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Gene therapy for haemophilia: Prospects and challenges to prevent or reverse inhibitor formation

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Abstract

Monogenic hereditary diseases, such as haemophilia A and B, are ideal targets for gene therapeutic approaches. While these diseases can be treated with protein therapeutics, such as factor VIII (F8) or IX (F9), the notion that permanent transfer of the genes encoding these factors can cure haemophilia is very attractive. An underlying problem with a gene therapy approach, however, is the patient's immune response to the therapeutic protein (as well as to the transmission vector), leading to the formation of inhibitory antibodies. Even more daunting is reversing an existing immune response in patients with pre-existing inhibitors. In this review, we will describe the laboratory and clinical progress, and the challenges met thus far, in achieving the goal of gene therapy efficacy, with a focus on the goal of tolerance induction.

Keywords

Haemophilia; factor VIII (F8); factor IX (F9); gene therapy; vectors; inhibitors; Immune tolerance

Introduction

Haemophilia A and B are x-linked hereditary diseases caused by a variety of mutations in the genes for pro-coagulant factors VIII (F8) and IX (F9), respectively. The frequency of haemophilia A is 1 out of 5000 males worldwide; haemophilia B is five-fold less frequent. These mutations lead to lower F8 or F9 protein expression and/or activity. The nature of the mutations in the *F8* gene, for example, range from frameshifts, missense mutations, nonsense mutations, inversions, large deletions to intron splicing errors (Mannucci & Tuddenham, 2001). The most severe forms of haemophilia result from nonsense mutations, large deletions, or inversions of *F8*. In severe haemophilia A, thrombin generation is so low that patients suffer from frequent spontaneous bleeding and bruising. Dependent on the severity of disease, joint and muscle haemorrhages can occur that lead to stiffness and to permanent damage (haemophilic arthropathy). Life-threatening intracranial bleeds can also occur with or without trauma.

Treatment of haemophilia A or B with plasma-derived or recombinant factors is routine, but a complication is the formation of antibodies ("inhibitors") to these proteins for which their immune systems have not been rendered fully tolerant as described below (Hoyer, 1994). These inhibitors can block the pro-coagulant activity of factors, catalytically inactivate them,

and/or accelerate their clearance from the circulation, thus rendering protein replacement therapy ineffective. As one might predict from the lack of complete tolerance, the frequency of inhibitor formation is higher in those patients with the severe forms of haemophilia A, with an overall frequency of 15–30% (Ehrenforth et al. 1992, Zhang et al. 2009). Inhibitor formation is less common in haemophilia B but can have more serious consequences because they are more commonly of the IgG1 isotype that can fix complement and lead to anaphylaxis, whereas IgG4 inhibitors that do not fix complement are more common against F8 (Bergamaschini, et al., 1996, Constantinescu et al., 1997; Ewenstein, et al., 1997; Fulcher, et al. 1987; Jadhav, M. & Warrier, I., 2000; Kasper, 1991; Warrier, et al. 1997).

Now that F8 and F9 concentrates no longer transmit human immunodeficiency virus or hepatitis viruses, formation of inhibitors is the most serious side effect in the treatment of haemophilia A and B, as it obviates this life-saving replacement therapy. Further, inhibitors prevent prophylactic factor administration, which is shown to prevent chronic haemarthropathy (Manco-Johnson et al., 2007). Reversing inhibitor formation is even more challenging than preventing it. Immune tolerance induction (ITI) can be attempted to reverse inhibitor formation clinically, and can be successful (Di Michele, 2011). However, it is expensive, requires use of central venous catheters, and may be less successful in African-Americans and patients with high titre inhibitors (Callaghan et al., 2011). Alternative approaches are needed.

Hence, a protocol to introduce a corrective clotting factor via gene therapy would be a very attractive treatment for haemophilia. Moreover, the ideal gene therapy protocol would not only replace the defective gene, but also might induce immune tolerance to the *F8* transgene to prevent or reverse inhibitor formation. Several other recent reviews are available with regard to gene therapy, in general (Mingozzi and High, 2011; Naldini, 2011; Doering et al., 2010; Kay, 2011), and for haemophilia, *per se* (Hough and Lillicrap 2005; High, 2011). This review will focus primarily on the issues involved in using gene therapy, as well as the immunological consequences, and approaches to prevent or reverse inhibitor formation.

