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Temporary amelioration of hyperlipidemia in low density lipoprotein receptor-deficient rabbits transplanted with genetically modified hepatocytes

(familial hypercholesterolemia/hepatocyte transplantation/gene therapy)

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ABSTRACT **Familial hypercholesterolemia is an inherited disease in humans that is associated with coronary artery disease and is caused by a deficiency of the receptor that mediates the internalization of low density lipoprotein (LDL). We have used an animal model for familial hypercholesterolemia, the Watanabe heritable hyperlipidemic (WHHL) rabbit, to design a therapeutic approach for this disease, which attempts to correct the hepatic defect in LDL receptor expression. Hepatocytes were harvested from WHHL rabbits, plated in primary cultures, and exposed to recombinant retroviruses capable of efficiently transferring a functional human LDL receptor gene. Genetically modified cells were harvested and infused into the portal vein of WHHL recipients, who were analyzed for metabolic consequences of human LDL receptor expression. Each animal exhibited a statistically significant decrease in total serum cholesterol 2–6 days after transplantation, with an eventual return to pretreatment levels. Proviral DNA sequences and virus-directed transcripts were detected in liver tissue 24 hr after transplantation. *In situ* hybridization demonstrated provirus expression in a small population of hepatocytes distributed in periportal sections of the liver. This study illustrates the potential of somatic gene therapy in ameliorating hyperlipidemia associated with familial hypercholesterolemia.**

Familial hypercholesterolemia (FH) is an autosomal dominant disorder in humans caused by a deficiency of the receptor that mediates the uptake of low density lipoprotein (LDL) (reviewed in ref. 1). Patients that inherit two abnormal LDL receptor alleles (i.e., homozygous deficient patients) have marked hypercholesterolemia from birth and suffer from accelerated and often life-threatening coronary artery disease in childhood. Current medical therapies are largely ineffective in improving the hypercholesterolemia or preventing the progression of coronary artery disease in these patients. Recent attempts at correcting the hepatic deficiency of LDL receptor in FH by orthotopic liver transplantation have been moderately successful (2, 3).

Another potentially effective approach to the treatment of FH is somatic gene therapy (4, 5). The liver is the primary organ responsible for the degradation of LDL and the only organ responsible for excretion of cholesterol (1), suggesting that the most appropriate target cell for LDL receptor gene transfer is the hepatocyte. Furthermore, it may be possible to achieve significant clinical improvement with only partial replacement of hepatic LDL receptor activity as suggested by the previously described correlation of residual LDL receptor activity and clinical severity of coronary artery disease in homozygous deficient patients (1, 6). Finally, the availability of an authentic animal model for FH, the Watanabe heritable hyperlipidemic (WHHL) rabbit, provides an opportunity to test experimental therapies *in vivo* (4, 7).

One approach we have considered in the design of liver-directed gene therapies is similar in concept to the well-described bone marrow-directed gene therapies. This method involves isolating hepatocytes from genetically deficient animals, transferring functional genetic material into the hepatocytes *in vitro*, and transplanting the genetically modified cells into the affected animals. In this report, we describe retrovirus-mediated gene transfer of a human LDL receptor gene into adult WHHL hepatocytes and transplantation of these genetically corrected cells into WHHL rabbits with consequent improvement in hypercholesterolemia.

MATERIALS AND METHODS

Animals. WHHL rabbits used in this study were derived from mating homozygous LDL-receptor-deficient rabbits. Wild-type New Zealand White rabbits were purchased from Dutchland Farms (Denver, PA). All animals were maintained on a Purina laboratory rabbit chow. Individual WHHL rabbits exhibited <2% variation in total serum cholesterol for a period up to 2 weeks prior to hepatocyte transplantation. In addition, baseline pretreatment serum cholesterol levels were indistinguishable between the two experimental groups: WHHL recipients transplanted with mock-infected cells (539 ± 46 mg/dl, mean ± 1 SD, $n = 6$) or LTR-LDLR-infected cells (543 ± 48 mg/dl, mean ± 1 SD, $n = 7$).

Recombinant Retrovirus. The retroviral vector (LTR-LDLR) used to produce amphotropic virus has been described (5) and is depicted in Fig. 1. The initial description of this vector stated that enhancer sequences in the 3′ long terminal repeat were replaced with homologous sequences from the myeloproliferative sarcoma virus (5). It has subsequently been discovered that, due to confusion regarding one of the DNA fragments used in the original construction of the vector, it actually contains Moloney enhancer and promoter sequences and an additional mutation (8) in the tRNA binding site; the rest of the vector is identical to the one described in the original reference (5). The original virus-producing cell line that was generated from this vector was subcloned prior to this study and shown to be free of replication-competent virus.

