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Adenovirus-Mediated Correction of the Genetic Defect in Hepatocytes from Patients with Familial Hypercholesterolemia

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Abstract—*Familial hypercholesterolemia (FH) is an inherited deficiency of LDL receptors that has been an important model for liver-directed gene therapy. We are developing approaches for treating FH that are based on direct delivery of recombinant LDL receptor genes to liver in vivo. As a first step towards this goal, replication-defective recombinant adenoviruses were constructed which contained either the* lacZ *gene or the human LDL receptor cDNA expressed from a β-actin promoter. Primary cultures of hepatocytes were established from two patients with homozygous FH and one nonFH patient, and subsequently exposed to recombinant adenoviruses at MOIs ranging from 0.1 to 5. Essentially all of the cells expressed high levels of the transgene without demonstrable expression of an early or late adenoviral gene product; the level of recombinant-derived LDL receptor protein in transduced FH hepatocytes exceeded the endogenous levels by at least 20-fold. These studies support the utility of recombinant adenoviruses for efficient transduction of recombinant LDL receptor genes into human FH hepatocytes without expression of viral proteins.*

INTRODUCTION

Familial hypercholesterolemia (FH) is an autosomal dominant disease caused by defects in the gene encoding LDL receptor (1). Patients who inherit two abnormal LDL receptor alleles provide an excellent model for the early development of gene replacement therapies. Homozygous FH is truly a lethal disease with patients developing life-threatening coronary heart disease (CHD) in childhood due to massive accumulations of LDL that are refractory to traditional pharmacological therapies. Metabolic correction has been achieved in FH homozygotes by orthotopic liver transplantation, indicating that selective correction of LDL receptor expression in hepatocytes by liver-directed gene therapy should be sufficient for improvement in hypercholesterolemia (2–4). The observation that FH homozygotes with residual LDL receptor activity have a better prognosis than those with null genotypes suggests that partial genetic correction may be therapeutic (1). Finally, the availability of an authentic animal model for FH, the Watanabe heritable hyperlipidemic (WHHL) rabbit, greatly facilitates the evaluation of new genetic therapies (5–7).

The early paradigm for gene therapy of FH was based on transplantation of autologous hepatocytes transduced with recombi-

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nant retroviruses ex vivo. The feasibility of this model of liver-directed gene therapy has been demonstrated in the WHHL rabbit (8); animals treated with ex vivo gene therapy realized a 25–40% diminution in serum cholesterol that lasted for the duration of the experiment (four months). Based on additional safety experiments in dogs (9) and baboons (10), a clinical protocol for the treatment of symptomatic FH homozygotes by ex vivo liver-directed gene therapy was designed and initiated. This approach, however, has several inherent limitations. The therapy requires a minimum of one major surgical procedure with the attendant risks. Furthermore, the efficacy is necessarily limited by the number of hepatocytes that can be harvested, manipulated ex vivo, and transplanted, which has never been greater than 5% of the total cells in liver (8). Finally, the complexity of the protocol severely restricts its application.

A more practical and potentially effective approach to gene therapy of FH is direct delivery of the gene to hepatocytes in vivo. This has been accomplished in the WHHL rabbit by infusion of synthetic molecular conjugates that are tropic for hepatocytes (11). However, the level of recombinant gene expression achieved in recipient animals was low and the effect was transient. Another potential approach to in vivo liver-directed gene therapy utilizes replication defective recombinant adenoviruses (12). Important advantages of adenoviruses for gene transfer to the liver include: (1) virus is easily purified at high concentrations (i.e., approximately 10^{13} particles/ml), and (2) gene transfer can be accomplished in nondividing cells. The utility of recombinant adenoviruses for in vivo gene therapy was first demonstrated in a mouse model of the liver metabolic disease caused by ornithine transcarbamylase deficiency (13). A similar approach has been used to deliver recombinant reporter genes to rat liver (14) and a human LDL receptor gene to mouse liver (15).

We describe in this report an important step toward the development of in vivo gene therapy of FH with recombinant adenoviruses. Primary cultures of hepatocytes from patients with homozygous FH were corrected without apparent toxicity by single exposure to low concentrations of recombinant virus.

