Atrial Natriuretic Peptide Gene Delivery Reduces Stroke-Induced Mortality Rate in Dahl Salt-Sensitive Rats

Kuei-Fu Lin, Julie Chao, Lee Chao

Abstract—Atrial natriuretic peptide (ANP) is a powerful hormone with hypotensive, natriuretic, diuretic, and many other beneficial effects. Direct infusion of ANP in therapeutics has limited success because of its short half-life in the circulation. Our previous studies have shown that ANP gene delivery attenuates hypertension, cardiac hypertrophy, and renal injury in Dahl salt-sensitive (Dahl-SS) rats. To investigate the potential therapeutic value of ANP gene delivery on salt-induced stroke and cerebrovascular disorders, an adenovirus harboring the human ANP gene (Ad.RSV-cANP) was injected into Dahl-SS rats on a high salt diet. A single intravenous injection of the ANP gene caused a significant reduction of blood pressure that lasted for more than 3 weeks. A maximal blood pressure reduction of 28 mm Hg was observed 2 weeks after gene delivery as compared with that of control rats injected with adenovirus harboring the LacZ gene under the control of the Rous sarcoma virus promoter (Ad.RSV-LacZ). Immunoreactive human ANP can be detected in the heart, lung, kidney, and brain of rats after gene delivery. The stroke mortality rate of Dahl-SS rats was significantly decreased (from 54% to 17% at 3 weeks and from 70% to 50% at 4 weeks after ANP gene delivery as compared with rats injected with control virus). ANP gene delivery also significantly attenuates salt-induced aortic hypertrophy as evidenced by reduced thickness of the aortic wall. This is the first study to demonstrate the potential of ANP gene delivery in reducing the mortality rate caused by cerebrovascular disorders and stroke. Successful application of this technology may have potential value in treating individuals with a high risk of stroke. (Hypertension. 1999;33[part II]:219-224.)

Key Words: gene delivery © rats, Dahl adenovirus blood pressure hypertension, aortic stroke

Stroke is the third leading cause of death in the United States, affecting more than 500 000 Americans annually. It is commonly due to thromboembolic occlusion or abrupt rupture of a cerebral artery, resulting in the focal death of brain tissue (cerebral infarction). Stroke patients, who have disturbances of motor strength and coordination, sensory discrimination, visual function, speech, memory, or other intellectual abilities, may cause a medical and social burden because their recovery is often incomplete.

Atrial natriuretic peptide (ANP) is a powerful hormone with hypotensive, natriuretic, diuretic, and other beneficial effects. Direct infusion of ANP in therapeutics has not been always practical because of its short half-life in the circulation. Recent evidence revealed that plasma levels of ANP were increased in patients with acute ischemic stroke. Binding sites for ANP have been found on brain microvessel endothelial cells and astrocytes, and elevated levels of cGMP have been noted in these cells after ANP binding. ANP acts directly on the central nervous system to inhibit water and sodium accumulation in ischemic brain edema; this action is probably related to its inhibitory effect on sodium transport in brain capillaries. In addition, ANP has been shown to modulate intracellular electrolyte content through activation of guanylyl cyclase. In cultured astroglia, ANP increases intracellular cGMP levels, thus regulating sodium content. ANP could act as a protective factor in the setting of ischemic stroke via both antiedema and vasodilator effects. Moreover, differential structural and functional characteristics of the ANP gene have been identified to make it a candidate for short tandem repeat, a quantitative trait locus linked to stroke in stroke-prone spontaneously hypertensive rats (SHRSP).

A severe lethal form of hypertension has been shown to develop in Dahl salt-sensitive (Dahl-SS) rats fed a high salt diet at an early age. Werber and colleagues observed a high stroke mortality in Dahl-SS rats fed an 8% NaCl diet. Our previous studies showed that ANP gene delivery attenuates hypertension, cardiac hypertrophy, and renal injury in Dahl-SS rats. To investigate the potential therapeutic value of ANP gene delivery on salt-induced stroke and cerebrovascular disorders, an adenovirus harboring the human ANP gene (Ad.RSV-cANP) was injected into Dahl-SS rats on a high salt diet. In this study, human ANP gene delivery not only resulted in a sustained reduction of blood pressure but also significantly reduced the stroke-induced mortality rate in Dahl-SS rats. The present study demonstrated the potential...
usefulness of ANP gene delivery in reducing the mortality caused by cerebrovascular disorders and stroke. These results also suggest that application of this technology in the treatment of persons with a high risk of stroke may deserve further consideration.

