Tolerance Induction in Hemophilia: Innovation and Accomplishments

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Abstract:

Purpose of review: Hemophilia is an X-linked blood coagulation genetic disorder, which can cause significant disability. Replacement therapy for coagulation factor VIII (hemophilia A) or factor IX (hemophilia B) may result in the development of high affinity alloantibodies ("inhibitors") to the replacement therapy, thus making it ineffective. Therefore, there is interest in directing immunological responses towards tolerance to infused factors.

Recent findings: In this review we will discuss latest advancements in the development of potentially less immunogenic replacement clotting factors, optimization of current tolerance induction protocols (ITI), pre-clinical and clinical data of pharmacological immune modulation, hepatic gene therapy, and the rapidly advancing field of cell therapies. We will also evaluate publications reporting data from pre-clinical studies on oral tolerance induction using chloroplast-transgenic (transplastomic) plants.

Summary: Until now, no clinical prophylactic immune modulatory protocol exists to prevent inhibitor formation to infused clotting factors. Recent innovative technologies provide hope improved eradication and perhaps even prevention of inhibitors.

Keywords: inhibitors, ITI, oral tolerance, gene therapy.

Introduction

Hemophilia is an X-linked monogenic blood coagulation disorder with significant morbidity, affecting primarily males [1]. Mutations in F8 and F9 genes lead to development of hemophilia A and B, respectively, of which hemophilia A is more prevalent (1:5,000 male births) [2,3]. Severity of hemophilia is based on residual activity of coagulation factors, where levels of <1% are classified as severe, 1-5% moderate, and 5-40% mild [4]. Factor replacement is used for therapy and prophylaxis since the 1950's. An increasing number of plasma-derived and recombinant FVIII and FIX products are available or in clinical development.

Development of inhibitory antibodies (inhibitors) is a major complication of replacement therapy, making it ineffective [5]. Inhibitors are high affinity alloantibodies directed against infused clotting factors, and are more common in hemophilia A patients [6]. Less than 5% of patients with severe hemophilia B develop inhibitors, mostly those with large F9 gene deletions [7]. Inhibitors are usually observed in previously untreated patients (PUP) within 50 days of exposure to factor product [6,8]. Bypassing agents are used to treat bleeding episodes, but the only way to eradicate inhibitors is through Immune Tolerance Induction (ITI). The mechanism of inhibitor formation is complex and not completely understood. Immune responses are initiated when antigen-presenting cells (APCs) encounter the "unfamiliar" replacement factor molecule and present it via HLA class II, marking it for elimination. Co-stimulatory signals via cell surface molecules such as cluster of differentiation 40 (CD40), or CD80/86 (which are found in dendritic cells and required for T cell survival), or ICOS ("inducible co-stimulator"), and secretion of pro-inflammatory cytokines are required for T cell activation [9]. $CD4^+$ T helper cells enable B cell activation

and differentiation into memory B cells or antibody producing plasma cells, which neutralize the infused clotting factor [10] (**Figure 1**).

Treatment of hemophilia – replacement therapies and bypassing strategies

Clotting factor replacement products can be used prophylactically or "on demand" in trauma or surgical intervention. Factor products have a relatively short half-life and require frequent administration [11,12,13]. One approach to increase systemic retention of clotting factors is pegylation. Pegylated factor products (Adynovate, Rebinyn) require less frequent administration than plasma derived or recombinant products [14]. IgG1 Fc conjugated clotting factor (Eloctate, Alprolix) can also prolong half life by binding to the neonatal Fc receptor (FcRn) and delaying lysosomal degradation [15-17]. There are also reports that Fc fusion products may be more tolerogenic, as suggested by pre-clinical studies and when used in ITI in a pediatric patient [18,19]. While the mechanism of tolerance by Fc-conjugated clotting factor is not completely understood, it has been suggested that upon degradation, the Fc portion is processed and presented by APCs (antigen presenting cells) by MHC II and promotes induction of regulatory T cells (Tregs) [20]. Fc fusion proteins also offer the ability for transplacental delivery of FVIII antigen, which can be exploited for perinatal tolerance induction. Transfer of maternal IgG to the fetal circulation is mediated by transcytosis upon binding to FcRn. Intravenous co-delivery of Fc fusions of A2 and C2 domains to pregnant hemophilia A mice during the third trimester suppressed inhibitor formation in the off-spring after challenge with FVIII at 2 months of age [21]. Furthermore, mechanistic studies with a model antigen revealed the ability to generate central tolerance and development of antigen-specific $CD4^+CD25^+FoxP3^+$ Treg.

