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Human gene therapy: present and future

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Summary

The hematopoietic system and the liver are two primary target organs for attempting somatic gene therapy of hereditary deficiencies. Several leading laboratories have recently been able to demonstrate that bone marrow cells from rodents and non-human primates can be successfully transduced with foreign genes, resulting in the functional expression of these genes in culture. The genetically reconstituted cells can subsequently be transplanted into X-irradiated recipients, and expression of the transduced genes is observed in the recipients for more than 6 months. Subsequently, gene transfer into peripheral T-lymphocytes in humans has been attempted, and the clinical trials are currently in progress. The liver is the other major organ under intensive investigation. Primary hepatocytes can be isolated from rodents, rabbits, and dogs, and successfully transduced with recombinant retroviruses. After autologous transplantation, long term survival of the engrafted cells in vivo has been observed. More recently, it has been shown that human hepatocytes can also be efficiently transduced with recombinant retroviruses. These experimental results have laid the foundation for somatic gene therapy of hereditary deficiencies in humans in the future.

Introduction

Although the development of gene transfer to mammalian cells in vitro and in vivo started more than a decade ago, human gene therapy was initiated recently, when the first experimental protocol proposed by Drs. French Anderson and Stephen Rosenberg to track "tumor infiltrating lymphocytes" with a marker gene that was used to treat melanoma was approved by the National Institutes of Health in January, 1989. The first therapeutic somatic gene therapy trial in humans, however, was the one conducted by Drs. French Anderson and Michael Blaese at the NIH to insert the human adenosine deaminase cDNA into peripheral blood lymphocytes followed by autologous transplantation of these genetically reconstituted cells back into a patient with severe combined immunodeficiency syndrome secondary to a deficiency of this enzyme. While this groundbreaking clinical trial is still ongoing, the preliminary results are encouraging and has resulted in the proposal by investigators throughout the country to attempt gene therapy of genetic disorders as well as various forms of cancer. There are currently more than 10 clinical protocols

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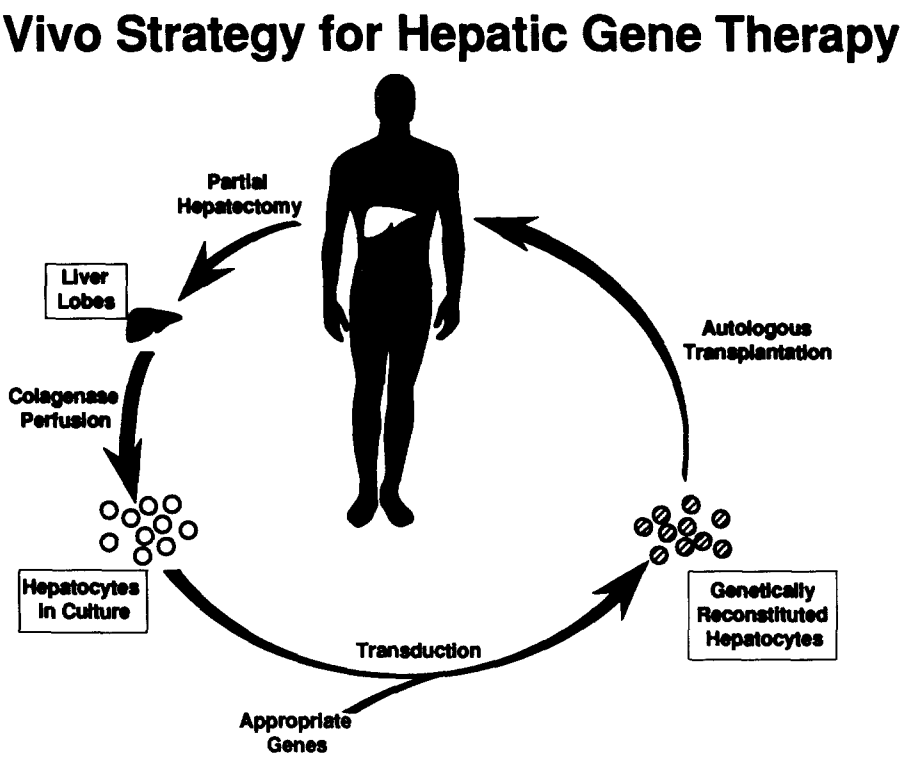

Figure 1. The approach for hepatic gene therapy is schematically outlined above.

approved by the NIH, and it is anticipated that the number will grow exponentially during the next few years.

