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Message Therapy: Gene Therapy That Targets mRNA Sequence and Stability

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Gene therapy, in its simplest form, involves the introduction of a gene encoding a normal version of a defective or a missing protein into cells. However, there are many types of acquired and inherited diseases or of dominantly acting inherited traits for which gene replacement is not possible. Treatment of infectious diseases such as hepatitis or HIV may require inactivation of a virus, and, in some cancers, a gene may be overly active or may have altered properties, owing to mutations. In such cases, it may be more appropriate to borrow the techniques of gene therapy to modify or to destroy specific mRNAs, to achieve a therapeutic endpoint. One form of message therapy involves specific modification of an mRNA, to take advantage of naturally occurring mRNA-editing activity. Other methods include targeted removal of a specific mRNA, typically by use of ribozyme or antisense technology.

mRNA Editing

Apolipoprotein B (apoB), a protein contained in lipoprotein particles secreted by the liver and the intestine, circulates in the human body in two forms, apoB100 and apoB48 (Young 1990). Human liver synthesizes only apoB100, whereas the intestine synthesizes apoB48. ApoB48 mRNA, which encodes the N-terminal 48% of the apoB100 open reading frame, is derived from apoB100 mRNA. ApoB48 is generated by specific post-translational editing of a cytidine residue, to change a CAA codon (glutamine) to a UAA stop codon. The editing process is performed by a complex of proteins, of which the catalytic subunit is designated the apoB mRNA-editing enzyme (apobec-1) (Ashkenas 1997; Chan et al. 1997). A similar editing process occurs for the glutamate receptor, expressed in the brain, in which an adenosine deaminase changes a CAG codon (arginine) to a CIG codon (glutamine), resulting in isoforms with different calcium-permeability properties (Smith and Sowden 1996; Ashkenas 1997).

Although apoB48 contains the N-terminal 48% of apoB100, the two proteins have divergent metabolic fates. The portions of apoB100 that mediate binding to the low density lipoprotein (LDL) receptor are absent in apoB48, as are the domains that bind apolipoprotein(a) (apo[a]) to form the atherogenic lipoprotein Lp(a) (Utermann 1995). The intestine secretes dietary lipids in the form of the apoB48-containing chylomicrons, which are converted in the plasma to chylomicron remnants, through lipolysis. The remnants are taken up into the liver via binding of apolipoprotein E (apoE) to the LDL receptor-related protein (LRP) (Herz and Willnow 1995). On the other hand, the liver secretes lipids in the form of very low density lipoprotein (VLDL), which contains both apoB100 and apoE. VLDL is converted to LDL, of which the sole apolipoprotein is apoB100, and, thus, is a ligand only for the LDL receptor. ApoE has a higher affinity for the LDL receptor than does apoB100, and the apoB48-containing lipoproteins have a much shorter half-life in the plasma than do the apoB100-containing lipoproteins (Young 1990).

The relatively slow clearance of plasma apoB100-containing lipoproteins and the requirement of the presence of apoB100 for synthesis of Lp(a) suggest that it might be clinically desirable to induce higher expression of apoB48, at the expense of apoB100. Ectopic expression of apobec-1 may be a promising strategy for message therapy, depending on whether (1) apobec-1 is sufficient to confer apoB mRNA-editing activity in the liver (i.e., is it the rate-limiting step in editing) and whether (2) a shift toward more apoB48 secretion will reduce plasma VLDL, LDL, and Lp(a) levels. Recombinant adenovirus was used as the gene-delivery vector because intravenous injection of adenovirus results in high-level transgene expression in mouse liver. Unlike humans, mice express apobec-1 in the liver (Funahashi et al. 1995) and have higher levels of circulating apoB48 and lower levels of LDL. Injection of a recombinant adenovirus, encoding

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rat apobec-1, into normal mice nearly eliminated apoB100 production (Teng et al. 1994) and reduced LDL levels. Both of these effects were transient, as commonly is seen when transgenes are expressed from a recombinant adenovirus, in immunocompetent mice (e.g., see Barr et al. 1995; Kozarsky et al. 1996; Wadsworth et al. 1997).