Bypassing agents can be used to restore hemostasis in patients with inhibitors. One experimental bypassing agent, which mimics the function of FVIII by binding to activated FIXa and FX, is the human, bispecific monoclonal antibody Emicizumab, recently approved as Hemlibra (Genentech Inc, San Francisco, CA). Emicizumab, when prophylactically administered once per week by the subcutaneous route, significantly reduced the number of bleeding episodes in patients with inhibitors [22,23]. No anti-drug antibodies were detected [24].

An alternative bypassing agent, Fitusiran (Alnylam Pharmaceuticals, Cambridge, MA) can be used to treat both hemophilia A and B [25]. Filtusiran is an RNA interference (RNAi) molecule that targets antithrombin, promoting blood coagulation, and is currently undergoing clinical trials.

Inhibitor formation and eradication - current ITI protocols

To date, the only clinically proven method to eradicate inhibitors and induce antigen specific tolerance to infused FVIII or FIX is ITI [26]. During ITI, patients receive daily, high dose (up to 200 IU/kg/day) infusions of clotting factors [27,28]. One of the working mechanistic theories of ITI is that repetitive, high doses of antigen can suppress activated T cell responses by overstimulation with antigen, followed by anergy and deletion [29], and has also been suggested to induce Tregs [10,30]. High antigen doses also eliminate antigen-specific memory B cells, as was demonstrated in hemophilia A mice [5,31,32]. Outcome of ITI is variable: it is successful in 60-70% of hemophilia A patients but only 30% of hemophilia B patients [5,33]. ITI in patients with FIX inhibitors often has to be discontinued due to severe allergic reactions to FIX or development of nephrotic syndrome [7,34,35,36]. The mechanism leading to nephrotic syndrome is poorly understood. This complication typically

occurs 8-9 months into high-dose ITI in patients with previous allergic phenotype, and likely reflects an immunotoxicity. Even in patients with successful outcome of ITI, inhibitors can reappear once they are re-exposed to regular factor infusions when antigen-specific T and memory B cells become re-activated [37,38]. This review will explore new approaches of treating hemophilia through developing novel tolerance induction protocols.

Immune modulatory drugs

An obvious approach to reduce inhibitor formation is the use of immune suppressive drugs, similar to their use for autoimmune diseases or in organ or cell transplantation. However, more desirable than general immune suppression is a targeted intervention promoting immune tolerance. Various approaches, including transient blockage of different pathways that promote co-stimulation to $CD4^+$ T cells, have been successfully employed in hemophilic mice [39].

Conceptually, shifting the balance of the T cell response from an effector to a Treg phenotype should induce immune tolerance (**Figure 2**). In this regard, the mTOR inhibitor rapamycin is ideally suitable, as antigen presentation in the presence of rapamycin causes programmed cell death of conventional $CD4^+$ T cells, while $CD4^+CD25^+FoxP3^+$ Treg are induced and expanded. Differential use of signaling pathways and metabolic requirements account for the ability of Treg to survive in the presence of rapamycin [40]. A 1-month regimen of FVIII and rapamycin tolerized hemophilia A mice to FVIII without compromising immune competence [41]. Unless peptide antigens are used, representing $CD4^+$ T cell epitopes, FVIII or FIX protein antigen dose during the regimen is a critical parameter. The effect of rapamycin can be enhanced through combination with other drugs, for example to manipulate dendritic cells

in a manner that augments Treg induction [40,42,43]. In an alternative method, co-administration of FVIII and rapamycin containing nanoparticles that are based on biodegradable polymers (Polylactic-co-glycolic acid, PLGA) prevented and reversed inhibitors in hemophilia A mice [44,45]. Interestingly, the antigen (peptide or protein) does not have to be co-packed with rapamycin into the nanoparticles.

