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Prospects of Chimeric RNA-DNA Oligonucleotides in Gene Therapy

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Key Words

Chimera · RNA-DNA oligonucleotide · Gene therapy · Targeted gene repair

Abstract

A strategy called targeted gene repair was developed to facilitate the process of gene therapy using a chimeric RNA-DNA oligonucleotide. Experiments demonstrated the feasibility of using the chimeric oligonucleotide to introduce point conversion in genes in vitro and in vivo. However, barriers exist in the low and/or inconstant frequency of gene repair. To overcome this difficulty, three main aspects should be considered. One is designing a more effective structure of the oligonucleotide. Trials have included lengthening the homologous region, displacing the mismatch on the chimeric strand and inventing a novel thioate-modified single-stranded DNA, which was demonstrated to be more active than the primary chimera in cell-free extracts. The second aspect is optimizing the delivery system. Producing synthetic carriers for efficient and specific transfection is demanding, especially for treatment in vivo where targeting is difficult. The third and most important aspect lies in the elucidation of the mechanism of the strategy. Investigation of the mechanism of strand exchange between the oligonucleotide molecule and double-stranded DNA in prokaryotes may greatly help to understand the mechanism of gene repair in eukaryotes. The development of this strategy holds great potential for the treatment of genetic defects and other purposes.

In the past decade, several strategies for gene therapy have been developed. The strategy of 'gene addition', through which the phenotype is corrected by addition of a functional gene, is a commonly used approach among conventional strategies. The high efficiency of viral vectors has allowed much progress in gene therapy to be made. However, barriers exist in the random integration, immune reaction and potential danger relative to viral diseases. Another strategy, called 'replacement', is an ideal method in the sense of 'gene therapy' in that the defective gene is removed and a normal gene is inserted at the same position. This molecular process is accomplished by homologous recombination, also called 'gene targeting'. However, researchers have experienced much difficulty with the technology and the inefficient targeting process in mammalian cells [39]. So, other strategies need to be developed to compensate for these shortcomings. To our relief, new strategies have been tried, including the one we discuss in this paper.

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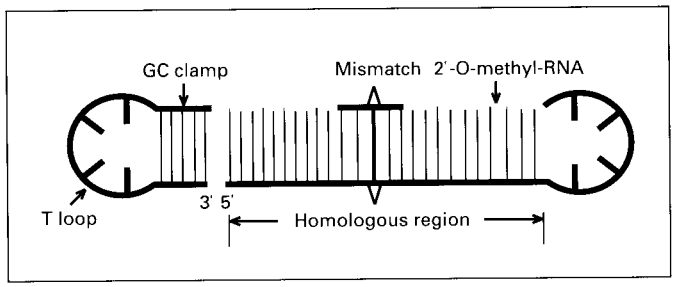

Fig. 1. Structure and character of an RDO. The single-stranded chimeric molecule is typically 68 nucleotides in length and includes twenty 2′-O-methyl-modified ribonucleotides in the 25-base pair segment homologous to the targeted gene. One mismatch site in the middle of the double-strand region is designed where a nucleotide change is sought. T loops at both ends and the 5-base pair GC clamp give the molecule much greater stability.

