C-Type Natriuretic Peptide Induces Redifferentiation of Vascular Smooth Muscle Cells With Accelerated Reendothelialization

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Abstract—We recently reported that C-type natriuretic peptide (CNP) occurs in vascular endothelial cells and acts as a vascular-type natriuretic peptide. In the present study, we stimulated the cGMP cascade in proliferating smooth muscle cells (SMCs), in which particulate guanylate cyclase-B, the specific receptor for CNP, is predominantly expressed, by use of an adenovirus encoding rat CNP cDNA (Ad.CNP). In the Ad.CNP-treated cultured SMCs, CNP caused the growth inhibition of SMCs at G1 phase with an early increase of p21 CIP1/WAF1 expression and subsequent upregulation of p16 INK4a. The expression of smooth muscle myosin heavy chain-2, which is the molecular marker of highly differentiated SMCs, was reinduced in the Ad.CNP-treated SMCs. The Ad.CNP-treated SMCs also reexpressed particulate guanylate cyclase-A, which shows high affinity to atrial and brain natriuretic peptide and is exclusively expressed in well-differentiated SMCs. CNP, which was overexpressed in rabbit femoral arteries in vivo at the time of balloon injury, significantly suppressed neointimal formation. Furthermore, an enhancement of the expression of smooth muscle myosin heavy chain-2 occurred in the residual neointima. In addition, early regeneration of endothelial cells was observed in the Ad.CNP-infected group. Thus, stimulation of cGMP cascade in proliferating dedifferentiated SMCs can induce growth inhibition and redifferentiation of SMCs with accelerated reendothelialization. (Arterioscler Thromb Vasc Biol. 2001;21:930-936.)

Key Words: adenovirus ■ C-type natriuretic peptide ■ gene therapy ■ vascular smooth muscle cells ■ endothelial cells

Recent evidence has revealed that vasoactive substances, especially those present in the blood vessels, not only regulate vascular tone but also modulate vascular growth. Vasoconstricting peptides (eg, angiotensin II [Ang II] and endothelin) promote vascular growth; conversely, vasodilating substances (such as natriuretic peptides and NO) inhibit vascular growth. Elucidation of the roles of these vasoactive molecules in vascular remodeling can provide opportunities to use them as a target of therapeutic interventions for vascular diseases.

The natriuretic peptide family consists of atrial, brain, and C-type natriuretic peptides (ANP, BNP, and CNP, respectively), which act as vasodilators and growth inhibitors of vascular smooth muscle cells (SMCs).1,2 These peptides elicit their biological effects via the elevation of intracellular cGMP by activating 2 biologically active natriuretic peptide receptors, namely, membrane-bound guanylate cyclase-A (GC-A) and guanylate cyclase-B (GC-B). We and others have demonstrated that ANP and BNP show high affinity to GC-A, whereas CNP selectively binds to GC-B.3,4 Although ANP and BNP are cardiac hormones secreted mainly from the atrium and the ventricle of the heart, respectively, we have demonstrated that CNP is produced in and secreted from vascular endothelial cells (ECs)5 to act as a local regulator of vascular tone and growth.6,7 We have also revealed that the endothelial secretion of CNP is stimulated by various cytokines and growth factors that are produced and activated in proliferative vascular lesions, especially transforming growth factor-β and tumor necrosis factor-α.5,8

In the pathogenesis of proliferative vascular lesions, alternation of differentiation of SMCs (from the “contractile” to “synthetic” phenotype) is considered to be important.9 We

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have found that the expression of biologically active natriuretic peptide receptors in SMCs is altered in accordance with the phenotypic change of SMCs. In SMCs of intact rat aortic media, which possess the contractile phenotype, GC-A was expressed more abundantly than was GC-B. In contrast, cultured rat aortic SMCs with the synthetic phenotype almost solely expressed GC-B. The gene expression of GC-B increased in balloon-injured rat carotid arteries in vivo. This evidence suggests that CNP can inhibit the growth of SMCs with the synthetic phenotype. Actually, we observed that CNP expression was augmented in human coronary atherosclerotic lesions.

