State-of-the-Art Lecture

Antisense Inhibition and Adeno-Associated Viral Vector Delivery for Reducing Hypertension

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Abstract Antisense oligodeoxynucleotides have been designed to inhibit the production of specific proteins. In models of hypertension, we have targeted the renin-angiotensin system at the level of synthesis (angiotensinogen) and the receptor (AT1 receptor). The design of antisense oligonucleotides requires choosing a site to inhibit mRNA processing or translation. The strategy we use is to make three oligonucleotides of antisense sequences, upstream and downstream from the AUG site and over the AUG site. The oligonucleotides are tested in a screening test. Antisense oligonucleotides to AT1-receptor mRNA and to angiotensinogen mRNA reduce blood pressure in spontaneously hypertensive rats when injected into the brain. They significantly reduce the concentration of the appropriate protein. The oligonucleotides are also effective when administered systemically. The decrease in blood pressure with antisense oligonucleotides delivered in blood or brain lasts 3 to 7 days. To prolong the action, direct injection of naked DNA and injection of DNA in liposome carriers have been tested. Viral vectors have been developed to deliver antisense DNA. The viral vectors available include retroviruses and adenovirus, but the adeno-associated virus (AAV) vector is the vector of choice for ultimate use in gene therapy. It offers safety because it is nonpathogenic, has longevity because it integrates into the genome, and has sufficient carrying capacity to carry up to 4.5 kb antisense or gene in a recombinant AAV. Using rAAV-antisense to AT1, mRNA, there is efficient transfection into cells and an inhibition of AT1 receptor number. In in vivo tests, rAAV-AS AT1-receptor when injected into the brains of SHR reduces blood pressure for more than 2 months. In young rats (3 weeks old), rAAV-AS AT1-receptor decreases blood pressure and slows the development of hypertension. While further experiments need to be done on dose-response relationships and on the cellular mechanisms of these effects, the results show the feasibility of AAV as a vector for antisense inhibition, which may ultimately be used in gene therapy for hypertension (Hypertension. 1997;29[part 2]:177-187.)

Key Words • antisense oligonucleotide • adeno-associated viral vector • AT1 receptor • angiotensin

Hypertension is one of the most important risk factors for myocardial infarction, stroke, congestive heart failure, end-stage renal disease, and peripheral vascular disease. Twenty-four percent (an estimated 43 million) of the adult, civilian, noninstitutionalized population of the United States has hypertension. The incidence of hypertension is slightly more prevalent among men than women. Many drugs are available for hypertension control, including new drugs that inhibit the Ang receptor or inhibit calcium channels. All present drug regimens suffer from one obvious disadvantage. Since the disease of hypertension is lifelong, treatments have to be repeated daily. In the case of drug treatment, compliance with a daily ration of pills by the patients is difficult to maintain. Our goal in developing a gene therapy approach to hypertension is to find effective ways of modifying genes chronically in vivo. We have used antisense sequences and delivery systems that allow a single dose of a specific gene antisense to have prolonged effects over several days, weeks, or months.

Although hypertension is a polygenic disease, single genes may play significant roles that can be modified by gene regulation. Lifton has contributed greatly to identifying rare examples of single-gene causes of hypertension and isolated various defects in the fundamental cellular processes, such as the Na+-Cl− transporter gene (Gitelman’s syndrome), epithelial Na+ channel subunits (Liddle’s syndrome and pseudohypoaldosteronism-I), and Na+-K+-2Cl− cotransporter, which is mutated in Bartter’s syndrome. Hypertension is a polygenic disease with genes overexpressing or underexpressing steroids, peptides, and lipids. In the expression of genes encoding components of the RAS, at least two mutations have been linked to hypertension. These include the angiotensin-converting enzyme (ACE) genes, D (deletion) mutation, and the angiotensinogen T235 variant. Inserting genes such as the renin gene to produce transgenic animals results in the animals developing hypertension. The opposite to gene insertion is gene knockout. Knockout genes for angiotensinogen production result in hypotension in mice. Antisense inhibition offers a different approach to knockout because it can be used in adult animals, whereas gene knockout must be done in embryos, and therefore the adult knockout animal is one that has survived development without a specific gene. A new idea with potential has been the production of transgenic rats with an antisense gene to angiotensinogen.

In SHR, Ang II is increased in the brain but normal in the periphery. In addition, AT1 receptors are overabundantly expressed in the brain of the hypertensive SHR compared with that of the normotensive rat.

The apparent increased activity of Ang in brain tissue of SHR gave us a starting point for testing the effectiveness of antisense inhibition in lowering blood pressure in hypertensive animals. Earlier experiments had shown that a single injection of the nonspecific Ang antagonist Sar1Ile5Ang II injected into the brain produced a brief but
Selected Abbreviations and Acronyms

AAV = adeno-associated virus  
AAV-AS = adeno-associated virus–antisense  
Ang = angiotensin  
AS-ODN = antisense oligodeoxynucleotide  
AT = angiotensin type II  
AVP = arginine vasopressin  
CMV = cytomegalovirus  
ITR = inverted terminal repeat  
LTR = long terminal repeat  
ODN = oligodeoxynucleotide  
pAAO = plasmid for angiotensinogen antisense  
paAT1 = plasmid for AT1-receptor antisense  
PCR = polymerase chain reaction  
RAAV = recombinant adeno-associated virus  
RAS = renn-angiotensin system  
RT-PCR = reverse transcription–polymerase chain reaction  
SHR = spontaneously hypertensive rats  
TGf = transforming growth factor  
TRH = thyrotropin releasing hormone  
VSMC = vascular smooth muscle cell  
WKY = Wistar Kyoto rats  
wtAAV = wild-type adeno-associated virus

significant decrease in blood pressure at a dose that was ineffective when given peripherally. Normotensive rats did not respond to central injections of Ang II antagonists. Therefore, it was hypothesized that in the SHR, the hypertension is associated with an overactive brain tissue system. We designed antisense oligonucleotides to specifically inhibit Ang II receptors or angiotensinogen to produce longer-lasting effects than a single injection of the pharmacological antagonist. The brain was convenient tissue to work on because of the relatively small volumes of oligonucleotides required.

