Prolonged Reduction of High Blood Pressure With an In Vivo, Nonpathogenic, Adeno-Associated Viral Vector Delivery of AT₁-R mRNA Antisense

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Abstract To produce a prolonged decrease in blood pressure, we have developed a non-pathogenic adeno-associated viral vector (AAV) with the antisense DNA for AT₁-R. AAV has many advantages over other viral vectors. AAV does not stimulate inflammation or immune reaction. AAV enters non-dividing cells and does not replicate. Therefore, it is an appropriate choice for gene therapy. Recombinant AAV was prepared with a cassette containing a cytomegalovirus promoter and the cDNA for the AT₁ receptor in the antisense direction. The cassette was packaged in the virus. Stable transfection of NG108-15 cells with the pAAV-AS (plasmid AAV) antisense to AT₁-R produced a significant reduction in AT₁ receptors. A single injection of rAAV-AS (viral vector) was made in adult spontaneously hypertensive rats, either directly in the hypothalamus (1 μL) or in the lateral ventricles (5 μL). The result shows that there is a significant decrease of blood pressure (±22±2 mm Hg) for up to 9 weeks after injection. Control injections of mock vector produced no change in blood pressure during the same time period in age-matched controls. In young spontaneously hypertensive rats (3 weeks), a single intracardiac injection of recombinant rAAV-AS reduced blood pressure and slowed the development of hypertension compared with controls (P<0.01). The results suggest that a prolonged reduction in high blood pressure can be achieved with AAV vectors delivering antisense to adult AT₁ receptors with a single administration. (Hypertension. 1997;29[part 2]:374-380.)

Key Words: recombinant adeno-associated virus (AAV) vector • AT₁ receptor • antisense • hypertension • NG108-15 cells • angiotensin
of a single injection of the rAAV-AS into the brain of adult SHR and into the heart of young SHR (3 weeks) and to compare its effect on the development of hypertension at adulthood and at early stage of development. The results are encouraging, showing significant long-term decreases in blood pressure in hypertensive rats.

Methods

Construction of Plasmid AAV

The AAV-derived vector can be adapted for several genes and promoters between the ITRs at each end (Fig 1). The AAV genome is removed, leaving only the ITRs. The vector has a 4.7-kb carrying capacity. Foreign genes, such as antisense DNA and marker genes, can be inserted together with foreign promoters. The plasmid AAV with the (gfp) gene was designed by Zoletukhin et al.\(^{10}\) (provided by N Muzyczka) and designated pAAV-gfp. The plasmid has a CMV promoter-driven gfp gene and also contains a neomycin (amgmoglycoside phosphotransferase) gene, neo\(^{d}\), driven by a thymidine kinase promoter, for selection by ganciclovir (G418) in transduced cells.

For the antisense vector, a 749-bp fragment (−183 to 566) of rat vascular AT\(_4\)R cDNA (provided by T J Murphy in the pKSCal8b plasmid)\(^{11}\) was amplified with PCR and ligated to pAAV vector in the antisense orientation in place of gfp and designated pAAV-AS Recombinant viruses (Fig 2) were produced using pAAV-AS and pAAV-gfp.

Method to Prepare Recombinant AAV

To prepare recombinant AAV, Human Embryonic Kidney (HEK293) cells were transfected with plasmid vector containing Ang receptor antisense and AAV terminal repeats (pAAV-AS), together with helper plasmid-delivering rep and cap genes (necessary for AAV replication) in trans using the calcium phosphate method. Eight hours after transfection, adenovirus was added at a multiplicity of infection of 5. When cells developed cytopathic effects (usually after 2 to 3 days), they were harvested in media and centrifuged at 900 g at 4°C for 15 minutes. Pellet was resuspended in 50 mmol/L Tris/Cl (pH 8.4) and 150 mmol/L NaCl and frozen-thawed three times. Cell debris was removed by centrifugation at 2000 g at 4°C for 10 minutes, and supernatant was subjected to heating at 56°C for 30 minutes to inactivate adenovirus. After cooling, precipitate was separated at 3700 g at 4°C for 10 minutes. CaCl\(_2\) (25 mmol/L) was added to supernatant and incubated at 4°C for 1 hour. After spinning at 13 300 g at 4°C for 20 minutes, the supernatant was removed and equal volume of ammonium sulfate (pH 7.0) saturated at 4°C, was added dropwise and incubated on ice for 30 minutes. The solution was centrifuged at 20 000 rpm in an SW28 rotor at 4°C for 30 minutes. The floating pellet was dissolved in HEPES-buffered saline and purified through 1.39 g/cm\(^3\) CsCl gradient with 1.5 g/cm\(^3\) CsCl cushion.

