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The Potential Role of Antisense Oligodeoxynucleotide Therapy for Cardiovascular Disease

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Abstract

Current drugs used in the treatment of cardiovascular disease are effective but compliance is poor and they are short acting (hours or one day). Gene therapy offers a way to produce long-lasting effects (weeks, months or years). Antisense inhibition is being developed for the treatment of hypertension, myocardial ischaemia and improved allograft survival in human vascular bypass grafts. We are currently using 2 strategies: (i) antisense oligodeoxynucleotides (AS-ODNs) which are delivered nonvirally and (ii) 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 (AT_1) receptors, angiotensinogen (ATG), angiotensin converting enzyme (ACE) and β_1 adrenoceptors effectively reduce hypertension in rat models. A single dose is effective for up to one month when delivered with liposomes. No adverse or toxic effects have been detected, and repeated injections are effective. For viral delivery, adeno-associated virus (AAV) is used with a construct to include a cytomegalovirus or tissue-specific promoter, antisense DNA to ATG, ACE or AT_1 receptors and a reporter gene. Results in rats and transgenic mice show significant prolonged reduction of hypertension, with a single dose administration of AAV-AS. Left ventricular hypertrophy is also reduced by antisense treatment. AS-ODNs to AT_1 receptors, ATG and β_1 adrenoceptors provide cardioprotection from the effects of myocardial ischaemia. The AT_1 receptor is more protective than losartan and does not increase plasma angiotensin as losartan does.

With the recent US Food and Drug Administration approval of fomivirsen for cytomegalovirus (CMV) retinitis, the first drug based on antisense technology has entered the market. This makes antisense technology a reality for therapeutic intervention. The antisense approach has the potential to provide longer-lasting and more specific effects than many of the currently available drugs and can, therefore, be developed for therapy of cardiovascular diseases (fig. 1). One area of research that we have initiated as an alternative therapy to current drug treatment for hypertension, is the preclinical development of antisense technology.[1] We have also used this antisense technology in the treatment of

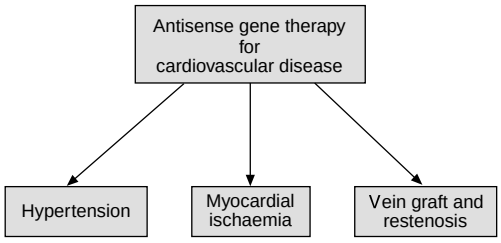

Fig. 1. The 3 areas of active research in the use of antisense gene therapy.

experimental myocardial ischaemia-reperfusion injury.[2] Others have used antisense technology to prevent cardiac allograft arteriopathy and extend the survival of patients.[3,4] The results of these experimental and early clinical studies are very promising for the potential use of antisense in cardiovascular disease.

1. The Need for New Drugs

Hypertension affects about 45 million people in the US alone.[5] There are excellent drugs that can control hypertension when taken on a daily basis (table I), but there are high costs associated with these treatments. Furthermore, there is the issue of poor compliance because of the cost or lack of tolerance to adverse effects, or both. The Joint National Committee on the Treatment of Hypertension (JNC-VI)[5] reports that, in spite of drugs, only about 23% of the population with hypertension have their blood pressure (BP) controlled. Poor compliance results in expensive clinical visits in which laboratory tests are carried out and treatment is frequently switched to alternate drugs that add to the overall cost of treatment.

Table I. Advantages and disadvantages of two antisense approaches

Adeno-associated virus	Antisense oligodeoxynucleotides
Advantages	
Delivers antisense to tissues	Can be used like a drug
Prolonged action (weeks/months/years)	Prolonged action (days/weeks) with a single dose
Can be tissue-target-directed	Reduces overactivity of excessive proteins but does not knockout normal physiology
Nonpathogenic, nontumorigenic, no safety issues	Specific for target protein
Non-inflammatory	No inflammatory reaction
Stable	Nontumorigenic, no safety issues
Disadvantages	
Needs gene switch to turn on and off	Not tissue specific
Slow latency of effect (days to 2 weeks)	Effect may not be antisense mechanism
	'Mild' effect
Requires systemic injection	Latency of 8-24h
	Not yet orally active

Apart from the financial cost, there is the human cost since hypertension that is not properly controlled leads to organ damage, particularly to the kidney, heart and blood vessels. End-organ damage markedly reduces quality of life, as well as life span. When a drug is taken once or twice daily, it may not uniformly control high BP. Many patients take their drugs in the morning, and therefore their hypertension control is at its poorest in the early morning.