Antisense inhibition has been developed, particularly in cell culture applications, to the point where it is being tested in clinical trials for HIV and cancer. The pros and cons of its use have been reviewed elsewhere. Before 1992, however, antisense had not been applied in vivo with any success. There was much concern about the efficiency of cellular uptake of oligonucleotides. As so often happens in science, three or four or more labs independently found that most AS-ODNs quickly migrate to the cell nucleus, and it is normally involved in DNA duplication. The role of RNA has been proposed. Based on studies of cellular uptake of labeled oligonucleotides, the picture emerged that AS-ODNs are acting in the cytosol and do not affect measurable mRNA levels. Indeed, there are several articles reporting antisense effects without detectable change in target mRNA levels.

Alternatively, the mechanism can be by reduction of mRNA. Decreased mRNA levels can occur by RNase H digestion of the RNA portion of the mRNA antisense DNA hybrid. RNase H is found in the cytoplasm as well as in the nucleus, and it is normally involved in DNA duplication. The role of RNase H is to cleave RNA that has bound to DNA. Activation of RNase H is advantageous because the enzyme leaves the AS-ODN intact so it is free to hybridize with another mRNA, making the reaction catalytic rather than stoichiometric. Besides inhibition of translation, other possible antisense mechanisms of action have been proposed. Based on studies of cellular uptake of labeled oligonucleotides, the picture emerged that most AS-ODNs quickly migrate to the cell nucleus, suggesting an intranuclear site of action.

Antisense DNA can hybridize to its target mRNA or pre-mRNA in the nucleus, forming a partially double-stranded structure that would inhibit its transport out of the nucleus into the cytoplasm, thus preventing translation. AS-ODNs targeted to intron-exon junction sites prevent the splicing process and consequently the maturation of the transcript. Therefore, antisense molecules might inhibit pre-RNA splicing or the transport of mRNA from the nucleus.

Table 1 lists the characteristics of AS-ODNs that need to be incorporated into their design and use. The concept of antisense inhibition assumes that a short DNA sequence in the antisense direction binds to the specific mRNA of the target protein in the cytoplasm and prevents either ribosomal assembly or read-through of the message. Most AS-ODNs are therefore targeted to the gene initiation codon (AUG) or part of the coding region downstream from it. Antisense oligonucleotides are 15 to 20 bases long, but longer or full-length DNA in the antisense direction is used in viral vectors. When designing antisense molecules, one has to consider two antagonistic factors, the affinity of oligonucleotide to its target sequence, which is dependent on the number and composition of complementary bases, and the availability of the target sequence, which is dependent on the folding of the mRNA molecule.

Several reports suggested that AS-ODNs targeted to different regions of the RNA have unequal efficiencies. These differences may be related to the predicted secondary structure of the target mRNA. The folding of the mRNA influences target- (sequence availability. The RNA double helices that are responsible for the secondary structure of the mRNA incorporate a weaker G-U base pairing next to A-U and G-C and are generally short and rarely perfect. Therefore, the design should avoid G repeats. Burgess and Farrell showed that when repeated G sequences appear in the oligonucleotide, the effects produced can be due to nonantisense mechanisms. Proper testing of antisense requires a sense ODN and a mismatch ODN control for each AS-ODN. An ODN that has strong Watson-Crick base pairing with 100% complementarity will form the more thermodynamically favorable structure with its target RNA.

AS-ODNs have several potential sites of action. AS-ODNs inhibit translation by hybridizing to the specific mRNA for which they are designed, and the hybridization prevents either ribosomal assembly or ribosomal sliding along the mRNA. This kind of action assumes that AS-ODNs are acting in the cytosol and do not affect measurable mRNA levels. Indeed, there are several articles reporting antisense effects without detectable change in target mRNA levels.

Table 1. Conditions for Antisense Oligonucleotide Inhibition

<table>
<thead>
<tr>
<th>Condition</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA sequence</td>
<td>is specific and unique</td>
</tr>
<tr>
<td>Uptake into cells</td>
<td>is efficient</td>
</tr>
<tr>
<td>Effect in cells</td>
<td>is stable</td>
</tr>
<tr>
<td>There is no nonspecific binding to proteins</td>
<td></td>
</tr>
<tr>
<td>Hybridization of the ODN is specific to the target mRNA</td>
<td></td>
</tr>
<tr>
<td>The targeted protein and/or mRNA level is reduced</td>
<td></td>
</tr>
<tr>
<td>The ODN is not toxic</td>
<td></td>
</tr>
<tr>
<td>There is no inflammatory or immune response induced</td>
<td></td>
</tr>
<tr>
<td>The ODN is effective compared with appropriate sense and mismatch ODN controls</td>
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</table>
clues to the cytoplasm. An alternate anti-gene strategy is to target the DNA with triplex-forming oligonucleotides to block DNA transcription. Effective AS-ODNs have been designed targeting exon-intron splicing sites or the major groove of the DNA, but triplex formation is corrected by DNA repair mechanisms.

