local tissue, we compared the cellular phenotypes of the adjacent tissue outside the graft wall between the control and sirolimus-coated groups. Compared with the types of cells found in the graft neo-intima, the adjacent tissue outside the ePTFE graft contained myofibroblasts, fibroblasts and smooth muscle cells (Figures 7 and 8). There were also few desmin-positive cells outside the grafts. At the boundaries...
between the graft and adjacent tissue in the high-dose sirolimus-coated group, the number of \( \alpha \)-SMA-positive cells was drastically reduced, signifying a decreased number of myofibroblasts compared with the control and low-dose group (see Figure 7G). Therefore, the distributions of cellular phenotypes in the adjacent tissue outside the graft wall were similar to those of the graft neointimas (see Figure 6G).

As shown in Figure 9, the semiquantitive scores of the control and low-dose group indicated similar cellular constitutions in the graft neointima and the tissue around the implanted graft wall. In the neointimal area, more myofibroblasts and contractile smooth muscle cells were detected in the sirolimus-coated group than in the control, but the results were not significantly different between the two groups. The high-dose sirolimus-coated group was excluded in this analysis due to the small sample size \( (n = 2) \), but the difference in distribution of cellular phenotypes is shown in Figures 6 and 7.

In summary, compared with the control group, the coating of the ePTFE graft with a low dose of sirolimus did not significantly change the distribution of cellular phenotypes of the neointima and adjacent tissue outside the implanted graft. However, in the high-dose sirolimus-coated group, the cellular constitutions of both the neointima and adjacent tissue were significantly different.

### Discussion

In this study, we demonstrated that sirolimus-coated ePTFE vascular grafts effectively suppress neointimal hyperplasia in the venous anastomosis site of an animal model.

The low-dose implanted grafts were tightly surrounded by adjacent tissue. Immunohistochemistry revealed a proliferation of myofibroblasts at the boundaries between the graft and adjacent tissue (Figure 8). Fibroblasts originating from the perigraft region were previously reported to infiltrate into the intragraft \([23, 24]\). Adhesion between the implanted material and the adjacent tissue is thought to be due to the normal foreign body response; myofibroblasts entering the graft wall produce a contractile force that pulls adjacent tissues together \([25, 26]\). In contrast, the majority of implanted grafts were not firmly attached to the surrounding tissue in the high-dose sirolimus-coated group, probably due to the inhibition of myofibroblast proliferation by sirolimus. An excess of sirolimus may retard the maturation of stable graft placements, which could delay cannulation.

The exact mechanism underlying the inhibition of neointimal hyperplasia by the sirolimus-eluting graft is unclear. We investigated the cellular migration and proliferation at the graft-venous anastomosis site. As shown in Figures

### Table 1. Determining the cellular phenotypes through immunohistochemistry

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<tr>
<th>Phenotype</th>
<th>( \alpha )-SMA</th>
<th>Vimentin</th>
<th>Desmin</th>
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<tr>
<td>Smooth muscle cells</td>
<td>+</td>
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<td>Myofibroblasts</td>
<td>+</td>
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<td>Fibroblasts</td>
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Fig. 6. Cellular phenotypes of graft neointima in the control group (A–C) and the low-dose (D–F) and high-dose (G–I) sirolimus-coated groups. Sections from each group were immunohistochemically stained with anti-\( \alpha \)-SMA (A, D and G), anti-vimentin (B, E and H), and anti-desmin (C, F and I) antibodies \((\times 200 \text{ magnification})\).
and 7, myofibroblasts and fibroblasts were found in the neointima in the lumen and the adjacent tissue outside the ePTFE graft. Fibroblasts in the neointima immediately adjacent to the suturing site migrated towards the centre of the graft lumen and differentiated into myofibroblasts, which are the major constituent cells of the neointima. These observations are consistent with the hypothesis that cell migration from the adventitia towards the graft lumen is the major contributor to neointimal hyperplasia [27]. In this process, fibroblasts outside the graft are transformed into myofibroblasts in the neointima. Neointima formation is quite similar to the wound healing process in that nascent fibroblasts migrate, proliferate and differentiate into myofibroblasts due to an external stimulus. As expected, we found that sirolimus effectively suppressed the migration and proliferation of myofibroblasts near the suturing sites. As blood vessels damaged by suturing are in direct contact with the drug-eluting ePTFE graft, the sirolimus released from the graft can effectively influence nearby stimulated cells not to migrate and proliferate even under high flow condition.