Biochemistry and expression of F8 and F9: A critical hurdle for gene therapy

For gene therapy (and tolerance) to be effective, one must be able to drive expression, presentation and secretion of a functional protein. In the case of clotting factors, there are several challenges to be considered. The *F8* gene on the X chromosome extends over 180 kilobases, with 26 exons that encode for a 250-kilodalton protein prior to glycosylation. Expression vectors for full length *F8* need to be capable of encoding over 8 kB of DNA, whereas B-domain deleted *F8* (which is fully functional) requires the expression of ~4.5 kB. The smaller *F9* gene requires expression of 1.4 kB of coding sequence. While *F8* is primarily made in the liver (e.g. in hepatocytes and endothelial cells), it is made in lesser amounts in other organs, including the lungs (Jacquemin et al., 2006). Vascular endothelial cells store *F8* and von Willebrand factor in Weibel-Pallade bodies, and both are released into the circulation after synthesis from these cells. However, retention sequences and the necessity for correct glycosylation put some limits on target cells *ex vivo*. For example, point mutations in the A3 and C2 domains of *F8* can lead to intracellular retention of the protein (Voorberg et al., 1996, Roelse et al. 2000; Summers et al., 2011). Thus, the use of tissue-specific promoters and the correct design for expression and secretion of *F8* can be achieved in a variety of cell types. *F9* is made exclusively in the liver by hepatocytes, but can be synthesized by a wide variety of cells following gene transfer.

An important distinction to remember is that for tolerance induction, expression of a full length and functional protein may not be required (Evans and Morgan, 1996; Lei and Scott, 2005), as will be discussed below.

Vectors for gene therapy: The choice is not easy

Gene therapies have evolved over the last decade. The first applications gaining public recognition involved retroviral gene therapy instead of bone marrow transplantation for the treatment of severe combined immunodeficiency disease (SCID) (reviewed in Hacein-Bey-Abina et al. 2010), a condition caused by gene mutations within the interleukin-2 receptor gamma chain, common to several critical cytokines in the immune system. Due to potentially fatal leukaemia as a complication arising from insertional mutagenesis by the SCID gene therapy vector, a moratorium on such trials was put in place. It was shown that the insertional mutagenesis in the SCID patients was non-random, due to the specific transgene in that vector, and facilitated by the underlying immunosuppression of SCID patients. Thereafter, clinical gene therapy trials were allowed to resume under heightened scrutiny. Further developments in the safety of vector vehicles and a better understanding of the immune system's involvement in gene therapy led to a resumption of clinical trials. Such clinical trials for haemophilia offer great hope for the treatment of this and other monogenic diseases.

Both viral and non-viral (e.g., “naked DNA”) vectors have been employed for transgene delivery, as integration of the genome is an important parameter. One criterion is simply the size of the inserted transgene. Since full length F8 requires a vector that can accept >8 kB of DNA, much of the clinical and pre-clinical work in the last decade utilized the smaller *F9* gene, cloned into adeno-associated viral (AAV) vectors. With naked DNA, the issues of concern include how to administer the transgene, determining the best dosage route, and how best to control the innate immune response stimulated by CpG motifs in the vector DNA (Vilaysane and Muruve, 2009; Avalos et al. 2010; Oberg et al. 2011.) As elaborated below, retroviral vectors convey the risk of insertional mutagenesis and recombination (Hacein-Bey-Abina et al. 2010). Therefore, the choice of the vector may depend not only on the target cell/organ and the size of the construct, but also on the side effects and immune consequences.

In this review, we will discuss several of the commonly used vectors and their successes and challenges.

Adenovirus and adeno-associated virus (AAV)

Despite their utility for delivery of large sequences of DNA with extremely high efficiency, therapeutic transgene engineering, adenovirus vectors are highly immunogenic, and their use may be limited by pre-existing antibodies due to endemic infections in mammals. Hence, the search for less-immunogenic viral vectors has had the greatest momentum in terms of gene therapy and tolerance experiments in mice and clinical trials in humans. While some effort has been directed at developing so-called “gutless” adenovirus (*i.e.*, helper-dependent viruses, devoid of all viral coding regions, that require a helper virus to supply critical viral proteins), much work in the last decade has utilized adeno-associated virus (AAV). Numerous AAV serotypes have been isolated from human and non-human primate tissues (Gao et al. 2002). These AAV vectors can be pseudotyped (*i.e.*, constructed with modified viral envelope proteins) with other serotypes to show improved transduction efficiency in various tissues, based on known tissue tropisms. Thus, dependent on the gene to be delivered, use of these vectors potentially allow for tissue-specific transgene targeting. For example, pseudotyping of AAV2 to other serotypes results in vector tropism for a variety of tissues like brain (AAV5), liver (AAV8) and muscle (AAV1) (Rabinowitz et al. 2004).

