Paclitaxel-coated expanded polytetrafluoroethylene haemodialysis grafts inhibit neointimal hyperplasia in porcine model of graft stenosis

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Abstract

Background. The main pathology of haemodialysis graft stenosis is venous neointimal hyperplasia at graft–venous anastomoses. Neointimal hyperplasia is also observed in cases of coronary artery in-stent restenosis. Paclitaxel is a chemotherapeutic agent used to treat cancer, and has been proven to inhibit neointimal hyperplasia of coronary artery in-stent restenosis. In this study, we examined whether a paclitaxel-coated haemodialysis graft could inhibit neointimal hyperplasia and prevent stenosis.

Methods. We dip-coated paclitaxel on expanded polytetrafluoroethylene (ePTFE) grafts at a dose density of 0.59 μg/mm². In vitro release tests showed an initial paclitaxel burst followed by a long-term slow release. Using ePTFE grafts with (coated group, n = 8) or without a paclitaxel coating (control group, n = 11), we constructed arteriovenous (AV) grafts connecting the common carotid artery and the external jugular vein in Landrace pigs.

Results. After excluding seven pigs for technical failure, cross-sections of graft–venous anastomoses obtained 6 weeks after placing the AV grafts were analysed. Percentage luminal stenosis, ratios of intima to media in whole cross-sections, areas of intima in the peri-junctional areas (within 2 mm above and 2 mm below the graft–venous junction), and the mean thickness of intima within venous sides of cross-sections, were 60.5% (range, 41.5–60.7), 13.0 (range, 0.7–5.1), 1.6 mm² (range, 0.2–8.0) and 0.3 mm (range, 0.1–2.2). All parameters were significantly different between the two groups (P < 0.05 by Mann–Whitney test).

Conclusion. Paclitaxel-coated ePTFE grafts could prevent neointimal hyperplasia and the stenosis of AV haemodialysis grafts.

Keywords: access; graft; haemodialysis; paclitaxel; polytetrafluoroethylene; stenosis

Introduction

Nowadays more than one million end-stage renal disease patients are sustained by haemodialysis. The long-term survival of these patients was impossible until durable access to the circulation was introduced, almost two decades after the first successful treatment of uraemia by haemodialysis [1]. Native arteriovenous (AV) fistulas and expanded polytetrafluoroethylene (ePTFE) grafts are the most common types of accesses. The proportions of access types used differ by country, but in the USA, for example, grafts represent almost 50% of the permanent vascular accesses used in the haemodialysis population [2].

Maintaining the patency of synthetic grafts needs careful surveillance and timely intervention, as almost 80% require a salvage procedure, i.e. angioplasty, surgical revision, or thrombectomy, for maintenance purposes within 1 year of placement, with a 2-year survival rate of around 50% [3]. Therefore, the cost...
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of vascular access care is high, especially among patients with a graft, and approaches over €7300 per patient-year during the first year of haemodialysis [4].

The major cause of this poor prognosis is the occurrence of thrombosis and stenosis in the venous anastomosis sites, or of draining of central veins, due to the aggressive development of venous neointimal hyperplasia [5,6]. Many efforts have been made to detect or prevent stenosis or thrombosis, e.g. anti-platelet agents, radiation, modification of graft shape, prophylactic angioplasty or meticulous monitoring for stenosis. But, no effective therapeutic interventions for haemodialysis vascular graft dysfunction exist at the present time [7].

Neointimal hyperplasia, which is responsible for stenosis at the venous anastomosis sites of haemodialysis grafts, is also the main pathology of restenosis, which develops after coronary artery stent insertion [5,8,9]. Several efforts have been made to prevent in-stent coronary artery restenosis, and of these, drug-eluting stents have been the most successful and are now used worldwide. Although several differences exist between haemodialysis graft stenosis and in-stent coronary artery restenosis, such as, vessel type and location, we considered that agents such as paclitaxel or rapamycin, which are eluted from stents and have been proven to be effective at preventing neointimal hyperplasia and in-stent restenosis of the coronary artery [10], would also be effective at preventing the neointimal hyperplasia of haemodialysis grafts. Thus, we coated ePTFE grafts with paclitaxel and devised an AV graft in a pig model to observe the effect of this coating on stenosis.

