Tolerance Induction by Viral *In Vivo* Gene Transfer

Eric Dobrzynski, PhD and Roland W. Herzog, PhD

Treatment of genetic disease by protein or gene replacement therapy is hampered by immune responses to the therapeutic protein. An excellent example is formation of inhibitory antibodies to coagulation factors in treatment of the X-linked bleeding disorder hemophilia. Experiments in murine and canine models of hemophilia B (deficiency in factor IX) have demonstrated sustained therapeutic levels of factor IX transgene expression following hepatic adeno-associated viral gene transfer in animals with deletion and nonsense mutations in the factor IX gene. This article reviews experimental evidence for induction of immune tolerance to the factor IX transgene product by hepatic adeno-associated viral gene transfer, which has been shown to limit T helper cell responses and to substantially reduce the risk of antibody responses. Tolerance induction is associated with activation of regulatory CD4⁺ T cells capable of suppressing antibody formation to factor IX protein. Hepatic administration of adeno-associated viral vector expressing ovalbumin in mice transgenic for a T cell receptor specific for this antigen provided direct evidence for induction of CD4⁺ T cell tolerance, including T cell anergy and clonal deletion. Taken together, these data indicate the potential for viral *in vivo* gene transfer not only to provide sustained systemic expression, but moreover to induce immunological hypo-responsiveness to the therapeutic gene product.

Keywords: Adeno-associated virus; Factor IX; Gene therapy; Hemophilia; T cell; Tolerance

A major concern in treatment of inherited protein deficiency by protein or gene replacement therapy is the risk for an immune response to the therapeutic protein which, by definition, differs from the mutation-carrying variant expressed by the recipient of therapy. For example, hemophilia is an X-linked bleeding disorder caused by mutations in the coagulation factor VIII (hemophilia A) or factor IX (hemophilia B) gene.

The most serious complication of current therapy (i.e., intravenous infusion of protein concentrate) is the potential for the formation of inhibitory antibodies to the intravenously infused clotting factor protein, which occurs at a frequency of 25%-30% in hemophilia A and 3%-4% in hemophilia B.¹ Immune tolerance protocols for elimination of inhibitors in patients include frequent high-dose infusion of factor concentrate along with immunoglobulin and/or immunosuppressive drugs.² Data from several Phase I clinical trials in gene therapy using different vectors and target organs have not found any inhibitor formation in patients.^{3,4} Even though no inhibitors have been observed in these clinical

trials, it should be noted that these patients were carefully selected based on previous history of inhibitor development during protein replacement therapy. In the case of muscle-directed factor IX gene transfer, only patients with missense mutations in the factor IX gene were enrolled in the trial.⁵

Inhibitor formation has been observed following gene therapy in pre-clinical animal studies in hemophilic mice or dogs.⁶ The risk of inhibitor formation in these animals is influenced by multiple factors, including the type and design of the gene transfer vector, route of administration, vector dose, transgene expression levels and the underlying native gene mutation. Another important difference between the preclinical animal studies and the human trials is that gene transfer was performed in naïve animals, while the human subjects may have developed immune tolerance due to repeated intravenous infusion of factor concentrates. Thus the prediction of inhibitor formation following the animal studies may be of particular relevance if gene transfer protocols were performed in previously untreated patients.

Reprint Requests: Roland W. Herzog, PhD, University of Florida, Progress Park, 13706 Innovation Drive, Room 201, Alachua, FL 32615-9586, Tel: 386-462-6139, Fax: 386-462-4099, E-mail: rherzog@ufl.edu

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Immune Responses to Coagulation Factors – General Considerations

Inhibitor formation against factor VIII or factor IX is dependent on CD4⁺ T helper cells. The T cell receptor of CD4⁺ T cells binds to a specific peptide antigen-major histocompatibility complex (MHC) class II complex expressed on the surface of antigen presenting cells (e.g., dendritic cells or macrophages). This process may lead to T cell activation, which through similar interaction between the activated T and B cells can lead to B cell activation and subsequent antibody formation. Signaling to the T cell through the T cell receptor is referred to as signal 1. In addition to T cell receptor signaling, activation of a naïve T cell requires co-stimulatory signals from the antigen presenting cells, also termed signal 2. If the T cell does not receive adequate stimulatory signals from the antigen presenting cells, tolerance may be induced.⁷ Expression of co-stimulatory molecules on the surface of antigen presenting cells is dependent on their activation status. Endogenous (e.g., cytokines) as well as exogenous (e.g., molecular structures associated with pathogens) activation signals have been described.⁷