MATERIALS AND METHODS

Recombinant Adenoviruses. The plasmids used to generate the recombinant, E1-deleted adenoviruses Ad.CB*lacZ* and Ad.CB*hLDLR* were constructed as follows. The plasmid CMVβA*lacZ* (10) was digested with SnaBI and NheI, and gagβAhLDLR (16) was digested with NheI and then partially digested with XhoI to isolate a fragments containing the β-actin promoter and either the *lacZ* gene or the human LDL receptor cDNA. These fragments were blunt-ended with Klenow. The plasmid pAdCMV-*lacZ* (17) was digested with SnaBI and NotI to remove the CMV promoter and *lacZ* gene (retaining the CMV enhancer), blunt-ended with Klenow, and ligated with inserts containing the β-actin promoter fused to either the *lacZ* or *LDLR* genes. The resulting vectors were designated pAdCB*lacZ* and pAd-CB*hLDLR*, respectively.

Plasmids were linearized with NheI and cotransfected into 293 cells with wild-type adenoviral DNA [strain sub360 (18), which contains a partial E3 deletion] that had been digested with XbaI and ClaI to remove the 5′ ITR. Recombinant adenoviruses were isolated following transfection (19), subjected to two rounds of plaque purification, and lysates were purified by cesium chloride centrifugation (20). The viral stocks were evaluated for titer by limiting dilution plaque assay on 293 cells and stored at −20°C after diluting fourfold with 10 mM Tris Cl, pH 8.1, 100 mM NaCl, 0.1% bovine serum albumin, and 50% glycerol. Titers of the glycerol stocks were: Ad.CB*lacZ*, 2.4×10^9 plaque-

forming units (PFU)/ml; Ad.CB*hLDLR*, 4×10^9 PFU/ml; wild-type Ad, 8×10^9 PFU/ml.

Isolation and Propagation of Hepatocytes. Hepatocytes were isolated from liver tissue by in situ perfusion of collagenase as described (21). Cells were plated at a density of 4×10^6 cells/10 cm^2 plate in Hormonally defined medium (HDM) supplemented with 10% FCS overnight and subsequently maintained in HDM alone.

Electron Microscopy. Plates of hepatocytes were fixed 24, 48, and 72 h after plating in 1.6% formaldehyde/2% gluteraldehyde in 0.1 M cacodylate buffer for 1 h at 4°C, then washed in 0.1 M cacodylate, and postfixed in 1.5% osmium tetroxide in 0.1 M cacodylate buffer for 30 min at 4°C. Samples were processed, stained, and analyzed by transmission electron microscopy as described (22).

Hepatocyte Infection. NonFH and FH hepatocytes plated in HDM (without serum) were infected with purified recombinant adenovirus [multiplicity of infection (MOI) = 0.1, 1, or 10] one to three days after plating; an estimate of cell number, based on inspection of the plate, was used to calculate the MOI. Two days following infection the cells were analyzed for *lacZ* or LDL receptor expression as described below.

Cytochemical Analyses. Plates of hepatocytes were analyzed for β-galactosidase expression using the X-gal histochemical stain (23) and for LDL receptor activity using an in situ activity assay based on the uptake of fluorescent labeled ligand. The LDL receptor assay was performed as follows. Hepatocytes were incubated with DMEM supplemented with 10% calf lipoprotein-deficient serum and 10 μg/ml of LDL labeled with the fluorescent probe 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchloridate (diI-LDL; gift of Bruce Auerbach, Parke-Davis, Ann Arbor, Michigan). Following a 4-h incubation, cells were rinsed with DMEM and diI-LDL uptake was visualized by fluorescent microscopy as described (16).

Expression of the adenoviral genes encoding DNA binding protein (DBP) and fiber protein was evaluated using immunocytochemical techniques with antibodies to the respective protein products, anti-DBP, a gift of A. Levine (Princeton University, Princeton, New Jersey), and anti-fiber (805F), from Chemicon International, Inc. (Temecula, California). The 293 cells plated on cover slips, infected with recombinant adenovirus (MOI = 1), and harvested 16 h postinfection were used as a positive control for early and late adenoviral gene expression. Hepatocytes were plated in 60-mm tissue culture dishes and infected the following day with wild-type adenovirus or with Ad.CB*lacZ* at a MOI of 1. The cells were harvested 18 h later by scraping with a rubber policeman, gently pelleted, suspended in OCT, and quick-frozen. Cells on cover slips (293 cells) or fresh-frozen sections (hepatocytes) were fixed by immersion in methanol at −20°C for 10 min and stored at −80°C. Samples were rehydrated in PBS and incubated serially with PBS containing 10% normal goat serum, primary antibodies diluted 1:20 (anti-DBP) or 1:10 (anti-fiber) in PBS/10% goat serum (30 min), and peroxidase-conjugated goat anti-mouse Ig (Jackson Immunoresearch, West Grove, Pennsylvania) diluted 1:200 in PBS/10% goat serum (60 min). Samples were washed in PBS, incubated with the AEC color reagent for 4 min, and stained in hematoxylin for 1 min.