The liver represents an attractive organ for targeting somatic gene therapy since there are a large number of diseases that result from hepatic deficiency states. The liver is a central organ in many metabolic pathways, and the hepatocyte as a cell is a factory for producing large quantities of secretable proteins. A major emphasis of this laboratory has been to develop technologies for the introduction of genes into hepatocytes in vitro and then transplant the transduced cells into the liver such that the transplanted cells continue to function as hepatocytes. The ultimate scheme of this type of therapy is depicted in Figure 1. By supplementing the liver with a gene that is deficient in specific disease states, it may one day be possible to cure the genetic disorder. We have focused on a common hepatic deficiency state known as alpha-1-antitrypsin deficiency that leads to early-onset lung disease.

Alpha-1-antitrypsin deficiency

AAT is a major protease inhibitor in human plasma and is a single polypeptide of approximately 50,000 daltons molecular weight [1-3]. It has a carbohydrate context of about 12% and contains 6-8 sialic acid residues [4,5]. It shows affinity to and inhibits a variety of serine proteases such as trypsin, chymotrypsin, thrombin, plasmin, kallikrein, elastase, collagenase, proteolytic enzymes from leukocytes and bacteria, but its most important inhibitor action is against neutrophil elastase [5,6]. Most of the serum AAT is of hepatic origin and accounts for most of the anti-neutrophil elastase activity in the lower respiratory tract [7]. Clinical importance of serum AAT was recognized following the observation that individuals with markedly deficiency serum AAT develop chronic obstructive pulmonary emphysema [8] and/or infantile liver cirrhosis [9].

The synthesis of AAT is controlled by an

autosomal and allelic system [5,10]. Over 75 different phenotypes of the protein have been classified [11]. The AAT locus has been assigned the term Pi (P for protease and i for inhibitor) and each phenotype a capital letter. Most individuals are homozygotes for PiM, which has a gene frequency of 0.95. Individuals homozygous for the PiZ phenotype have serum AAT levels of only 10-15% of that of the PiM individuals. The level of AAT in heterozygous individuals of the MZ phenotypes is about 50-60% of normal [10], suggesting that the defect resides with the AAT gene itself and not with a trans-regulatory mechanism. The genetic deficiency is inherited as an autosomal co-dominant trait, and the gene frequency of the PiZ phenotype in Caucasians of Northern European ancestry is 0.02-0.03 [5,12]. PiZ is more common in the United States, with a gene frequency of 0.03-0.04 [13]. It was observed clinically that reduction in serum AAT levels in ZZ homozygotes (12% of normal) and SZ heterozygotes (35% of normal) predisposed these individuals to development of pulmonary emphysema, such that the ZZ homozygotes have a risk 30 times greater than that of the general population [5]. It has been estimated that about 1/4000 and 1/800 among the Caucasian population in the United States are ZZ homozygotes and SZ heterozygotes, respectively. With the total annual birth rate of 4 million and a population of about 250 million, the statistics would suggest that approximately 1,000 ZZ homozygotes and 5,000 SZ heterozygotes are born in the United States every year, and that there are 60,000 ZZ homozygotes and 300,000 SZ heterozygotes in the country. Although there is a broad range in the age of onset and the severity of the disease state, 80-90% of ZZ homozygotes will develop panacinar emphysema, most prominently in the lower lung zone [5].

