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Long-Term Improvement of Hypercholesterolemia After ex Vivo Gene Therapy in LDLR-Deficient Rabbits

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Familial hypercholesterolemia (FH) is an inherited disorder in humans that is caused by a deficiency of low density lipoprotein receptors (LDLRs). An animal model for FH, the Watanabe Heritable Hyperlipidemic rabbit, was used to develop an approach for liver-directed gene therapy based on transplantation of autologous hepatocytes that were genetically corrected ex vivo with recombinant retroviruses. Animals transplanted with LDLR-transduced autologous hepatocytes demonstrated a 30 to 50 percent decrease in total serum cholesterol that persisted for the duration of the experiment (122 days). Recombinant-derived LDLR RNA was harvested from tissues with no diminution for up to 6.5 months after transplantation.

MANY INBORN ERRORS OF METABOLISM are caused by inherited defects of liver function. One potential therapeutic approach for this group of diseases is the use of somatic gene transfer to permanently correct the genetic defect in hepatocytes. We used FH as a model for developing liver-directed gene therapies. FH is an autosomal dominant disorder caused by defects in the function or expression of LDLRs (*1*). Individuals with the disease have severe hypercholesterolemia and develop premature coronary heart disease. Homozygous deficient individuals are refractory to conventional therapies; however, orthotopic transplantation of a liver expressing normal amounts of LDLR has resulted in substantial metabolic improvements (*2*). The success of orthotopic liver transplantation suggests that hepatic reconstitution of LDLR expression by gene transfer would achieve therapeutic efficacy.

The existence of an animal model of FH, the Watanabe Heritable Hyperlipidemic (WHHL) rabbit, provides an experimental setting for the development of genetic therapies (*3*). One experimental approach, referred to as ex vivo gene therapy, involves the removal of a portion of the liver from an LDLR-deficient animal and the establishment of primary cultures of hepatocytes. Recombinant retroviruses are used to transduce a functional LDLR gene into the cultured hepatocytes that are then harvested and transplanted into the animal from which they were derived. The feasibility of a cell-based therapy for FH is supported by studies that demonstrated transient metabolic improvements after transplantation of allogeneic hepatocytes (for example, WHHL hepatocytes expressing human LDLR or hepatocytes derived from a wild-type rabbit) (*4, 5*).

A critical step in the development of an effective ex vivo gene therapy in the WHHL rabbit was the production of recombinant retroviruses capable of efficiently transducing a wild-type rabbit LDLR gene into primary cultures of WHHL hepatocytes. A full-length cDNA for LDLR was isolated from a λgt11 library derived from liver mRNA of a New Zealand White rabbit (*6*). The cDNA was cloned into a retroviral vector that expresses the recombinant gene from sequences of the chicken β-actin promoter, and amphotropic retroviruses were produced (*7*). Viral stocks derived from the *lacZ*-expressing vector BAG were used in control experiments (*8*).

The efficacy of ex vivo gene therapy in the WHHL rabbit was tested with the following experimental strategy (*9*). We subjected a group of 12 WHHL rabbits to 30% partial hepatectomy and perfused the excised liver lobes with collagenase to release hepatocytes. The recovered hepatocytes were placed in primary culture and exposed to either the LDLR virus or the *lacZ* virus. Retrovirally transduced hepatocytes were subsequently harvested (2×10^8 cells or 2% of the total cells in a liver) and injected into the spleen of the animal from which the cells were originally derived. Hepatocytes were labeled with ^{111}In before we transferred them into a separate animal in order to assess their trafficking in vivo after intrasplenic injection; greater than 95% of the infused cells immediately entered the portal circulation and seeded into the liver. This analysis does not permit a precise determination of the cells remaining in the spleen; this number could easily vary fivefold, between 1 and 5%. The two experimental groups subjected to further analyses included WHHL rabbits transplanted with autologous hepatocytes transduced with either the LDLR virus ($n = 5$) or the *lacZ*-expressing virus ($n = 7$).

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Transduced hepatocytes were analyzed for gene transfer and expression. We obtained a minimal estimate of gene transfer by incubating the LDLR-transduced hepatocytes with fluorescent-labeled LDL and visualizing the cultures by fluorescence microscopy (7) or by subjecting the *lacZ*-transduced cells to the chromogenic X-gal assay (*8*). Transduction efficiency ranged from 10 to 30% of the hepatocytes for each virus. A quantitative estimate of recombinant gene expression in the cell population was provided through the use of a ribonuclease (RNase) protection assay (*4*). Total cellular RNA was isolated and hybridized with cRNA probes specific for the recombinant and endogenous LDLR transcripts. The hybridization mixtures were digested with RNase A and fractionated in a nondenaturing polyacrylamide gel. An autoradiograph of a representative experiment is presented in Fig. 1A. Recombinant-derived *lacZ* or LDLR RNAs (migrating as bands between 172 and 180 bp) were detected in transduced hepatocyte cultures from each animal at amounts approximately equal to endogenous LDLR RNA (detected as a broad band between 70 and 75 bp).