Western Blot Analysis. Hepatocytes were rinsed with PBS, scraped from the dish using a rubber policeman, and pelleted cells were stored at −70°C. Cells were solubilized by suspension in buffer containing 20 mM Tris Cl, pH 8, 1 mM $CaCl_2$, 0.15 M NaCl, 1% Triton X-100, and a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml leupeptin, 0.2 mg/ml soybean trypsin inhibitor, 0.1 mg/ml chymostatin, and 0.1 mg/ml aprotinin). Following a 1-h incubation on ice, insoluble material was removed by centrifugation at 4°C, and the protein concentration of the supernatant

was determined using the BioRad DC kit. Equal amounts of protein (68 μg in FH2 experiment and 90 μg in FH3 experiment) were loaded in each lane of an SDS–6% polyacrylamide gel in the absence of boiling or reducing agents. Purified β-galactosidase (Sigma Chemical Co., St. Louis, Missouri) was included as a positive control, and for comparison, 100 μg (in FH2 experiment) or 200 μg (FH3) of an extract prepared from normal human liver as previously described (24) was also included. The gel was transferred to nitrocellulose and the filters probed (24). The antibodies used were a monoclonal anti-β-galactosidase antibody (IgG1) from Sigma, diluted 1:500, and a monoclonal anti-human LDLR antibody (IgG1) from Amersham (Arlington Heights, Illinois), diluted to 1 μg/ml. Following incubation with a peroxidase-conjugated goat anti-mouse IgG antibody diluted 1:1000, the filters were washed, then incubated with ECL (Amersham) according to the manufacturer's directions, and exposed to Kodak XAR-5 X-ray film.

RESULTS AND DISCUSSION

The design of safe and efficient methods for delivering recombinant genes to hepatocytes in vivo would greatly enhance the utility of liver-directed gene therapy. Previous attempts using liposomes (25) or DNA/protein complexes (11, 26) resulted in levels of expression that were inadequate for therapeutic effects. Direct infusion of recombinant retroviruses into the portal vein after partial hepatectomy is associated with gene transfer in only a few percent of hepatocytes (27). Experiments with rats and mice indicate that infusion of recombinant adenoviruses in the absence of partial hepatectomy or injury to the liver is associated with high-level transgene expression in the majority of hepatocytes (13–15, and unpublished data). In this report, we explore the feasibility of using recombinant adenoviruses to correct a genetic deficiency of LDL receptor in primary hepatocytes of patients with FH.

Hepatocytes were isolated from liver tissue of a normal donor (Z8) and two FH patients (FH2 and FH3). A summary of the salient features of the patients and the cell isolations is provided in Table 1. A portion of a donor liver from a nonFH patient that was surgically reduced prior to transplantation was used as a source of normal hepatocytes. Hepatocytes from two FH patients (designated FH2 and FH3) were isolated from freshly resected liver that was used for ex vivo gene therapy; additional plates of cells not suitable for transplantation were used in these experiments. The recovery of hepatocytes from the collagenase perfusions ranged from 4.4 to 20 × 10^6 cells/g wet weight of tissue with a viability of 92–93%. Cells were maintained in culture for four days. Representative electron photomicrographs of cells from FH3 are presented in Fig. 1. Greater than 95% of the cells retained ultrastructural features specific to hepatocytes for the duration of the experiment, confirming the purity of the preparation and the stability of the cultures in terms of hepatocyte differentiation. This experience with hepatocytes from FH patients is consistent with our previous studies with liver from four nonFH patients (21).

Table 1. Summary of Hepatocyte Isolations

				Cell isolation		
Patient	Age	Sex	Clinical status	Liver mass (g)	Cell (*N*)	Viability (%)
Z8	37	M	NonFH donor	156	0.7×10^9	92
FH2	12	M	FH	230	4.6×10^9	92
FH3	7	F	FH	187	2.9×10^9	93