Methods

Materials

Dahl-SS rats (male, 4 weeks old; Sprague-Dawley Harlan, Indianapolis, IN) were used in this study. Rats were divided into 2 groups. The first group was fed a standard rat chow (0.4% NaCl; Harlan Teklad). The other group was fed a high salt diet (4% NaCl; Harlan Teklad). All rats had free access to water. Throughout the study period, all animals were housed in a room that was kept at constant temperature (25±1°C) and humidity (60±5%) and was lighted automatically from 8:00 AM to 8:00 PM. All procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Resources, National Academy of Sciences, Bethesda, MD).

Preparation of Replication-Deficient Adenovirus Vector Ad.RSV-cANP

Plasmid RSV-cANP was constructed as previously described,\(^\text{19}\) in which the expression of human ANP cDNA (456 bp) was under the control of the Rous sarcoma virus long-terminal repeat (RSV-LTR) and followed by a Simian virus 40-polypeptide A signal sequence. The transcription unit of RSV-cANP-poly A (1618 bp, including the RSV-LTR, the human ANP cDNA, and a SV40-polypeptide A signal sequence, was released from the RSV-cANP plasmid by Sal I digestion. Plasmid pAd.RSV-cANP was constructed by inserting the released fragment into the adenovirus shuttle vector pAdLink.1 (adenoviral capacity, 8 kb) at a Sal I site. The pAd.RSV-cANP plasmid DNA was purified using a Qiagen plasmid DNA kit.\(^\text{18}\) The purified DNA was sent to the Institute for Human Gene Therapy, Wistar Institute (Philadelphia, PA) for generation of adenovirus Ad.RSV-cANP harboring the RSV-cANP-poly A transcription unit. Adenovirus harboring the LacZ gene under the control of the Rous sarcoma virus promoter (Ad.RSV-LacZ) was purchased from the Institute for Human Gene Therapy.

Intravenous Delivery of Adenoviral Vectors Ad.RSV-cANP and Ad.RSV-LacZ

Twenty-seven Dahl-SS rats fed a high salt diet containing 4% NaCl for 4 weeks were randomly divided into 3 groups and were intravenously injected with either Ad.RSV-cANP (n=13) or Ad.RSV-LacZ (n=7) at a dosage of 2.4×10\(^{10}\) plaque forming units per rat through the tail vein. Seven Dahl-SS rats on a 4% NaCl diet did not receive any adenovirus injection.

Blood Pressure Measurement

The systolic blood pressure of rats was measured with a manometer-tachometer (Nautime KN-210; Nautime Seisakusho Co) with the tail-cuff method.\(^\text{19}\) Unanesthetized rats were placed in a plastic holder mounted on a thermostatically controlled warm plate, which was maintained at 33°C to 35°C during the measurement. An average of 10 readings was taken for each animal.

Tissue Preparation

At 3 days after gene delivery, rats from each group were anesthetized intraperitoneally with pentobarbital at a dose of 50 mg/kg body weight and perfused with normal saline (0.9% NaCl) by cardiac puncture. Tissues including heart, lung, kidney, and brain were homogenized in normal saline with a Polytron homogenizer (Brinkmann Instruments). The homogenate was centrifuged at 600g for 10 minutes. The supernatant was incubated in 0.5% sodium deoxycholate and then centrifuged at 10 000g for 30 minutes. Total protein in the supernatant was determined by the method of Lowry et al.\(^\text{20}\) Tissue extracts were subjected to radioimmunoassay (RIA) for human ANP. At 4 weeks after Adenovirus gene delivery, all surviving animals were euthanized. Brains and thoracic aortas were immediately removed. Serial coronal brain sections (2 mm in thickness) cut with razor blades were used for the evaluation of infarction area. Thoracic aortas (8 mm in length) were used for histological investigations.