Upon activation, CD4⁺ T cells differentiate into T helper cells. The type of T helper cell that is being activated depends, among other factors, on the cytokine milieu present during the interaction between the T cell and the antigen presenting cells. Subsets of T helper cells can be differentiated by their cytokine expression pattern and include Th1, Th2 and Th3 cells. Typically, Th1 cells produce interferon (IFN)- γ , tumor necrosis factor (TNF)- α and interleukin (IL)-2; Th2 cells produce IL-4, IL-5, IL-10 and transforming growth factor (TGF)- β ; and Th3 cells produce high amounts of TGF- β . Type 1 T regulatory (Tr1) cells produce large amounts of IL-10 and are immune suppressive. Th1, Th2 and Th3 cells are all capable of activating B cells and promoting antibody formation, however, the particular subclass of immunoglobulin produced differs. Th1 cells promote IgG2a formation in mice (equivalent to IgG1 in humans), Th2 cells cause IgG1 formation in mice (IgG4 in humans) and Th3 cells drive IgA formation (typically secreted to mucosal surfaces). Inhibitory antibodies are typically comprised of IgG1 and/or IgG2a in mouse models and IgG1 and/or IgG4 in humans. Additionally, Th1 cells provide help for CD8⁺ T cells which, in contrast to CD4⁺ T cells, are MHC class I restricted and differentiate into cytotoxic T lymphocytes upon activation. This is more of a concern in gene-based treatment, because activation of cytotoxic T lymphocytes could lead to destruction of cells expressing the therapeutic protein.⁸

Gene Protocols May Differ in Their Risk for Immune Responses

There are a variety of vectors capable of transferring a functional coagulation factor gene. The choice and design of the vector can have a profound effect on the likelihood and specifics of an immune response. For example, the capsid of adenoviral vectors elicits a potent innate immune response

which causes vector dose-limiting toxicity.⁹ Adenoviral and lentiviral vectors (if pseudotyped with envelope from vesicular stomatitis virus-G) efficiently transduce professional antigen presenting cells, thereby increasing the risk of cellular and antibody-mediated immune responses to the transgene product.^{10,11}

While all gene therapy protocols and vector systems, not unlike conventional protein-based therapy, bear some risk of inhibitor formation, several lines of research have established that this risk can be reduced by restricting transgene expression to hepatocytes and/or by limiting the interaction of the vector with antigen presenting cells.^{10,12-17} Incorporation of a tightly regulated hepatocyte-specific promoter has been shown to limit antibody formation to secreted transgene products in the context of adenoviral or lentiviral vectors.^{10,12}

Studies from two laboratories on retrovirus-mediated gene transfer to livers of neonatal mice and dogs with hemophilia A or B resulted in sustained curative levels of factor VIII and factor IX.¹⁸⁻²⁰ Further analysis with a human factor IX transgene showed that this approach can lead to induction of robust immune tolerance to the therapeutic gene product.²¹ This work takes advantage of differences in the immune system of neonatal compared to older animals, which provides a window of time when the immune system is not fully matured and immune tolerance induction is facilitated.

Our approach toward treatment of hemophilia B is based on *in vivo* gene transfer with adeno-associated viral vectors. These are derived from a single-stranded, naturally replication-deficient and non-pathogenic member of the parvovirus family with a 4.7 kb single-stranded DNA genome.²² The vector contains a factor IX expression cassette, but is devoid of viral coding sequences. Adeno-associated viral vectors can efficiently transfer genes to non-dividing target cells such as muscle fibers and hepatocytes. Intramuscular, as well as hepatic administration, has led to sustained systemic factor IX expression and partial correction of hemophilia B in small and large animal models.^{14,23-30} Both strategies were subsequently tested in Phase I/II clinical trials.^{5,31,32} Hepatic gene transfer was typically carried out by injection of vector into the portal vein or the hepatic artery. A comparison of both protocols in several animal models of hemophilia B indicated a reduced risk of inhibitor formation by the hepatic route.^{6,13,14,30} Sustained factor IX expression was observed even in animals with a factor IX gene deletion or nonsense mutation.^{14,27,28} Once the adeno-associated viral (serotype 2) vector is infused into the liver, a tropism toward hepatocytes ultimately results in factor IX expression in approximately 5% of hepatocytes, with vector dose-dependent levels of expression.^{29,33} We now asked the question of whether this observed sustained expression in the absence of inhibitor formation is linked to induction of immune tolerance to the transgene product.