Animals were killed at various times after transplantation, and we harvested the organs for an analysis of recombinant gene expression using the RNase protection assay (Fig. 1B). Substantial quantities of retrovirally derived LDLR RNA were detected in spleen and liver of animals transplanted with *lacZ* or LDLR-transduced hepatocytes. Quantitative analysis of these data estimated amounts of recombinant-derived LDLR RNA at 4% of the endogenous amount in the spleen and 2% of the endogenous amount in the liver (Fig. 1B, animals W185, W117, and W169). Similar amounts of recombinant transcripts were achieved in liver and spleen of animals transplanted with *lacZ*-transduced cells (Fig. 1B, animals W98 and W204). The amount of recombinant RNA in both liver and spleen was stable for at least 6.5 months after cell transplantation. This is the amount of genetic reconstitution that one would expect, based on the number of cells transplanted and the efficiency of gene transduction in vitro. We transplanted 2% of the number of hepatocytes in a liver. Evaluation of hepatocytes before transplantation revealed a transduction efficiency of 20% (on average). Functional studies in vitro have indicated that the transduced cells over express LDLR activity so that the amount of activity in the population is at least equal to that found in normal hepatocytes. The expected reconstitution based on the number of cells transplanted and the efficiency of complementation therefore agree with that measured by RNase protection assays (2 to 4%), assuming that most of the cells engraft.

We analyzed splenic tissues from transplant recipients (W98, W204, and W185) for the presence of hepatocytes by in situ hybridization using cRNA probes to the hepatocyte-specific gene that encodes serum albumin (Fig. 2) (*10*). Expression of albumin was detected in a variable number of cells in spleens from transplant recipients with antisense probes spanning two nonoverlapping areas of the albumin RNA (Fig. 2, A and C). This specific signal was not detected when the antisense probes were hybridized to spleens of nontransplanted animals or when the corresponding sense probes were hybridized to spleens of transplant recipients (Fig. 2, B and D). The presence of cells expressing this hepatocyte-specific function confirms the persistence of transplanted hepatocytes that have retained some degree of differentiated function. The frequency and distribution of albumin-expressing cells were extremely variable within the spleen (*10*).

We evaluated the metabolic efficacy of ex vivo gene therapy by measuring its effect on total serum cholesterol. Baseline amounts of serum cholesterol were established with samples obtained from each rabbit 10 days before transplantation, and serial determinations were made for variable periods of time after transplantation (Fig. 3). Transplantation of LDLR-transduced hepatocytes was associated with a decline in serum cholesterol, ranging from 156 to 445 mg per deciliter of serum (308 ± 107 mg/dl; mean ± SD; n = 5), corresponding to a decrease of 25 to 45%. This effect occurred within 24 to 48 hours of transplantation and persisted in each animal for the duration of the experiment. No consistent change in serum cholesterol was noted after transplantation of *lacZ*-transduced cells. We subjected these data to statistical analysis using repeated measures analysis of variance (*11*); insufficient data points restricted these analyses to a 60-day period after transplantation. Significant differences in serum cholesterol (P < 0.001) were noted at all time points when the two experimental groups were compared directly. Comparisons to concentrations before treatment revealed significant decreases in the LDLR group (P < 0.001) but not in the *lacZ* group (P = 0.3678). The decline in