RIA for Human ANP

The level of human ANP in each tissue extract was determined by a RIA specific for human ANP. Ten micrograms of human synthetic ANP (Ser 99–Tyr 126; Sigma Chemical Co) was labeled with 1 mCi of \(^{125}\)I–iodine that had been iodinated with iodogen for 10 minutes at room temperature. The iodinated ANP in 250 mmol/L sodium phosphate buffer, pH 7.0, was separated on a reverse-phase C18 high performance liquid chromatography column in an acetonitrile gradient \(^{125}\)I–ANP–labeled tracer that was eluted from the column at 19 to 20 minutes after injection was identified by antibody titration. Serial dilutions of standard ANP (10 to 1280 pg) or tissue extracts (100 µL) were incubated with goat anti-human ANP antisera (1:1500 dilution; Sigma) in a solution containing 0.01 mol/L PBS, pH 7.4, 0.3% BSA, 0.1% Triton X-100, 0.1 mmol/L EDTA and 0.1% sodium azide, and \(^{125}\)I–ANP–labeled tracer (10 000 cpm in 100 µL) in a total volume of 400 µL for 18 to 24 hours at 4°C. The reaction was stopped by adding 800 µL 25% polyethylene glycol in PBS containing 0.1% sodium azide and 400 µL 1% bovine gammaglobulin in PBS containing 0.1% sodium azide. The radioactivity of the precipitate was determined in a gamma counter.

Monitoring of Stroke Development

The rats were monitored daily for the occurrence of stroke. The symptoms associated with stroke development have been previously described for SHRS.\(^\text{21,22}\) Initially, SHRS develop convulsive repetitive forearm movement followed by inappropriate posture during which rats sit with legs hyperextended in a “kangaroo-type” posture. In this study, the symptoms associated with Dahl-SS rats were often lethargy and poor grooming. There is no typically fixed period between the onset of the first behavioral symptom of stroke and death. Some animals died abruptly after the first behavioral symptom of stroke, whereas others were euthanized at a point at which death was likely to occur within 1 day.

Confirmation of Infarction Area of Stroke Brain

Serial coronal brain sections (2 mm in thickness) were stained with 2, 3, 5-triphenyltetrazolium chloride (TTC; Sigma). Brain slices were immersed in normal saline containing 2% TTC at 37°C for 30 minutes. TTC, a colorless salt, is reduced to form an insoluble red formazan product in the presence of a functioning mitochondrial electron transport chain. Thus, the infarcted region lacks staining and appears white, whereas the normal noninfarcted tissue appears red.

Morphological and Histological Investigations

Segments of the thoracic aorta (8 mm in length) were preserved in 4% buffered formaldehyde solution and paraffin-embedded. Five-micrometer-thick sections were cut and stained with hematoxylin-eosin and analyzed microscopically and morphometrically. Measurements of the thickness of the aortic wall were performed according to the guidance of Dr Jo Anne Simson, Professor Emeritus in the Department of Anatomy and Cell Biology, Medical University of South Carolina. Ten measurements taken from different positions of each aorta were averaged. All sections were evaluated by independent personnel with no prior knowledge of the group from which rats were obtained.

Statistical Analysis

Repeated blood pressure measurements at each time point were taken after gene delivery for comparison between control and experimental groups, and the data shown in the figures were analyzed with the use of either unpaired Student’s t test or ANOVA and Fisher’s protected least significant differences test. Group data are expressed as
mean±SEM. Survival curves were constructed using Kaplan-Meier analysis. Statistical significance of these data was measured by ANOVA and χ², with a SAS software package. Values of blood pressures and other parameters were considered significantly different at P<0.05.

Results

Blood Pressure Reduction after Intravenous Injection of the Human ANP Gene

Dahl-SS rats (4 weeks old) were fed a high salt (4% NaCl) diet or normal rat chow (0.4% NaCl) as controls for 4 weeks until blood pressure differences between these 2 groups were greater than 45 mm Hg. Dahl-SS rats on the high salt diet were divided into 3 subgroups. Two groups were intravenously injected with either adenovirus Ad.RSV-cANP carrying the ANP gene or control virus Ad.RSV-LacZ containing the LacZ gene through the tail vein. The third group was not given any adenovirus injection. Blood pressures of these rats were monitored weekly for 3 weeks after gene delivery. Figure 1 shows systolic blood pressures of Dahl-SS rats on a 4% NaCl diet after intravenous injection of Ad.RSV-cANP and Ad.RSV-LacZ. Blood pressure values are expressed as mean±SEM (n=6 in each group except that at 3 weeks after gene delivery n=4 in 4% NaCl and Ad.RSV-LacZ plus 4% NaCl groups). Standard error is represented by bars. *P<0.05, †P<0.01 vs high salt and Ad.RSV-LacZ groups.

