Effective treatment of familial hypercholesterolaemia in the mouse model using adenovirus-mediated transfer of the VLDL receptor gene

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Liver directed gene transfer with adenoviral vectors is being considered for the treatment of several metabolic diseases, including familial hypercholesterolaemia (FH). Gene replacement therapy of human low density lipoprotein (LDL) receptor gene into the murine model of FH transiently corrected the dyslipidaemia; however, humoral and cellular immune responses to LDL receptor developed — possibly contributing to the associated hepatitis and extinguishing of transgene expression. We evaluated an alternative strategy of ectopic expression in the liver of the very low density lipoprotein (VLDL) receptor, which is homologous to the LDL receptor but has a different pattern of expression. Infusion of recombinant adenoviruses containing the VLDL receptor gene corrected the dyslipidaemia in the FH mouse and circumvented immune responses to the transgene leading to a more prolonged metabolic correction.

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Familial hypercholesterolaemia (FH) is an autosomal dominant disorder caused by defects in the low density lipoprotein (LDL) receptor gene[1], which leads to elevated serum LDL. Homozygotes, with two mutant LDL receptor genes, have massive elevations in serum LDL that cause premature coronary heart disease, frequently leading to death by myocardial infarction before the age of 20. Current treatments for FH include removal of LDL by apheresis, and orthotopic liver transplantation, which restores a normal complement of hepatic LDL receptors[1]. The first form of gene therapy developed for this disorder was an *ex vivo* approach using recombinant retroviruses in animal models[2,3] and in humans[4,5]. Recombinant adenoviruses have been used *in vivo* as a vector for liver directed gene replacement therapy in animals. The *in vivo* approach has been effective in lowering plasma cholesterol levels in rabbit[6,7] and mouse models[8] of FH; however, expression of adenovirus-encoded LDL receptor has only been transient.

Immune responses of mice curtail the duration of transgene expression in animals treated with recombinant adenoviruses. Administration of recombinant adenovirus to the livers or lungs of immunodeficient mice results in long-term transgene expression[9–11]. Experiments in animals deficient in T cell subsets along with adoptive transfer techniques have shown that the loss of transgene expression is largely due to the effects of $CD8^+$ T cells, a subset of which are cytolytic T lymphocytes (CTLs). This is partly the result of an immune response to adenovirus proteins, which are expressed in mice at a low levels, despite deletions in the E1 region of the adenovirus genome[12,13]. Whereas the transient nature of expression of a transgene using recombinant adenovirus in an immunocompetent animal renders it ineffective for long-term *in vivo* gene replacement, the duration of expression is sufficient to determine the effects of a particular transgene on metabolism. Thus, genes in addition to the LDL receptor can be examined to determine their *in vivo* functions, as well as their effectiveness in treating hypercholesterolaemia.

We considered transfer of the gene encoding very low density lipoprotein (VLDL) receptor by recombinant adenovirus as a possible therapeutic intervention in FH and other dyslipidaemias, as this receptor mediates uptake of VLDL, the precursor to LDL. VLDL receptor cDNAs from rabbits[14], humans[15], and mice[16,17] have been cloned. This receptor is homologous to the LDL receptor, but has a different pattern of expression as well as a different ligand specificity. The VLDL receptor is expressed in peripheral tissues including heart and skeletal muscle, adipose tissue, kidney, and brain, but not in the liver, which is the major site of LDL receptor expression. Mice genetically deficient in VLDL receptor have normal serum lipids, suggesting that the endogenous gene product does not significantly contribute to systemic lipoprotein metabolism or that the germline interruption has induced expression of a compensatory gene[18]. It has been suggested that VLDL receptor binds to apolipoprotein E which is found in VLDL and intermediate density lipoprotein (IDL), but not in LDL, although this has not been conclusively shown[19]. In contrast, the LDL receptor binds apoB100 (contained in VLDL, IDL, and LDL) and also apoE.