Currently, the three regions that are considered to be the best targets for designing effective AS-ODNs are the 5' cap region, the AUG translation initiation codon, and the 3' untranslated region of the mRNA. Since most mRNAs have an AUG initiation codon, targeting 12 to 15 of the neighboring bases should produce inhibitory ODNs. Routinely, all designed oligonucleotides must be checked with the GenBank for existing sequences to avoid any homology with other mRNAs. Essentially, antisense design boils down to trial-and-error testing in a model first. A general rule suggested by our own experience is that for a 15-mer oligonucleotide, three different sites should be tested with the expectation that at least one will work. Obviously, it is desirable to have a rapid screening test in vitro or in vivo for the specific protein that the AS-ODN has been designed to inhibit. Controls are sense ODN and mismatch (one or more nucleotides different from AS) or scrambled, where the entire sequence is random.

Stability of Oligonucleotides

Oligonucleotides in their natural form as phosphodiester oligonucleotides are subject to rapid degradation in the blood, intracellular fluid, or cerebrospinal fluid by exonucleases and endonucleases. The half-life of phosphodiester oligonucleotides is in the range of minutes in blood and tissue culture media. The half-life of oligonucleotides is somewhat longer in cerebrospinal fluid, and intact oligonucleotides can be detected 24 hours after injection into the cerebral ventricles. Several chemical modifications have been proposed to prolong the half-life of oligonucleotides in biological fluids and enhance their uptake while retaining their activity and specificity.

The most widely used modified oligonucleotides are phosphorothioates, in which one of the oxygen atoms in the phosphodiester bond between nucleotides is replaced with a sulfur atom. These phosphorothioate oligonucleotides have greater stability in biological fluids than normal oligonucleotides. The half-life of a 15-mer phosphorothioate oligonucleotide is 9 hours in human serum, 4 days in tissue culture media, and 19 hours in cerebrospinal fluid. Phosphorothioate oligonucleotides can be synthesized with automated DNA synthesizers, but the product may contain impurities unless purified on an affinity gel. Oligonucleotides should be checked to ensure that they are pure.

One or more of the oxygen atoms in the phosphodiester bond can be replaced with a variety of other compounds, such as methyl groups (methylphosphorothioate), alkyl phosphonester, phosphoramidate, or boranophosphate, all of which expand the half-life of oligonucleotides in vivo experiments. Some clever new designs are being tried in which the ODN has a dumbbell shape produced by a hairpin extension at the 3' end. It is hoped that these third- or fourth-generation ODNs will provide longer-lasting stability, enhanced uptake kinetics, and affinity for the target.

Cellular Uptake of Oligonucleotides

To hybridize with the target mRNA, AS-ODNs have to cross the cell membrane. Saturable uptake of oligonucleotides reaches a plateau within 50 hours, occurs rapidly, and, depending on the cells, the uptake can be efficient. Uptake is faster for shorter oligonucleotides than for longer ones. Decreasing the temperature prevents oligonucleotide uptake, indicating that there is an active uptake mechanism. An 80-kD oligonucleotide-binding protein has been proposed to be the receptor molecular for oligonucleotide uptake. An efflux mechanism has also been described indicating temperature-dependent secretion of the oligonucleotides from the cells to the extracellular space.

Pharmacology of AS-ODNs

Antisense inhibition can be considered pharmacologically a drug-receptor interaction in which the oligonucleotide is the drug and the target sequence is the receptor. For binding to occur between the two, a minimum level of affinity is required, which is provided by hydrogen bonding between the Watson-Crick base pairs and base stacking in the double helix that is formed. To achieve pharmacological activity, a minimum number of 12 to 15 bases can provide the minimum level of affinity. Longer sequences are more specific, but above 20 bases, problems of cell uptake begin to reduce the effectiveness of ODNs.

One of the main advantages of antisense inhibition is the specificity of the AS-ODN target-sequence interaction provided by the Watson-Crick base pairing. An oligonucleotide 12 to 15 nucleotides long is specific enough statistically to be complementary to a single sequence. Increasing the length of the antisense should result in a higher level of specificity, but it decreases its uptake into cells. With viral vectors, however, the uptake problem is overcome because the virus freely enters cells by binding to viral receptors on cell membranes. Therefore, in a viral vector a full-length DNA antisense sequence can be used. The mechanism of action of antisense DNA is different from that of the AS-ODN. The antisense DNA produces an antisense mRNA that competes negatively with mRNA in the cytoplasm.

Toxicity

AS-ODNs can inhibit protein synthesis in cultured cells in nanomolar doses. The therapeutic window for AS-ODNs is rather narrow; when testing for the optimal dose, small increments in the high nanomolar range should be tested. High concentrations may produce nonspecific binding to cytosolic proteins and give misleading results.