The gradient was fractionated, and aliquots were hybridized with \(^{32}\)PdCTP-labeled random primed AAV probe. Positive fractions were pooled and concentrated using Centricon-30.

Virus Titer Assay

Titer of the virus was assayed by dot-blot analysis. Two samples of 2 \(\mu\)L of virus each were diluted in 200 \(\mu\)L of DMEM. One sample was digested with 10 U of DNase I at 57°C for 30 minutes, the second sample served as a control. The above samples were diluted 1 1 with 20 mmol/L Tris/Cl (pH 8.0), 20 mmol/L EDTA (pH 8.0), 1% SDS, and digested with 200 \(\mu\)g of proteinase K at 37°C for 1 hour. Samples were phenol/chloroform extracted and ethanol-precipitated with addition of 40 \(\mu\)L glycerol as the carrier. Precipitates were dissolved in 0.4 mmol/L NaOH, 10 mmol/L EDTA (pH 8.0), and loaded on the Zeta-probe membrane together with AAV DNA standards. Membranes were hybridized in 7% SDS, 0.25 mmol/L sodium phosphate buffer (pH 7.1), 1 mmol/L EDTA (pH 8.0) at 65°C for 10 minutes and then hybridized with \(^{32}\)PdCTP-labeled random primed AAV probe at 65°C overnight. Membranes were washed in 1% SDS, 0.4 mmol/L sodium phosphate buffer (pH 7.1), and 1 mmol/L EDTA and autoradiographed. The intensity of the DNase-digested samples was compared with standards, and virus titer was calculated based on the equation 1 ng DNA=1×10\(^{10}\) DNA bases=2×10\(^{6}\) copies of 5-kb ssDNA and on the fact that infectious viruses are 1% of the total virus particles.

Cell Culture

Mouse neuroblastoma×rat glioma hybrid cells (NG108-15) were grown in DMEM with 1-glutamine and 4.5 g/L glucose supplemented with 10% fetal bovine serum, 100 mmol/L hypoxanthine, 0.4 mmol/L amnomophen, 16 mmol/L thymidine, 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin in an incubator (Queene Systems, Inc) with humidified atmosphere of 5% CO\(_2\) and 95% air at 37°C. Cells were used between passages 35 and 50. The incubator was replaced every day except the day immediately following subsequent cultivation, when it was not changed.

Transfection of NG108-15 Cells by the pAAV-AS

NG108-15 cells were plated (day 1) in six-well plates and transfected the following day (day 2) at a confluence of ≈75% Plasmid (2 \(\mu\)g) and 10 \(\mu\)L lipofectamine (2 mg/mL) were added to 100 \(\mu\)L Opti-MEM each. The two solutions were gently mixed and kept for 30 minutes at room temperature. The mixture was then filled up to 1 mL with Opti-MEM, gently mixed, and added to cells previously washed with Opti-MEM. Cells were placed into the incubator. After 5 hours, the normal medium (heat-inactivated fetal bovine serum) was given to the cells. On day 3, the cells were subcultivated and pAAV-AS-transfected cells were selected for G418 (600 \(\mu\)g/mL) resistance. This concentration of G418 is required to kill 100% of nontransfected cells.