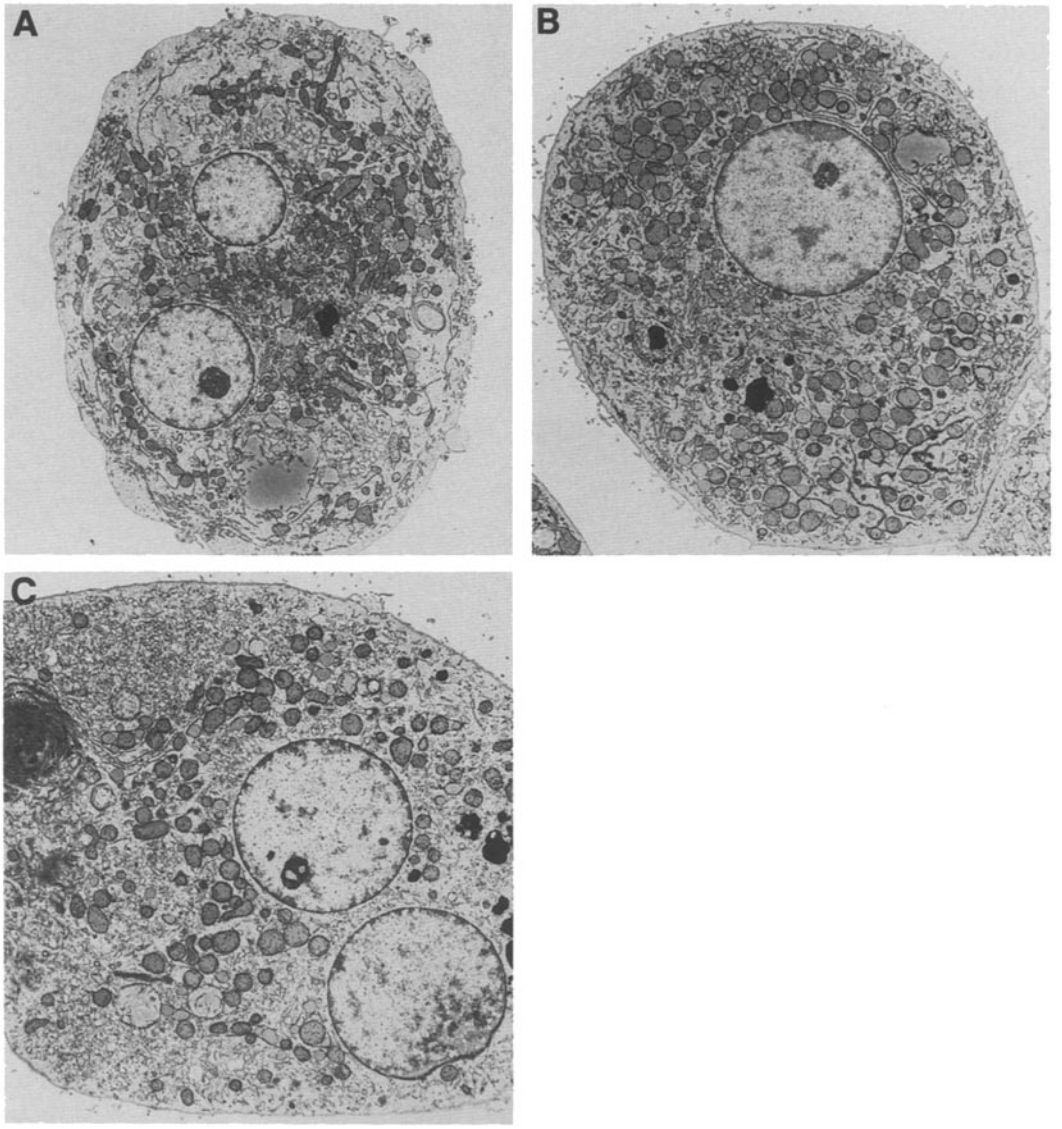

Fig. 1. Electron micrographs of cultured FH hepatocytes. Cultures of hepatocytes from FH3 were harvested one (panel A), two (panel B), and three (panel C) days after plating, fixed, and analyzed by transmission electron microscopy. Photomicrographs of representative cells are presented.

Conditions required for efficient adenovirus-mediated gene transfer were established with normal hepatocytes using the Ad.CB*lacZ* virus. Purified virus was diluted into HDM and added directly to the cultured hepatocytes 24–72 h after plating; cells were harvested for evaluation of transgene expression 24 h later. Figure 2 presents X-gal histochemistry of normal hepatocytes that were mock infected (panel A) or infected with Ad.CB*lacZ* at an MOI of 0.1 (panel B) and 1.0 (panel C). The efficiency of gene transfer was directly proportional to the concentration of virus added to the medium with essentially 100% of the cells expressing high levels of *lacZ* when cells were exposed

