Adenovirus-Mediated Expression of β-Adrenergic Receptor Kinase C-Terminus Reduces Intimal Hyperplasia and Luminal Stenosis of Arteriovenous Polytetrafluoroethylene Grafts in Pigs

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Background—Hemodialysis vascular access dysfunction is the single most important cause of morbidity in kidney hemodialysis patients. Failure of an arteriovenous polytetrafluoroethylene (PTFE) graft, the most common form of hemodialysis access, is primarily due to intimal hyperplasia and thrombosis at the venous anastomosis.

Methods and Results—This study was aimed at evaluating the efficacy and safety of an adenoviral vector (Ad2/βARKct) encoding the carboxyl terminus of β-adrenergic receptor kinase (βARKct) in a pig model of arteriovenous PTFE graft failure. Transduction of the external jugular vein with Ad2/βARKct (5E9, 5E10, or 5E11 particles per vein) did not result in systemic toxicity, as measured by clinical and pathological assessments. Ad2/βARKct significantly reduced neointimal hyperplasia in the graft/vein anastomosis. It also improved the graft patency rate and angiographic score, as measured histologically and angiographically, compared with vehicle or empty viral vector controls.

Conclusions—Our results suggest that local administration of adenoviral vectors encoding βARKct into the jugular vein represents a viable strategy to treat AV graft hemodialysis vascular access failure. (Circulation. 2005;111:1679-1684.)

Key Words: thrombosis ■ hyperplasia ■ grafting ■ hemodialysis ■ gene therapy

Hemodialysis vascular access dysfunction is the single most important cause of morbidity in the hemodialysis population, which is currently over 230,000 in the United States and growing at a rate of 5% per annum.1 The most common vascular procedure performed in chronic hemodialysis patients in the United States is the arteriovenous (AV) polytetrafluoroethylene (PTFE) graft, which accounts for ≈65% of all hemodialysis access.2 These grafts have a dismal primary patency rate of 50% at 1 year and 25% after 2 years.3 Intimal hyperplasia at the venous anastomosis is the primary cause of thrombosis and consequent vascular access dysfunction of AV PTFE hemodialysis grafts.3-5 Despite the magnitude of the problem, there are currently no effective therapies for the prevention or treatment of venous intimal hyperplasia in PTFE grafts in these patients.

Although the precise mechanism is not fully understood, smooth muscle cell migration and proliferation are known contributors to the intimal hyperplasia observed in PTFE dialysis grafts.4-6 Gβγ-signaling has been shown to play a critical role in the pathogenesis of vascular proliferative disorders including arterial restenosis after angioplasty and vein graft failure.7-9 G-protein–coupled receptors regulate cell proliferation via activation of mitogen-activated protein kinases (MAPKs).10 For example, after receptor activation by stimuli, the Gβγ-subunit of the inhibitory G protein can transduce the inhibitory signal of smooth muscle cell proliferation by activating the p21ras-(Ras)-dependent p42/p44 MAPK, which may serve as a critical controller for pathological vascular intimal hyperplasia.10 G-protein–coupled receptors occupied by ligand are desensitized by the G-protein–coupled receptor kinases (GRKs).11 GRK2 (also named β-adrenergic receptor kinase [βARK]) is one of the 7 GRKs identified, binds to and subsequently phosphorylates ligand-bound adrenergic receptors. This Gβγ-dependent phosphorylation triggers desensitization and internalization of the receptors.11 Inhibition of Gβγ-signaling by overexpression of the carboxyl terminus of β-adrenergic receptor kinase (βARKct) has been shown to effectively reduce intimal hyperplasia in rat and rabbit models of restenosis after balloon injury and vein graft failure.7-9 Other cytotoxic and cytostatic gene therapy strategies that target neointima formation in general and vascular smooth muscle cells in particular have also shown efficacy for the treatment of smooth muscle proliferative disorders in animal models.10,12-19 In the present study, we evaluated the efficacy and safety of adenovirus-mediated expression of βARKct in a pig model of AV PTFE graft failure. Our results suggest that local administration of adenoviral vectors encoding βARKct...
represents a novel strategy to treat AV graft hemodialysis vascular access failure.

