

Somatic gene therapy for hypertension

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Abstract

Gene therapy for hypertension is needed for the next generation of antihypertensive drugs. Current drugs, although effective, have poor compliance, are expensive and short-lasting (hours or one day). Gene therapy offers a way to produce long-lasting antihypertensive effects (weeks, months or years). We are currently using two strategies: a) antisense oligodeoxynucleotides (AS-ODN) and b) antisense DNA delivered in viral vectors to inhibit genes associated with vasoconstrictive properties. It is not necessary to know all the genes involved in hypertension, since many years of experience with drugs show which genes need to be controlled. AS-ODN are short, single-stranded DNA that can be injected in naked form or in liposomes. AS-ODN, targeted to angiotensin type 1 receptors (AT₁-R), angiotensinogen (AGT), angiotensin converting enzyme, and β 1-adrenergic receptors effectively reduce hypertension in rat models (SHR, 2K-1C) and cold-induced hypertension. A single dose is effective up to one month when delivered with liposomes. No side effects or toxic effects have been detected, and repeated injections can be given. For the vector, adeno-associated virus (AAV) is used with a construct to include a CMV promoter, antisense DNA to AGT or AT₁-R and a reporter gene. Results in SHR demonstrate reduction and slowing of development of hypertension, with a single dose administration. Left ventricular hypertrophy is also reduced by AAV-AGT-AS treatment. Double transgenic mice (human renin plus human AGT) with high angiotensin II causing high blood pressure, treated with AAV-AT₁-R-AS, show a normalization of blood pressure for over six months with a single injection of vector. We conclude that ODNs will probably be developed first because they can be treated like drugs for the treatment of hypertension with long-term effects. Viral vector delivery needs more engineering to be certain of its safety, but one day may be used for a very prolonged control of blood pressure.

Key words

- Gene therapy
- Hypertension
- Antisense
- Vectors

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Introduction

Although many excellent pharmacological agents are available commercially for the treatment of hypertension, the problems of cardiovascular disease related to hypertension continue to affect millions of people throughout the world.

Part of the problem is expense. Many of the drugs are expensive, and therefore, unavailable to poor segments of all societies. Another problem is detection. Hypertension is going undetected in about 40% of the population of the United States, according to the NHANES III Report (1). Of those in whom hypertension has been detected, about

53% receive treatment with the full benefit of the multiple drugs available. However, it is estimated that only 27% of treated hypertensive patients fully comply with their treatment (1). Clearly, there is a need for rethinking our approach to the treatment of hypertension. There are many indications that nonpharmacological means are effective. However, it is difficult to achieve compliance for exercise, weight loss and low-salt diets in the general population. For treating hypertension on a world scale, we need something akin to an immunization against hypertension. Since hypertension is not a single gene disease, except in very few cases (2), we need to develop ways that would improve hypertension control by providing longer-lasting effects with a single dose and reducing side effects that lead to poor compliance. To do this we have been developing a bilateral approach based on somatic gene therapy.

Somatic gene therapy

Somatic gene therapy uses recombinant DNA, introduced into non-germ line cells to express messenger RNA and the appropriate protein. Alternatively, as we are using antisense, somatic gene therapy involves recombinant antisense DNA to express an antisense mRNA which will inhibit an overexpressed protein that is critical to the disease. Since hypertension is a multigene disease, the question is how to decide on the candidate genes for gene therapy. We have ignored the difficulties of this approach by concentrating on the genes that have been shown to be successful targets of current drugs. These include beta-receptors, angiotensin converting enzyme, angiotensin type 1 receptor (AT_1 -R) and some that follow logically, including angiotensinogen (AGT). Transfer of the antisense genes to somatic cells is achieved by an *in vivo* approach. It would be possible to try an *ex vivo* approach in which target cells are removed from the host, transduced *in vivo* and then reimplanted

as genetically modified cells. However, this strategy has no obvious applicability to hypertension, where the cause of the disease lies in the blood vessels and not in one specific organ. The *in vivo* approach has its own challenges. One challenge is to provide sufficient antisense DNA, either alone or in a vector, to produce a sufficient concentration for uptake in a large number of cells. To do this we have developed strategies for hypertension gene therapy with a) antisense oligodeoxynucleotides (AS-ODN) and b) viral vectors to deliver antisense DNA.

Antisense oligonucleotides

Gene transfer can be carried out with direct injection of "naked DNA". In using ODN we have found that direct injection is effective, but the efficiency of uptake is greatly increased by delivering the ODN in cationic liposomes.

