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Unique strategies for therapeutic gene transfer in haemophilia A and haemophilia BWFH State-of-the-Art Session on Therapeutic Gene Transfer Buenos Aires, Argentina

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Summary

Gene therapy of haemophilia has been initiated through a number of approaches including expression in muscle, liver and omental implanted fibroblasts, or i.v. injection of an expression construct under the control of a ubiquitous promoter. In all these approaches, the goal was to have factor VIII (FVIII) or factor IX (FIX) synthesized so that it restored the levels of the missing protein in blood. The three talks in this session are totally, or at least in part, directed at strategies that may be clinically effective even in the absence of correction of the missing plasma clotting factor, although the haematopoietic stem cell or blood outgrowth endothelial cell therapy could achieve plasma correction as well. Two of the approaches achieve localized coagulation factor expression without necessarily correcting the systemic defect – one is with synthesis of FVIII or FIX within the joint space and the other is with the local release of FVIII (or FIX) by platelets at the site of vascular injury. All of the three approaches have demonstrated efficacy in small animal models and are now the subject of larger animal studies. None has yet to progress to human trials.

Keywords

factor IX; factor VIII; FVIII inhibitor; gene therapy; haemophilia A; haemophilia B

Gene therapy of haemophilia has been initiated through a number of approaches including expression in muscle [1], liver [2] and omental implanted fibroblasts [3], i.v. injection of an expression construct under the control of a ubiquitous promoter [4]. The follow summarizes three talks given at the World Federation of Haemophilia Meeting in Buenos Aires, Argentina on gene therapy of hemophilia that emphasize new approaches or strategies for gene therapy of hemophilia.

Intraarticular gene therapy for haemophilia

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Bleeding-induced joint destruction is the major morbidity complicating factor VIII and factor IX deficiency. Standard and appropriate therapy to maintain the health of joints requires restoring factor activity throughout the entire circulating blood volume. Although bleeding into six joints (knees, ankles and elbows) is estimated to account for 80% of haemarthroses [5], few therapies are directed locally to the joints. Gene therapy has been pioneered for rheumatoid arthritis and osteoarthritis [6–8] diseases, which share many pathological features with bleeding-induced arthropathy [9]; the approaches have primarily sought to modulate inflammatory or angiogenic cytokine expression in the joint space. While multiple gene delivery approaches to achieve *systemic* correction of haemophilia A and B have been investigated, clinical success has yet to be achieved [2,10–12]. *Local* therapy directed to joints could potentially address the major complication of haemophilia while circumventing some barriers to systemic expression, for example if the requirement for the total therapeutic protein expression was decreased or if the immune presentation of potentially immunogenic gene delivery vectors or clotting factor differed quantitatively or qualitatively.

A series of investigations now have explored the hypothesis that extravascular clotting factor in the joint tissues in haemophilia can contribute to local haemostasis and protection from joint deterioration independently of circulating plasma clotting factor [11,12]. The hypothesis can be modelled *in vivo* because haemophilic mice develop bleeding-induced arthropathy within joints that mirror human haemophilic synovial changes closely, including synovial proliferation, neoangiogenesis and cartilage erosion [11,13]. A haemophilic mouse synovitis histopathology grading system has been validated by Valentino and Hakobyan [14]. A joint haemorrhage model consisting of a single puncture of the knee joint capsule with a 30-G needle to induce bleeding of joint vasculature has been standardized in FIX knockout (FIX^{-/-}) and FVIII knockout (FVIII^{-/-}) mice. Haemostatically normal mice do not develop synovitis, but greater than 95% of haemophilic mice develop synovitis after the haemostatic challenge [11,15].