Alternate AAV serotypes may have a different immunogenicity profile, dependent in part on the experience and genetic background of the host. Studies in mice by Herzog and colleagues have shown that expression of the transgene and avoidance of immune responses

is both serotype and mouse strain-restricted, and thus likely to be controlled by human leucocyte antigen (HLA) type in humans (Nayak & Herzog, 2010). Gene therapy for haemophilia with AAV vectors is most efficient with hepatic delivery (Mingozzi *et al.* 2003). Further, there is evidence that this route leads to the generation of regulatory T cells ("Tregs"), which are able to suppress the immune response to the transgene *per se* (Cao *et al.* 2009).

In addition, a number of collaborating laboratories have pioneered gene therapy for *F9* and *F8* with AAV vectors with serotypes 2, 5, 6 and 8, not only in haemophilia A mice but also in dogs and in non-human primates, due in part to better liver delivery (cf. Jiang *et al.* 2006; Manno *et al.* 2006; Mingozzi *et al.* 2007).

Sabatino *et al.* (2011), using AAV8, have demonstrated long-term expression of *F8* and reduction (>90%) of bleeding episodes for over two years in vector-treated haemophilia A dogs. Finn *et al.* (2009, 2010) also reported that AAV vectors encoding canine *F8* can lead to sustained transgene expression when delivered into the liver, at least in some dogs with pre-existing *F8* inhibitors. They were able to achieve a shortening of clotting times, a reduction of bleeding episodes, and a disappearance of inhibitors over time in two different haemophilia A dog colonies. Immune tolerance was confirmed upon repeated challenges with canine *F8* protein. The reasons for the variation between individual dogs are not clear, and differences in immune response modifier genes may play a role in addition to stochastic factors pertaining to antigen presentation, and the immune response.

In an interesting ovine model, Nathwani and colleagues took advantage of the long gestation period of sheep and the ability to intervene during fetal life to deliver *F9* *in utero* (David, *et al.* 2011). They were able to deliver human *F9* in such a way as to achieve long-term expression of the transgene with no apparent immune response. This approach is encouraging because sheep, like humans and (unlike rodents), achieve immunological competence by the end of the second trimester. While there are clear risks to the invasiveness of fetal delivery, such an approach could prevent inhibitor development in the first place, especially in patients predicted to be at high risk based on genetic analysis (mutation, HLA) and family history. In a similar vein, neonatal gene therapy for *F8* delivery has been achieved by several groups (Xu *et al.* 2007; Matsui *et al.*, 2009; Ponder, 2011).

Despite the success of AAV-mediated *F9* gene transfer into skeletal muscle or even liver tissue of dogs, mice, and/or sheep (resulting in sustained transgene expression and correction of bleeding), expression in humans has been of much shorter duration. Clinical trials in patients with haemophilia B have suggested that AAV capsid-specific cytotoxic (CD8) T cell response is responsible for the elimination of AAV-transduced hepatocytes, resulting in therapeutic failure (Mingozzi & High, 2011). This may be in part due to pre-existing immunity to the vector.

Therefore, pre-clinical studies in non-human primates have been initiated by High and colleagues to assess the safety of short-term immunosuppression administered around the time of AAV vector administration, with the purpose of blocking capsid-specific T-cell responses until viral capsid is degraded and cleared from transduced cells. These studies showed that immunosuppressive drugs interfering with Treg induction may increase the risk of anti-transgene immune responses, while regimens based on the immunosuppressive drugs, mycophenolate mofetil (MMF) and rapamycin used in transplantation, are safe in the context of AAV-mediated liver gene transfer (Mingozzi *et al.*, 2007). Rapamycin, which inhibits the response to interleukin-2, has been shown to selectively expand regulatory T cells in humans (Battaglia *et al.*, 2006). Based on these results, a clinical trial of AAV-*F9* liver gene transfer in haemophilia B subjects has recently been started, in which vector was

administered under a 12-week immunosuppressive regimen of MMF and rapamycin (ClinicalTrials.gov ID: NCT00515710). Another study has been initiated in which an AAV8 vector expressing *F9* is being tested; preliminary results from this study show some efficacy at the low vector dose, and dose escalation is underway (ClinicalTrials.gov ID: NCT00979238; Nathwani et al, 2011a, b; Ponder, 2011.)

