Therapeutic Potential of Small Interfering RNA for Central Nervous System Diseases

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RNA interference (RNAi) is an evolutionarily conserved mechanism to degrade messenger RNA (mRNA) that has sequence homology to small double-stranded RNA (dsRNA). It is believed that the normal biological function of RNAi is to protect the host cells from RNA viruses and transposons, which can jeopardize the genome. Production of dsRNA in the host cell signals a series of events that ultimately results in the degradation of complementary mRNA. Recently, small interfering RNA (siRNA) has been used to study the function and significance of a vast number of genes in a variety of cell types. In the future, siRNA may have tremendous potential as gene-specific therapeutic agents for the treatment of many diseases. We discuss the potential role of siRNA as a novel therapeutic strategy for several central nervous system (CNS) diseases.

MECHANISMS OF RNAi

The mechanisms of RNAi were initially described in plants, when RNA for pigment-producing genes was introduced into petunias to enhance color.1,2 Unexpectedly, these petunias had near total color loss, and this paradoxical effect was termed cosuppression. Two years later, a similar observation was seen in fungi and referred to as quelling.3 Like many other genetic discoveries, the realization that these potent suppressive effects were mediated by dsRNA was made in Caenorhabditis elegans and termed RNAi.4 RNAi is a posttranscriptional gene-silencing mechanism. As a means to minimize the effect of potentially dangerous dsRNA, not typically expressed in eukaryotes, its presence activates a pathway that degrades mRNA containing complementary sequences to dsRNA. Dicer, a cytoplasmic ribonuclease III–like enzyme, recognizes dsRNA and cleaves it into 21 to 23 nucleotide fragments that contain 2 to 3 nucleotide overhangs (Figure). These siRNAs bind to a multiprotein complex called RNA-induced silencing complex (RISC), in which the dsRNA fragments are linearized and the strands separated. The single-stranded RNA bound to the RISC, known as the guide strand, can now hybridize to complementary mRNA. An enzyme within the RISC, argonaute 2, cleaves the mRNA so that translation of the transcript is no longer possible. The guide strand within the RISC can repeatedly bind complementary mRNA, amplifying the degradation of the target mRNA.

Scientists are now using the RNAi pathway to target self-genes. This has been accomplished by delivering plasmid or viral vectors to cells that are processed into short hairpin DNA that contain a 21-base dsRNA sequence that can be cleaved by Dicer into siRNA. Alternatively, synthetic siRNA readily transfects cells and was shown to be capable of directly activating the RISC and degrading the target mRNA. The largest obstacle to RNAi is delivery of the siRNA to the target cells, in particular to cells in the CNS that are protected by the blood-brain barrier. However, this is rapidly being overcome by using viral vector– or peptide-conjugated siRNA to target the siRNA to specific cell populations.5,6 In addition, new strategies to modulate or bypass the blood-brain barrier are being developed, which should enhance the possibility of efficiently delivering siRNA to the CNS.7

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The potential of siRNA as a therapeutic option for neurologic disease was first observed in a mouse model for spinocerebellar ataxia type 1,4 which is a dominantly inherited, progressive neurodegenerative disease caused by an expanded polyglutamine (CAG) sequence in the ataxin 1 protein. To target the ataxin 1 protein, plasmids containing various short hairpin RNA (shRNA) sequences within the human ataxin 1 complementary DNA sequence were tested in neuronal cell lines. Two sequences that flanked the CAG repeat region coding for the polyglutamine sequence decreased ataxin 1 levels. These shRNA sequences were subsequently inserted into recombinant adenovirus-associated virus to determine if ataxin 1–specific siRNA had a biological effect in the transgenic mouse model for spinocerebellar ataxia type 1. Intracerebellar injection resulted in enhanced motor coordination, improved neuropathologic findings, and complete loss of ataxin 1 inclusions in transfected Purkinje cells, demonstrating a clear therapeutic benefit.

Spinocerebellar ataxia type 3, also known as Machado-Joseph disease, is also an autosomal dominant neurodegenerative disease that results from an expanded CAG repeat sequence within the ataxin 3 gene. Initial attempts to inhibit ataxin 3 by RNAi targeting various sequences within the ataxin 3 gene resulted in efficient but not allele-specific suppression of the mutant and normal ataxin 3.5 Subsequently, siRNAs were developed that targeted a single nucleotide polymorphism in the ataxin 3 gene, which is in linkage disequilibrium with the expanded CAG repeat, typically segregating with the disease allele. Development of synthetic siRNA that had the single mutation at position 7 failed to differentiate the mutant and normal alleles. However, siRNA that added a mutation at position 8 within the siRNA sequence, so that it contained 2 mismatched bases to the normal sequence and a single mismatch to the mutant allele, demonstrated significant inhibition of the mutant allele while maintaining expression of the normal allele. In addition, siRNAs were developed that shifted the mutant base to the center of the siRNA sequence, which demonstrated allele-specific suppression. These siRNA sequences were subsequently inserted in plasmid and adenovirus vectors as shRNA and demonstrated similar suppression capabilities in vitro, showing that efficacy is maintained when the siRNA is produced by different modes and delivery.

