Accelerated Reendothelialization With Suppressed Thrombogenic Property and Neointimal Hyperplasia of Rabbit Jugular Vein Grafts by Adenovirus-Mediated Gene Transfer of C-Type Natriuretic Peptide

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Background—Vein graft disease limits the late results of coronary revascularization. C-type natriuretic peptide (CNP) inhibits the growth of vascular smooth muscle cells. Given the effects of CNP on cGMP cascade, we hypothesized that transfected CNP genes modulate endothelial repair and thrombogenicity in the vein graft.

Methods and Results—Autologous rabbit jugular vein grafts were incubated ex vivo in a solution of adenovirus vectors containing CNP gene (Ad.CNP) or Escherichia coli lac Z gene (Ad.LacZ) and then interposed in the carotid artery. Reendothelialization, mural thrombi formation, and intima/media ratio were evaluated on the 14th and 28th postoperative days. More reendothelialization was seen in Ad.CNP-infected grafts than in Ad.LacZ-infected grafts both at 14 days (0.81 ± 0.05 versus 0.30 ± 0.14, *P* < 0.01) and at 28 days (0.96 ± 0.04 versus 0.45 ± 0.08, *P* < 0.001). The mural thrombus area was smaller in Ad.CNP-infected grafts than in Ad.LacZ-infected grafts. Neointimal thickening was significantly suppressed in the Ad.CNP group. The in vitro wound assay with human coronary artery endothelial cells revealed significant potentiation of the wound repair process by CNP and atrial natriuretic peptide administration.

Conclusions—Infected Ad.CNP accelerated reendothelialization and suppressed thrombosis and neointimal hyperplasia. The method may potentially prevent vein graft disease in patients undergoing coronary artery revascularization. (Circulation. 2002;105:1623-1626.)

Key Words: natriuretic peptides ■ viruses ■ grafting ■ genes

Accelerated atherosclerosis within grafted vein grafts allows only 38% to 45% of patency by the end of 10 postoperative years. During the first month after bypass surgery, vein graft attrition results from thrombotic occlusion, followed by late graft failure caused by neointimal hyperplasia.  

Endothelium-dependent relaxation is demonstrated to be reduced in saphenous vein (SV) grafts compared with internal mammary artery (IMA) grafts.  

Endothelial production of nitric oxide (NO) and prostacyclin is lower in veins than in arteries, and NO synthesis is further reduced in bypass grafting.  

We reported reduced expression of guanylate cyclase A, the receptor for atrial natriuretic peptide (ANP) and brain natriuretic peptide, and less production of cGMP stimulated by ANP in SV compared with IMA.

We previously demonstrated that natriuretic peptides inhibit vascular growth through the cGMP-dependent pathway.  

Furthermore, C-type natriuretic peptide (CNP) is secreted from endothelial cells to act as an endothelium-derived relaxing peptide for vascular remodeling.  

We also reported that endothelial CNP expression is progressively reduced in accordance with the severity of human coronary atherosclerosis.  

Together with the recent findings that show that the cGMP pathway is involved in promotion of neovascularization and inhibition of tissue factor or plasminogen activator inhibitor-1 expression, in this study, we investigated the effect of adenoviral gene transfer of CNP on vein graft patency in rabbit jugular vein–carotid artery interposition graft procedures.

**Methods**

Construction of Recombinant Adenoviruses

We constructed a recombinant replication-defective adenoviral vector encoding the rat CNP cDNA, Ad.CNP, as previously reported.

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1623
We also constructed an adenoviral vector, Ad.lacZ overexpressing Escherichia coli β-galactosidase (lacZ) gene.

Animal Preparation
Male Japanese white rabbits weighing between 2.5 and 3.5 kg underwent unilateral interposition carotid jugular vein grafts, as previously reported.12 The right jugular vein was cannulated with a 24-gauge catheter. A 2F Fogarty balloon catheter was inserted, and after the balloon was inflated with 0.2 mL of air, the intima of the jugular vein was denuded by three passages of the catheter. The vein was ligated again just above the incision, and 150 µL of sorbitol-added lactated Ringer’s saline (Otsuka Pharmaceutical) containing 5×10⁶ PFU of Ad.CNP (n=10) or Ad.lacZ (n=10) was infused and incubated for 30 minutes. Internal pressure of the vein was ~30 mm Hg. The vein segment was harvested and anastomosed into the ipsilateral carotid artery in a reverse end-to-end fashion. Fourteen or 28 days after the procedure, the animals were killed by overdose of sodium pentobarbital.