Retroviral and lentiviral gene therapy

The first step in the development of retroviral gene therapy for haemophilia was reported in 1995 (Chuah et al. 1995; cf. Chuah et al. 2004). This group engineered *F8* cDNA into a murine tumour virus vector and successfully expressed *F8* in a variety of cells. Subsequently, Evans and Morgan (1998) utilized retroviral transduction of haematopoietic stem cells to express *F8* in haemophilic mice, albeit transiently. Interestingly, despite the fact that human *F8* protein or activity was undetectable, many of these mice were rendered tolerant to subsequent injections of *F8* protein, a point we shall return to later. This suggests that stem cells expressing *F8* may mature into tolerogenic antigen-presenting cells in the thymus or periphery to present *F8* epitopes.

A phase I retroviral gene therapy trial in severe haemophilia patients with intravenous administration of B-domain deleted *F8* was first reported in 2003 (Powell et al., 2003). Though well tolerated, expression was of short duration, presumably because retroviral vectors do not infect non-dividing cells.

Safety has a concern with retroviral gene therapy. Fischer and colleagues (summarized in Hacein-Bey-Abina et al. 2010; Fischer et al., 2010) used retroviral gene therapy with a cytokine gene (*IL2RG* chain) to treat immunodeficient (SCID) patients. Five of 20 children developed leukaemia, leading to a temporary moratorium on retroviral gene therapy trials in France and the United States. Evaluation of the human subjects with leukaemia, as well as animal models of retroviral-mediated insertional mutagenesis (Dave et al., 2004) indicated that the leukaemia risk derived primarily from the specific transgene sequences delivered in the SCID patients, particularly in the context of a retroviral vector, and it is not a generic risk of gene therapy. In the search for a safer form of gene therapy, these results led to the use of transduction of specific cell types, such as haematopoietic lineage cells *ex vivo*, and efforts to overcome issues of transgene inactivation. Other studies have focused on the use of insulated self-inactivating retroviral or lentiviral vectors driven by weaker promoters to transduce haematopoietic stem cells (Moayeri et al. 2004, 2005; Ramezani & Hawley 2009). In general, these approaches have led to prolonged expression times and apparent tolerance to the transgene in reconstituted, non-myeloablated haemophilic mice. Whether this approach will be effective in hosts with pre-existing inhibitors has not been determined.

Belay et al., (2010) used alternative cellular targets for retroviral gene therapy, specifically human bone marrow mesenchymal stem cells. They achieved good expression of *F8* *in vitro* and sustained expression in immunodeficient mice *in vivo*; however, it is not known whether such expression can be maintained in immunocompetent hosts.

Avoiding the immunological sequelae of gene (and protein) therapy for haemophilia

One of the major challenges in haemophilia therapy is the formation of inhibitors to the life-saving protein therapeutics *F8* and *F9*. Hence, the lack of tolerance in haemophilia patients to these proteins, combined with the inherent immunogenicity of *F8*, for example (Skupsky et al. 2009) continue to be critical hurdles. Moreover, the immunogenicity of the vector *per se* had long been ignored by molecular biologists (cf. Check, 2005). Approaches to negate or minimize immunogenicity have been attempted in animal models (Skupsky et al 2009; Navarrete et al., 2010) but these may not lead to the induction of immune tolerance. A more

daunting problem is how to reverse pre-existing immunity in patients with inhibitors. Recent progress using different cellular “Trojan horses” (such as B cells) and microRNA, discussed below, lend hope that these hurdles will be overcome.

Immune tolerance induction (ITI) has been in practice clinically for three decades (Brackmann et al. 1996). Despite its large cost (which can be as high as £1 million per patient), ITI can be effective, but has variable success (summarized by Di Michele, 2011). Furthermore, ITI generally is more difficult to achieve in patients with inhibitor titres >10 Bethesda Units because the success rate is relatively low in those patients (Zhang et al., 2009; Di Michele, 2011). Prophylaxis with F8 can be used successfully to prevent inhibitor production and is clearly the most desirable approach from the point of immune tolerance and also prevention of haemophilic arthropathy (Manco-Johnson et al. 2007; Coppola et al., 2008; Kurnik et al., 2010; Oldenburg, 2011; C. Koenigs, University Clinic, Frankfurt, Germany, personal communication). It is widely accepted that tolerance is easier to induce in naïve recipients (Scott, 1994). Based on the relative developmental maturity of the immune system, attempts to induce tolerance in the perinatal period could be considered in patients at high risk for developing inhibitors (Scott, 1994; L. Hoyer, American Red Cross, Rockville, MD, USA, personal communication).