![Fig 1 Schematics of wild-type AAV (wtAAV) and recombinant AAV vectors (rAAV)](http://hyper.ahajournals.org/)

Wild type AAV

rAAV-GFP

rAAV-AS

Neo integration

Replication

Capid

Fig 1 Schematics of wild-type AAV (wtAAV) and recombinant AAV vectors (rAAV) in rAAV-gfp vector, almost all of the parental AAV genome has been deleted, except the terminal repeats, and replaced with gfp, driven by a CMV promoter, and neo\(^{d}\) genes. In rAAV-AS vector, the gfp gene is replaced by a 760-bp fragment of the AT-R gene with cDNA in antisense orientation. ITR indicates AAV inverted terminal repeat, rep and cap, wtAAV genes encoding replication (Rep) and capsid (Cap) proteins, CMVp, human CMV early promoter, gfp, A vector GFP gene, and neo\(^{d}\), neomycin phosphotransferase gene from Tn5.
NG108-15 in 4 to 7 days. Three weeks after selection, binding experiments were started on pAAV-AS-transfected and non-transfected control cells.

**GFP Detection**

NG108-15 cells were grown on a cover slide, embedded in a tissue culture dish. Pictures were taken using a laser-scanning confocal system, BioRad MRC-600 mounted on an Olympus IMT-2 inverted microscope.

**Detection of Neomycin Resistance Gene in Transfected NG108-15 Cells by PCR and RT-PCR**

To confirm that the transfected cells expressed pAAV-AS, RNA and DNA from NG108-15 cells transfected with pAAV-AS after 2 weeks on G418 selection were isolated by the method of Chomczynski. Control consisted of cells treated with lipofectamine only during transfection. One hundred nanograms of DNA was analyzed by PCR. Presence of neomycin resistance gene was detected using a set of primers yielding a 757-bp fragment, the 5' primer was 5'-GAATTGCAAGAACCCGTTCG-3' and a 3' primer was 5'-CGATAGAAGGCGATGCGCTGC-3'. Amplification was performed for 40 cycles with annealing at 65°C. As a positive control, 1 pg of the plasmid containing neo gene was used.

DNA was analyzed by PCR. Presence of neomycin resistance gene was amplified.

**Membrane Preparation**

Confluent NG108-15 cells were washed twice with ice-cold phosphate-buffered saline, then dislodged and homogenized (Potter-Elvehjem homogenizer) in ice-cold Tris-HCl buffer (20 mmol/L Tris, 5 mmol/L EDTA, 150 mmol/L NaCl) and centrifuged at 51500 g for 30 minutes at 4°C. The pellet was resuspended in the same buffer and homogenized again. The protein content was diluted to 1 mg/mL, and the membranes were used immediately for experiments.

**Binding Assay**

One hundred micrograms of membrane proteins were incubated with 0.2 nmol/L of the radiolabeled Ang II antagonist 125I-[Sar3, Ile8]Ang II in the absence and presence of 1 µmol/L cold Ang II, PD123319, or losartan to determine the total, non-specific AT1, and AT2 receptor binding, respectively. The membrane proteins were incubated in triplicate with the receptor ligands in 500 µL Tris-HCl buffer containing 0.1% bovine serum albumin (BSA), 10 µmol/L o-phenanthroline, and 0.1% BSA for 90 minutes at room temperature. At the end of the incubation procedure, membrane-bound ligand was collected on Whatman GF/B filter paper using a Brandel cell harvester (Biomedical Research and Development Laboratories, Inc.) and the radioactivity was determined with a Beckman γ-counter. An AT1-R saturation assay using a range of 0.01 to 10 nmol/L 125I-[Sar3, Ile8]Ang II performed on control, nontransfected cells revealed Kd = 10 µmol/L and Bmax = 2.8 pmol/mg protein (based on two experiments done in triplicate).

For the binding experiment, 0.2 nmol/L radiolabeled ligand was used, based on the saturation curve.

**Protein Determination**

Protein content was measured by the method of Bradford using BSA as a standard.

**Animals**

Adult (weight, 250 to 275 g) and young male (weight, 55 g, 3-week-old) SHR were purchased from Harlan (Indianapolis, Ind). The rats were kept in cages in a 12 hour light-dark cycle. They were fed standard laboratory rat chow and tap water ad libitum.

**Blood Pressure Measurement**

SBP was measured with an electrophysionometricanometer (Narco Bio-Systems, division of International Biomedical, Inc) using the indirect tail-cuff method. Unanesthetized rats were placed in a plastic holder that was mounted on a heating strip that was thermostatically controlled to maintain 37°C during measurement. Ten readings per rat were taken and the SBPs calculated. The adult rats were recorded for 1 month (n=7 experimental animals and 7 controls). After 1 month, SBP measurements were continued with four experimental rats.