Thirty minutes before the animals were killed, 3 mL of 1.5% Evans blue dye (Sigma Chemical Co) was infused through the ear vein. The grafted carotid arteries were harvested after perfusion fixation with 10% phosphate-buffered formalin.

Planimetric Analysis of Reendothelialization and Vessel Morphometry
The grafts were incised longitudinally and photographed for planimetric analysis of the reendothelialized area. The area that was stained blue was interpreted as representing areas still denuded.
Gly 22) -ANP (4–23) (10 ng/mL), ANP quantified by counting the number of cells in 5 successive 125-
°/H11002 tute) (10 ng/mL). Control cultures and cultures containing vascular
endothelial growth factor (VEGF 165; Sigma) (10 ng/mL), ANP
were also evaluated, as previously reported.11

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Endothelial Cells

Promotion of Wound Repair of Cultured

Wound Assays

Confluent monolayers of human coronary endothelial cells (HCAECs; Clonetics; Walkersville, Md) were grown to confluency in 6-well plates, and half of the cells were scraped off and the cultures were further incubated in MCDB-131 medium containing 1% or 2.5% FCS.13 Control cultures and cultures containing vascular endothelial growth factor (VEGF 165; Sigma) (10 ng/mL), ANP (human ANP [1–28]) (10−10 mol/L), CNP (CNP-22; Peptide Institute) (10−11 to 10−7 mol/L), and des-(Gin5, Ser19, Gly20, Leu21, Gly22)-ANP (4–23) amide (C-AMF [4–23]; Sigma) (10−7 mol/L) were incubated at 37°C for 3 days. Migration and proliferation were quantified by counting the number of cells in 5 successive 125-μm sections from the wound edge. Effects of CNP on cell proliferation were also evaluated, as previously reported.11

Statistical Analysis

All values are expressed as mean±SEM. Factorial ANOVA followed by Bonferroni/Dunn was used to determine significant differences in multiple comparisons.

Results

Neointimal Thickening

In Ad.CNP-treated grafts, CNP immunostaining was detected both in the intima and the media, as shown in the Figure (A). The tissue level of cGMP was significantly higher in Ad.CNP-treated grafts (4.58±0.23 fmol/mg) than Ad.lacZ-treated grafts (0.89±0.41 fmol/mg, n=4, P<0.05). Ad.lacZ-treated grafts showed considerable neointimal thickening by

28 days (the ratio of intima to media: 14 days, 1.22±0.13; 28 days, 1.48±0.12). On the other hand, in the Ad.CNP-treated group, neointimal thickening was significantly suppressed (14 days, 0.43±0.11; 28 days, 0.47±0.06; P<0.05 versus Ad.lacZ-treated group).

Reendothelialization

The Figure (B) shows Evans blue dye staining of the adenovirus-infected vein grafts. The endothelialized areas in Ad.lacZ-infected grafts were 0.30±0.14 on day 14 and 0.46±0.08 on day 28. In contrast, in the Ad.CNP-infected group, 0.81±0.05 on day 14 and 0.96±0.01 of the whole intimal area on day 28 were reendothelialized. Thus, CNP overexpression in grafted rabbit jugular veins remarkably accelerated reendothelialization (P<0.05 both on day 14 and day 28 versus the Ad.LacZ-infected group).

Thrombotic Occlusion and Mural
Thrombus Formation

In the Ad.LacZ-infected group, mural thrombi were formed in 4 of 5 rabbits by 14 days and 5 of 5 (with total thrombotic occlusion of one case) by 28 days. In contrast, in the Ad.CNP-infected group, no thrombus formation was detected by 14 days, but by 28 days, thrombus formation was detected in 1 of 5 rabbits (Figure, B). The mural thrombus area was smaller in Ad.CNP-infected grafts than in Ad.LacZ-infected grafts (14 days: 0 versus 0.20±0.09; 28 days: 0.01±0.01 versus 0.31±0.17).