So far, there is little information on the molecular mechanism of the phenotypic modulation of SMCs. Recently, however, it has been demonstrated that activation of the cGMP cascade by overexpression of the constitutively active form of cGMP-dependent protein kinase induces a change in the morphology of cultured SMCs with the synthetic phenotype to that observed in differentiated SMCs with the contractile phenotype.

In this context, we hypothesized that when the CNP gene is locally overexpressed in proliferating SMCs to augment cGMP production within SMCs, it may elicit an antiproliferative effect more preferentially on SMCs with the synthetic phenotype, thus suppressing the proliferation of SMCs effectively and modulating the phenotypic state of the SMCs. To examine this hypothesis, in the present study, we overexpressed the CNP gene by adenovirus-mediated gene transfer and investigated the effects on the growth characteristics and differentiation state of proliferating SMCs as well as endothelial regeneration in vitro and in vivo.

**Methods**

**Construction and Purification of Recombinant Adenoviruses**

We constructed an adenoviral shuttle vector overexpressing the CNP gene, pAC.CMV/CNP, by inserting the 384-bp SalI/XbaI fragment of the rat CNP cDNA, which we cloned into pAC-CMVpLPα, containing a constitutive cytomegalovirus (CMV) gene promoter and enhancer, pUC polylinker, and a fragment of the simian virus 40 genome that includes the small T-antigen intron and the polyadenylation signal. A recombinant replication-defective adenoviral vector, Ad.CNP, was generated by cotransfection of pJM17 and pAC.CMV/CNP into 293 cells. We also constructed an adenovirus vector, Ad.LacZ, overexpressing the *Escherichia coli* LacZ gene by the same method.

**Cell Culture and In Vitro Overexpression of CNP**

Cultured rat aortic SMCs were obtained and cultured as previously described. Quiescent SMCs were plated in 6-well plates at 2.0 × 10^5 cells per well. After overnight incubation, SMCs were infected with Ad.CNP or Ad.LacZ at a multiplicity of infection (MOI) of 20. The infected cells were cultured in the presence of 5% FCS for 6 days thereafter. In parallel, daily repetitive administrations of CNP (10^-8 M) were performed for comparison. CNP-like immunoreactivity (LI) in cultured medium was measured by our specific radioimmunoassay. The amount of cGMP was measured as previously described. Viable cell counts were determined by hemocytometer measurement after staining by trypan blue. On the sixth day of culture, relative rates of DNA synthesis were assessed by [3H]thymidine incorporation, as previously reported. Bovine arterial ECs (BAECs) were obtained and cultured as previously reported. Quiescent BAECs were plated in 6-well plates at 2.0 × 10^5 cells per well. BAECs were incubated with CNP for 2 days, and [3H]thymidine incorporation was determined. Cell numbers at the same time points were evaluated by modified MTT assay with use of the cell viability assay reagent SF (Nakalai Tesque). For the cells used, the real counted cell number was linearly proportional to a 450-nm optical density (OD_{450}) value ranging between 0 and 2.6. The amino acid sequence of CNP is identical in humans, pigs, and rats, and we confirmed no significant species difference of the CNP effect on the growth of SMCs.

**Cell Cycle Analysis**

For cell cycle analysis, SMCs were infected with Ad.CNP or Ad.LacZ at an MOI of 20 for 48 hours in the presence of 10% FCS. Then, SMCs were arrested at the G0/G1 phase of the cell cycle by incubating them in defined serum-free (DSF) medium supplemented with 5 mg/mL insulin, 5 mg/mL transferrin, and 5 ng/mL sodium selenite for 48 hours. After that, the cells were stimulated for proliferation with 5% FCS. After 24 hours of growth induction, the cells were stained with propidium iodide (125 mg/mL) and were analyzed with a Becton Dickinson FACScan and CellFit software.