Although serum AAT levels in individuals of the ZZ and SZ phenotypes are only 12% and 35% of the level in the normal MM phenotype, the molecular weight, amino acid composition, carbohydrate content, and specific activity to inhibit elastase are very similar to those of the normal protein [14-16]. Subsequent peptide mapping experiments and amino acid composition analysis of tryptic and CNBr fragments of the proteins demonstrated the replacement of a glutamic acid in the M type protein by a lysine in the Z type protein [17,18]. The substitution was subsequently confirmed by establishing the amino acid sequence of the two peptides [19]. A similar substitution from a second glutamic acid (M) to valine (S) in a CNBr fragment of the two proteins has also been observed [20,21].

We have previously cloned the gene for human AAT and determined its nucleotide sequence, from which the amino acid sequence of the protein has been deduced [22,23]. From the gene sequence it was postulated that the underlying mutation in the gene responsible for the deficiency syndrome was a G to A transition in the codon for the glutamate residue [24]. This observation has been confirmed by cloning and sequencing of the mutant chromosomal hAAT gene.

One approach to treating deficient patients is to infuse hAAT into their blood. Crystal and colleagues at the National Institutes of Health initiated clinical trials several years ago and demonstrated that therapeutic levels of hAAT can be reached in the lungs of patients weighing 100 kg by weekly infusions of 6 grams of purified human plasma AAT [25]. It is too early to know whether the protocol will be effective to arrest further pulmonary degradation and deterioration of lung function [26]. Nevertheless, this biochemical restoration of hAAT levels in the lung has stimulated a number of biotechnology firms to produce the human protein through recombinant means. Bacterial and yeast systems have been used to produce massive quantities of human AAT without the carbohydrate side-chains. Although the resulting protein is perfectly functional to inactivate neutrophil elastase, its half-life in blood is reduced dramatically (from days to hours), as it is efficiently cleared by the kidney [27]. Systems using mammalian cells will yield the properly glycosylated protein, but the production levels are too low to be practical. The most innovative approach has been the attempt to produce this protein in the milk of transgenic

farm animals, and it has recently been reported that a level of 60 g/l of human AAT in the milk of transgenic sheep has been achieved [28].

At the present time, the only reliable source of human AAT is still spent human plasma. This biological product has been approved by the Food and Drug Administration as an "orphan drug" for treatment of AAT deficiency, and the supply is obviously limited. While this problem might be resolved in the future by the transgenic farm animal technology, whether "enzyme replacement" can be the effective treatment of chronic disorders by infusion of massive quantities of recombinantly produced protein for the lifetime of the patients without side-effects still remains an open question.

Liver directed somatic gene therapy for alpha-1-antitrypsin deficiency

In vitro AAT gene delivery

Theoretically, introduction of the functional human AAT gene into somatic cells of deficient individuals would potentially provide a population of cells capable of synthesizing and secreting AAT into the circulatory system. The protein would then diffuse into the alveolar spaces of the lung and provide the necessary anti-protease function to prevent tissue destruction by polymorphonuclear neutrophil elastase. It is important to note that this form of genetic therapy could only be effective in ameliorating the pulmonary manifestations of AAT deficiency, and would not be expected to have any efficacy against the hepatic disease which might be caused by excessive accumulation of the mutant protein in the liver [29].

Somatic gene therapy will require highly efficient means for transfer of genes into primary cell cultures and live animals. Recombinant retroviruses are particularly well suited for this purpose. Moloney Murine Leukemia Virus (MoMLV) represents an excellent vector system in that it has the properties of being able to efficiently infect a wide variety of cells leading to stable integration of the proviral genome into the chromosome of the host cell. Most important, it is possible to entirely remove all of the genes coding for viral proteins and replace these sequences with recombinant genes of interest, without affecting the ability of the virus to infect cells and stably integrate into the host genome [30]. Such defective retroviruses can carry a recombinant gene such as hAAT into target cells for functional expression of the human protein. While the recombinant viruses are capable of infecting and transforming somatic cells, they are incapable of producing any viral proteins and cannot form infectious viral particles for subsequent propagation [31,32]. We have reported on the construction of recombinant retroviruses capable of transducing the human AAT gene into different cell types including primary hepatocytes.

