Retroviruses: delivery vehicle to the liver

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Liver-directed gene therapy holds great promise for the treatment of inherited metabolic disease. Two strategies have emerged. *Ex vivo* gene therapy involves the transplantation of autologous hepatocytes transduced with recombinant retroviruses while in culture. The feasibility of this approach has been demonstrated in several animal models, and a human trial has been initiated. An alternative strategy uses recombinant viruses to deliver the transgene directly to hepatocytes *in vivo*.

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Introduction

Inborn errors of metabolism comprise a diverse group of rare, disabling disorders that collectively contribute to substantial morbidity in our society. A large number of these disorders are caused by defects in enzymes that are primarily expressed in the liver and which are involved in intermediary metabolism [1]. Three examples include: ornithine transcarbamylase deficiency (OTCD), which is characterized by an accumulation of ammonia leading to episodes of coma; familial hypercholesterolemia (FH), which is caused by a defect in low density lipoprotein (LDL) receptor and is characterized by an extreme elevation in LDL cholesterol leading to the development of premature coronary heart disease; and phenylketonuria (PKU), which is caused by a defect in phenylalanine hydroxylase and is characterized by mental retardation if not properly managed. Several strategies have been developed for treating inborn errors of metabolism: first, counseling and/or patient education; second, metabolite intervention either by pharmacologic replacement of the product, dietary restriction, or by utilizing an alternative enzymatic pathway; third, activation or replacement of the dysfunctional protein; and finally, organ transplantation to replace the defective liver function. Despite extensive research in the development of these and other innovative therapies most of these diseases are essentially refractory to anything except palliative therapy.

Several metabolic diseases have been treated by orthotopic liver transplantation. In many situations, this was performed in an attempt to treat secondary consequences of the primary molecular defects that lead to generalized destruction of liver function. However, orthotopic liver transplantation has also been indicated in lethal metabolic diseases as a means of correcting the primary genetic defect in liver function. In this application, the liver is structurally normal and the transplant is performed to replace a single defective metabolic derangement. Orthotopic liver transplantation for metabolic disease has been performed in a limited number of patients with the following diseases: FH, α-1-antitrypsin deficiency, Crigler–Najjar syndromel; and a few glycogen-storage diseases [2]. In pediatric cases, the five-year survival rate following liver transplantation for metabolic diseases is approximately 83%. Mortality can occur in the perioperative period, while chronic immunosuppressive therapy and the ever-present threat of rejection contributes to ongoing morbidity. Because of these unavoidable complications, it is difficult to justify a liver transplant in all but the most severe conditions. A more attractive approach would be to establish methods for selectively reconstituting the expression of the functional gene in the liver by somatic gene therapy.

An alternative approach to organ transplantation is *ex vivo* gene therapy. The process of *ex vivo* gene transfer entails harvesting the appropriate tissue, isolating the target cell, growing the cells *in vitro*, introducing the gene of interest into the cells with recombinant retroviruses, and placing the genetically modified cells back into the patient from which they were derived. An important early paradigm of *ex vivo* gene therapy has been strategies based on the transplantation of genetically modified hepatocytes.

The first step of liver-directed *ex vivo* gene therapy is the isolation of viable hepatocytes from biopsied tissue. Important limitations in the process of *ex vivo* gene therapy directed to the liver is the inability to expand isolated hepatocytes in culture. It is possible to establish primary cultures but impossible to passage these cells. Thus, to obtain therapeutic quantities of hepatocytes for genetic modification, it is necessary to resect significant portions of tissue and genetically modify the cells soon after isolation.

Abbreviations

FH—familial hypercholesterolemia; **LDL**—low density lipoprotein; **LTR**—long terminal repeat;
OTCD—ornithine transcarbamylase deficiency; **PKU**—phenylketonuria; **WHHL**—Watanabe heritable hyperlipidemic.

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Hepatocyte isolation

Hepatocytes have been isolated from a variety of species including mouse, rat, rabbit, dog, baboon and human tissue [3,4,5••,6,7•,8•,9••,10–16]. The general approach is to perfuse liver tissue with a neutral protease to release individual cells. Fractions of cells enriched for hepatocytes are obtained by differential sedimentation. The enriched suspension of hepatocytes are plated in culture and cultivated in a hormonally defined media. (See the references [3,4,5••,6,7•,8•,9••,10–17] both for examples of the numerous species from which hepatocytes have been isolated, and for published experiences of the isolation and *in vitro* cultivation of hepatocytes.) Despite strategies for enrichment, hepatocyte cultures are never pure; one frequently recovers cultures that contain greater than 95% hepatocytes but there are always a number of contaminating cells within these cultures (e.g., endothelial cells, Kupffer cells, ito cells or biliary epithelial cells). The quality of hepatocyte preparations, which is most frequently measured in terms of total yield and viability, varies considerably.

Extensive literature exists on the primary cultivation of hepatocytes *in vitro*. A variety of conditions have been established for maintaining hepatocytes in culture. Despite tremendous efforts it has been impossible to establish conditions necessary to passage and expand adult hepatocytes. In addition, it is difficult to maintain the differentiated state of the cells for prolonged periods in culture. In a typical experiment, hepatocytes can be maintained in culture for approximately 14 days, during which time the cells undergo several rounds of cell division.