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Abbreviations: LDL, low density lipoprotein(s); WHHL, Watanabe heritable hyperlipidemic; FH, familial hypercholesterolemia; PCR, polymerase chain reaction.

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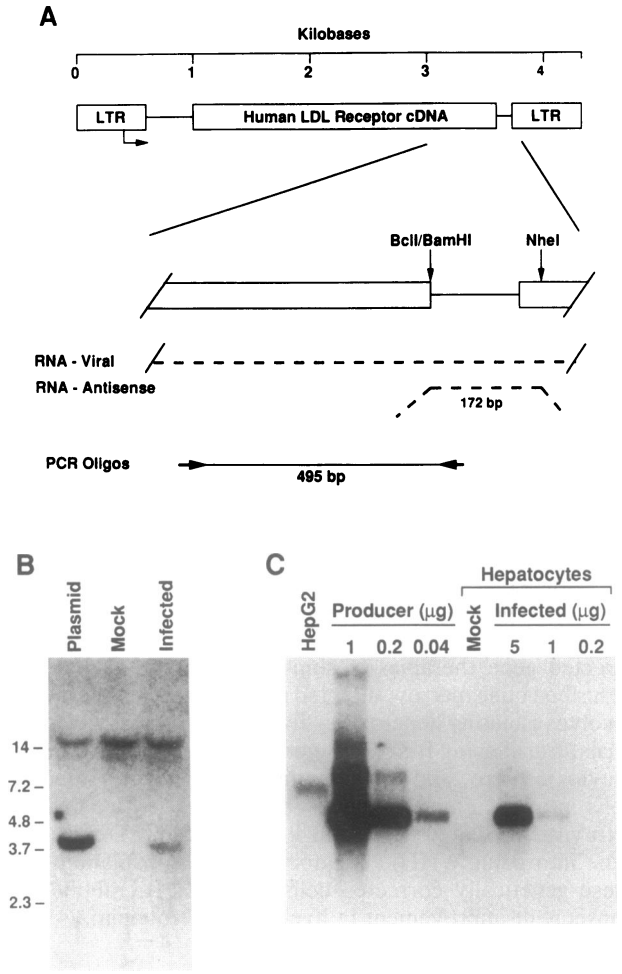

FIG. 1. Retroviral vector and *in vitro* characterization of transduced WHHL hepatocytes. (*A*) Retroviral vector. Vector sequences involved in the production of appropriate probes are expanded in the area below the vector. The RNA probe complementary to viral sequences in the recombinant transcript is noted; a 172-base-pair transcript should be protected from digestion with RNase A. The 5′ and 3′ PCR oligonucleotides (Oligos) are derived from human LDL receptor cDNA sequences and flanking viral sequences, respectively, as shown; a 495-base-pair fragment is amplified with these oligonucleotides. bp, Base pair(s); LTR, long terminal repeat sequences. (*B*) Southern blot analysis. Total cellular DNA was isolated and analyzed (10 μg per lane) for proviral sequences. Lanes: "Plasmid," DNA from mock-infected hepatocytes plus 1.25 pg of LTR-LDLR plasmid; "Mock," DNA from mock-infected hepatocytes; "Infected," DNA from hepatocytes infected with LTR-LDLR virus. Molecular size standards (in kilobases) are indicated at left. (*C*) RNA blot analysis was performed on total cellular RNA. Samples were derived from HepG2 (5 μg), the virus-producing cell line (1, 0.2, and 0.04 μg), mock-infected hepatocytes (5 μg), and hepatocytes infected with the LTR-LDLR virus (5, 1, and 0.2 μg). HepG2 cells were grown in medium containing lipoprotein-deficient serum prior to harvest for RNA.

Hepatocyte Isolation and Infection. Rabbit hepatocytes were prepared and plated in primary culture as described (5). Two days after the cultures were established, the medium was changed to virus-containing medium (supplemented with Polybrene at 8 μg/ml) that was harvested from the virus-producing cells (5). Mock-infected hepatocytes were exposed to the same type of medium supplemented with Polybrene but void of LDL receptor-expressing virus. Twelve to 18 hr later, the cells were harvested for transplantation and assayed for gene expression. Cells were detached from the tissue culture plates by incubation in 0.1% trypsin/EDTA for 10–20 min at 37°C.