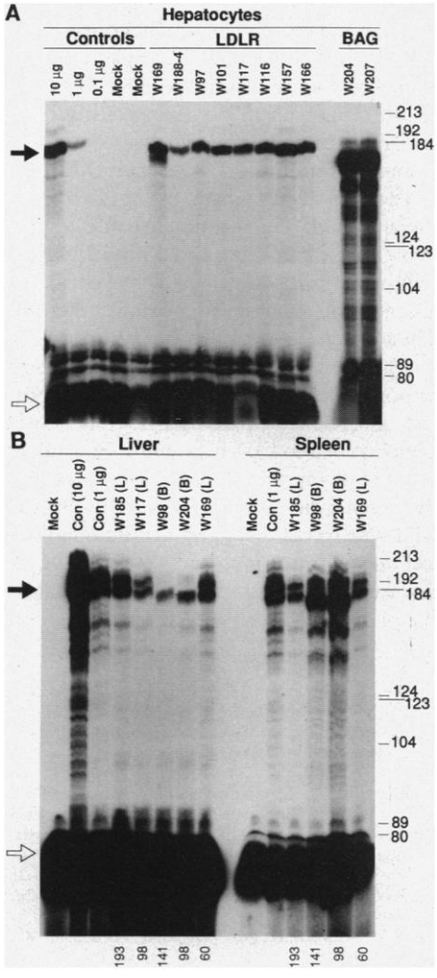

Fig. 1. Preparations of total cellular RNA were analyzed for the presence of recombinant LDLR RNA with a quantitative RNase protection assay (*4*). Proviral-derived RNAs were detected with an antisense RNA probe (3Z-env); protected transcripts migrated as a series of bands between 172 and 180 bp. Antisense RNA (3Z-wLDLR) that specifically detects endogenous WHHL LDLR RNA as a broad band between 70 to 75 bp was used as an internal control in each assay. Radioactivity in the resulting bands was quantified with a Beta Scope 630 (Betagen). The closed arrow indicates the location of the 3Z-env–protected band, and the open arrow indicates the location of the 3Z-wLDLR–protected band. Molecular mass markers are indicated in base pairs in the right margins. (**A**) Controls represent RNA (10 µg) from uninfected WHHL hepatocytes analyzed alone (mock) or in combination with 10 µg, 1 µg, or 0.1 µg of RNA from NIH 3T3 cells infected with the LDLR virus. Additional samples include RNA (10 µg) from WHHL hepatocytes infected with either LDLR or BAG. Animals W97, W101, W116, W166, and W207 experienced complications during the second surgical procedure and could not be included in subsequent experiments (*9*). (**B**) RNA isolated from the livers (100 µg) of rabbits transplanted with LDLR (L) or BAG hepatocytes (B) were analyzed. Controls included RNA from an untransplanted WHHL liver (100 µg) analyzed alone (mock) or in combination (con) with 10 or 1 µg of RNA from NIH 3T3 cells infected with the LDLR virus. Analyses were also performed on splenic RNA (50 µg) of animals transplanted with LDLR or BAG hepatocytes. Controls included RNA (50 µg) from an untransplanted spleen analyzed alone (mock) or in combination with 1 µg of RNA from NIH 3T3 cells infected with the LDLR virus. Times after transplantation that tissues were harvested are listed at the bottom in days.

cholesterol stabilized in the LDLR group and did not significantly change during the time of analysis.

Thus, a 30% improvement in serum cholesterol was achieved with a 2 to 4% reconstitution of LDLR expression. It is not surprising that serum cholesterol in the WHHL rabbit would be a sensitive biological assay for hepatic gene transfer. The rabbit has virtually no functional LDLR activity (<2% of normal activity). Our experiment, therefore, increased hepatic LDLR activity from essentially 0 to approximately 2 to 4% of normal. The absolute increment in LDLR activity is small; however, the relative increase is great. There are some data in individuals with FH that also bear on this issue (*12*). Several groups have described in FH homozygotes an inverse relation between residual LDLR activity and baseline serum cholesterol. The amount of residual LDLR activity in FH homozygotes is also related to prognosis.

A potential complication of gene therapy for FH is the development of an immunological response to the product of the transplanted gene. Sera from transplant recipients were analyzed for the formation of antibodies to wild-type rabbit LDLR protein. Lysates from human FH fibroblasts infected with rabbit LDLR-expressing virus were used as a source of antigen in a protein immunoblot analysis of sera. The positive control in this experiment was polyclonal antiserum made in New Zealand White rabbits to purified LDLR protein (*13*). Antibodies to the rabbit LDLR protein were not detected in sera from transplant recipients with this technique (*14*).

The results presented in this report have implications for the general application of this technology to humans, as well as for its specific application to the treatment of FH. Long-term therapeutic efficacy of ex vivo gene therapy in the WHHL rabbit was demonstrated with a legitimate metabolic end point, the persistent diminution of total serum cholesterol. Although the genetic reconstitution and resulting metabolic correction in WHHL rabbits were not complete, similar genetic and metabolic end points in homozygous FH individuals may be therapeutic, especially in those individuals that express low amounts of residual LDLR activity (*12*).

The major advantage of ex vivo gene therapy with autologous hepatocytes, as demonstrated in this animal model, is that long-term function can be achieved in the absence of immunosuppressive therapy. These experiments also address the potential complication of destructive immunological responses to the recombinant LDLR protein in recipients that had not previously been exposed to LDLR proteins. Functional stability of the transduced cells in vivo and the absence of a serological response to the wild-type receptor suggest that immunological rejection may not be a confounding or limiting problem in some FH individuals.