**Methods**

**Construction of Recombinant Adenoviral Vectors**

Ad2/CMVEV and Ad2/β-gal4, which encode no transgene and β-galactosidase, respectively, were generated as described previously. Ad2/βARKct was also constructed with an E1-deleted Ad2/E4ORF6 backbone (wild-type E2 and E3, and deletion of E4 except for ORF6; kindly provided by Dr W. Koch, Jefferson Medical College, Philadelphia, Pa). Ad2 nucleotide sequences between 357 and 4021 were replaced with the human cytomegalovirus enhancer-promoter, the coding sequence of the last 195 carboxyl terminal amino acids of the bovine adrenergic receptor kinase 1, and the SV40 polyadenylation signal sequence.

**Construction of AV PTFE Grafts and In Vivo Gene Transfer**

All animal studies were performed under protocols approved by the Institutional Animal Care and Use Committee of Genzyme Corporation and in accordance with the *Guide for the Care and Use of Laboratory Animals* (US DHHS publication No. NIH 86-23). AV PTFE grafts in pigs were surgically constructed, as described previously with modifications. Briefly, female Yorkshire pigs (4 to 6 months, weight 25 to 30 kg, female) were injected intramuscularly with glycopyrrolate (0.01 mg/kg) and Telazol (3 to 5 mg/kg). The animals were then intubated and put on isoflurane (1% to 3%) to maintain a suitable anesthetic plane. Blood samples were collected for a complete blood count (CBC), serum chemistry, and analysis of coagulation parameters. Heparin (100 IU/kg) was given as a bolus intravenously. One AV shunt between the left common carotid artery and right external jugular vein was created. A longitudinal incision (~10 cm) was made in the middle line of the neck. The left common carotid artery and right external jugular vein were exposed and dissected free of surrounding tissues. A thin-walled IMPRA ePTFE vascular graft, 15 cm in length and 6 mm in internal diameter with the 2 ends at a 25° to 30° angle, was prepared. The external jugular vein was clamped with 2 bulldog clamps ~4 cm apart. The blood was withdrawn from the clamped segment of the jugular, followed by rinsing with a heparin-saline solution (~2 mL) to remove any residual blood. Two milliliters of PBS supplemented with 10% sucrose, alone or containing adenoviral particles, was injected into the rinsed vein segment. While the clamped vein segment was incubated with PBS with or without adenoviral vectors for 30 minutes, the carotid artery was clamped, and an arteriotomy (9 to 10 mm) was made. The PTFE graft was anastomosed end-to-side to the artery with polypropylene 6-0 (Prolene) suture. PBS with or without adenoviral vectors was withdrawn from the clamped segment of the jugular, followed by construction of the PTFE/vein graft. These animals were euthanized 5 days after gene transfer. The transduced vein segments were harvested and stained with the β-galactosidase substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), as described previously. The tissue segments were embedded in paraffin blocks and sectioned. The total number of smooth muscle cells in a given histological section and those expressing β-galactosidase were counted under light microscopy.

**Statistical Analysis**

Data are expressed as mean ± SEM. The number of animals examined is indicated by n. Data were analyzed by ANOVA, followed by Newman-Keuls test. A probability of <0.05 was considered statistically significant.

**Results**

**Gene Transfer Into Jugular Veins**

PBS supplemented with 10% sucrose alone (2 mL) or containing Ad2/β-gal4 (5E11 particles) was infused into the clamped external jugular vein segment in 4 pigs and allowed to bathe the vein for 30 minutes, followed by construction of the PTFE/vein graft. These animals were euthanized 5 days later, and the extent of transgene expression in the transduced vein segment was determined. Histological sections revealed that endothelial cells and patches of smooth muscle cells in the medial area expressed nuclear-localized β-galactosidase in the Ad2/β-gal4–treated segment of the jugular vein (Figure 1). Approximately 4.9 ± 2.4% of the cells stained positively for β-galactosidase expression. In contrast, no cells showed no angiographic narrowing.
expressing β-galactosidase were observed in the contralateral external jugular vein samples. These results suggest that adenoviral vectors are capable of transducing vascular endothelial and smooth muscle cells.

Effects of Ad2/βARKct on Minimal Inner Diameter of PTFE/Vein Graft

In a safety and efficacy study, 8 pigs were enrolled in each of the 5 experimental groups (vehicle, Ad2/CMVEV at 5E11 particles, and Ad2/βARKct at 5E9, 5E10, and 5E11 particles). All pigs recovered from surgery and the in vivo gene transfer procedure. Other than minor skin redness at the site of surgery, no complications were observed. Twenty-eight days after the procedure, the majority of the PTFE/vein grafts treated with vehicle were occluded at the graft/vein anastomosis, with a patency rate of 37.5% (Figure 2). All the grafts in pigs that received Ad2/CMVEV (5E11 particles per vessel) were occluded. In animals treated with Ad2/βARKct at 5E9, 5E10, and 5E11 particles per vessel, the patency rate was 75%, 37.5%, and 75%, respectively. Although a dose response was not observed, these results suggest a trend that transduction of the vein segment with Ad2/βARKct increases the patency rate of AV PTFE grafts.