What is needed are long-acting, antihypertensive, therapeutic agents that have few or no adverse effects and an extended effect with a single administration. Ideally, patients with chronic disease would like a single treatment whose effects last for several days, months or even years. This one-shot approach, if it were to result in sustained 24-hour decrease in BP, would significantly reduce heart attacks, stroke and end-organ damage.

For myocardial ischaemia, the current treatment is limited. Angiotensin converting enzyme (ACE) inhibitors are supported by data from the Survival and Ventricular Enlargement (SAVE) trials,[6] but again they must be taken daily. Therefore, the need for a new generation of long-acting drugs has led us to develop antisense gene therapy.

2. Strategies for Antisense Gene Therapy

There are 3 main strategies for applying antisense approach to hypertension and ischaemic heart disease: (i) oligodeoxynucleotides (ODNs); (ii) naked or plasmid DNA (pDNA); and (iii) virus vector delivered antisense complementary DNA (cDNA).

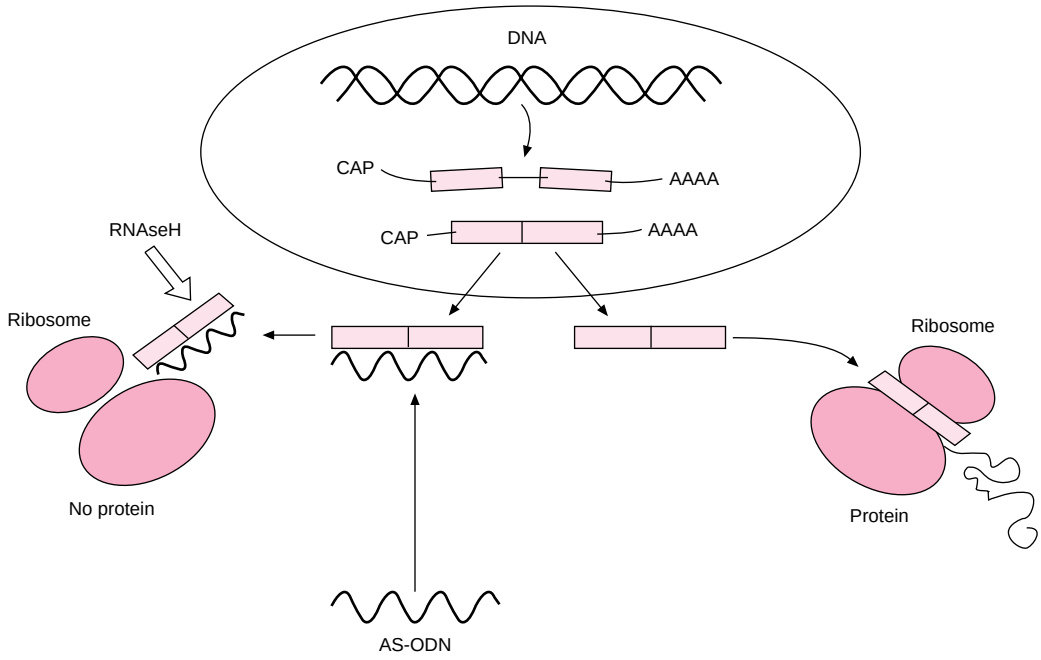

Fig. 2. The mechanism of antisense action. Antisense oligodeoxynucleotides (AS-ODN) are synthesised, single strands of DNA designed to hybridise with messenger RNA (mRNA). The hybridisation can take place in the nucleus to inhibit transcription or in the cytoplasm to inhibit translation. Binding of antisense ODN to mRNA can have 2 effects. The binding stimulates RNAseH which hydrolyses the mRNA, thereby reducing the total amount of mRNA, or the binding prevents the mRNA from being translated through the ribosome and thereby reduces the amount of specific protein synthesised.