Direct targeting of B cells to eliminate anti-FVIII formation has been tested in humans in a Phase II clinical study. However, the B cell depleting monoclonal anti-CD20 (Rituximab) showed only a modest effect in reducing existing inhibitors and preventing anamnesis after repeated exposure. [46]. One limitation may be that antibody-producing plasma cells do not express CD20 and are therefore not targeted. Addition of a specific tolerance-inducing step may also be necessary to prevent inhibitor relapse. A recent study in hemophilia A mice found that anti-CD20 and rapamycin can be combined for more effective reversal of inhibitors [47]. This approach was superior to using either drug alone or the combination of rapamycin and Treg therapy.

Tolerance induction via gene therapy

Gene therapy with adeno-associated viral (AAV) vectors has resulted in sustained correction of monogenic disorders in multiple recent gene therapy trials The first FDA approved gene therapy drug for a genetic disease (Luxturna), to correct an inherited form of blindness, is based on an AAV vector [48,49]. AAV is a small, non-pathogenic, non-integrating, replication deficient parvovirus with vast tissue tropism. It has a packaging capacity ~5kb, and can transduce non-dividing cells *in vivo* [50]. The liver is an ideal target for gene delivery for hemophilia A and B. Both FVIII and FIX is synthetized and secreted by the liver; FIX is

produced by hepatocytes while FVIII is primarily made in endothelial cells [51,52]. Due to the small size of the F9 cDNA, a powerful expression cassette F9 can be easily packaged when incorporated into the AAV genome. Several clinical trials for hemophilia B with liver directed AAV delivery either ongoing or completed [49,53,54] [55,56]. Follow-up in both long-term and a recent trial by Spark Therapeutics demonstrated sustained expression of near normal FIX activity after gene transfer [48,53]. By using the hyperactive FIX-Padua variant, it was possible to use a vector dose 5-10-fold lower compared to previous trials without compromising therapy [53]. Packaging of FVIII into AAV is more challenging due to its large size. Nonetheless, use of the B-domain deleted FVIII transgene, optimization of small promoters and use of codon-optimization substantially improved gene expression. In a first successful clinical trial by BioMarin for severe hemophilia A, seven patients received IV injection of a high dose (6×10^{13} vector genomes [vg]/kg) of such a cassette packaged into AAV5. All patients showed sustained expression of functional FVIII one year after gene transfer [58], with no spontaneous bleeds. Rather than targeting the natural site of FVIII expression (endothelial cells), gene expression in this approach occurs in hepatocytes from a cell type-specific promoter.

Importantly, the liver represents a tolerogenic site of gene expression, as it is constantly exposed to foreign proteins from the gut and circulation [59]. Immune tolerance to FVIII and FIX induced by hepatic gene transfer depends on induction and expansion of antigen-specific $CD4^+CD25^+FoxP3^+$ Tregs, which actively suppress antibody and T cell responses [60,61]. At the same time, activation induced cell death is required to induce tolerance [61]. Thus, tolerance is maintained upon subsequent IV delivery of factor product or secondary gene transfer [62-64]**.** Multiple studies in hemophilic dogs demonstrate that hepatic AAV gene

transfer suppresses inhibitor formation and can even eradicate pre-existing inhibitors [65-67]. This was also successful and safe in hemophilia B mice with pre-existing IgE/anaphylactic reactions against FIX [63]. Tregs were found to be critical for both induction and maintenance of tolerance. Tolerance can also be achieved by hepatic gene transfer using lentiviral vectors with optimized regulation of gene expression using microRNA targets that eliminate expression in certain antigen presenting cells and/or cell-type-specific promoters [68-70]. Interestingly, tolerance is established by targeted expression to hepatocytes, liver sinusoidal endothelial cells, or myeloid cells alone. Avoiding expression in plasmacytoid dendritic cells appears crucial to the success of these approaches [70,71].