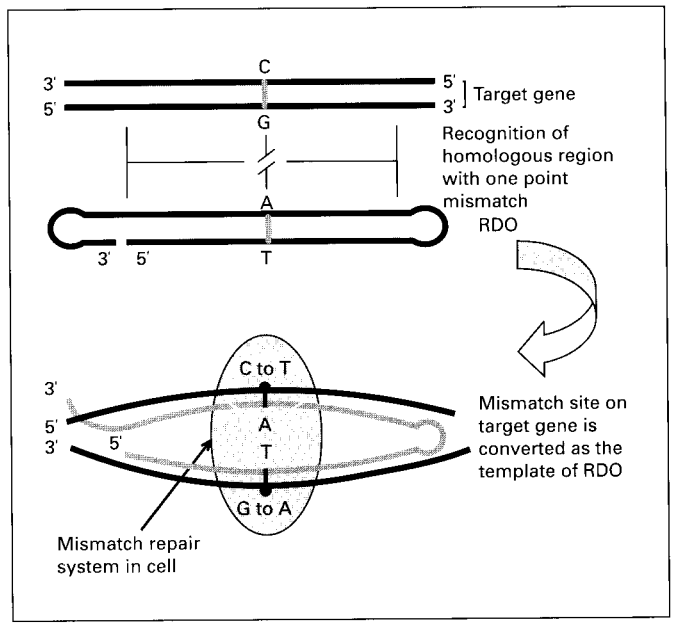

Fig. 2. The process of targeted gene repair. RDOs are introduced to the target gene. They locate the target region by homologous pairing. The mismatch site seems to be recognized and converted by a cellular-inherent repair system, for which researchers are trying to discover the functional components and how they work.

The Basis of a Newly Developed Strategy

Kmiec's group has done research work in homologous recombination for many years. They noticed that gene transcription could activate homologous pairing of nucleosomal DNA [22, 23]. RNA, a product of transcription, plays a role in extending the half-life of DNA molecules during the initial phase of recombination. They observed that the length of the complementary sequence required for stable DNA pairing might be reduced when one of the molecules contains RNA [21]. As DNA pairing is the rate-limiting step during homologous recombination, they tried to control this phase to improve the frequency of gene targeting. Since gene targeting is a rare event, they also formulated the idea that a single point mutation be repaired rather than an entire dysfunctional gene. Thus, an alternative strategy based on gene targeting was formed, known as targeted gene repair or targeted gene correction/conversion [20].

Against this background, a novel chimeric RNA-DNA oligonucleotide (RDO) molecule (fig. 1) was designed as a tool to facilitate the process of gene conversion [40]. It is a single 68-mer oligonucleotide folded into a duplex. In the duplex, 25 base pairs of a double-strand region are constructed to be complementary to the DNA sequence of the target gene except for one mismatched base pair. One of the strands comprises all DNA residues. The other comprises 5 DNA residues in the middle, flanked by 10 2′-O-methyl-RNA residues on both sides. The modification of RNA residues helps to resist RNase H-mediated degradation. Two single-strand ends are composed of hairpin caps of four T residues each and a GC clamp. The 5′ and 3′ ends of the molecule are juxtaposed and sequestered. All these structures contribute to enhance the resistance to nucleases in cells.

The hypothesis of the working process of RDO is described below (fig. 2). First, chimeras are introduced to the target gene. They locate the homologous region by homologous pairing. As the chimera is designed to contain a base pair which creates a mismatch with the target site in the gene, it will be recognized by the cell's inherent DNA repair machinery. As a result, the target gene site is rectified as the template of the RDO.

Compared with conventional strategies, targeted gene repair has several advantages: (1) the genetic defect is corrected within its normal chromosomal context, avoiding position effects, transgenic silencing or other concerns that may arise during developing transgenic events; (2) unlike viral vectors, this approach does not elicit an immune response, and (3) the molecule is synthetic and produced like a drug.

Trials of the Feasibility of Gene Therapy in vitro and in vivo

Dysfunctional genes resulting from single point mutations provide the objects of this strategy. At first, an episomal gene (plasmid vector) containing human liver/bone/kidney alkaline phosphatase (AP) cDNA, whose gene product was available to be monitored by direct biochemical and histochemical assays, was chosen [41]. Proper RDOs were introduced into CHO cells with extra chromosomal plasmids containing AP genes with single point mutations. Corrections of the point mutations were accomplished with a frequency approaching 30%. The relatively high frequency ensured the possibility of repairing a point mutation on an extrachromosomal plasmid by an RDO. Soon, targeted gene repair of genomic DNA was successful. A point mutation responsible for sickle cell anemia in the genomic DNA of cultured lymphoblasts was corrected [12]. Mutations in the β-globin gene in HeLa and other epithelial cells were also repaired [35]. Prominently, the frequency of RDO-directed site-specific nucleotide exchange in the AP gene in the genome of the HuH-7 cell line was as high as 40% [25]. It was shown that targeted gene repair by the chimera molecule can be achieved not only in transcribed genes but also in nontranscribed ones. Furthermore, considering the possibility that RDO causes nonhomologous recombination events, which would lead to spurious mutation or random integration, researchers sequenced hundreds of bases surrounding the target site and found no alteration [12, 38]. Southern blot analysis was also carried out which excluded the random integration of chimeric molecules [41]. So, the safety and fidelity of this strategy were confirmed.

