Kidney International, Vol. 65 (2004), pp. 1897–1905

Local overexpression of C-type natriuretic peptide ameliorates vascular adaptation of porcine hemodialysis grafts

JORIS I. ROTMANS, HENCE J.M. VERHAGEN, EVELYN VELEMA, DOMINIQUE P.V. DE KLEIJN, MIEKE VAN DEN HEUVEL, JOHN J.P. KASTELEIN, GERARD PASTERKAMP, and ERIK S.G. STROES

Department of Vascular Medicine, Academic Medical Center, Amsterdam, The Netherlands; Experimental Cardiology Laboratory, University Medical Center, Utrecht, The Netherlands; and Department of Vascular Surgery, University Medical Center, Utrecht, The Netherlands

Local overexpression of C-type natriuretic peptide ameliorates vascular adaptation of porcine hemodialysis grafts.

Background. Outflow obstruction at the outflow tract of arteriovenous grafts contributes significantly to the poor patency rates of dialysis grafts in vivo. We addressed the potential of local periadventitial gene therapy at the outflow tract for improving access patency in a validated porcine model of arteriovenous grafts using an adenoviral vector encoding murine C-type natriuretic peptide (Ad.CNP).

Methods. Gene transfer efficiency and optimal virus concentration were determined using Ad.LacZ on porcine jugular veins in vivo ($N = 2$). Next, in 14 pigs, arteriovenous grafts were implanted bilaterally between the carotid artery and the jugular vein, followed local venous transduction with Ad.CNP (right) and Ad.mock (left). Transduction efficiency of Ad.CNP was evaluated by reverse transcription-polymerase chain reaction (RT-PCR) and cyclic guanosine monophosphate (cGMP) measurements ($N = 2$). Fourteen days after gene transfer, arteriovenous grafts were excised for histologic analysis ($N = 12$).

Results. Ad.LacZ transduction (1 × 10E10 IU) of porcine veins resulted in evident expression of β-galactosidase, mainly in the adventitia. At termination, intima/media ratio was decreased by 37% in CNP-treated veins, predominantly due to medial thickening (Ad.CNP 3.1 ± 0.6 mm^2 vs. Ad.mock 1.70 ± 0.3 mm^2; $P < 0.01$) rather than decreased intimal hyperplasia (NS). Adventitial delivery of CNP resulted in increased external elastic lamina (EEL) (Ad.CNP 11.8 ± 1.4 mm vs. Ad.mock 9.4 ± 1.0 mm; $P = 0.04$) and luminal area (Ad.CNP 10.7 ± 1.4 mm^2 vs. Ad.mock 8.8 ± 1.7 mm^2; $P = 0.05$) at the venous anastomosis.

Conclusion. Overexpression of CNP enhances venous medial thickening and increases outward remodeling in the outflow tract of porcine arteriovenous grafts. These findings underscore the potential of local gene-therapeutic interventions in preventing luminal narrowing at the outflow tract of hemodialysis grafts.

Key words: arteriovenous graft, C-type natriuretic peptide, gene therapy, intimal hyperplasia, vascular remodeling, hemodialysis access.

Received for publication October 3, 2003
and in revised form November 7, 2003
Accepted for publication December 3, 2003

© 2004 by the International Society of Nephrology

Hemodialysis access complications constitute a major cause of morbidity in hemodialysis patients. In the United States, approximately 70% of the 230,000 hemodialysis patients depend upon an expanded polytetrafluoroethylene (ePTFE) graft for permanent vascular access. The current 1- and 2-year primary patency rates of ePTFE grafts are as low as 50% and 25%, respectively [1]. The failure of hemodialysis access grafts is predominantly due to flow impairment at the venous outflow tract, predominantly due to the rapid development of an intimal hyperplastic response in a localized region at the venous anastomosis [2]. This intimal hyperplasia is characterized by vascular smooth muscle cells (VSMCs), microvessel formation, and matrix deposition [3, 4]. Recent studies have elegantly demonstrated the pivotal role of adventitia, and in particular the periadventitial fibroblasts as active participants in the process of vascular remodeling and neointima formation [5, 6]. Unfortunately, no effective measures have been reported to be able to improve the poor patency rates of ePTFE arteriovenous grafts [1].

C-type natriuretic peptide (CNP) is the most recently identified member of the natriuretic peptide family [7]. CNP is an endothelium-derived peptide that binds to the transmembrane receptor natriuretic peptide receptor-B (NPR-B) [8], resulting in the synthesis of cyclic guanosine monophosphate (cGMP) [9]. CNP has been shown to inhibit the migration of VSMC [10], constrictive remodeling [11], and inhibits thrombus formation after vascular injury [12]. In this respect, CNP has emerged as a novel regulator of vascular tone and growth.

Intimal hyperplasia often constitutes a localized abnormality, which is difficult to inhibit using systemic approaches. Previously, pretreatment of the vessel (e.g., oligodenucleotides or adenoviral transduction) have proven valuable in preventing intimal hyperplasia [13, 14]. More recently, Khurana et al [15] have described paintbrush-assisted, periadventitial gene delivery as an easily applicable, promising method for localized vascular

use, which offers a feasible intervention during vascular surgical procedures.

In the present study, we validated the anti-migratory effects of CNP in porcine fibroblasts in vitro. Subsequently, we evaluated the feasibility and transduction efficiency of localized paintbrush-assisted, periadventitial adenoviral vector gene therapy at the venous outflow tract of arteriovenous ePTFE-grafts in pigs. Finally, we compared the composition of the venous outflow tract of either adenoviral vector encoding CNP (Ad.CNP) or Ad.mock-transduced vessels.

METHODS

Vein fibroblasts isolation and culture

Fibroblasts were isolated from the epigastric vein of Landrace pigs. After excision under sterile conditions, the adventitia was stripped from the media with two tweezers and finely minced. The adventitial fragments were digested overnight at 37°C in 6 mL of phosphate-buffered saline (PBS) containing 30 mg of collagenase A (Roche, Basel, Switzerland). After centrifuging the suspension at 1500 rpm for 5 minutes, collagenase was removed and replaced by Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS), 25 mmol/L HEPES, penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells were grown in a humidified atmosphere of 5% CO_2/95% air at 37°C. Fibroblasts at the 3rd-5th passage were used in the present study.

