Hepatocyte-directed Gene Transfer in Vivo Leads to Transient Improvement of Hypercholesterolemia in Low Density Lipoprotein Receptor-deficient Rabbits*

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Familial hypercholesterolemia is an inherited disease in humans, caused by a deficiency of low density lipoprotein (LDL) receptors, that we have used as a model for developing liver-directed gene therapies. Our strategy is to reconstitute hepatic LDL receptor expression in vivo by administering a DNA-protein complex that is capable of targeting the delivery of functional LDL receptor genes to hepatocytes. Infusion of this DNA-protein complex into the peripheral circulation of a rabbit animal model for familial hypercholesterolemia resulted in hepatocyte-specific gene transfer and a temporary amelioration of hypercholesterolemia. This noninvasive approach to gene therapy should have applications in the treatment of a wide spectrum of human diseases.

Familial hypercholesterolemia (FH) is an inherited disease in humans that we have used as a model for developing new approaches to gene therapy based on gene transfer directed to the hepatocyte. This disease, which is caused by a deficiency in the receptor for LDL, is associated with hypercholesterolemia and premature development of coronary heart disease (1). Transplantation of a normal liver into a patient with FH can correct the hyperlipidemia, suggesting that reconstitution of hepatic LDL receptor expression should be sufficient for phenotypic improvement (2–4).

The availability of an authentic animal model for FH, the Watanabe heritable hyperlipidemic (WHHL) rabbit, has greatly facilitated the development of innovative therapies (5–7). We previously used the WHHL rabbit to develop a genetic therapy in which hepatocytes were harvested, genetically modified ex vivo with retroviruses, and transplanted into allogeneic WHHL recipients (8, 9). Transplantation of genetically modified cells was associated with temporary amelioration of hypercholesterolemia suggesting short term efficacy (9). The clinical utility of ex vivo gene therapy in the liver may be limited, in part, by the morbidity of the invasive procedures used to harvest and transplant hepatocytes. A more direct approach is to deliver genes to hepatocytes in vivo.

One possible strategy for obtaining hepatocyte-specific gene transfer in the WHHL rabbit is suggested by earlier experiments performed in the rat (10–12). In this system, the cellular specificity of gene transfer is mediated by interactions between the gene transfer substrate and the hepatocyte-specific asialoglycoprotein receptor. This gene transfer substrate is constructed in the following manner. A high affinity ligand for the asialoglycoprotein receptor, asialoorosomucoid (ASOR), is covalently attached to poly-L-lysine, and the protein conjugate is complexed to a DNA vector that is capable of directing transcription in a mammalian cell (10–12).

In this report we have utilized targetable DNA-protein complexes to develop a method for directly introducing functional LDL receptor genes into hepatocytes of WHHL rabbits in vivo. Analysis of WHHL rabbits following injection of DNA-protein complexes containing a LDL receptor gene revealed rapid clearing of the complex from the circulation because of hepatocyte specific uptake mechanisms and the subsequent appearance of recombinant derived LDL receptor RNA in liver. This was associated with statistically significant decreases in total serum cholesterol for at least 6 days following in vivo gene transfer. These studies demonstrate the potential of in vivo gene transfer for developing effective and noninvasive approaches to liver-directed gene therapies.

MATERIALS AND METHODS
Vector and DNA-Protein Complexes

The vector was constructed in a single three-part ligation using fragments that were cloned in a directional manner. The three fragments used in this construction are described below.

Fragment A—An XbaI to BglII fragment (3.6 kb) of plasmid MTRV.JT was isolated which contains the following functional elements: a 231-bp fragment of genomic DNA spanning the polyadenylation signal of the bovine growth hormone gene, β-lactamase, the prokaryotic origin of replication from PUC 19, and an eukaryotic transcriptional unit expressing xanthine-guanine phosphoribosyltransferase (XGPT).

Fragment B—Sequences spanning an enhancer located 5' to the mouse albumin gene (−12 to −9 kb) were excised on an EcoRV to BglII fragment and fused in reverse orientation to sequences spanning the mouse albumin promoter (−282 to +21) (13). The 5' end of the fragment contains a synthetic Sall site attached to position +21 of the albumin promoter sequence.

