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COMMENTARY

Clinical progress in gene therapy: Sustained partial correction of the bleeding disorder in patients suffering from severe hemophilia B

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The field of gene therapy has gained momentum in recent years thanks largely to the clinical successes for the treatment of monogenetic hereditary diseases. Since the early years of gene therapy, hemophilia had been widely regarded as an important target disease in its own right and a trailblazer for the field at large (Axelrod et al., 1990; Kay & High, 1999). The disease and the underlying genetics are well understood. Moreover, therapeutic efficacy can easily be ascertained based on robust clinical endpoints (i.e. circulating clotting factor levels, bleeding episodes) and there are several well-established and validated hemophilic animal models available for pre-clinical testing.

Hemophilia A and B are due to deficiencies in coagulation factors VIII (FVIII) and IX (FIX), respectively, and are characterized by uncontrolled bleeding episodes primarily in the joints and muscles but also in the vital organs which can be life-threatening. Current treatment is based on protein substitution therapy using either purified plasma-derived or recombinant clotting factors. Though this treatment has improved the patients' quality of life it is not curative and some patients develop inhibitory antibodies against the clotting factors that render further substitution therapy ineffective. What makes hemophilia a particularly attractive target disease for gene therapy is that it is

not necessary to normalize clotting factor levels in the blood to obtain a therapeutic effect. Indeed, even clotting factor levels slightly above the 1% threshold are considered therapeutic and can convert severe hemophilia into a moderate form. People with moderate hemophilia have 1% to 5% of the normal clotting factor in their blood. They tend to have bleeding episodes after injuries and some without obvious cause (i.e. “spontaneous” bleeding episodes). People with mild hemophilia have 5% to 50% of the normal clotting factor in their blood and most of these patients usually have problems with bleeding only after serious injury, trauma or surgery. In many cases, mild hemophilia is not diagnosed until an injury, surgery or tooth extraction results in prolonged bleeding. The first episode may not occur until adulthood. Based on encouraging preclinical studies in hemophilic mouse or dog models, achieving sustained elevated clotting factor levels above the 1% or 5% threshold by gene therapy seemed within reach. However, pursuing this goal was much more elusive than had initially been anticipated. Though several gene therapy trials for hemophilia A or B were initiated, using either viral or non-viral vectors, none of these studies resulted in sustained therapeutic levels of clotting factors in the plasma of the treated patients (Matrai et al., 2010; High 2011). The failures of these trials casted some doubts whether gene therapy would ultimately be effective for the treatment of hemophilia .

Nevertheless, a glimmer of hope was apparent following the muscle-directed and liver-directed gene therapy trials in hemophilia B patients when adeno-associated viral vectors (AAV) were used based on serotype 2. In particular, AAV2-based FIX gene delivery resulted in sustained FIX expression in the transduced muscle but the levels attained were insufficient to achieve FIX expression levels in the plasma above the 1% threshold (Kay et al., 2000; Jiang et al., 2006). In contrast, following liver-directed gene therapy with an AAV2 vector that expressed FIX from a robust liver specific promoter, therapeutic FIX expression levels could be achieved up to 12% of normal levels in one of the subjects treated at the highest vector dose (i.e. 2×10^{12} vector genomes/kg) (Manno et al., 2006). These levels were consistent with what could be attained by AAV2-based liver-directed gene therapy in preclinical hemophilia B dog studies. Unfortunately, in this case, FIX expression levels declined to basal 1 month after gene therapy. This decline could possibly be attributed to a vector-dose dependent AAV2 capsid-specific T cell response that eliminated the FIX-transduced hepatocytes (Mingozzi et al., 2007). This T-cell response coincided with the emergence of liver inflammation and transient, self-limiting liver toxicity. The reason why the dog model did not faithfully reproduce this T cell response that was observed in human subjects may be due, at least in part, to the presence of pre-existing anti-AAV2 T cell immunity in humans following natural exposure to infectious wild-type AAV and helper viruses. These previous clinical studies strongly suggested that AAV-based approaches may ultimately yield sustained circulating FIX expression levels if the T-cell immune response against the FIX-transduced cells could be prevented or contained.

An important milestone has now been reached in a recent gene therapy clinical trial for hemophilia B (Nathwani et al., 2011). This clinical trial builds upon this prior work and on encouraging pre-clinical data in non-human