Phosphodiester oligonucleotides are degraded to their naturally occurring nucleotide building blocks relatively quickly; therefore, no toxic reaction is expected from even high doses of phosphodiester. Studies on phosphorothioate oligonucleotides in rats show that after intravenous injection, phosphorothioated oligonucleotides are taken up from the plasma mainly by the liver, fat, and muscle tissues. Phosphorothioate oligonucleotides are excreted through the urine in 3 days, mainly in their original form. An apparent mild increase in plasma lactate dehydrogenase, and to a lesser extent indicators of a possible transient liver toxicity, has been noted with very high doses of phosphorothioated oligonucleotides. Whole new classes of oligonucleotide backbone modifications of phosphorothioate oligonucleotides are being developed to avoid the possible liver toxicity in humans.
Antisense and Hypertension

The role of candidate genes in the pathogenesis of hypertension can be studied by inhibition of the specific, overexpressed target gene with AS-ODNs. This approach is being developed in rat models of hypertension. Table 2 lists all the current in vivo studies of antisense inhibition in which blood pressure has been measured.

The first study on antisense and hypertension was by Gyurko et al., who used synthetic AS-ODN, delivered intracerebroventriculally, in SHR with established hypertension. Based on data indicating an overactive brain RAS is involved in the hypertension of this rat model, we applied antisense inhibition to the RAS (Fig 1). We designed an AS-ODN constructed to bases -5 to +13 of angiotensinogen mRNA (18 mer) and a 15-mer oligonucleotide to bases +63 to +77 of AT2-receptor mRNA. The AT2 receptor-AS-ODN was phosphorothioated, and the angiotensinogen mRNA AS-ODN was a phosphodiester. In both cases, hypertension was significantly reduced by the application of a single dose of 50 μg AS-ODN. The mean arterial blood pressure recorded chronically was reduced by up to 35 mm Hg, in most cases lowering the high blood pressure to normotensive levels. These effects were measured 8 and 24 hours after treatment. The effects began at 8 hours and were significant at 24 hours. The single dose of AS-ODN to the AT2-receptor mRNA produced a long-lasting, 7-day decrease in blood pressure. AT1 receptors were measured to test that the targeted protein had been decreased after this treatment. AT1-receptors were significantly reduced in the paraventricular nucleus and in the anterior third ventricle area. After administration of antisense to angiotensinogen mRNA, brain Ang II levels were significantly reduced (P < 0.05), indicating inhibition of the brain renin-Ang II synthesis system. Blood pressure in these animals also decreased significantly for several days. The results indicated that by inhibiting the brain RAS by AS-ODNs to the major substrate or the major receptor, high blood pressure was effectively lowered. Follow-up studies investigated how much decrease there was in the receptor and angiotensinogen levels with these oligonucleotides and also investigated the other effects of Ang II.

Ambuhl et al. quantified the reduction in Ang receptor after AS-ODN against mRNA for the AT1 receptor. We used autoradiography, quantitative analysis, and membrane binding. Control injections contained sense or scrambled oligonucleotides or saline vehicle. Three daily injections of AS-ODN into the third ventricle of Sprague-Dawley rats decreased AT1-receptor numbers significantly by 25% in hypothalamic tissue block AT1 receptors were not affected by the AT1 antisense.

Table 2. Use of Antisense Oligodeoxynucleotides to Reduce Hypertension In Vivo

<table>
<thead>
<tr>
<th>Antisense Target mRNA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensinogen (phosphorodiester) and Ang</td>
<td>Gyurko et al., 1993</td>
</tr>
<tr>
<td>AT2 receptor (phosphorothioate)</td>
<td>Phillips et al., 1994</td>
</tr>
<tr>
<td>Angiotensinogen (phosphorothioate) ICV</td>
<td>Wielbo et al., 1996</td>
</tr>
<tr>
<td>Angiotensinogen (phosphorothioate) in Sendai virus and liposomes via hepatic vein</td>
<td>Tomita et al., 1994</td>
</tr>
<tr>
<td>Angiotensinogen (phosphorothioate) delivered in hypothalamus intraventricularly</td>
<td>Wielbo et al., 1996</td>
</tr>
<tr>
<td>c-fos (phosphorothioate) ICV brain stem</td>
<td>Suzuki et al., 1994</td>
</tr>
<tr>
<td>TRH (phosphodiester) ICV</td>
<td>Suzuki et al., 1995</td>
</tr>
</tbody>
</table>

ICV indicates intracerebroventricular.
oligonucleotide occurred. Much of it was close to the site of injection and the surrounding brain nuclei. This reflects the rapid uptake of ODN by brain cells. No decreases in mRNA were found, and it was concluded that the angiotensinogen antisense inhibited mRNA translation and not transcription.

Injections in the brain, however, are obviously not practical for hypertension therapy. A more practical route is by oral intake or injection into the peripheral blood. Tomita et al. demonstrated a decrease in high blood pressure by AS-ODN against rat angiotensinogen mRNA by delivering three antisense ODNs into the portal vein. The ODNs were encapsulated in liposomes that contained viral agglutinins. The viral carrier, in this case, was a Sendai virus that had been shown to be efficient in promoting fusion with target cells by Kaneda et al. The effect of the ODNs was to produce a transient decrease in plasma angiotensinogen levels in SHR. This decrease in protein was correlated to a decrease in blood pressure and a reduction in hepatic angiotensinogen mRNA. Plasma Ang II concentration was also decreased in these rats. The reduction in blood pressure, however, was shorter lasting (1 to 4 days) than the reductions in hormone (1 to 7 days). This discrepancy has not been explained. Recently Wielbo et al., in a follow-up study of injecting antisense centrally, succeeded in showing a reduction in blood pressure by a single intravenous injection of angiotensinogen ODN encapsulated in a liposome (cholesterol plus phospholipids). Using an FITC label, they showed that the intravenous route delivered the ODN mostly to the liver. Liver angiotensinogen was reduced after antisense treatment. This study opens the possibility of effective direct delivery through intravenous injection with antisense prepared in a liposome. Preliminary work in a different model of hypertension indicates that naked DNA is also effective.