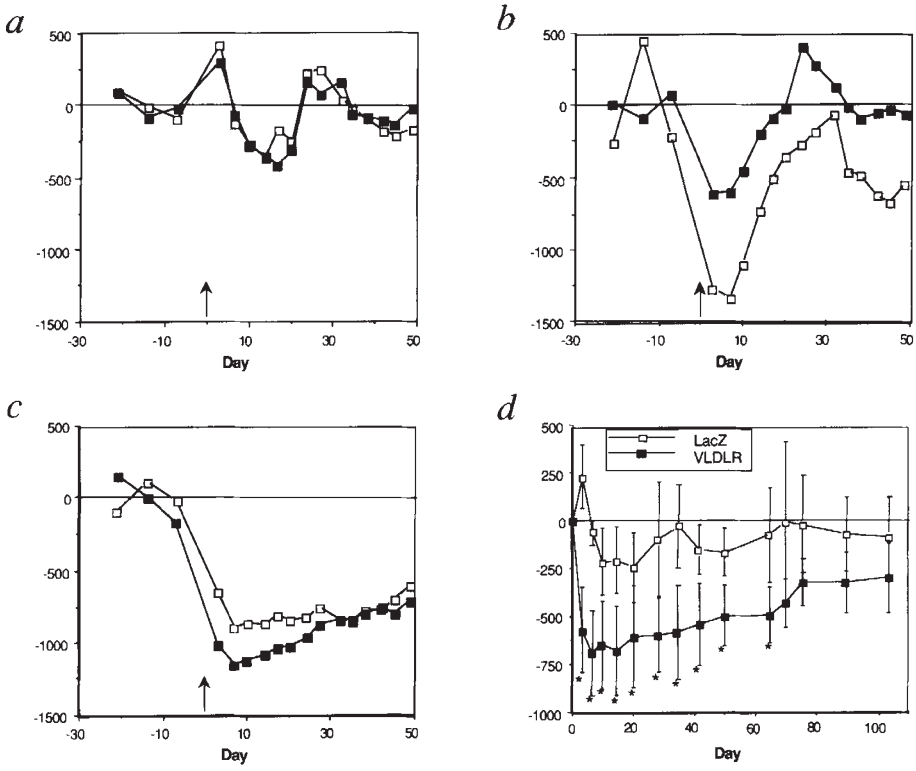

Fig. 1 Plasma cholesterol levels in LDL receptor knockout mice following infusion of recombinant adenovirus. In *a*, *b* and *c*, each line represents results from individual mice in a single experiment. Mice were infused with H5.010CMV*lacZ* (*a*), H5.010CB*hLDLR* (*b*), or H5.010CMV*VLDLR* (*c*). Pre-infusion plasma cholesterol levels were (*a*) 934 and 973 mg/dl, (*b*) 1554 and 966 mg/dl, and (*c*) 1186 and 1453 mg/dl for open and filled squares, respectively. *d*, Average results ± standard deviation from two separate experiments for mice infused with H5.010CMV*lacZ* ($n = 9$) or with H5.010CMV*VLDLR* ($n = 10$). Average pre-infusion cholesterol levels were 870 mg/dl and 946 mg/dl, respectively. Asterisks indicate $P < 0.05$.

The mouse model of FH, deficient in LDL receptor by a germline interruption of the gene[8], was used in this study to investigate the host responses to gene replacement therapy with recombinant adenoviruses that may limit the utility of the treatment. Gene replacement therapy with the LDL receptor was compared and contrasted to the alternative therapy, of ectopic expression of the VLDL receptor in the liver. We analysed the efficacy of each therapy, the duration of transgene expression, and the resulting host immune responses.

Gene transfer of the LDL receptor

Gene replacement therapy using recombinant adenoviruses to reconstitute LDL receptor expression in liver has been evaluated in mouse[8] and rabbit[6,7] models of FH. Expression of the recombinant LDL receptor gene and its associated effect on serum lipids was transient in the rabbit model lasting less than 3 weeks; the mouse model was analysed only at early time points. Recombinant adenoviruses used in the rabbit study were examined in the mouse model of FH to facilitate a more detailed analysis of host responses to therapy. Mice were infused intravenously with 1×10^{11} particles of E1 deleted recombinant adenoviruses encoding either *lacZ* (H5.010CMV*lacZ*) or human LDL receptor (H5.010CB*hLDLR*), and serum lipids were followed over time (Figs 1, 2).