Construction and purification of recombinant adenovirus

To construct a recombinant adenovirus, cDNA of murine CNP was inserted into the *Hin*dIII and *Eco*RI sites of a cytomegalovirus (CMV) promoter containing adenoviral shuttle vector based on adenovirus serotype 5 (AdApt.mCNP). The CNP cDNA-containing vector was cotransfected with a cosmid vector containing the remaining part of the Ad5 genome into PER.C6^{TM} cells. The recombinant virus (Ad.CNP) was produced intracellularly and subsequently purified via plaques and CsCl ultracentrifugation. Similarly, adenoviral constructs and viral vectors carrying β-galactosidase DNA (Ad.LacZ) or without insert (Ad.mock) were generated as described [16]. Procedures and handling of tissues exposed to recombinant vectors were approved by the National Institute for Genetic Modified Organisms.

Fibroblast transduction

Subconfluent porcine fibroblasts in 6-wells plates were transduced with Ad.CNP or Ad.mock at a multiplicity of infection (MOI) of 500 in DMEM containing 2% FCS. After overnight incubation, the culture medium was replaced by DMEM containing 10% FCS. Three days after transduction, 5×10^{-4} mol/L isobutylmethylxanthine (IBMX) (Sigma Chemical Co., St. Louis, MO, USA) was added to the culture medium to block phosphodiesterase activity. The cells were incubated for additional 30 minutes at 37°C. The cells were then homogenized in lysis buffer [0.1% sodium dodecyl sulfate (SDS), 1% Triton in PBS] for cGMP measurements, or in Tripure Isolation Reagent (Roche, Basel, Switzerland) for RNA isolation.

Migration assay

To investigate the effect of CNP-22 (the biologically active form of CNP on migration of porcine vein fibroblasts), a Boyden chamber assay was performed using a 24-well chemotaxis chamber containing polycarbonate filters with 8 μm pore size (Costar Corp., Cambridge, MA, USA). Therefore, cultured fibroblasts were trypsinized and suspended at a concentration of 5.0×10^5 cells/mL. A volume of 100 uL of fibroblast suspension and 10^{-7} mol/L human CNP-22 (American Peptide Company, Sunnyvale, CA, USA) were placed in the upper compartment and 600 μL of medium supplemented with 5% FCS as chemoattractant was placed in the lower compartment. After 8 hours of incubation, the filters were removed, and the number of cells migrating to the lower surface of the filter was evaluated microscopically by counting the number of stained nuclei per high-power field (×100). The same experiment was performed using Ad.CNP (MOI 500)-transduced fibroblasts, 3 days after transduction. Ad.mock-transduced fibroblasts were used as a control. Three independent experiments were performed in quadruple, and each sample was counted randomly in four different areas in the center of the membrane. Migration activity was calculated as the mean number of migrated cells observed in four high power fields.

Protocol in vivo experiments

This study was approved by the ethical committee on Animal Experimentation of the University Medical Center Utrecht and conforms with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, 1996). The experimental protocol is shown in Figure 1. For this study, 16 specified pathogen-free (SPF) female Landrace pigs were used. Transduction efficiency and optimal virus concentration were determined using Ad.LacZ on jugular veins ($N = 2$). Next, in 14 pigs, arteriovenous grafts were implanted bilaterally between the carotid artery and the jugular vein, followed by in vivo gene transfer. These pigs were transduced with Ad.CNP (right venous anastomosis) and Ad.mock (left venous anastomosis). Biologic activity of CNP was evaluated by cGMP measurements combined with reverse transcription-polymerase chain reaction (RT-PCR), 3 days after transduction ($N = 2$). Histologic examination

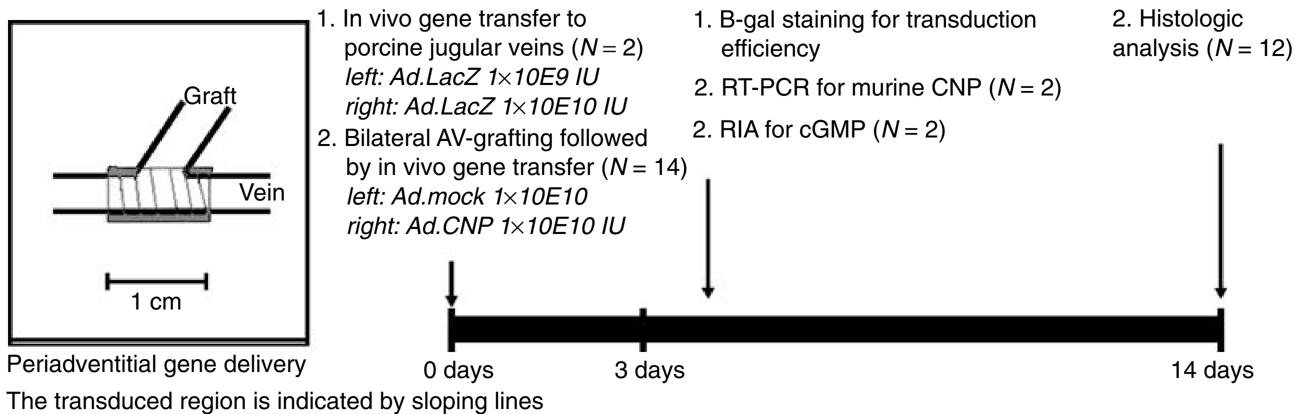

Fig. 1. Experimental protocol. The amount of virus applied to the jugular vein is expressed in infectious units (IU). Abbreviations are: AV, arteriovenous; Ad.CNP, adenoviral vector encoding murine C-type natriuretic peptide; β-gal, β-galactosidase; RT-PCR, reverse transcription-polymerase chain reaction; RIA, radioimmunoassay.

was performed in the remaining 12 pigs, 14 days after gene transfer.