Gene transfer into hepatocytes using retroviruses

Several approaches have been developed for transferring genes into cultured hepatocytes. The traditional approaches of transfection such as calcium phosphate transfection, electroporation, and lipofection have been used; however, expression of the recombinant gene has been extremely low and transient [18–21]. Furthermore, these approaches are associated with substantial *in vitro* toxicity. The most successful strategy has been to use recombinant retroviruses.

Retroviruses are enveloped viruses containing a single-stranded RNA genome [22]. A typical retrovirus expresses three essential structural genes: *gag*, encoding a collection of proteins involved in virus assembly; *pol*, encoding a multifunctional protein containing several activities including reverse transcriptase and an integrase; and *env*, encoding a glycoprotein that spans the envelope and contains a domain on the surface of the virion that is recognized by a cell surface receptor. Retroviruses bud from the surface of infected cells in a non-lytic manner. Infection begins with endocytosis of the virus and delivery of the RNA genome into the cytoplasm through a fusion event between the virion envelope and the membrane of the endosome that is mediated by the fusigenic envelope protein. The RNA is converted to a double-stranded DNA intermediate through the sequential activity of reverse transcriptase, RNase H, and DNA polymerase. The DNA is very efficiently integrated into the genome of the recipient cell in a relatively random manner. The integrated form of the virus, called the provirus, contains repeated sequences, termed long terminal repeats (LTRs), at both ends. The provirus contains a single transcriptional unit that is initiated at the 5′ LTR. This full-length transcript, called the genomic RNA, has two functions: first, it contains a sequence, called ψ, that enables it to be packaged into virions; and second, it can be translated, yielding the Gag and Pol proteins. The genomic transcript undergoes processing to form the subgenomic transcript that can be translated into the Env protein.

Several strategies have been suggested for using retroviruses to transduce recombinant genes. One approach is to splice a recombinant gene into a complete retroviral genome. The resulting recombinant virus will transduce the recombinant gene; however, it will remain infectious, with recipient cells producing recombinant viruses. Several investigators have suggested strategies for generating recombinant retroviruses that are replication defective. The general approach is to generate a packaging cell line that can provide *in trans* all the proteins necessary to form virions. This is accomplished by transfecting into a mouse fibroblast cell line a retroviral genome from which the ψ sequences have been deleted. The genomic transcript can express *gag*, *pol* and *env*; however, it is not packaged into virions. A cell line that produces recombinant virus is generated by transfecting into the packaging cell line a retroviral vector in which the *gag*, *pol* and *env* sequences have been replaced with a recombinant gene. The genomic transcript from the retroviral vector contains ψ sequences, and thus it is incorporated into virions of the packaging cell line. Resulting viruses transduce the recombinant gene and are replication-defective because they do not transduce *gag*, *pol* and *env*. Packaging cell lines are available with different tropisms based on the envelope proteins that they express [23–25]. Ecotropic cell lines express viruses whose tropism is restricted to rodents, while virus from amphotropic cell lines have extremely wide host ranges and are capable of infecting most mammalian cells, including humans.

A critical step in the development of *ex vivo* gene therapy for the liver is to stably transfect a large proportion of cultured hepatocytes before transplantation of the corrected autologous cells. The limitations of primary hepatocyte cultures described above provide difficult challenges in achieving this. First, hepatocytes cannot be passaged, and thus selection and expansion of the transduced cells is not possible. Furthermore, the cells undergo few cell divisions and remain differentiated and viable for only a short period of time. Therefore, gene transfer must occur during a short window after plating and it must be extremely efficient. The most promising approach has been with recombinant retroviruses. Wolff *et al.* [16] were the first to demonstrate transduction of hepatocytes using a *neo*-expressing retrovirus and adult rat hepatocytes. Success-

ful transduction was further demonstrated using *lacZ*-expressing retroviruses in rat hepatocytes and *neo*-expressing retroviruses with newborn mouse hepatocytes [13,15]. Under optimal circumstances it is possible to achieve transduction efficiencies that approach 30–50% with a single exposure to virus. It is critical that the cells are plated at subconfluent densities so that they can undergo at least one round of cell division. The most important factor in achieving efficient gene transfer is the time after plating that the cells are exposed to virus. We and others have identified a window between 48–72 h after plating during which the cells are most susceptible to infection. The mechanisms for this are unclear; however it may relate, in part, to the fact that the cells are most actively cycling during this time. Retrovirus-mediated gene transfer has now been demonstrated in cells from a variety of species including adult and newborn mouse, rat, adult and newborn rabbit, dog, baboon, and human [6,7•,8•,11,14–16,26]. In addition, a variety of potentially therapeutic recombinant genes have been transduced and expressed in hepatocytes. These include genes expressing the LDL receptor, ornithine transcarbamylase, phenylalanine hydroxylase, factor IX, and UDP glucuronosyltransferase (JM Wilson and F Askari, unpublished data)[4,5••,6,11,14,26].