**RNA Isolation, Reverse Transcription–PCR, and Northern Blot Analyses**

Gene expressions of smooth muscle myosin heavy chain (SM)-1, SM-2, GC-A, and GC-B in SMCs were analyzed by reverse transcription and polymerase chain reaction (PCR), and gene expressions of p21(CIP/WAF1) and p16(INK4a) in SMCs were analyzed by Northern blot analysis. For RNA extraction, SMCs were arrested at the G0/G1 phase by incubating them in DSF medium for 48 hours. Then, the cells were infected with Ad.CNP or Ad.LacZ at a MOI of 20 and stimulated for proliferation with 5% FCS. RNA extraction was performed at 0, 2, 4, and 6 days after the infection, with the medium changed on alternate days. cDNA was synthesized from 2-μg RNA templates with oligo(dT) priming by SuperScript II reverse transcriptase (Life Technologies, Inc). PCRs were initiated by adding 50 pmol each of a gene-specific primer set to 1 μL cDNA in 50 μL PCR buffer and 2.5 U Taq DNA polymerase (Takara). Each cycle consisted of optimized conditions (30 seconds at 94°C, 30 seconds at 60°C, and 2 minutes at 72°C) by use of a thermal cycler (ASTEC). After 40 cycles of PCR with the use of Taq DNA polymerase, aliquots of each PCR reaction mixture were size-fractionated by agarose gel electrophoresis. The sequence of each primer set was as follows: for SM-1, sense 5'-AAAGTTCGCGATGTG-3' and antisense 5'-CAGGCCAAAGTCGATGAAGT-3'; for SM-2, sense 5'-CTGCAGAGCATATTGATGCGAAGAAA-3' and antisense 5'-GCTCACTGAGAATGTCCTTG-3'; for GC-A, sense 5'-GAGAAACAGGCACAA-3' and antisense 5'-TATCCCTGAAGTGCTCATT-3'; and for GC-B, sense 5'-AAGCTTACTGGAAGAGGAGA-3' and antisense 5'-TATCCACCAAGGTTAGAG-3'. The p21(CIP/WAF1) cDNA probe was obtained by cDNA synthesis and the PCR method by using total RNA of SMCs with the sense (5'-GAGCCACAGGCCACCATTGTC-3') and antisense (5'-CTCCCGTGGGCACTCAGG-3') primers corresponding to nucleotides 84 to 103 and 587 to 607, respectively, of rat p21(CIP/WAF1) cDNA. The p16(INK4a) probe was a gift provided by Dr Devid Beach (Howard Hughes Medical Institute, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The GAPDH probe was purchased from Clontech Laboratories, Inc.

**Protein Extraction and Western Blot Analysis**

Protein levels of p16(INK4a) were evaluated by Western blot analysis. SMCs were made quiescent by incubating them in DSF medium for 48 hours. Then, the cells were infected with Ad.CNP or Ad.LacZ at an MOI of 20 and were stimulated for proliferation with 5% FCS. Protein extraction was performed 0, 4, and 6 days after the infection by using a Laemmli buffer (62.5 mmol/L Tris, pH 6.8, 2% SDS, and 10% glycerol). The protein concentration was measured by using BCA protein assay reagent (Pierce). After it was boiled, 50 μL protein extract was subjected to SDS-PAGE on 12.5% gel. The blots were then transferred to nitrocellulose membranes (Amersham Pharmacia Biotechnology).
Balloon Angioplasty and Adenovirus Infection of Rabbit Femoral Arteries