We also examined the minimal luminal diameter of the patent PTFE/vein grafts. All the patent grafts in the vehicle group showed stenosis, as measured by angiography. In contrast, 2, 1, and 1 patent graft in pigs that received Ad2/βARKct at 5E9, 5E10, and 5E11 particles per vessel, respectively, showed no angiographic stenosis. The percentage of stenosis was calculated as the minimal luminal diameter of the graft at the anastomosis site divided by the luminal diameter of the most adjacent point of the normal vein segment (downstream of the anastomosis and proximal to the heart) that showed no angiographic narrowing. Completely occluded grafts were calculated as 100% stenosis. Data are mean±SEM (n=8). *P<0.05 compared Ad2/CMVEV.

Effects of Ad2/βARKct on Intima Formation

The effect of adenovirus-mediated expression of βARKct on the hyperplastic response to PTFE grafting was also assessed histologically. The external jugular vein segment proximal to, including, and distal to the PTFE graft anastomosis site (~5 cm) was cut into 5 segments (2 from the vein region upstream of the graft/vein anastomosis, 1 from the graft/vein anastomosis region, and 2 from the vein region downstream of the graft/vein anastomosis). As reported previously,22 neointimal lesions were predominantly located at the graft/vein anastomosis region (Figure 4). The gross view of the graft/vein

Figure 1. Representative gross view (A, B) and photomicrographs (C, D) of vein/PTFE graft and untreated contralateral external jugular venous segment. Ad2/β-gal4 (5E11 particles/vein) was infused into external jugular vein and allowed to incubate for 30 minutes, and AV PTFE graft was then created. Five days later, vein/graft segments and contralateral external jugular vein were harvested and stained with X-Gal.

Figure 2. Angiograms of external jugular vein/PTFE grafts that were treated with PBS (A), Ad2/CMVEV at 5E11 particles/vein (B), or Ad2/βARKct at 5E9 (C), 5E10 (D), or 5E11 (E) particles/vein. PBS alone or adenoviral vectors were infused into external jugular vein and allowed to incubate for 30 minutes, and AV PTFE graft was then created. Twenty-eight days later, angiogram was obtained.

Figure 3. Statistical analysis of percentage of stenosis of external jugular vein/PTFE grafts that were treated with PBS (vehicle), Ad2/CMVEV (EV, 5E11 particles/vein), or Ad2/βARKct (βARKct 5E9, 5E10, or 5E11 particles/vein). Percentage of stenosis was calculated as minimal luminal diameter of graft at anastomosis site divided by luminal diameter of most central normal vein below anastomosis. Completely occluded grafts were calculated as 100% stenosis. Data are mean±SEM (n=8). *P<0.05 compared Ad2/CMVEV.
anastomosis region revealed that neointima tissues had overgrown into the PTFE graft with resultant thrombosis (Figure 5). The graft/vein anastomosis region of the grafts that were angiographically occluded was completely filled with tissue. The lumen of the grafts that showed angiographic stenosis was partially blocked by tissue outgrowths. These results were consistent with the angiographic assessment. Thrombotic occlusions are complex and multifactorial, although neointimal hyperplasia is a major determinant. Neointimal hyperplasia was measured histologically at the time of euthanasia of the animal rather than that of the thrombotic event. We did not observe any newly formed thrombotic plaques in the histological sections; however, it is possible that the thrombotic plaque might have become a part of the neointima with cellular infiltrates and vascularization after thrombotic occlusion.

Examination of histological sections revealed that the graft/vein anastomosis region was filled with fibrous and fibromuscular neointima (Figure 6). Intimal hyperplasia was present in both the vein and graft portion. Neointimal lesions were minimal in the upstream and downstream vein segments. Quantitative analysis showed that in the graft portion of the anastomosis, the neointima area–to–graft area ratio was comparable in pigs treated with vehicle or Ad2/CMVEV (Figure 7). The neointima-graft ratio was significantly (P<0.05) lower in animals treated with Ad2/βARKct than in those treated with vehicle or Ad2/CMVEV. In the venous portion of the graft/vein anastomosis, the ratio of neointima area to medial area was also significantly reduced in animals treated with Ad2/βARKct compared with those treated with vehicle or Ad2/CMVEV (Figure 7). These results suggest that local delivery of Ad2/βARKct into the external jugular vein inhibits neointimal hyperplasia in a pig model of AV PTFE graft failure.