The initial retrovirus construction used in these studies contained the complete open reading frame of a full-length human alpha-1-antitrypsin cDNA cloned into the retroviral vector pZOPNEO-SV(X). This construct contains the packaging signal, MoMLV long terminal repeat (LTR) sequences, and the bacterial Tn5 neomycin resistance gene. Plasmid DNA was introduced into X2 cells in order to produce "helper free" recombinant retroviruses. NIH3T3 cells infected with the recombinant virus acquired the provirus in their genome. The virally transformed cells contained significant levels of human AAT mRNA and immunoreactive hAAT protein [33]. The human AAT protein was subsequently shown to be properly glycosylated, secreted into the medium, and functional in terms of its inhibitory activity against purified human neutrophil elastase [33].

N2 is a MoMLV-derived virus containing LTRs, packaging signal sequences, and the bacterial Tn5 neomycin resistance gene. The human AAT cDNA along with its own promoter to direct liver expression was inserted into this vector. The internal promoter was comprised of the transcriptional regulatory elements as well as the CAP site of the human AAT gene (-1200 to +45). This promoter was previously shown to exhibit tissue specific expression in hepatoma cells and in

the liver of transgenic mice. The recombinant retroviral vector NATAAT (N2 with the AAT minigene) was introduced into the GPE86 ecotropic packaging cell line, and the supernatant was used to infect the amphotropic packaging cell line GPAM12. Clones have been isolated and expanded. A clonal line with the highest titer (1 x 10^6 cfu/ml) was used to transduce primary hepatocytes. After the infection phase, cell culture media were collected and analyzed for human AAT secreted by the hepatocytes using a specific radioimmunoassay. Transduced hepatocytes secreted only 50ng/2x10^6 cells/day of human AAT, suggesting that certain enhancer elements might not be present on the tissue-specific promoter fragment of the hAAT gene used in this construct. Since the level of human AAT expression was disappointingly low with this construct, we compared the strengths of a number of cellular and viral promoters in primary hepatocytes and found that the human intermediate-early cytomegalovirus promoter-enhancer was the strongest in the primary cell culture system [34].

The human AAT cDNA was placed into the LNCX retroviral vector [35] such that the AAT cDNA is under the transcriptional control of the human CMV promoter. The retroviral vector (LN/CMV-hAAT) was then introduced into the GPE86 ecotropic or GPAM12 amphotropic packaging cell line. Clones producing titers of 5 x 10^6 have been isolated for both the amphotropic and ecotropic packaging lines. The ecotropic line was used for transduction of primary mouse hepatocytes. The optimal conditions for retroviral transduction of mouse hepatocytes was concomitantly developed with an ecotropic retroviral vector encoding the bacterial beta-galactosidase (ß-gal) gene. High titer viral supernatants were incubated with cultured primary mouse hepatocytes at various times after isolation, and plated at various densities. Forty eight hours after viral transduction the cells were stained with X-gal. Maximal infection efficiency was achieved at 48 hours after isolation and at the plating density of 2 x 10^4 cells per square cm. Under these optimal conditions 25-30% of infected hepatocytes stained blue. The next experiment was to transduce the hepatocytes with the vector in vitro and measure secreted AAT in the supernatants with a radioimmunoassay using an antibody that does not cross-react with the mouse analog. After transduction, human AAT expression was determined to be 20 to 30 ug/2 x 10^6 cells/day, which was 2-3 orders of magnitude higher than expression with the internal AAT promoter used in the previous construct.

