

Oligonucleotide-based Gene Correction Strategies: Applications to Neuromuscular and Cardiovascular Diseases

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Gene augmentation is an attractive and viable approach in treatment of inherited diseases, despite its limitations, ie, the amount of viral genome in replication-defective viral vectors is often too small for larger copy DNAs to be inserted. In addition, most viral vectors, when assessed *in vivo*, can elicit a host immune response, especially in cases of introduction of a gene copy where protein is completely absent, may have potential mutagenic effect on the host genome, or may be down-regulated. Therefore, alternative therapeutic approaches are being investigated, such as chimeraplasty, in which a mutated allele that already exists in an affected individual can be corrected. Although the only gene defects that can be corrected by chimeraplasty are point mutations, and the correction frequencies are variable, it has been observed that intracellular delivery of oligonucleotides is likely to be more efficient than that of plasmid DNA or viral vectors, because corrected genes are expressed from their autologous promoters, ensuring thus correct spatial and temporal expression, and host immune response is not elicited. Another strategy in the therapy of inherited diseases, such as Duchenne muscular dystrophy, is the application of antisense RNA oligonucleotides, or splicomers, to the exclusion of exons whose mutation leads to production of deficient essential proteins. Here we report the recent progress made and problems encountered in each of these fields, and discuss the potential of nucleotide-based gene correction strategies in treatment of neuromuscular and cardiovascular diseases.

Key words: apolipoproteins; cardiovascular diseases; dystrophin; gene engineering; gene targeting; gene therapy; hyperlipidemia; muscular dystrophies; neuromuscular diseases; point mutation; risk factors; RNA, antisense; RNA splicing

The conventional approach to the treatment of inherited disease by gene therapy is that of gene augmentation. That is the supply, in *trans*, of a functional copy of the gene that is defective in the diseased state. This is mostly achieved by the introduction of functional copy DNAs (cDNAs) or, if the gene is short enough, genomic DNA sequences, into plasmid or viral vectors. The viruses that are in widespread use in gene delivery research include adeno-associated viruses, retroviruses, lentiviruses (such as human immunodeficiency virus, simian immunodeficiency virus, and equine infectious anemia virus), and most commonly, adenoviruses. Each virus family has distinct advantages over others, but they also have their drawbacks. For example, adenovirus has the ability to infect quiescent or non-dividing cells. This feature makes it a far more attractive tool for engineering skeletal muscle cells than, for example, retrovirus, which cannot. However, retroviruses have the advantage over adenoviruses of being able to insert the therapeutic transgene into the host genome, which often results in more sustainable levels of expression. With this in mind, attempts are being made to engineering chimeric adenovirus-retrovirus viruses, which can combine the advantages of several virus families (Roberts et al, submitted for publication). Due to their

often pathogenic nature, it is important that the viral vectors are used simply as delivery vehicles, and that they only perform one transduction event. This is achieved by deleting large amounts of the viral genome, ensuring that the subsequent viruses are "replication defective". This has the added advantage of creating more space in the genome to insert foreign (ie, therapeutic) DNA. A major drawback of many viruses is the limited amount of nucleic acid that can be successfully packaged into infectious virus particles, so large cDNAs, for example dystrophin, cannot be inserted in their complete state, and efforts have to be made to establish which, if any, regions of the coding sequence may be deleted while still generating a functional protein.

Even when a complete gene delivery system has been generated, problems are encountered when its therapeutic benefit is assessed *in vivo*. Most viruses, even though replication-defective, will still elicit a host immune response, whether cell-mediated or humoral. Furthermore, in cases of inherited disease in which a protein is completely absent in the diseased state, the introduction of a therapeutic copy of that gene is itself likely to induce an immune response, which is clearly undesirable, and will result in no therapeutic benefit whatsoever. If the chosen viral

vector does not integrate its genome into that of the host, there is likely to be a severely reduced longevity of expression, especially in rapidly dividing cells, but if integration does occur, there are serious risks associated with insertional mutagenic effects on the host genome. Moreover, if all of these problems can be overcome, there is the potential for heterologous promoters to be down-regulated within a short time, leading to a loss of therapeutic gene expression.