Fifteen male Japanese White rabbits weighing 2.5 to 3.0 kg were used. All animals used in the present study received humane care in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication No. 85-23, revised 1985). Bilateral femoral arteries were exposed through oblique incisions in the groin. After a 2-cm segment of the femoral artery was isolated with 2 vascular clamps, a 2F Fogarty balloon catheter was inserted from the deep femoral artery, and after the balloon was inflated with 0.2 mL of air, the intima of the femoral artery was denuded by 3 passages of the catheter. After the catheter was removed, the lumen was washed with 5% Evans blue dye in PBS, and 200 μL serum-free medium containing 5 x 10⁵ plaque-forming units of Ad.CNP or Ad.LacZ was instilled into the vascular lumen from the deep femoral artery with a 1-mm metal-tipped arterial cannula, which was ligated in place. The solution was instilled for 30 minutes at a pressure of 100 mm Hg. Two weeks after the procedure, the animals were killed by an overdose of pentobarbital sodium. To evaluate intimal and medial areas of the arteries, femoral arteries were harvested after fixation by perfusion of 10% phosphate-buffered formalin into the abdominal aorta. The arteries were subjected to the usual histological examination, and intimal and medial areas were measured by digital planimetry in at least 5 histological sections from each artery. For immunohistochemical analysis, femoral arteries were harvested without fixation and were frozen immediately on dry ice after being embedded in OCT compound (Miles Laboratories). Immunostainings for CNP, SM-1, and SM-2 were performed with mouse-specific monoclonal antibodies for each substance, as previously reported.

In Vivo Vasomotor Reactivity

Vasomotor reactivity of the arterial segment subjected to balloon injury and gene transfer was evaluated 2 weeks after the injury. A 3F end-hole infusion catheter (Target Therapeutics) was inserted into the left femoral artery and advanced to the origin of the infected right iliac artery under fluoroscopic guidance. This catheter was used for the infusion of vasoactive drugs and selective angiography of the femoral artery. Angiography was performed before and immediately after the drug administration with 1 mL of nonionic contrast media. To assess vasomotor reactivity, Ang II or acetylcholine was delivered via the 3F catheter at a dosage of 0.015 or 0.15 pmol/kg, respectively, for 2 minutes.

Evaluation of Reendothelialization

Thirty minutes before the animals were killed, they received an intravenous injection of 6 mL of 0.5% Evans blue dye delivered PBS, and the ear vein to identify the remaining nonendothelialized area. The area of intimal surface that was stained blue after the application of Evans blue dye was interpreted as identifying the portion of the arterial segment that remained endothelium deficient.

Drugs

CNP (CNP-22) and Ang II were obtained from Peptide Institute Inc and Sigma Chemical Co, respectively.

Statistical Analysis

All values are expressed as mean±SEM. Factorial ANOVA followed by the Fisher protected least significant difference test was used to determine significant differences in multiple comparisons.

Results

We infected cultured rat aortic SMCs with Ad.CNP at an MOI of 20. CNP-LI was detected in the conditioned media of SMCs treated with Ad.CNP on the second day after the treatment and increased thereafter. The level of CNP-LI in the medium was 162±55 fmol/mL (n=6) on the fourth day and 62±21 fmol/mL (n=6) on the sixth day after the infection, which was 1500 to 4000 times higher than it was in cultured BAECs, as we previously reported. In consequence of the marked increase of CNP production, cGMP production was augmented in the Ad.CNP-infected SMCs. CNP concentration in the conditioned media of the Ad.CNP-infected SMCs was 3.1±0.4 pmol/10⁶ cells on the fourth day, when CNP production was maximal, and 2.2±0.3 pmol/10⁶ cells on the sixth day, whereas it could not be detected in untreated or Ad.LacZ-infected SMCs (<0.03 pmol/10⁶ cells). After 6 days of incubation in the presence of 5% FCS, remarkable growth suppression was observed in the Ad.CNP-infected SMCs (2.8±0.6 x 10³ cells per well) compared with untreated SMCs (8.4±0.5 x 10³ cells per well) and the Ad.LacZ-infected SMCs (7.8±0.5 x 10³ cells per well). In the Ad.CNP-infected SMCs, [¹H]thymidine incorporation on the sixth day of incubation showed 34% reduction compared with that in the Ad.LacZ-infected SMCs (2100±70 versus 3190±160 cpm per well, respectively; P<0.05).