Hepatocyte Transplantation. Animals were prepared for laparotomy with i.v. pentobarbital anesthesia. By using sterile surgical technique, the abdomen was opened through a 3-cm midline incision, a loop of jejunum was externalized, and a tributary of the superior mesenteric vein was identified and secured with ligatures. A prewarmed suspension of hepatocytes (1–2 × 10^8 cells in a volume of 10 ml containing 100 units of heparin) was introduced into the mesenteric vein through a 27-gauge needle over 5 min. The needle was then removed, hemostasis was achieved by tying the ligature, the loop of jejunum was returned to the abdomen, and the abdominal incision was closed in two layers. The animals received amoxicillin (5 mg/kg of body weight, subcutaneously) once daily on the day of surgery and for 5 days after the operation. Venous samples were subsequently obtained through a marginal ear vein and analyzed for total cholesterol as described (9).

Molecular Analysis of Gene Transfer and Expression. *Blot hybridization.* Total cellular RNAs and high molecular weight DNAs were prepared and analyzed by blot hybridization using human LDL receptor cDNA as the probe (5).

Polymerase chain reaction (PCR). Proviral sequences were detected in DNA isolated from liver tissue by using PCR (10). Thirty cycles of the reaction were performed with a program that included denaturation at 94°C for 1 min and annealing/elongation at 72°C for 2.5 min. Oligonucleotides used in this reaction have the following sequences: 5′ probe, AGGTCAGCTCCACAGCCGTAAGGACACAGC; 3′ probe, GGCTCGTACTCTATAGGCTTCAGCTGGTGA. Their location within the vector is illustrated in Fig. 1*A*.

RNase protection assay. RNA probes were generated from transcription plasmids constructed in the following manner. Moloney sequences in the retroviral vector between the synthetic *Bam*HI site at nucleotide 7674 and the *Nhe* I site at nucleotide 7846 (see ref. 11 for numbering) were cloned between the *Bam*HI and *Xba* I sites of pGEM-3Z (f+) (called 3Z-env); transcription from the SP6 promoter produces antisense RNA that is specific for the virus-directed transcript. This *Bam*HI–*Nhe* I fragment was also cloned into pGEM-4Z in order to produce sense RNA (4Z-env) from the same promoter. Antisense RNA spanning rabbit LDL receptor coding sequences was used as an internal control in RNase protection experiments. An *Sma* I–*Xho* I restriction fragment from a rabbit LDL receptor cDNA clone (nucleotides 2475–2572; ref. 12) was cloned between the *Sma* I and *Sal* I sites of pGEM-4Z (4Z-rLDLR). Transcription vectors were linearized by digestion with appropriate restriction endonucleases (3Z-env, *Eco*RI; 4Z-env, *Hin*dIII; and 4Z-rLDLR, *Hin*dIII) and used as templates in transcription reactions according to the recommendations of the manufacturer (Promega).

RNase protection studies were performed on total cellular RNA according to the method of Melton *et al.* (13). Radioactivity in the resulting bands was quantified with a Betascope 630 (Betagen, Waltham, MA).

In situ hybridization. In situ hybridization was performed by a modification of the method described by Pinter and Lugo (14). Linearized transcription probes were used as templates in *in vitro* transcription reactions using SP6 polymerase and uridine 5′-[α-[^{35}S]thio]triphosphate (Amersham). Radiolabeled antisense RNA probes were isolated and hybridized with cryostat sections at 45°C for 16 hr in the presence of 50% formamide. The slides were washed at high-stringency conditions, coated with Kodak emulsion NTB-2, and exposed for 4 weeks.

RESULTS

Retrovirus-Mediated Gene Transfer into Adult WHHL Hepatocytes. The recombinant virus LTR-LDLR was used in the

present studies to infect adult WHHL hepatocytes. After infection, hepatocytes were harvested for transplantation and analyzed for retrovirus transduction and human LDL receptor expression.