Fragment C—This Sall to XbaI fragment (3.0 kb) was derived from
a previously published retroviral vector LTR-LDLR (8). It includes the entire cDNA for human LDL receptor (2.6 kb) along with 430 bp of additional 3' sequence derived from the retroviral genome (nucleotides 7816–8113 of the Moloney murine leukemia virus genome; see Ref. 14 for numbering). The 5' end of the LDL receptor cDNA was converted to a Sall site in preparation for this construction.

Targeted DNA-protein complexes were prepared as described previously (12). In brief, human orosomucoid isolated from pooled human plasma was desialated with neuraminidase and subsequently coupled to poly-L-lysine (M, = 59,000) using 1-ethyl(3-)-3-dimethylaminopropylcarbodiimide. The ASOR/poly-L-lysine conjugate was purified by molecular sieve chromatography as described previously and complexed to plasmid DNA using an agarose gel retardation assay to determine optimal conjugate to DNA ratios for each plasmid coupled to poly-L-lysine. ASOR/poly-L-lysine conjugate complexes were synthesized as follows: ASOR was labeled with [35S]-methionine in the presence of recombinant LDL receptor RNA using a quantitative RNase protection assay (9). RNA derived from the p9-12alb(h)LDLR vector was detected with an antisense RNA probe that was synthesized from the previously described vector 3Z-env (9). This RNA probe is complementary to vector-specific sequences in the 3'-untranslated region of the recombinant transcript and is detected as a 172-bp band. Antisense RNA that specifically detects endogenous WHHL LDL receptor RNA was used as an internal control in each assay. The transcription vector used to synthesize this RNA probe (3Z-wLDLR) was constructed in the following manner. A restriction fragment from a WHHL LDL receptor cDNA clone, spanning the Smal site at position 211 to the NarI site at 514, was isolated (see Ref. 7 for numbering). The NarI site was converted to a HindIII site with synthetic linkers, and the revised fragment was ligated with the HindIII to Smal backbone fragment of pGEM3Zf(+)(Promega). Endogenous WHHL LDL receptor RNA is detected with the 3Z-wLDLR probe as an 80-bp band. Transcription vectors were linearized with EcoRI (3Z-env) or MnlI (3Z-wLDLR) and used as templates in transcription reactions according to the recommendations of the manufacturer (Promega). RNA probes were gel purified prior to use (17).

Total cellular RNA prepared from liver was hybridized with equal quantities of 3Z-env and 3Z-wLDLR probe (5 × 10^5 cpm of each probe/assay) and analyzed for protection to digestion with RNase A as described previously (9). Samples were electrophoresed through a 6% polyacrylamide/urea denaturing gel. Radioactivity in the resulting bands was quantified with a Beta Scope 630 (Betagen, Waltham, MA).

Metabolic Consequences of in Vivo Gene Transfer

WHHL rabbits were derived from mating homozygous LDL receptor-deficient rabbits and were purchased from Dr. Mahlan at New York University. Experiments were conducted in accordance with the guidelines of the Committee on Use and Care of Animals from the University of Michigan and Albert Einstein College of Medicine. Six WHHL rabbits were enrolled in a two-treatment cross-over study in which they received DNA-protein complexes containing either p9-12alb(h)LDLR or p9-12albCAT plasmid (4.0 mg of total DNA in each complex/dose). Animals were approximately 4 months of age at the initiation of the experiment and were maintained in a 12-h light/dark cycle on a Purina laboratory rabbit chow. DNA-protein complexes were injected into the marginal ear vein over a 10-min time period. Important features of the animals, including average pretreatment cholesterol level, sex, and weight, are summarized below: A, 818 mg/dl, male, 2.8 kg; B, 365 mg/dl, female, 2.6 kg; C, 622 mg/dl, male, 2.9 kg; D, 440 mg/dl, male, 2.7 kg; E, 688 mg/dl, male, 2.8 kg; and F, 710 mg/dl, female, 2.8 kg.