Hepatocellular transplantation

An in vitro method for gene transfer requires that the genetically altered hepatocytes be returned to living animals. In order to test various hepatocyte transplantation technologies, we devised a system for heterologous transplantation that allowed detection of functioning donor cells in recipients for long durations and without the complications of rejection of the engrafted cells by the host's immune system. A transgenic mouse line which expressed the human AAT gene in the liver was made in C57/BL6 background for transplantation into regular inbred recipients. The advantage of this system is that the mice were syngeneic and the survival of hepatocytes after heterologous transplantation could be followed indirectly using a radioimmunoassay for human AAT in recipient mouse sera over long periods of time. Initially, we attempted heterologous hepatocyte transplantation by implantation of hepatocytes on inert matrices. Hepatocytes from transgenic mice were seeded on Gelfoam (a macrocarrier composed of cross-linked Type I collagen) and the matrix was implanted adjacent to the liver of non-transgenic recipients. Although the animals had measurable levels of human AAT for 7 days, there was none present at 14 days in any of the mice. While human AAT expression could be prolonged for 5-10 additional days when an angiogenesis factor (endothelial cell growth factor) was used to precoat the gelfoam in order to promote blood vessel recruitment to the graft in vivo, histological examination of the implemented matrices at one month, with or without the angioenesis factor,

could not identify any surviving hepatocytes (unpublished results). Repeated experiments with similarly negative results led to the inevitable conclusion that neovessels were not suitable for the long term survival of hepatocytes in vivo, so that alternative methodologies for hepatocyte transplantation must be explored.

From the above experiments, we reasoned that donor hepatocytes might need to be returned to the recipient liver in order to achieve long-term survival. Thus we attempted to inject donor hepatocytes directly into the portal vein of recipient mice, hoping that the donor cells would be delivered to the liver by circulating blood. Expression of hAAT was observed for 18 months in the recipients after injecting 5 x 10^5 transgenic donor hepatocytes into the portal vein [36]. The appearance of hAAT in transplant recipients was clearly due to expression of the transgene from donor hepatocytes, as C57/BL6 recipients transplanted with non-transgenic donor hepatocytes had no detectable hAAT in their serum. Identical results were also obtained by direct injection of 2 x 10^6 donor hepatocytes into the spleen (Figure 2). These results demonstrated not only the ability of the transplanted cells to survive long-term in vivo, but also that they remained functional as hepatocytes, since the transgene expression is hepatocyte specific [37]. Assuming that the average hAAT production per cell was unchanged, the serum level of hAAT observed after transplanting into the spleen corresponded to the survival of 2-8 x 10^5 hepatocytes, which was equivalent to 10%-90% of injected cells. To directly compare the efficacy of portal vein and splenic injection for hepatocyte transplantation, experiments using both methods were done with cells from the same transgenic donor. For splenic transplantation, the level of expression was directly proportional to the number of cells injected. Although on a per cell basis splenic and portal vein injection again gave comparable results, the mortality rate of injecting into the portal vein was 40% using 5 x 10^5 cells and 100% with 2 x 10^6 cells, whereas it was only 10% after injecting 2 x 10^6 cells into the spleen. Thus, we concluded that splenic injection was the preferred route for hepatocyte transplantation in mice.

Having demonstrated long-term survival and function of transplanted hepatocytes, it was important to localize the cells in vivo. A second transgenic mouse line containing the *E. coli* ß-gal gene was generated to enable donor cells to be identified histologically after transplantation. The transgenic ß-gal line contained 20 copies per haploid genome of the *E. coli* ß-galactosidase gene controlled by the 1.2 kb hAAT promoter, and ß-gal expression in these animals occurred almost exclusively in hepatocytes. X-gal staining of liver sections or cultured hepatocytes from the transgenic ß-gal mice showed that approximately 10% of cells stained blue, while extensive evaluation of liver sections or cultured hepatocytes from non-transgenic littermates never identified a single blue cell. Expressing cells were scattered randomly throughout the liver, a distribution pattern identical to that observed for hAAT in the transgenic hAAT mice [36].