Southern blot analysis of total cellular DNA was used to directly measure the efficiency of gene transfer in the infected population of cells. These studies demonstrated unrearranged proviral sequences in infected hepatocytes with a relative copy number equal to 0.05–0.1 provirus per cell (Fig. 1*B*). Duplicate plates of hepatocytes were analyzed for virus-directed transcripts by using RNA transfer blots with a probe that is relatively specific for human LDL receptor RNA (Fig. 1*C*). This cDNA probe detected abundant human LDL receptor transcripts in infected WHHL hepatocytes as well as in the human hepatoblastoma cell line HepG2. The retrovirus-derived transcript is substantially shorter than the endogenous human transcript because the long 3′ untranslated region from the endogenous gene was deleted in the retroviral vector. The infected population of hepatocytes had levels of LDL receptor RNA that exceeded endogenous levels in the human hepatoblastoma cell line by 5- to 6-fold (Fig. 1*C*).

Transplantation of Genetically Modified Hepatocytes. Hepatocytes from a single WHHL rabbit were harvested, plated into culture, and mock infected or infected with the LTR-LDLR virus. After infection, the cells were harvested and transplanted into WHHL recipients by means of the portal vein (1–2 × 10^8 cells per animal, which is 1–4% of the total number of hepatocytes found in the liver). Animals that received LTR-LDLR-infected cells (three donors into seven recipients) demonstrated significant decreases in serum cholesterol on days 2–5 following transplantation ($P < 0.001$, Student's t test) when compared to pretreatment levels (Fig. 2). The peak effect was noted 3 days after transplantation and was associated with a decline in total serum cholesterol to 70 ± 3% of pretreatment values (mean ± 1 SD, $n = 7$). Transplantation of mock-infected WHHL hepatocytes (three donors into six recipients) had no statistically significant effect on serum cholesterol ($P > 0.6$, Student's t test) when compared to pretreatment levels (Fig. 2). A direct comparison between the control group and the experimental group using the Student's t test revealed statistically significant decreases in serum cholesterol on days 2–6 of the rabbits transplanted with genetically modified hepatocytes ($P < 0.001$). Decreases in lipoproteins that are known ligands for LDL receptor (i.e., very low density lipoproteins, intermediate density lipoproteins, and LDL) contribute to the overall diminution in total cholesterol (data not shown).

Tissues were harvested from transplant recipients in order to perform detailed molecular and cellular analyses of gene transfer and expression. Separate animals were euthanized for tissue collection at 10 min, 24 hr, and 19 days after transplantation with genetically modified hepatocytes.

Liver tissues were analyzed for proviral DNA sequences by using PCR (Fig. 3) because standard hybridization techniques lacked the necessary sensitivity. Proviral DNA sequences were detected in animals transplanted with infected hepatocytes and sacrificed 10 min and 24 hr after transplantation; proviral DNA was no longer detected in liver harvested 19 days after transplantation. Similar analysis of DNA from other tissues such as lung failed to detect proviral DNA, suggesting that most of the hepatocytes seed in sinusoids (data not shown).

RNase protection analysis was used to detect and quantify the recombinant transcript in total cellular RNA from liver tissue. The recombinant RNA was detected with an antisense probe that is complementary to viral sequences in its 3′ untranslated region (Fig. 1*A*). Another probe that is specific for endogenous rabbit LDL receptor RNA was incorporated into each assay as an internal control. Analysis of RNA from LTR-LDLR-transduced murine fibroblasts demonstrated protection of the 3Z-env probe but not the 4Z-rLDLR probe (172 base pairs) (Fig. 4, lane tRNA + VP), whereas assays of control WHHL RNA revealed no protection of the 3Z-env probe but substantial protection of the 4Z-rLDLR probe (98 base pairs) (Fig. 4, lane WC). Each probe produced a band whose intensity varied in proportion to the amount of total cellular RNA used in the initial hybridization. These studies confirm the specificities of the probes and support the use of the assay for quantifying viral and endogenous LDL receptor transcripts. Infected hepatocytes expressed the recombinant transcript at levels equal to the endogenous LDL receptor transcript (data not shown). Virus-directed RNA was also

