Molecular Therapy
Commentary

Transient expression of factor VIII and achronic high-fat diet induces ER stress and late hepatocyte oncogenesis

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Liver-directed gene therapy using adeno-associated virus (AAV) for delivery of therapeutic transgenes has posed particular challenges over the past 25 years, and there are no FDA-approved products to date. Reasons include lack of efficacy or toxicity or both, despite acceptable efficacy and toxicology results in animals, including non-human primates. The experience with AAV-mediated gene therapy for hemophilia is a case in point. The clotting factor VIII gene was cloned in 1984, but production of the full-length recombinant protein is challenging. Despite this, two therapeutic recombinant factor VIII proteins were approved and marketed by 1993. A subsequent B-domain deleted (BDD) version of factor VIII (FVIII) proved more efficient to manufacture in cell lines than full-length FVIII., However, it was later recognized that high levels of FVIII expression are responsible for an endoplasmic reticulum (ER) stress response in cells.[1] With the enigmatic toxicity seen in AAV-factor VIII gene therapy trials, all of which use the BDD factor VIII, investigation redoubled into whether an ER stress response may be responsible for the variable and diminishing levels of factor VIII observed in the clinical trials.

In this issue of *Molecular Therapy*, Kapelanski-Lamoureux and colleagues further investigated their previously identified unfolded protein response in mice receiving only the BDD FVIII plasmid transiently by hydrodynamic injection.[2] Unexpectedly, when fed a high-fat diet to induce liver inflammation, they found an association with the development of hepatocellular tumors at 65 weeks in 100% of the mice receiving the FVIII BDD plasmid, 58% of mice receiving a plasmid expressing a more foldable hyperglycosylated BDD FVIII, and no mice receiving a plasmid encoding an easily foldable protein (Figure 1). Importantly, the transgenes were expressed transiently and not delivered with AAV, thus removing that vector and its propensity for integration from being implicated in the oncogenic process.

The field of liver-directed gene therapy is dominated by phenomenology and observation, not so much by mechanism, thus obscuring the reasons behind the variability, lack of predictability of response, and hepatocyte toxicity that can occur.[3] Originally, in hemophilia clinical trials, we thought hepatocyte dose-related toxicity was attributable to a cytotoxic T cell response to viral peptides presented on the cell surface in the context of unique HLA class 1 molecules[4] despite a tolerogenic environment. Later, FVIII expression was also considered the culprit, yet the clinical trial findings were unclear, and the toxicity in participants with hemophilia A appeared multifactorial. Using a stronger promoter, one group was able to establish an ER stress response in hemophilic mice treated with AAV5-BDD factor VIII[5] but has not detected it in a limited number of liver biopsies sampled from trial participants a few years after the therapy was administered.[6]

At least three groups are actively evaluating AAV-FVIII in the clinic. One is in phase 2 (Spark),[7] the second one is in phase 3 (Pfizer),[8] and the third has completed phase 3 (BioMarin),[9] which is conditionally approved in the EU and has filed for approval in the US. In all studies, FVIII expression rises to therapeutic or supranormal levels, falls over the first year, and then appears to slowly decline year over year in some or most trial participants in a steady fashion.[9,10] The consequence is that transgenic FVIII levels will approach and drop below the therapeutic threshold in many patients, and they will lose the benefit of therapy in a matter of years. The cause(s) of this effect are not understood but do not follow the kinetics nor can be solely ascribed to a cytotoxic T cell response that has been attributed to the decline in FIX in some patients with hemophilia B.[4,11] In participants with hemophilia B, reactive steroids can prevent the loss of FIX[11] but have much less of an effect on preserving FVIII levels.[9,10]

The implications of the new observations of Kapelanski-Lamoureux and colleagues are worth considering against this backdrop. AAV vectors delivering therapeutic levels of transgenes integrate at a rate of about 1 in 1,000 hepatocytes, leading to over 100 million integration events in adult human livers containing ~140 billion hepatocytes. Integration tends to be in regions of open chromatin and has not led to hepatocellular carcinoma in humans to date. However, total numbers of treated patients and time from infusion are both small. Likewise, non-human primate studies have all been negative for hepatocellular carcinoma (HCC) but are relatively short term and do not take into account the substantial rates of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) in individuals consuming Western diets, which are independent risk factors for HCC.[12] Adding a transient unfolded protein response as a risk factor for HCC independent of AAV