Then, targeted gene correction in vivo in mice was reported. The liver was chosen as the target organ because many dysfunctional secretory factors or enzymes in hepatocytes result from point mutations. Another important reason was that RDOs can be delivered to hepatocytes via asialoglycoprotein receptors by tail vein injection. With this method, researchers induced a mutation in a rat hepatic factor IX allele at a frequency of 40% [24] and inserted a base pair into the UDP-glucuronosyltransferase-1 gene in Gunn rat hepatocytes [27]. Although the insertion rate was less efficient than was base exchange for factor IX, the 20–25% insertion efficiency was associated with an improvement of symptoms.

Because of its accessibility, point conversion in skin and muscle has also been successful. Researchers designed an RDO molecule to repair a point mutation in the tyrosinase gene in cultured melanocytes and in albino mouse skin by direct injection [1, 2]. The changed genotype and phenotype lasted for several months, indicating the permanence and stability of the converted gene. Similarly, by directly injecting RDOs into muscles, some researchers repaired the defects in dystrophin genes in skeletal muscles of animals, including mice and dogs [5, 33].

Experiments in plants by RDO-mediated changes in genotype and phenotype hold great potential for plant engineering, such as improving the quality or quantity of crop products [6, 42, 43]. In addition, through plant experiments, it was demonstrated that the converted gene can be passed to progeny by Mendelian transmission [43]. A mutant green fluorescence protein gene in maize was site-specifically repaired by RDO. Then, plants containing the modified genes were regenerated. Progeny analysis indicated Mendelian transmission of the corrected gene. There are also studies demonstrating the genetic stability of converted genes in animals, but they have not been tested through several generations.

We have been interested in gene therapy and the regulation of β-thalassemia for many years. Even though we achieved success using chemical medicine such as myleran to reactivate the fetal gene [30], as well as viral vectors containing β-globin gene with fragments of locus control region to achieve expression of the transferred gene [28], we found that the RDO-mediated gene therapy strategy had greater potential for some types of β-thalassemia. We introduced a βE gene, a point mutant β-globin gene which is very common in Chinese populations [8], into MEL cells. We successfully corrected the point mutation with the highest correction efficiency of 1.9%. Furthermore, a targeted single point exchange in the Gγ-globin gene, which is associated with elevated Gγ-globin gene expression in the adult stage, was introduced into HeLa and CMK cells by RDO. This kind of mutagenesis may increase γ-globin in adult patients so it can be used to alleviate symptoms of β-thalassemia and sickle cell anemia [29]. By using a modified DNA strand of RDO, we have achieved a positive result from targeted mutagenesis of the nucleotide associated with hereditary persistence of fetal hemoglobin in hematopoietic stem cells purified from human umbilical cord blood [Yin, W.X., et al., unpubl. data]. This suggests the possibility of this strategy being applied for the treatment of patients suffering from β-thalassemia. However, before clinical application, we need to collect more data from animal models.

Defining Problems with This Strategy

Over the 4-year period of the development of this strategy, we have seen not only great potential but also significant limitations. With experimental data accumulating, it has become clear that this method is more challenging in some types of cell lines than in others. Published frequencies of gene repair vary greatly. Thus, it is difficult for us to anticipate the result before each trial. How should the efficiency be improved to obtain stable and reproducible results? Viewing the entire repair process, we postulate that three main elements are involved, as discussed below.