To compare the potential therapeutic value of extravascular clotting factor replacement within the joint, intraarticular (i.a.) haemorrhage was induced by joint capsule needle puncture; at the same time, the mice received human FIX via the needle into the joint space (i.a.) or were alternatively treated with FIX intravenously (i.v.). Examining joint histopathology 2 weeks after the injury, FIX injected in the joint coincident with bleeding protected haemophilia B mice from synovitis at doses that were 80–90% lower than doses required i.v. to achieve the same protection. The experimental design was reproduced using FVIII^{-/-} mice. Factor VIII delivered locally in the joint prevented synovitis using doses 80–90% lower than required i.v. to achieve the same degree of protection [12].

Similar to human haemophilia, haemophilia A mice develop neutralizing antibodies (inhibitors) after protein replacement more frequently than haemophilia B mice. Following exposure to FVIII i.a., when compared with i.v. exposure, FVIII^{-/-} mice developed both a lower incidence and lower titre of inhibitors. The efficacy of i.a. FVIII and FIX has been

examined also in joints in which the normal anatomy was disrupted. Synovitis was induced in haemophilic mice by joint capsule injury. Clotting factor given coincident with a subsequent induced haemarthrosis in the inflamed joint prevented additive pathological changes resulting from the 'recurrent' injury. Taken together, the results suggest that clotting factor's action to protect joints need not occur solely via circulating factor (i.e. through action at the intraluminal surface of the blood vessel) and support the potential efficacy and safety of a strategy to confer endogenous factor expression to tissues within the joint space.

To examine joint-directed gene therapy, human FIX packaged in different serotype capsids of adenoassociated virus (AAV2, AAV5 or AAV8) was delivered directly to the left knees of FIX^{-/-} mice; the right knee received only normal saline [12]. After 4 weeks of AAV expression, bilateral knee bleed was induced by needle puncture. Two weeks later, at the time of killing, 100% of negative control knees that did *not* receive gene therapy had histological evidence of bleeding-induced synovitis. In the AAV-treated knees, significantly less synovitis was demonstrated using each of the AAV serotypes; FIX antigen and activity were detectable in synovial fluid and by immunohistochemistry and quantitative PCR of joint tissues. In the case of the AAV5 vector, protection was significant at an i.a. dose of 2.5×10^9 particles per animal, i.e. ~20-fold lower than the dose of AAV.FIX that was associated with transient systemic FIX levels followed by a cytotoxic lymphocyte response against transduced hepatocytes in a human clinical trial [2]. The studies suggest that multiple joints could be treated while using a total vector particle number that is within the range of virus load that has proven to be immunologically well tolerated in muscle- and liver-directed human clinical trials.

In subsequent experiments, mice have been treated with IA FIX gene therapy vector as late juveniles, subjected to repeated induced joint haemorrhages during adulthood, and examined at timepoints as late as 6 months after the gene therapy. Limbs treated with the AAV.FIX not only demonstrate less acute and chronic synovial inflammation, but also fewer chronic bone changes, compared with untreated contralateral (injured control) limbs of the same animal. The results in haemophilic animals support further exploration of clotting factor gene delivery to joint as an adjunct to systemic protein or gene therapies for prevention of early and late outcomes of haemophilia. In addition, further studies using these reagents may yield more global insights into potential extravascular roles of FVIII and FIX in normal haemostasis and wound healing following haemorrhage [16].

Stem cell-based gene delivery – ex vivo approaches using gene-modified cells

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Current replacement therapy for haemophilia is effective and safe. However, the expenses of factor concentrates are prohibitive for most health systems in developing countries, and therefore 80% of the world's haemophiliacs currently have no access to high-quality haemophilic care. Gene- and cell-based therapies are considered promising approaches to treat haemophilia patients and would avoid frequent replacement therapy, with a considerable improvement in the quality of life for these patients.

Several strategies have been proposed for gene therapy for haemophilia. These strategies are based on both *in vivo* and *ex vivo* approaches. The *in vivo* delivery studies using non-viral or viral vectors, such as, AAV, and retroviral have demonstrated very encouraging preclinical data [17–21], and earlyphase clinical trials [1,2,4] were safe. However, to achieve the therapeutic success of these strategies, there remain challenges on both efficacy and safety issue such as potential side effects related to vector-mediated cytotoxicity, unwanted immunological responses [22,23] and the risk of insertional mutagenesis.