FIG. 1. Cellular distribution of DNA-protein complex in liver tissue. Rabbits were injected with radiolabeled DNA-protein complex and liver tissue was removed and analyzed for uptake of radioactivity. Panel A, analysis of liver tissue from a WHHL rabbit that received ASOR-labeled complex (4.0 mg of DNA; specific activity = 0.85 × 10^5 cpm/μg). Panel B, analysis of liver tissue from a WHHL rabbit that was injected with saline. In each case, magnification is × 100.
In Vivo LDL Receptor Gene Transfer in WHHL Rabbits

RESULTS AND DISCUSSION

A vector that expresses normal human LDL receptor was constructed [p9-12alb(h)LDLR] for in vivo gene transfer experiments in WHHL rabbits. Transcriptional elements from the mouse albumin gene, shown to confer liver specific expression in transgenic animals, were used to drive expression of a full-length cDNA for human LDL receptor (13). The vector contains an enhancer sequence located upstream of the albumin gene (−9 to −12 kb) fused to the albumin promoter (−282 to +21, Ref. 13). LDL receptor sequences in p9-12alb(h)LDLR were replaced with sequences encoding for the prokaryotic gene chloramphenicol acetyltransferase (CAT) to generate a vector called p9-12albCAT. This vector was used as a negative control in metabolic experiments. DNA-protein complexes were synthesized with either p9-12alb(h)LDLR (called LDLR complex) or p9-12albCAT (called CAT complex).

To study the organ and cellular distribution of DNA-protein complex uptake in vivo, it was necessary to introduce a radiisotope into the DNA-protein complex without perturbing its structural configuration. This was accomplished by synthesizing LDLR complexes with [35S]-labeled ASOR. Radiolabeled LDLR complexes were injected into the marginal ear vein of WHHL rabbits which were euthanized 10 min later and analyzed for uptake of radioactive activity in various organs. DNA-protein complex is rapidly cleared from the plasma and primarily taken up by the liver after 10 min (85% of total recovered radioactivity, data not shown). Similar kinetics and organ distribution of uptake have been demonstrated in rodent models in which the DNA or protein moieties of the complex were labeled (11). This suggests that radiolabeling the ASOR component may be a valid method for tracking the complex.

This approach to in vivo gene transfer was further characterized with respect to the cellular distribution of DNA-protein complex uptake within the liver. The hepatocyte is the predominant cell type in the liver and the desired target for gene transfer when using the strategy described in this study. However, nonparenchymal cells of the liver, such as endothelial cells and Kupffer cells, could potentially serve as alternative targets for gene transfer. Livers harvested 10 min after injection of radiolabeled LDLR complex were fixed, sectioned, and subjected to autoradiographic analysis in order to visualize the cellular distribution of radioactivity. Radioactive signal, detected as silver grains, was 100-fold greater in experimental tissues (Fig. 1A) than it was in liver tissue from saline injected rabbits (Fig. 1B). The majority of this signal (>90%) was seen as discrete grains located over hepatocytes, while some signal was seen infrequently over Kupffer cells as either aggregates (data not shown) or single grains (Fig. 1A). This nonparenchymal cell uptake may represent larger forms of the DNA-protein complex that has been phagocytosed by Kupffer cells.

The intracellular fate of the recombinant gene was studied in WHHL rabbits after administration of unlabeled LDLR complex. Animals were euthanized various times after in vivo gene transfer and liver tissue was characterized with respect to different times of exposure (left side, 2 day exposure and right side, 5 day exposure). The undigested probes (2 x 10^6 cpm/lane) were electrophoresed in lanes 32-env and 32-wLDLR. Molecular weight markers (γ-ATP-labeled pBR322/pX HaeIII fragments) and their corresponding sizes in base pairs are presented in the far right lane.
to the abundance and structural integrity of the recombinant gene as well as the level of recombinant derived RNA.

