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Molecular Therapy

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AAV Vector Biology in Primates: Finding the Missing Link?

Liver-targeted gene transfer using vectors based on adeno-associated virus (AAV) has been successfully used to treat several animal models of genetic and acquired disease. Attractive features of AAV vectors include relatively weak activation of the innate immune system and the ability to direct high levels of gene transfer and long-term expression upon *in vivo* delivery to relatively quiescent cell types, such as hepatocytes. Nonetheless, clinical progress has been slow. Thus far, two phase I/II trials have been performed, both using factor IX gene transfer to the livers of adult male patients with severe hemophilia B.[1,2] In part, the unexpected occurrence of T-cell responses to input capsid, resulting in transaminitis in several patients, prevented rapid adaptation of the protocol to other diseases. Memory CD8$^+$ T cells in humans stemming from natural infection with wild-type AAVs are a likely culprit.[1] It should be pointed out that the vast majority of preclinical studies have been conducted in mice. However, laboratory mice are typically not natural hosts for the parent viruses from which AAV vectors are derived, and several attempts to reproduce T cell–mediated destruction of AAV-transduced hepatocytes failed in murine models.[3]

Large animals are better suited for translational studies to test safety, for scale-up of efficacy, and to conduct long-term follow-up, and thus may help address questions that mouse studies cannot answer. However, such studies are expensive, and there is probably not a perfect model that will answer all questions. Regulatory agencies favor studies in a natural model of disease such as hemophilia B dogs, which supported the initial clinical trials on muscle- and liver-directed gene therapy with AAV2 vectors.[1] An alternative approach is to use animals that are evolutionarily closer to humans and therefore more likely to faithfully represent the cellular machinery involved in transduction and other aspects of vector and host biology. Rhesus macaques, in particular, have enabled major advances in pharmaceutics and vaccine development, and they have provided a large portion of the preclinical studies in support of the more recent and still ongoing phase I/II clinical trial of AAV8-mediated, liver-targeted gene transfer for hemophilia B. In this issue of *Molecular Therapy*, four articles test the utility of the nonhuman primate model to address potential roadblocks to sustained therapeutic gene expression from AAV vectors in the liver, including immune responses to vector and transgene product and stability of the vector genome.[4–7]

Nonhuman primates are naturally infected with AAVs. Studies in macaques facilitated modeling of the effects of preexisting antibodies on AAV gene transfer, stemming from prior natural infection, and correctly predicted their negative impact on liver gene transfer in humans even at low titers.[8,9] Indeed, patients in the current hemophilia B trial were prescreened for the presence of neutralizing antibodies against AAV8 before enrollment. Additional critical insights from the macaques included scale-up of efficacy with peripheral vein delivery, although the increase in factor IX transgene expression from a self-complementary vector genome was not as profound as was first observed in mice.[10,11]

At the same time, it has been puzzling that nonhuman primate studies failed to model the destruction of transduced hepatocytes by capsid-specific cytotoxic T lymphocytes, despite natural infection with AAV and the resulting preexisting immunity. The study by Li *et al.*[4] in this issue shows that, although AAV capsid-specific CD8$^+$ and CD4$^+$ T cells can be detected in the blood of naturally infected macaques and humans, these T cells exhibit significant differences in their differentiation status and function. The authors suggest that this may explain the species differences seen in sustained hepatic gene transfer. Central memory cells, more common in humans, expand more vigorously upon restimulation with AAV capsid antigen than the effector memory T cells, which are more common in nonhuman primates. Additionally, human capsid-specific T cells were found to produce interferon-γ, a cytokine

that upregulates the major histocompatibility complex I (MHC I) molecules that are critical for $CD8^+$ target cell recognition, whereas those from nonhuman primate T cells did not. These findings raise several questions. Are there differences between rhesus macaques and humans in the diversity, distribution, or replication of the AAV genome—all of which may influence T-cell responses? Are there differences between the helper viruses that affect the response to native AAV? Are differences in the immune systems of the two species impacting the T-cell response, such as the markedly higher number of different MHC I molecules in rhesus macaques?

Preexisting immunity to AAV vectors in humans can possibly be avoided altogether by performing gene transfer in young children.[12] Furthermore, many genetic metabolic diseases manifest at a young age, making it essential to have effective interventions for treatment that can be initiated at an early age and achieve long-term efficacy. However, AAV vector genomes persist predominantly in episomal forms, thereby increasing safety by reducing the risk of insertional mutagenesis. Ironically, this feature may hinder extension of therapeutic protocols to pediatric patients because episomal genomes may be lost in a growing tissue. This problem is highlighted by the study by Wang *et al.*, who report that AAV8-mediated hepatic gene transfer in infant primates is safe and efficient but less stable when compared to adolescent animals, with a significant loss of genomes and transgene expression over time.[6] The authors studied nonhuman primates because the growth rate of infant nonhuman primates is similar to the growth trajectory of humans. Potential solutions to this problem lie in novel approaches to targeted integration (such as zinc-finger nucleases or transcription activator–like effector nucleases) or in protocols based on sequential administration of different serotype vectors. Interestingly, Mattar *et al.* achieved sustained therapeutic factor IX expression in macaques after hepatic *in utero* gene transfer with self-complementary AAV vectors.[7] Despite substantial loss of vector genomes within the first month after birth, subsequent expression was quite stable, with persistence of episomal and integrated vector forms.

Immune responses may be directed not only against the vector but also against the transgene product. Because of the tolerogenic nature of hepatic gene expression, liver gene transfer with AAV is being considered for immunomodulatory therapy for lysosomal storage and other genetic diseases.[13] This concept, based largely on findings in mice, has received a boost by Nietupski *et al.*, who provide the first strong evidence for tolerance induction in nonhuman primates.[5] In this study, liver-restricted expression of the lysosomal enzyme α-galactosidase A from AAV8 vectors resulted in humoral immune tolerance, meaning a lack of antibody formation even after subsequent immunization with exogenous enzyme.

A disappointing finding by Nietupski *et al.* is that levels of α-galactosidase A were 1–2 logs lower than those achieved in male mice at the same vector dose by weight. Although equivalent vector copies were found in mouse and primate liver, messenger RNA levels in primates were much lower. Transcriptional silencing could not be explained by DNA methylation because AAV genomes were not found to have been methylated.[5] Unlike in mice, expression in the monkeys could not be elevated by androgens, local delivery to the liver, immune suppression, a self-complementary vector, or pharmacological approaches. More experiments are needed to elucidate why high levels of expression can be achieved in primate liver only with certain transgenes.

In conclusion, nonhuman primate models yield important data that aid translation of gene therapy protocols but also have serious limitations for predictions of outcomes in humans. Albeit slow and expensive, more small-scale early-phase clinical trials are needed. Collaborations between multiple centers—as has been the case in the hemophilia trials—make this approach more feasible and greatly facilitate progress.

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