**Delivery of Antisense in Rats**

**ICV Injection**

The adult rats were anesthetized with pentobarbital (65 mg/kg IP) and placed in a stereotactic instrument. After removal of scalp skin and cleaning of the exposed dorsal cranium, a hole was made on the coordinates of -1 mm posterior to the bregma and ~1 mm lateral to the bregma. A Hamilton syringe with a fine needle (33 gauge) was lowered 5 mm into the brain, and in the experimental group (n=7), 5 µL of rAAV-AS viral vector (2X 10^9 infectious particles/µL) was injected into the lateral ventricles. In the control group (n=7), mock rAAV (5 µL) was injected in the same way. Mock virus was AAV containing gfp in place of antisense DNA. In all other respects, the construct was the same as the rAAV-AS. The needle was removed and the hole sealed by bone wax. Skin flaps were sutured, and the animals were returned to their cages for recovery. Blood pressure was recorded 1 week before injection and then at weekly intervals after the injection of rAAV-AS.

**Systemic Injection**

For the intracardiac injections in the young rats, blood pressures were measured from 3 weeks of age. The young SHR were divided into two groups. For the rAAV-AS intracardiac injection (n=6), each animal was anesthetized with methoxyflurane (met- fane), and the injection was made by passing a needle below the sternum at a slight angle dorsally and laterally to the left side of the animal. A pulse felt through the needle indicated that the injection site was in the heart. rAAV-AS 25 µL (2X 10^9 infectious particles/µL) was injected into the heart. In one rat, the injection failed to enter the heart. This rat was monitored by SBP recordings with the other groups but was not included in the final analysis. Control rats (n=5) received intracardiac injection of vehicle (0.9% saline).

Losartan injections were made in a separate group of 4-week-old SHR (n=5). Each animal received a dorsal subcutaneous injection of losartan 10 mg/kg. The injections were given every 2 days for 2 weeks.

**Expression of rAAV-lacZ Vector In Vivo**

To test the duration of the vector in the brain tissue, rAAV-lacZ was injected into the lateral ventricles of Sprague-Dawley rats. Animals were killed 3 days, 1 week, or 1 month after injection. The hearts were perfused with saline and the brains removed and frozen. Sections were cut on a Microtome Cryostat (International Equipment Company) (30 µm) and incubated with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) solution. The presence of β-galactosidase indicated by blue staining was observed directly by light microscopy.

**Statistical Analysis**

Data were analyzed with the use of standard statistical methods. Repeated blood pressure measurements were taken after gene delivery, and animals were compared with controls with their baseline measurements ANOVA and Neumann-Keuls tests were applied. Group data are expressed as mean±SEM. Signifi-
Results

Expression of GFP in Cultured Cells

The first test was to assess transfection efficiency of pAAV-gfp in different cell lines. These included ATOO (mouse pituitary cells) and L929 (mouse fibroblasts). Expression of GFP in the cells was measured 24 hours after transfection by comparing green fluorescence of the transfected cells to cells treated only with lipofectamine. The results showed that there was a 50% transfection efficiency in the cells.

Transfection in NG108-15 (rat glioma–mouse neuroblastoma hybrid cells) was also efficient as measured by GFP expression. Fig 2 illustrates examples of transfected NG108-15 cells expressing GFP.

Detection of the Neomycin Resistance Gene in Transfected NG108-15 Cells by PCR and RT-PCR

Presence of the neomycin resistance gene in host DNA was confirmed by PCR of DNA extracted from NG108-15 cells transfected with pAAV-AS, 2 weeks after the experiment (Fig 3). A 757-bp fragment was also detected in RNA extract of pAAV-AS–transfected cells after RT-PCR was performed (Fig 3). No visible band was present in control cells.

Effect of pAAV-AS Transfection on Ang II Receptor Binding in NG108-15 Cells

The pAAV-AS plasmid–transfected NG108-15 cells showed 41% reduction in AT1-R number assayed at 0.2 nmol/L [125I]-[Sar1, Ile8] Ang II compared with the nontransfected cells (1.9±0.3 fmol/mg versus 3.2±0.6 fmol/mg; n=8; P<.001). The AT2-R number was not changed. Fig 4 shows the significant reduction in AT1-R number determined at 3 to 5 weeks after transfection (four consecutive passages).