Methods

Paclitaxel coating on an expanded polytetrafluoroethylene (ePTFE) graft and its in vitro release kinetics

Paclitaxel (Genexol®, Samyang Genex Inc., Korea) was loaded onto ePTFE vascular grafts (IMPRA, BARD Inc.) using a dipping method. Briefly, dry paclitaxel was dissolved in acetone (Fisher Scientific) at 2 mg/ml or 10 mg/ml, and ePTFE vascular grafts were dipped vertically into these solutions and incubated for 30 min at 37°C. The paclitaxel-loaded ePTFE vascular grafts were then fixed and maintained under vacuum overnight to completely remove the solvent. Loaded amounts of paclitaxel on grafts were measured as follows. Drug-coated grafts were soaked in polypropylene tubes containing 5 ml MeOH and shaken for 30 min, and then the methanolic solution was analysed by high-performance liquid chromatography (HPLC; Agilent 1100 Series, USA). HPLC analysis was performed using a mobile phase of water:acetonitrile (60:40 v/v) at a flow rate of 1.0 ml/min, a 4.6 × 150 mm C18 reverse-phase column and a UV detector set at 227.4 nm. Under these conditions, paclitaxel was eluted at 8.07 min. The corresponding median dose densities of paclitaxel at these concentrations were 0.59 (0.57–0.60) and 4.20 (3.44–4.57) μg/mm².

For in vitro release studies, we used a solution of phosphate-buffered saline (PBS, pH 7.4) containing 0.05% (w/v) Tween-20 (Hayashi Pure Chemical Ind., Ltd, Japan), a non-ionic surfactant, as paclitaxel is poorly soluble in PBS alone. Individual ePTFE vascular grafts of 1.5 cm length were placed in polypropylene tubes containing 5 ml of release medium and incubated in a 37°C/100 rpm hybridization incubator (FineMould Precision Ind., Korea) for 8 weeks. At designated times, the medium was removed completely from these tubes, and they were then stored for analysis. The medium was replaced with 5 ml of fresh release medium. The amounts of paclitaxel released to medium were determined by HPLC.

Animals and operative procedure

Nineteen male Landrace pigs, weighing 50 ± 5 kg, received a single ePTFE-AV graft, either with (the coated group, n = 8) or without a paclitaxel coating (the control group, n = 11), between the common carotid artery and the external jugular vein. Mean paclitaxel loading on coated grafts was 0.59 μg/mm² (range, 0.57–0.60). Animals were euthanized 6 weeks post-operatively. The study protocol was approved by the Institutional Animal Care and Use Committee of the Laboratory Animal Research Center at the Samsung Biomedical Research Institute. Animal care facilities at the centre are accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) and conform to the Guidelines for the Care and Use of Laboratory Animals, published by the US National Institute of Health (NIH Publication No. 85–23, revised 1996).

Before the operation and at the end of the experiment, animals were fasted overnight and anaesthesia was induced with intramuscular ketamine HCl (20 mg/kg) and xylazine HCl (2 mg/kg). They were then intubated and ventilated with a mixture of O₂ and air (1:2) containing enflurane (2%). An ear vein was used to continuously administer 0.1 mg/kg of vecuronium bromide. Animals were monitored using a patient monitor (M1205A Omnicare, Hewlett-Packard) and an anaesthesia gas monitor (602–3A Poet IQ, Criticare system).

In the present study, we adopted the animal model proposed by Rotmans et al. [11]. Starting 7 days pre-operatively, pigs were administered acetylsalicylic acid 100 mg/day until termination. Clopidogrel (PLAVIX® Sanofi-Synthelabo) 75 mg was added on pre-operative day 1 and continued at a dose of 75 mg/day until termination.

The common carotid artery and the external jugular vein were exposed through a longitudinal incision in the lateral side of the neck along the sternocleidomastoid muscle. Heparin 100 IU/kg was given intravenously before vessel manipulation. All AV grafts were created by experienced vascular surgeons. The common carotid artery was clamped using atraumatic clamps, and an 8 mm arteriotomy was performed. An end-to-side anastomosis was created at a 45° angle using a continuous 6-0 polypropylene suture. Reinforced, thin-walled, ringed ePTFE grafts (6 mm in diameter by 15 cm in length) were used, and were sterilized before use in ethylene oxide. Venous anastomoses were created in a similar fashion. At termination 6 weeks after index operation, pigs were anaesthetized as described earlier. Proximal carotid arteries supplying grafts were cannulated
and ligated beyond the cannula, and grafts and adjacent vessels were perfused with saline for 3 min. Subsequently, the grafts were perfused with formalin at physiologic pressure (i.e. 100 mmHg) for 2 min. Both sides of the arteries and veins were then ligated, thus allowing pressure fixation of the vessels. Grafts and adjacent vessels were then excised and immersed in formalin for at least 24 h.