Non-viral gene delivery, using cationic liposomes such as DOTAP and DOPE, has been successfully used by our group to deliver β_1 -adrenoceptor antisense oligodeoxynucleotides (β_1 -AS-ODN) to act as novel beta blockers with prolonged effects. By optimizing the liposome/ODN ratio and the incubation procedure, we are able to produce antihypertensive effects with a single dose of β_1 -AS-ODN for up to 33 days. The beauty of the β_1 -AS-ODN is its specificity. The β_1 -AS-ODN reduces β_1 -adrenoceptors but does not affect β_2 -adrenoceptors. Secondly, the β_1 -AS-ODN does not cross the blood-brain barrier, and therefore the novel β_1 blocker, based on antisense, will have no central nervous system side effects. The strongest uptake sites are in the heart and kidney where the β_1 -adrenoceptors play a significant role. In the heart they control the force of contraction and this is reduced by the β_1 -adrenoceptor. However, the heart rate is not affected. This is in contrast to the effects of currently available beta blockers that have both β_1 and β_2 actions, and secondly, reduce heart rate as well as heart contractility. There-

fore, the specificity offered by the ODN provides a more precise and accurate way of controlling the mechanisms contributing to high blood pressure without the side effects of bradycardia (3). Furthermore, since the effect lasts for 30 days with a single injection, the AS-ODN is greatly superior to any currently available drug, all of which have to be taken on a daily basis.

We have also established that AGT is an effective AS-ODN for hypertension therapy. In human hypertension, the AGT gene has been shown to be linked to and play a role in the disease (4). However, there is no currently available drug to inhibit AGT. We have designed antisense, targeted to AGT mRNA and tested it *in vivo* and *in vitro* (5). When given *iv* the AGT-AS-ODN would reduce blood pressure significantly when delivered with a liposome. These studies have been confirmed by other studies independently, showing that AGT-AS-ODN reduces blood pressure for up to 7 days with a single systemic dose (6). A similar story is true for the effects of AT₁-AS-ODN. This has been tested centrally with *icv* injections and peripherally. It has been tested in spontaneously hypertensive rats (SHR) and also in 2 kidney-1 clip animals and environmentally induced hypertension. In all cases the antisense produces a decrease in blood pressure within 24 h of administration. The effect lasts for up to 7 days and there is no effect on heart rate (7). The distribution of antisense is to be in blood vessels, kidney, liver and heart. Most of the uptake is in the kidney and liver. A reduction in AT₁-R after treatment with the AT₁-AS-ODN reveals reductions in the protein in kidney, aorta and liver (8).

In summary, the ODN has proved to be useful in demonstrating in the preclinical setting the power of AS-ODN to specific targets in reducing blood pressure for several days with a single administration. Laboratory data indicate that these effects are the result of uptake of the antisense into cells

where they migrate to the nucleus and inhibit the production of protein, most likely through translational inhibition of messenger RNA. This could occur by the hybridization of ODN with specific mRNA, preventing the passage of the mRNA through the ribosome. Alternatively, DNA hybridization to RNA will in some tissues stimulate the production of RNase-H for the specific sequence of mRNA bound to the ODN (Figure 1). Other useful features of ODN are that they can be produced cheaply and in large quantities. Secondly, they do not cross the blood-brain barrier and therefore, when given peripherally, will not have central effects. Third, they are most effective when delivered in the right combination of ODN to cationic liposome. Treatment of rats with liposomes has not shown any liver toxicity in our hands (9).

Delivery by adeno-associated virus plasmid

To produce very prolonged effects, we use antisense delivery by viral vector. Several viral vectors are available but the adeno-associated virus (AAV) is both safe for use in humans and large enough to carry antisense genes with tissue-specific promoters. Therefore, we are developing antisense therapy using the AAV as a vector. To construct a viral vector requires the design and production of plasmids and gene packaging into the vector. The plasmid should be effective, but for a shorter time than the viral

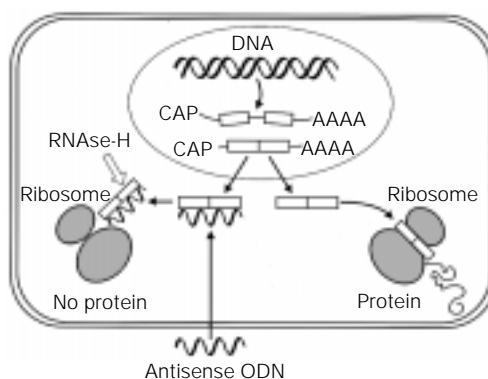


Figure 1 - Antisense oligodeoxynucleotide (AS-ODN) inhibition. An AS-ODN is designed to hybridize to specific mRNA which will either prevent translation of the specific protein (by preventing read-through of mRNA in the ribosome), or stimulate RNase-H which will destroy the mRNA hybridized to the AS-ODN. Either or both mechanisms will reduce levels of the specific protein.

