Correction of the genetic defect in hepatocytes from the Watanabe heritable hyperlipidemic rabbit

(retroviral vector/gene transfer/familial hypercholesterolemia)

JAMES M. WILSON*, DAVID E. JOHNSTON†, DOUGLAS M. JEFFERSON†, AND RICHARD C. MULLIGAN*

*Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142; and †Departments of Medicine and Physiology, Tufts-New England Medical Center, Boston, MA 02111

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ABSTRACT Familial hypercholesterolemia is an inherited disease in humans that is caused by a defect in the receptor for low density lipoproteins (LDLR). The existence of an animal model for this disease, the Watanabe heritable hyperlipidemic (WHHL) rabbit, makes it an attractive candidate for developing new therapies that involve gene transfer into liver. As a first step toward the development of these therapies, we report the use of retrovirus-mediated gene transfer to correct the genetic defect in hepatocytes isolated from WHHL rabbits. A series of retroviral vectors that express the gene for human LDLR were constructed, each differing in the transcriptional elements used to drive LDLR expression. Helper-free amphotropic virus stocks representing each construct were then used to infect primary cultures of hepatocytes that were isolated from newborn WHHL rabbits. The efficiency of transduction, as measured by Southern analysis of integrated proviral sequences, ranged from 20% to 100%. Expression of human LDLR was analyzed by blot hybridization analysis of total cellular RNA and by biochemical and in situ analyses of transduced cultures for receptor function. The vector in which the expression of LDLR was driven by the viral long terminal repeat sequence produced the greatest quantity of LDLR RNA and protein in WHHL hepatocytes; LDLR activity approached normal levels in these cultures.

New therapies that involve the transfer of normal genetic material into genetically abnormal tissue are being considered in the treatment of several inherited diseases. Previously, most attention has been placed on diseases treatable by gene transfer into bone marrow, such as the severe-combined immunodeficiency syndromes (1) and thalassemias (2). However, recent advances in hepatectomy transplantation (3) and the demonstration of efficient gene transfer into cultured hepatocytes (4–6) suggest that new therapeutic approaches based on gene transfer may also be possible for the treatment of inherited diseases that have their origin in the liver.

One such disease, familial hypercholesterolemia, is an autosomal dominant disorder in humans that leads to elevated levels of serum cholesterol and the premature development of coronary artery disease (reviewed in ref. 7). The primary genetic defect in this disorder is an abnormality in the expression or function of the receptor for low density lipoproteins (LDLR). Patients with two abnormal alleles for LDLR have severe hypercholesterolemia that is refractory to conventional medical therapy; they often die of myocardial infarction before the age of 20 (7). Liver transplantation has been performed in several receptor-negative patients and has successfully corrected the deleterious metabolic consequences of the primary receptor deficiency state (8). However, the relatively high morbidity and mortality of this surgical procedure along with the requirement for life-long immunosuppressive therapy restrict the use of this therapeutic approach to a very limited patient population.

In 1973, Watanabe identified a strain of rabbits, subsequently called the Watanabe heritable hyperlipidemic (WHHL) rabbits, that demonstrated clinical and metabolic abnormalities similar to those associated with familial hypercholesterolemia in humans (9). The WHHL rabbit was subsequently shown to be deficient in functional LDLR activity due to an in-frame deletion of a portion of the LDLR structural gene (10). This animal model was instrumental in delineating key steps in the regulation of cholesterol metabolism in vivo (reviewed in ref. 11). The existence of such an animal model for an important inherited disease of the liver has prompted us to investigate the feasibility of developing a genetic therapy for familial hypercholesterolemia. As a first step, we describe here the successful use of retrovirus-mediated gene transfer to correct the genetic defect in hepatocytes from WHHL rabbits.