**Tissue preparation and morphometric analysis**

Specimens were cut from the arterial and venous sides in 5 mm blocks and embedded in paraffin. Five micrometre thick sections of veins and arteries 1 cm proximal and distal to anastomoses were then prepared. Serial sections were taken around anastomoses to obtain cross-sections at the centre of the graft–vein anastomosis, which were also perpendicular to the blood flow, as shown in Figure 1A and B, since stenosis of haemodialysis graft is most prominent at the junction between a haemodialysis graft and a draining vein [6]. All sections were stained with haematoxylin and eosin (H&E), and the most representative cross-sections were chosen. These were further stained with Masson’s trichrome using an immunohistochemical method (described subsequently). Intima and media were manually traced on Masson’s trichrome stained slides that differentiate media. Area measurements were performed by computer-assisted morphometry, on captured images obtained using a colour camera attached to a light microscope. The areas and thicknesses of intima and media were manually traced.

To compare stenosis and intimal hyperplasia between the control and coated groups, several parameters were measured as follows. Percentage luminal stenosis was calculated as the area of intima divided by the total lumen area inside grafts and vascular media (region ‘a’ in Figure 1B). The intima–media ratio was calculated as the area of total intima divided by area of total media in venous anastomosis cross-sections (region ‘a’ in Figure 1B). Areas of intima in the peri-junctional areas 2 mm into the graft side and 2 mm into the venous side from the graft–vein junction (region ‘b’ in Figure 1B) were also measured. Average intima thicknesses were calculated as areas of intima divided by the circumferential length of intima within venous sides of cross-sections (region c in Figure 1B).

**Immunohistochemical staining**

Sections of paraffin-embedded tissues were immunostained with mouse antihuman α-smooth muscle actin antibody (DAKO, Glostrup, Denmark). Briefly, sections were deparaffinized and hydrated through a xylene and graded alcohol series. After antigen retrieval by heating the tissues in 10 mM sodium citrate buffer twice for 7 min, sections were serially incubated in 0.3% H2O2 for 30 min, in normal blocking serum for 30 min, in primary anti-serum diluted in buffer (1:100) for 18 h, in biotinylated secondary antibody for 30 min, in streptavidin–horseradish peroxidase conjugate for 30 min and in peroxidase substrate solution (diaminobenzidine) for 2 min. The sections were then counterstained with haematoxylin.

**Statistics**

The differences between control and coated-group parameters were examined using the Mann–Whitney test (SPSS 12.0 for Windows, SPSS Inc.). P-values of <0.05 were considered statistically significant.

**Results**

**In vitro release test**

Figure 2 shows the *in vitro* paclitaxel release profiles of the coated ePTFE grafts. Paclitaxel was loaded onto ePTFE vascular grafts using two different solution concentrations, as described earlier, namely, at 2 mg/ml (low dose, 0.59 μg/mm²) and at 10 mg/ml (high dose, 4.20 μg/mm²). The release curves obtained were parallel and their gradients were proportional to the amount of total drug loaded. Cumulative paclitaxel releases in PBS–Tween-20 from 0.59 and 4.20 μg/mm².
paclitaxel-loaded ePTFE grafts are shown in Figure 2A. In general, a burst of paclitaxel release is followed by a longer lasting, slower sustained release (Figure 2B). Even in the high-dose case (4.20 mg/mm²), the initial paclitaxel burst was high, but after 3 weeks, more than 50% of the initial loading remained.

Animal experiments and morphometric analysis

Of the 19 pigs (11 in the control group and eight in the coated group) that received AV grafts, three pigs in the control group and one pig in the coated group died before 4 weeks after surgery due to haemorrhage around graft–venous anastomoses. In one pig from the control group, the graft–venous anastomosis area was replaced by fibrotic tissue, and therefore, the graft–venous junction was not formed. In one pig in the control group and another in the coated group, graft–venous junctions could not be identified on prepared slides, probably due to poor specimen handling. Therefore, after excluding five pigs from the control group and two pigs from the coated group, histological and morphometric analyses were performed on specimens from six pigs that had received a coated AV graft and from six pigs that had received a non-coated AV graft.

The cross-sections of graft–venous anastomosis 6 weeks after surgery from the six uncoated control grafts and the six coated grafts are shown in Figure 3, in the upper and lower panels, respectively. Slices of grafts were sometimes folded or detached during the process of tissue preparation but their locations were easily traced by considering adjacent sections. Intima was recognized as pale blue stained inside the vascular media, which appeared as a layer of thick red fibres that were more evident at high power magnification. Uncoated grafts showed luminal stenosis caused by intimal growth on veins and peri-junctional grafts. Changes in coated grafts were minimal and sharply contrasted to controls. Hyperplastic tissue at the venous anastomosis in both groups consisted of myxoid matrix and spindle cells, the myoblastic nature of which was demonstrated by a positive reaction to α-smooth muscle actin immunostaining.