Arteriovenous graft implantation and adenoviral transduction of porcine jugular veins

Starting 6 days before the operation, all pigs received acetylsalicylic acid 80 mg/day. Clopidogrel 225 mg was added 1 day prior to operation and continued at a dose of 75 mg/day until termination. ePTFE arteriovenous grafts were created bilaterally between the carotid artery and the internal jugular vein in 14 pigs, weighing 54 kg ± 0.9 kg, as previously described [17]. In short, through a longitudinal incision in the midline of the neck, the common carotid artery and the internal jugular vein were dissected bilaterally. The artery was clamped with atraumatic clamps and an arteriotomy was made. An end-to-side anastomosis was created at an angle of 45° by use of a continuous suture of 8–0 polypropylene (Prolene; Ethicon, Somerville, NJ, USA). All ringed ePTFE-grafts were 5 mm in diameter and 7 cm in length (W.L. Gore & Associates, Flagstaff, AZ, USA). The venous anastomosis was created in a similar fashion. After finishing both anastomoses, adenoviral transduction was performed using a small synthetic paintbrush with a flat-tip diameter of 0.25 inch. Viruses were applied, 30 minutes after incubation in 37°C water bath to improve transduction efficiency [18]. The vectors were applied directly to the outer venous surface at the venous anastomosis by lightly brushing the 50 μL vector solution containing 1×10^{10} IU into the tissue [15].

Tissue preparation and histologic analysis

Two weeks after the procedure, blood flow through the proximal and distal vein was measured with a perivascular flow probe (Transonic Systems, Ithaca, NY, USA). Next, grafts were perfused with formalin at physiologic pressure (100 mm Hg). After 2 minutes, both sides of the arteries and veins were ligated allowing pressure fixation of the vessels. Subsequently, grafts and adjacent vessels were excised and immersed in formalin for at least 24 hours. The fixated veins were cut in 5 mm blocks and embedded in paraffin. Five micrometer thick sections were prepared of the vein 1 cm proximal and distal to the anastomosis and at the center of the anastomosis. For morphometric analysis, sections were stained for general morphology (Elastin von Gieson). With the highest magnification that allowed visualization of the entire vein section in one field, the area and thickness of the intima and media, and the luminal area of the venous anastomosis, were manually traced. The circumference of the venous part of the anastomosis was determined by tracing the length of the external elastic lamina (EEL).

For immunohistochemical analysis, sections were incubated in 1.5% hydrogen peroxide in methanol to block endogenous peroxides. Next, sections were incubated in boiling 10 mmol/L citrate acid for 15 minutes and subsequently preincubated with 10% horse serum (Vector Laboratories, Burlingame, CA, USA). Serial sections from each graft were stained with antibodies against α-smooth muscle actin, at 1:1500 dilution (Sigma Chemical Co.), and Ki-67 for cellular proliferation, at 1:100 dilution (Immunotech, Marseille, France). Subsequently, sections were incubated with a biotinylated anti mouse IgG antibody (Vector Laboratories) for 1 hour. Immunoreactive materials were visualized by the use of streptavidin-labeled horseradish peroxidase (HRPO), diaminobenzidine in 0.05 mol/L Tris-Cl mixed with 0.01 mol/L imidazole, and 0.1% hydrogen peroxide. Sections were counterstained with hematoxylin. A proliferation index was defined as the number of positive cells divided by the sum of Ki-67 negative and positive cells and expressed as a percentage.

Apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) method by using an in situ cell death kit (Roche).

Radioimmunoassay cGMP

To examine biological activity of CNP after Ad.CNP transduction, cGMP levels were determined using a Radioimmunoassay Kit with ^{125}I-labeled cGMP (Amersham International, Buckinghamshire, UK). Therefore, cultured fibroblasts were homogenized in 0.5 mL lysis buffer containing 0.1% SDS, and 1% Triton in PBS. For detection of cGMP levels, 3 days after in vivo gene transfer, the transduced grafted veins (1 cm) were frozen in liquid nitrogen and ground using a pestle and mortar. cGMP extraction was performed according to manufacturer's protocol.

RNA isolation and RT-PCR

Total RNA was isolated from cultured fibroblasts and grafted veins using 1 mL Tripure Isolation Reagent (Roche) according to manufacturer's protocol.

Prior to the cDNA synthesis for the RT-PCR assay, 250 ng of RNA was treated with DNase to remove adenoviral DNA from the samples. PCR was performed with reactions carried out for 35 cycles of 94°C denaturation for 30 seconds, 54,2°C annealing for 30 seconds, and 72°C elongation for 30 seconds. PCR primers that only recognize murine CNP sequences were used to detect adenoviral transferred CNP synthesis. Each reaction contained 14 μL cDNA, 200 μmol/L dioxynucleoside triphosphate (dNTP), reaction buffer, 2.5 U Taq DNA polymerase and 1 μmol/L of each primer. Aliquots of each PCR reaction mixture were size-fractionated by agarose gel electrophoresis. The amount of β-actin mRNA was used as a positive control for cDNA synthesis. The following oligonucleotides were used as primers: murine CNP (forward primer 5′-TACAAAGGCGGCAACA AG-3′, reverse primer 5′-GCAATAAACAAGTTCGG ATCTC-3′) and porcine β-actin (forward primer 5′-ACCACCTTCAACTCGATCATG-3′, reverse primer 5′-GTGATCTCCTTCTGCATCCTG-3′).

Statistical evaluation

Data are presented as mean ± standard error of the mean. SPSS 11.0 was used for all statistical calculations. To ascertain the significance of differences we performed the Wilcoxon test. P values < 0.05 were considered significant.