Tolerogenic cell therapies

Dramatic advances have been made in recent years in cancer immunotherapy using gene modified lymphocytes. Similar concepts may apply to tolerance induction in hemophilia [72]. For example, primary B cells can be engineered to induce tolerance to FVIII or FIX [73-75]. However, production of vectors that efficiently transduce human B cells remains a limitation. Alternatively, engineering of Treg is an emerging and promising concept [76,77]. Substantial progress has been made with *ex vivo* expansion of human Treg, and gene transfer technologies for their engineering are available and approved for clinical use [78-81]. In hemophilic mice, transplant of autologous ex vivo expanded Treg promoted tolerance induction to FVIII and FIX, likely by reducing the ability of dendritic cells to provide co-stimulation and by promoting induction of endogenous Treg [82]. Nonetheless, an antigen-specific approach, requiring fewer Treg for transplant, would be more desirable. Antigen

specificity of Tregs can be re-directed to FVIII by transducing them with T cell receptors (TCR) specific for a FVIII MHC II-peptide complex. Tregs expressing an HLA-DRA DRB1*0101 restricted T cell epitope specific to the C2 protein of FVIII were indeed shown to suppress antibody formation against FVIII *in vivo* upon transplant into "humanized" hemophilia A mice [83]. Utilizing chimeric artificial receptors (CARs) would eliminate MHC restrictions and therefore the need to customize the receptor for individual patients. In a first proof-of-principle study, a single-chain variable fragment (scFv) of a FVIII-A2 domain epitope immunoglobulin was isolated from a phage library and linked to 2nd generation intracellular signal transduction domains to direct a response to FVIII upon gene transfer to human $CD4^{+}CD25^{+}CD127^{lo}$ Treg. Effectiveness of this approach was demonstrated in hemophilia A mice [77] (**Figure 2**). *In vitro* studies suggest that these CAR-Treg still require antigen presenting cells for stimulation although they do not rely on recognition of peptide-MHC complexes [77]. Since these studies on engineered FVIII-specific Treg utilized human cells transplanted into mice for *in vivo* evaluation, durability of this approach awaits further study, as does evaluation in pre-immune animals compared to prevention of inhibitor formation.

Oral tolerance

Perhaps more readily acceptable as a prophylactic regimen to prevent inhibitor formation in PUPs could be to induce systemic immune tolerance for hemophilia A and B by delivering the antigen orally [84,85]. This concept, relies on the ability of the gut to control unwanted responses to food antigens through a complex immune regulatory mechanism [86-88]. A growing body of evidence now supports the usefulness of oral tolerance to combat food allergies [89,90]. Transgenic plant cells producing high amounts of clotting-factor in

chloroplasts ("transplastomic" plants) provides low production costs and bioencapsulation via the plant cell wall [91]. Commensal bacteria in the gut are able to degrade cell wall components, thus releasing the antigen. Expression of a FVIII or FIX fusion protein that includes a transmucosal carrier such as CTB (cholera toxin B subunit) assures effective delivery across the gut epithelium [92].