To compare stenosis and intimal hyperplasia in the two groups quantitatively, we measured several parameters in the sample cross-sections, i.e. percentage of luminal stenosis, the intima–media ratio at the cross-section of graft–venous anastomoses (region ‘a’ in Figure 1B), intimal areas in peri-junctional areas 2 mm into the graft side and 2 mm into the venous side from the graft–vein junction (region ‘b’ in Figure 1B), and the average thickness of intima in venous lumen (region ‘c’ in Figure 1B). In the third case of the control group, the whole lumen at the cross-section was unidentifiable and was excluded from comparison (case ‘C’ of Figure 3), although comparisons of intimal areas in the peri-junctional area 2 mm into the graft–side and 2 mm into the venous side from the graft/vein junction were possible. Therefore, the number of cases included in the comparison was five for the control group and six for the coated group, except for peri-junctional intimal area comparisons, in which six cases were used for the control group.

The median values of these parameters were 60.5% (range, 41.5–60.7), 13.0 (range, 8.6–20.4), 23.7 mm² (range, 10.8–32.1) and 2.1 mm (range, 1.1–3.0), respectively, in the control group, whereas the corresponding median values of these parameters in the coated group were 10.4% (range, 1.0–17.8), 1.0 (range, 0.7–5.1), 1.6 mm² (range, 0.2–8.0) and 0.3 mm (range, 0.1–2.2), respectively. As shown in Figure 4, all of these parameters showed that stenosis or intimal hyperplasia at the graft–venous anastomosis site was significantly reduced by the paclitaxel coating ($P < 0.05$ by Mann–Whitney test for all the parameters).

Intimal hyperplasia at arterial graft anastomosis was minimal in the control group, and thus, no comparison was made with the coated group.

Paclitaxel concentrations from peripheral venous blood samples immediately, and 1 and 3 days after the construction of the AV graft were measured by HPLC in three pigs in the coated group. All these were
below the detection limit of 0.14 μg/ml. Furthermore, no systemic or local signs attributable to the toxic effects of paclitaxel were noted in coated-group animals used in the analysis.

Discussion

This study shows for the first time, that paclitaxel-coated ePTFE grafts effectively prevent stenosis in haemodialysis grafts. This result is comparable with the effect of paclitaxel-eluting stents for preventing in-stent coronary artery restenosis. In several respects, the stenosis of haemodialysis grafts differs from in-stent coronary artery restenosis, as in the former, stenosis is located in or originates from a draining vein, whereas in the latter, it originates from an artery. Moreover, stenosis of haemodialysis grafts is mainly located at the junction between the haemodialysis graft and the draining vein, whereas in the case of in-stent coronary artery restenosis it is located mainly in the middle of the inserted stent [6,8]. Despite these differences, the main pathology, namely, neointimal hyperplasia, is similar in both types of stenosis, and is composed of smooth muscle cells, macrophages and endothelial cells with an accompanying matrix in both lesion types [5,8,9,12]. Therefore, paclitaxel eluting from both inside and outside a coated ePTFE graft or attached to a graft was also found to be effective at preventing cells from encroaching the graft from the venous side or from the adventitia to eventually form microvessels or a matrix and cause stenosis.

Although little or no stenosis was observed at the anastomosis sites of paclitaxel-coated AV grafts, and this contrasted dramatically with control-group specimens, it was difficult to demonstrate this difference quantitatively and objectively, since it was not easy to obtain a cross-section specimen precisely at the midline of the venous anastomosis site and perpendicular to the flow of the draining vein. Therefore, we measured several parameters, such as percentage of luminal stenosis, ratio of intima to media in whole cross-sections, area of intima in the peri-junctional areas, and mean intimal thickness within the venous side of the cross-section. And, all these parameters of the paclitaxel-eluting graft were significantly better than those of the control.

Previous reports have described the prevention of haemodialysis graft stenosis via local drug delivery, such as, of an injectable biodegradable copolymer mixed with paclitaxel [13] or of a sirolimus-eluting stent [14] implanted over a venous anastomosis. Both studies show that the local delivery of such drugs could be effective at reducing intimal

Fig. 3. Cross-sections of graft–venous anastomoses 6 weeks after surgery from six uncoated control grafts (upper panel, A–F) and six coated grafts (lower panel, G–L) (Masson trichrome stain, original magnification ×12.5). Intima can be recognized as pale blue stained areas inside vascular media, which appears as layers of thick red fibres and is more evident at high-power magnification. Uncoated grafts show luminal stenosis caused by intimal growth on veins and peri-junctional grafts. Changes in coated grafts were minimal in sharp contrast to the control.
hyperplasia and stenosis. The results of the present study are consistent with that of the aforementioned studies, but our study represents the first attempt to deliver a drug locally in the form of a drug-coated graft.