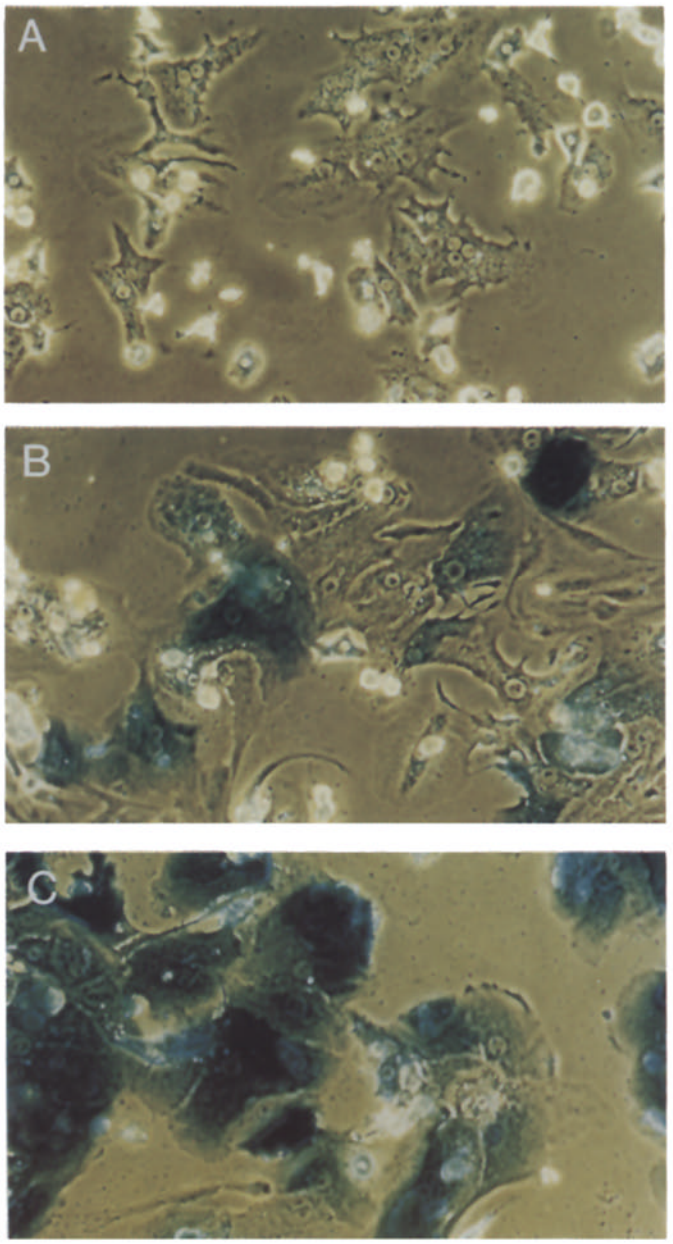

Fig. 2. X-gal histochemistry of infected hepatocytes. Normal human hepatocytes were infected two days after plating with Ad.CB*hLDLR* at a MOI of 1 (A), or with Ad.CB*lacZ* at a MOI of 0.1 (B) or 1 (C), and stained one day postinfection with X-gal.

to virus at an MOI of 1 without apparent cytopathic effects. Additional experiments have demonstrated that cytopathology does not occur until the MOI is equal to or greater than 50 (data not shown).

Hepatocytes from the two FH patients were exposed to Ad.CB*lacZ* and Ad.CB*hLDLR* at MOIs equal to 1 and 10, and whole cell lysates were analyzed for β-galactosidase and LDL receptor by Western blot analysis

(Fig. 3). Analysis with antibody to β-galactosidase revealed a predominant band at 116 kDa that was specific to lysates from Ad.CMV*lacZ*-infected cells and comigrated with purified β-galactosidase enzyme. Analysis with a monoclonal antibody to human LDL receptor revealed a 130-kDa band in lysates from liver and cultured hepatocytes of a normal donor. This was not present in cultured hepatocytes from either FH patient, indicating their mutations are associated with quantitative defects in LDL receptor expression. In contrast, extremely high quantities of LDL receptor protein were detected in lysates of FH hepatocytes exposed to the Ad.CB*hLDLR* virus; the level of recombinant LDL receptor protein in cells infected at an MOI of 10 exceeded the levels in nonFH liver or hepatocytes by at least 20-fold.