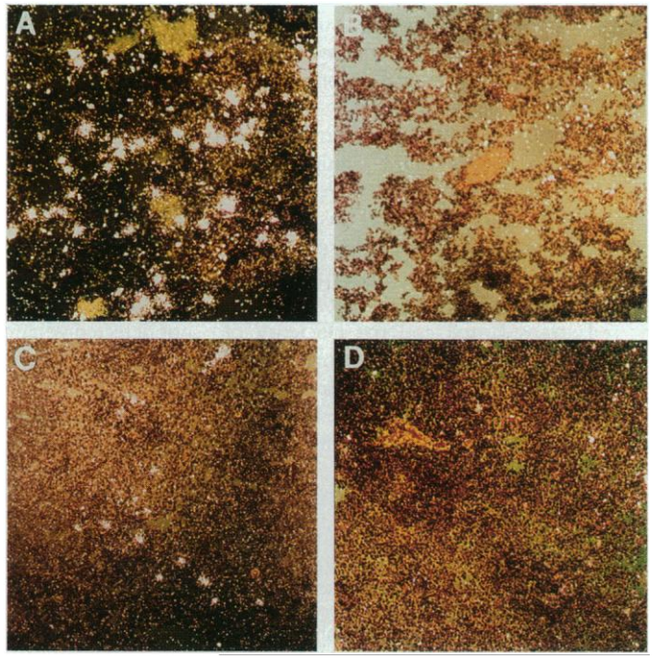

Fig. 2. Albumin expression in spleen of transplant recipients. Cryostat sections of spleen (10 μm) were hybridized with ^{35}S-labeled cRNAs from rat albumin with the method of Pinter and Lugo (*15*) with modifications as described (*4*). One probe was generated from a pGEM4Z vector that contains a Pst I to Hind III fragment from rat albumin cDNA spanning nucleotides 922 to 1266 (rat probe); the other probe contains a consensus albumin sequence spanning nucleotides 826 to 909 (consensus probe) (*16*). Slides were counterstained with hematoxylin and eosin. Tissues were derived from a WHHL rabbit transplanted with LDLR-expressing hepatocytes and killed 193 days later (W185). Dark-field views of representative areas are presented. Sections were hybridized to antisense (**A**) and sense (**B**) consensus albumin probes (magnification, ×18) and antisense (**C**) and sense (**D**) rat albumin probes (magnification, ×9).

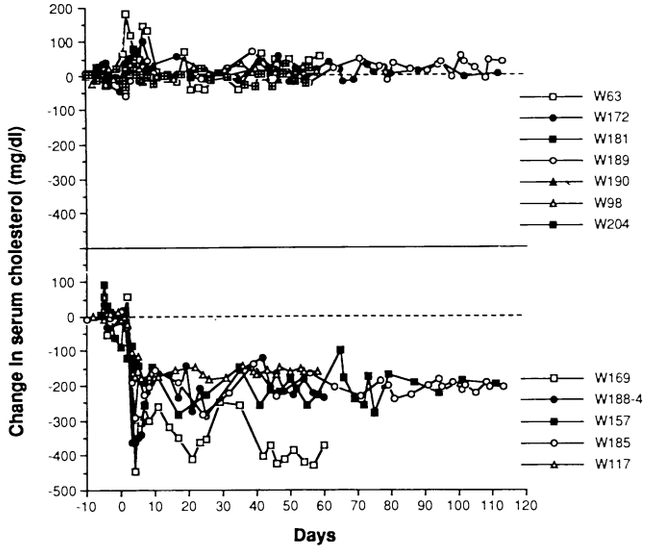

Fig. 3. Effect of hepatocyte transplantation on total serum cholesterol. Baseline amounts of cholesterol were established from determinations made 10 days before transplantation. Assays were performed with the method of Trinder (*17*). Sera were analyzed from each recipient for variable periods of time after transplantation (60 to 121 days). Multiple determinations were made each week. The data are presented as change in serum cholesterol from baseline versus time after transplantation. At the initiation of the study, the animals were 4.0 to 5.0 months old and massed 2.8 to 3.0 kg. (**Top**) WHHL rabbits that received *lacZ*-transduced cells. Baseline serum cholesterol amounts from these animals before treatment were as follows: W63, 534 mg/dl; W172, 685 mg/dl; W181, 501 mg/dl; W189, 565 mg/dl; W190, 478 mg/dl; W98, 671 mg/dl; and W204, 697 mg/dl. (**Bottom**) WHHL rabbits that received LDLR-transduced hepatocytes. Baseline serum cholesterol concentrations from these animals before treatment were as follows: W169, 988 mg/dl; W188-4, 806 mg/dl; W157, 754 mg/dl; W185, 678 mg/dl; and W117, 614 mg/dl.