Not all of the above problems are insurmountable, and the use of gene augmentation remains an attractive and viable option as a potential therapy for inherited disease. However, the search for alternative approaches to genetic medicine has continued, and within the past five years, a new technique has been developed that has the potential to overcome the majority of the problems associated with gene augmentation. Rather than introducing a new functional copy of a gene to replace the defective one, the technique of chimeraplasty is designed to correct the mutated allele that already exists in the affected individual. There is no restriction to the size of the gene that can be corrected. There is no risk of the generation of replication-competent pathogens. There is no risk associated with insertional mutagenesis. The corrected gene is expressed using its autologous promoter, in the right place, at the right time, and to an appropriate level. The barrier to the efficacy of the method is that the only defects that can be corrected are point mutations.

In a further approach, oligonucleotides can be used to achieve gene correction at the level of pre-mRNA splicing. For example, if a gene contains a nonsense mutation which would lead to a pathological phenotype arising from the deficiency of an essential protein, the exclusion, during splicing, of the exon carrying such a mutation would be predicted to restore the reading frame, allowing production of a protein lacking the sequences encoded by that exon. If the absence of those sequences does not itself render the protein inactive, the disease phenotype will be at least partially overcome. The splicing mechanism is dependent on RNA-RNA base-pairing interactions between the small nuclear ribonucleoproteins (snRNPs) and sequences surrounding each exon in the pre-messenger RNA. The disruption of this base-pairing by competition using modified antisense RNA oligonucleotides (splicomers) would be predicted to induce exon skipping. As with gene correction by chimeraplasts, the expression of the gene continues to be under the control of all the relevant spatial and temporal elements, and will not result in undesirable effects, such as insertional mutagenesis or host immune response.

Chimeraplasty

The use of chimeric RNA-DNA oligonucleotides (called chimeraplasts) to induce and correct point mutations was first described in 1996 by Eric Kmiec and co-workers (1), at the Thomas Jefferson University in Philadelphia, Pa, USA. In this landmark study, chimeraplasts were used to correct a non-functional allele of a human alkaline phosphatase gene on an ex-

pression plasmid in Chinese hamster ovary (CHO) cells. The rate of correction was demonstrated by histochemical staining for the presence of functional alkaline phosphatase, by an allele-specific hybridization technique, and by direct sequencing of polymerase chain reaction (PCR) products, to be in the order of 30-40%.

The design of the chimeraplast used in those first experiments has remained largely unchanged to the present day. It consisted of 68 nucleotides (*nt*) that could base-pair to give a double-stranded molecule, in which one strand was comprised entirely of DNA, and the other strand comprised two runs of 10 *nt* of 2'-O-methyl (2'-O-Me) RNA, separated by a five *nt* stretch of DNA (Fig. 1A). A short region predicted to have a high melting temperature (a GC clamp) was found at the 3' end, and four T residues separated the double-stranded regions, allowing the energetically favorable formation of tight hairpins.

The base, which is to be mutated, lies at the centre of the stretch of five DNA nucleotides on the chimeric strand, and has its complement at the centre of the all-DNA strand. This ensures that the chimeraplast is capable of intramolecular base-pairing along its entire length.

This has implications for the mechanism of chimeraplast action (Fig. 1B). It is presumed that invasion of the host genome is initiated and stabilized by

