Vascular smooth muscle cells (VSMCs) are the main peripheral target for hormone angiotensin II (Ang II) acting through angiotensin II type 1 receptors (AT₁-R). In many models of hypertension the renin-angiotensin system (RAS) is overactive and contributes to the hypertension. Inhibiting the RAS by reducing Ang II production with angiotensin-converting enzyme (ACE) inhibitors or antagonizing AT₁-R are current therapeutic approaches for controlling hypertension. To develop more specific, longer-lasting approaches to inhibiting RAS, we have previously designed antisense (AS) oligonucleotides to AT₁-R and angiotensinogen mRNA. These oligonucleotides can be delivered in vivo and produce significant decreases in blood pressure for 3 to 7 days with a single injection. To produce longer-lasting antisense inhibition to reverse or ameliorate high blood pressure, we are delivering AAV-AT₁-R-AS in viral vectors. Iyer et al showed that a single injection of retrovirus AT₁-R-AS vector in neonate spontaneously hypertensive rats (SHR) prevented hypertension from developing for 90 days. Furthermore, Martens et al demonstrated that treatment with this recombinant retrovirus not only attenuates the development of hypertension for up to 120 days, it also prevents renovascular and cardiac pathophysiological changes in the treated SHR.

For human therapy, the challenge is to produce viral vector delivery of AT₁-AS to reverse hypertension. There are several viral vectors to choose from, including retroviruses, adenovirus, herpes simplex virus, lentivirus, and adeno-associated virus (AAV). All have certain advantages and disadvantages, but the AAV offers several attractive advantages and few disadvantages. AAV is safe to use and does not induce any pathogenic or inflammatory response. The AAV is a defective parvovirus that cannot replicate in cells without the presence of a helper wild-type adenovirus or herpes virus. Recombinant AAV (rAAV) has the capability of gene transfer with stable long-term expression. In this study we report the results of using AAV vector that we constructed to deliver AT₁-R mRNA antisense with a cytomegalovirus (CMV) promoter. The objectives of the present study were to (1) demonstrate in vitro the effectiveness of the plasmid AAV (pAAV) as a vector for transgenic expression in VSMCs; (2) study the biological effect of...
between passages 12 and 15 for transfection or transduction; they were then kept for 48 hours in case of transfection or split every week for 8 weeks for transduction experiments.

Transfection of VSMCs With the Plasmid pAAV
VSMCs were plated (day 1) in 6-well plates and transfected the following day (day 2) at a confluence of ~60% to 70% using lipofectamine reagent (Gibco BRL) according to manufacturer protocol; 2 μg of the plasmid (pAAV-AS) and 10 μL lipofectamine (2 mg/mL) were used per well. Cells were used 48 hours after transfection to measure AT1-R by a ligand-binding method.

Green Fluorescent Protein Detection
VSMCs were grown and transfected using lipofectamine reagent (Gibco BRL) on a cover slide and embedded in a 35-mm tissue culture dish. The same field of cells was observed under visible light and phase-contrast ring of Axiovert 135 (Zeiss, Germany) and then under UV light with a green fluorescent protein (GFP)-specific filter. The amount of the green epifluorescent area (UV light and GFP-specific filter) as a percentage of total area (visual light, phase-contrast ring) was estimated by 2 independent investigators. Photographs were taken 48 hours after transfection using a Nikon N6006 camera attached to Axiovert 135.

Transduction of VSMCs With rAAV
VSMCs were plated in 10-cm plates to be ~60% confluent on the next day. On the day of the experiment, cell medium was removed, each plate was washed with 5 mL of Opti-MEM, cells were covered with 5 mL Opti-MEM, and recombinant AAV was added at MOI of 5. After 3 days the medium was changed to DMEM with 10% FBS and antibiotics. Control cells were treated in the same manner, except that no virus was added. Cells were passaged once a week. Transgene expression was studied from the third day, and AT1-R binding was done up to 8 weeks.