Expression of rAAV-lacZ Vector In Vivo After Gene Delivery

Fig 5 shows the presence of β-galactosidase in the paraventricular nucleus and supraoptic nucleus 1 month after injection of rAAV-lacZ in adult SHR. Many labeled cells were found spread over other tissues including cortex, amygdala, piriform cortex, and periventricular sites.

Hypotensive Effect of ICV rAAV-AS Gene Delivery in Adult SHR

The effect of rAAV-AS delivered by a single injection (5 μL) in the lateral cerebral ventricles of adult SHR was monitored weekly for 9 weeks. The results show that compared with the baseline levels (181 mm Hg), the treated rats had a significant reduction in blood pressure (23±2 mm Hg; P<.05) 1 week later (Fig 6). A persistent reduction in blood pressure continued to be maintained for up to 9 weeks. The controls were tested weekly for 4 weeks.
Their blood pressures showed no reduction from baseline, and over the 4-week period, the hypertension was well established.

Compared with the mock vector control rats, there were significant differences at all times for all 4 weeks tested \((P < .05)\). The original protocol was to test the animals for 1 month, and the control animals were killed after 1 month, but because 66.6% of the experimental group had maintained significantly lower blood pressure than their baseline or control levels, they were recorded for an additional 5 weeks. At 9 weeks, the blood pressure still had not reached the original baseline of the treated group. No effect on heart rate due to the vector was observed. The animals did not appear unhealthy and had no reduction in body weight.

**Hypotensive Effect of Intracardiac rAAV-AS Gene Delivery in Young SHR**

Intracardiac injection of rAAV-AS at the dose used (25 \(\mu\)L; \(2 \times 10^9\) infectious particles/mL) slowed the development of hypertension in young SHR (Fig 7). We compared the effects of the rAAV vector−carrying antisense with a nontreated control. The SBP of the rAAV-AS animals was significantly below the level of the control animals for 5 weeks after the rAAV-AS injection \((P < .01)\). Losartan given to a separate group of SHR by subcutaneous injections every 2 days for 2 weeks also lowered blood pressure compared with untreated controls \((P < .05)\) (Fig 8). However, the effect of rAAV-AS lasted longer with a single injection than the losartan-treated group with multiple injections.

**Discussion**

The results show that gene delivery with a recombinant AAV vector of an antisense DNA to the AT\(_1\)-R has potential for chronically reducing hypertension in adult animals and slowing the development of hypertension in young animals. Decreased blood pressure was sustained and persisted for over 2 months in the adult SHR, and hypertension development was slowed in young SHR. These
findings, while preliminary, suggest the feasibility of rAAV-AS for gene therapy of hypertension. Obviously, the intracranial route of injection would be unacceptable for treatment of human hypertension, but the experiment in young rats with intracardiac injections suggests that the rAAV-AS will also be effective through systemic delivery. We are exploring nasal sprays and intramuscular injections as alternate routes.

Further testing requires not only systemic routes of delivery, but also higher titers and vector modification with tissue-specific promoters. The CMV promoter has been shown to stimulate gene expression in different parts of the brain for up to 3 months. In the present study, AAV with a CMV promoter was effective for at least 1 month. At that time, brains were expressing β-galactosidase in several cells, including hypothalamic cells. In non-brain tissue, such as blood vessels, more specific promoters need to be tested.

The experiments are a development from our previous work using antisense oligonucleotides. In the first report of the use of antisense oligonucleotides to reduce hypertension, we demonstrated that AT1-R mRNA-directed antisense and angiotensinogen mRNA-targeted antisense when injected into SIIR were both effective in reducing blood pressure significantly. The reduction in blood pressure was simultaneous with a reduction in receptor binding in key regions of the brain that have been associated with cardiovascular regulation. In the original report, the effects on blood pressure were only measured for 24 hours after injection. In a later study, we demonstrated that the effects lasted for up to 7 days with a single administration of AS-ODN. While the brain offers several advantages from a research point of view for studying the feasibility of antisense inhibition, the ultimate goal is to provide a safe, efficient method of delivery for humans. Therefore, in subsequent studies we injected antisense systemically and demonstrated that angiotensinogen antisense ODN injected intra-arterially in liposome encapsulation was taken up by the liver and reduced the levels of angiotensinogen with simultaneous reduction in blood pressure in SHR. A similar but more complex study was carried out by Tomita et al., who delivered three antisense oligonucleotides to angiotensinogen via the hepatic portal vein and also demonstrated a transient decrease in blood pressure.