Figure 1. Model for induction of immune tolerance to a therapeutic gene product by hepatic adeno-associated viral-mediated gene transfer. Vector is infused into the hepatic circulation resulting in gene transfer and transgene expression in hepatocytes (green stain). Subsequent presentation of transgene product-derived peptides via MHC class II molecules (likely through professional antigen presenting cells) to naïve CD4⁺ T cells causes induction of T cell anergy and deletion of transgene product-specific T cells or causes activation of regulatory T cells. The latter suppress activation of any remaining responsive CD4⁺ T cells. As a consequence of these mechanisms, there is a lack of T helper cell responses, in turn causing failure to activate B cells and CD8⁺ T cells. Ultimately, antibody or cytotoxic T cell responses are not observed or are limited.

Tolerance Induction to Factor IX by Hepatic Adeno-Associated Viral Gene Transfer

The ability to induce tolerance to a therapeutic protein in an adult animal by *in vivo* gene transfer would provide new perspectives and possibilities to the field of gene replacement therapy. Our initial experimental system to address these questions was hepatic gene transfer of a human factor IX cDNA by adeno-associated viral administration to immune competent adult mice of different strain backgrounds.¹⁵ From studies with other routes of administration, it was clear that human factor IX represented a neo-antigen to which these animals were not tolerant. One could now test whether immunological unresponsiveness to human factor IX after hepatic adeno-associated viral gene transfer was due to tolerance or ignorance. To this end, mice were challenged by subcutaneous administration of human factor IX in complete Freund's adjuvant several weeks after gene transfer. Control mice did not receive gene transfer or were injected with an adeno-associated viral vector expressing an irrelevant gene product (green fluorescent protein). While controls formed antibodies to human factor IX, hepatic adeno-associated viral-human factor IX transduced mice failed to respond to the immunization.¹⁵

Additional studies demonstrated antigen-specific tolerance induction and suggested that a level of expression of approximately 30 ng/ml plasma was required for tolerance induction. Some strains of mice showed an antibody response to human factor IX after low dose vector administration, but were tolerized at higher vector doses or if a stronger promoter was chosen. A detailed dose escalation revealed that levels of transgene expression as determined by the combination of vector dose, promoter strength and mouse strain determined whether immune tolerance was achieved.¹⁵ Induction of

immune tolerance was also documented in hemophilia B mice with a factor IX gene deletion, albeit with a lower success rate, suggesting that endogenous factor IX expression may facilitate tolerance induction. Furthermore, data in this experimental system revealed a correlation between lack of B cell and lack of T helper cell responses affecting Th1 and Th2-dependent responses.^{15,34}

In general, mechanisms leading to T cell unresponsiveness include T cell anergy (a state of unresponsiveness associated with lack of IL-2 cytokine expression), clonal deletion of antigen-specific T cells or activation of regulatory T cells (T_{reg}). Whether a T cell is tolerized or activated to become an effector cell depends on the activation status of the antigen presenting cells that interact with T cells. Activation of antigen presenting cells is dependent on "danger" or inflammatory signals. Thus, the context of antigen presentation (e.g., type of tissue, inflammatory signals provided by gene transfer vector or injury) plays a significant role in T cell activation. Depending on the target tissue, local cytokine concentrations may also skew T cell activation. For example, it is known that antigen presentation in lymphoid tissues associated with mucosal surfaces can induce T_{reg}, meaning that CD4⁺ T cells activated in this context differentiate into T helper cells that suppress immune responses through secretion of certain cytokines such as IL-10 or TGF- β (more information can be found in literature on oral and nasal tolerance). Examples for such induced T_{reg} include Tr1 and Th3 cells with immune suppressive properties. On the other hand, there are also "naturally occurring" regulatory T cells that are generated through stimulation with self-antigens. These CD4⁺CD25⁺ T cells constitutively express the α chain of the IL-2 receptor (CD25) and are critical for prevention of autoimmune disease.³⁵ If gene transfer resulted

in activation or generation of CD4⁺CD25⁺ T_{reg}, this could be significant for maintaining tolerance.