As cited above, several approaches to deliver *F9* or *F8* genes systemically have led to success when delivered in haematopoietic stem cells, although sustained expression has been difficult to achieve. Nonetheless, these efforts can lead to tolerance to the clotting factors (Evans and Morgan, 1998; Moayeri et al. 2005; Matsui et al. 2011). *It is therefore important to consider that tolerance to the genetically expressed factor should be the primary goal.* Indeed, achievement of tolerance to clotting factors could be recommended as a precursor to attempts to achieve high-level expression of functional F8. This would also promote acceptance of administered product for short-term therapy.

A variety of approaches, primarily in mouse models for haemophilia, lend insight for future application of gene therapy for inhibitor formation. As noted earlier, hepatic delivery is not only an effective route for cell-appropriate expression of clotting factors, but liver transplantation has been found to be tolerogenic of proteins that are synthesized by the engrafted organ (Calne et al, 1969; Tiegs and Lohse, 2010). Using AAV serotypes expressing human F9, Herzog and colleagues (Cao et al. 2007; Nayak et al. 2009) provided the first definitive evidence for induction of CD4+CD25+ regulatory T cells, specific to the transgene product, by hepatic gene transfer into naïve animals. These CD25+ Tregs were able to suppress subsequent immune responses to F9 in secondary recipient mice (see below).

However, hepatic gene transfer is not always tolerogenic for a variety of reasons. For instance, with transfer of *F8* DNA by bolus intravenous injection (which leads to expression in the liver), the expression is evanescent and is accompanied by the production of high titred F8 inhibitors. This is probably due to the stimulation of an innate immune response to CpG dinucleotide motifs in the naked DNA (typical of bacterial nucleic acid), via Toll-like receptor 9 (Vilaysane & Muruve 2009). Miao and colleagues have used this challenging model to develop methods that counteract this immune response, *e.g.* by blocking co-stimulation (Peng et al. 2008) and/or the additional administration of immunosuppressive drugs cited above (Miao et al. 2006, 2010), thereby leading to long-term expression and apparent specific tolerance to the transgenically expressed *F8*. In some cases, Tregs are responsible for this tolerance to the ectopically expressed *F8* and these regulatory cells can be expanded with IL-2 immune complexes (Miao, 2010; Liu et al. 2011).

Annoni et al. (2009) showed that intravenous injection of lentiviral vectors expressing a green fluorescent protein (a marker for gene transfer in tissues) led to hepatic expression of the transgene. This expression however then disappeared, and a concurrent appearance of CD8 cytotoxic T cells and antibodies against the transgene product was observed (Annoni et al. 2009; Naldini, 2011). This group then engineered a microRNA (*MIR142*) that suppressed expression of the transgene in haematopoietic lineage cells. Interestingly, when this modified lentiviral vector encoding miRNA was introduced, not only was there persistent expression of F8, but tolerance was also induced, mediated by CD25+ Tregs. Further modification of this approach was recently published by Matsui et al. (2011). They modified (pseudotyped) the vector to express a lentiviral envelope glycoprotein, in conjunction with a liver-restricted promoter and a miRNA-regulated *F8* transgene: this protocol achieved sustained, therapeutic levels of F8. These modifications to the lentiviral delivery system effectively restricted *F8* transgene expression to the liver. This suggests that directing expression into non-professional antigen-presenting cells may be a key to introducing tolerance.

Additional approaches for the induction of tolerance to therapeutic F8 have involved oral delivery (requiring relatively large and highly expensive doses), presentation on immature dendritic cells and/or treatment with non-depleting anti-CD3 (Qadura et al. 2008; Waters et al. 2009). These attempts however have not been successful in regard to the modulation of therapeutic protein immunogenicity *per se*.

Choice of cellular targets for gene therapy and/or tolerogenic presentation

Despite progress in the development of gene therapy vectors and delivery systems, the major challenge remains the induction of tolerance to the transgene as well as the vector. For example, the use of haematopoietic and other stem cells as delivery systems, cited earlier, has the potential to induce tolerance, at least in naïve recipients. In addition, methods to avoid presentation to the immune system by professional antigen-presenting cells, cited above (Annoni et al. 2009), offer great promise especially if they favour the generation of regulatory T cells. Furthermore, transgenic expression in cells lacking class II major histocompatibility complex (MHC) molecules or co-stimulatory molecules has been used to avoid an immune response to the transgene. Several obvious examples generated in the recent years take advantage of these properties, including expression in red cells, platelets and B-lymphocytes discussed below. These cellular carriers may be successful for entirely different reasons, but all have been applied in animal models with pre-existing inhibitors.