METHODS

Isolation, Maintenance, and Infection of Hepatocytes. Newborn New Zealand White (NZW) rabbits and WHHL rabbits (3–5 days old, weighing 50–80 g) were used as the source of hepatocytes. Newborn WHHL rabbits were derived from matings between homozygous deficient males and females and were kindly provided by J. Knapka (National Institutes of Health). Four WHHL rabbits from two litters (two rabbits per litter) were used in these studies (named WHHL 1–4). Newborn NZW rabbits purchased from Pine Acres Rabbity (West Brattleboro, VT) were used as controls. Hepatocytes from newborn rabbits were prepared as described for adult rats (6) except the collagenase perfusions were performed retrograde (12). Cells were plated at a density of 3–4 × 10^4 cells per cm^2 onto plates with a polycationic matrix (Primaria; Falcon, Oxnard, CA) in hormonally defined medium (13) supplemented with 10% fetal bovine serum; 4–6 hr later the medium was replaced with fresh hormonally defined medium, which was subsequently changed every 24 hr during the duration of the experiment.

Hepatocyte cultures were infected for 12 hr with viral stocks (5 ml per 10-cm plate) containing Polybrene (8 µg/ml). Unconcentrated viral stocks were prepared from the producer cells as described (6).

DNA and RNA Analyses. DNA and RNA were prepared and analyzed as described (6, 14). Hybridization filters were probed with a 1.9-kilobase (kb) LDLR cDNA fragment (HindIII to EcoRI fragment of pT2Z1) that was labeled to high specific activity with [32P]dCTP by using the random primer method (15). RNA transfer blots were stripped and reprobed

Abbreviations: LDLR, receptor for low density lipoproteins; WHHL, Watanabe heritable hyperlipidemic; Mo-MLV, Moloney murine leukemia virus; LTR, long terminal repeat sequences; NZW, New Zealand White; AcLDL, acetylated LDL; Dil, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate.

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with a cDNA probe for human γ-actin (HindIII to BamHI fragment of pHF-1; ref. 16).

**Cytochemical Analyses.** Hepatocyte cultures were analyzed for expression of viral-directed β-galactosidase and endogenous glucose-6-phosphatase activity by using cytochemical stains as described (6). Cultures were analyzed for the presence of LDLR or the receptor for acetylated LDL (AcLDL) by incubating the cultures in hormonally defined medium containing fluorescent labeled LDL or AcLDL [labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) at 10 μg/ml; Biomedical Technologies, Stoughton, MA] for 6–8 hr, followed by three rinses with phosphate-buffered saline and fixation in phosphate-buffered saline containing 0.5% glutaraldehyde (17, 18).

**Assay of LDL Degradation.** Five-day-old cultures of hepatocytes plated in 35-mm dishes were assayed for degradation of $^{125}$I-labeled LDL ($^{125}$-LDL) [10 μg/ml, 0.15 μCi/μg (1 Ci = 37 GBq) obtained from Biomedical Technologies] by using the procedure described by Goldstein et al. (19).

**RESULTS**

**Generation of Recombinant Retroviruses Encoding Human LDLR.** In an attempt to generate recombinant retroviruses capable of expressing large amounts of LDLR, four different retroviral vectors were tested (Fig. 1). In the vector LTR-LDLR, transcription should initiate at the 5' LTR and give rise to a single full-length viral transcript that expresses LDLR. In the remaining vectors, expression of LDLR should be driven by transcripts initiated from transcriptional control sequences located internal to the proviral transcriptional unit (see legend to Fig. 1 for details of the constructions). Each of these latter vectors differs in the transcriptional elements responsible for transcription: BA-LDLR contains a 267-bp segment of the chicken β-actin gene extending from −266 to +1 (23); H4-LDLR contains a 704-bp segment of the human histone H4 gene extending from −696 to +8 (24); and TK-LDLR contains a 256-bp segment of the thymidine kinase gene from herpes simplex virus extending from −200 to +56 (25). Each of the three vectors also contains a deletion of viral transcriptional enhancer sequences located in the 3' LTR, so as to reduce the amount of viral transcription after reverse transcription and integration of the recombinant provirus (26). Human LDLR coding sequences for all four vectors were derived from a full-length human LDLR cDNA insert. Virus-producing cell lines were isolated for each vector by using the amphotropic packaging cell line ϕ-Crip as described (26). Individual virus-producing clones were tested for production of virus that transmitted the correct proviral structure by Southern analysis as described (6). Cell lines transmitting the highest number of proviral copies to recipient cells were chosen for this study. All virus-producing cell lines were maintained in culture for 4–6 wk prior to their use in order to test for the presence of helper virus. None of the cell lines yielded any detectable helper virus nor transferred the packaging functions (data not shown).