Ex vivo delivery of therapeutic transgenes provides a safer strategy by avoiding systemic distribution of viral vectors. A clinical trial that used autologous skin fibroblasts, genetically modified with the FVIII transgene, implanted into the greater omentum of severe haemophilia A patients, was well tolerated and a safe procedure [3]. However, as only the short-term modest levels of FVIII were detected, it suggested that the viability of the transplanted cells as well FVIII expression levels is a critical requirement to achieve a sustained therapeutic benefit. The problems in scaling up this strategy to reach therapeutic levels of FVIII are a major obstacle of this strategy.

The use of haematopoietic stem cells (HSC) provides an alternative strategy to deliver the therapeutic coagulation factor. Preclinical studies in haemophilia A murine model with expression of FVIII in blood cells [24,25] or platelets [26,27] demonstrated encouraging results. Dr Wilcox demonstrates that in haemophilia A dogs, the use of autologous transplant of modified HSC expressing FVIII is feasible [28]. An inconvenient of these HSC-based strategies for haemophilic gene therapy is the use of myeloablative conditioning to facilitate engraftment in the bone marrow niches.

Another alternative for *ex vivo* haemophilic gene therapy with a non-invasive cell isolation, and without the need of myeloablative regimen is the use of autologous endothelial progenitor cells isolated from peripheral blood known as blood outgrowth endothelial cells (BOECs) [29]. Dr Lillicrap's group [30] has demonstrated that FVIII can be delivered from BOECs genetically modified *in vitro* utilizing a lentiviral vector that contains the FVIII transgene. In adult FVIII knockout immunocompetent mice, therapeutic levels of FVIII in the circulation for >6 months after subcutaneous implantation of BOEC-modified progenitor cells were observed. A similar strategy has been evaluated in a preclinical study with normal and haemophilia A dogs using the omentum as an alternative site for the implantation of FVIII-expressing BOECs. Preliminary results showed evidence that the implanted cells have the ability to produce and secrete FVIII for over a year [31]. The presence of low levels of inhibitory and non-inhibitory antibodies to FVIII in this canine model indicates that a short course of immune suppression may be required for sustained transgene expression.

More recently, the generation of induced pluripotent stem (iPS) cells from somatic cells [32] holds the possibility of alternative source of cells that can be genetically modified for the treatment of haemophilia [33]. The rapid advancements in the field of iPS cell technology since 2006 are remarkable, when Takahashi and Yamanaka [32] showed that ectopic expression of defined transcription factors was sufficient to reprogram fibroblasts to a pluripotent state. This represents an alternative for the generation of pluripotent cells without using human embryonic cells. There are substantial challenges for clinical implementation

of iPS cell generation such as the fully maturation to the desired cell, the efficacy on the use non-integrating methods and the risk of tumour formation. However, given the efforts of several research groups that have been dedicated to the improvement of the quality of their reprogramming and the significant achievements of the past few years, it is possible that iPS cell technology will be used for clinical applications.

Platelet-based gene therapy of haemophilia A and B

Robert R. Montgomery, Qizhen Shi, David A. Wilcox and Guowei Zhang, Blood Research Institute of Blood Center of Wisconsin

To date, the gene therapy approaches for either FVIII or FIX have directed protein synthesis to various somatic cells [34,35]. These approaches have targeted the ultimate replacement of FVIII or FIX in plasma where FVIII and IX normally carry out their support in haemostasis but do not become activated until vascular injury perturbs the need for activation of haemostasis locally. These approaches are intended for those patients who do not have inhibitory antibodies. In some cases, the development of inhibitory antibody may reduce the number of cells producing FVIII or IX.