2.1 Antisense Oligodeoxynucleotides (AS-ODNs)

An antisense ODN (AS-ODN) is a single-stranded synthetic DNA, usually with backbone modification with a specific sequence to hybridise to a specific messenger RNA (mRNA). Hybridisation of the ODN to mRNA inhibits the mRNA from sliding through the cytoplasmic ribosome and prevents translation. The hybridisation will also stimulate RNAse-H which destroys the specific sequence of hybridised mRNA. The effect is a reduction in the protein (fig. 2). Variations on this concept can be designed to inhibit transcription using triple helix–forming ODNs in the nucleus. However, in practice the design of AS-ODNs is complicated because the structure and secondary structure of RNA is not understood well enough to be predictable. The RNA structure influences the affinity of an ODN to bind to its RNA target. The affinity constants will vary with the length of the ODN and its position on the RNA scaffold. Several alternative sites for ODN binding are available in the nucleus or the cytoplasm. Alternatively, pre-mRNA sites can be targeted to inhibit transport from the nucleus.

The most commonly used method of constructing AS-ODN is to focus the design around the initiation codon (AUG) at the 5′ end of the mRNA. This is based on the assumption that the single-stranded mRNA at the AUG site allows entry into the ribosome. Therefore, it is both a site which can be hybridised with an ODN and a site where the hybridisation would prevent ribosomal translation of the mRNA. Alternatively, the terminal sequence (UAA) and other downstream sites can be targeted for antisense, but rationale design, based on melting temperatures, sequence interactions, secondary

structures, GC repeats and many other factors is not yet available. Although computer programs that predict optimal structure/basepair probability matrix/temperature algorithms are useable, the only sure way to make effective AS-ODNs is the empirical approach of trial and error. Usually 3 or more sequences encompassing the AUG site of ODNs 15 to 20 mer will yield an effective AS-ODN *in vivo*.

2.2 Plasmid DNA

The second strategy is to construct a pDNA containing a cassette with a promoter and cDNA in the antisense orientation. While success with naked DNA is being reported to deliver genes such as vascular endothelial growth factor (VEGF),[7,8] it has not been reported yet for antisense. A plasmid with a CMV promoter and angiotensinogen (ATG) antisense produced a decrease in BP for up to 8 days but it was no better than with a straight-forward AS-OND.[9]

2.3 Viral Vectors

To achieve a very long-lasting effect (months or years) in reducing hypertension, DNA in the antisense direction can be delivered in viral vectors.[10-12]

There are several vectors to choose from, including herpes simplex virus (HSV1), adeno-associated virus (AAV), lentivirus, adenovirus and retroviruses.

Adenovirus vectors have many advantages in theory but have not proved to be effective over the long term, primarily because they stimulate inflammatory responses. Until third generation 'gutless' adenoviruses become available that do not activate inflammatory reaction, the adenovirus vector is limited to research use.

The primary limitation of retroviral vectors is that they are effective only in dividing cells and not in nondividing cells. This may be quite appropriate for fast growing tissue, such as neointima. Further, they may activate tumourgenic genes and be transmitted in the germ line which would make them unacceptable for human use.

Of all the available vectors, the AAV has a number of advantages for use as a DNA vector. It has no known pathogenicity. It has adequate size for carrying DNA antisense, and it produces stable, long term, transgene expression since it is able to integrate into the genome. We have produced the recombinant AAV (rAAV) antisense vector targeting the angiotensin 1 (AT_1) receptor by packaging a cassette containing a CMV promoter and AT_1-receptor cDNA in the antisense direction into recombinant AAV. A significant decrease in the number of AT_1 receptors in vector-transfected vascular smooth cells compared with untransfected cells was noted with this approach.[10] The expression of the antisense vector continued for over 9 weeks in culture. We have successfully used AAV-delivered antisense to AT_1-receptor mRNA to decrease BP in young and adult spontaneously hypertensive rats (SHR).[11,12]

3. AS-ODN Approach

Of the different strategies for therapy based on gene modification, it seems most likely that the AS-ODN will be adopted for clinical therapy first. The cost of manufacturing short AS-ODNs has been rapidly falling as production techniques have been upscaled. Each year the price per nucleotide has gone down.

AS-ODNs can be administered like a drug, with an effect lasting several days and a dose response. They can be given in single doses repeatedly and without toxic effect or losing their efficacy. Stopping treatment is an option when needed. The effect of AS-ODN therapy is mild. They do not produce a total blockade of protein production because the AS-ODN competes for the numbers of copies of mRNA being produced by the nucleus. Whether the presence of antisense DNA increases RNA copy number or not, is not clear. What is clear is that antisense inhibition of transcription is unlikely to produce 100% decrease in mRNA. The competing production of mRNA versus the inhibition of translation or increase of degradation produces a decrease of protein that may range from 15 to 50%. Nevertheless, this is usually sufficient for a physiological effect and may be advantageous for two reasons.