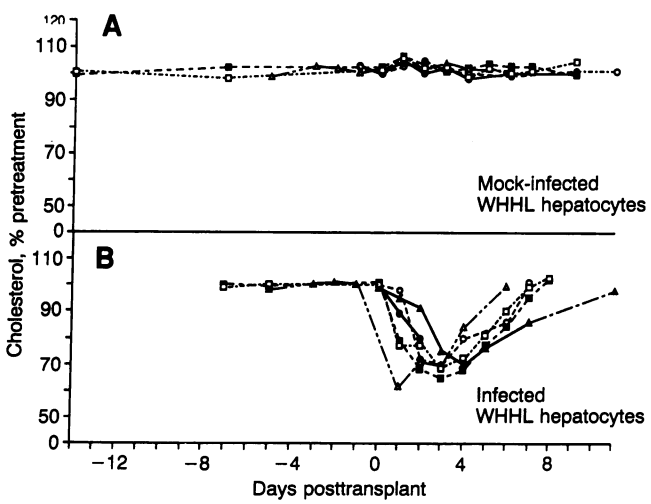

FIG. 2. Effect of hepatocyte transplantation on cholesterol metabolism in WHHL rabbits. Donor hepatocytes were transplanted into WHHL rabbits. Total serum cholesterol, presented as the % pretreatment level, was measured as a function of time following transplantation, which occurred on day 0. Data are plotted for each animal. (*A*) Mock-infected WHHL hepatocytes were transplanted into six WHHL recipients. (*B*) LTR-LDLR-infected WHHL hepatocytes were transplanted into seven recipients.

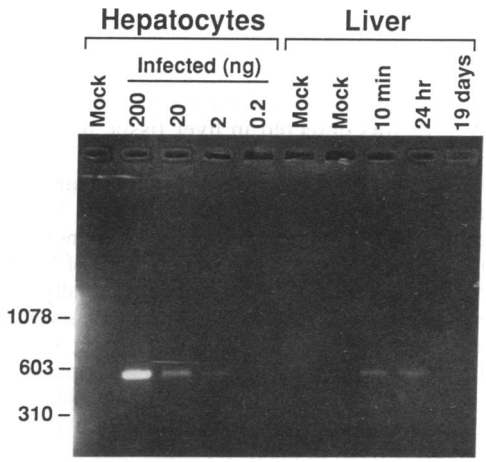

FIG. 3. Analysis of liver tissue for proviral DNA sequences by PCR. Total cellular DNA was isolated from hepatocytes and liver tissues and analyzed for proviral sequences using PCR. Hepatocyte DNA was from mock-infected (200 ng) and LTR-LDLR-infected (200, 20, 2, and 0.2 ng) hepatocytes. Liver DNA from each tissue was isolated on separate occasions with identical results; representative assays are presented. Samples were from control WHHL rabbits (Mock) and livers harvested from transplant recipients 10 min, 24 hr, and 19 days after the transplant. Sizes of the bands (in nucleotides) are indicated at left.

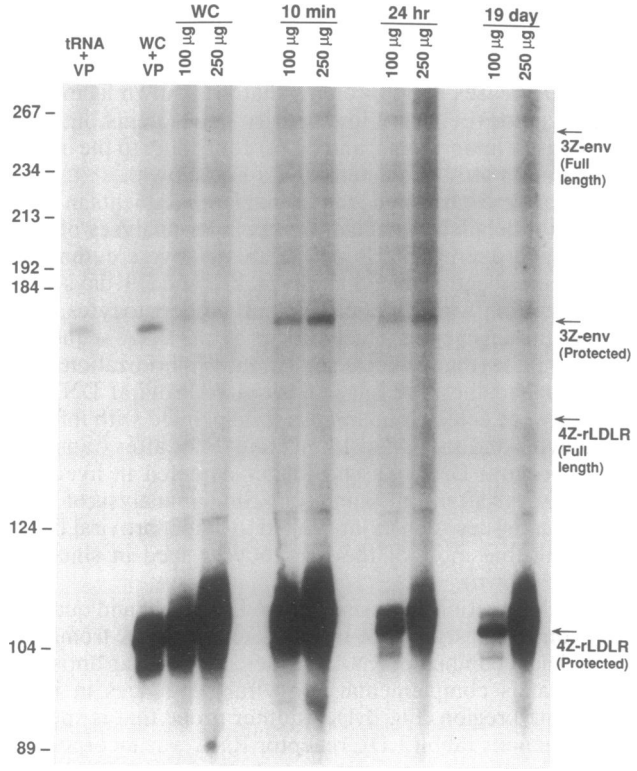

FIG. 4. RNase protection assays. Total cellular RNA samples were hybridized with ^{32}P-labeled RNA probes complementary to viral sequences (3Z-env) and endogenous LDL receptor sequences (4Z-rLDLR) and analyzed for protection from digestion with RNase A. The locations of the full-length and protected RNA probes are noted at right. Individual lanes are indicated at the top of the autoradiograph. tRNA (100 μg) was supplemented with 100 ng of viral producer (VP) RNA; RNA from the liver of a control WHHL (WC) rabbit (100 μg) was supplemented with viral producer RNA (100 ng); and liver RNA (100 μg and 250 μg) was obtained prior to transplantation (WC) as well as 10 min, 24 hr, and 19 days after transplantation with genetically modified hepatocytes. Molecular size markers (in base pairs) are indicated at left.