vector. This is illustrated with the adeno-associated-based vector for AGT-AS cDNA (Figure 2). A plasmid containing AAV terminal repeats was prepared with a cassette consisting of a CMV promoter, the rat AGT cDNA based on the sequence by Lynch et al. (10). The cDNA is oriented in the antisense direction. In addition, the cassette contains an internal ribosome entry site and as a marker, the green fluorescent protein (GFP) gene (11). This vector designated pAAV-AGT-AS was transfected into rat hepatoma H-4 cells. A control plasmid, pAAV-AGT-S, contains the cDNA in the sense direction. Transgene expression was assayed by using RT-PCR of the antisense gene and green fluorescence to detect the GFP gene. At 48 h after transfection, there was clear dominant expression of GFP in the H-4 cells. AGT levels in the cells were measured by radioimmunoassay and compared between sense- and antisense-treated cells. There was a significant reduction of AGT (120 ± 14 vs 230 ± 20 ng/mg protein, $P < 0.01$). Transgene expression was detected by RT-PCR in the H-4 cells starting at 2 h and continuing for at least 72 h.

Testing the plasmid *in vivo*, where the sense and antisense plasmids were injected *iv* into SHR rats, showed that AGT-AS expression was positive in heart and lung at 3 days and in lung at 7 days. Expression in the kidney was absent or weak. A separate set of rats were injected with the antisense and their blood pressure was followed for 10

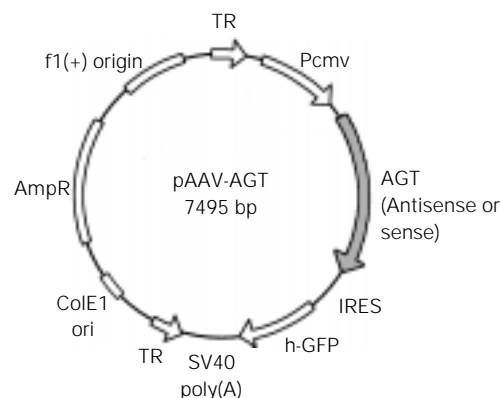
days. The SHR were 24 weeks of age when injected with 3 mg/kg plasmid. Within 24 h there was a significant drop in blood pressure and for the following 6 days the blood pressure remained at a lower level than in sense-treated rats. The drop in blood pressure correlated to a drop in plasma AGT levels which was significant at days 3 and 5 after injection. The decrease in blood pressure with injection of plasmid could be prolonged by injecting the plasmid with cationic liposomes (DOTAP/DOPE). However, the prolongation was only one day more, but the reduction in blood pressure was more profound and this was correlated to a greater decrease in plasma AGT levels when the antisense was given with the liposome.

Recombinant AAV vector delivery of antisense to angiotensinogen

To test the effect of the AGT-AS viral vector we prepared both plasmids with neomycin resistance gene and tested the vector first in vascular smooth muscle cells (VSMC) (12) and then *in vivo* by systemic injection into SHR (13).

VSMC are the main peripheral target for angiotensin II acting on AT_1 -R. The hypothesis that the renin-angiotensin system is overactive in hypertension has led to the development of AT_1 -R blockers, such as Losartan. Losartan, given in human hypertension at a dose of 3 mg/kg, can be taken orally, but it has to be on a daily basis. To test whether an AAV delivery of an AT_1 -R inhibitor with antisense would produce a more prolonged effect, we injected both adult and 5-day-old SHR. Five-day-old SHR were chosen because the quantity of recombinant AAV-AS (rAAV-AS) required is less than that needed for adults. However, at this stage hypertension has not developed. Hypertension in SHR develops between the 8th and 10th week after birth. Therefore, injecting 5-day-old SHR allowed us to observe if the development of hypertension would be re-

Figure 2 - A plasmid containing AAV terminal repeats was prepared with a cassette containing a CMV promoter (Pcmv), rat AGT cDNA in the antisense direction, an internal ribosome entry site (IRES), and the gene for green fluorescent protein (GFP). TR, Terminal repeat.



duced. A single injection of AAV-AGT-AS, or of rAAV-AT₁-AS in 5-day-old SHR, prevented the development of hypertension at week 10 (13). As the control groups treated with saline or sense continued, the difference in blood pressure became more apparent (up to 40 mmHg) (Figure 3).