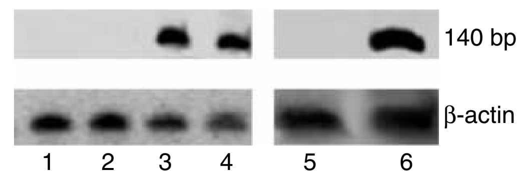

Fig. 2. Gene expression 3 days after transduction with adenoviral vector encoding murine C-type natriuretic peptide (Ad.CNP) or Ad.mock, analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Left panel shows gene expression in cultured porcine fibroblasts. (lanes 1 and 2, Ad.mock-transduced fibroblasts; lanes 3 and 4, Ad.CNP-transduced fibroblasts). Right panel shows the gene expression after in vivo gene transfer (lane 5, Ad.mock-transduced vein; lane 6, Ad.CNP-transduced vein). The amount of β-actin mRNA was used as internal standards.

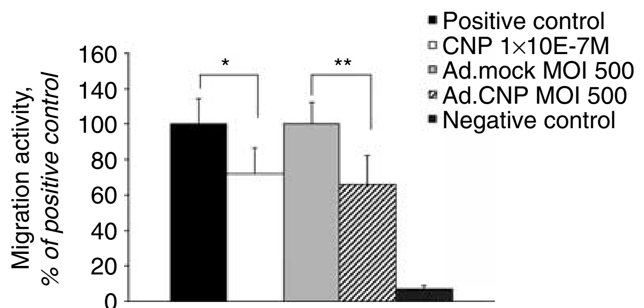

Fig. 3. Effect of C-type natriuretic peptide (CNP)-22 and adenoviral vector encoding murine C-type natriuretic peptide (Ad.CNP) transduction on the migration of cultured porcine vein fibroblasts. Values are expressed as the mean percentage ± SEM of migrated cells, as compared with the positive control and Ad.mock, respectively. The negative control represents fibroblast migration without fetal calf serum (FCS) stimulation. $^{*}P = 0.05$; $^{**}P = 0.04$.

RESULTS

In vitro experiments

First, we examined the efficiency of Ad.CNP transduction of porcine fibroblasts. Gel electrophoresis of PCR-amplified products showed clear bands, corresponding to the size of the murine CNP PCR product (Fig. 2). Three days after transduction, cGMP production was increased in Ad.CNP-transduced fibroblasts, when compared to Ad.mock-transduced fibroblasts (227 ± 22 fmol/mg protein vs. 123 ± 12 fmol/mg protein, respectively, $P = 0.05$, $N = 4$). The Boyden chamber assay showed that CNP-22 and Ad.CNP transduction, significantly inhibited FCS-stimulated migration of porcine fibroblast (Fig. 3).

In vivo experiments

Ad.LacZ transduction of porcine veins resulted in expression of β-galactosidase in the adventitia and, to lesser extent, in the medial VSMCs, 3 days after gene transfer (Fig. 4C). As determined by the amount of β-galactosidase–positive cells in multiple cross-sections of Ad.LacZ-transduced veins, a viral titer of 1×10^{10} IU/cm was superior to a titer of 1×10^{9} IU/cm (235 ± 75 positive cells/mm^2 vs. 164 ± 17 positive cells/mm^2, respectively).

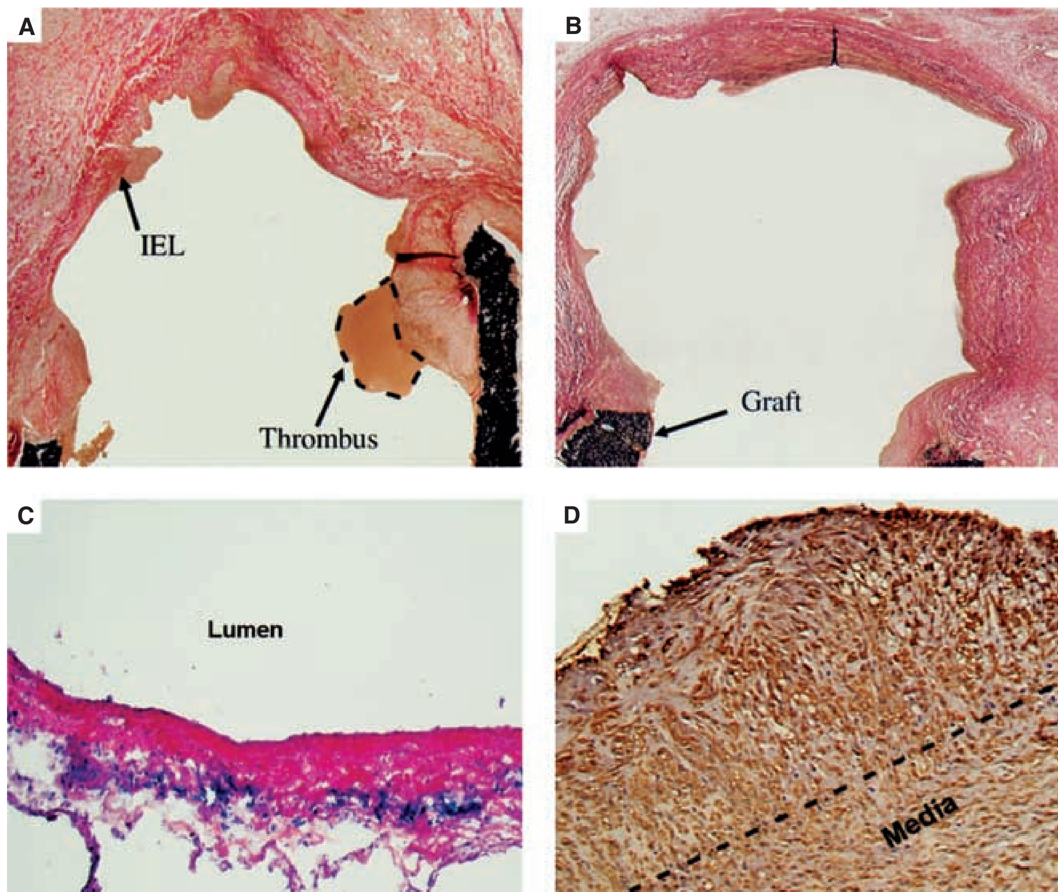

Fig. 4. Representative Elastin von Gieson–stained 5 μm section of an adenoviral (Ad.mock)-transduced (*A*) and adreoviral vector encoding murine C-type natriuretic peptide (Ad.CNP)-transduced (*B*) vein (original magnification 12.5×). (*C*) Detail of Ad.LacZ-transduced vein, 3 days after transduction (original magnification 100×). (D) Detail of α-smooth muscle cell actin–stained section. The dotted line indicated the boundary of the neointima area (original magnification 200×). The intima predominantly consists of vascular smooth muscle cells (VSMC).