**Expression of LDLR in Hepatocytes.** Cells used for the infection studies were hepatocytes isolated from three NZW rabbits and four WHHL rabbits (see Methods). Collagenase perfusions routinely produced 40–80 × 10⁶ cells per animal with >90% viability. Cells plated at subconfluent densities formed aggregates (5–20 cells per aggregate) that covered ~20% of the dish when visualized 6 hr after plating. The primary cultures underwent marked proliferation after 36 hr in culture, achieving confluence by day 3 or 4.

$^{1}$ϕ-Crip is an amphotropic packaging cell line in which the gag-pol and env genes are expressed from separate defective proviral DNAs. Virus-producing cell lines made from ϕ-Crip do not produce replication-competent virus or transfer the packaging function (O. Danos and R.C.M., unpublished data).
cultures was equivalent to that seen in pure secondary cultures of endothelial cells derived from bovine aorta (Fig. 2d).

To optimize the conditions for infection of the hepatocytes, we utilized a high-titer amphotropic virus (BAG) encoding Escherichia coli β-galactosidase (28). Cells transduced by the BAG virus can be detected by a simple cytochemical reaction that stains the cell’s cytoplasm blue (28). Optimal transduction of WHHL hepatocytes was achieved with cells from newborn animals that were plated at subconfluent density and exposed to virus 36 hr after the initial plating (data not shown). The matrix substrata had little effect on transduction efficiency (data not shown). An example of WHHL hepatocytes infected with the BAG virus and analyzed for β-galactosidase expression is shown in Fig. 2a.

Having optimized the conditions for infection, NZW and WHHL hepatocytes were infected with the four different LDLR virus preparations 2 days after being placed in culture and were analyzed for gene transfer and LDLR expression on day 5. Integration of the recombinant proviral sequences into the cellular DNA isolated from infected hepatocytes was detected by Southern blot analysis (Fig. 3). DNA from transduced hepatocytes was digested with Kpn I and ana-
Genetics: Wilson et al.

Fig. 3. Southern analysis of transduced hepatocytes. Hepatocyte cultures were mock-infected (control) or infected with BA-LDLR (BA), H4-LDLR (H4), TK-LDLR (TK), or LTR-LDLR (LTR) and analyzed for integration of proviral sequences. Lanes labeled p contained DNA (10 ng) from mock-infected cultures that were supplemented with 7.5 ng of the appropriate retroviral plasmid (this represents approximately one copy of plasmid per cell); lanes labeled g represent genomic DNA (10 ng) isolated from infected populations of hepatocytes. The upper panel is a representative experiment of hepatocytes from a NZW rabbit, whereas the lower panel is from WHHL rabbit 1. Selected molecular size markers (expressed in kb) are noted along the left hand border.

Lyzed by the method of Southern (29) using the LDLR cDNA as a probe. Kpn I has unique recognition sites in the LTR sequences; consequently, each integrated provirus should yield a common restriction fragment irrespective of the site of integration. Each virus-producing cell line efficiently transmitted proviral sequences without rearrangement in hepatocytes from NZW and WHHL rabbits. The relative copy number of integrated provirus varied from a maximum of 1–2 copies per cell for cultures infected with the H4-LDLR virus to a minimum of 0.1–0.2 copy per cell for cultures infected with the LTR-LDLR virus.

Additional experiments were performed to show that the viral DNA detected in Fig. 3 was integrated into hepatocyte DNA. DNAs from transduced hepatocytes were digested with EcoRI (a restriction enzyme that has a single site in the proviral DNA) and subjected to Southern analysis using a LDLR probe. If the viral DNA existed as an integrated provirus no distinct EcoRI fragments should be detected because the outer borders of these fragments are located in flanking DNA and therefore are heterogeneous. In fact, no EcoRI fragments were detected when this analysis was done, suggesting that the majority of viral DNA was integrated into hepatocyte chromosomal DNA (data not shown).