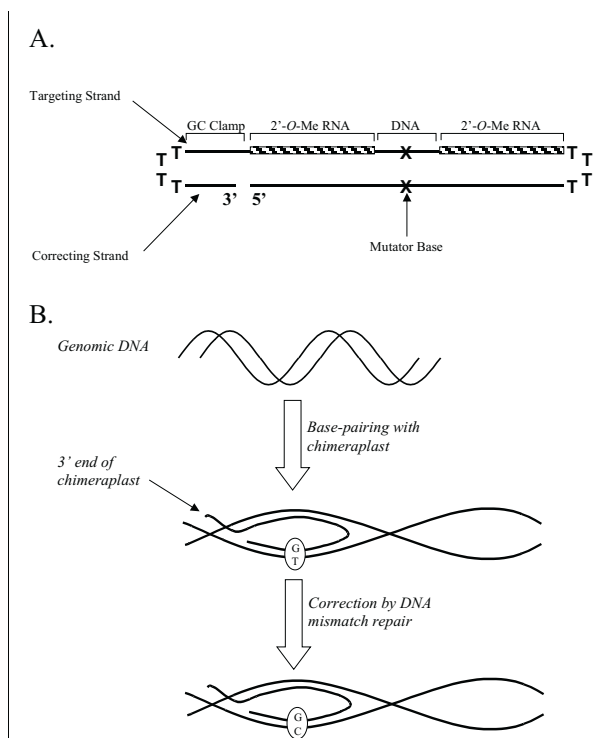


Figure 1. A. The structure of a typical chimeraplast, in which the chimeric (targeting) strand is entirely base-paired to the all-DNA (correcting) strand. B. Schematic representation of chimeraplast action. Strand invasion by the chimeraplast is initiated by RecA, and stabilized by base-pairing of the targeting strand to the upper strand of the genomic DNA. The base-pairing of the correcting strand to the lower strand of the genomic DNA creates a mismatch, which is repaired. This example depicts a T to C mutation.

the chimeric strand; the T_m of a hybrid between DNA and 2'-O-Me RNA is higher than that of either DNA-DNA or DNA-RNA, and will thus be more stable. Indeed, if the chimeric strand is replaced by one that is comprised entirely of DNA, the ability of the oligonucleotide to induce gene correction is reduced by a factor of 2, compared to the "classical" design (2). Following base-pairing of the chimeric strand to the genomic target, the DNA strand is then able to base-pair with the complementary strand. Whether this occurs solely by Watson-Crick base-pairing, or involves an element of Hoogsteen base-pairing is not clear. If Watson-Crick base-pairs are used exclusively, there is clearly no prospect for the chimeraplast to bind both intra- and intermolecularly. After the invasion of the genome by the chimeraplast, the point mutation in the genomic DNA that is mismatched with the central base on the all DNA strand of the chimeraplast is corrected. If only the chimeric strand is mismatched with the genomic DNA, the conversion frequency is reduced by 95%, compared to a chimeraplast that has mismatches on both strands. Indeed, if the mismatch is on the all-DNA-strand only, the efficiency of correction improves by as much as 50% (2). This is presumably a function of a greater complementarity, and hence stability of the chimeric (targeting) strand with the genome. It is not altogether clear as to the mechanism by which the invasion and correction occurs, but recent evidence suggests that the initial invasion is mediated by RecA gene and its mammalian homologues. RecA is unable to catalyze the invasion of duplex DNA by single stranded oligonucleotides, but if the complement of the oligonucleotide is also included in the reaction, a displacement loop (D-loop) is formed that is stable following the dissociation of RecA (3). It is proposed that chimeraplasts are able to form a "complement-stabilized" D-loop, and that the mismatch that exists between the chimeraplast and the genome, along with the recognition of the unusual four-stranded structure, leads to repair of the region (2). Mismatch repair proceeds via excision of approximately 100 bases in the vicinity of the D-loop, followed by DNA replication by DNA polymerase δ , with the all-DNA strand of the chimeraplast as a template. The process is dependent on a number of proteins, including MSH2, and is much more efficient than homologous recombination. The dependence of MSH2 in chimeraplasty has been demonstrated in cell-free extracts, but the role of DNA polymerase δ remains unclear due to the lack of effect of its inhibitor, aphidicolin (4).