Mice infused with *lacZ* virus demonstrated a characteristic but not significant fluctuation in serum cholesterol (Fig. 1*a*) reflected in minor changes of all lipoprotein fractions (Fig. 2, first column). A summary of the results of nine animals treated with *lacZ* virus is shown in Fig. 1*d*. Mice infused with the LDL receptor virus demonstrated large decreases in plasma cholesterol which lasted for approximately 2 weeks. Cholesterol levels decreased 3-fold (from 966 to 353 mg/dl) and 7-fold (from 1554 to 219 mg/dl) in the two animals studied, and returned to baseline by 3 weeks post-infusion (Fig. 1*b*). The decrease in serum cholesterol is reflected in coordinate diminution in serum LDL (Fig. 2, middle row).

Mice were sacrificed at various times following infusion of virus, and liver tissue was harvested for direct analysis of transgene expression using X-gal histochemistry to detect *lacZ* expression and immunofluorescence to measure LDL receptor expression (Fig. 3). Tissues harvested 3 days post-infusion of virus demonstrated either β-galactosidase (Fig. 3, left column) or LDL receptor protein (center column) in at least 80% of hepatocytes; in each experiment the vector specific signal was substantially higher than that seen in animals before gene transfer (data not shown) or following infusion with identical quantities of an adenovirus expressing an irrelevant gene (Fig. 3, top row). For both *lacZ* and the LDL receptor, transgene expression diminished to undetectable levels by day 21 (Fig. 3) and was associated with the development of a self limited mononuclear infiltrate in liver that peaked at day 10 and resolved by day 21 (Fig. 4 and data not shown). The infiltrate consisted of portal as well as lobular inflammation, accompanied by the presence of apoptotic bodies, as previously reported for mouse liver[9]. The extent of pathology was indistinguishable between the *lacZ* and LDL receptor infused mice. The time course of LDL receptor expression is consistent with the initial large decline in plasma cholesterol and subsequent return to baseline and is similar to previous experiments in rabbits[6,7] and in mice[9,20].

Immune responses to the LDL receptor

Previous studies have implicated cellular immune responses to viral proteins in cells infected with E1 deleted viruses as contributing to the apparent extinction of transgene expression[12,13,21]. This concept was extended in the current study to evaluate both cellular and humoral immune responses to the transgene product with a focus on LDL receptor as a target. Plasma samples from both LDL receptor knockout and normal C57BL/6 mice treated with adenovirus were used in Western blots to detect antibodies to aden-