Therefore, a titer of 1×10^{10} IU/cm was used for the Ad.CNP and Ad.mock transductions.

Three days after in vivo gene transfer of Ad.CNP, mRNA of murine CNP was detected by RT-PCR (Fig. 2). Simultaneously, a 56% increase in cGMP content was observed in tissue extracts of Ad.CNP-treated vein ($N = 2$), when compared to Ad.mock-treated veins (320 and 340 fmol/cm vein vs. 210 and 215 fmol/cm, respectively).

Two weeks after graft implantation and gene transfer, all grafts were patent. In none of the pigs, neither systemic nor local side effects of adenoviral transduction were observed. Flow measurements of the grafted vein revealed no significant differences between the Ad.CNP and Ad.mock-transduced veins (900 ± 57 mL/min vs. 808 ± 67 mL/min, respectively, $P = 0.2$). Histologic analysis showed that CNP gene transfer did not inhibit intimal hyperplasia at the venous anastomosis (Table 1). Representative sections from the venous anastomosis are shown in Figure 4. By contrast, the medial area of Ad.CNP-transduced veins was increased in all pigs, resulting in a 37% decrease in intima/media (I/M) ratio, when compared to Ad.mock-transduced veins. This was accompanied by an increased circumference of the venous part of the anastomosis ($P = 0.04$) and luminal area of the venous anastomosis in Ad.CNP-treated veins ($P = 0.05$). In three out of 12 pigs, the luminal area at the venous anastomosis was not larger in the Ad.CNP-transduced veins (nonresponders).

Table 1. Morphometric analysis of Elastin von Gieson–stained sections obtained from the venous anastomosis

Venous anastomosis	Ad.CNP	Ad.mock	*P* value
Intima area	2.53 ± 0.9	1.94 ± 0.5	0.53
Medial area	3.1 ± 0.6	1.70 ± 0.3	0.002
Intima/media ratio	0.73 ± 0.1	1.15 ± 0.2	0.034
External elastic lumina	11.8 ± 1.4	9.4 ± 1.0	0.04
Luminal area	10.7 ± 1.4	8.8 ± 1.7	0.05

Ad.CNP is adenoviral vector encoding murine C-type natriuretic peptide. Intima, media and lumina values are expressed in square millimeters and are the mean area ± the standard error of the mean (SEM). The exteral elastic lumina is expressed in millimeters and is the mean length ± the standard error of the mean (SEM).

Proliferation rates and the amount of apoptotic cells at the venous anastomosis did not differ between Ad.CNP and Ad.mock-transduced veins (proliferation rate Ad.CNP 9.8% ± 3% positive cells vs. Ad.mock 10.1% ± 3% positive cells, $P = 0.89$; apoptotic cells Ad.CNP 4.3 ± 2 cells per 10 high power fields vs. Ad.mock 5.1 ± 2 cells per 10 high power fields, $P = 0.59$).

In each graft, one stenotic lesion was observed, with maximum size at the center of the venous anastomosis. A continuous decrease in lesion area was observed up to 1 cm away from the anastomosis. At these remote locations, no significant differences in intimal and medial areas were observed between the Ad.CNP and Ad.mock-transduced veins (proximal vein, intimal area Ad.CNP 0.48 ± 0.1 vs. Ad.mock 0.63 ± 0.3, $P = 0.94$; medial area Ad.CNP 2.6 ± 0.2 vs. Ad.mock 2.1 ± 0.4, $P = 0.18$; distal vein, intimal area Ad.CNP 0.38 ± 0.3 vs. Ad.mock 0.42 ± 0.2, $P = 0.87$; medial area Ad.CNP 2.7 ± 0.5 vs. Ad.mock 3.0 ± 0.8, $P = 0.80$).

DISCUSSION

In the present study we show that periadventitial application of Ad.CNP in a bilateral arteriovenous ePTFE graft model is associated with successful transduction, as demonstrated by the presence of murine CNP-mRNA and increased cGMP content in the treated vein compared to the mock-transduced contralateral vein. In this model, local overexpression of CNP results in a significant decrease of the I/M ratio compared to the control vein, predominantly due to increased medial thickening. Despite the increase in medial thickening of the Ad.CNP transduced vein, the increase in EEL (i.e., outward remodeling) in the Ad.CNP-transduced vein resulted in an increase of the luminal area at the venous anastomosis of CNP-treated veins.

The periadventitia as gene therapeutic target

Recent studies have elegantly demonstrated the pivotal role of the adventitia, and in particular periadventitial fibroblasts in neointima formation and vascular remodeling. Activated adventitial fibroblasts may infiltrate the injured media of arterialized vein grafts [19]. Also in a porcine model, Shi et al [20] demonstrated translocation of adventitial fibroblast to the subendothelial space of vein grafts.

The interest in gene therapeutic approaches in order to attenuate intimal hyperplasia has multiple rationales. First, systemic therapies have not been successful in treating intimal hyperplasia thus far, most likely due to insufficiently high concentrations of the therapeutic compounds in the area of interest. In this respect, local adenovirus-mediated gene therapy offers a promising tool in the treatment of localized vascular disorders. Second, genes of interest can be transferred to the adventitia of target vessels by local transfer, applicable during vascular surgical procedures [21, 22]. In this respect, the paintbrush-assisted, periadventitial gene delivery technique has been validated in rabbit and canine carotid arteries [15], resulting in targeted and efficient gene transfer with an optimal transduction time of only 10 minutes. In the present study, we have validated this technique in porcine arteriovenous grafts. Three days after Ad.LacZ transduction, β-galactosidase–positive cells could be demonstrated in the adventitia and to lesser extend in the media.

