Hepatic gene therapy for haemophilia B

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Summary. Early retroviral-mediated factor IX gene transfer into deficient dogs showed that constitutive expression of low levels of factor IX which has led to persistent improvement of clinically relevant parameters such as the WBCT and PTT. Conversely, in vivo adeno-viral mediated delivery of the factor IX cDNA into hepatocytes of haemophilia B dogs has resulted in greater than wild-type plasma concentrations of clotting factor with complete, albeit transient normalization of haemostasis for a short time. An immune response directed against the vector transduced cells presented a big obstacle to clinical application. However, the future of gene therapy for factor IX deficiency appears bright with the development of fully adeno-viral gene-deleted vectors, rAAV and lentiviral vectors which seem to offer safety, therapeutic levels of factor IX and relatively long-term persistence. We must proceed with cautious optimism as these vector systems undergo further scrutiny.

Keywords: Liver, viral vectors, hepatocyte.

A number of different cell types including keratinocytes, hepatocytes, fibroblasts, myoblasts and endothelial cells have been targeted for factor IX gene replacement therapy [1–5]. Following gene transfer, these cells have produced functional factor IX because of their ability to gamma-carboxylate the appropriate ‘glu’ domains and secrete the functional protein. Early efforts employed an ex vivo gene transfer method in rodents and involve transfer of the factor IX gene into the cells via a recombinant retroviral vector and subsequent transplantation of the cells back into the animal [6]. Two major problems encountered in these studies have been the rather low concentration of circulating protein and/or the loss of transgene expression over time. The inability to achieve long-term expression is primarily the result of loss of the transduced cells or inactivation of the expression vectors. More recent efforts have used direct transduction of hepatocytes in vivo using different recombinant viral vectors.

The liver produces virtually all of the circulating factor IX and a significant proportion of factor VIII; thus, it will undoubtedly be an important target for gene therapy of the haemophiliacs. The liver represents an excellent tissue for gene therapy for several reasons: (1) Many genetic disorders are the result of hepatic enzyme deficiencies that require liver-specific enzymes or cofactors that are needed for proper metabolic function. (2) The liver is a major synthetic organ and is responsible for production of a large number of circulating proteins including factor VIII, factor IX and other coagulation factors. (3) Gene transfer into this organ may be the best way for achieving high-level production of haematogenously delivered proteins. (4) The liver is the only organ in the body besides bone marrow that can regenerate itself after partial ablation. (5) The liver provides a large cellular target for direct in vivo gene transfer using intravenous infusion of cell specific vectors. With the availability of a factor IX deficient canine model [2] and more recently a mouse model [7, 8] that can be used for preclinical trials, the liver represents an excellent target organ for gene therapy for haemophilia. We have approached the hepatocyte as a target for factor IX gene therapy, and I will discuss our efforts towards this goal.

In vivo hepatic gene transfer of recombinant retroviral vectors

We developed a direct method of hepatic gene transfer in the mouse that was accomplished by partial hepatectomy followed by portal vein infusion of viral particles [9]. Partial hepatic ablation is needed to stimulate cell division which is a requirement for proviral DNA integration into the host chromosome. In order to determine the frequency of gene transfer into hepatocytes, a retroviral vector that encodes the E. coli beta-galactosidase gene was used as an intracellular reporter protein. After gene transfer, the hepatocytes were isolated and stained with 5-bromo-4-chloro-3-imidoyl-6-D-galactopyranoside (X-Gal).

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Hepatocytes expressing the recombinant enzyme stain blue and we found that 1–2% of hepatocytes were transduced by this method.

The relative simplicity of this method lent itself to testing a number of different retroviral vectors in the mouse. Moreover, we hypothesized that it may be possible to perform in vivo delivery of retroviral vectors into a large animal model. Thus, we performed a number of studies of direct in vivo hepatic gene transfer into the hepatocytes of dogs. Similar to the mouse experiments, our first step was to use the retroviral vector that expressed the beta-galactosidase gene. A two-thirds partial hepatectomy was performed in normal dogs and at the same time a catheter was placed into the splenic vein of the animal so that retrovirus could be infused without additional surgery. Recombinant virus containing the beta-galactosidase gene virus was infused at 24 h, 48 h and 72 h post-hepatectomy. The frequency of hepatocyte transduction varied between 0.3% and 1% and was similar to what had been observed in the mouse [10].

Hepatic gene therapy for canine haemophilia B

Direct hepatic gene transfer was adapted for trials in the Chapel Hill haemophilia B canine model. These animals have no detectable factor IX antigen or activity that results from a missense mutation in the catalytic domain [11]. Three animals that varied in weight from 5.3 to 7.5 kg (ages 11 to 14 weeks) were infused with a retroviral vector that contained the canine factor IX cDNA under the transcriptional control of the mouse Moloney LTR promoter enhancer [10]. About 1.5 × 10⁹ viral particles were infused into the portal vasculature as was done in the normal dog studies described above. The plasma canine factor IX concentrations were determined by an immunologic ELISA assay and by a biological assay. Assuming a wild type animal has 11.5 μg/ml we were able to detect factor IX concentrations in the range of 2 to 10 ng/ml. Haemostatic parameters included the measurement of the whole blood clotting times (WBCT) and partial thromboplastin times (PTT).