The first is that a small percentage decrease in protein may have a substantial effect on a disease

state when it is due to an increase in a specific protein synthesis. This certainly seems to be the case for antisense targeted to the mRNA of AT_1 receptors, ATG or ACE. Lowering levels of these proteins, even by as little as 16%, significantly reduces the high BP levels that these proteins maintain.

Secondly, such proteins have a normal physiological function and to obliterate them by total inhibition induces compensatory side effects or physiological disturbances due to imbalance from the normal cellular homeostasis.

Thus, antisense therapy may be viewed as a mild inhibition rather than a knockout approach. Since the effects of antisense are long-lasting, compared with drugs, this advantage is particularly important for the well-being of future patients.

3.1 Backbone Modification

The ODN sequence in the natural form is a phosphodiester, but phosphodiesters do not have prolonged activity *in vivo* because of breakdown by nucleases. The most frequently used modification to protect the ODN is the phosphorothioation of bases in which a sulphur atom substitutes the phosphodiester oxygen atom (see fig. 3). Phosphorothioate internucleotide linkage creates a nuclease resistant ODN, but the cost is a lower binding affinity for the RNA target compared with the phosphodiester form. There have been numerous developments in this area to improve AS-ODN nuclease resistance and the reader is referred to Phillips[13] for more detailed information.

3.2 Uptake and Distribution of AS-ODNS

Zamecnik and Stephenson[14] applied antisense for the first time to the inhibition of Rous sarcoma virus replication. Nevertheless, AS-ODNs were considered suspect because of claims that there was very poor uptake into cells. Further concerns were that interference of AS-ODNs with other cell protein synthesis may produce nonspecific effects. Since all of these early studies were *in vitro*, it was probably differences of cell type and tissue culture conditions *in vitro* that added enough complications and contradictions to make the whole area of

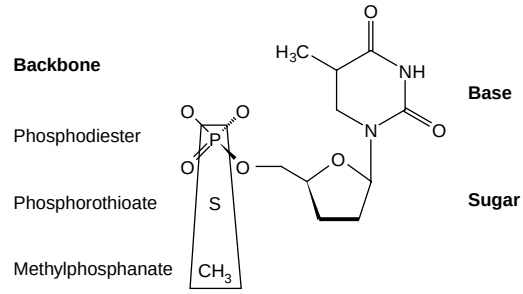

Fig. 3. Backbone modifications of antisense oligodeoxynucleotides (AS-ODN). The naturally occurring phosphodiester is short-lived. By replacing the phosphodiester oxygen atoms with sulphur or methyl phosphanate, the AS-ODN is protected and has a prolonged action.

antisense technology seem murky. A number of negative articles delayed the use of AS-ODNs *in vivo* for many years.

From 1992 to 1993 the first *in vivo* studies independently showed that application of AS-ODN uptake into brain tissue[15-17] or blood vessels[18] was not a problem. Not only was there evidence of adequate uptake, but there were also dramatic physiological effects. Using fluorescein isothiocyanite (FITC)-labelled ODNs, AS-ODN was shown to be taken up rapidly into adrenal cells and transported directly to the nucleus.[19] This transport to the nucleus occurred within 60 minutes of uptake and continued in the presence of ODNs for up to 8 hours. At 6 hours there is an efflux of ODN from the nucleus and cell so that a point of equilibrium occurred and was maintained for 24 to 72 hours. This appears to be the basis of the long term effects of AS-ODNs compared with currently available drugs.

By delivering AS-ODN in liposomes, the time for uptake is prolonged and *in vivo*, new data indicates extremely prolonged actions so that a single dose of AS-ODN to the β_1 adrenoceptor reduces BP for up to 33 days.[20]

4. Gene Therapy for Hypertension

The goal of gene therapy for hypertension is to provide treatment that decreases BP for a much

longer time period than can be achieved with current drugs, and with an equivalent or improved adverse effect profile. Although many risk factors for the development of hypertension have been identified, the exact pathophysiological mechanism remains largely undefined. Hypertension is known to be a multifactorial disorder that is likely to involve a wide array of genes. Candidate genes that make attractive targets for investigation of potential therapeutic intervention include those regulating vasoconstrictors, such as renin-angiotensin family, adrenergic receptor, endothelin and neuropeptide Y genes, and those regulating vasodilators, including genes for kallikrein, atrial natriuretic peptide, nitric oxide and calcitonin gene-related peptide. Nevertheless, as drug therapy has been successful by targeting a limited number of gene products, the same parsimonious approach can be taken with gene therapy. We have performed several studies to assess the potential for gene therapy targeting mRNA for AT_1 receptor and β_1 adrenoceptor. We chose these targets based on successful drugs in use.