In contrast, the daily repetitive administration of CNP (10⁻⁸ mol/L) caused significant but less production of cGMP (0.15±0.04 pmol/10⁶ cells on the fourth day and 0.46±0.02 pmol/10⁶ cells on the sixth day). Accordingly, after 6 days of incubation, the extent of growth suppression by exogenous CNP was also less pronounced (cell numbers were 5.9±0.9 x 10³ cells per well for CNP-treatment versus 4.8±0.5 x 10³ cells per well for control [P<0.05]; [¹H]thymidine incorporation was 3370±133 cpm per well for CNP treatment versus 4000±86 cpm well for control [P<0.05]).

In the cell cycle analysis by flow cytometry, in SMCs stimulated with 10% FCS, 66% of the cells were in the G1 phase. In SMCs incubated for 48 hours under the serum deprivation, 95% of the cells were arrested in the G1/G0 phase. By stimulating the cells for 24 hours in the presence of 5% FCS, 76% of the Ad.LacZ-infected SMCs were in the G1 phase. In contrast, 86% of the Ad.CNP-infected SMCs were still in the G1 phase. Then, entry into the S phase was inhibited in 10% of the Ad.CNP-infected SMCs.

We next examined the expression of 2 cyclin-dependent kinase (CDK) inhibitors, p21CIP1/WAF1 and p16INK4a, during the 6-day observation period of growth inhibition in the Ad.CNP-infected SMCs. p21CIP1/WAF1 mRNA expression was augmented by 3-fold in the Ad.CNP-infected SMCs compared with the Ad.LacZ-infected SMCs on the second day after the infection. The augmented level of p21CIP1/WAF1 expression remained during the observation period. In contrast, at 0, 2, or 4 days after infection, p16INK4a mRNA was not detected in SMCs infected with Ad.CNP or Ad.LacZ, but it was detected in the Ad.CNP-infected SMCs on the sixth day after infection (Figure 1A). Late induction of p16INK4a expression was also confirmed at the protein level (Figure 1B).

To evaluate the effect of stimulation of the cGMP cascade on SMC phenotypic modulation, we further analyzed the expression of SM-2, which is demonstrated to be preferentially expressed in well-differentiated SMCs. SM-2 mRNA expression was not detected in the control Ad.LacZ-infected SMCs throughout the 6-day observation. In contrast, in the Ad.CNP-infected SMCs, SM-2 mRNA was detected on the sixth day after infection. Furthermore, in the Ad.LacZ-infected SMCs, GC-A mRNA was not detected, but GC-B mRNA was highly expressed. In the Ad.CNP-infected SMCs, GC-B expression was attenuated. Conversely, the gene ex-
pression of GC-A became detectable (online Figure I; please see http://atvb.ahajournals.org).

Figure 2 illustrates CNP expression in rabbit femoral arteries 2 weeks after balloon injury by immunostaining with the anti-CNP monoclonal antibody that we originally developed.16 Although our antibody recognizes endogenous rabbit CNP and exogenously transfected rat CNP, more intense CNP immunostaining was observed throughout the injured vessel walls in Ad.CNP-infected vessels compared with Ad.LacZ-infected vessels, which indicates the significant expression of CNP by Ad.CNP infection. As shown in online Figure II (please see http://atvb.ahajournals.org) and the Table, after 2 weeks of observation, femoral arteries that were subjected to balloon injury showed apparent neointimal thickening. In the arteries infected with Ad.LacZ after balloon injury, similar neointimal thickening was shown. In contrast, neointimal thickening was markedly suppressed, and the ratio of intimal area to medial area showed 53% reduction in the Ad.CNP-infected arteries compared with the Ad.LacZ-infected arteries after balloon injury.