Galli et al. injected AS-ODN to angiotensinogen mRNA in 2-kidney, 1 clip rats. These rats have high circulating Ang II, which causes the hypertension. The AS-ODN in a single dose (100 μg IV) lowered blood pressure significantly for several (∼17) days.

Antisense TRH receptor has also been shown to reduce arterial blood pressure in SHR. Here, an intrathecal approach was used to deliver AS-ODN complementary to TRH receptor mRNA. The rats were WKY and SHR. The antisense was an 18-base phosphodiester, and the rats received 100 μg per day for 3 days. Twenty-four hours after the last injection, the magnitude of the response to an intrathecal TRH injection (10 μg) was significantly reduced in the antisense-treated group. In separate experiments, mean resting arterial blood pressure was significantly reduced by the antisense treatment in SHR but was not affected in WKY. The blood pressure in these rats was relatively low (156±4.8 mm Hg) but was reduced to 119±8.8 mm Hg (P<.01). No efforts were made to measure a change in the TRH receptors after the treatment in this study. Without showing that TRH was reduced, one cannot be sure that the antisense has reduced blood pressure by the mechanism of inhibiting receptor protein synthesis.

In another study by the same group, Suzuki et al. injected antisense to c-fos mRNA in the rostral ventral medullar of SHR rats, and mean arterial pressure in anesthetized rats was significantly reduced at 6 hours compared with sense-treated controls. In this experiment, the protein (c-fos) was shown to be reduced in the brains of the antisense-treated rats. In all the studies on SHR in which WKY normotensive controls have been used, including our own, no effects of antisense have been found on the WKY. This indicates that antisense is useful for reducing overactive hormonal systems, such as the RAS, but does not interfere with the normal physiological functions of the hormonal system.

Several studies report applying AS-ODNs to cell cultures relevant to hypertensive mechanisms. Smooth muscle cell proliferation is a critical feature of many forms of hypertension. The oncogene c-myc and the protein myosin are believed to be involved in this process. Simmons et al. used antisense to nonmuscle myosin heavy chain or c-myc phosphorothioated ODNs to inhibit proliferation of smooth muscle cells in vitro. The growth suppression correlated with reductions in the concentrations of nonmuscle myosin heavy chain and c-myc RNA and their corresponding proteins. Sense controls were without effect. The effects were long lasting, up to 72 hours. TGF-β is also implicated in the faster growth observed in VSMCs from SHR compared with normotensive controls. AS-ODN complimentary to TGF-β1 mRNA significantly suppressed VSMC number in SHR but not in WKY. Plasmid, an activator of TGF-β, did not stimulate DNA synthesis in the presence of the AS-ODN. This cellular study suggests that endogenous TGF-β plays a role in the hypertension of SHR through the increased growth of VSMCs.

Important pathophysiological events, such as arterial restenosis after balloon catheterization, have been effectively influenced with AS-ODNs. Inhibition of two cell-cycle regulatory enzymes, cyclin-dependent kinase 2 and cell division cycle 2 kinase, with AS-ODNs resulted in a nearly complete abolition of neointima formation, which is thought to be responsible for arterial restenosis. Application of c-myc AS-ODNs in a pluronic gel to the outside of balloon-catheter–injured blood vessel resulted in a 75% decrease in c-myc expression and significantly reduced neointima formation. This result also suggests the remarkable penetrating ability of the oligonucleotides by which they reached the neointima of the rat carotid from the outside of the vessel.

The outcome of cerebral infarction can be influenced with AS-ODNs. Synthesis of NMDA receptors, which are thought to be responsible for neuronal death after cerebral vascular occlusion, can be inhibited by antisense treatment. Such an inhibition reduces the volume of the infarction produced experimentally by the occlusion of the middle cerebral artery.

Delivery of Antisense

Table 3 shows the currently used methods of delivering antisense for hypertension and some of their advantages and disadvantages.

Naked DNA

Direct injection of the antisense DNA has been used in the experiments described above. For injections into the brain, naked DNA appears to be very successful. In a number of studies using different AS-ODNs, there has been efficient uptake and effective reduction in protein and inhibition of the physiological parameters studied. Uptake is so efficient that one difficulty with intracerebroventricular injections is that the DNA tends to be taken up close to the site of injection and not to spread to other parts of the brain. While this has little impact for hypertension therapy, it is an important consideration in antisense strategies for
TABLE 3. Vectors for Gene Transfer in Hypertension