A test of the effects of heightened apobec-1 expression on Lp(a) metabolism was not possible in normal mice, because they do not express apo(a) and because apo(a) binds to human, but not to mouse, apoB100. However, a transgenic mouse that expresses both human apoB100 and human apo(a) has been generated and thus makes Lp(a) (Linton et al. 1993; Callow et al. 1994). This transgenic mouse was injected with a recombinant adenovirus expressing apobec-1; as a consequence, there was increased editing of both the transgenic human and the endogenous mouse apoB mRNAs (Hughes et al. 1996). This lowered LDL levels and, furthermore, lowered Lp(a) levels, suggesting that apobec-1 may be a therapy for the reduction of Lp(a), thus reducing the risk of atherosclerosis in humans.

To determine whether apobec-1 could confer apoB mRNA-editing activity on a liver that, like human liver, normally does not express apobec-1, recombinant adenovirus was used in normal rabbits (Hughes et al. 1996) or in LDL receptor–deficient rabbits (Kozarsky et al. 1996). In both studies, the livers of rabbits edited apoB mRNA, after injection with a recombinant adenovirus encoding apobec-1, demonstrating that hepatic apobec-1 expression is sufficient to confer editing activity. A rabbit model of the human disease familial hypercholesterolemia (FH) was used to determine whether hepatic expression of apobec-1 would be therapeutic, that is, would result in lowered LDL levels. The rabbit model was the LDL receptor–deficient Watanabe heritable hyperlipidemic (WHHL) rabbit, which, like FH patients, is defective in LDL-receptor activity and exhibits high levels of circulating LDL and premature atherosclerosis. Expression of apobec-1 in WHHL-rabbit liver via a recombinant adenovirus resulted in increased levels of circulating apoB48 and in increased apoB48 content in VLDL. The VLDL cholesterol levels were reduced, accompanied by alterations in VLDL size and by modest reductions in total plasma cholesterol (Kozarsky et al. 1996). Evidence of a therapeutic effect of apobec-1 overexpression, in the context of increased plasma LDL levels, was confirmed by use of the LDL-receptor knockout mouse. In the mouse, increased apobec-1 activity in the liver was correlated with an ~30% reduction in apoB-containing lipoprotein cholesterol and with significantly decreased LDL levels, as measured by nondenaturing gradient-gel electrophoresis (Teng et al. 1997).

Gene therapy by introduction of the apoB mRNA editase into the liver has the potential to be useful in a variety of hypercholesterolemic conditions, for which the lowering of plasma LDL and Lp(a) levels is the goal. One potential advantage, in comparison with the overexpression of LDL receptors, for example, is that hepatic expression of apobec-1 is not likely to result in sudden increases in cholesterol uptake into the liver, as has been observed in animals overexpressing the LDL-receptor transgene (Kozarsky et al. 1994). Of even more importance, in the case of LDL receptor–deficient FH, apobec-1 would not stimulate an immune response (it normally is expressed in the intestine), whereas the LDL receptor might, particularly in an FH patient who expresses no LDL receptor protein (Kozarsky et al. 1996). However, there is the potential drawback that mice and rabbits that are transgenic for apobec-1 can develop hepatocellular carcinoma and dysplasia (Yamanaka et al. 1995). Because normal mice express apobec-1 in the liver without developing dysplasia, it is possible that these complications could be avoided by reduction of apobec-1 transgene expression. These hepatic symptoms may arise from editing of other mRNA substrates; a few mRNAs with sequences homologous to those that determine editing specificity in apoB mRNA have been identified. Two of these mRNAs, encoding a tyrosine kinase (Yamanaka et al. 1995) and a novel translational repressor (Yamanaka et al. 1997), indeed are edited in the livers of apobec-1 transgenic mice. The alterations in these and other genes are likely to be responsible for the development of dysplasia; thus, specificity of the apoB mRNA-editing activity is crucial to the determination of the specificity of gene therapy. Careful control over expression levels and/or engineering of the editing enzyme may be needed in order to make this strategy safe and effective.