detected in liver tissue harvested 10 min and 24 hr after transplantation with genetically modified hepatocytes (Fig. 4). Quantitative analysis of this experiment indicated that the viral transcript was present at 1–3% of the level of endogenous LDL receptor mRNA (data not shown). No recombinant transcript was detected in liver tissue harvested at 19 days.

Recombinant gene expression was also characterized at the cellular level by *in situ* hybridization (Fig. 5). The RNA probe complementary to virus-specific sequences provided excellent specificity for this *in situ* detection of human LDL receptor mRNA. *In situ* hybridization was initially performed with the antisense probe on liver that was harvested 24 hr after transplantation of genetically modified hepatocytes. Approximately 1 in 500 to 1 in 1000 cells contained a high density of cytoplasmic grains (10- to 100-fold over background). These positive cells were distributed as single cells in the periportal areas of the liver, suggesting that the infused hepatocytes lodge in sinusoids soon after leaving the portal venule (Fig. 5 *A* and *B*). Hybridization signals were no longer detected in tissues that were analyzed 19 days after transplantation of genetically modified hepatocytes (data not shown). Specificity of this method is supported by the absence of hybridization over background when the antisense probe was hybridized with control WHHL liver (data not shown) or when the sense probe was hybridized with liver from a transplant recipient harvested 24 hr after transplantation (data not shown).

DISCUSSION

We have used an animal model of FH, the WHHL rabbit, to develop therapeutic approaches that involve the transfer of LDL receptor genes into hepatocytes. The strategy described in this report involves isolating hepatocytes from a WHHL rabbit, transducing a functional LDL receptor gene into these cells *in vitro*, and transplanting the genetically modified cells into another WHHL rabbit.

The availability of an authentic animal in combination with sensitive assays for *in vivo* gene expression provide the opportunity to critically evaluate the efficacy of this proposed therapy. The molecular basis and metabolic consequences of the WHHL mutation have been the subject of extensive investigation (4, 7, 15–20). An in-frame deletion in the LDL receptor gene leads to the expression of a dysfunctional receptor molecule that has virtually no detectable activity (15–21). Exposure of WHHL hepatocytes to the recombinant virus produced a mixed population of cells in which 10–20% of the hepatocytes expressed levels of human LDL receptor mRNA (Fig. 1) and protein (5) that exceeded normal endogenous levels by 5- to 10-fold; net expression of human LDL receptor in these cultures was equal to the normal level. Gene replacement was achieved by transplanting a small number of this mixed population of genetically modified WHHL hepatocytes (≈2% of the total hepatocytes in an adult rabbit liver). Functional replacement of LDL receptor activity therefore should be ≈2% of normal in transplant recipients. This prediction is in excellent agreement with quantitative analyses of RNA from liver harvested 10 min and 24 hr after transplantation, which detected the recombinant transcript at 1–3% of the level of the endogenous transcript. This rather modest amount of genetic correction, however, led to a substantial improvement in hypercholesterolemia to 70% of pretreatment levels. This finding is consistent with previous studies of homozygous deficient patients in which the level of residual LDL receptor activity was shown to directly correlate with serum cholesterol levels and progression of coronary artery disease (1, 6). For example, receptor-negative patients (<2% of control receptor activity) have more severe coronary disease and are less responsive to conventional therapies than receptor-defective patients (>2% control residual receptor activity). This also suggests that functionally converting a patient from receptor-negative to receptor-defective status may be metabolically and clinically efficacious.