It is possible that the model systems that have been used to assess the efficiency and mechanism of chimeraplast activity are dependent on plasmid (ie, supercoiled) DNA for activity, and that the mechanism by which gene correction by chimeraplasts occurs in the mammalian genome is somewhat different. Whether or not that is true remains to be seen, but there is clear evidence from an increasing number of studies that chimeraplasts can be effective in mammalian cell cultures and whole animal models. The first demonstration of the technique in a genomic context came again from Kmiec's lab (5). Chimeraplasts were designed to correct the sickle cell anemia

mutation (β^S) in β -globin to the wild-type (β^A) allele. When introduced to lymphoblastoid cell-lines that were homozygous for the β^S allele, correction to β^A was observed at a detectable level, as adjudged by genomic Southern hybridization, PCR-restriction fragment length polymorphism (RFLP), and direct sequencing of PCR products. Importantly, this was achieved in the absence of any significant increase in random mutations, demonstrating the safety of the technique as a potential genetic medicine. Shortly afterwards, the correction of an alkaline phosphatase gene in a genomic context was performed, with an intracellular correction efficiency in excess of 40% (6).

The next "quantum leap" in the method came the following year, with the first demonstration of the success of chimeraplasty in a whole animal. Chimeraplasts designed to correct the mutation in the clotting factor IX, which causes hemophilia B, were introduced into rat models by tail-vein injection. Correction of the mutant to the wild-type allele was observed at levels of up to 40%, after administration of two doses of chimeraplast. A single dose of less oligonucleotide yielded a correction frequency of about 11% (7).

It is evident from this, and other studies, that the liver is particularly amenable to the uptake of chimeraplasts (7,8), but other organs, including skin (9) and muscle (10,11), are also well suited as targets for the technique, and can result in potentially therapeutic levels of correction, approaching 20% in the dystrophin-deficient *mdx* mouse (10). Liver targeting has also been improved by the use of galactosylated delivery agents, such as percutaneous ethanol injection, which bind to the asialoglycoprotein receptor on the surface of hepatocytes (12). The technique has also been demonstrated in plants, showing the potential to introduce herbicide resistance into tobacco (13) and maize (14,15), by modification of the acetolactate synthase and acetohydroxyacid synthase, respectively. Whereas this correction has been observed only at very low levels, the application of chimeraplasty to genetic modification in this way may overcome many of the problems associated with the introduction of exogenous genes into random sites in the plant genome. As a result, the technique may become of enormous agronomic benefit.

Application of Chimeraplasty to Treatment of Cardiovascular Diseases

Cardiovascular diseases currently causes 38% of premature deaths in men and 30% of premature deaths in women in the United Kingdom, and the latest figures predict that by 2010, cardiovascular diseases will account for 40% of all premature deaths worldwide (16). The molecular pathology of cardiovascular diseases is extremely heterogeneous, and arises from both genetic and environmental factors, including dietary fat intake. There are a number of genotypes in the human population that can predispose to cardiovascular diseases, and in particular atherosclerosis – the deposition of cholesterol in plaques on artery walls (atheromas), leading to occlusion of the artery, and subsequent increased risk of heart attack.

A major cause of atherosclerosis is that of type III hyperlipoproteinemia, which arises as a result of a deficiency in the levels of the functional isoform of Apolipoprotein E (ApoE3), observed in individuals who are homozygous for the ApoE2 allele. The ApoE protein is a major component of several lipoproteins, including high density lipoprotein (HDL) and very low density lipoprotein (VLDL), and is cleared via the interaction of ApoE with the low density lipoprotein (LDL) receptor (LDLR), and the LDL receptor-related protein (LRP) on the surface of liver cells. ApoE2 shows a substantially reduced interaction with both of these receptors, compared with ApoE3, which leads to reduced clearance of cholesterol and its esters from the circulation, and consequently to increased risk of atherosclerosis.

ApoE3 differs from ApoE2 by a C to T point mutation, such that amino acid 158 is arginine and cysteine, respectively. This introduces the prospect of correction of ApoE2 to ApoE3 by chimeraplasty. We have developed a CHO cell line expressing the human ApoE2 gene. The cells were transfected with varying amounts of an E2-E3 chimeraplast, complexed with a 22 kDa linear polyethylenimine. After treatment for as little as 48 hours, with 200 nmol/L chimeraplast, we were able to observe correction of the ApoE2 allele to ApoE3 in as much as 40% of the isolated CHO-E2 DNA, as determined by PCR-RFLP analysis (17). In addition, treated cells were clonally selected and passaged repeatedly. DNA isolated from cells that had been passaged for 20 generations showed that some of the clones were ApoE2, some were ApoE3, and some were a mixture of E2 and E3. This shows that the correction is stably retained, and occurs with high efficiency *in vitro*. Preliminary experiments suggest that the same chimeraplast, after intraperitoneal injection, is capable of inducing the E2 to E3 correction in the liver of an ApoE knockout mouse that is also transgenic for the human ApoE2 gene (18).