In our hepatic adeno-associated viral-factor IX gene transfer approach, adoptive transfer studies provided evidence for activation of regulatory CD4⁺ T cells capable of suppressing antibody formation to human factor IX.¹⁵ No suppression was observed when CD4⁻ cells were transferred. Experiments in knockout mice showed that tolerance induction was not dependent on $\gamma\delta$ -T cells or CD8⁺ T cells, which may be involved in regulation during oral tolerance induction.^{36,37} Further analysis of CD4⁺ T_{reg} is under active investigation. Other experiments suggested a requirement for Fas-Fas ligand mediated apoptotic cell death for maintenance of antigen-specific tolerance.¹⁵

Direct Evidence for CD4⁺ T Cell Tolerance in Transgenic Mice

A precise immunological characterization of tolerance induction in the factor IX gene transfer system faces several limitations. Antigen-specific T cells are rare and cannot be specifically identified. These limitations can be overcome by use of a mouse that is transgenic for a specific T cell receptor. The model we chose is based on BALB/c mice that express the chicken ovalbumin (ova)-specific T cell receptor on 80%-90% of their CD4⁺ thymocytes.³⁸ This T cell receptor is specific for the ova peptide 232-339 and is encoded by the rearranged V α 13 and V β 8.2 genes. The ova peptide is presented to the T cell receptor by the MHC class II molecule I-A^d. The high number of ova specific CD4⁺ T cells allows for extensive *in vitro* and *in vivo* studies not possible in non-transgenic mice. Lymphocytes isolated from lymphoid organs from naïve DO11.10 mice are responsive to *in vitro* cell culture containing ova protein or peptide. Physically tracking these cells is made possible by staining with clonotypic KJ1-26 monoclonal antibody specific to the DO11.10 T cell receptor followed by flow cytometry. This experimental approach has been successfully used to uncover mechanisms of oral tolerance induction.^{39,40}

Hepatic administration of an adeno-associated viral vector resulted in vector dose-dependent systemic expression of ova similar to experiments with factor IX.⁴¹ Lymphocytes from the spleen, thymus, inguinal and portal lymph nodes were harvested and examined at various time points after gene transfer. Re-stimulation of lymphocyte cultures with ova showed a reduction in IL-2 cytokine release compared to control mice (transduced with adeno-associated viral-green fluorescent protein vector) as early as 10 days after gene transfer. Lack of cytokine production correlated with lack of lymphocyte proliferation, which was largely reversed by addition of exogenous IL-2 indicating T cell anergy.⁴¹ Numbers of ova-specific CD4⁺ T cells were significantly reduced among thymocytes, splenocytes and lymph node cells by 1-2 months after gene transfer. By 2 months, the proportion of ova-specific cells was reduced among CD4⁺ T cells. Together, these data suggest clonal deletion and negative selection against ova-specific CD4⁺ T cells. Both thymic and

peripheral deletion/selection mechanisms may play a role in reducing numbers of ova-reactive T cells. Evidence for T cell anergy and increased apoptotic cell death of ova-specific CD4⁺ T cells was also obtained after adoptive transfer of DO11.10 transgenic CD4⁺ T cells to non-transgenic BALB/c mice followed by hepatic adeno-associated viral-ova injection.⁴¹

Are Naturally Occurring Regulatory T Cells Involved?