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10. The following tissues were analyzed by in situ hybridization to the rat albumin probe: W98 (4 sections from one tissue block), W204 (44 sections from four tissue blocks), and W185 (40 sections from three tissue blocks). The following tissues were analyzed by in situ hybridization to the consensus albumin probe: W204 (28 sections from three tissue blocks) and W185 (23 sections from three tissue blocks). There was substantial variation in the frequency of albumin-expressing cells. Review of 114 microscopic fields (×20 magnification) from the spleen of animal W185 revealed the following distribution of albumin-expressing cells with the rat albumin probe: zero cells per field, 25.8%; one to ten cells per field, 69.7%; and greater than ten cells per field, 4.5%. The same analysis with the same tissue revealed the following distribution of albumin-expressing cells with the consensus albumin probe: zero cells per field, 12.5%; one to ten cells per field, 68.7%; and greater than ten cells per field, 18.8%.
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Isolation of an Active Human Transposable Element

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Two de novo insertions of truncated L1 elements into the factor VIII gene on the X chromosome have been identified that produced hemophilia A. A full-length L1 element that is the likely progenitor of one of these insertions was isolated by its sequence identity to the factor VIII insertion. This L1 element contains two open-reading frames and is one of at least four alleles of a locus on chromosome 22 that has been occupied by an L1 element for at least 6 million years.

TRANSPOSABLE ELEMENTS ARE A common feature of the genomes of eukaryotes (*1*). Retrotransposons (class I and class II) are a specific group of transposable elements that can be transcribed into RNA, reverse transcribed into cDNA, and then reintegrated as cDNAs into the genome at a new location. Examples of class II retrotransposons are the F, G, and I factors of *Drosophila melanogaster* and the long interspersed elements (LINE-1 or L1) of mammals (*2–4*). Class II retrotransposons do not contain long terminal repeats, have at least one open-reading frame (ORF), and have an A-rich tract at their 3′ ends. These retrotransposons are flanked by duplications of the insertion site that vary in length. The amino acid sequence derived from one of the reading frames contains a region similar to reverse transcriptases of retroviruses and class I retrotransposable elements (*5, 6*). Although retrotransposition by way of an RNA intermediate has been documented only for the I factor of *D. melanogaster* (*7*), the structural similarity of class II elements implies that they are all retrotransposons.

The L1 element is common in all mammalian genomes (*3*). In humans, L1 elements (full-length and truncated) compose about 5% of the genome and number 50,000 to 100,000 copies (*8*), most of which are truncated at the 5′ end (*9*). About 3500 copies are full-length (*10*). It has been proposed that rare L1 elements are capable of retrotransposition; however, no active individual sequences have been isolated.

A consensus human L1 element constructed from genomic L1 sequences predicted two ORFs that were not seen in any of the individual sequences used to construct the consensus (Fig. 1A) (*9*). On average, the nucleotide sequence of a genomic L1 element differs from the consensus sequence by 5% (*9*). In contrast, L1 cDNAs from a human embryonal tumor cell line differ in nucleotide sequence from the L1 consensus sequence by <1% (*11*). This suggests that the consensus sequence reflects the sequence of actively transcribed and potentially retrotransposable L1 elements.

Evidence that some L1 elements in the human genome are retrotransposable was provided by de novo transposition of truncated L1 DNA into exon 14 of the factor VIII gene producing hemophilia A in two individuals (*12*). Each new insertion contained the 3′ portion of an L1 element, including much of the second ORF in the consensus sequence (ORF2) (Fig. 1A) and a polyadenylic acid tail, and was flanked by a perfect target-site duplication (*13*). We report here the isolation of the L1 element that is the likely progenitor of the insertion from patient JH-27. It represents the first-identified active transposable element in humans.

From a region of the factor VIII insertion in patient JH-27 [nucleotides (nts) 4250 to 4269], we made a 20-nt oligomer (JH-27 oligomer) that differed at three positions from the consensus L1 sequence (Fig. 1A). (Nucleotide positions are numbered such that 1 represents the first nucleotide in the progenitor allele L1.2B.) When the JH-27 oligomer was hybridized to Bam HI-digested genomic DNA from 106 individuals, four to ten fragments of single-copy intensity were observed in the DNA from each individual (Fig. 1B) (*14*). Analysis of ge-

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