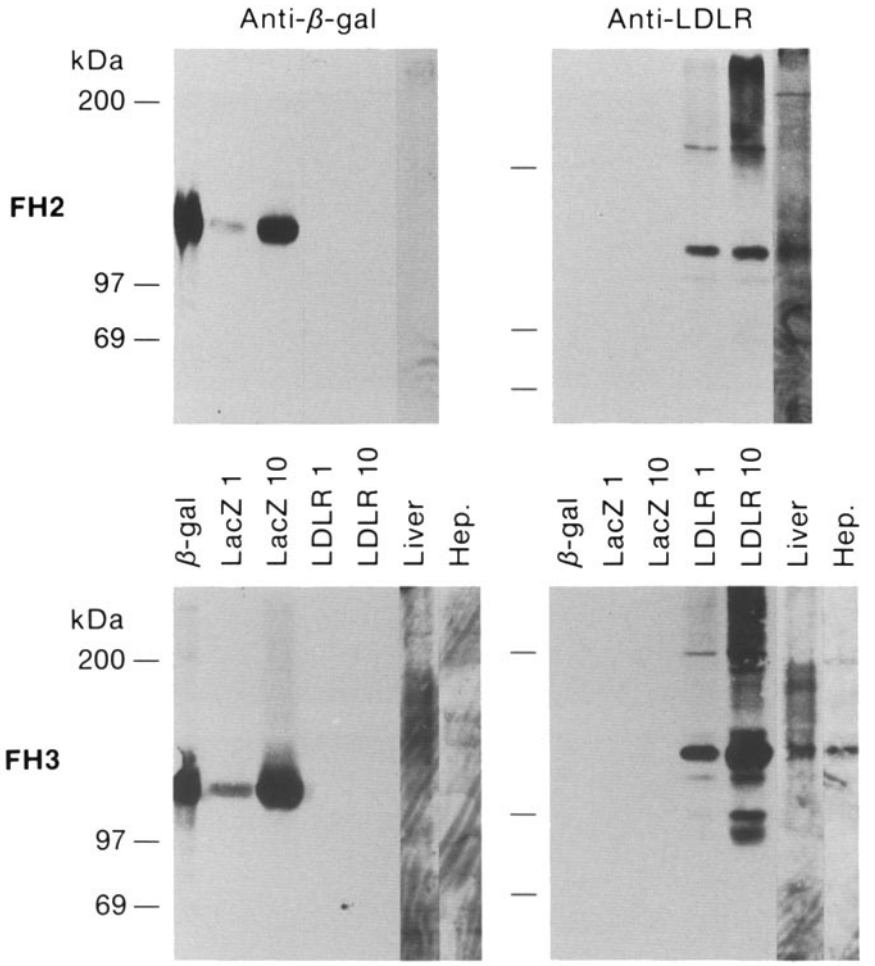

Fig. 3. Western blot analysis of infected hepatocytes. Lysates were prepared from infected cells and were subjected to SDS-PAGE followed by transfer to nitrocellulose. Filters were incubated with either an anti-β-galactosidase monoclonal antibody (left panels) or with an anti-human LDL receptor monoclonal antibody (right panels). β-gal, purified β-galactosidase; *lacZ* 1 and *lacZ* 10, FH hepatocytes infected with Ad.CB*lacZ* at MOIs of 1 and 10, respectively; LDLR 1 and LDLR 10, FH hepatocytes infected with Ad.CB*hLDLR* at MOIs of 1 and 10, respectively; Hep, normal human hepatocytes; liver, extract prepared from normal human liver. In each experiment, lanes derived from normal hepatocytes and liver were from the same blot, but exposed three times longer than lanes derived from FH hepatocytes.

FH hepatocytes were analyzed for functional LDL receptor activity using an in situ assay that is based on the receptor-mediated uptake of fluorescent-labeled LDL. Figure 4 presents fluorescent micrographs of FH3 hepatocytes that were infected with Ad.CB*lacZ* at an MOI of 10 (panels A and B) and Ad.CB*hLDLR* at an MOI of 1 (panels C and D) and 10 (panels E and F). No fluorescence was detected in the Ad.CMV*lacZ*-infected cells, with 75% of the cells demonstrating high levels of LDL uptake after exposure to Ad.CB*hLDLR* virus at an MOI equal to 10.