Metabolic improvements associated with hepatocyte transplantation, however, were not permanent. There are two potential explanations for the apparent deterioration in LDL receptor function *in vivo*. There could be destruction and actual loss of the genetically modified hepatocytes. Alternatively, the cells may engraft, but expression from the recombinant gene may extinguish. In an attempt to differentiate between these possible mechanisms, the integrated provirus was used as a specific and sensitive marker of the genetically modified cells *in vivo*. Proviral DNA was detected in liver of transplant recipients prior to and during the period of metabolic improvement. The provirus was no longer detected in liver tissue after the total cholesterol returned to baseline. This suggests that cell loss is an important factor in the deterioration of *in vivo* LDL receptor function. Specific mechanisms responsible for the loss of hepatocyte engraftment remain unclear. It is possible that graft rejection due to major histocompatibility complex incompatibility of allogeneic cells may contribute to the disappearance of genetically modified cells. Alternative explanations include toxicity due to constitutive overexpression of LDL receptor or graft rejection based on an immunologic response to the human LDL receptor protein.

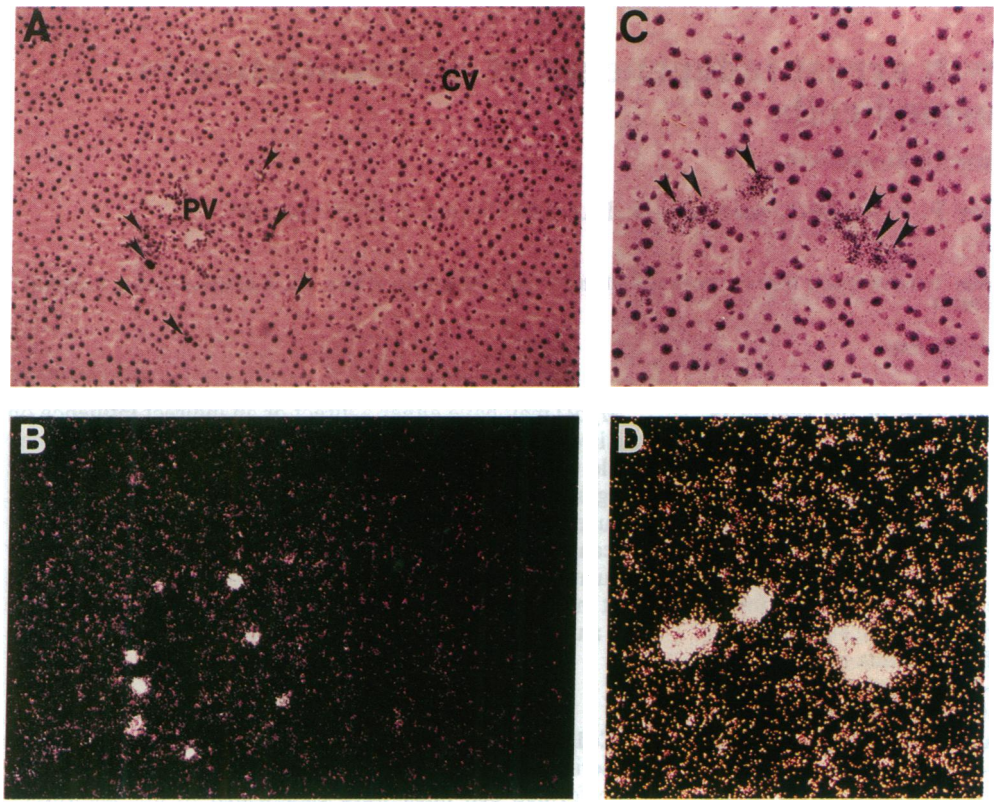

FIG. 5. *In situ* hybridization of liver from transplant recipients. Cryostat sections of liver were hybridized with the virus-specific RNA probe. These tissues were derived from a WHHL rabbit 24 hr after transplantation with LTR-LDLR-transduced hepatocytes. Slides were counter stained with hematoxylin. Bright-field (*A*) and dark-field (*B*) views of an area demonstrating periportal distribution of transplanted cells are shown. PV, portal vessels and a probable biliary ductule; CV, central vein. Cells that are clearly positive in the dark field are indicated by arrowheads in the bright field. Higher power magnification of transplanted hepatocytes visualized on a bright field (*C*) and a dark field (*D*) are shown. (*A* and *B*, ×40; *C* and *D*, ×140.)

In summary, we have used an animal model of FH, the WHHL rabbit, to develop a therapeutic approach in which a normal LDL receptor gene is introduced into genetically deficient hepatocytes, which are transplanted into recipient animals. This intervention resulted in a temporary amelioration of the profound hypercholesterolemia that is characteristic of this disorder. More complete and long-term normalization of the hyperlipidemia could potentially be accomplished with greater expression of the transgene in autologous hepatocytes.

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