SM-1 immunostaining was detected in the Ad.LacZ- and Ad.CNP-infected blood vessels. However, in Ad.LacZ-infected arteries, SM-2 staining was barely detected in the neointima. In contrast, in Ad.CNP-infected vessels, SM-2 staining was positive in reduced neointimal lesions, especially in the outer half of the neointima and the whole media (Figure 3). Reflecting the reinduction of the expression of SM-2, vascular reactivity to the vasoconstrictor (Ang II) was partially restored in the Ad.CNP-infected injured artery compared with the Ad.LacZ-infected blood vessel. As shown in Figure 4, in the injured portion of the femoral artery, Ang II produced more pronounced vasoconstriction in the Ad.CNP-treated group (percentage of luminal narrowing 44%) than in the Ad.LacZ-treated group (percentage of luminal narrowing 22%). Furthermore, although ECs were not detected in Ad.LacZ-infected vessels, regenerated ECs covered the lesion in Ad.CNP-infected vessels (Figure 2, inset). By 14 days, Evans blue dye staining disclosed accelerated reendothelialization of the Ad.CNP-treated balloon-injured femoral artery compared with the Ad.LacZ-treated injured vessel (Figure 5). Being compatible with accelerated reendothelialization in the Ad.CNP-treated group, endothelium-dependent vasorelaxation by acetylcholine infusion was more pronounced in the Ad.CNP-infected femoral arteries compared with the Ad.LacZ-infected femoral arteries (Figure 5).

**Table**

<table>
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<th>Media, mm²</th>
<th>Neointima, mm²</th>
<th>I/M Ratio</th>
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<tr>
<td>Untreated</td>
<td>0.40±0.03</td>
<td>0.35±0.06</td>
<td>0.88±0.19</td>
</tr>
<tr>
<td>Ad.LacZ</td>
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<td>0.26±0.03</td>
<td>0.81±0.07</td>
</tr>
<tr>
<td>Ad.CNP</td>
<td>0.27±0.02*</td>
<td>0.10±0.01†</td>
<td>0.38±0.02†</td>
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Values are mean±SEM (n=5). I/M indicates ratio of intima to media. *P<0.05 vs untreated; †P<0.01 vs untreated and P<0.05 vs Ad.LacZ.

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Figure 1. A, Gene expression of p21\(^{CIP1/WAF1}\) and p16\(^{INK4a}\) in cultured SMCs infected with Ad.LacZ or Ad.CNP. After SMCs were made quiescent, the cells were infected with Ad.CNP or Ad.LacZ and cultured in the medium containing 5% FCS. At 0, 2, 4, and 6 days after the treatment, cells were harvested, and Northern blot analysis was performed. Total RNA (20 μg) was used for analysis. B, Protein level of p16\(^{INK4a}\) in cultured SMCs infected with Ad.LacZ or Ad.CNP. The experimental conditions were the same as described above. Western blot analysis was performed to detect p16\(^{INK4a}\) expression by using 50 μg of whole-cell lysates.