Expression of Human ANP after Gene Delivery

Expression levels of human ANP in Dahl-SS rats were analyzed by a RIA specific for human ANP. Immunoreactive human ANP was detected in the heart, lung, kidney, and brain 3 days after intravenous injection of the human ANP gene (Figure 2). Linear displacement curves for immunoreactive ANP in the heart, lung, brain, and kidney of Dahl-SS rats were parallel with the standard curve of human ANP, indicating their immunological identity (Figure 2). Serial dilutions of the heart, lung, brain, and kidney extracts from control rats injected with Ad.RSV-LacZ showed a lack of parallelism with the human ANP standard curve (data not shown). These results indicate that goat anti-human ANP antibody has some cross-reactivity with rat ANP; however, human and rat ANPs are not immunologically identical and are distinguishable in the RIA.

Mortality Rate of Dahl-SS Rats with Stroke

Dahl-SS rats began to show symptoms of stroke (including lethargy, poor grooming, convulsive repetitive forearm movement, and semiplegia) at 5 ½ weeks after high salt loading. Some animals died rapidly after the appearance of the first behavioral symptom of stroke. Figure 3 shows Kaplan-Meier survival plots for Dahl-SS rats after ANP gene delivery. At 3 weeks after ANP gene delivery (51 days after high salt loading), the survival rates were 100% in control (0.4% NaCl diet), 83% in the Ad.RSV-cANP group (4% NaCl), and 46% in high salt loading (4% NaCl diet alone and Ad.RSV-LacZ) groups (Figure 3). At 4 weeks after adenovirus injection (58 days after high salt loading), 70% of...
Dahl-SS rats fed a high salt diet with or without LacZ adenovirus injection died from stroke. Cumulatively, 50% of Dahl-SS rats in the Ad.RSV-cANP group survived. The Kaplan-Meier plots were analyzed statistically by $\chi^2$ ($P < 0.05$). Pathological changes in coronal brain sections including hemorrhage, edema, and focal infarction were observed in Dahl-SS rats with stroke at 4 weeks after gene delivery. Figure 4 shows that focal infarction regions in the brain from animals of the high salt plus Ad.RSV-LacZ group were stained white with 2% TTC. Similar staining results were also seen in the high salt alone group (data not shown). After ANP gene delivery, brain sections of Dahl-SS rats appeared reddish and relatively normal (Figure 4).

**Human ANP Gene Delivery Reduced Salt-Induced Aortic Thickening in Dahl-SS Rats**

The thickness of the aortic wall was significantly reduced in the Ad.RSV-cANP group at 4 weeks after gene delivery as
compared with those in the Ad.RSV-LacZ and the high salt alone groups (153.5 ± 2.2 μm versus 202.8 ± 18.1 and 198.4 ± 12.2 μm, respectively, mean ± SEM, n = 5, P < 0.05; Figure 5), whereas the aortic wall of Dahl-SS rats on a 0.4% NaCl diet was 136.7 ± 3.5 μm thick. These results indicate that human ANP gene delivery can attenuate, at least in part, salt-induced aortic hypertrophy in hypertensive Dahl-SS rats.

**Discussion**

The present study shows that a continuous supply of ANP by adenovirus-mediated gene delivery into Dahl-SS rats fed a high salt diet resulted in a reduction of the stroke-induced mortality rate, sustained delay in blood pressure increase, and partial attenuation of aortic hypertrophy. A single injection of adenovirus carrying the human ANP gene caused a significant reduction of blood pressure that lasted for more than 3 weeks. The expression of human ANP was detected in tissues relevant to cerebral, cardiovascular, and renal function, such as the brain, lung, heart, and kidney. The ability of ANP gene transfer to protect against structural changes in cerebral vasculature and in the aortic wall in hypertensive Dahl-SS rats may provide significant information in future therapeutic applications of human ANP gene delivery in salt-induced stroke or cerebrovascular disorders.