Sandelin and co-workers (Chang et al. 2006, 2008) used a lentiviral vector to express *F9* in haematopoietic stem cells thus providing erythroid cell delivery following non-myeloablative conditioning. Although initial F9 levels were low, there was therapeutic benefit in terms of clotting times. Other groups (Shi et al. 2006, 2008; Yarovoi et al. 2003; Gewirtz et al. 2008) transgenically engineered *F8* in megakaryocytes, leading to expression of clotting factor in platelets. These cells act as a “Trojan horse” to bring F8 to sites of injury without priming of the immune system. When F8-containing platelets were recruited to sites of vascular injury, they were able to unload F8 to drive haemostasis. Not only did these mice have normal bleeding times (suggesting that platelet released F8 could function in the clotting cascade), but also the platelets survived in the presence of passive antibody treatment or transfer of immune spleen cells. Recently, Shi was able to express *F8* driven by the *IIb* promoter in bone marrow cells to correct bleeding in haemophilic mice with inhibitors (Q. Shi, Blood Center of Wisconsin, Milwaukee, WI, USA, personal communication). If this can be reproduced in larger animals that are hyperimmune to F8, it will be a major step forward in gene therapy for F8 in inhibitor positive patients, as well as in previously untreated patients.

Our laboratory has utilized both a stem cell delivery and B cells to deliver F8 domains for tolerance induction via B cell presentation. The focus was to induce tolerance to the A2 and C2 domains of F8, the major targets of inhibitory antibodies, rather than attempt to express full length F8. Thus, we cloned each domain in frame with an IgG carrier and used a retroviral-based vector to transduce B cells. When these transduced B cells were transferred to either naïve or primed haemophilic recipient mice, the immune response to subsequent F8 challenge was reduced. In fact, in primed mice, inhibitor titres declined within one month (Lei and Scott, 2005). This tolerogenic effect required the presence of CD25 Treg cells for both its induction and maintenance. Subsequent studies by Skupsky et al. (2010) demonstrated that these Tregs could block the expansion of specific T cells *in vitro*, similar to the regulatory effects described by Miao and Herzog and their collaborators (*op cit.*) in their model systems. Thus, efforts to generate and expand *specific* T regulatory cells should be considered in all efforts to generate tolerance to both the transgene (*F9* or *F8*) and vector-derived epitopes.

It is also worthwhile to consider the use of lymphocyte depletion techniques to facilitate tolerance. Anti-CD3 has been employed by Waters et al. (2009) to create a “window” for gene therapy and *F8* delivery. Zhang et al (2011) employed partial depletion of B cells with anti-CD20 (Rituxan) to facilitate tolerance induction in an adaptation of ITI in haemophilic mice. This was based on the hypothesis that the remaining B cells are more tolerogenic antigen-presenting cells and may function as B regulatory cells (or “Bregs”), analogous to Tregs (Mauri, 2010). Whether Rituxan therapy in haemophilia patients will be effective and will work via the same mechanism remains to be determined.

Finally, a novel approach that may be effective in preventing responses to clotting factor transgenes and vector epitopes is based on the recent discovery of regulatory epitopes in IgG described by De Groot et al (2008). These peptides, called Tregitopes, were discovered based on motifs predicted to be MHC class II binding; indeed, these were conserved, promiscuous epitopes and that common to IgG isotypes in several species. When delivered with antigen, they have been shown to be suppressive for immune responses to cognate antigens and to reverse or prevent autoimmunity and transplant rejects (A.S. De Groot., Univ. Rhode Island, Providence, RI, USA, personal communication). Incorporation of Tregitopes into AAV or other gene therapy vectors could be useful to prevent the immune response to both the transgene and the viral vector *per se* (Mingozzi & High 2011).

Perspectives for the future

Gene therapy for haemophilia A and B has seen enormous progress during the last decade. The realization that there is an immune response to the transgene as well as to the vector is now well appreciated. This is important not only for haemophilia but also for other monogenic diseases. Thus, the combination of novel vectors, miRNA technology and approaches to modulate the immune response to both the transgene and vector epitopes bode well for the future. While reversing an ongoing inhibitor response remains a challenge, current understanding of specific B and T cell recognition and regulation by Tregs suggest that this next hurdle can be overcome.

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