In addition to ApoE, there are a number of other targets for chimeraplasty that may bring about a reduction in the levels of cholesterol in the bloodstream. For example, lecithin cholesterol acyl transferase (LCAT) is an enzyme which esterifies cholesterol, and is a key enzyme in lipoprotein metabolism and HDL maturation. This process of reverse cholesterol transport removes cholesterol from atherosclerotic plaques in the arteries, and LCAT is thus described as being anti-atherogenic. Chimeraplasts can be used to reverse mutations such as G30S, which causes total LCAT dysfunction, but can also increase the activity of LCAT by introducing changes such as S208A and S216A, which has been demonstrated to increase LCAT activity by as much as 16-fold (19).

Apolipoprotein AI (ApoAI) is one of the main components of the HDL, acts as a primary acceptor of free cholesterol, and activates LCAT in the circulation. Although cases of hypercholesterolemia arising from ApoAI deficiency have been reported, our approach focusses on the atheroprotective nature of two mutations of ApoAI (ApoAI_{Milano}; R173C and ApoAI_{Paris}; R151C). These mutations induce ApoAI to

dimerize, which increases the rate of uptake of the HDL particles by the liver. These gain-of-function mutations are currently being introduced by chimeraplasty.

The technique of chimeraplasty is widely applicable to the correction of genetic diseases arising from point mutations. Nevertheless, there are a number of obstacles that remain to be overcome. For example, whereas the liver appears to be readily amenable to chimeraplast uptake following intravenous delivery, and the skin can be treated by topical application or subcutaneous injection, diseases arising from the deficiency (or in some cases, over-activity) of proteins that are produced in other organs remain problematic.

Another allele of apolipoprotein, ApoE4, varies from the E3 allele by a C112R change. It is involved in dominant hyperlipidemia and restenosis (which could be treated by liver-directed chimeraplasty), and is also a major risk factor for Alzheimer's disease and other neurodegenerative disorders. While research in our laboratory is investigating the suitability of ApoE4 as a target for correction by chimeraplasty in cultured cells, little progress has been made in the delivery of therapeutic oligonucleotides to the brain.

The question that remains unanswered is why the efficiency of chimeraplast-mediated gene correction appears to be so variable. Even in cultured cells, where the problems associated with efficient delivery are largely irrelevant, others (20) and we have observed wide variations in the targeting efficiency. For example, the conversion of ApoE2 to ApoE3 occurs readily (up to 40% with a single treatment), but in the case of the LCAT gain-of-function mutations, the correction is not observed, even after direct cellular injection of the chimeraplast. There is no reason to suppose that the quality of one set of oligonucleotide reagents is any different than the other. The possibility exists that the efficiency of correction is dependent on the guanine/cytosine content of the target site. It might be reasonable to assume that the binding affinity of the targeting (chimeric) strand of a chimeraplast for the genome may be higher with increased guanine/cytosine content, which could be expected to result in increased efficiency of correction. This can be seen in the analysis performed recently, in which the targeting strand was composed entirely of 2'-O-Me RNA, and with no mismatch between it and the genomic sequence (2). However, the chimeraplasts that are intended to target LCAT S216A and ApoE2-E3 have guanine/cytosine contents of 72% and 64%, respectively, suggesting that, if anything, the LCAT gene should be corrected with higher efficiency than ApoE. That this is not the case suggests alternative reasons for the variability, based perhaps on the chromatin structure in the region of the target gene.