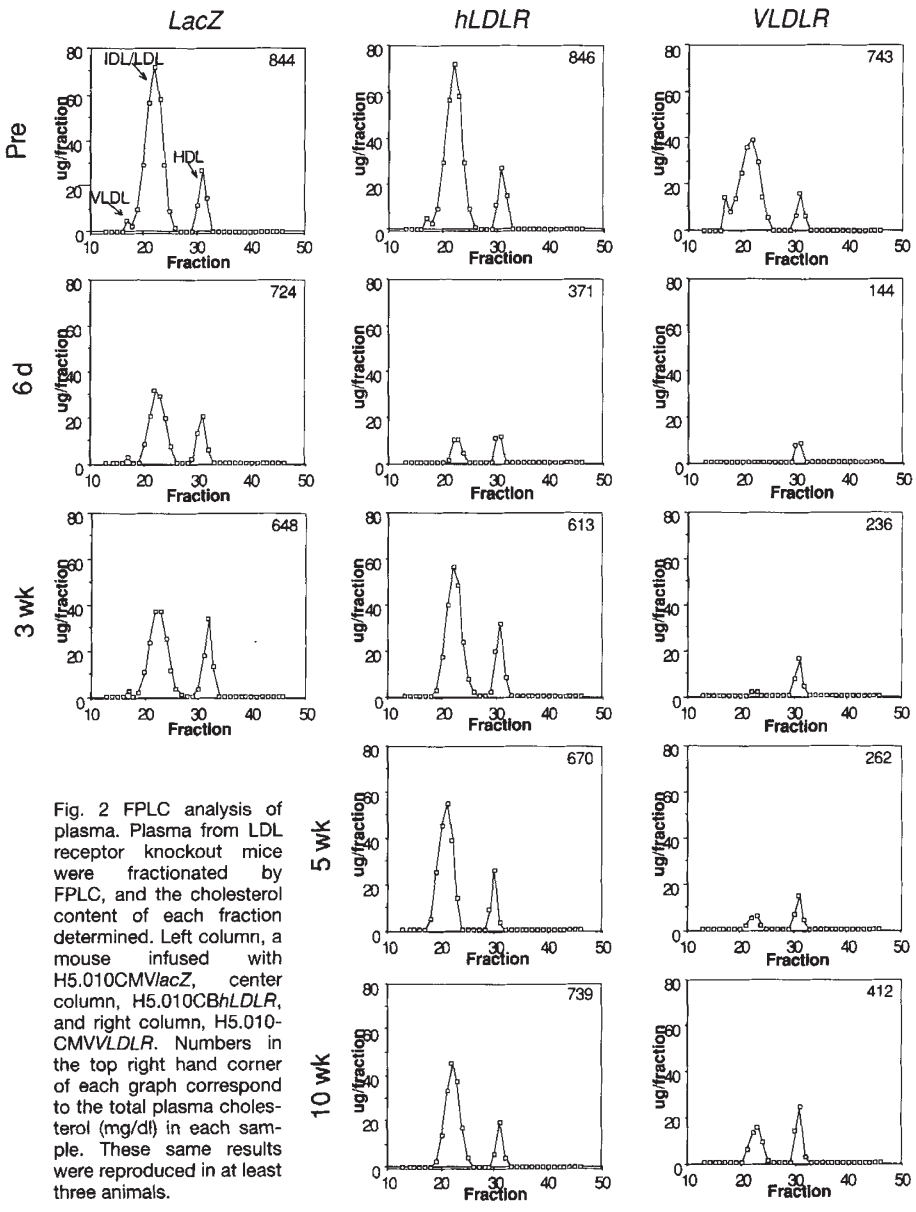

Fig. 2 FPLC analysis of plasma. Plasma from LDL receptor knockout mice were fractionated by FPLC, and the cholesterol content of each fraction determined. Left column, a mouse infused with H5.010CMV*lacZ*, center column, H5.010CB*hLDLR*, and right column, H5.010-CMV*VLDLR*. Numbers in the top right hand corner of each graph correspond to the total plasma cholesterol (mg/dl) in each sample. These same results were reproduced in at least three animals.

ovirus as well as to the transgene products. All mice infused with particles of recombinant adenovirus developed antibodies to adenovirus capsid proteins (Fig. 5*d*), with major bands corresponding to hexon, penton, and fiber. All mice infused with LDL receptor virus developed antibodies that recognized the human LDL receptor protein (Fig. 5*b*), with LDL receptor knockout mice (Fig. 5, lanes –/–) consistently developing higher titer antibodies than C57BL/6 mice (Fig. 5, lanes +/+). Antibodies from LDL receptor knockout mice cross-reacted with mouse LDL receptor protein, whereas antibodies from C57BL/6 mice (which express normal mouse LDL receptor) did not (data not shown). Animals of each genotype produced antibodies to β-galactosidase following infusion of *lacZ* virus (Fig. 5*a*). These data demonstrate that animals can generate a humoral immune response specific for the transgene product as well as to the injected adenovirus. It also provides indirect evidence of antigen specific activation of T helper cells, which is required for development of T-cell dependent, antibody-secreting B cells.