The mechanism by which ANP decreases the stroke mortality rate of Dahl-SS rats is not clear at the present time. Two considerations are relevant to our present observation. First, ANP acts directly on the blood-brain barrier and microvessel receptors of the brain, resulting in a reduced sodium transport and an antiedema effect. It has been shown that ANP exerts a regulatory effect on the intracellular electrolyte content in various cells. Brain tissue reportedly contains many ANP-specific receptors involved in the regulation of guanylyl cyclase. ANP has been shown to raise intracellular cGMP levels in cultured astroglia, suggesting that ANP prevents the swelling of astroglia in brain tissue through the regulation of sodium transport. After high salt loading, sodium influx from the blood to the brain across the blood-brain barrier may therefore be interrupted by ANP. Because sodium transport has been shown to be a rate-limiting step in edema formation, ANP may delay edema formation by inhibiting sodium transport in brain capillaries. Therefore, the effect of ANP on sodium transport is the primary reason for the antiedema effect of ANP on the brain. The reduction of the stroke mortality rate in Ad.RSV-cANP–treated rats may be attributable to the antiedema effect of ANP on the brain. Also, the expression of exogenous human ANP in the kidney significantly increased urinary sodium excretion in salt-loaded Dahl-SS rats, which may indirectly reduce sodium concentration in the circulation and sodium influx into the brain via the blood-brain barrier. In addition, the ability of ANP to protect Dahl-SS rats against stroke may be secondary to the decrease in blood pressure. Two types of stroke occurred in Dahl-SS rats: hemorrhagic and ischemic. In this study, hemorrhagic stroke was found in approximately 70% of salt-loaded animals (data not shown), suggesting that salt-induced hypertension may ultimately cause the rupture of cerebral blood vessels. Because ANP gene delivery may prevent salt-induced cell swelling in brain microvessels through the regulation of sodium transport, the protective effect of ANP on the brain could account for the lower incidence of stroke and the reduced mortality rate we saw in salt-hypertensive rats.

Extensive investigations in the past decade have shown that Dahl-SS rats are more vulnerable to vascular injuries (eg, intimal and medial hyperplasia, thrombotic formation with periarterial massive infiltration of inflammatory cells, and renal damage). As demonstrated in the present study, high-salt loading caused severe aortic thickening in Dahl-SS rats injected with control virus, which was evidenced by a marked increase in the thickness of aortic wall. Also, long-term high salt intake caused a significant increase in elastic layers and cell size in the media of aorta. To reduce these lesions, ANP gene delivery was shown to partially but significantly attenuate salt-induced aortic hypertrophy. ANP may mediate these effects by inhibiting vascular smooth muscle cell enlargement and proliferation in the aorta due to its antimitogenic and antcell hyperplastic properties. These combined effects may be responsible for the reduction of thickness of the aortic wall and the attenuation of aortic hypertrophy.

In the present study we observed a significant weight loss in Dahl-SS rats injected with control adenovirus beginning at the fifth week after high salt loading but not in rats injected with Ad.RSV-cANP. At 3 weeks after ANP delivery, the survival rate in the ANP group was twofold higher than that of high salt groups with or without Ad.RSV-LacZ injection. Cumulatively, 70% of the salt-loaded Dahl-SS rats with or without the Ad.RSV-LacZ injection suffered from stroke and died as compared with 50% of rats receiving ANP gene delivery at the end of the experiment. Although adenovirus-mediated gene delivery can achieve a high level of expression within 1 week, it only lasted about 1 month mainly because of immunosurveillance by the host. Furthermore, systemic gene delivery via intravenous injection is relatively limited in its ability to target end organs like the brain because of the obstacle of the blood-brain barrier. Intracisternal or intracerebroventricular injection of adenoviral vectors carrying desirable genes may provide a more promising and effective expression efficiency in the brain for achieving local gene transfer. However, safety is an important consideration in central injection of adenovirus. Injury to the brain stem can occur during the administration of the virus into the cisterna magna of rats. To accomplish effective adenovirus-mediated gene transfer in the central nervous system, an alternative method is to open the vascular endothelium by osmotic disruption of the blood-brain barrier and to inject vectors via the carotid artery. In terms of therapeutic applications, human ANP gene delivery with adenovirus–associated virus or improved adenovirus may prolong the protective effects on salt-induced stroke or cerebrovascular diseases and offer a better alternative for long-term and high-efficiency gene expression.

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References