The use of ODNs to deliver antisense has several advantages (table I). The difference from current drugs is that their effects are long-lasting with a single dose and they are designed to be highly specific so that there should be few, if any, adverse effects. In a series of studies, starting in 1993, Gyurko et al.[17] demonstrated that hypertension (systolic BP >150 mmHg) could be significantly decreased by a single injection of AS-ODN, specific for selected sequences of ATG mRNA or AT_1 receptor mRNA. In the early studies, injections were made directly into brain tissue to study hypertension of neurogenic origin.[17] In other studies AS-ODN was administered intravenously.[21] The application of AS-ODN was enhanced by delivery with a liposome carrier[21] or Sendai virus.[22] These studies showed that a single injection of AS-ODN to AT_1 receptors was effective in producing a significant drop in BP for up to 7 days.[23] Similarly, Makino et al.[24] have reported a sustained (7 days) reduction in hypertension in spontaneously hypertensive rats (SHR) with a single injection of AS-ODN directed at ATG mRNA given intravenously.

Further studies in our laboratory demonstrate that AS-ODNs are effective in different models of hypertension and can be given repeatedly, without toxic effect. The reduction in BP was maintained for several days with each repeated injection (fig. 4). Pharmacological dose-response curves have been shown with the ODN approach. Further, weekly repeated doses of AS-ODN in cationic liposome did not produce any liver toxicity as measured by changes in liver enzymes, AST or ALT. The distribution of intravenous AS-ODN in rats with AS-ODN directed at AT_1 receptor mRNA was in the aorta, kidney, adrenal gland and liver.[25] Because ODNs do not cross the blood-brain barrier there are no central effects. This may be advantageous, since several antihypertensive drugs have CNS adverse effects which patients can not tolerate. One such class of drugs is β_1 blockers. A β_1 adrenoceptor AS-ODN has been developed in our laboratory that has no central effects, but specifically inhibits β_1 adrenoceptors in the heart and kidney.[20]

In adult transgenic mice, which have elevated BP due to increased angiotensin II, a single systemic injection of rAAV-AT_1-AS produces a normotensive state for more than 6 months (unpublished data from ongoing study). Throughout this time, the rAAV is present and expressing antisense mRNA to compete with the AT_1 receptor mRNA and reduce the number of receptors.

Before viral vectors can be useful clinically, they need more engineering to ensure their safety, tissue specificity and control. In some situations it may be necessary to switch off the vector. In most cases of cardiovascular disease, it is desirable to have tissue-specific promoters to limit expression to heart or blood vessels.

5. Gene Therapy for Myocardial Ischaemia-Reperfusion Injury

Myocardial ischaemia followed by reperfusion injures heart tissue and reduces cardiac function. During ischaemia, AT_1 receptors are upregulated, suggesting that angiotensin II may have a role in the ischaemia-reperfusion injury to heart performance.[26]