Antimigratory effects of CNP have been shown in human [23] and rat VSMCs [10]. In order to validate the biological activity of murine CNP in a porcine model, we evaluated the effect of CNP on migration of porcine adventitial fibroblasts. Adenoviral delivery of CNP as well as CNP-22 showed antimigratory effects on porcine adventitial fibroblasts. The presence of the NPR-B receptor on fibroblasts [24] supports the potential of these cells to respond to CNP stimulation. In addition, CNP is also secreted from cardiac fibroblasts [25], which are more responsive to CNP than cardiac smooth muscle cells [26]. Periadventitial delivery of Ad.CNP to porcine veins in vivo resulted in mRNA synthesis of murine CNP, with a simultaneous increase in cGMP levels, indicating biological activity of CNP.

Effect of adventitial delivery of CNP

CNP significantly decreased the intima to media ratio at the venous outflow tract compared to the mock transduced contralateral vein. However, CNP did not attenuate initimal hyperplasia in the outflow tract. These results are consistent with a recent study by Morishige et al [11], who also showed no effect on intimal hyperplasia of adenoviral gene transfer of CNP in a porcine model of coronary balloon angioplasty. The absence of a significant decrease of intimal area in vivo, indirectly suggesting "insufficient" inhibition of migration, can be due to insufficient CNP levels in vivo after adventitial transduction. In contrast, several studies in rabbit [12, 27] and rat [28] models, have reported suppressed intimal hyperplasia after adenoviral gene delivery of CNP. These conflicting findings may simply be due to heterogeneity of the species used in these studies. However, other differences in the study design may also have contributed to this apparent discrepancy. In the rat and rabbit studies, adenoviral delivery was performed intraluminally. Use of the latter approach can be expected to result in higher CNP concentrations compared to the concentration after periadventitial gene delivery, mainly due to the smaller cellular density in the adventitia. Also, the distance between the adventitia and subendothelial space may prove to be a barrier preventing adequate CNP delivery. Of note, the latter issue was contradicted by previous data in rabbits, in which adventitial administration of 10 μmol/L of

CNP-22 was able to prevent intimal hyperplasia after carotid artery injury [29].

Notably, we cannot exclude that decreased periadventitial Ad.CNP transduction and increased CNP diffusion distance in pig veins has limited the effect of Ad.CNP transduction on intimal hyperplasia in the present model.

In contrast to the intimal hyperplasia response, local delivery of CNP did result in an increased medial thickening, which has been associated with vein-graft arterialization [30, 31]. The construction of an arteriovenous graft invokes an injury response of medial VSMCs, characterized by an increase in both apoptosis and proliferation rates. Overall, these processes determine successful remodeling of vein grafts during the arterialization phase [32]. The increased medial thickening upon Ad.CNP transduction can be due to decreased apoptosis and/or increased proliferation of medial VSMCs. At termination 14 days after gene transfer, no significant differences between proliferation and apoptosis of VSMCs could be observed between CNP and mock-transduced sides. However, the maximum apoptotic and proliferative rates after vein grafting have been reported to vary from several hours [33] to 1 week [34]. Therefore, we cannot exclude that due to the obligatory single time point observation at 2 weeks we may have missed a significant effect of CNP on VSMC apoptotic or proliferation rates at an earlier time point. The exact value of increased medial thickness in Ad.CNP treated veins is unknown. Feinfeld et al [35] reported a correlation between media thickness of the vein at the venous outflow tract and the number of months undergoing hemodialysis. These structural adaptations can be expected to enable vein grafts to withstand high arterial pressure. However, a direct relation between early medial thickening and long-term graft patency remains to be established.

Venous outward remodeling is essential for adequate maturation of the vein distal to the graft. Notably, the luminal area is an important determinant for graft thrombosis [1]. Whereas luminal area is determined by wall thickness and total vessel area, outward remodeling is defined as an increase in vessel area encompassed by the EEL. In the present study, a clear increase in luminal area was observed at the anastomosis of CNP treated veins. Of note, this increase occurred in spite of a significant increase in media thickness, clearly indicating the presence of outward remodeling in the Ad.CNP-transduced veins. These findings concur with previous data, showing decreased constrictive remodeling after adenoviral delivery of CNP in porcine coronary arteries [11]. The mechanism of this effect remains unclear. In comparison, matrix metalloproteinase inhibitors also induce inhibition of constrictive remodeling in favor of outward remodeling [36], predominantly due to a decrease in adventitial collagen [37]. Since CNP also inhibits collagen synthesis [25, 38], decreased adventitial collagen accumulation could have contributed to vascular remodeling upon adventitial CNP overexpression in the present study. In addition, the increase in luminal area can relate to the antagonistic effects of natriuretic peptides at various sites in the renin-angiotensin-aldosterone cascade [39]. Since angiotensin II has a pivotal role in the process of constrictive remodeling [40], inhibition of this system can also contributed to the beneficial effects on outward remodeling during CNP overexpression.

The important question whether the increased medial thickening and enhanced outward remodeling after Ad.CNP transduction will positively affect long-term patency of the arteriovenous grafts needs to be addressed in future long-term studies in the present model. In the end, the potential impact of this finding needs to be validated in humans.

Study limitations

In the present study, we used an adenoviral vector encoding murine CNP. Biochemical properties of murine CNP might differ from porcine CNP. However, the amino acid sequence of CNP is identical in human, pig, rat, and mouse [41, 42]. Furthermore, we have shown production of biologic active CNP in the present model by measuring increased levels of cGMP after Ad.CNP transduction in vivo, as well as by demonstrating antimigratory effect of murine CNP using porcine fibroblasts in vitro.

Exposure of adenoviral vectors per se has been shown to induce immunologic and inflammatory reactions, which can promote the formation of intimal hyperplasia [43]. In the present study, we observed intimal and medial areas after Ad-mock transduction comparable to those without adenoviral exposure [17]. Hence, periadventitial paintbrush-assisted adenoviral exposure is unlikely to contribute to intimal hyperplasia formation in the present model.