When high-dose (2.41 μg/mm²) sirolimus-coated grafts were implanted, the luminal area of the venous anastomosis site increased and the neointimal hyperplasia decreased markedly. However, despite the high drug loading, some degree of neointimal hyperplasia was still observed on the luminal surface of the graft walls. When we investigated paclitaxel-coated vascular grafts in our previous studies, we

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**Fig. 7.** Cellular phenotypes of tissue outside the graft wall in the control group (A – C), and the low-dose (D – F) and high-dose (G – I) sirolimus-coated groups. Sections from each group were immunohistochemically stained with anti-a-SMA (A, D and G), anti-vimentin (B, E and H) and anti-desmin (C, F and I) antibodies (×200 magnification).

**Fig. 8.** Cellular phenotypes of tissue adjacent to the ePTFE grafts obtained by staining sequential sections of a low-dose sirolimus-coated vascular graft (Figure 3K) with anti-a-SMA (A), anti-vimentin (B), and anti-desmin (C) antibodies; (a) ePTFE graft wall, (b) myofibroblasts, (c) fibroblasts and (d) contractile smooth muscle cells (×400 magnification).
found little neointimal hyperplasia on the luminal surface of the graft walls [28, 29]. These results indicate that paclitaxel effectively suppressed the migration and proliferation of myofibroblasts, whereas sirolimus had a weaker inhibitory effect on neointimal hyperplasia in the luminal surface of ePTFE grafts, even at a high loading concentration.

Besides inhibiting neointimal hyperplasia, these sirolimus-eluting ePTFE grafts make the use of polymers as drug-containing reservoirs unnecessary. Almost all drug-eluting cardiac stents use polymers for controlled drug release. Although the exact mechanisms underlying long-term histological responses such as delayed healing and late stent thrombosis remain unclear, these tissue reactions are significantly associated with localized hypersensitivity due to the presence of inflammatory cells such as eosinophils, lymphocytes and giant cells in sirolimus-eluting stents. Furthermore, the hypersensitive tissue reaction is likely due to the polymers rather than the drugs, which are completely eluted by 3 months [30]. Therefore, the polymers remaining on the implant may induce inflammation, resulting in late increases in neointimal formation [31]. In contrast, sirolimus-coated ePTFE grafts were fabricated by a dipping method without any polymer, and any residual drug was completely released 6 weeks after implantation in our pig model. In a previous study, paclitaxel as an anti-proliferative agent was found to effectively suppress inflammation around implanted grafts as well as the stenosis of vascular grafts [32]. Likewise, sirolimus-eluting ePTFE grafts effectively inhibited neointimal hyperplasia in venous anastomoses. In addition, there was little inflammation evident on H&E staining (shown in Supplementary data). Therefore, possible long-term histological reactions of the tissue adjacent to the polymers are not an issue when using sirolimus-coated grafts. Additional and more elaborate experimental analyses may be necessary to confirm the presence of inflammation and its relationship to the amount of sirolimus coated on the ePTFE grafts.

The ideal vascular access should have a high patency rate with little intervention and a short maturation time. All of the low-dose sirolimus-eluting ePTFE grafts were patent for 6 weeks and tightly bound by the adjacent tissue without infection, which could shorten the maturation time of implanted grafts, thereby allowing early cannulation.

**Conclusions**

Sirolimus-eluting ePTFE grafts effectively suppressed neointimal hyperplasia. However, the inhibitory effect of sirolimus-eluting haemodialysis grafts on neointimal hyperplasia was weaker than that of paclitaxel-coated grafts observed in previous studies.

**Supplementary data**

Supplementary data are available online at http://ndt.oxfordjournals.org/

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