These positive results with antisense oligonucleotides stimulated us to test viral vector delivery to produce long-term hypotension with antisense inhibition. We chose to use the AAV because of its many advantages compared with other vectors such as retroviruses or adenoviruses. The properties of AAV vectors include safety, because AAV is nonpathogenic and does not replicate. It produces no viral genes that stimulate inflammatory responses. It has site-specific integration in the genome of chromosome 19. It can be concentrated and is stable in tissue. The vector can easily be modified to carry foreign genes and tissue-specific promoters. Therefore, we constructed an rAAV with antisense to AT1-R. The in vitro studies indicated efficient transfection of foreign genes (gfp and lacZ) into cultured cells. The PCR and RT-PCR detection of neomycin resistance gene confirmed that the pAAV-AS was being expressed in the transfected cells. There was a decrease in AT1-R number in the NG108-15 cells in the pAAV-AS–transfected cells. It is not known at this stage whether rAAV integrates into the genome and is expressing AS mRNA or whether the expression is episomal. Further studies with low-molecular-weight DNA will resolve this. The rAAV-AS induced a prolonged decrease in blood pressure for as long as we recorded it in this experiment (2 months). More experiments are needed to confirm the result, but taking the data from adult and young SHR together, the rAAV-AS does appear to be effective in chronically lowering blood pressure β-Gal gene expression from the rAAV-lacZ lasted at least 1 month, suggesting that the rAAV AS was also expressed for this time. Kaplitt et al. and McCowan et al. have reported that the rAAV vector with CMV promoter expresses β-galactosidase in the brain for up to 3 months. Therefore, it seems likely that the rAAV-AS was expressing the antisense DNA during the 2-month period. In the young rats given rAAV-AS by intracardiac injection, it is not known in which tissue the rAAV was sequestered and expressed. Further analysis of RT-PCR in multiple tissue samples is necessary to determine the fate of the AAV. At present, the data suggest that the reduction in blood pressure compared with controls and the retardation of hypertension development over 5 weeks was due to AT1-R decrease. The data obtained with the AT1-R antagonist losartan indicate that it is the blockade of AT1-Rs that underlies the reduction in blood pressure. The advantage of the rAAV-AS for possible therapy is that it can be effective when given once rather than repeated for several weeks. The experiment is being repeated with younger SHR to test if there is a critical period in the development of AngII-mediated peripheral resistance that, when inhibited, would permanently prevent the development of hypertension. Several studies indicate this is possible. We showed that levels of Ang II are elevated in 4-week-old SHR compared with Wistar-Kyoto rats. Harrap et al. found that ACE inhibition in 6- to 10-week-old SIIR prevents hypertension and proposed a critical period spanning those weeks. Our data show that the rise in blood pressure in the control rats is very rapid in weeks 3, 4, and 5 of life. By 6 to 10 weeks, hypertension is established, so it would seem that the early weeks of age would be more vulnerable to Ang II–receptor changes.

Several recent studies have applied gene therapy to hypertension since the antisense studies began in 1993. Phillips et al.
et al.23 showed that human ANP, delivered as a naked DNA construct, fused to the Rous sarcoma virus 3' long-terminal repeat and injected intravenously into SHR through the tail vein, produced decreased blood pressure in 4-week-old SHR that lasted for 7 weeks. The maximal reduction in pressure was 21 mm Hg. These results are strikingly similar to the present results in young SHR using quite different gene-modification procedures one to introduce a gene associated with vasodilation and diuresis (ANP) and the other an antisense to inhibition of a vasoconstrictor (Ang II). Both approaches raise the feasibility of gene therapy for treatment in human hypertension with safe delivery systems and open new possibilities for research.

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References

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