Transgene and AT1-R Gene Expression by RT-PCR
To check the time course of the neo gene expression in the cells and the effect on AT1-R gene expression, RNA from control VSMCs and cells transfected with plasmid pAAV-AS was isolated from 2 to 48 hours using PureScript reagent (Gentra). Control consisted of cells treated with lipofectamine only during transfection. RNA from VSMCs treated with virus rAAV-AS was prepared every week after transduction for 8 consecutive weeks. RNA (1 to 3 μg) was digested with DNase I (RNase free) for 10 minutes at 37°C in the presence of 5 U of RNase inhibitor. After heat inactivation of DNase, RNA was reversed transcribed for 50 minutes at 42°C using SuperScript II Reverse Transcriptase and oligo(dT) as a primer (Gibco BRL). The reaction was stopped by heating samples for 15 minutes at 70°C. Next, 1 μL from the reverse transcription (RT) reaction was added to 49 μL final-volume PCR reaction, and amplification was performed using a set of neo primers yielding a 757-bp fragment. The 5′ primer was 5′-GGATTGCAGCGAGGTCTCTCCG-3′ and the 3′ primer was 5′-CGATAGAAAGCCAGTGCGTCG-3′. Amplification for transfected cells was performed for 40 cycles with annealing at 65°C. RNA from transduced cells after RT was subjected to 35 cycles of PCR with the first set of neomycin primers as above and then nested PCR with 5′-GCTATTCGCGCTATGACTG-3′ and 3′ primer of 5′-GCGATACCGTAAAGCACGA-3′, yielding a 688-bp product. In parallel, 1 μL from the RT reaction was added to 49 μL final-volume PCR reaction, and amplification was performed using a set of AT1-R primers yielding a 719-bp fragment. The 5′ primer was 5′-TGACACCACTGATCATGATG-3′ and the 3′ primer was 5′-GCGATACCGTAAAGCACGA-3′. Amplification for transfected cells was performed for 40 cycles with annealing at 65°C. For normalization, the sample RNA was also amplified using GAPDH primers.
The 5′ primer was 5′-CCCATCATACCATCTTCCAGGA-3′ based on exon 3 sequence, and the 3′ primer was 5′-CGCTGCTTCACCACCTTTCT-3′ based on exon 7 sequence. Amplification products were analyzed on 1% agarose gel stained with ethidium bromide.

The first experiment was to assess transfection efficiency of pAAV-gfp in VSMCs. Expression of GFP in the cells was observed 48 hours after transfection by comparing the area showing green fluorescence with a total area of cells. In pAAV-GFP control cells treated with lipofectamine, the field under UV light with a GFP-specific filter was black. In pAAV-gfp transfected cells, the amount of green epifluorescent area (UV light using GFP-specific filter) was determined with a Beckman γ-counter. Protein content was measured in cell lysates.

Expression of GFP in VSMCs

The 5′ primer was 5′-CCCATCATACCATCTTCCAGGA-3′ based on exon 3 sequence, and the 3′ primer was 5′-CGCTGCTTCACCACCTTTCT-3′ based on exon 7 sequence. Amplification products were analyzed on 1% agarose gel stained with ethidium bromide.

Intracellular Calcium Measurement

Intracellular calcium concentration ([Ca2+]i) in VSMCs was measured using imaging fluorescence microscopy. Briefly, the cells were loaded with fura 2 by incubation with 5 μmol/L membrane-permeable fura 2 acetoxymethylester (fura 2-AM dissolved in 1 mmol/L DMSO stock) in Tyrode’s solution for 30 to 60 minutes at 37°C. The culture dishes containing cells were then centered in the optical field of a ×40 oil immersion fluorescence objective of an inverted microscope (Nikon Diaphot).

Randomly selected cells were illuminated alternately with UV light of 340 and 380 nm wavelength using an IonOptix chopper-based, electronically controlled, dual-excitation imaging fluorescence system. Cell fluorescence (emitted light) was collected through a 510-nm barrier filter before acquisition by an ICCD camera (Phillips FTM800). Fluorescence signals were digitized on-line using an IBM-PC compatible computer and IonOptix fluorescence image acquisition and analysis software. The fluorescence signals, F340 and F380, were background subtracted during the experiment. The signals are reported here as changes in F340/F380. This gives a relative indication of [Ca2+]i.

Statistical Analysis

Data were analyzed with the use of standard statistical methods. ANOVA and Neuman-Keuls tests were applied. Group data are expressed as mean±SEM. Significance was set at the P<0.05 value.