A major limitation of adenoviral gene transfer is the transient nature of transgene expression, which might explain the absence of a CNP effect on intimal hyperplasia in porcine arteriovenous grafts. Significant transgene expression in grafted jugular veins is maintained for at least 7 days [44]. Other vectors like adeno-associated viruses might prolong the expression of transferred genes [45]. However, the need for long-term exposure remains to be established. Thus, a recent study suggested sustained effects after a single dose of CNP being able to inhibit intimal hyperplasia in a balloon-injury model in rabbits [46]. Hence, the effect of long-term CNP exposure in the present model remains to be evaluated.

CONCLUSION

Periadventitial gene delivery is a feasible approach for the venous anastomosis of arteriovenous grafts. Local

overexpression of CNP reduced the I/M ratio and increased the luminal area in the outflow tract of porcine arteriovenous grafts. These data support the potential of gene therapeutic strategies in order to attenuate luminal narrowing at the outflow tract of hemodialysis arteriovenous grafts.

ACKNOWLEDGMENTS

This study was supported by a grant from the Dutch Kidney Foundation (CSP 6001). The ePTFE-grafts were supplied by W. L. Gore & Associates (Flagstaff, AZ, USA). Clopidogrel was supplied by Sanofi-Synthelabo (Maassluis, The Netherlands). We thank Crucell Holland (Leiden, The Netherlands) for generously supplying the adenoviral vectors. Dr. Z.S. Katusic from the Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Rochester, MN, USA, is greatly acknowledged for his comprehensive instructions for the periadventitial brushing technique. Machiel Nagtegaal is acknowledged for his excellent assistance with the pathology work-up.

Reprint requests to Erik S.G. Stroes, M.D., Department of Vascular Medicine, Academic Medical Center, Meibergdreef 9, Room F4.250, 1105 AZ, Amsterdam, The Netherlands.
E-mail: E.S.Stroes@amc.uva.nl

REFERENCES

1. Schwab SJ, Harrington JT, Singh A, *et al*: Vascular access for hemodialysis. *Kidney Int* 55:2078–2090, 1999
2. Kanterman RY, Vesely TM, Pilgram TK, *et al*: Dialysis access grafts: Anatomic location of venous stenosis and results of angioplasty. *Radiology* 195:135–139, 1995
3. Roy-Chaudhury P, Kelly BS, Miller MA, *et al*: Venous neointimal hyperplasia in polytetrafluoroethylene dialysis grafts. *Kidney Int* 59:2325–2334, 2001
4. Swedberg SH, Brown BG, Sigley R, *et al*: Intimal fibromuscular hyperplasia at the venous anastomosis of PTFE grafts in hemodialysis patients. Clinical, immunocytochemical, light and electron microscopic assessment. *Circulation* 80:1726–1736, 1989
5. Sartore S, Chiavegato A, Faggin E, *et al*: Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: From innocent bystander to active participant. *Circ Res* 89:1111–1121, 2001
6. Zalewski A, Shi Y, Johnson AG: Diverse origin of intimal cells: Smooth muscle cells, myofibroblasts, fibroblasts, and beyond? *Circ Res* 91:652–655, 2002
7. Sudoh T, Minamino N, Kangawa K, *et al*: C-type natriuretic peptide (CNP): A new member of natriuretic peptide family identified in porcine brain. *Biochem Biophys Res Commun* 168:863–870, 1990
8. Koller KJ, Lowe DG, Bennett GL, *et al*: Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP). *Science* 252:120–123, 1991
9. Komatsu Y, Itoh H, Suga S, *et al*: Regulation of endothelial production of C-type natriuretic peptide in coculture with vascular smooth muscle cells. Role of the vascular natriuretic peptide system in vascular growth inhibition. *Circ Res* 78:606–614, 1996
10. Ikeda M, Kohno M, Yasunari K, *et al*: Natriuretic peptide family as a novel antimigration factor of vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 17:731–736, 1997
11. Morishige K, Shimokawa H, Yamawaki T, *et al*: Local adenovirus-mediated transfer of C-type natriuretic peptide suppresses vascular remodeling in porcine coronary arteries in vivo. *J Am Coll Cardiol* 35:1040–1047, 2000
12. Ohno N, Itoh H, Ikeda T, *et al*: Accelerated reendothelialization with suppressed thrombogenic property and neointimal hyperplasia of rabbit jugular vein grafts by adenovirus-mediated gene transfer of C-type natriuretic peptide. *Circulation* 105:1623–1626, 2002
13. O'Brien T: Adenoviral vectors and gene transfer to the blood vessel wall. *Arterioscler Thromb Vasc Biol* 20:1414–1416, 2000
14. Smith RC, Walsh K: Local gene delivery to the vessel wall. *Acta Physiol Scand* 173:93–102, 2001
15. Khurana VG, Weiler DA, Witt TA, *et al*: A direct mechanical method for accurate and efficient adenoviral vector delivery to tissues. *Gene Therapy* 10:443–452, 2003
16. Havenga MJ, Lemckert AA, Grimbergen JM, *et al*: Improved adenovirus vectors for infection of cardiovascular tissues. *J Virol* 75:3335–3342, 2001
17. Rotmans JI, Velema E, Verhagen HJ, *et al*: Rapid, arteriovenous graft failure due to intimal hyperplasia: A porcine, bilateral, carotid arteriovenous graft model. *J Surg Res* 113:161–171, 2003
18. Kossila M, Jauhiainen S, Laukkanen MO, *et al*: Improvement in adenoviral gene transfer efficiency after preincubation at +37 degrees C in vitro and in vivo. *Mol Ther* 5:87–93, 2002
19. Kalra M, Miller VM: Early remodeling of saphenous vein grafts: Proliferation, migration and apoptosis of adventitial and medial cells occur simultaneously with changes in graft diameter and blood flow. *J Vasc Res* 37:576–584, 2000
20. Shi Y, O'Brien JEJ, Mannion JD, *et al*: Remodeling of autologous saphenous vein grafts. The role of perivascular myofibroblasts. *Circulation* 95:2684–2693, 1997
21. Laitinen M, Pakkanen T, Donetti E, *et al*: Gene transfer into the carotid artery using an adventitial collar: Comparison of the effectiveness of the plasmid-liposome complexes, retroviruses, pseudotyped retroviruses, and adenoviruses. *Hum Gene Ther* 8:1645–1650, 1997
22. Schneider DB, Sassani AB, Vassalli G, *et al*: Adventitial delivery minimizes the proinflammatory effects of adenoviral vectors. *J Vasc Surg* 29:543–550, 1999
23. Kohno M, Yokokawa K, Yasunari K, *et al*: Effect of natriuretic peptide family on the oxidized LDL-induced migration of human coronary artery smooth muscle cells. *Circ Res* 81:585–590, 1997
24. Chrisman TD, Garbers DL: Reciprocal antagonism coordinates C-type natriuretic peptide and mitogen-signaling pathways in fibroblasts. *J Biol Chem* 274:4293–4299, 1999
25. Horio T, Tokudome T, Maki T, *et al*: Gene expression, secretion, and autocrine action of C-type natriuretic peptide in cultured adult rat cardiac fibroblasts. *Endocrinology* 144:2279–2284, 2003
26. Doyle DD, Upshaw-Earley J, Bell EL, Palfrey HC: Natriuretic peptide receptor-B in adult rat ventricle is predominantly confined to the nonmyocyte population. *Am J Physiol Heart Circ Physiol* 282:H2117–H2123, 2002
27. Qian JY, Haruno A, Asada Y, *et al*: Local expression of C-type natriuretic peptide suppresses inflammation, eliminates shear stress-induced thrombosis, and prevents neointima formation through enhanced nitric oxide production in rabbit injured carotid arteries. *Circ Res* 91:1063–1069, 2002
28. Ueno H, Haruno A, Morisaki N, *et al*: Local expression of C-type natriuretic peptide markedly suppresses neointimal formation in rat injured arteries through an autocrine/paracrine loop. *Circulation* 96:2272–2279, 1997
29. Gaspari TA, Barber MN, Woods RL, Dusting GJ: Type-C natriuretic peptide prevents development of experimental atherosclerosis in rabbits. *Clin Exp Pharmacol Physiol* 27:653–655, 2000
30. O'Brien JEJ, Ormont ML, Shi Y, *et al*: Early injury to the media after saphenous vein grafting. *Ann Thorac Surg* 65:1273–1278, 1998
31. Fann JI, Sokoloff MH, Sarris GE, *et al*: The reversibility of canine vein-graft arterialization. *Circulation* 82:IV9–18, 1990
32. Davies MG, Hagen PO: Pathophysiology of vein graft failure: a review. *Eur J Vasc Endovasc Surg* 9:7–18, 1995
33. Rodriguez E, Lambert EH, Magno MG, Mannion JD: Contractile smooth muscle cell apoptosis early after saphenous vein grafting. *Ann Thorac Surg* 70:1145–1153, 2000
34. Westerband A, Crouse D, Richter LC, *et al*: Vein adaptation to arterialization in an experimental model. *J Vasc Surg* 33:561–569, 2001
35. Feinfeld DA, Batista R, Mir R, *et al*: Changes in venous histology in chronic hemodialysis patients. *Am J Kidney Dis* 34:702–705, 1999
36. Sierevogel MJ, Pasterkamp G, Velema E, *et al*: Oral matrix metalloproteinase inhibition and arterial remodeling after balloon dilation: An intravascular ultrasound study in the pig. *Circulation* 103:302–307, 2001