As a tool to facilitate this repair process, the structure of the RDO is one of the keys to successful gene targeting. Although the structure of the RDO mentioned above has been shown to be effective in the repair process, it is not the best one possible. Modifications and variations of this structure have been tested which resulted in some improvements in the frequency of success (as discussed below). So, much attention should be paid to work in this field.

Another rate-limiting element concerns RDO delivery. Some liposome delivery systems and nonliposome delivery systems have been demonstrated to be able to facilitate this process. However, the efficiency of RDO delivery from outside the cell to inside the nucleus is consequently reduced in the multistep transport process. Besides cell toxicity generated by carrier complexes, there are three barriers to RDO delivery: the plasma membrane, the uncertain fate of RDO molecules in the cell and blockage from entering the nucleus if they fortunately survive both endocytotic processing and nucleases in cells. More difficulty exists in experiments in vivo because we have not found the most suitable means for RDO delivery to target cells. Exceptions are muscle and skin cells, which are easy to reach by direct injection, and hepatocytes, which may be specifically transfected via asialoglycoprotein receptors on their surfaces [3, 4]. As for hematopoietic stem cells, which are target cells for many genetic blood diseases, there are no specific transfection methods available yet.

The most troublesome and rate-limiting element is the inherent repair activity of cells. As a matter of fact, frequencies of gene repair vary greatly in different types of cells even though delivery effects of RDOs seem similar [35]. We know that targeted gene correction depends on the inherent recombination and repair ability of cells. However, the mechanism has not been clearly elucidated yet, which makes it difficult to control the process.

No matter what difficulties we are faced with, we and other RDO researchers have indeed focused our work on these problems.

Optimizing the Structure of RDOs

In addition to controlling the purity and concentration of RDO molecules, modifying the structures of the molecules plays an important role in gene correction. In the past, researchers usually designed RDO molecules as mentioned above, with the same length and structure. Then it was found that the frequency of gene repair could be improved by increasing the length of the homologous region [26]. The chimera with a 25-nucleotide homology region was about 40 times more active than the chimera containing a 15-nucleotide homology region, and when the homology region was extended to 35 nucleotides, activity increased by an additional 10-fold. Additionally, a series of structurally diverse chimeric oligonucleotides were used to analyze structure-activity relationships [14]. It was shown that the chimera containing the mismatch on the DNA strand directed gene conversion with a higher efficiency than the one containing a mismatch on the chimeric strand, or even when compared with the original double-mismatched molecule. The authors of that study revealed that the two strands of an RDO play different roles: the RNA-containing strand is involved in strand pairing and the stability of the complex, while the DNA-containing strand directs mismatch repair activity. Recently, the same authors reported that the DNA strand of the RDO molecule can itself direct gene repair in cell-free extracts [16]. To avoid nucleases, several linkages at both ends of the single-stranded oligonucleotide were modified by thioate. This novel molecular structure was about 3 times better at directing repair activity than the original chimera. These results imply that we may try to perfect the structure of the oligonucleotide molecule to improve the efficiency to some degree.

Delivery Systems of RDOs

Various liposomes, including DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl-ammonium salts), lipofectamine, lipofectin, superfectin, cytofectin and others have been used for the encapsulation and condensation of RDO molecules. Several articles have reported the results of cellular uptake of RDO and localization of RDOs in cells. Researchers have incubated target cells with various

ratios of fluorescence end-labeled RNA-DNA molecules and synthetic carriers. Observation of the localization of fluorescence revealed that it varied among the types of targeted cells and liposomes used. So, it is imperative to select suitable carriers before gene repair is carried out.

