Antisense Oligonucleotides Shed New Light on the Pathogenesis and Treatment of Spinal Muscular Atrophy

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Spinal muscular atrophy (SMA) is one of the most common autosomal recessive disorders that cause infant mortality. SMA is caused by loss of the survival of motor neuron (SMN) protein, resulting in the degeneration of motor neurons in the spinal cord.[1,2] In type 1 SMA, the most severe form, onset of disease occurs before 6 months of age and death occurs within the first 2 years of life. There is currently no effective treatment for SMA. Using systematic administration of an antisense oligonucleotide (ASO) to neonatal mice, Hua *et al*. now show that this approach has a robust ability to rescue a mouse model of severe SMA, significantly extending the life span of affected mice.[3] The new data also reveal a role for liver-produced insulin-like growth factor 1 (IGF1) in the development of SMA pathogenesis. These findings not only raise questions concerning the involvement of peripheral tissues, such as liver, in SMA pathogenesis but also provide new leads for developing potential drug candidates to treat this devastating disease.

The SMN protein has diverse functions in RNA metabolism and is expressed ubiquitously throughout the body. However, it remains unknown why and how a deficiency in SMN protein primarily affects the motor system. In addition to its involvement in forming nuclear "gem" structures and participating in RNA splicing, SMN protein is also present in the cytoplasm, where it forms complexes with various ribonucleoproteins and messenger RNAs (mRNAs) and is crucial for mRNA transport in axons.[1,2] Whether motor neuron degeneration is caused by impaired SMN function during RNA splicing, mRNA transport, or other cellular processes is unknown, and the downstream targets of SMN remain to be identified. The human genome contains two SMN genes—*SMN1* and *SMN2*—and SMA is often caused by homozygous loss of *SMN1*. Although *SMN2* encodes a functional protein, the gene contains a single silent nucleotide transition in exon 7, leading to frequent skipping of this exon during RNA splicing. As a consequence, the *SMN2* gene generates only approximately 10% of the correctly spliced transcript, a level insufficient to support long-term motor neuron survival.[1,2]

One strategy for developing a potential treatment for SMA is to boost the production of correctly spliced transcripts from the *SMN2* gene.[2] An intronic splicing silencer, termed ISS-N1, in intron 7 of the SMN gene has previously been identified.[4] ASOs that blocked ISS-N1 were shown to increase the inclusion of exon 7 in *SMN2* in patient fibroblasts. In a previous study, Hua *et al.* identified an optimal 18-mer termed ASO-10–27 that targeted ISS-N1 and promoted efficient inclusion of *SMN2* exon 7 (ref. 5). They demonstrated that ASO-10–27, when infused via intracerebroventricular (i.c.v.) injection into mice with mild SMA, increased inclusion of *SMN2* exon 7 in spinal cord motor neurons. Hua *et al.* have now extended their work by administering ASO-10–27 via subcutaneous (s.c.) injection into neonatal mice with severe SMA.[3] Two doses of ASO-10–27 injected between postnatal day 0 (P0; newborn) and P3 extended the median life span of the mice from 10 days to 108 days. One additional injection on both P5 and P7 further extended the life span to 137 days. These changes in life span were dose dependent; at the highest dose (two injections of 160 μg of ASO-10–27 per gram of body weight), the median life span was extended to 248 days, a 25-fold increase relative to the untreated control. In addition, early intervention was important: delay of ASO-10–27 treatment to between P5 and P7, even when an elevated dose was administered, increased the life span only marginally. This finding is similar to what was observed in two previous SMA gene therapy studies that used mouse models of severe SMA.[6,7] In those studies, expression of wild-type SMN in the central nervous system (CNS) of neonatal mice through adeno-associated virus (AAV)–mediated gene delivery extended the median life span from 15 days to 157 days in one study and by more than 250 days in another. These studies therefore raise hope that real progress can be made to treat this as yet untreatable disease.

In addition to extended life span, Hua *et al.* showed that many disease-associated symptoms, including reduced myofiber size and altered neuromuscular junction architecture, were improved in the treated mice.[3] Behavior and motor function in ASO-treated mice were also improved in a dose-responsive manner. However, although ASO-treated mice continued to gain body weight, they never reached the same weight as the control heterozygous mice. Moreover, the fraction of mice with ear and tail necrosis continued to increase with age. Finally, unlike most of the heterozygous control mice, the majority of ASO-treated mice with SMA failed to survive beyond 500 days. The exhibited growth abnormality and lasting necrosis may ultimately contribute to the reduced long-term survival benefit provided by ASO for these mice. One can argue that the treatment only transiently elevates SMN expression as a result of the relatively short half-life of ASO *in vivo* (~22 days in liver).[3]

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However, in an AAV gene therapy study for mice with SMA, treated mice exhibited a similar premature death,[7] suggesting that ASO injection or AAV-mediated gene delivery is not sufficient to completely correct the disease phenotype.