<table>
<thead>
<tr>
<th>Vector</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naked DNA</td>
<td>Administered directly into tissue by injection</td>
<td>Difficult to control sites of uptake</td>
</tr>
<tr>
<td>Gyurko et al., 1995</td>
<td></td>
<td>Some tissues preferential, eg, liver, kidney, blood vessels</td>
</tr>
<tr>
<td>Wang et al., 1995</td>
<td></td>
<td>DNA copy number unquantifiable</td>
</tr>
<tr>
<td>Lin et al., 1995</td>
<td></td>
<td>DNA copy number unquantifiable</td>
</tr>
<tr>
<td>Phillips et al., 1994</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liposome-encapsulated</td>
<td>Easy to produce</td>
<td>May facilitate uptake</td>
</tr>
<tr>
<td>oligonucleotides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wielbo et al., 1996</td>
<td></td>
<td>Preferential uptake in certain tissues, eg, liver</td>
</tr>
<tr>
<td>Tomita et al., 1994</td>
<td></td>
<td>Toxic to some tissues, eg, brain</td>
</tr>
<tr>
<td>Retrovirus</td>
<td>Efficient transfer</td>
<td>Only useful in dividing cells</td>
</tr>
<tr>
<td>Liu et al., 1995</td>
<td></td>
<td>May damage non-target dividing cells</td>
</tr>
<tr>
<td>Katovich et al., 1996</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Produced without difficulty in high titters</td>
<td>Invokes inflammatory response to high titters</td>
</tr>
<tr>
<td>Willard et al., 1992</td>
<td></td>
<td>Copy number not controlled</td>
</tr>
<tr>
<td>AAV</td>
<td>Integrates into the genome</td>
<td>Does not integrate into the genome (episomal)</td>
</tr>
<tr>
<td>Wu et al., 1994</td>
<td></td>
<td>Difficult to produce high titters</td>
</tr>
<tr>
<td>Phillips et al., 1994</td>
<td></td>
<td>Small loading capacity (4-5 Kb)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Effective</td>
</tr>
</tbody>
</table>

FIG 2 Schematic of wtAAV and rAAV vectors. In rAAV-gfp vector, almost all of the parental wtAAV vectors have been deleted except for the terminal repeats and replaced with gfp, driven by a CMV promoter (p) and the neomycin resistance (neo) gene has been inserted with a thymidine kinase promoter (TKp). The neo serves for selection in vitro and as a reporter gene in vitro or in vivo when PCR and RT-PCR are used to detect it. In pAAV-AS vectors, the gfp gene is replaced by a 750-bp fragment of the ATu receptor gene with cDNA in antisense orientation CMVp indicates human CMV early promoter, gfp, A Vicente GfP gene, and neo', neomycin phosphotransferase gene from Tn5. Other promoters have been substituted for CMV including AVP, neuron specific enolase, and glial fibrillary acid protein promoters.

Wielbo et al. [43] compared liposome-encapsulated antisense and naked DNA given intra-arterially. They found that only liposome encapsulation was effective, whereas naked DNA was not, under the same conditions. Twenty-four hours after 50 µg liposome-encapsulated AS-ODN, blood pressure decreased 25 mm Hg. Empty liposomes showed no effect, and liposome-encapsulated scrambled ODN had no significant action Unencapsulated AS-ODN also had no significant effect on blood pressure Confocal micrographs of rat liver tissue 1 hour after intra-arterial injection of 50 µg unencapsulated FITC antisense or liposome-encapsulated FITC-conjugated antisense showed intense fluorescence in liver tissue sinusoids with the liposome-encapsulated ODN Levels of protein (angiotensinogen and Ang II in the plasma) were significantly reduced in the liposome-encapsulated ODN group Antisense alone, lipids alone, and scrambled ODN in liposomes were without effect on protein levels

Liposome development with cationic lipids allows high transfection efficiency of plasmid DNA. The short, single-stranded AS-ODNs are not actually encapsulated but are complexed with micromolar vesicles by electrostatic interactions. This simplifies the production of antisense delivery system and allows for a variety of routes of delivery, including aerosol nasal sprays and parenteral injections

Viral Vectors

There are several viruses that have been tested for gene delivery, and each has its advantages but does not fit perfectly to the description of the "ideal viral vector". To be the perfect vector, a virus should fulfill all of the following criteria

1. The vector should be safe. This means that it cannot be a virus known to cause disease, or it has to be rendered harmless. The viral vector should not elicit an immune or inflammatory response. It should not integrate into the genome, randomly carrying the risk of disrupting other cellular genes and mutagenesis. The virus also has to be replication deficient for the prevention of the spread to other tissues or infection of other individuals.

An ideal vector would deliver a defined gene copy number into each infected cell...
2. In addition, the vector must be efficiently taken up in target tissue. The virus has to infect the target cells with high frequency to achieve biological effect.

3. To be practical, the vector should be easy to manipulate and produce in pure form. The virus should be able to accommodate the gene of interest, along with its regulatory sequences, and the recombinant DNA has to be packaged with high efficiency into the viral capsid proteins.

**Retroviruses**

These have been used primarily because of their high efficiency in delivering genes to dividing cells. Retroviruses permit insertion and stable integration of single-copy genes. Although effective in cell culture systems, they randomly integrate into the genome, which raises concerns about their safety for practical use in vivo. Retroviruses can only act in dividing cells, which makes them ideal for tumor therapy but less desirable where other cells are dividing that need to be protected. In hypertension research, they are being investigated in developing SHR. Researchers at the University of Florida delivered a retrovirus vector (LNSV) containing an antisense DNA to AT receptor mRNA. Injections in the heart of 6-week-old SHR resulted in effective long-term inhibition of AT receptor mRNA and significant inhibition of the development of hypertension. Several measures indicated that the AT receptors in vessels were reduced in responsiveness to the treatment.