Antisense Oligonucleotide-mediated Modulation of Pre-mRNA Splicing

The use of antisense RNAs to modulate splicing of pre-mRNA was first described in a β -thalassemia, in which a point mutation in the second intron of the

β -globin gene generates a cryptic exon that leads to a frameshift, and hence non-functional β -globin. Antisense RNAs were shown to be able to block the inclusion of this cryptic exon, and restore functional β -globin production (21,22). This technique has also been used to modulate the alternative splicing pathway of the interleukin-5 receptor, such that production of the membrane-bound isoform, which is involved in the pathogenesis of asthma, is suppressed (23). Given their ability to modulate pre-mRNA splicing, we would now propose that oligonucleotides used in this way be called splicomers.

Modulation of Exon Skipping in Dystrophin Deficiency by Splicomers

Duchenne muscular dystrophy is a lethal muscle-wasting disease of childhood. It is caused by frame shifting or nonsense mutations in the Duchenne muscular dystrophy gene, which lead to absence of the protein dystrophin. In-frame deletions, on the other hand, allow variable expression of truncated dystrophin proteins, which are associated with the milder allelic disease, Becker muscular dystrophy. Present approaches to develop treatments for Duchenne muscular dystrophy are focussing on the delivery of recombinant dystrophin cDNA molecules to skeletal muscle, ie, gene augmentation therapy, or the upregulation of potentially compensating proteins, such as utrophin or integrin. However, gene augmentation therapy has encountered technical difficulties, as described above. This offers a novel strategy towards treatment of Duchenne muscular dystrophy, which circumvents the need to deliver large recombinant genes or to use viral vectors. Its primary aim is to evaluate the modulation of splicing of endogenous dystrophin pre-mRNA transcripts by blocking splice sites adjacent to frame-shifting mutations using splicomers. The approach was designed to induce the splicing machinery to skip mutant exons in Duchenne muscular dystrophy and to restore the reading frame of resulting mRNA molecules. The ultimate aim of the work is to evaluate direct *in vivo* administration of optimized splicomer/vector formulations in the *mdx* mouse as a preclinical model for antisense gene therapy of Duchenne muscular dystrophy.

It has been shown previously that splicomers are capable of altering the selection of exons during splicing of dystrophin pre-mRNA *in vitro* (24) and in cultured muscle cells (25). This was shown to be achievable at only a low level, and resulted in exclusion of other exons, in addition to that targeted (exon 23). More recently, we adopted an intelligent approach to splicomer design. This has involved detecting the most favorable interaction between a radio-labeled synthetic pre-mRNA fragment and a stepwise array of splicomers, in addition to employing a more empirical screening approach. We have analyzed a range of overlapping splicomers spanning across the region flanking the intron boundaries of *mdx* exon 23. The pattern of splicing of dystrophin pre-mRNA in treated *mdx* cultures was analyzed by reverse transcriptase-PCR-RFLP of a region extending from exon 18 to

exon 30. None of the splicomers that target the 3' splice site of exon 23 were effective in inducing exon skipping. Similarly, not all of those targeting the 5' splice site appeared to be active. However, splicomers that hybridize to the 3' half of the 5' splice site were capable of causing exon 23 to be skipped with high efficiency and specificity, and sequencing demonstrated that exon 22 has been joined correctly to exon 24, using the correct splice junction. Dose analyses indicated that over 50% skipping could be obtained under optimal conditions. Similar results have been reported recently (26), but the oligonucleotides used also induced skipping of exon 24. We are currently pursuing *in vivo* studies of the use of splicomers on the *mdx* mouse model of Duchenne muscular dystrophy.

Conclusion

The use of chimeraplasts and splicomers to restore the production of proteins that are deficient in cases of inherited disease shows a great deal of potential as an alternative approach to genetic medicine. There are clearly still some problems to overcome, such as uptake by organs other than the liver, muscle, and skin, and the determination of why some chimeraplast-mediated gene correction works and some does not. But the fact that the corrected genes are expressed from their autologous promoters, ensuring correct spatial and temporal expression, and the observation that systemic intracellular delivery of oligonucleotides is likely to be more efficient than that of plasmid DNA and viral vectors, without eliciting an immune response in the host, ensures that these strategies will be powerful weapons in the fight against genetic disorders in the future.

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Received: March 23, 2001

Accepted: May 30, 2001

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