To localize transplanted hepatocytes, the transgenic ß-gal mice were crossed with the transgenic hAAT mice to generate compound heterozygous animals which expressed both transgenes. Hepatocytes obtained from these mice were transplanted into C57/BL6 recipients by either portal vein or splenic injection. Serum hAAT in the recipients was monitored for 1-2 months after transplantation until the levels were stabilized. Livers and spleens of the recipients

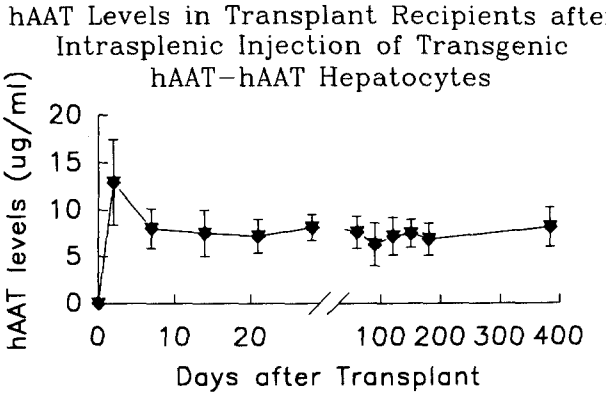

Figure 2. Freshly isolated hepatocytes from hAAT-hAAT transgenic mice were isolated and 2 x 10^6 cells were transplanted into the spleen of C57Bl/6 recipients. Serum hAAT concentrations are shown +/- the SEM for 5 recipients. (Used with permission from the Proc Natl Acad Sci USA.)

were then sectioned and stained with X-gal. Blue hepatocytes were identified within the liver sections after splenic or portal vein injection. More important, the blue donor hepatocytes were scattered within the parenchyma and distributed throughout the section, providing unambiguous evidence that the transplanted cells have established themselves to be permanent grafts within the normal liver parenchyma in the recipients. Extensive histological examination of spleens after splenic injection, using X-gal straining or a hepatocyte specific glucose-6-phosphatase assay, failed to identify transplanted hepatocytes in most recipients and identified only a few small clusters in others. We estimated that, on average, half of the surviving hepatocytes were localized to the liver, where the frequency of donor cells was calculated to be as high as 1 in 400 of all hepatocytes. Whether or not the remainder of the transplanted hepatocytes resided elsewhere was unclear. Inaccuracy in counting blue cells could contribute to this discrepancy. Alternatively, our assumption that the expression of hAAT per cell was unchanged by the transplantation procedures could be incorrect. However, the fact that the ß-gal protein was equally well expressed in periportal and perivenous regions of transgenic ß-gal mouse livers made this latter possibility less likely. Despite these caveats, it was clear that a large fraction of the surviving hepatocytes localized in the recipient liver.

The canine model for somatic gene therapy

The next critical step was to determine whether hepatocytes that underwent in vitro manipulation and retroviral transduction could be successfully transplanted back into the animal and function in a manner similar to the transgenic mouse hepatocytes. We selected the canine model to develop technologies for autologous transplantation of virally transduced hepatocytes because the size of a young dog parallels that of a human infant, and the technology of autologous hepatocyte transplantation is directly applicable to human patients in the future. Because AAT is a secretable marker, it offers an advantage for testing various gene transduction and transplantation protocols, since success of the method can be easily determined by serum measurement of human AAT.

Prior to the transplantation of transduced primary canine hepatocytes, however, scale-up methodologies for the preparation of large numbers of hepatocytes from partial hepatectomized liver tissues as well as their transplantation back into the same animals must be established. A midline abdominal incision was made and the right lateral lobe of the liver isolated and removed. In some animals, at the time of partial hepatectomy, a vascular access catheter was cannulated into a mesenteric or splenic vein. A subcutaneous injection port connected to the catheter was placed over the right lateral portion of the abdominal wall. The isolated liver lobe was immediately cannulated and hepatocytes isolated by collagenase perfusion [38]. The optimal conditions for retroviral transduction of canine hepatocytes were determined. The optimal time of infection was 32 to 36 hours after isolation and the optimal density of plating was 2×10^6 cells/100 mm culture dish. Under these conditions, about 25% of the cells stained with X-gal when a ß-galactosidase containing retroviral vector was used for transduction. For transplantation experiments, about 2×10^9 hepatocytes were cultured on 1000 plates. Approximately 4 to 5 liters of fresh viral supernatant was prepared for each experiment. Culture conditions used here selected against non-hepatocyte liver cells. By both morphology and staining with antibody to the hepatocyte-specific enzyme marker glutamine synthetase, most of the cells (about 99%) were hepatocytes. After retroviral gene transduction, the cultured hepatocytes were harvested, washed, filtered, and then transplanted back into the animal. The animals underwent single direct splenic injection or multiple infusions via the portal vasculature.