Total cellular DNA isolated from liver tissue was analyzed for p9–12alb(h)LDLR DNA sequences by blot hybridization (Fig. 2A). DNA was restricted with BamHI to excise a 1.4-kb fragment from the plasmid, and the filter was probed with vector-specific sequences. Liver tissue harvested 10 min after injection of the DNA-protein complex demonstrated very high levels of the intact fragment along with some partially degraded plasmid DNA; comparison to plasmid controls indicated that this tissue contained approximately 1000 copies of plasmid/cell. This estimate of gene targeting is in agreement with the level of gene delivery to the liver expected from the injection of 4 mg of plasmid (16 kb in size) into a rabbit that has 2–4 × 10^10 hepatocytes. Analysis of livers harvested at later time points revealed ongoing degradation of the plasmid and a progressive decline in the abundance of the intact plasmid from 100 copies/cell at 4 h to 1 copy/cell at 24 h (Fig. 2A); plasmid DNA was less than 0.1 copy/cell in liver harvested 48 h after gene transfer (data not shown). These experiments indicate that the DNA-protein complex remains intact in the blood and capable of efficiently delivering large quantities of DNA to the liver; the plasmid DNA is apparently degraded following uptake by the liver.

Liver tissues were analyzed for the presence and abundance of recombinant human LDL receptor transcripts using a quantitative RNase protection assay (9). A representative experiment is shown in Fig. 2B. An RNA probe (3Z-env) complementary to vector-specific sequences in the 3′ untranslated region of the recombinant transcript was used to detect vector-derived RNA. RNase digestion of the resulting duplex produces a protected fragment of 172 bp. An RNA probe (3Z-LDLR) complementary to endogenous LDL receptor RNA was used as an internal control in this assay (the WHHL mutation does not affect the concentration of endogenous LDL receptor RNA (7)). Endogenous LDL receptor RNA was specifically detected in this assay as an 80-bp protected fragment. Recombinant derived LDL receptor RNA was first detected at 4 h, reached a maximum at 24 h, and declined to undetectable levels by 72 h after gene transfer. Quantitative analysis of this assay was used to estimate the abundance of vector-derived RNA relative to the endogenous LDL receptor transcript. Maximal levels of recombinant LDL receptor RNA, achieved 24 h after gene transfer, were estimated to be 2–4% of endogenous.

Experiments were performed to determine the metabolic effects of hepatocyte-directed gene transfer in vivo. WHHL rabbits were injected with LDLR complex or CAT complex and analyzed for changes in total serum cholesterol. Injection of LDLR complex into WHHL rabbits led to a transient decrease in total serum cholesterol. To rigorously document the metabolic effect of this treatment, six WHHL rabbits were entered into a protocol that involved a two-treatment (injection of LDLR complex or CAT complex), two period, cross-over design, with repeated measurements of total serum cholesterol within each period (Fig. 3). Animals A–C received LDLR complex on day 0 and the CAT complex on day 12; the treatment order was reversed in animals D–F. The data were analyzed using a repeated measures analysis of covariance with the mean of the three base-line measurements as the covariate (19). This permitted a comparison between the two treatments as well as an assessment of possible interactions between the two treatments in terms of carry-over and time trends.

Injection of the LDLR complex led to an immediate but transient decrease in total serum cholesterol by 153 ± 53 mg/DL (mean ± 1 S.D., n = 6) which was approximately 25–30% of the pretreatment value; this did not occur in animals injected with the CAT complex. Cholesterol returned to base line, following transient LDL receptor expression, at a rate equal to 32 ± 10 mg/dl/d (mean ± 1 S.D., n = 6). This agrees with previous experiments in WHHL rabbits subjected to LDL apheresis in which cholesterol was acutely lowered by 200 mg/dl followed by a return to base line at a rate equal to 32 ± 10 mg/dl/d (mean ± 1 S.D., n = 6) (20). Direct comparison between treatment with LDLR complex and CAT complex revealed statistically significant differences (p < 0.001) lasting 6 days and reaching a maximum on the second day after gene transfer. Experiments performed on separate animals demonstrated expression of CAT in liver of rabbits injected with the CAT complex. No evidence of carry-over from one treatment to another was noted. Areas under the cholesterol time curve were also analyzed. Treatment with LDLR complex was associated with a highly significant decrease in the area under the cholesterol time curve (p < 0.01) compared to injection of the CAT complex. Again, there was no evidence of any carry-over or period effects between the two treatments.