CBC, Serum Chemistry, Coagulation Analysis, and Anatomic Pathology

Blood samples were collected from all animals before surgery and euthanasia for CBC, serum chemistry, and coagulation analysis. After surgery and delivery of adenoviral vectors into the jugular vein, most parameters examined were within the

![Figure 4](image1.png)

**Figure 4.** Photomicrographs of upstream vein regions (A, B), graft/vein anastomosis region (C), and downstream vein regions (D, E) of external jugular vein/PTFE graft. External jugular vein segment proximal and distal to PTFE graft anastomosis site (~5 cm) was cut into 5 segments.

![Figure 5](image2.png)

**Figure 5.** Gross view of representative anastomosis site of external jugular vein/PTFE grafts. PBS (2 mL) supplemented with 10% sucrose alone (A) or containing Ad2/CMVEV at 5E11 particles/vein (B) or Ad2/βARKct at 5E9 (C), 5E10 (D), or 5E11 particles/vein (E). Adenoviral vectors were infused into external jugular vein and allowed to incubate for 30 minutes, and AV PTFE graft was then created. Twenty-eight days later, graft was harvested.

![Figure 6](image3.png)

**Figure 6.** Photomicrographs of anastomosis site of external jugular vein/PTFE grafts. PBS (2 mL) alone (A), Ad2/CMVEV at 5E11 particles/vein (B), or Ad2/βARKct at 5E9 (C), 5E10 (D), or 5E11 particles/vein (E) were infused into external jugular vein and allowed to incubate for 30 minutes. AV PTFE graft was then created. Twenty-eight days later, graft was harvested.

![Figure 7](image4.png)

**Figure 7.** Quantitative analysis of neointima formation in anastomosis site of external jugular vein/PTFE grafts that were treated with PBS (2 mL) alone (A), Ad2/CMVEV at 5E11 particles/vein (B), or Ad2/βARKct at 5E9 (C), 5E10 (D), or 5E11 particles/vein (E). Data are mean±SEM (n=8). *P<0.05 and **P<0.01 compared with vehicle; †P<0.05 and ††P<0.01 compared with Ad2/CMVEV.
normal physiological range (data not shown). In 1 animal that received Ad2/βARKct at a dose of 5E10 particles, total bilirubin increased to 25 mg/dL (normal range 0 to 1.1 mg/dL) before euthanasia. This increase could be an artifact, because all other serum chemical parameters were normal for this particular animal. Activated partial thromboplastin time and thrombin time in all animals before surgery increased owing to intravenous administration of heparin. The activated partial thromboplastin time remained slightly higher in all the animals before euthanasia owing to the residual effect of the heparin administered at the time of surgery. A comprehensive necropsy revealed no macroscopic changes that were indicative of systemic toxicity. Microscopic examination of all sampled organs and tissues by an independent pathologist indicated that administration of Ad2/βARKct into the jugular vein did not result in any notable pathological alterations.

**Discussion**

The pig model of AV PTFE graft failure has been characterized by other investigators. The size and histological structure of the pig vessels are similar to those of humans. More importantly, the histological lesions of venous neointimal hyperplasia in pigs are very similar to those in human PTFE dialysis grafts. In the present study, we observed extensive formation of neointima composed of smooth muscle cells, myofibroblasts, inflammatory cells, and microvessels at the graft/vein anastomosis. Moreover, there was neointimal overgrowth into the graft lumen. Microvessels were also formed in the adventitia, which suggests adventitial remodeling. These results are consistent with previous reports that the histological lesions of venous neointimal hyperplasia in this model closely resemble the lesions characteristic of human dialysis PTFE grafts; however, the graft patency rate was lower than that reported in a similar pig model. We noted that Rotmans et al constructed 2 bilateral PTFE AV grafts in each pig in their study, whereas only a single AV shunt between the left common carotid artery and right external jugular vein was created in the present studies because of concerns about cerebral ischemia. It is conceivable that the different structure and resultant flow velocity may have contributed to the discrepancy in patency rate.