This concept was successfully employed to prevent inhibitor formation and anaphylaxis against FIX in hemophilia B mice using initially tobacco, and subsequently lettuce plants [93,94], upon repeated oral delivery (2x/week for 2 months) over a wide dose range. Intact antigen could be stored for at least 2 years in form of powder of freeze-dried plant cells. Production of clinical grade material was efficient and could be scaled-up in a hydroponic system [94]. Repeated oral delivery to hemophilia B dogs suppressed antibody formation against FIX, so that correction of coagulation was consistently achieved upon weekly IV delivery of human FIX over an 8-week period [95]. Experiments in mice also showed the ability to reverse the pathogenic antibody response against FIX [87]. Mechanistic studies showed increases in subsets of dendritic cells in the immune system of the small intestine that are known to promote Treg induction, antigen-specific up-regulation of immune suppressive cytokines in response, and induction of $CD4^+CD25^+FoxP3^+$ as well as latency-associated peptide $(LAP)^+$ Tregs that actively suppressed antibody formation upon adoptive transfer [87]. Oral delivery of combination of either heavy chain and C2 or heavy chain and light chain antigens (derived from B domain deleted, BDD, FVIII) suppressed inhibitor formation against FVIII in different strains of hemophilia A mice, again at low antigen doses [96,97].

Conclusion/Summary:

In conclusion, a number of recent innovative and diverse approaches have been developed to induce immune tolerance to coagulation factors. These include fusion proteins, oral tolerance, immune modulatory drugs, *in vivo* gene therapies, cell therapies, among others. Until now, no clinical prophylactic immune tolerance protocol exists to prevent inhibitor formation in PUPs, which may change with the emergence of these new technologies, pending further studies and risk-benefit analysis. Complexity and costs also vary substantially for these diverse approaches, representing additional factors that may affect feasibility for routine clinical use.

Key points:

- Formation of inhibitory antibodies against coagulation factors is a major problem in the treatment of hemophilia
- Diverse and innovative approaches to tolerance induction have emerged in recent years
- Gene therapies are being developed that can simultaneously achieve correction of hemophilia and immune tolerance
- Immunomodulatory drugs and oral tolerance may accomplish prophylactic tolerance induction
- Tolerogenic lymphocyte therapies, foe example using engineered regulatory T cells, hold promise

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Conflicts of interest: RWH is member of the scientific advisory board of AGCT (Applied Genetic Technologies Corporation) and has received royalty payments from Spark Therapeutics as well as grant support from Novo Nordisk and Bayer in the past.

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Figure legends:

Figure 1. Overview of key cellular components that modulate inhibitor formation in hemophilia. Professional antigen presenting cells (APCs) such as dendritic cells present peptides derived from FVIII or FIX via their MHC II molecules to $CD4^+$ T cells. These may differentiate into T helper cells and aid in the activation of B cells (which in turn produce the inhibitory antibodies). Peptide presentation by APCs to $CD4^+$ T cells may also result in the induction of Tregs (regulatory $CD4^+$ T cells) that actively suppress immune responses. Inhibitor development is largely dependent on $CD4^+$ T cell help, which can, however, be suppressed by Tregs. Factors that lead to $CD4^+$ T cell anergy (unresponsiveness) or their deletion also contribute to the prevention of the immune response. It is unclear whether Tregs can directly interact with and suppress activated B cells from differentiating into memory B cells or directly suppress inhibitor-producing plasma cells. High antigen doses directly inhibit memory B cells, leading to down-regulation of inhibitor formation.

Figure 2. Experimental approaches to promote tolerance to clotting factor therapy that may serve to replace or supplement conventional ITI. *In vivo* gene transfer into the tolerogenic liver microenvironment using either AAV or lentiviral vectors promoted tolerance in several pre-clinical models. The immunomodulatory drug rapamycin, either alone or encapsulated in nanoparticles, is tolerogenic when co-administered with antigen. Fc-conjugation of FVIII/FIX may not only increase half-life in circulation but also facilitate tolerance induction. Oral administration of lettuce-encapsulated clotting factor leads to tolerance, which is mediated by the induction of at least two types of Tregs. Cellular therapy with either *ex vivo*

expanded Tregs, engineered Tregs expressing specific TCRs, or CAR-Tregs that have receptor specificity to clotting factor without MHC restriction, have all been shown to promote tolerance in animal models of hemophilia.