In the rAAV-AGT-AS-treated SHR, measures of plasma AGT levels showed a corresponding lack of increase in AGT in the antisense-treated groups compared to the significant increase of AGT in the control animals. Correlation of AGT with blood pressure was significant ($P < 0.05$) in the control treated animals and not significant in the antisense-treated animals. This shows that AGT in the SHR is correlated with an increase in blood pressure. The AAV was expressed in kidney, heart and liver throughout the time of the reduction in blood pressure. Thus, we concluded that the early treatment with a single dose of rAAV-AGT-AS, given systemically, prevents the full development of hypertension in adult SHR by a prolonged reduction in AGT levels. Similarly, the results with the rAAV-AT₁-AS showed a reduction in hypertension development correlated with a consistent reduction in AT₁-R in VSMC.

Advantage of AAV vector

The AAV vector offers many advantages: firstly, the AAV vector has sufficient carrying capacity for an antisense transgene with a strong promoter for expression. Secondly, the AAV is very safe because it is a non-proliferating, dependent virus that induces no known pathology. Thirdly, an important requirement for treating a chronic disease such as hypertension is prolonged effect. The AAV is very stable for continuous expression of transgenes. Fourthly, the AAV does not induce an inflammatory response or activate immune responses which counteract its benefits. Lastly, AAV transduces non-dividing cells so that it is an appropriate

vector for adult tissue.

Other vectors

Other vectors are being tested for hypertension gene therapy. Adenovirus vectors have been used with kallikrein gene insertion (14). However, the adenovirus synthesizes proteins which trigger the immune system and cause inflammation which limits use in human therapy so far. Iyer et al. (15) reported that a retrovirus with antisense AT₁-R injected into newborn SHR prevents the development of hypertension in the adults. Several months after the vector was injected, hypertension failed to develop. However, PCR analysis of the vector DNA showed that the virus had disappeared by 30 days indicating that the long-term effect was due to exposure to antisense for the AT₁-R at a critical period. In all respects, the SHR had become as normal as their normotensive controls (16). Retroviruses are appropriate for dividing cells but are limited by their lack of effect in non-dividing cells and therefore are not suitable for hypertension therapy in adults. Retroviruses may be useful in treating cardiomyopathy, restenosis and vascular remodeling, where cells are actively dividing. Lentivirus vectors, which can infect slowly dividing cells, are just beginning to be explored for therapeutic value. They offer large gene-carrying capacity and are easily produced. The disadvantage is the risk of uncontrolled infection and the potential for

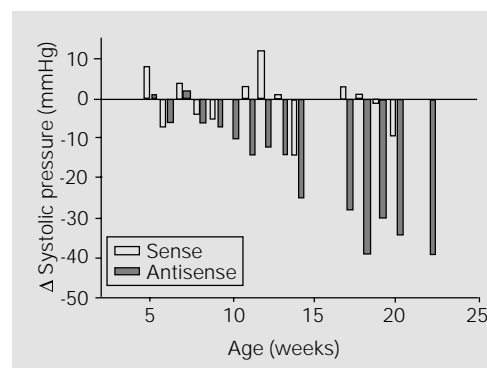


Figure 3 - Change in systolic pressure in spontaneously hypertensive rats (SHR) injected with rAAV-AGT compared to saline-injected SHR.

neoplastic changes.

In addition to the choice of vectors, the control of transgenes needs to be engineered and new promoters need to be explored. The ideal promoter will be active for prolonged periods to maintain transgene expression and specific for a tissue cell type. Furthermore, vectors need mechanisms to switch them on or off as required. This can be tested with the tetracycline transactivator system, by which a transgene can be activated in the presence (or absence) of tetracycline.

Conclusion

Both antisense oligonucleotides and antisense DNA delivered in a vector have advantages for gene therapy.

Antisense ODN: can be used like a drug; has an action that lasts for days or weeks; is specific for a target protein; reduces overactive protein, but permits normal physiology.

The AAV vector with antisense DNA: has very prolonged action (weeks/months) with a single dose; is safe, non-pathogenic and non-inflammatory.

However, there are many technical problems with respect to choice of gene, vector

delivery, efficiency of transfer, the route of administration and uptake, lack of toxicity, lack of immune response, as well as clinical effectiveness that must be understood before gene therapy for hypertension (or any disease) is acceptable. Equally clear is that the gene therapy approach is attractive as long as we have diseases that cannot be cured. The rAAV-AS strategy, in which DNA in the antisense direction expresses antisense mRNA to compete with sense mRNA, appears to be effective for reducing high blood pressure in models of hypertension. Its development could provide a new generation of antihypertensive agents that would be administered in a single dose for prolonged effects lasting several months (17). Such gene therapy would have high specificity and no pathology. Alternatively, antisense oligonucleotides are effective, and could be used like long-acting drugs to provide sustained control of hypertension with infrequent administration. It seems that AS-ODN will be clinically acceptable first because of our familiarity with drug treatments. The viral vector approach will come later, when all the basic science will have been done to assure the patient that it is safe.

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