As previously reported, the majority of intimal hyperplasia in the pig model occurred at the graft/vein anastomosis, whereas minimal neointimal lesions were observed in the upstream and downstream vein segments. It has been suggested that neointimal lesions in the upstream and downstream vein may occur more frequently if the PTFE graft is maintained for longer than 28 days. In addition, the animals did not have uremia and were not subjected to dialysis, which was different from patients who undergo dialysis. Despite these differences, the marked similarities between pig and human neointimal lesions indicate that this pig model is clinically relevant to human PTFE graft failure.

The adenoviral vectors were delivered locally into the external jugular vein and withdrawn after incubation of the clamped venous segment. Therefore, the amount of vectors that could have leaked into circulation should be minimal. To assess systemic toxicity associated with local delivery of adenoviral vectors to the vessel wall, CBC, serum chemistry, necropsy, and organ/tissue histopathology were analyzed for all pigs. These examinations revealed no detectable systemic toxicity associated with treatment with Ad2/βARKct or Ad2/CMVEV, except that total bilirubin increased to 25 mg/dL (normal range 0 to 1.1 mg/dL) before euthanasia in 1 animal treated with Ad2/βARKct (5E10 particles). This increase could be artifactual, because all other serum chemical parameters were normal for this particular animal. Histopathologic examination of the liver also revealed no pathological alterations. Considering that systemic delivery of adenoviral vectors at high doses is known to cause potentially fatal liver injury, careful monitoring of liver toxicity is essential for future preclinical and clinical studies.

Hemodialysis vascular access dysfunction, primarily due to intimal hyperplasia at the venous anastomosis, presents a major challenge for the management of hemodialysis patients using an AV PTFE graft. The present study is the first to evaluate a gene therapy approach for the treatment of AV PTFE graft failure in a clinically relevant large-animal model. Histological assessment showed that adenovirus-mediated expression of βARKct significantly reduced venous neointimal hyperplasia at the vein/graft anastomosis site. There was also a trend that the AV PTFE graft patency rate, as measured by angiography, was higher in animals that received Ad2/βARKct at 5E9 and 5E11 particles per vessel than in those administered vehicle or Ad2/CMVEV. It was lower in pigs treated with Ad2/βARKct at 5E10 than in pigs treated with Ad2/CMVEV but not vehicle. Quantitative analysis suggested that the percentage of stenosis was lower in animals that received Ad2/βARKct at 5E9 and 5E11 particles per vessel than those that received vehicle or Ad2/CMVEV. These results indicate that transduction with Ad2/βARKct inhibits venous neointimal hyperplasia of AV PTFE grafts. This inhibition of intimal hyperplasia translates into a gain in minimal luminal diameter and an increase in graft patency rate.

Because βARKct is believed to attenuate cellular Gβγ-signaling in affected vascular smooth muscle cells, a “by-stander” is not expected. However, the present studies demonstrated that adenovirus-mediated expression of βARKct reduced neointima formation even though only \( \approx 5\% \) of the smooth muscle cells in the venous wall were transduced, as measured by X-Gal staining. Adenoviral vector–mediated transgene expression in vivo is known to peak 2 to 3 days after delivery and gradually decreases to lower levels owing to host immune responses or promoter shut-off. Because transgene expression in the vessel wall was measured 5 days after gene transfer in the present studies, the percentage of cells transduced initially could be higher. It is also possible that the percentage of smooth muscle cells transduced was underestimated by X-Gal staining. The precise mechanism by which transduction of a small percentage of the smooth muscle cells resulted in a significant reduction of neointima formation remains to be determined.

A trend was noted that Ad2/CMVEV, an empty viral vector that expresses no transgene, worsened intimal hyperplasia, stenosis, and graft occlusion compared with vehicle controls. Pathological examination of the anastomosis histological sections revealed no notable difference between the
animals that received Ad2/CMVev or Ad2/βARKct and their vehicle-treated counterparts. We did not find any significant inflammatory cell infiltrates; however, adenoviral vectors are known to cause an inflammatory response at the vessel wall.\(^{17,26}\) Inflammation might have occurred earlier but resolved 28 days after vector delivery, the time of termination of the in-life phase of the present animal studies. Thus, it is possible that the preinflammatory properties of the adenoviral vectors might have contributed to worsening of intimal hyperplasia.

In summary, in a pig model of AV PTFE graft failure with relevance to clinical manifestations, local delivery of Ad2/βARKct did not result in systemic toxicity. Transduction of the external jugular vein with Ad2/βARKct reduced neointimal hyperplasia in the graft/vein anastomosis and improved graft/vein patency rate. These results suggest that local administration of Ad2/βARKct may represent a viable strategy to treat hemodialysis vascular failure.

References


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