primates (Nathwani et al., 2006). In this trial, patients suffering from severe hemophilia B (<1% FIX) were injected by peripheral vein administration with an AAV serotype 8 vector (AAV8) encoding a codon-optimized FIX. This AAV8 serotype can efficiently transduce hepatocytes, does not interact as efficiently with antigen-presenting cells as AAV2 and has limited cross-reactivity with pre-existing anti-AAV2 antibodies (Gao et al., 2002, Vandenberghe et al., 2006, VandenDriessche et al., 2007). The vector design was based on a self-complementary (sc) configuration that results in enhanced hepatic transduction (McCarthy, 2001, 2003; Nathwani et al., 2006). This scAAV design is more efficient possibly because it obviates the need for second strand synthesis or re-annealing of positive and negative AAV strands to generate transcription-competent double-stranded DNA templates. Subjects received low (2×10^{11} vg/kg), intermediate (6×10^{11} vg/kg) or high (2×10^{12} vg/kg) scAAV8-FIX vector doses, with two participants in each cohort. All subjects expressed FIX above the 1% threshold for several months after gene therapy. In particular, sustained FIX levels varied between 2 to 11% of normal levels in all the treated subjects. Four of the six discontinued FIX prophylaxis and remained free of spontaneous bleeding episodes, though most of these subjects required prophylaxis to prevent bleeding upon trauma. The other two participants required less frequent FIX protein infusions but FIX infusions were still required possibly because of the preexisting hemophilic arthropathy. It is particularly encouraging that no inhibitory antibody responses against the FIX protein itself could be detected in any of the treated subjects. This may reflect the ability of the FIX-transduced hepatocytes to induce immune tolerance against the transgene product (Mingozzi et al., 2003). However, since patients were pre-selected for lack of inhibitors following protein substitution therapy there may be an intrinsic bias against this risk. Ultimately, it will be worthwhile to evaluate this gene therapy approach in patients that have a higher intrinsic risk of developing inhibitors.

Though this is the first study that yields sustained FIX expression levels after gene therapy, immune mediated clearance of AAV-transduced hepatocytes remains a concern. In particular, one subject who received the highest vector dose developed grade III liver toxicity related to the vector itself, resulting in a significant increase in serum transaminase levels and a concomitant decrease of FIX levels from 7% to 3%. This was associated with the detection of AAV8 capsid-specific T cells. The other subject had a slight increase in liver enzyme levels concomitant with an increase in AAV8 capsid-specific T cells and a slight decrease in FIX level. However, other factors, unrelated to the gene therapy itself, may also have contributed to the slight increase in liver enzymes. Consequently, these T cell responses were reminiscent of the AAV2-specific T cell response in the previous liver-directed gene therapy trial. This indicates that serotype switching does not suffice to prevent such capsid-specific T cell responses. Nevertheless, this AAV-specific T cell response could be controlled with a short course of glucocorticoids which rapidly restored liver enzyme level to normal and maintained FIX levels in the range of 3-11% in these two high dose subjects.

There are still a number of unresolved questions that were prompted by this original study. One intriguing and unexplained finding is that two of the

participants that received the mid-dose had an elevated AAV8 capsid-specific T cell response, in the absence of any liver toxicity. Though liver toxicity is associated with a peripheral capsid-specific T cell response (i.e. in one of the subjects who received the highest dose), not all capsid-specific T cell responses measured in the peripheral blood are associated with hepatocyte destruction and liver toxicity. This suggests that peripheral T cell responses may not necessarily reflect the actual local T cell response or repertoire in the liver itself. This possible dichotomy may reflect differences in the liver micro-environment and cytokine milieu. Alternatively, the level of presentation of AAV8 capsid-derived antigenic peptides on MHC class I hepatocytes may not have sufficed to trigger T-cell mediated hepatocyte destruction in these two subjects who received the mid dose compared to the highest dose subject. Finally, one must formally consider the possibility that the transaminase elevation in the fifth subject was not related to vector-specific host immune responses. Future AAV-based gene therapy trials are required in a larger number of patients to address some of these outstanding questions.

Though sustained therapeutic FIX levels were obtained in this trial, these levels are not sufficient to achieve a *bona fide* cure of hemophilia and to prevent bleeding in the face of trauma or injury. Consequently, improved vector designs are warranted to achieve full hemostatic correction in patients with severe hemophilia B. Indeed, since scAAV has a limited cloning capacity of 2.5 kb a truncated liver-specific promoter was used to accommodate the FIX gene in the scAAV backbone. This truncated promoter was less potent than its non-truncated counterpart. Though the use of higher vector doses may be required to further increase FIX levels this may require additional fine-tuning of the immunosuppressive regimens to contain inadvertent T cell responses and hepatocellular injury. Moreover, the proportion of functional infectious vector particles *versus* empty particles could be further improved. Last but not least, The AAV capsids themselves could be engineered further to minimize the risk of inducing dose-limiting T cell responses and liver toxicity

This clinical trial demonstrates unequivocally that gene therapy can result in a sustained therapeutic effect in hemophilia B patients yielding FIX levels sufficient to convert severe to moderate or even mild hemophilia. It is tempting to speculate that the same approach could ultimately also be used for gene therapy of the more common hemophilia A form. However, it is more challenging to accommodate the larger FVIII transgene into an AAV vector without compromising vector titer and efficiency. Nevertheless, we have recently overcome this hurdle using a codon-optimized FVIII (Ward et al., 2011) driven from a robust minimal liver-specific promoter. These clinical trial findings have important implications not just for gene therapy of hemophilia but also for other monogenetic disease. The study clearly underscores the potential of AAV for liver-directed gene therapy and further strengthens the case for using the latest generation AAV vector platforms for clinical gene therapy in other tissues. Hemophilia has once again become the trailblazer for the gene therapy field.

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