The whole blood clotting time in haemophilia B dogs is about 45–50 min whereas the treated animals had reductions in the whole blood clotting times of more than 50% to 18–22 min. The WBCT in normal animals is 6–8 min. The PTT was also significantly shortened in these animals. Thus, with as little as 0.1% of the normal concentration of factor IX there was a dramatic improvement in biochemical parameters of haemostasis. Most importantly, we were able to demonstrate that production of recombinant factor IX persists. In the first animal treated, the plasma factor IX concentration and haemostatic parameters have remained stable over 24 months.

Surgical partial hepaetectomy is a rather extreme measure to perform in patients. Thus, we developed a two viral system in which a recombinant adenoviral vector was constructed that expressed a non-secreted urokinase molecule which selectively destroyed hepatocytes. As a result, during liver regeneration it was possible to transduce a larger percentage of hepatocytes initially in the range of 2% [12, 13] and now more than 10% (Patijn and Kay, unpublished).

A better method would be to induce hepatocytes to proliferate without first having to induce hepatic damage. This has been accomplished at low levels using KGF [14] near the time of retroviral administration. Recently our laboratory has used hepatocyte growth factor and has been able to achieve stable gene transfer into 30% of hepatocytes (Patijn and Kay, unpublished).

The ultimate method would be to use a retroviral vector that can transduce hepatocytes in vivo without the need to stimulate proliferation. The invention of a recombinant lentiviral vector which transduces non-dividing cells has been created and been used to transduce hepatocytes in vivo [15] and will be useful for haemophilia gene therapy.

Adenoviral-mediated hepatic gene therapy

Recombinant adenoviral vectors will transduce non-replicating cells; hence there is no requirement for partial hepatectomy for gene transfer. These vectors have the advantage of being able to be concentrated to high titers up to 10¹⁵ particles/ml. However, the adenoviral genome remains extra-chromosomal, a fact that has major implications for long-term stable gene expression. Studies were initiated to evaluate the adenoviral vector as a means of gene transfer into the liver of animals.

Using a recombinant vector that encodes the beta-galactosidase gene, we were able to transduce virtually all hepatocytes in the mouse after portal vein infusion of about 100 particles per cell without evidence of toxicity [16]. Moreover, a dose response challenge revealed that the proportion of transduced cells was directly related to the amount of adenovirus infused. If the dose were increased to about 500 particles per cell, the mortality rate rose to greater than 90%. In spite of the high transduction rates, the expression from transduced hepatocytes diminished over time in the mouse. At 16 weeks post-treatment only 0.5–10% of the hepatocytes contain beta-galactosidase, and this is due to in part to loss of the adenoviral genome over time.

Adenoviral-mediated hepatic gene therapy for haemophilia B

The high efficiency of gene transfer makes this vector attractive for the treatment of haemophilia. The vector
can transduce 100% of hepatocytes with more than one copy per cell. We have constructed adenoviral vectors that express canine factor IX and used these to treat haemophilia B dogs. We infused 2.4 x 10^{12} viral particles into the portal vasculature of three haemophilia B dogs weighing 14-17 kg [17]. The animals produced two to three times the wild-type level of factor IX which normalized their haemostatic abnormalities as determined by monitoring the WBCT, PTT, and secondary bleeding times. As predicted from the mouse experiments, expression was transient and the haemostatic parameters slowly returned to their pre-treatment levels over a period of 2 months concomitant with a fall in plasma factor IX. This appears to result from an immune response directed against cells containing the vector. The first generation adenoviral vectors produce small amounts of viral antigens that are recognized by the immune system. There have been two major strategies to attempt to eliminate this problem. The first involves immunomodulation strategies. Preferably, methods that would have limited immune suppression are more desirable than generalized, long-term immunosuppression with agents such as cyclosporine and FK506. To this end, we have investigated the use of co-stimulatory blocking agents such as soluble CTLA4 Ig (blocks CD28-B7 interaction) [18] and anti-CD40 ligand (blocks CD40-CD40 ligand interaction) [19]. In mouse studies, these agents can be given for very short periods of time, resulting in transient immunosuppression but long-term adenoviral-mediated gene expression lasting at least a year. In some situations [19], the vector can be administered a second time because the immunological agents can block the humoral response directed against the viral particle which usually limits secondary gene transfer.

The second approach is to remove most or all of the viral genes that remain in the vector. These future generation vectors have less toxicity and less immunogenicity and show promise for long-term expression in mouse systems. There are still some unresolved issues related to the issue of whether or not specific sequences either in the transgenic DNA and/or vector DNA are required for stabilization of the vector in cells in vivo [18].

Recombinant AAV-mediated gene transfer for the treatment of haemophilia B

Adeno-associated viruses are single-stranded DNA viruses that cannot replicate in the absence of a helper virus such as adenovirus. The viral genes are removed from the vector. To do this, the therapeutic cDNA is placed between the viral ITRs and the viral proteins are supplied in trans by cotransfection with a plasmid DNA and coinfection with adenovirus. The adenovirus can then be removed by physical methods and the recombinant AAV isolated by gradient fractionation. We have recently demonstrated that recombinant AAV vectors can transfer the factor IX cDNA into liver at levels that result in up to 2 µg/ml of biologically active factor IX in mouse plasma for more than 1 year [20]. The vector lacks all viral genes and has no detectable toxicity. Interestingly, there are more than three copies of the vector per liver cell, but by in situ hybridization only 5% of the cells contain the vector. This suggests that the vector has contaminated in the cells. Our preliminary data show that the vector integrates into hepatocytes in a head to tail concatamer. This suggests that life-long therapeutic concentrations of hFIX may be obtainable with a single administration of vector. Studies in larger animals will be required as well as further toxicity testing before application in humans.

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