Results

Expression of GFP in VSMCs

The first experiment was to assess transfection efficiency of pAAV-gfp in VSMCs. Expression of GFP in the cells was observed 48 hours after transfection by comparing the area showing green fluorescence with a total area of cells. In pAAV-GFP control cells treated with lipofectamine, the field under UV light with a GFP-specific filter was black. In pAAV-gfp transfected cells, the amount of green epifluorescent area (UV light and GFP-specific filter) as a percentage of total area (visual light, phase-contrast ring) was estimated by 2 independent investigators as being ≈80% (Figure 2).

Time Course of the Neomycin Resistance Gene Expression in Transfected VSMCs by RT-PCR

Time course of the neomycin resistance gene expression in host cells from 2 hours to 48 hours after the start of transfection was shown by RT-PCR of RNA extracted from VSMCs transfected with pAAV-AS (Figure 3, top). The transgene band of the expected size of 757 bp was weak but was visible starting at 2 hours of transfection and increased at later time points. No visible band was present in control cells treated with lipofectamine only.

Time Course of the AT1-R Gene Expression in Transfected VSMCs by RT-PCR

Time course of the AT1-R gene expression from 2 hours to 48 hours after the transfection was shown by RT-PCR of RNA extracted from VSMCs transfected with pAAV-AS (Figure 3, middle). Starting at 5 hours, AT1-R mRNA was decreased compared with control cells treated with lipofectamine only. After 48 hours AT1-R mRNA in transfected cells was 42±7% (n=3, normalized by GAPDH expression).

Effect of pAAV-AS Transfection on Ang II Receptor Binding in VSMCs

The pAAV-AS plasmid transfected VSMCs showed a 74% decrease in AT1-R number assayed at 0.2 nmol/L 125I-[Sar1,Ile8]Ang II compared with the nontransfected cells in Opti-MEM (14.9±4.8 versus 56.7±8.4 fmol/mg, n=3; P<0.005) or 63% reduction compared with the nontransfected cells treated with lipofectamine (14.9±4.8 versus 40.2±5.1 fmol/mg, n=4; P<0.005). Figure 4 shows the...
significant reduction in AT₁-R number determined 48 hours after transfection.

**Effect of pAAV and rAAV-AS on Ang II–Stimulated Increase in Intracellular Calcium in VSMCs**

The effects of AT₁-R-AS was tested on changes in [Ca²⁺]ᵢ as a measure of second-messenger activity in the Ang II signaling pathway. In both transfected by plasmid pAT₁-R-AS (Figure 5A) and transduced by virus rAAV-AS VSMCs (Figure 5B), there was a significant decrease in the Ang II–stimulated change in [Ca²⁺]ᵢ. In plasmid or virus-treated cells there was a significant attenuation of the Ang II response: Ang II only increased [Ca²⁺]ᵢ 72 ± 6 or 113 ± 22% versus 308 ± 15% or 278 ± 18% in the control, respectively (n = 157 cells for plasmid-treated cells, n = 202 cells for virus-transduced VSMCs; difference between control and treated group is statistically significant at P < 0.01).

At the same time, AT₁-R-AS as a plasmid or virus had no effect on depolarization-stimulated (80 mmol/L KCl) or α-adrenergic receptor–dependent increases (1 μmol/L phenylephrine) in [Ca²⁺]ᵢ. There was no difference between the R₉₀₀ and R₉₀₀₀ of control or treated cells.

**Time Course of the Neomycin Resistance Gene Expression in Virus-Transduced VSMCs by RT-PCR**

The time course of the neomycin resistance gene expression in host cells was shown by RT-PCR of RNA extracted from VSMCs transduced with virus rAAV-AS. Neomycin resistance gene was expressed from 1 week to at least 8 weeks after the transduction (Figure 6). No visible band was present in control cells. After that time, however, both control and transduced cells became difficult to passage and culture was discontinued.

**Effect of rAAV-AS on Ang II Receptor Binding in VSMCs**

Starting from the 2nd week after treatment, the rAAV-AS virus-transduced, nonselected VSMCs showed significant reduction in AT₁-R number assayed at 0.2 nmol/L ¹²⁵I-[Sar¹,Ile⁸]Ang II compared with the nontransduced cells, with a mean decrease of 21% (Table). This continued for 8 weeks.