Fortunately, the techniques for synthesizing DNA carriers are developing rapidly. Several kinds of cationic, neutral and anionic liposomes provide diverse formulations possessing different respective characteristics. Besides lipid-based systems, researchers use protein-based methods to delivery DNA molecules [31]. Peptides such as poly-*L*-lysine, histone and especially protamine have demonstrated their ability to increase the delivery efficiency by condensing DNA and enhancing uptake [9, 10]. Polymers such as PEI (polyethylenimine) and polyamidiamine dendrimers are also used widely for their simplicity and versatility in transfection. PEI, one of the polymers gaining prominence in delivering DNA, is thought to protect DNA from degradation in the endosome by promoting early release as well as facilitating entry into the nucleus [17]. A significant improvement in cell-specific transfection is achieved by conjugating these chemical materials with ligands, such as epidermal growth factor for cells expressing epidermal growth factor receptors [36]. Through incubating carrier complexes with ligands, such as asialoorosomucoid or galactocerebroside, which bind to liver-specific asialoglycoprotein receptors, transfection of liver cells will be much more efficient [3, 4]. As for nuclear targeting, nuclear localization signals are added to synthetic DNA delivery systems to enhance efficiency by specific recognition [7]. Although the approaches mentioned above were tested with plasmid DNA, they shed new light on RDO research.

The RDO molecule differs from plasmid DNA in terms of both molecular size and structure. In one study, a fluorescent-labeled pure 68-mer DNA analogue of RDO, with the same length and structure as a chimeric molecule, was followed to reveal its intracellular fate [37]. The results showed that a negatively charged liposome formulation called AVE™-3, which was coupled to protamine, was effective in nuclear targeting. Nuclear localization of oligonucleotides could be observed with the AVE™-3 formulation. When protamine was absent, no fluorescent-labeled oligos could be found in the nucleus.

RDO delivery to hepatocytes has been achieved with a receptor (asialoglycoprotein)-mediated method [3, 4]. Liposome formulations were prepared to encapsulate the oligonucleotides. Galactocerebrosides, as ligands to recognize the asialoglycoprotein receptors on hepatocytes, were incubated in the formulations for targeted delivery. Three different liposomes were used for preparation of the formulation, i.e. an anionic liposome, a neutral liposome and a cationic liposome. All three formulations were efficient in delivering the chimeric molecules and promoting A-to-C conversion at the Ser^{365} position in the rat factor IX gene. However, the anionic liposome formulation was the most efficient vehicle in vitro. Additionally, attaching lactose in the same role as galactocerebroside to modify the nonliposome carrier PEI was demonstrated to be highly effective in RDO delivery in vivo. Using this system, a mean rate of A-to-C conversion of 48% in genomic DNA for the rat factor IX gene in the liver was achieved. These results suggest that a simple, stable and reliable delivery system for hepatocytes has been established. So, it is hopeful that targeted delivery of RDO to other tissues can be fulfilled via antibodies through specific recognition of antigens on cell surfaces.

Establishment of a Cell-Free Extract System for the Study of RDOs

Variability among different cell types has prompted researchers to develop a simple in vitro reaction system using nuclear extracts as a means of rapidly and reproducibly measuring the conversion frequency [11, 19, 34]. Cell-free extracts make it possible to avoid variability caused by tissue culture, transfection and RDO delivery to the nucleus. A shuttle vector is constructed containing an antibiotic-resistant gene whose resistance function is impeded by a point mutation. The appropriate RDO molecule, designed to repair the mutation, is added to the reaction mixture. After the reaction of gene repair, plasmid DNAs are isolated, purified and electroporated into *Escherichia coli* cells which are selectively cultured on plates for analysis. This process is called 'the genetic readout assay'. The frequency determined by this method appears to mimic chromosomal gene conversion except that it is lower than the latter. The reason may be that some proteins are excluded or inactivated during the preparation of nuclear extracts. However, this system provides researchers with a controllable condition to investigate the mechanism of the targeted repair process.