Oligonucleotide Therapeutics

An alternative strategy for mRNA-targeted gene therapy is the use of site-directed oligonucleotide therapeutics, such as ribozymes or antisense oligonucleotides. Both agents target RNA in a sequence-specific manner and have the potential to modify RNA or to abrogate translation by mediation of RNA degradation. Antisense oligonucleotides bind with target RNA and inhibit translation through several possible mechanisms, such as by inhibition of ribosomal readthrough or by activation of the cellular enzyme RNaseH. Ribozymes are enzymatic RNA molecules capable of catalyzing sequence-specific RNA cleavage (Cech 1992). RNA cleaved by a ribozyme becomes unstable and is not translated. Sequence specificity is conferred by standard Watson-Crick base pairing. By alteration of the ribozyme or the antisense target-recognition sequences, any cellular or viral RNA may be targeted, in principle. Diseases associated with aberrant or viral expressed RNA, such as can-

cer, restenosis, and HIV, are the current focus of oligonucleotide-therapy research. However, recent reports of RNA repair or alternate splicing, with oligonucleotides, expand the future possibilities to include diseases associated with loss of protein function through genetic mutation. Ribozymes and antisense oligonucleotides also may be useful for the direction of alternate processing or for the repair of mRNA targets. Sullenger and Cech (1994) demonstrated this by adapting the splicing activity of a group I ribozyme, to recognize and to correct a mutated *lacZ* gene in *E. coli.* Jones et al. (1996) modified the system so that targeted mRNA would be tagged by the ribozyme with a novel exon 3, to allow an assessment of efficacy and specificity. Results indicated a high degree of target-specific tagging, but many additional nontargeted cellular mRNA were tagged. Efforts are underway to improve the site specificity of this technology.

Because of their small size, multiple antisense oligonucleotides or ribozymes, aimed at multiple regions of a target RNA, can be incorporated into current gene-therapy vectors. The engineering of such redundancy into the vectors could allow multiple mediators of a disease to be suppressed and might prevent the emergence of escape mutants, in viral targets (such as HIV). Target-specific effects of antisense oligonucleotides have been demonstrated in many cell and animal model systems (Agrawal 1996; Akhtar and Agrawal 1997). There are numerous reports of ribozyme efficacy in cell-culture models of human disease (Christoffersen and Marr 1995), with significant focus on cancer and viral targets.

The potential to target virtually any mRNA has allowed several groups to identify the growth factors, cell-cycle regulators, and apoptosis mediators necessary for tumor growth and metastasis. Czubayko et al. (1994) used ribozymes to demonstrate a crucial role for the growth factor pleotrophin (PTN) in human melanoma cells. Melanoma cell lines stably transfected with a plasmid expressing either of two ribozymes targeted to PTN showed significant reduction in PTN secretion, reduced soft agar colony–forming ability, and an inability to form tumors in athymic nude mice. Specificity of the ribozymes was demonstrated by comparison of the efficacy in cell lines expressing a cDNA for either PTN or a closely related growth factor (midkine [MK]) with 50% homology to PTN but lacking the PTN target sites. Whereas both ribozymes reduced PTN and the colony-forming ability in PTN-expressed cells, neither ribozyme had an effect on colonies in MK-expressing cells. In addition, no ribozyme efficacy was observed with inactive ribozyme constructs. The group then used the ribozyme constructs to establish a set of human melanoma cell lines with different levels of PTN. A correlation was observed between ribozyme-mediated PTN levels and angiogenesis and metastasis (Czubayko et al. 1996).