In the first experiments, the animals developed shock symptoms which included lethargy, pallor, hypotension, poor perfusion, and hematemesis. Most of the episodes were transient and resolved within minutes after the infusion was stopped. Two of the first seven experimental animals died

and necropsy revealed serosanguineous ascites, small bowel hemorrhage, and spleen and liver infarction. The liver vasculature and portal vein were engorged and thrombosed with hepatocytes. In the surviving animals, there were no associated long term effects. These animals were sacrificed without unusual findings at necropsy.

Three additional heterologous transplants were performed with freshly isolated (without plating) hepatocytes by three different methods while monitoring the portal vein and femoral artery pressures in each animal. During transplantation, if portal vein pressure increased before a systemic drop in blood pressure it would suggest a portal vascular mechanical obstruction, whereas early hypotension would suggest the infusion of a vasoactive material. No difficulties were associated with the transplantation of 1 x 10^9 hepatocytes into each animal. Liver and spleen biopsies were obtained at the end of the transplant and histologic examination of the liver identified hepatocytes in the portal vasculature. In order to attempt to replicate the shock conditions, components of the media were infused, and after infusion of fetal calf serum in dog #8, the mean arterial pressures dropped from the low 80's mmHg to mid 30's mmHg for several hours. Serum infusions into normal dogs also elicited a transient clinical shock response as mentioned above. This suggested that the shock episodes were vasoactive in origin, leading to a secondary mechanical obstruction of the portal vasculature. This prompted more extensive washing of hepatocytes prior to the successful transplantations.

In two animals (A1 and A2), hepatocytes transduced with the LN/CMV-hAAT retroviral vector were autologously transplanted via the splenic vein. Immunohistochemical staining for human AAT in infected cultured cells revealed a transduction frequency of about 25%, which is in good agreement with hepatocytes transduced with the β-galactosidase vector. In vitro production of hAAT in the culture media of hepatocytes after viral transduction reached 50 to 75 μg/2 x 10^6 cells/48 hours at 4 to 6 days after retroviral infection. After trypsinization, trypan blue exclusion revealed 97% and 76% hepatocyte viability in A1 and A2, respectively. Transplantation of virally transduced hepatocytes was performed three times over a 24 hour period for A1 (4 x 10^8 cells) and twice over a 12 hour period for A2 (6.4 x 10^8 cells). During the transplantation procedure, both animals suffered several minor shock episodes as described above but recovered quickly and continued to live without any apparent side effects for the duration of the experiments. The in vivo production of hAAT was determined by periodic measurements of serum hAAT in the transplanted animals as shown in Figure 3. The peak hAAT concentration was 4.6 μg/ml in animal A1 and 1.2 μg/ml in animal A2, obtained 6 to 8 days after transplantation. The greater expression of hAAT in A1 correlated with a greater proportion of viable hepatocytes that were transplanted. Unfortunately, the serum hAAT concentration began to fall at 10 days, reaching undetectable levels after day 47 in A1 and day 35 in A2.