Transduced cultures were first analyzed for LDLR expression by blot hybridization analysis of cellular RNA. (Fig. 4). A faint band with an apparent molecular size equal to 3.5 kb was detected in mock-infected cultures (Fig. 4, lane con); this band probably represents endogenous LDLR RNA. The predominant RNA species in cultures infected with BA-LDLR, H4-LDLR, and TK-LDLR were the transcripts initiated at the internal promoter. The relative abundance of these RNAs consistently varied in a vector-dependent manner as follows: BA-LDLR > H4-LDLR > TK-LDLR > putative endogenous signal. As expected, very little transcription initiated from the LTR of these vectors was detected since the enhancer deletion that is present in the 3′ LTR of the starting plasmid is transferred to the 5′ LTR during proviral passage into hepatocytes (26). Cultures infected with the LTR-LDLR virus produced a single very intense band representing a transcript initiated at the LTR. All blots were stripped and reprobed with a human γ-actin cDNA probe to control for variation in the amount of RNA that was loaded. There was no detectable variation in the intensity of the γ-actin band, suggesting that equal quantities of undegraded RNA were loaded (data not shown).

Biochemical activity of the exogenous LDLR was assessed in situ by visualizing the uptake of Dil-LDL. Representative fluorescent micrographs are presented in Fig. 2 g–l. Mock-infected NZW rabbits exhibited a uniformly high level of fluorescence in all cells (Fig. 2g), whereas mock-infected WHHL hepatocytes showed very little fluorescence (Fig. 2h). WHHL hepatocytes infected with the LTR-LDLR virus had the greatest amount of LDL uptake with ≈20% of the cells showing high levels of fluorescence (Fig. 2i). BA-LDLR-infected WHHL hepatocytes demonstrated a population of cells with moderate activity (Fig. 2i), whereas H4-LDLR-infected WHHL hepatocytes showed a low level of activity in virtually all cells (Fig. 2j); the activity of LDLR in TK-LDLR-infected cells was barely over background (Fig. 2k).

Transduced hepatocytes were also analyzed for degradation of 125I-LDL in an attempt to quantify the amount of human LDLR expressed (Table 1). Activity of LDLR was greatest in hepatocytes infected with the LTR-LDLR virus: hepatocytes from a NZW rabbit showed an increase in LDLR activity from 170 ng/mg per 5 hr in mock-infected cells to 274 ng/mg per 5 hr in transduced cells, whereas cells from WHHL rabbits exhibited an increase in activity from 30–40 ng/mg per 5 hr in mock-infected cells to 155 (WHHL 1) and 84 (WHHL 3) ng/mg per 5 hr in transduced cells. The level of LDLR activity in LTR-LDLR transduced hepatocytes is ≈700 ng/mg per 5 hr (4-fold greater than the activity of the endogenous receptor in NZW rabbits) when corrected for the

Table 1. Quantitative analysis of 125I-LDL degradation in transduced hepatocytes

<table>
<thead>
<tr>
<th>Virus</th>
<th>125I-LDL degradation,* ng/mg of protein per 5 hr</th>
<th>WHHL 1</th>
<th>WHHL 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>170 ± 8</td>
<td>38 ± 5</td>
<td></td>
</tr>
<tr>
<td>BA-LDLR</td>
<td>201 ± 10</td>
<td>42 ± 3</td>
<td></td>
</tr>
<tr>
<td>H4-LDLR</td>
<td>194 ± 9</td>
<td>30±</td>
<td>47 ± 3</td>
</tr>
<tr>
<td>TK-LDLR</td>
<td>188 ± 17</td>
<td>44 ± 8</td>
<td></td>
</tr>
<tr>
<td>LTR-LDLR</td>
<td>274 ± 6</td>
<td>155±</td>
<td>84 ± 13</td>
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Degradation rates were also measured in the presence of a 50-fold excess of unlabeled LDL. Under these conditions NZW hepatocytes (mock-infected and transduced) had degradation rates that ranged from 50 to 60 ng/mg per 5 hr, whereas WHHL hepatocytes had degradation rates that ranged from 10 to 20 ng/mg per 5 hr. Data are presented as mean ± SD (N = 3 for WHHL 3 and N = 4 for NZW).