**Adenoviruses**

Adenoviruses vectors have been tested successfully in their natural host cells, the respiratory endothelia, as well as other tissues such as vascular smooth and striated muscle and brain. Adenovirus is a double-stranded DNA with 2700 distinct adenoviral gene products. The virus infects most mammalian cell types because most cells have membrane receptors. Viruses enter the cell by a receptor-induced endocytosis and translocate to the nucleus. Most adenovirus vectors in their current form are episomal, that is, they do not integrate into the host DNA. They provide high levels of expression, but the episomal DNA will invariably turn inactive. In some species, e.g., mice, this may be a long time compared with their life span, but in humans it is a limitation of the virus as a vector. Repeated infections result in inflammatory response with consequent tissue damage. This is because the adenovirus expresses genes that lead to immune cell attacks. This further limitation makes current recombinant adenovirus vectors unsuitable for long-term treatment, and several gene therapy trials using adenovirus have failed to produce acceptable results. Preliminary studies with adenovirus vectors for delivery of AT, receptors in mRNA antisense have been tested in rats and reduce developing hypertension in SHR. The adenovirus is easy to produce and therefore useful for animal studies of mechanisms. However, the adenovirus as a vector has too many limitations at present to be successful in human gene therapy. Further engineering of the adenovirus may eventually avoid these limitations.

**Adeno-Associated Virus**

The AAV has been gaining attention because of its safety and efficiency. It has been successfully used for delivering antisense RNA against α-globin and HIV LTR, and it is our vector of choice for delivering antisense targeted to the AT-1 receptor in hypertensive rat models.

AAV is a parvovirus, discovered as a contamination of adenoviral stocks. It is a ubiquitous virus (antibodies are present in 85% of the US human population) that has not been linked to any disease. It is also classified as a dependovirus, because its replication is dependent on the presence of a helper virus, such as adenovirus. Five serotypes have been isolated, of which AAV-2 is the best characterized. AAV has a single-stranded linear DNA that is encapsulated into capsid proteins VP1, VP2, and VP3, to form an icosahedral virion of 20 to 24 nm in diameter.

The AAV DNA is approximately 4.7 kilobases long. It contains two open reading frames and is flanked by two ITRs (Fig 2). There are two major genes in the AAV genome: rep and cap. Rep codes for proteins responsible for viral replication, whereas cap codes for capsid protein VP1-3. Each ITR forms a T-shaped hairpin structure. These terminal repeats are the only essential cis components of the AAV for chromosomal integration. Therefore, the AAV can be used as a vector with all viral coding sequences removed and replaced by the cassette of genes for delivery. Three viral promoters have been identified and named p5, p19, and p40, according to their map position. Transcription from p5 and p19 results in production of rep proteins, and transcription from p40 produces the capsid proteins. For more powerful expression, we have inserted a CMV promoter. Other promoters are being tested that are specific to certain cells, including AVP promoter for cells synthesizing AVP, neuron-specific enolase, and glial fibrillary acid protein.

Upon infection of a human cell, the wtAAV integrates to the q arm of chromosome 19. Although chromosomal integration requires the terminal repeats, the viral components responsible for site-specific integration have been recently targeted to the rep proteins. With no helper virus present, AAV infection remains latent indefinitely. Upon superinfection of the cell with helper virus, the AAV genome is excised, replicated, packaged into virions, and released to the extracellular fluid. This fact is the basis of rAAV production for research.

There are several factors that prompted researchers to study the possibility of using rAAV as an expression vector. One is that the requirements for delivering a gene to integrate into the host chromosome are surprisingly few. It is necessary to have the 145 bp ITRs, which are only 6% of the AAV genome. This leaves room in the vector to assemble a 4.5-kb DNA insertion. While this carrying capacity may prevent the AAV from delivering large genes, it is amply suited to delivering small genes and antisense cDNA. Capacity is sufficient to ensure a specific gene response, a potent promoter, and a marker and/or selecton gene, such as a neomycin resistance gene.

The second characteristics that makes AAV a good vector candidate is its safety. There is a relatively complicated rescue mechanism. Not only adenovirus (wild type) but also AAV genes are required to mobilize the rAAV. The spread of rAAV vectors to nontarget areas can be limited to certain tissues. AAV is not pathogenic and is not associated with disease. The removal of viral coding sequences in producing a rAAV minimizes immune reactions to viral gene expression, and therefore rAAV does not evoke an inflammatory response (in contrast to the recombinant adenovirus).
The final cassette also contains promoters such as CMV promoter. The AAV is grown in cells in the presence of adenovirus as a helper virus, and the cassette is packaged in the adenovirus. The AAV is taken up by cells and expressed for several weeks. The result shows that rAAV injected into the brain is taken up by cells and expressed for several weeks. The brain is taken up by cells and expressed for several weeks.

**Expression of Antisense Sequences With Vectors**

The general concept for antisense gene delivery in the AAV vector and the steps involved are shown in Fig 3. To illustrate these steps, a brief description is given that is applicable to hypertension. Further details are presented in Reference 72.