Huntington disease is another dominantly inherited neurodegenerative disease in which an expanded polyglutamine plays a critical role in pathogenesis. Long CAG repeats within the huntingtin gene generate a protein that is predisposed to aggregation and toxic to neurons, particularly in the cerebral cortex and striatum. The huntingtin gene is expressed in all cells, but its function remains unknown. Mice deficient in the huntingtin gene are embryonic lethal, indicating that the huntingtin gene product is critical for development.10-12 Chen et al13 used a novel strategy to target the huntingtin gene by inserting shRNA into a transposon system, which inserts efficiently into almost any genome sequence, thus allowing continuous repression of the target gene. Using this system, expression of the normal huntingtin gene was suppressed in various cell lines, but suppression of the mutant huntingtin gene has yet to be addressed.

**ALZHEIMER DISEASE**

Alzheimer disease (AD) is a neurodegenerative disorder that affects 20 million to 30 million people worldwide and accounts for 60% to 70% of cognitive impairment in elderly patients in Western Europe and North America. One of the critical elements for the use of siRNA as a therapeutic agent in the treatment of diseases is the identification of relevant and specific target genes. The molecular characterization of lesions and the discovery of genes involved in the pathogenesis of familial and/or sporadic AD have revealed several targets for siRNA. The β-amyloid precursor protein gene (APP) was the first gene directly associated with AD in which a dominantly inherited mutation results in an early-onset, aggressive form of the disease.14 There are currently approximately 20 APP missense mutations near the cleavage site of secretases, suggesting that altered cleavage of APP negatively affects β-amyloid deposition. This genetic diversity illustrates why the identification of pathognomonic APP mutations with siRNA is a critical initial step before suppressing a specific mutant allele. Miller et al15 generated shRNA plasmids specific for the Swedish APP allele that contains 2 adjacent point mutations. By altering the position of the mutations within the siRNA sequence, the mutant APP allele could be suppressed to varying degrees in vitro while maintaining the normal APP expression. Since there are transgenic mice that express this Swedish APP mutation,16-18 the analysis of mutant and normal APP expression following treatment...
will ultimately determine the potential of these APP-specific siRNAs as therapeutic tools.

Similarly, a mutant allele of the tau protein was targeted in vitro using shRNA plasmids, demonstrating that a single nucleotide mutation could be used to suppress mutant tau by siRNA. However, the position of the mutation within the siRNA sequence was critical for selective inhibition of the mutant allele. Dominantly inherited mutations in the tau gene may be targets in neurodegenerative diseases other than AD. Frontotemporal dementia with parkinsonism linked to chromosome 17 has mutations in tau, resulting in altered sequence or aberrant splicing. Abnormal expression of tau is also associated with progressive supranuclear palsy and corticobasal ganglionic degeneration. Additional targets for AD include the apolipoprotein E ε4 allele (ApoE4), the secretases, and the presenilin genes. Together with increasing age, ApoE4 is considered the most significant risk factor for AD. Suppression of ApoE4 with siRNA could potentially reduce the probability of developing AD or delay its onset, at least in heterozygotes. Because ApoE4 plays a critical role in cholesterol and triglyceride transport, the risk-benefit ratio of gene targeting in ApoE4 homozygotes is less clear. More than 150 mutations have been identified in the presenilin genes, which result in an early-onset aggressive form of AD. Presenilin is the catalytic subunit of γ-secretase, and the mutations in presenilin enhance production of the highly self-aggregating Aβ42 peptide, which can be reversed by suppressing mutant presenilin with siRNA. This leads to the question of whether the secretasces could be the point of therapeutic intervention. Because β-secretase-deficient mice have no apparent deficits, this particular enzyme may be a good therapeutic target. Kao et al demonstrated that suppression of β-secretase with siRNA inhibited Aβ production, as well as the neurotoxicity associated with oxidative stress in neuronal cultures from wild-type and Swedish APP mutant mice. The relatively large number of targets for therapeutic intervention in AD and the relative ease of developing mutant or allele-specific siRNA make AD a good candidate for developing therapeutic siRNA.

MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of unknown etiology. The neurologic deficits appear to be mediated by demyelination and axonal transection. Therefore, targeting both immunologic and neurologic proteins may be necessary for therapeutic intervention in MS. Diseases such as MS may be more easily modulated with siRNA than other CNS diseases because systemic administration can readily target immune cells and potentially the site of active lesions due to blood-brain barrier breakdown. Several inflammatory mediators appear to play a critical etiological role. Specifically, it was demonstrated that systemic administration of interferon gamma causes disease exacerbations. Also, immunomodulation with agents that promote an anti-inflammatory cytokine profile are approved and currently being used for MS therapy. In experimental autoimmune encephalomyelitis (EAE), a murine model of MS, the disease is mediated by CD4+ T cells that produce IFN-γ, and suppression of IFN-γ by CD4+ T cells prevents their ability to transfer disease. Interestingly, mice genetically deficient in IFN-γ develop severe EAE, suggesting that systemic loss of IFN-γ results in immune dysregulation that may actually enhance EAE. Since IFN-γ is expressed by a variety of immune cells, siRNA specific for a transcription factor, T-bet, which appears to specifically regulate IFN-γ expression in CD4+ T cells, was used as a strategy to specifically target encephalitogenic T cells in EAE. Transfection of myelin-specific T cells in vitro with T-bet-specific siRNA prevented these cells from inducing EAE following transfer into naive mice. More important, intravenous injection of T-bet-specific siRNA at the time of EAE induction prevented the onset of disease, indicating that inhibition of a transcription factor critical to the development of the pathogenic T cells was sufficient to prevent disease. Using T-bet-specific siRNA to treat established EAE will determine the feasibility of using this siRNA as a therapeutic tool for MS. Other immune cell markers that are easily targeted in the periphery, such as interleukin 23 and osteopontin, which appear to play a critical role in immune-mediated demyelinating disease and whose deficiency does not appear to result in systemic immunosuppression, may be potential targets for siRNA therapy in MS.

Permanent neurologic deficits appear to result from axonal damage in MS, and currently no therapeutic agents enhance axonal repair. Recent studies inhibiting Nogo-A or its receptor by monoclonal antibodies or peptide inhibitors in experimental acute spinal cord injury enhanced functional recovery. Nogo-A is a potent inhibitor of neurite outgrowth and probably plays a critical role in negatively regulating regeneration and plasticity in the adult CNS. Suppression of Nogo-A with siRNA may be beneficial in the repair of axons in MS. Since there is episodic blood-brain barrier breakdown in EAE and MS, systemic administration of siRNA may be sufficient for targeting the areas of the lesions. In addition, the glial reaction to injury results in the production of many other inhibitory molecules that prevent axonal repair. Other CNS proteins that contribute to demyelination and Wallerian degeneration may also be targets for RNAi in MS.

CNS TUMORS

Malignancy in the CNS remains one of the most difficult tumors to treat. In vitro studies with glioma cell lines have recently identified several proteins that are overexpressed by these tumor cells and appear to play a critical role in tumor survival, proliferation, invasion, and angiogenesis. Down-regulated Notch-1 and its ligands, which are overexpressed in primary human gliomas, inhibit proliferation and induce apoptosis. In addition, transfection of glioma cells with Notch-1-specific siRNA before injection into mice enhances survival. Notch signaling is critical in developmental cell fate and adult nervous system plasticity; thus, the suppression of Notch-1 and its ligands may have unforeseen adverse effects.

Proteases that enhance extracellular matrix degradation play a critical role in tumor invasion and progression. Cathepsin B, urokinase-type plasminogen activator receptor, and matrix metalloproteinase have been targeted with siRNA both in vitro and in vivo. Both tumor invasion and angiogenesis were inhibited, and preestablished intracranial tumors in mice demonstrated significant regres-
tion following intracranial injection of plasmids expressing these siRNAs. These proteases are abundantly expressed and play central roles in normal biological functions such as immune surveillance; therefore, they should be studied cautiously and the risk-benefit ratio evaluated carefully in potentially fatal illness such as brain tumors.

CONCLUSIONS

Most pharmacotherapies that are currently being used for treatment of neurodegenerative or inflammatory CNS disorders target molecules that are localized downstream in the pathogenic cascade. As a consequence, their effects are often not specific and are moderate with regard to disease modulation. Furthermore, these agents are often associated with numerous adverse events. The siRNAs have 2 major advantages over conventional therapies. They can be used diagnostically to identify gene mutations underlying a clinical phenotype, and they can directly target the gene product involved in the etiology of a clinical disease. With our ever-accumulating knowledge of signaling pathways, it will soon be possible to design therapeutic interventions with siRNA that are specific for certain pathologic conditions and have relatively few systemic adverse effects.

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