Recently, attention has increasingly focused on the role of naturally occurring CD4⁺CD25⁺ T_{reg}.³⁵ This subpopulation consists of approximately 10% of the total CD4⁺ T cells, is required to prevent autoimmunity and can efficiently suppress activation of CD4⁺CD25⁻ T cells.^{35,42} Activation of the CD4⁺CD25⁺ T_{reg} cells has been reported in oral and intravenous tolerance models.⁴³ T_{reg} cells have also been reported to prevent immune-mediated pathology in several disease models, including autoimmune thyroiditis, *Helicobacter hepaticus*-induced colitis and pulmonary hyper-inflammation.⁴⁴⁻⁴⁶ CD4⁺CD25⁺ T_{regs} nonspecifically suppress CD4⁺CD25⁻ T cells *in vitro*, but are thought to act in a more antigen-specific manner *in vivo*.⁴⁷ Interestingly, by 60 days after adeno-associated viral-ova hepatic gene transfer to DO11.10 transgenic mice, the percent of CD4⁺CD25⁺ T cells increased (compared to control mice) in the thymus, spleen and lymph nodes.⁴¹ *In vitro* assays were used to confirm T_{reg} function of these cells. This increased population of CD4⁺CD25⁺ cells likely contributes to ova-specific T cell anergy, thereby helping to maintain T cell tolerance towards the ova transgene product. Using the adoptive T cell transfer of CD4⁺CD25⁺ T cells, Gross et al. reported that these cells prevented an immune response to adeno-associated viral gene transfer to skeletal muscle.⁴⁸ Our laboratory is currently investigating a potential role of regulatory CD4⁺CD25⁺ T cells in prevention of antibody and cellular immune responses to the transgene product in the context of hepatic adeno-associated viral gene transfer.

Potential Hurdles for Clinical Application

Despite these encouraging results, which document the possibility of using hepatic gene transfer for sustained therapeutic gene transfer and induction of immune tolerance, several aspects of the biology of gene transfer may limit success of this strategy in clinical application. It is at least theoretically possible that observations on tolerance induction in rodent models will not translate to humans. Already, there are differences in the success rate of tolerance induction in different strains of mice, indicating a role of the genetic background.^{10,15} Nonetheless, data from canine models of hemophilia suggest that hypo-responsiveness of the immune system to factor IX antigen after hepatic gene transfer is not limited to mice, but may also be seen in other species.¹⁴ We would also like to point out that selection criteria for matching donors and recipients are less stringent in liver transplants compared to other organs in human patients and that hepatic allografts are possible even across incompatible HLA barriers supporting the idea of using liver as a tolerogenic organ of gene transfer.^{49,50}

A second concern is the impact of the antigen itself. For example, tolerance induction by hepatic gene transfer is possibly more difficult for factor VIII than for the antigens described above. T cell responses to factor VIII in humans are complex, involving a large repertoire of epitopes.^{51,52} The prevalence of inhibitor formation in hemophilia A in conventional protein-based therapy is substantially higher than in hemophilia B (20%-30% vs. 3%-4%) and avoiding antibody responses to factor VIII after hepatic gene transfer in murine and canine models has been more challenging than in factor IX gene transfer.⁵³⁻⁵⁵

Third, published studies were performed in naïve animals, while human patients (perhaps with the exception of young children) typically have antigen-experienced T cells to the transgene product (and often to the viral vector as well due to natural infection). For example, in contrast to our experience in hemophilia B mice and dogs, expression of factor IX following adeno-associated viral-mediated hepatic gene transfer in patients with severe hemophilia B was not sustained, despite absence of an antibody response to factor IX.⁵⁶ Ongoing studies are designed to test the hypothesis that reactivation of memory T cells to adeno-associated viral capsid was responsible for loss of transduced hepatocytes.⁵⁶ The impact of memory T cells on tolerance induction and strategies to block such responses should be explored.

Conclusions

Experiments described above demonstrate that adeno-associated viral-mediated gene transfer to the liver can induce immune tolerance to the transgene product. Other laboratories have now shown that these observations extend beyond factor IX gene transfer and have implications for treatment of other diseases, such as lysosomal storage disorders.⁵⁷ Antigen levels play a critical role in tolerance induction, which involves a combination of mechanisms, including T cell anergy, deletion and activation of regulatory T cells. Additional experiments should enable us to further define subsets of regulatory CD4⁺ T cells involved. During translation of this approach to clinical application, one has to keep in mind that data in pre-clinical models developed thus far were from animals naïve to vector and transgene products prior to gene transfer, while human subjects may have memory T cells. Nonetheless, robust tolerance induction in animal models of human disease is encouraging for going forward with *in vivo* gene therapy protocols.

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Author Affiliations

Eric Dobrzynski, PhD

Department of Pediatrics

*University of Pennsylvania Medical Center
and The Children's Hospital of Philadelphia
Philadelphia, Pennsylvania*

Roland W. Herzog, PhD

Department of Pediatrics

*University of Pennsylvania Medical Center
and The Children's Hospital of Philadelphia
Philadelphia, Pennsylvania*

Current affiliation:

*University of Florida
Gainesville, Florida*