Mechanism of Gene Repair

Studies in cell-free extract systems have provided clues as to how the RDO mediates gene repair: (1) the alignment reaction of the RDO with its homologous sequence

in the target gene is catalyzed by the RecA protein in bacteria and the RecA-like (hRad51) protein in mammalian cells, and (2) the nucleotide conversion is promoted by the mismatch repair binding protein, mutS, in bacteria, and its homolog, hMHS2, in mammalian cells. Experiments have shown that gene repair activity seriously decreases in the reaction system lacking the above functional proteins [11]. Another protein, P53, also seems to be related to repair activity [32]. It seems that P53 can inhibit homologous recombination and bind to three-strand DNA substrates, which mimic early recombination intermediates [13]. It was found that nuclear extracts made from embryonic fibroblasts of the P53–/– mouse showed a higher gene correction than those of the isogenic P53+/+ mouse [11]. These results suggest that the recombination process is a rate-limiting step in gene conversion by RDO in cells.

Even though RDO researchers have discovered some factors affecting the activity of gene repair, the molecular mechanism of gene repair by chimeric oligonucleotides has not been thoroughly revealed. Although we do not know exactly how the chimera interacts with the target DNA and introduces the gene conversion, it is obvious that one activity catalyzed by repair or recombination proteins might be homologous pairing between the chimera and its target. It has been demonstrated that the *E. coli* RecA protein can mediate joint molecule formation between RDOs and single-strand targets [18]. A subsequent experiment revealed that chimeras participate in the strand exchange with short double-stranded DNA [14]. Chimeric oligonucleotides were incubated with RecA protein, $ATP_{\gamma}S$ and ^{32}P-labeled 46-base pair double-stranded DNA fragments. These short double-stranded DNAs were partially complementary to the chimeras. Electrophoresis in a nondenatured 12% polyacrylamide gel identified the expected strand exchange product, a complement-stabilized Y arm structure joint molecule. Two mechanisms were considered to account for the strand exchange. One was a three-strand exchange mechanism that depends on the RecA protein separating the strands of the duplex. Once a single strand becomes part of the presynaptic filament, it can initiate strand exchange with an homologous duplex. In this process, the chimera with a single-strand overhang or hairpin loop might be unwound by the RecA protein as the first step. An alternative mechanism for joint formation would be a four-strand exchange between two duplexes homologously aligned in a RecA filament. The short length of the chimera and the juxtaposition of ends might facilitate the strand exchange process. The latter explanation is supported in a newly published paper [15] which reports evidence for a four-strand exchange catalyzed by the RecA protein. The researchers found that the RecA protein recombines short hairpin substrates with an homologous stem provided that one of the hairpins possesses a chimeric RNA-DNA backbone. These results indicated that gene repair in eukaryotic reactions might involve a complementary stabilized D loop, a four-strand joint molecule. Each strand of the chimera was hybridized to a complementary strand of the target DNA.

Although there are differences between gene repair in eukaryotes and strand exchange in prokaryotes, studies of strand exchange reactions involving a chimeric RDO have greatly helped in understanding the mechanism of gene repair by RDOs.

Future Directions in RDO Research

With the experimental data accumulated in bacteria, animals and plants, we can understand more thoroughly the process of the RDO strategy. All the basic research work being carried out now is preparing for therapeutic applications or similar functions. The novel single-strand oligonucleotide, a new generation of RDO [16] as a tool to facilitate this process, has thrown new light on this field. Further work will include testing and applying it in gene repair in cell lines and in entire organisms.

In addition to the problems discussed in this paper, we should further consider therapeutic problems. Is this strategy restricted only to disorders with a single point mutation? Could it be extended to other genetic diseases, similar in genetic dysfunction?

With our greater insight into the etiologies of major diseases, including tumors, blood disorders and cardiovascular diseases, much effort should be expended on treating these diseases. The most important thing is that only when we thoroughly understand the molecular process of the RDO strategy can we control it and apply it to treat diseases effectively and safely.

Experiments in plants have provided new findings on characteristics of the strategy utilizing RDOs. At the same time, they show great potential for RDOs in gene engineering. Applications of RDOs should have great utility in the area of plant functional genomes, which will produce important commercial effects.

All that is needed is to try, test and improve.

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