In practice, it is difficult to construct an AV graft animal model which consistently shows substantial stenosis without thrombosis. Moreover, it requires time to observe stenosis after constructing an AV graft, and its development depends on the animal model used [11,12]. If thrombosis occurs too early after AV graft construction, it will not permit the development of stenosis and will confound result interpretations. The occurrence of thrombosis may depend on the location of constructed AV grafts and the anticoagulation or antiplatelet therapy administered [11,12]. After several attempts, we eventually adopted the model proposed by Rothmans et al. [11] and achieved a satisfactory consistent animal model of haemodialysis graft stenosis.

It is well known that paclitaxel exerts potent inhibitory effects on smooth muscle cell proliferation and migration, and that it can prevent neointimal hyperplasia found in in-stent restenosis of the coronary artery when delivered locally [15]. However, there is concern about the safety of paclitaxel as it is a toxic anti-neoplastic drug. The loading of paclitaxel used in our experiment was 0.59 mg/mm², which is substantially less than the 1.0–3.1 mg/mm² used in coronary artery stents [16,17]. However, the total dose implanted in the present experiment was 1.8 mg, which is far greater than the 54–146 mg introduced by a coronary artery stent [17], due to the surface area difference. Nevertheless, these doses are <1% of the dose commonly used to treat malignancies, and elution of paclitaxel into the systemic circulation from coated grafts occurs over a period of several weeks as compared with only several hours in the case of paclitaxel chemotherapy. Lower dose paclitaxel coatings may also be effective at preventing the stenosis of haemodialysis grafts; this aspect requires further experimentation. In the context of reducing the total dose of coated paclitaxel, it could be argued that coating only the venous end of grafts may be a better option than coating whole grafts, since this is the main site of graft stenosis.

Fig. 4. Comparison of stenosis and intimal hyperplasia in the control and the coated group. (A) Percentage luminal stenosis was calculated as the area of intima divided by total lumen area (region ‘a’ in Figure 1B). (B) Intima-media ratio was calculated as the area of total intima divided by the area of total media (region ‘a’ in Figure 1B). (C) Intimal areas in peri-junctional areas 2 mm into the graft side and 2 mm into the venous side from the graft-vein junction (region ‘b’ in Figure 1B). (D) Average intimal thicknesses were calculated by dividing intimal area by intimal circumference (region ‘c’ in Figure 1B). (C) The number of animals included in the comparisons was five in the control group and six in the coated group, but for the peri-junctional intimal area comparison, six cases were used in the control group. The third case in the upper panel (case C) of Figure 3 was excluded from comparisons A, B and D since whole lumen could not be identified in cross-sections. All comparisons between the control and coated groups were significantly different (*P<0.05 by the Mann-Whitney test).
However, the fact that stenosis also develops at arterial anastomosis sites and in the middle of grafts, and that a paclitaxel coating on whole graft could prevent inflammatory reactions and swelling around AV grafts, needs to be taken into consideration. Moreover, these inflammatory reactions can sometimes be severe enough to cause morbidities and can lead to unnecessary treatment, because their effects are similar to those of infections [18,19].

When designing a drug-eluting system on a medical device, incorporating the drug in a polymer coating matrix is often preferred, since it allows more sustained and predictable drug elution. However, there is always a concern that the polymer per se may induce an inflammatory reaction or neointimal proliferation [20]. Moreover, electron microscopy demonstrated that ePTFE, which is the most commonly used material for haemodialysis grafts, has a rougher surface than the stainless steel of a bare cardiac stent and that it contains numerous surface pits. These observations suggest that a paclitaxel coated by dipping and solvent evaporation on ePTFE can adhere to the pitted polymer surface and thus be released in a controlled manner. As was expected, in vitro release kinetic experiments showed an initial burst followed by a sustained drug release over a period of more than 8 weeks. Although we should consider the fact that this experimental condition may differ from in vivo release under high blood flow, this release pattern is encouraging. Naturally, further comparative experimentation on in vitro/in vivo release profiles is needed.

In conclusion, paclitaxel coated-ePTFE grafts were found to prevent neointimal hyperplasia and the stenosis of AV haemodialysis grafts, especially at graft–venous anastomoses. Further experiments are necessary to improve the efficiency of paclitaxel-coated ePTFE grafts and to confirm their safeties.

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