Hepatocyte transplantation

Strategies for harvesting and transplanting the genetically modified hepatocytes are currently being developed. For purposes of *ex vivo* gene therapy it is necessary to design approaches that enable the *ex vivo* manipulated cells to be efficiently harvested and transplanted, and allow the cells to stably engraft and continue expressing the recombinant gene. Initial attempts to transplant hepatocytes used unmarked syngeneic cells that were introduced into sites such as the dorsal fat pad or spleen [27,28]. Long-term engraftment of these cells was documented by demonstrating persistence of cells with hepatocyte morphology in the ectopic sites. These strategies are not suitable for therapeutic application because the number of cells that can be engrafted are extremely limited. An approach of more relevance to gene therapy is to demonstrate correction of hepatocyte dysfunction by virtue of cell transplantation. This has been attempted in several animal models. Transplantation of syngeneic hepatocytes into the portal circulation of rats following 90% partial hepatectomy resulted in improved survival, indicating short-term function of the cells until the remnant liver regenerated [29]. Rats genetically deficient in albumin achieved transient levels of detectable serum albumin when transplanted with allogeneic hepatocytes into the portal vein or peritoneal cavity [30]. The production of albumin was prolonged when the recipient was immunosuppressed or the donor cells were purged of macrophages. Stable correction of defective bilirubin metabolism was achieved in the Gunn rat transplanted with congenic hepatocytes [31]. Similar principles have been applied with transgenic mice [32–34]. Hepatocytes from animals carrying a liver-specific transgene have been transplanted into congenic recipients. Engraftment of the donor hepatocytes, detected by virtue of transgene expression, has been demonstrated for at least one year. The first trials of hepatocyte transplantation in humans were conducted in Japan. Allogeneic hepatocytes were transplanted into the spleens of patients suffering from fulminant liver failure [35].

Lessons learned from models of hepatocyte transplantation have now been applied to models of *ex vivo* gene therapy. The major difference is that the cells used in the transplantation studies described above were freshly isolated, whereas those used in *ex vivo* gene therapy must be cultured for at least three days — during their transduction with retroviruses — before they are harvested for transplantation. Anderson *et al.* [3] achieved short-term engraftment of syngeneic hepatocytes that were transduced with a *neo* virus and transplanted onto a sponge implanted into the peritoneal cavity of rats. We have used the human disease FH as a model for developing *ex vivo* gene therapies for the liver. The availability of an animal model of this disease, termed the Watanabe heritable hyperlipidemic (WHHL) rabbit, has been extremely useful in developing gene-therapy strategies. Transplantation of allogeneic hepatocytes transduced with a retrovirus containing the human LDL receptor gene into WHHL rabbits led to temporary amelioration of hypercholesterolemia [14]. Loss of *in vivo* efficacy of the therapy was the result of destruction of the transplanted cells, presumably because of an immune response directed at the allogeneic cells or the human LDL-receptor protein. These problems were overcome in a second series of experiments in which autologous hepatocytes transduced with retroviruses containing a normal rabbit LDL-receptor gene were transplanted into the portal circulation of WHHL rabbits [5••]. Recipient animals realized a prolonged improvement in serum cholesterol; analysis of liver tissue demonstrated high levels of recombinant-derived LDL-receptor RNA for the duration of the experiment (6.5 months). The practicality of this approach has been demonstrated in larger animals, including dogs and baboons [8•,9••].

In vivo gene transfer using retroviruses

Recent studies have demonstrated the feasibility of using recombinant retroviruses to deliver genes to hepatocytes *in vivo* [36]. The major problem is that hepatocytes are normally quiescent and efficient retroviral infection requires a population of target cells that are dividing. Hatzglou *et al.* [37] injected recombinant viruses into the peritoneal cavity of developing rats at a time when the fetal liver is actively growing. Using a recombinant virus with a liver-specific promoter, they were able to demonstrate transduction of hepatocytes. Similar results have been achieved in adult rats when the virus is infused into animals following injury to the liver, such as partial hepatectomy, to stimulate cell division in the target hepatocytes. Infusion of retrovirus into the portal circulation

of rats following partial hepatectomy resulted in stable transduction in a small population of hepatocytes based on PCR analyses [38]. The efficiency of hepatocyte transduction is increased 1–5% when the liver is temporarily devascularized and infused with virus 24 h after partial hepatectomy [39].

Conclusions

Gene transfer directed to hepatocytes has emerged as an important early paradigm of gene-replacement therapies. Recombinant retroviruses have been used in both *ex vivo* and *in vivo* approaches to liver-directed gene therapies. The rational and feasibility of *ex vivo* gene therapy for liver metabolic disease has been established. While this form of therapy will probably have important applications, it is complicated and impractical for broad use. In addition, the efficiency is limited by the number of autologous cells that can be harvested and re-infused into the portal circulation. Importantly, the duration of therapeutic effect achieved using this approach is unknown because the gene is not inserted into a stem cell. *In vivo* gene therapy provides the potential of more efficient gene transfer and the feasibility of repeated treatments. However, approaches for stimulating hepatocyte division that are more practical and less morbid than partial hepatectomy will need to be developed.

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