Molecular analysis of SMN2 expression in Hua and colleagues' study showed that s.c. injection led to dose-responsive inclusion of exon 7 in various tissues, with the strongest effect occurring in the liver.[3] Exon 7 inclusion increased only moderately in the CNS. In contrast, i.c.v. injection of the same ASO led to exon 7 inclusion exclusively in the CNS, not in other tissues. The fact that systemic ASO administration resulted in a much more prolonged life span than i.c.v. injection suggests that tissues other than the CNS are involved in SMA pathogenesis. Because all mice with severe SMA exhibited reduced body weight, and the liver is the major organ that produces IGF1, which modulates animal growth, Hua *et al.* examined serum IGF1 levels in SMA mice.[3] Indeed, IGF1 levels in SMA mice were significantly reduced relative to those of heterozygous controls. Close examination revealed, however, that the expression of IGF-binding protein acid-labile subunit (IGFALS) rather than the expression of IGF1 itself was affected in SMA mice. IGFALS binds to IGF1 and IGF-binding protein 3 to form a stable ternary complex, extending the half-life of IGF1 from 10 minutes to more than 12 hours.[8] ASO administration rescued expression of *Igfals* in the liver and restored serum IGF1 levels. Because *Igfals* expression correlated with SMN deficiency, Hua *et al.* hypothesize that an early deficiency in circulating IGF1 may contribute to SMA pathogenesis.[3] This hypothesis is supported by observations that local increases in IGF1 in either the spinal cord or muscle increase the survival of mice with severe SMA.[9,10] *Igf1*-null mice are also phenotypically similar to SMA mice, exhibiting a similar small size, severe muscle dystrophy, and early death.[11] The gain in animal body weight in the current study of Hua *et al.* appeared to be greater than that observed in the two AAV gene therapy studies, suggesting a more efficient rescue of *SMN2* splicing in the liver and increased IGF1 production. The current study thus suggests that IGF1 signaling in the liver is also involved in SMA pathogenesis.

ASO injection during the neonatal period clearly provides therapeutic benefits to the SMA mice. Because only two injections were sufficient to generate a long-term survival advantage, the current data suggest that a transient early restoration of SMN expression can have a profound effect on treated animals. This raises the prospect for translation into large animals and clinical application. A study using AAV-mediated gene delivery in nonhuman primates is encouraging.[6] A newborn cynomolgus macaque transduced with intravenous AAV injection showed robust expression of the reporter gene within the dorsal root ganglia and motor neurons along the entire neuraxis. ASO administration via i.c.v. or s.c. injection may have similar potential for success in large animals. However, the window for therapeutic intervention during development may be limited. In mice, only early intervention between P0 and P3 resulted in significant correction of phenotypic abnormalities.[3] Formation of the mouse blood–brain barrier could block efficient CNS delivery of ASO via s.c. administration. Whether it is possible to introduce the ASO into the CNS of older animals via other routes and whether such a maneuver could be carried out in time to rescue already damaged motor neurons in patients are some of the issues that must be resolved.

The new strategy demonstrated by Hua *et al.* offers some obvious advantages over AAV-mediated gene delivery; it would be much easier and more cost-effective to prepare good manufacturing practice–grade ASO than AAV in sufficient quantities for clinical application. In addition, the application of ASO in the clinic would not be burdened by regulatory issues concerning vector safety. Whereas immune responses against AAV capsid protein could prevent repeated virus administration, ASO is not expected to elicit such responses. Considering the importance of timing for therapeutic intervention, prenatal diagnosis or screening of newborns for SMN mutations remains critical for the success of this strategy. One issue raised by the current study is why the CNS correction of *SMN2* splicing by i.c.v. injection extended the life span of SMA mice for only 6 days, whereas s.c. injection extended the animal life span by more than 200 days. Is a transient increase in SMN expression in multiple tissues besides the CNS necessary to achieve long-lasting correction of the disease phenotype? Another issue is whether IGF1 levels are reduced in the livers of SMA patients and how *Igfals* expression is affected by SMN deficiency. Is it mediated through defective RNA splicing because of the lack of functional SMN protein or through other mechanisms, such as reduced transcription or IGFALS protein stability? Deriving hepatocytes from induced pluripotent stem cells from SMA patients may provide a powerful tool for resolving this issue.[12,13]

In summary, Hua *et al.* have identified a new and potentially easier approach than viral vector–mediated gene delivery to treat SMA. This study also uncovers potential functional roles of peripheral organs such as the liver in the progression of the disease. Further refinement of these discoveries should improve our understanding of SMA and allow development of new approaches to treat this disease in humans. Because aberrant alternative splicing has been shown to play critical roles in the pathogenesis of several other human diseases,[14] the strategy described in this study may also be applicable for the treatment of those splicing-relevant diseases.