We have performed experiments with six different preparations of p9–12alb(h)LDLR containing complexes (16 different injections), and three preparations of a control complex (four separate injections). None of these experiments differed.

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qualitatively from the results presented in Fig. 3. Finally, none of the animals developed antibodies to human ASOR when measured 2 weeks after the second injection by Western blot analysis (data not shown).

Many therapies for FH attempt to decrease serum LDL-cholesterol by increasing hepatic expression of LDL receptor (1). This has been accomplished in homozygous patients by orthotopic liver transplantation from donors who express normal levels of LDL receptor (2–4). More recently, we have demonstrated short term metabolic efficacy in the WHHL rabbit by transplanting allogeneic wild-type hepatocytes or WHHL hepatocytes genetically corrected ex vivo with recombinant retroviruses (9). We describe, in this report, a pharmacological approach to the treatment of FH in which functional LDL receptor genes are delivered directly to hepatocytes in vivo using soluble DNA-protein complexes.

_in vivo_ gene transfer has several potential advantages over organ/cell transplantation in the treatment of metabolic diseases of the liver. One advantage is that the therapeutic gene is expressed in a cell and organ that has not been manipulated ex vivo. Recombinant gene expression will occur in a cellular environment that is more physiologically relevant. In addition, the capacity of this approach to reconstitute hepatic gene expression is theoretically greater than the capacity of cellular therapies which are limited by the number of cells that will engraft (21). Finally, the therapeutic gene can be delivered to the appropriate cell in a noninvasive way with little apparent morbidity.

An important feature of this gene delivery system relates to the cellular specificity of gene transfer and expression. DNA-protein complexes made with the p9-12alb(h)LDLR vector should direct hepatocyte-specific gene expression in vivo based on both the cellular specificity of gene uptake and the transcriptional specificity of gene expression. It will be difficult, however, to develop a strategy in which either step is absolutely cell-specific. For example, analysis of liver following injection of radiolabeled DNA-protein complex indicated uptake predominantly in hepatocytes with a variable amount of label always located over nonparenchymal cells. The long term consequences of misdirected gene transfer and/or expression will need to be studied.

While our initial results are encouraging, the technology of hepatocyte-directed gene transfer must be further developed if it is to realize its full potential in the management of diseases such as FH. In order to improve the efficacy of _in vivo_ gene therapy it will be necessary to increase the level of recombinant gene expression which, in this study, was 2–4% of the endogenous level of LDL receptor expression. The 20–30% decrease in serum cholesterol resulting from 2–4% of normal LDL receptor gene expression will likely be therapeutic but not curative. Another apparent limitation is that the expression of the recombinant gene in WHHL rabbits was temporary. Nevertheless, transient gene expression may be adequate in several clinical settings such as in the treatment of acute illness with a single dose of the complex or in the treatment of chronic disease with repeated doses of the complex. It will be preferable in many diseases such as FH to achieve prolonged or permanent recombinant gene expression following a single treatment. One strategy to accomplish this, which has been successful in rats, is to stimulate liver regeneration immediately following _in vivo_ gene transfer (12). Another potential problem with this technology may be the development of an immune response to the gene transfer substrate after repeated injections. We are encouraged, however, that none of the animals developed antibodies to ASOR after two injections of DNA-protein complex.

This study illustrates a novel approach to gene therapy that in principle resembles more traditional forms of pharmacological therapy. The actual drug is a nonviral complex of protein and DNA that is capable of targeting the delivery of functional genes to specific cells _in vivo_. Somatic gene therapy of this type should provide a noninvasive, clinically practical approach to the treatment of a wide spectrum of disease states.

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