Figure 2. Immunohistochemical analysis of CNP expression in balloon-injured rabbit femoral arterial walls infected with Ad.LacZ (left) and Ad.CNP (right). The infected blood vessels harvested 2 weeks after the balloon injury were stained with a monoclonal antibody specific to CNP. M indicates media; *, neointima. Inset shows regeneration of ECs over the neointima in the Ad.CNP-infected group. Original magnification ×360 and ×810 (inset).
Using cultured BAECs, we also confirmed in vitro that CNP at a dose of $10^{-11}$ to $10^{-10}$ mol/L significantly enhanced the proliferation of BAECs treated with 5% FCS. $[^{3}H]$Thymidine incorporation was as follows: $8.01 \pm 0.13 \times 10^4$ cpm per $2 \times 10^4$ cells in the vehicle-treated group, $8.81 \pm 0.15 \times 10^4$ cpm per $2 \times 10^4$ cells in the CNP $(10^{-11}$ mol/L)–treated group ($P<0.05$), and $9.06 \pm 0.09 \times 10^4$ cpm per $2 \times 10^4$ cells in the CNP $(10^{-10}$ mol/L)–treated group ($P<0.05$ versus vehicle, $n=8$). Cell numbers (OD$_{450}$ values) were $1.73 \pm 0.03$ in the vehicle group, $1.92 \pm 0.07$ in the CNP $(10^{-11}$ mol/L)–treated group ($P<0.05$), and $1.92 \pm 0.03$ in the CNP $(10^{-10}$ mol/L)–treated group ($P<0.05$ versus vehicle, $n=6$). CNP potentiated the proliferation of BAECs in a time-dependent manner. CNP $(10^{-10}$ mol/L) treatment caused $119.0 \pm 9.1\%$ increase over the control value 1 day after the treatment and $133.3 \pm 5.8\%$ increase over the control value 2 days after the treatment ($P<0.05$, $n=4$). In contrast, the same treatment of CNP $(10^{-11}$ to $10^{-10}$ mol/L) administered to SMCs for 2 days under 5% FCS culture had no significant effect on the cell number increase of SMCs (OD$_{450}$ values were $1.61 \pm 0.07$ in vehicle, $1.59 \pm 0.05$ in CNP $(10^{-11}$ mol/L), and $1.51 \pm 0.05$ in CNP $(10^{-10}$ mol/L)).

**Discussion**

In the present study, we have demonstrated that overexpression of CNP inhibits the growth of cultured SMCs with the synthetic phenotype through the elevation of cGMP. Repetitive administration of synthetic CNP causes less profound growth inhibition. Therefore, continuously secreted CNP from the Ad.CNP-infected SMCs exerts significant growth-inhibitory action on SMCs by the efficient activation of cGMP production in an autocrine/paracrine fashion.

Overexpression of CNP has been shown to cause growth inhibition of SMCs at the $G_1$ phase. Recently, it has been demonstrated that p21$^{CIP1/WAF1}$ binds to the CDK2-cyclin E or cyclin A complex and inhibits the progression of the cell cycle.
In the present study, p21 CIP1/WAF1 expression was augmented in the Ad.CNP-infected SMCs 2 days after the infection by Ad.CNP. Thus, it is suggested that the augmentation of gene expression of p21 CIP1/WAF1 is associated with the significant growth inhibition of SMCs infected with Ad.CNP. Recently, we reported that CNP and 8-bromo cGMP augments the gene expression of a novel homeobox gene, Gax (growth arrest–specific homeobox), which is demonstrated to activate p21 CIP1/WAF1 expression and decrease CDK2 activity. 18

In the present study, we also investigated the influence of stimulation of cGMP cascade on the state of differentiation of SMCs. We examined the expression of myosin heavy chain isoforms, which were proposed to be important molecular markers for SMC differentiation. 15 It is reported that SM-1 is constitutively expressed at all stages of SMCs and that SM-2 is expressed only in differentiated SMCs. 15 We observed that the gene expression of SM-2 was reexpressed in cultured SMCs and also in SMCs in the neointima infected with Ad.CNP. Redifferentiation occurred in the medial SMCs and in SMCs of the neointima in the proximity of the media, which were supposed to cease migration and proliferation at the earliest time among the neointimal SMCs. In addition, we also demonstrated that the expression of GC-A was reinduced in cultured SMCs infected with Ad.CNP. GC-A is almost completely downregulated during the phenotypic change of SMCs from contractile to synthetic. 10 We further observed restoration of vascular reactivity accompanied with the suppression of luminal narrowing. Therefore, from our in vitro and in vitro results, it can be speculated that activation of the cGMP cascade by overexpression of CNP in SMCs might induce redifferentiation of the cells and alter the phenotype of the SMCs from synthetic to contractile.