Previous studies indicated that mice also develop a cytotoxic T lymphocyte (CTL) response to adenoviral proteins following intravenous infusion of the recombinant virus, and suggested that this response at least partially accounts for the limited duration of transgene expression[13]. Our study analysed animals following infusion with the LDL receptor adenovirus for activation of CTLs to both viral antigens and the transgene product, human LDL receptor. CTLs to specific targets were detected in a standard ^{51}chromium (^{51}Cr) release assay (see Fig. 6) in which MHC compatible target cells were infected with either recombinant adenovirus or vaccinia viruses that express single relevant gene products. Splenocytes from C57BL/6 mice infused with the LDL receptor recombinant adenovirus were evaluated for their ability to lyse targets infected with recombinant adenovirus, to measure activity to viral proteins (Fig. 6*a*), or with vaccinia virus expressing

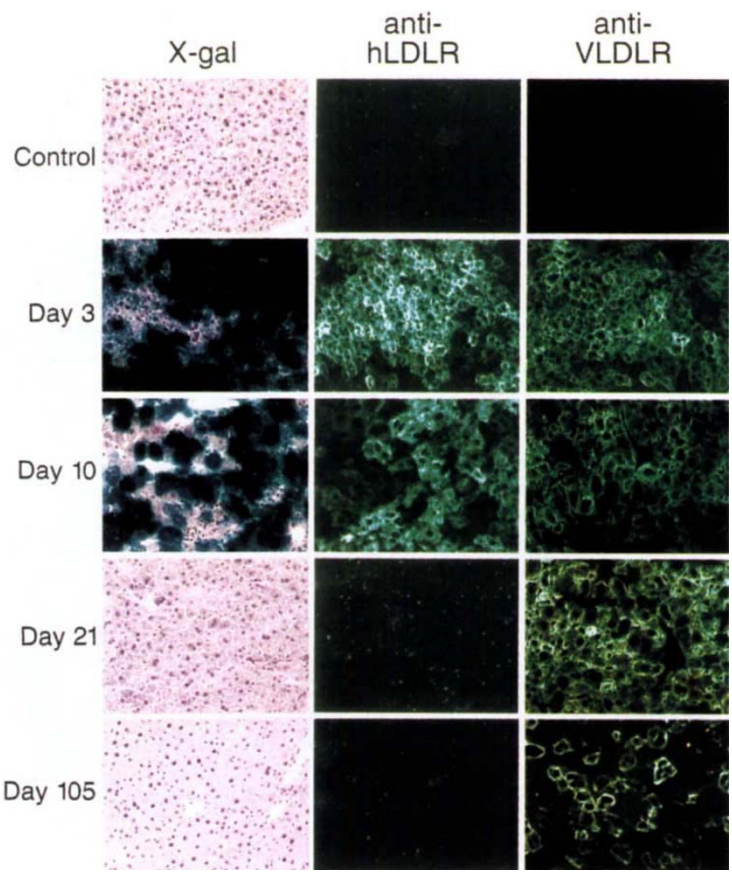

Fig. 3 Transgene expression in livers of LDL receptor knockout mice. Fresh-frozen liver tissues from mice killed on the indicated days post-infusion were stained with X-gal (first column), with anti-LDL receptor antibody (middle column) or with anti-VLDL receptor antibody (right column). Control row: left and right panels, day-3 mouse infused with H5.010CB*hLDLR*; center panel, day-3 mouse infused with H5.010CMV*VLDLR*. Rows labelled Day 3 through Day 105, left column, mice infused with H5.010CMV*lacZ*; center column, mice infused with H5.010CB*hLDLR*; right column, mice infused with H5.010CMV*VLDLR*.

LDL receptor, to measure activity to LDL receptor protein (Fig. 6*b*). Cytolytic activity was demonstrated with lymphocytes from animals infected with the human LDL receptor virus to target cells infected with the same virus. No cytolysis was detected to mock infected targets supporting the specificity of the assay (Fig. 6*a*). These same effector cells demonstrated significant cytolytic activity to targets infected with LDL receptor vaccinia virus that was not present when infected with a control vaccinia (Fig. 6*b*). These experiments provide strong evidence for the presence of activated CTL to human LDL receptor in C57BL/6 mice following gene therapy.