**Discussion**

This study was carried out in 2 stages, first with AAV-based plasmid and second with recombinant AAV. The results show that AAV-based plasmid can efficiently transfect VSMCs. The reporter gene, green fluorescent protein, was present in approximately 80% of cells. In other cell lines, such as NG108-15, ATt-20, and L929 using the same plasmid and transfection method, we observed a similar high efficiency of transfection. AAV-based plasmid carrying AT₁-R antisense...
and neo' gene is expressed in these cells as early as 2 hours after the start of the transfection. The pAAV AT1-R-AS decreased AT1-R mRNA starting at 5 hours. AT1-R number determined by ligand binding 48 hours after transfection showed a statistically significant decrease in antisense-transfected cells compared with control cells. The decrease in receptor number correlates well with the transfection efficiency in these cells as shown by the expression of green fluorescent protein. As a test of the physiological effect of receptor number, a small amount, leading to a disproportionally larger physiological change. We hypothesize that pAAV-based AT1-R antisense is effective in reducing the activity of endogenous VSMC Ang II. Also, when rAAV-AT1-R-AS was studied, the AT1-R number and [Ca2+]i were reduced. We have noticed previously that antisense reduces receptor number by a small amount, leading to a disproportionally larger physiological change. We hypothesize that there is a threshold for effect or a lack of spare receptors. Without an irreversible blocker of AT1-R we have not been able to test this hypothesis. The expression of the transgene was prolonged and present in the cells for at least 8 weeks after a single exposure to rAAV.

The goal of this research is to establish new ways of reducing blood pressure by gene manipulation to achieve long-lasting effects without toxicity or side effects. Although current ACE inhibitors and angiotensin receptor blockers (ARBs) are very effective in antihypertension therapy, they are drugs foreign to the body. As such, they have side effects. Additionally, they must be taken daily, which increases noncompliance problems. The antisense approach is a biological one in which the DNA can be integrated into the genome and constantly produces antisense mRNA. Inhibiting angiotensin activity is only one approach to gene manipulation. Other methods such as increasing gene expression of vasodilator proteins such as bradykinin and atrial natriuretic peptide or increasing nitric oxide synthase are also being studied and proving effective in animal models.21,22

We have used AAV-AT1-R-AS to reduce blood pressure in SHR for several weeks by delivery to the brain.10,23 The mechanism of action, however, is quite different from peripheral application, where the most likely target site is blood vessels. Recently, Martens et al12 showed that intracardiac injection of retrovirus AT1-R-AS in neonate SHR prevented hypertension from developing and normalized blood vessel vascular reactivity and voltage-dependent K⁺ current density.

The properties of recombinant AAV vectors include safety because they are nonpathogenic and do not replicate. AAV can transduce nondividing cells, whereas retrovirus cannot. AAV vectors target homologous chromosomal genes at high frequencies and introduce particular modifications without additional mutations.24 Adenoviruses produce viral genes that stimulate inflammatory responses. For treatment of cardiovascular diseases such as hypertension, it is important to maintain transgene expression for clinically significant amounts of time. Therefore, AAV has positive potential for ultimate use in gene therapy.

We constructed an rAAV with antisense to AT1-R. The RT-PCR detection of neomycin resistance gene confirmed that the rAAV-AS was being expressed for several weeks in transduced VSMCs without selecting the cells. The results indicate efficient uptake, long-lasting expression, and effective inhibition of Ang II–induced Ca²⁺ response. The mechanism for AS-AT1-R appears to be through integration of rAAV into the genome. There is extensive evidence that this occurs in the wild-type AAV13 and recent evidence that rAAV is also integrated.25 The transgene expression would produce specific AS mRNA targeted to AT1-R mRNA and hybridize with endogenous AT1-R mRNA, reducing translation of the AT1-R protein. The reduction in receptor number reduces the response to Ang II as shown by the reduced [Ca2+]i. Although these experiments are in vitro, the results suggest that rAAV-AT1-R-AS will be effective in vivo, in VSMCs of blood vessels, for reducing the vasoconstrictive effects of Ang II. In those forms of hypertension, where there is either increased Ang II or increased receptor responsiveness or both, the results support that the rAAV AT1-R-AS will likely produce prolonged attenuation of Ang II–induced vasoconstriction.

### Number of AT1-R in VSMCs Transduced With rAAV-AS

<table>
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<tr>
<th>AT1-R Assayed on Each Consecutive Week After rAAV Treatment</th>
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References
Antisense Inhibition of AT$_1$ Receptor in Vascular Smooth Muscle Cells Using Adeno-Associated Virus-Based Vector
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