There could be several possible explanations for the loss of hAAT production in the serum after transplantation. The first possibility was that the animal made antibodies to hAAT leading to a fall in serum levels. This was not the case in A1,

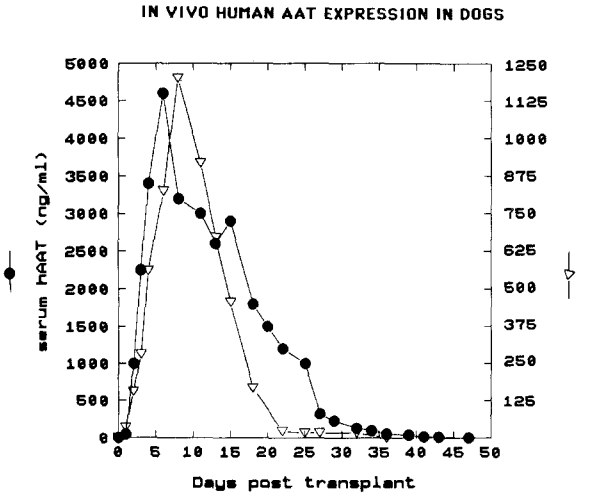

Figure 3. In vivo hAAT production in Dogs A1 and A2 after transplantation of transduced hepatocytes. The serum concentration of hAAT was determined pre and post transplantation of 3.8 x 10^8 and 6.4 x 10^8 hepatocytes in animals A1 (filled circles) and A2 (open triangles), respectively. (Used with permission from the Proc Natl Acad Sci USA.)

since the serum was assayed and found not to contain antibodies to hAAT. The other possibilities were that the cultured hepatocytes survived for only a short period after transplantation or that the expression of the hAAT transgene became turned off in vivo. To determine whether the transduced cells were still present after serum hAAT levels became undetectable, on days 47 and 120 post-transplant animal A1 underwent open liver biopsy. Multiple liver wedges were obtained from several portions of each lobe. In addition, hepatocytes were prepared from one lobe and cultured in the usual manner. Genomic DNA was isolated from cultured hepatocytes one week after isolation and from each tissue sample representing different liver lobes. These genomic DNA samples were subjected to analysis by a semi-quantitative polymerase chain amplification assay using a NEO specific set of primers. This assay showed that about 1/400 living hepatocytes contained proviral DNA. The total number of viable cells transplanted in animal A1 was approximately 4×10^8. If one assumes that the liver mass of the animal is 3.5% of the total body weight and that there are 10^8 hepatocytes per gram of tissue, the total number of hepatocytes in this dog was about 2×10^{10}. Assuming 50% survival, the transplanted cells would represent about 1% of the original total liver mass. However, only 25% of the cells were virally transduced in our hands, so only 0.25% of the liver mass represented transduced cells. This general estimate was in qualitative agreement with the observed number of transduced cells detected by the PCR assay.

The observations that serum hAAT concentrations eventually fell in transplanted animals while the transduced hepatocytes remained viable in vivo strongly suggested that the transcription of the transgene was shut off in vivo. This issue of promoter shutoff in the liver can be resolved by using promoters derived from liver-specific cellular genes that are highly expressed under normal in vivo conditions. Some of these promoters such as albumin [39,40] and transthyretin [41] have been demonstrated to direct the expression of marker genes at high levels in livers of transgenic mice. With the development of technology for large scale hepatocyte isolation, retroviral transduction, and autologous transplantation procedures in a dog model, the strategy of hepatocyte transduction followed by autologous transplantation offers an excellent approach to the genetic correction of certain hepatic deficiencies in man.

Human gene therapy: the future

Somatic cell gene transfer methodologies have additional applications which include include therapies for solid malignant tumors. There currently are several approved human trials under way which utilize gene transfer of either interleukin-2 or tumor necrosis factor mini-genes into tumor infiltrating lymphocytes followed by autologous transplantation of these cells into the patient. The secretion of these molecules into the tumor may result in enhanced immunologic destruction of the tumor. Other immunologic methods that utilize gene transfer into tumor cells to make them more immunologic are also under study. Gene transfer into somatic cells will have widespread application for many inherited and acquired disorders. There is little doubt that "human gene therapy" as a discipline will be a major component of "molecular medicine" that will be common clinical practice well into the next century.

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