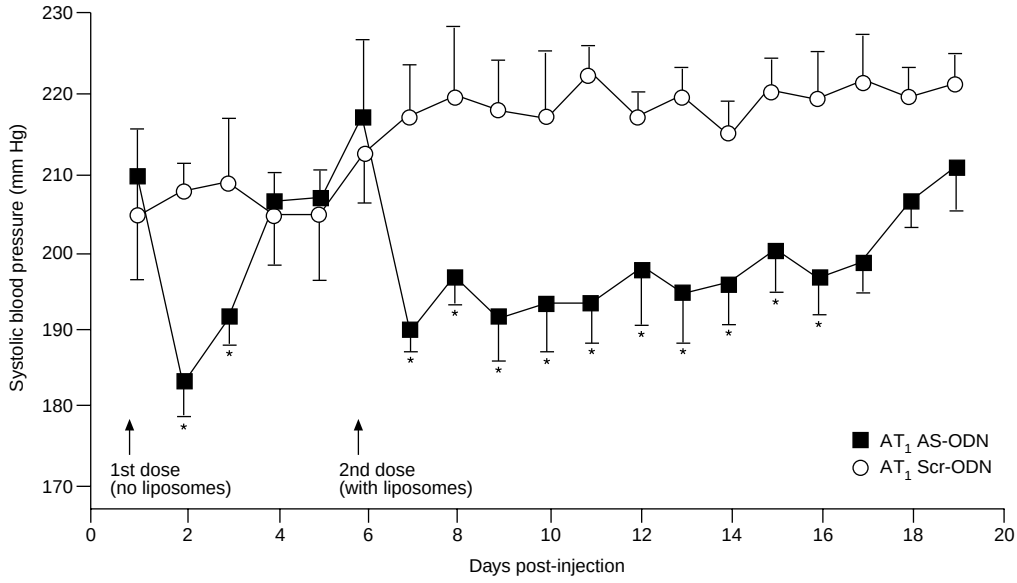

Fig. 4. Reduction of high blood pressure (BP) by antisense oligodeoxynucleotide (AS-ODN). This model is renovascular hypertension in which BP is very high because of elevated levels of angiotensin. The AS-ODN is designed to reduce (but not abolish) the number of angiotensin 1 (AT_1) receptors synthesised. It was injected intravenously. The first dose reduced BP for 3 days. A second dose, this time with liposomes to deliver the antisense, reduced BP significantly for over 10 days. The very high baseline BP probably requires higher doses of AS-ODN to normalise pressure. **Scr-ODN** = scrambled ODN.

To test if AS-ODN could be beneficial we investigated AS-ODN to AT_1 receptor and compared its effects with the AT_1 receptor antagonist, losartan.[2] We (Yang et al.[26]) hypothesised that suppression of AT_1 receptor expression by AS-ODN would protect cardiac tissues during ischaemia-reperfusion. To test this hypothesis, AS-ODN, which had successfully decreased BP in hypertensive models, was injected into Sprague-Dawley rats in liposome carriers DOTAP/DOPE (1,2-dioleoyl-3-trimethylammonium propane/1,2-dioleoyl-phosphatidyl-ethanolamine).[26] Hearts from AS-ODN– or scrambled ODN (Scr-ODN)–treated rats were excised 24 hours later, perfused *in vitro* and subjected to global ischaemia followed by reperfusion. Another group of rats was given losartan 4 to 6 hours before excising the hearts. Ischaemia-reperfusion resulted in a 2- to 3-fold increase in myocardial AT_1 receptor expression, a marked increase in coronary perfusion pressure and left ventricular end diastolic pressure, and a marked decrease in developed left ventricular pressure (indicating severe left ventricular dysfunction).[2] Administration of AS-ODN, but not Scr-ODN, significantly preserved cardiac function and blocked the increased AT_1 receptor expression following ischaemia-reperfusion (fig. 5). Use of losartan also improved cardiac function following ischaemia-reperfusion but the beneficial effects of AS-ODN on cardiac dynamics were more significant than those of losartan. As expected, plasma angiotensin II level increased after losartan, but unexpectedly did not increase after AS-ODN.

Thus, the use of AS-ODN directed at AT_1 receptor mRNA in the ischaemia-reperfusion injury abolished the increase in myocardial AT_1 receptor expression and protected against cardiac dysfunction. The increase in plasma angiotensin II level in the losartan-treated group is consistent with the up-regulation of the renin angiotensin system following AT_1 receptor blockade. The absence of an increase in plasma angiotensin II level in the AS-ODN–

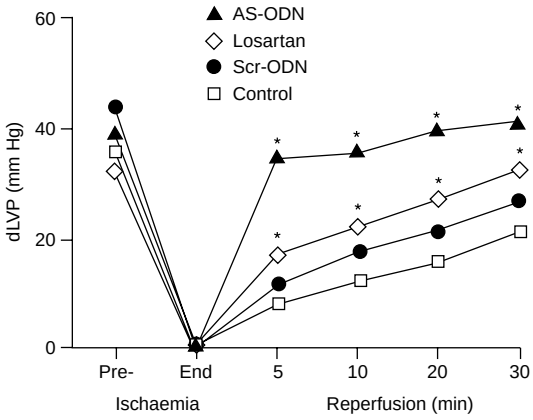

Fig. 5. Protection by antisense oligodeoxynucleotide (AS-ODN) to angiotensin 1 (AT_1) receptor messenger RNA (mRNA) in a model of ischaemia-reperfusion. The figure shows that the developing left ventricular pressure (dLVP), a measure of cardiac function, is significantly improved with AS-ODN compared with control (vehicle) and scrambled ODN (Scr-ODN). Losartan, a AT_1-receptor antagonist, was also effective but not as effective as AS-ODN (reproduced from Yang et al.,[2] with permission).

treated rats is an interesting observation. Although the precise basis for the absence of an increase is not known, it may relate to the AT_1 receptors in the kidney controlling renin release. We would suggest that if patients are receiving AS-ODN treatment to AT_1 receptor mRNA, they would benefit in the case of myocardial ischaemia.