*Analyses were performed on selected cultures of one NZW rabbit and two WHHL rabbits (WHHL 1 and WHHL 3).

1Single determination.
actual number of cells that were transduced.\textsuperscript{5} Hepatocytes infected with viruses that express LDLR from a transcript driven by an internal promoter (i.e., BA-LDLR, H4-LDLR, and TK-LDLR) exhibited little to modest increases in 125I-LDL degradation.

**DISCUSSION**

The existence of the WHHL rabbit offered us a unique opportunity to begin to experimentally assess the feasibility of liver-directed genetic therapies. First, the genetic defect in the rabbits responsible for the hyperlipidemic state has been identified (10), and the corresponding normal gene (i.e., for LDLR) has been cloned (30). Second, correction of the metabolic consequences of the defect will most likely necessitate the transfer of a functional gene into hepatocytes (11) rather than the other more easily transplanted somatic cells. Finally, the WHHL rabbit appears to be an authentic animal model for familial hypercholesterolemia, a life-threatening human disease (7, 9–11).

The demonstration in this study that the human LDLR gene can be efficiently transferred to and expressed in hepatocytes deficient in LDLR expression represents an important first step toward a genetic therapy for familial hypercholesterolemia. Recombinant retroviruses possessing amphotropic host range were shown to efficiently infect NZW and WHHL rabbit hepatocytes, thus obviating the need to biochemically select the transduced cells. This high level of transduction may ultimately prove to be critical for the successful development of hepatocyte transplantation strategies, since the prolonged growth of cells in vitro necessary to effect any selection may deleteriously affect their subsequent long-term performance in vivo.

Our testing of a number of different retroviral constructs was prompted by several conflicting reports regarding the transcriptional activity of particular viral vectors (4–6). All four vectors tested in the current study led to the efficient infection of hepatocytes, at levels consistent with their titers on murine fibroblastic cells. However, RNA and protein analyses of transduced cells revealed consistent vector-dependent differences in the level of LDLR gene expression. Specifically, RNA transfer analysis of transduced cells revealed that the LTR-LDLR construct gave rise to \( \approx 7 \)-fold more RNA than the BA-LDLR vector, which, in turn, gave rise to \( \approx 10 \)-fold more RNA than the H4-LDLR or TK-LDLR vector. Although these differences are likely due to inherent variation in the strengths of the transcriptional elements present in the different vectors, the stability of each chimeric RNA may also vary and may contribute significantly to the observed differences. Analysis of transduced hepatocytes for production of functional receptor confirmed the RNA data. In WHHL hepatocytes infected by the LTR-LDLR virus, the overall number of receptors approached the level found in normal hepatocytes. Since only 20% of the cells were infected, the actual level of receptor expressed in transduced cells was 4- to 5-fold greater than the level expressed in normal hepatocytes. Assuming the titers of the LTR-LDLR virus stocks can be improved, this overexpression of LDLR may dramatically reduce the absolute number of cells that will need to be reintroduced into a recipient to obtain a correction of the metabolic consequences of LDLR deficiency.

Having demonstrated efficient transfer and expression of the LDLR gene in WHHL hepatocytes in vitro, the next, more difficult step will be to transplant transduced hepatocytes into recipient WHHL rabbits in a way that they function and persist. Previously described methods for hepatocyte transplantation such as intrasplenic transplantation (31) and intraperitoneal transplantation of microcarrier-attached hepatocytes (3) may be attempted with transduced WHHL hepatocytes. However, improved methods of hepatocyte transplantation will have to be developed before this approach could provide a long-term genetic cure of inherited diseases such as familial hypercholesterolemia. The development of methods for directly delivering the functional gene to hepatocytes in vivo may, in the long run, provide a more effective and less morbid approach to the genetic treatment of liver-specific inborn errors of metabolism.

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\textsuperscript{5}Southern analysis (Fig. 3 and data not shown) demonstrated a relative copy number of proviral integrants per cell equal to 0.2 for the NZW rabbit, 0.2 for WHHL 1, and 0.1 for WHHL 3.

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