A recent new strategy has been developed by two research groups – one under the direction of Morty Poncz at Children's Hospital of Philadelphia, and three research groups in Milwaukee at the Blood Research institute under the direction of Qizhen Shi and Bob Montgomery and at the Medical College of Wisconsin under the direction of David Wilcox. The Philadelphia group uses the GPIb α -promoter with FVIII, and the Milwaukee groups use the α IIb-promoter with both FVIII and FIX. Most of this discussion will be focused on the studies in Milwaukee.

Ever since the discovery that FVIII and von Willebrand factor (VWF) are two separate proteins that circulate in blood as a non-covalent complex, there have been studies to characterize the importance of this relationship. As both FVIII and VWF are released in parallel after DDAVP, we explored the DDAVP response in severe haemophilia and severe von Willebrand's disease after replacement therapy and found that the DDAVP releasable pool of FVIII was dependent on both VWF and FVIII being synthesized *in vivo* [36]. Studies then demonstrated that if FVIII was expressed in an endothelial cell or a megakaryocyte, the FVIII was stored together with VWF in the Weibel-Palade body and α -granule respectively [27,37,38]. This brought up the feasibility of using platelet-directed expression of FVIII as a means of gene therapy for haemophilia A. Transgenic mice and bone marrow transduced with the FVIII cDNA under the control of the platelet α IIb-promoter resulted in platelets with FVIII co-localized with VWF in platelet α -granules. Not only was this approach effective for cessation of bleeding in the FVIII KO mouse, but this approach was also effective even in the presence of high titre inhibitory antibodies to FVIII [27]. Furthermore, bone marrow transduced with a lentiviral 2bF8-construct conferred the same protection as the transgenic approach [39], and the presence of inhibitory antibodies did not preclude the engraftment and subsequent efficacy of 2bF8-lentiviral transduced HSC [40]. Using double KO mice with neither FVIII nor VWF, FVIII storage and release were present in the platelet from both mice, but the amount of stored FVIII was significantly increased in the presence of VWF. In FVIII KO mice, following BMT with HSC transduced

with 2bF8, no immune response has been demonstrated to FVIII unless high-dose FVIII was given in the presence of adjuvant. Thus, gene therapy with platelet-directed FVIII expression is an attractive strategy for an ex vivo approach in haemophilia A.

In contrast when FVIII was targeted to endothelial cells with the Tie2-promoter, plasma levels and storage were absolutely dependent on the presence of VWF, but the efficacy in the presence of inhibitory antibodies was clearly abrogated compared with the 2bF8 approach.

As haemophilia B might similarly be benefitted by platelet delivery, FIX was similarly targeted to the megakaryocyte/platelet and stored in platelet α -granules. In contrast to 2bF8, 2bF9 targeting also resulted in small amounts of FIX in plasma that might contribute to efficacy. HSC transduced with 2bF9 lentivirus also conferred protection in the FIX KO mouse, but unlike 2bF8, there was not significant haemostatic benefit in the presence of FIX inhibitory antibodies. Similar to the 2bF8 approach, 2bF9 has not yet been associated with an immune response in FIX KO mice.

There are clearly many challenges to overcome with a lentiviral mediated gene therapy approach. Haemophilic patient groups have demanded that large animal models are necessary to establish safety and efficacy for new genetic approaches. Nevertheless, this therapeutic approach is exciting, particularly for haemophilia A patients with inhibitory antibodies.

Conclusions

While gene therapy trials have been developed for haemophilia, it is still not sufficiently developed to become a routine clinical approach for therapy. These three approaches offer potential unique new strategies for (i) *ex vivo* gene therapy using HSCs or BOECs, (ii) targeted protein expression in affected haemophilic joints or (iii) the delivery of clotting factors to vessel injury sites by platelets. Two of these approaches are specifically being developed so that they offer hope for haemophilic patients even in the presence of inhibitory antibodies. The safety of these approaches still needs to be explored further in small and large animal models before advancing to the bedside, but unique approaches like these may offer future hope for success.

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