We constructed plasmids for both AT1-receptor antisense (paAT1) and angiotensinogen antisense (paAo) into the AAV-derived expression vector. Initially we used a plasmid containing AAV genome and 750 bp DNA inserted into the AAV in the antisense direction downstream from the promoter. The plasmid was transfected with lipofectamine into NG108-15 cells (for the paAT1) or hepatoma H4 cells for the paAo. In both cases, there were significant reductions in the appropriate proteins, namely AT1 receptor and angiotensinogen. To test that the cells expressed AAV, we used the rep gene product as a marker. Immunocytochemical staining with a rep protein antibody showed that the majority of cells in culture fully expressed the vector. A further development of the AAV was the insertion of more powerful and specific promoters than the p40 promoter. AAV with CMV promoter and neomycin resistance (neo) gene as a selectable marker is now being used in our current experiments on antisense AT1-receptor mRNA. The AAV cassette contains either full-length DNA in the antisense direction or markers, green fluorescent protein (gfp) gene or lacZ gene. The NG108-15 cells with AAV plasmid containing the neo gene were selected by antibiotic, G418 (600 µg/mL), and the selected clones viewed for gfp expression. Two weeks after transfection, all of the cells were expressing gfp (Fig 4). There is a latency for AAV integration of 1 to 2 weeks, then expression increases. Transfection efficiency of this AAV/gfp in different cell lines including AT120 (mouse pituitary cells), L929 (mouse fibroblasts), HEK239 (human embryonic kidney cells), and NG108-15 (neoblastoma cells) is over 50%. Expression in vivo was tested by direct injection into the brain. An AAV with an VP to drive a lacZ gene was constructed. The vector expressed β-galactosidase in neurons of the paraventricular nucleus and supraoptic nucleus. The expression was in magnocellular cells, which normally express AVP (Fig 5). The expression was observed at 1 day, 1 week, and 1 month with no diminution of signal. This is an example of how AAV can be developed for specific tissue and/or cell gene expression and shows that AAV vectors can deliver foreign genes into adult brain for long periods of time.

**Functional Tests**

To prepare the antisense vector, a large fragment of the Ang receptor cDNA (750 bp) was amplified using PCR and ligated to an AAV-derived vector in the antisense orientation, in place of gfp. The resulting vector (rAAV-AS) contained AAV ITRs, a CMV promoter (CMVp), the DNA encoding AT1 receptor mRNA in the antisense direction, and a neomycin resistance gene (neo). The rAAV-AS vector was used to transform Escherichia coli.
ery applicable to humans

...injection of rAAV-AS in young SHR reduced blood pressure and slowed the development of hypertension. This drop in blood pressure was prolonged in 4 rats for 9 weeks, whereas controls had no reduction in blood pressure. This was considerably longer than the longest effect observed with antisense oligonucleotide (Fig 5). The results demonstrate that rAAV-AS in a single application is effective in chronically reducing hypertension. Furthermore, intracardiac injection of rAAV-AS in young SHR (3 weeks old) reduced blood pressure and slowed the development of hypertension. The results show the feasibility of the rAAV-AS in reducing hypertension. They encourage further research on gene regulation in hypertension and exploration of the most effective routes of delivery applicable to humans.

Summary

Long-term suppression of synthesis of individual proteins is desirable in conditions in which overexpression of endogenous genes or expression of foreign genes is thought to be the cause of the disease. Expression of antisense vectors produces antisense RNA, which can hybridize with the target mRNA and prevent its translation by either promoting its degradation, preventing its transport from the nucleus to the cytoplasm, or interfering with ribosomal function.

There are many problems to overcome in the use of antisense technology as a treatment for hypertension that are related to uptake efficiency in various tissues and its stability and activity once inside the cell. However, AS-ODNs to either AT1-receptor mRNA or angiotensinogen mRNA are effective in lowering hypertension in the SHR to normotensive levels. A single injection in the brain or systemically in lipid-encapsulated form is effective for up to 7 days. The antisense approach appears to be working through the expected mechanisms of inhibiting mRNA translation as the appropriate proteins are reduced in correlation to the physiological effect. More models of hypertension need to be tested, and the effectiveness of repeated doses to maintain reductions in blood pressure needs study. The long-term goal of prolonged blood pressure control by antisense may lie in the delivery of antisense DNA with viral vectors. The case for using rAAV as the vector of choice is based on safety, simplicity, and effectiveness. The carrying capacity is large enough for antisense cDNA. Results with AAV vectors in vitro demonstrate uptake in specific cells (NG108-15) and expression, separation from adenovirus, and heat inactivation to eliminate any residual adenovirus.

The vector was tested for AT1-receptor inhibition in vitro. Selection of the vector-transfected cells was based on the presence of the neomycin resistance gene. The cells had a significant (P < 0.1) decrease in Ang II AT1 receptor number compared with the control, untransfected cells. No effect was seen on the AT2 receptors.

To test for effectiveness in vivo, rAAV-AS plasmid vector was microinfused into the lateral ventricles of adult male SHR. Control rats received AAV with GFP reporter gene but without the AS gene (“mock” vector) in vehicle, which was artificial cerebrospinal fluid. Blood pressure was measured by the tail-cuff method. There was a significant decrease in systolic blood pressure in the group of rats that received the rAAV-AS vector. No effect was observed in the controls. Systolic blood pressure decreased by 23±2 mmHg in the first week after administration. This drop in blood pressure was prolonged in 4 rats for 9 weeks, whereas controls had no reduction in blood pressure. This was considerably longer than the longest effect observed with antisense oligonucleotide (Fig 5). This result demonstrates that rAAV-AS in a single application is effective in chronically reducing hypertension. Furthermore, intracardiac injection of rAAV-AS in young SHR (3 weeks old) reduced blood pressure and slowed the development of hypertension. The results show the feasibility of the rAAV-AS in reducing hypertension. They encourage further research on gene regulation in hypertension and exploration of the most effective routes of delivery applicable to humans.

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