6. Gene Therapy for Blood Vessel Grafts and Restenosis

One of the first *in vivo* uses of antisense in cardiovascular disease was the application of ODNs to the cell cycle regulatory enzyme, cyclin-dependent kinase (cdk) and the oncogene (*c-myb*).[27] Antisense phosphoediester ODN to these and cdc2 targets, using a combination of liposome and Sendai virus delivered to the carotid arteries after balloon catheterisation, inhibited the intimal hyperplasia associated with restenosis.[28] Antisense has been targeted to intercellular adhesion molecules (ICAM-1), a mediator of T cell adhesion,[29] cdc2 kinase,[18,30] platelet-derived growth factor,[10,31] and pivotal cell cycle transcription factor (E2F),[32] and these have all proven to be useful in reducing neointimal formation. Nevertheless, these experiments are a long way from treating coronary artery restenosis in humans, since most experiments are carried out in the carotid artery.

One positive result of this work is that antisense has been used to improve allograft survival. Bypass vein grafts frequently fail because they develop neointimal hyperplasia and accelerated atherosclerosis. However, during surgery the donor veins can be treated *ex vivo* by soaking in a solution of AS-ODN for 10 to 15 minutes. Mann and Dzau[4] have demonstrated that there is cellular uptake and intracellular distribution of ODN after transfection with a virus-liposome complex. Recently, they reported data on a group of 41 patients, 16 of whom had untreated grafts, 17 of whom were randomly assigned grafts treated with E2F antisense and 8 who also received grafts treated with control Scr-ODN. The patients were followed for 60 weeks. The antisense-treated group had fewer graft occlusions, revisions or critical stenosis than the control groups. There were no safety concerns, and the study proved AS-ODN in humans is feasible and beneficial.

7. Summary

AS-ODNs are being developed in 3 areas of cardiovascular research: hypertension, myocardial ischaemia, and prevention of restenosis and atherosclerosis in vein allografts. Because they have prolonged and highly specific effects, AS-ODNs may be useful alternatives to current drugs in hypertension therapy where compliance and stable control are problems. The approach may also be more beneficial than current drugs to improve cardiac performance after ischaemia-reperfusion. AS-ODNs are being tested clinically in cardiac allograft transplantation. They appear to be well tolerated and effective in humans, prolonging the life of vein grafts without complications, reducing restenosis and atherosclerosis.

We have provided a brief overview of the antisense approach and discussed its potential benefits. Preclinical studies indicate that antisense therapy can produce long-lasting efficacy with few adverse

effects in hypertension and in heart disease. Antisense to the β_1 adrenoceptor provides a promising new long-lasting β-blocker.[33] Since abnormalities in the renin-angiotensin system have been identified, not only in hypertension and myocardial ischaemia but also in adverse disease states such as congestive heart failure, atherosclerosis, skin wounds and intimal hypertrophy that occurs after angioplasty, the antisense approach targeted to the renin-angiotensin components may also be applicable to modify the outcome of these disease states. It is likely that the antisense strategy, directed at other mediators such as endothelin, thromboxane A_2 and oxidised low density lipoprotein receptor mRNA will expand the arsenal of ODNs for therapy.

Antisense delivered by viral vectors is still a long way from being clinically acceptable because more engineering of the vectors is required for tissue specificity and the requirement to switch genes on and off. Of all the virus vectors currently available, the AAV appears to be the safest and most stable.

Delivery of AS-ODNs can be as simple as giving a drug, and eventually orally active delivery and nasal spray delivery will be developed. The cost of ODN manufacture has been steadily declining. Patient use of ODNs would be less frequent than current drugs which should make for better compliance. If there are fewer adverse effects, less frequent dosage administration and clinical efficacy, as predicted, AS-ODNs will become the drugs of the future.

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