Ribozymes also are attractive therapeutics against viral RNA targets, and efficacy has been demonstrated, against viral RNA expressed by the influenza A virus (Tang et al. 1994), the bovine leukemia virus (Cantor et al. 1993), and HIV (Sarver et al. 1990). AIDS, resulting from HIV infection, remains a significant medical concern, and enormous effort has been made in the development of ribozymes as therapeutics against this virus. The first reports of the efficacy of ribozymes against HIV emerged shortly after the discovery of the ribozyme. Sarver et al. (1990) targeted a plasmid-encoded ribozyme to the gag region of HIV. Upon infection with HIV, $CD4^+$ HeLa cells, transfected with the ribozyme plasmid, showed a reduction in viral gag mRNA and an inhibition of viral replication, as measured by a reduction in the p24 viral envelope antigen. Such early efforts, using transient plasmid transfections, cell lines, and laboratory-adapted HIV isolates, increasingly have given way to the use of stable viral vectors for the expression of ribozymes, primary hematopoietic cells, and clinical isolates of the virus (Wong-Staal 1995; Bauer et al. 1997). In one report, retroviral constructs expressing a hairpin ribozyme conferred resistance to multiple strains of HIV, in peripheral blood lymphocytes (Leavitt et al. 1994). Specificity was demonstrated by the lack of inhibition of a closely related virus, HIV-2, which lacks the U5 long terminal repeat target site. Hammerhead ribozymes targeting HIV U5 or tat regions also have proved effective (Dropulic et al. 1992; Sun et al. 1995). Other investigators have shown that a hammerhead-ribozyme dimer targeting two sites, in HIV tat and rev mRNA, protects T cells. In this case, the mechanism was demonstrated by inclusion of an inactive ribozyme construct, which showed significantly less protection (Zhou et al. 1994).

Several groups are assessing the technical challenges involved in isolating and transducing hematopoietic progenitor cells (HPC) with ribozyme-expressing vectors against HIV. Gene therapy directed to HPC, which gives rise to all lymphocytic and myelocytic cell lineages, may offer an attractive strategy for a number of genetic diseases. Yu et al. (1995) transduced HPC, obtained from fetal cord blood, with a retroviral vector expressing a hairpin ribozyme and demonstrated protection against a macrophage-tropic strain of the HIV virus, in the differentiated progeny. More recently, Bauer et al. (1997) transduced HPC, obtained from the peripheral circulation of adults, with the tat and rev dimer-ribozyme retrovirus described above and observed significant protection against a primary clinical isolate and a monocytotropic lab isolate of HIV.

The ability to downregulate mRNA coding for cell-cycle regulators, apoptotic mediators, growth factors, etc., makes oligonucleotide gene therapeutics attractive for applications to such diseases as cancer, restenosis, and other proliferative disorders. In viral diseases, ribo-

zymes and antisense oligonucleotides are attractive for their potential to target multiple viral RNAs, including the viral genome, prior to reverse transcription and integration, in the case of RNA viruses such as HIV. The capabilities of oligonucleotide gene therapy, demonstrated against viral and acquired diseases, likely will be extended to heritable genetic diseases. Some of these diseases, such as Huntington disease and related neurodegenerative disorders, are characterized by the expression of mutant mRNAs encoding dominant negative proteins of unknown function (Paulson and Fischbeck 1996). Ribozymes or antisense oligonucleotides could readily be designed to target these mRNAs and may offer important strategies for the treatment of these diseases.

Conclusions

Gene therapy at the mRNA level has several advantages over gene therapies in which the primary purpose is the addition of a protein product. First, message therapy is versatile; it is able to be widely applied to both inherited and acquired diseases. It is performed on somatic cells, most of which are able to be transduced by use of one or more current vectors, and it avoids the complicated ethical issues that would be involved if transgenes were introduced into germ cells. Furthermore, these therapies have the potential to be reversible and thus can be active only when needed. Inactivation may occur by the deliberate obtaining of transient expression, through introduction of a nonintegrating vector into cells with a limited half-life or by use of regulatable promoters. In addition, message therapy can circumvent limitations in the duration of transgene expression, by avoidance of the development of an immune response to the transgene, either because the protein product is normally expressed in patients (but in a different tissue of the body, as with apobec-1) or because the transgene directs synthesis of an RNA/oligonucleotide and not of a protein. Limitations primarily involve the specificity of the mRNA modifications, which may be controlled by careful regulation of levels of expression (apobec-1) or by direct engineering of specificity (ribozymes and oligonucleotides). Thus, mRNA-directed therapy may be a useful addition to the collection of strategies for gene therapy for human disease.

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