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Multifunctional Theranostic Nanoparticles for Brain Tumors

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Antiangiogenic approaches have been extensively exploited to provide a rationally designed therapy for the treatment of brain cancer. The brain tumor endothelium, with characteristics of high proliferation, high permeability, and high expression of proangiogenic factors (such as vascular endothelial growth factor, VEGF), is a particularly appealing therapeutic target for this strategy. Many antibody drugs, which primarily target the interaction between VEGF and its receptors, have been investigated in clinical trials but have shown only modest effects. Recent research published by Agemy *et al.*[1] has alternatively harnessed a tumor-homing peptide (CGKRK) to specifically deliver multifunctional theranostic nanoparticles composed of iron oxide nanoworm (NW) and mitochondria-targeted cytotoxic peptide $_{D}[KLAKLAK]_{2}$ to glioblastoma multiforme (GBM) endothelium. Moreover, with the help of a tumor-penetrating peptide (iRGD), the NWs were capable of infiltrating the tumor tissue after extravasation for tumor cell eradication. This strategy was evaluated in lentivirus-induced, transplantable, and orthotopic brain tumor models. Complete tumor ablation was achieved in the first model and significant prolongation of survival was observed in the latter two models, suggesting promise for eventual clinical application.

The CGKRK tumor-homing peptide was previously identified using a combination of *in vivo* and *ex vivo* phage display systems.[2] The peptide was found to accumulate in the endothelium and parenchyma of tumors and dysplastic foci with high specificity after intravenous administration. Moreover, the peptide was demonstrated to home to the endothelium of different types of subcutaneously grown transplanted tumors. The homing property was especially apparent in a subcutaneous tumor model implanted by Matrigel in combination with VEGF and basic fibroblast growth factor. It is believed that the combination of these two angiogenesis-related growth factors is sufficient to elicit the expression of the binding moieties for this homing peptide, which are strategically exploited as the target in antiangiogenic therapy. Agemy *et al.* harnessed the tumor-homing property of the CGKRK peptide to deliver a previously identified proapoptotic peptide, $_{D}[KLAKLAK]_{2}$, as a chimeric peptide.[3] The peptide was covalently linked to an iron oxide NW with a 5K–polyethylene glycol linker. The $_{D}[KLAKLAK]_{2}$ is designed in such a way that this α-helix-forming, cationic peptide disrupts the membrane once the cationic amino acids are attached to the head groups of anionic phospholipids in the membrane. However, the peptide preferentially disrupts the mitochondria membranes because of their higher content of anionic phospholipids than that of cytoplasm membranes, which contain mostly zwitterionic phospholipids. Therefore, the peptide gives rise to minimal toxicity outside of the cell. Once internalized, the peptide causes cell death by destabilizing the mitochondria membranes and subsequently inducing mitochondria-dependent apoptosis.[3] The exploitation of elongated iron oxide NWs, which have been demonstrated to be more effective, in terms of targeting capacity, than spherical nanoparticles, additionally provided a signal for diagnostic imaging with magnetic resonance imaging.

Agemy and colleagues assembled this multifunctional theranostic nanoparticle from a collection of state-of-the-art elements. The tumor accumulation of the NWs coated with the chimeric peptide composed of CGKRK and $_{D}[KLAKLAK]_{2}$ indicated that the function of the targeting peptide was not compromised in the chimeric form or after conjugation to the NWs. The $_{D}[KLAKLAK]_{2}$ peptide maintained mitochondrial targeting and apoptosis-inducing capacity in the NWs as well. These results suggest that the elegantly designed nanoparticles include all the essential elements of an advanced theranostic reagent without compromising the individual functionalities of the components. Indeed, the half-maximal inhibitory concentration (IC_{50}) of free $CGKRK_{D}[KLAKLAK]_{2}$ was much higher (>200-fold) than that of the NW-linked peptide, suggesting that coating of the peptide on the NWs enhanced its activity, probably because of the increased local concentration.

To evaluate the delivery specificity and therapeutic efficacy of the NWs *in vivo*, Agemy and colleagues chose a GBM model, the most frequent primary and malignant brain tumor model, by targeting the endothelium—the very supply for tumor survival and growth.[4] They studied three mouse GBM models that closely resemble human GBMs in their aggressiveness and diffusion pattern into normal brain tissue. The first model was created by injection of a lentiviral vector carrying the H-Ras V12 oncogene and a short hairpin RNA targeting p53 into the hippocampus of mice. This tumor shows all the features of GBM, including hypervascularity.[5] After systemic administration every other day for 3 weeks, the tumors were eradicated in 9 of 10 mice treated, with no histologically detectable

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