The recombinant adenoviruses used in this study were disabled by deleting E1a and E1b, which are normally required to activate the expression of other viral genes. It is possible, however, that this block in activation can be overcome by cellular factors or by exposing the cells to high MOIs of replication defective adenovirus. Experiments in human bronchial xenografts using the same viruses have demonstrated a block in transi-

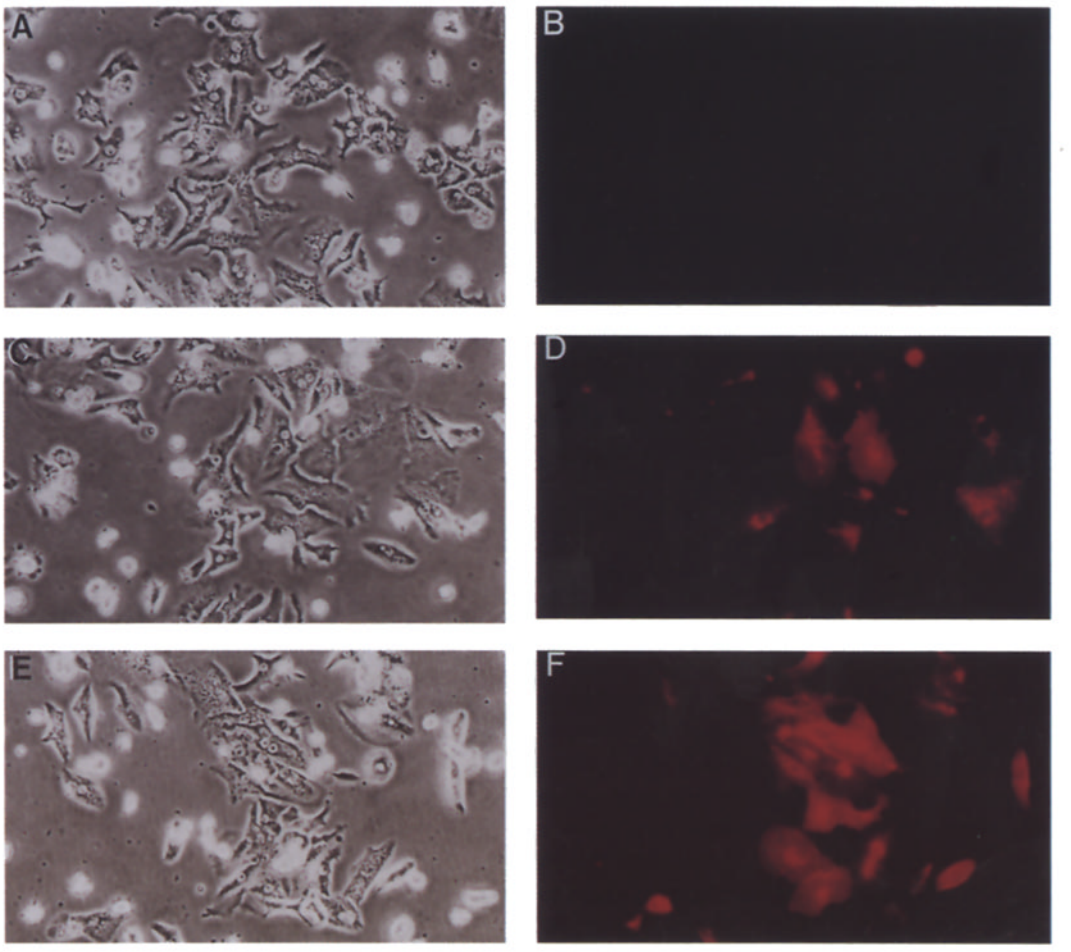

Fig. 4. LDL uptake assay of infected FH hepatocytes. FH hepatocytes were infected two days after plating with Ad.CB*lacZ* at a MOI of 10 (A, B), or with Ad.CB*hLDLR* at a MOI of 1 (C, D) or 10 (E, F). Twenty-four hours after infection, cells were incubated with medium containing 10% calf lipoprotein-deficient serum and 10 μg/ml dil-LDL for 4 h. Panels A, C, and E are phase-contrast pictures, and panels B, D, and F are the corresponding fields viewed using fluorescence optics.

tion from early to late transcriptional units with high level expression of the early gene E2a (20). Similar techniques of immunocytochemistry were used to study the expression of adenoviral proteins in human hepatocytes exposed to adenoviruses. Expression of the early gene E2a and the late gene L5 was evaluated using antibodies to the corresponding gene products, the 72-kDa DNA binding protein and the fiber protein, respectively (Fig. 5). In each case, cells were harvested for analysis 16–18 h after exposure to virus. The 293 cells exposed to Ad.CMV*lacZ* (which replicates due to 293 cells providing the E1 gene products in *trans*) demonstrated DBP and fiber protein in virtually all cells (Fig. 5A–C); panel B shows punctate nuclear staining characteristic of DBP and panel C shows dark cytoplasmic staining characteristic of fiber. Human hepatocytes infected with wild-type Ad5 demonstrated high levels of DBP and trace levels of fiber in subpopulations of cells (Fig. 5D–F); no viral proteins were detected in human hepatocytes infected with the recombinant virus (Fig. 5G–I).