Promotion of Wound Repair of Cultured
Endothelial Cells

The Table depicts the effect of CNP on the repair process of wounded cultured HCAECs. VEGF as the positive control significantly promoted the wound healing of HCAECs. The administration of CNP significantly potentiated this wound

<table>
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<th>Treatment</th>
<th>1</th>
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<th>3</th>
<th>4</th>
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<tr>
<td>Control (2.5% FCS)</td>
<td>60±3</td>
<td>107±7</td>
<td>142±10</td>
<td>159±13</td>
<td>163±14</td>
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<td>134±9*</td>
<td>177±12*</td>
<td>200±16*</td>
<td>207±17*</td>
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<td>ANP (10−10 mol/L)</td>
<td>77±5*</td>
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<td>175±11*</td>
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<td>209±13*</td>
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<tr>
<td>C-ANF[4–23] (10−10 mol/L)</td>
<td>66±6</td>
<td>115±9</td>
<td>141±12</td>
<td>148±13</td>
<td>150±14</td>
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<tr>
<td>VEGF (10 ng/mL)</td>
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<td>180±8*</td>
<td>240±11*</td>
<td>267±16*</td>
<td>272±17*</td>
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<tr>
<td>(n=9)</td>
<td></td>
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<tr>
<td>Control (1% FCS)</td>
<td>57±2</td>
<td>80±3</td>
<td>93±4</td>
<td>94±4</td>
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<tr>
<td>CNP</td>
<td></td>
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<tr>
<td>10−15 mol/L</td>
<td>64±4</td>
<td>91±4</td>
<td>106±4</td>
<td>109±5*</td>
<td>109±5*</td>
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<tr>
<td>10−14 mol/L</td>
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<td>133±7*</td>
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<td>10−13 mol/L</td>
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<td>119±4*</td>
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<tr>
<td>10−12 mol/L</td>
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<td>98±5</td>
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<tr>
<td>10−11 mol/L</td>
<td>62±4</td>
<td>84±6</td>
<td>90±6</td>
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Values are cumulative cell numbers shown as mean±SEM. *P<0.05 vs corresponding controls.

Computerized planimetry (NIH Image version 1.61) was used for the analysis. The areas of both reendothelialization and thrombosed vessel wall were calculated as the ratio to the total luminal area in the whole graft. The vessels were then stained with elastin/van Gieson. Intimal thickness and medial thickness were measured by the same digital planimetry in at least 3 histological sections from each graft and at 5 equally divided segments in each section. Immunostaining for CNP was performed with our specific CNP monoclonal antibody, as previously reported.8 cGMP levels in grafted veins were determined as previously reported.4
repair process. ANP at the same concentration also exerted the similar action, whereas C-ANF (4–23), which is the agonist for the clearance receptor of natriuretic peptides and lacks the potency to stimulate cGMP production, had no significant effect. As shown in the Table, the effect of CNP on promotion of wound repair was dose-dependent within lower doses (10^{-5} to 10^{-11} mol/L). Higher doses of CNP (10^{-9} to 10^{-7} mol/L) had no significant effect. The same pattern of CNP effect was observed in cell number increase, which was inhibited by a cGMP-dependent protein kinase inhibitor (RP8-CPT-cGMP) (data not shown).

Discussion

In reversed autologous vein bypass grafting of the common carotid artery in rabbits, the present study demonstrates that CNP overexpression in the vein graft caused not only suppression of intimal thickening but also acceleration of reendothelialization with less thrombus formation, which indicates that CNP gene transfer at the time of vein grafting prevents the sequence of vein graft failure, for example, to reduce early thrombosis, reduce intima formation, and prevent atherosclerosis.

In this study, we observed early reendothelialization in Ad.CNP-infected grafts. Furthermore, in the in vitro wound assay, both CNP and ANP significantly promoted the wound-repair process to a similar extent. In contrast, C-ANF (4–23), which does not stimulate cGMP production, did not exert a significant effect. These findings of ours, together with the previous report on NO, indicate that elevation of cGMP production in endothelial cells promotes their migration and/or proliferation. This is the first demonstration that the gene transfer of CNP, the vasodilator and the stimulator of cGMP genesis, can accelerate reendothelialization.

This study also revealed that the vein graft infected with Ad.CNP was less susceptible to thrombus formation. The mechanism of suppression of thrombus formation by CNP overexpression is not clear at present. Early reendothelialization observed in this study can be one of the mechanisms. Recently, it has been demonstrated that natriuretic peptides and NO can suppress the expression of plasminogen activator inhibitor-1 or tissue factor. Overexpression of CNP can also directly modulate the expression of molecules regulating blood coagulation and fibrinolysis.

This study demonstrated that gene transfer of the soluble factor CNP into the graft to prevent both early and late occlusion, with endothelial recovery, is a promising strategy to circumvent graft failure, without obvious systemic complications such as decrease of blood pressure or bleeding tendency.

References

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