It is reported that the p16 INK4a protein inhibits binding of CDK4 and CDK6 to cyclin D and induces dephosphorylation of retinoblastoma protein, resulting in G1 growth arrest. 19 Urashima et al 20 have reported that p16 INK4a not only suppresses proliferation but also induces differentiation in transformed cells from acute lymphoblastic leukemia. In the present study, the significant growth suppression occurred at least 2 days after the infection, which was parallel to the augmented expression of p21 CIP1/WAF1. However, reinduction of the expression of SM-2 and GC-A was detected only 6 days after the infection, with the associated induction of p16 INK4a expression. Therefore, although Yang et al 21 reported that G0/G1 arrest and also cell differentiation were induced in malignant cells by the overexpression of the p21 CIP1/WAF1 gene, our results suggest that the expression of p21 CIP1/WAF1 is not enough for the induction of differentiation of SMCs and that additional expressions of CDK inhibitors may be required for the redifferentiation of SMCs.

There have been few reports on therapeutic transfer of a functional gene whose product can specifically inhibit proliferating SMCs. Because CNP preferentially affected SMCs with the synthetic phenotype that express GC-B abundantly, CNP gene transfer proposed in the present study may give a new aspect to the strategy of gene therapy targeted to proliferating SMCs to induce redifferentiation.

Interestingly, in the present study, early regeneration of ECs was observed in Ad.CNP-infected injured blood vessels. The mechanism(s) by which accelerated endothelialization was achieved is not clearly understood at present. Because the SMCs in the reduced neointima seemed to recapture the differentiated phenotype in the present study, the production of postulated inhibitors of EC migration, eg, transforming growth factor-β or fibronectin, 22 could be reduced. The direct action of CNP secreted from SMCs for the modulation of endothelial repair may also be possible, inasmuch as we have
also demonstrated the potentiating action of CNP on endothelial proliferation in vitro. Indeed, it has also been reported that NO can induce angiogenesis in vivo.\textsuperscript{23} The difference of CNP action on the proliferation of SMCs and ECs has been investigated further. Because the regeneration of ECs is known to reduce or prevent intimal thickening,\textsuperscript{24} the accelerated reendothelialization can further contribute to the reduction of the neointimal formation in the Ad.CNP-infected group.

Ueno et al\textsuperscript{25} have reported that when the adenovirus vector expressing CNP was applied to balloon-injured rat carotid arteries in vivo, neointimal formation was markedly reduced in an infection site–specific manner without an increase in plasma CNP level. The results of the present study, which shows suppression of neointimal formation in balloon-injured rabbit femoral arteries infected with Ad.CNP, are compatible with their report. In the present study, we further demonstrated that overexpression of CNP not only can suppress the proliferation of SMCs but also can induce the redifferentiation of SMCs and the acceleration of endothelial regeneration in vitro and in vivo. We must admit that because we used cultured rat SMCs and bovine ECs in experiments in vitro and because we examined the growth of rabbit SMCs and ECs in experiments in vivo, there might be some difference in the effect of CNP on the growth of SMCs and ECs from different species.

In conclusion, the overexpression of CNP caused activation of the cGMP cascade, which regulated the expression of cell cycle–regulating genes, including p21CIP1/WAF1 and p16 INK4a , and induced selective G\textsubscript{1} growth inhibition of proliferating SMCs with alteration of the phenotype from synthetic to contractile. In addition, growth inhibition and redifferentiation of SMCs were accompanied by the acceleration of reendothelialization in vivo. Our results suggest that CNP can be useful as an autocrine/paracrine growth modulator for gene therapy to recapture vascular integrity in proliferative vascular lesions.

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References


C-Type Natriuretic Peptide Induces Redifferentiation of Vascular Smooth Muscle Cells With Accelerated Reendothelialization


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