37. Sierevogel MJ, Velema E, van der Meer FJ, *et al*: Matrix metalloproteinase inhibition reduces adventitial thickening and collagen accumulation following balloon dilation. *Cardiovasc Res* 55:864–869, 2002
38. Canaan-Kuhl S, Ostendorf T, Zander K, *et al*: C-type natriuretic peptide inhibits mesangial cell proliferation and matrix accumulation in vivo. *Kidney Int* 53:1143–1151, 1998
39. Davidson NC, Barr CS, Struthers AD: C-type natriuretic peptide. An endogenous inhibitor of vascular angiotensin-converting enzyme activity. *Circulation* 93:1155–1159, 1996
40. Strawn WB, Ferrario CM: Mechanisms linking angiotensin II and atherogenesis. *Curr Opin Lipidol* 13:505–512, 2002
41. Tawaragi Y, Fuchimura K, Tanaka S, *et al*: Gene and precursor structures of human C-type natriuretic peptide. *Biochem Biophys Res Commun* 175:645–651, 1991
42. Ogawa Y, Itoh H, Yoshitake Y, *et al*: Molecular cloning and chromosomal assignment of the mouse C-type natriuretic peptide (CNP) gene (Nppc): Comparison with the human CNP gene (NPPC). *Genomics* 24:383–387, 1994
43. Newman KD, Dunn PF, Owens JW, *et al*: Adenovirus-mediated gene transfer into normal rabbit arteries results in prolonged vascular cell activation, inflammation, and neointimal hyperplasia. *J Clin Invest* 96:2955–2965, 1995
44. Channon KM, Fulton GJ, Gray JL, *et al*: Efficient adenoviral gene transfer to early venous bypass grafts: Comparison with native vessels. *Cardiovasc Res* 35:505–513, 1997
45. Maeda Y, Ikeda U, Ogasawara Y, *et al*: Gene transfer into vascular cells using adeno-associated virus (AAV) vectors. *Cardiovasc Res* 35:514–521, 1997
46. Yasuda S, Kanna M, Sakuragi S, *et al*: Local delivery of single low-dose of C-type natriuretic peptide, an endogenous vascular modulator, inhibits neointimal hyperplasia in a balloon-injured rabbit iliac artery model. *J Cardiovasc Pharmacol* 39:784–788, 2002