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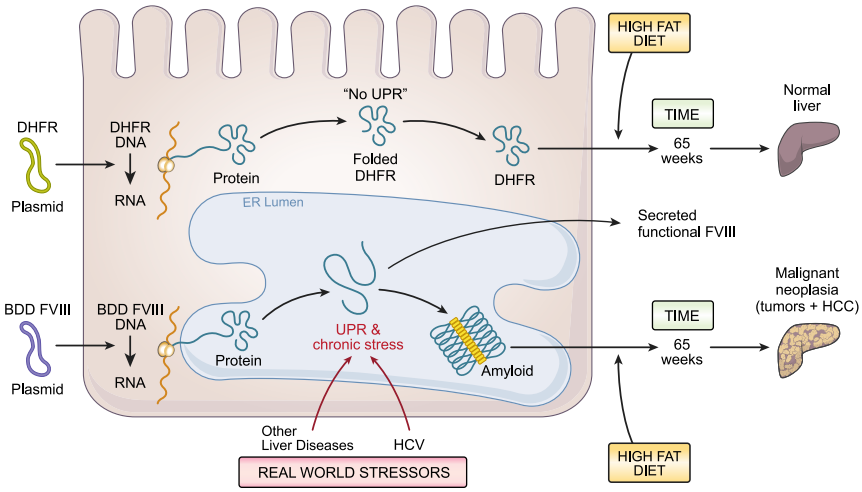

Figure 1. Kapelanski-Lamoureux et al. show that transient expression of dihydrofolate reductase (DHFR) versus BDD FVIII lead to vastly different outcomes after 65 weeks of high-fat diet

Whereas DHFR leads to well-folded DHFR without an unfolded protein response (UPR), just a brief expression of BDD FVIII with the addition of lengthy ER stress leads to malignancy and neoplasia in the form of tumors including hepatocellular carcinoma (HCC). Many patients are subject to additional real-world stressors including other liver diseases such as hepatitis C virus (HCV).

integration and NAFLD/NASH, in a population where a substantial number of patients have recovered from decades of hepatitis C infection, which is also a risk factor for HCC, is a cause for concern.

Clearly, the disparate results between durability of FVIII and FIX AAV gene therapy require scientific inquiry. While this new publication is intriguing and extends the multiple-hit hypothesis leading to HCC, it is premature to extrapolate these epigenetic changes leading to malignancy to human recipients of AAV-FVIII gene therapy. On the other hand, protein misfolding induced by FVIII transgenes has been recognized for decades, and the underlying inflammatory consequences of the unfolded protein response, coupled with other insults such as an increased lipid burden and changes resulting from multi-decade hepatitis C virus (HCV) infections, must be taken into consideration when analyzing the benefit/risk balance in treatments for patients with hemophilia A. The study thus has potentially far-reaching general implications beyond gene therapy for hemophilia. Results suggest that hepatic expression of proteins that misfold and thus induce cellular stress followed by a second insult on the liver such as high-fat diet may predispose to malignancy.

A global registry, monitoring every patient who receives AAV gene therapy for hemophilia, is a requirement to detect low-incidence events as early as possible. The World Federation of Hemophilia (WFH), in collaboration with the International Society for Thrombosis and Haemostasis, and other medical and lay organizations, plus the pharmaceutical companies developing gene therapy, have all come together to generate a core dataset designed to capture all patients who receive AAV gene therapy globally: the WFH Gene Therapy Registry.[13]

In the meantime, it is also clear that improved AAV-FVIII vectors are needed to provide less variability, more predictability of responses, longer durability, and, in conjunction with immunomodulatory agents, the ability to redose. This research is required to cure hemophilia, a dream since the disorder was first scientifically recognized over 200 years ago. At least one contribution receiving minimal attention is engineering an FVIII that has substantially improved secretion over the BDD FVIIIs used in clinical gene therapy studies today.[14,15]

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