In summary, we show that recombinant

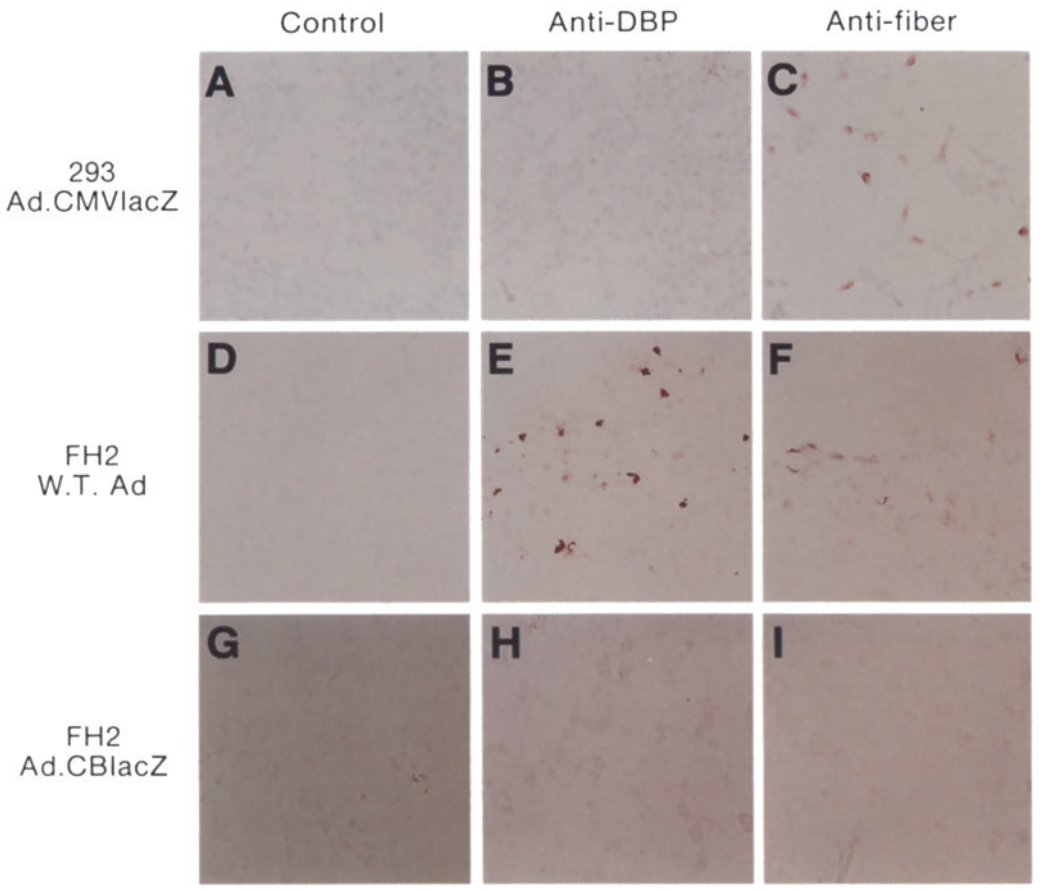

Fig. 5. Immunocytochemical analysis of transduced hepatocytes for viral protein expression. The 293 cells infected with Ad.CMV*lacZ* [a recombinant adenovirus in which *lacZ* is expressed from the CMV promoter (17), which replicates in 293 cells] at an MOI of 1 (panels A–C) and FH2 hepatocytes infected with either wild-type adenovirus (Ad5; panels D–F) or with the recombinant Ad.CB*lacZ* (panels G–I) at an MOI of 1 were harvested 16–18 h after infection. Samples were stained with no primary antibody (control; panels A, D, and G), or with antibodies recognizing the adenoviral gene products, the 72-kDa DNA binding protein (DBP; panels B, E, and H) or the fiber protein (panels C, F, and I).

adenoviruses based on human Ad5 are capable of very efficiently transducing genes into human hepatocytes without apparent cytopathic effects or expression of adenoviral proteins. This approach was used to fully correct the genetic defect in hepatocytes isolated from patients with FH. The encouraging results in human hepatocytes along with previous studies in rodents suggest that recombinant adenoviruses may be useful in the development of in vivo liver-directed gene therapies in humans. However, several important issues remain regarding the utility of recombinant adenoviruses for gene therapy of FH. The effects of high-level constitutive expression of LDL receptor and its ability to catabolize LDL have to be addressed. In addition, it will be necessary to develop strategies for achieving prolonged transgene expression in vivo.

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