Hepatic expression VLDL receptor corrects hypercholesterolaemia of FH

A recombinant adenovirus was constructed encoding the human VLDL receptor cDNA under the control of the CMV promoter (designated H5.010CMV*VLDLR*). The VLDL receptor has been shown *in vitro* to bind VLDL[14,19], a precursor of LDL that contains apoE. The VLDL receptor is not expressed in liver and usually plays little role in circulating lipoprotein levels[18]. We reasoned that high-level expression of VLDL receptor in liver may significantly diminish LDL levels in FH by increasing the catabolism of its precursor, VLDL. Problems of immune responses to the therapeutic gene product that could subvert the effectiveness of gene replacement therapy for deficiency states could be avoided, since extrahepatic VLDL receptor is expressed in extrahepatic sites in both FH and non FH.

VLDL receptor containing adenovirus was infused intravenously into LDL receptor knockout mice to achieve high level expression of this lipoprotein receptor in liver. Two mice showed large decreases in cholesterol levels (Fig. 1*c*) that were similar in magnitude to the declines seen in LDL receptor adenovirus-infused mice (Fig. 1*b*), with maximum decreases of more than 4-fold (from 1186 to 288 mg/dl and from 1453 to 299 mg/dl). Surprisingly, plasma cholesterol levels did not return to baseline by 3 weeks post-infusion (compare Fig. 1*b* to 1*c*). The average change in plasma cholesterol observed for ten LDL receptor knockout mice treated with VLDL receptor virus was statistically significant ($P < 0.05$) through 9 weeks post-infusion when compared to data from mice infused with identical quantities of *lacZ* virus (Fig. 1*d*). Sera from individual mice were analysed by FPLC to determine the effects of VLDL receptor expression on lipoprotein fractions. On day 6 post-infusion, VLDL and LDL fractions were undetectable (Fig. 2); over time, the LDL fraction slowly recovered, although even at 10 weeks post-infusion, the peak height was slightly lower than the HDL peak height (Fig. 2). VLDL remained undetectable although minor differences may escape detection because of limitations in the sensitivity of the cholesterol assay. The LDL peaks mirrored the total plasma cholesterol levels, and confirmed that the prolonged lowering of plasma cholesterol was accompanied by sustained decreases in VLDL and LDL levels.

To further characterize the effects of hepatic VLDL receptor expression on lipoprotein metabolism, turnover studies were performed in LDL receptor knockout mice 5 days after infusion of recombinant adenovirus. Mice were injected with both ^{125}I-labelled LDL and ^{131}I-labelled VLDL, and the rate of disappearance of each isotope from the plasma was monitored. Infusion of LDL receptor adenovirus led to accelerated clearance of LDL as compared to infusion

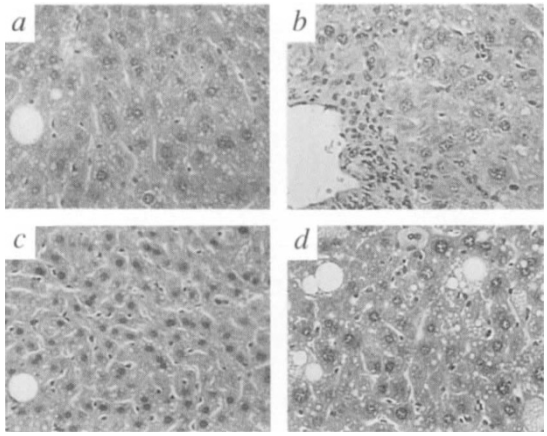

Fig. 4 Histopathologic analysis of liver tissue. Livers were harvested from LDL receptor knockout mice following infusion of recombinant adenovirus or of buffer, and formalin-fixed tissues were stained with haematoxylin and eosin. *a*, Mouse infused with PBS, day 154 post-infusion; *b*, VLDL receptor infused mouse, day 7; *c*, LDL receptor infused mouse, day 154; *d*, VLDL receptor infused mouse, day 154.

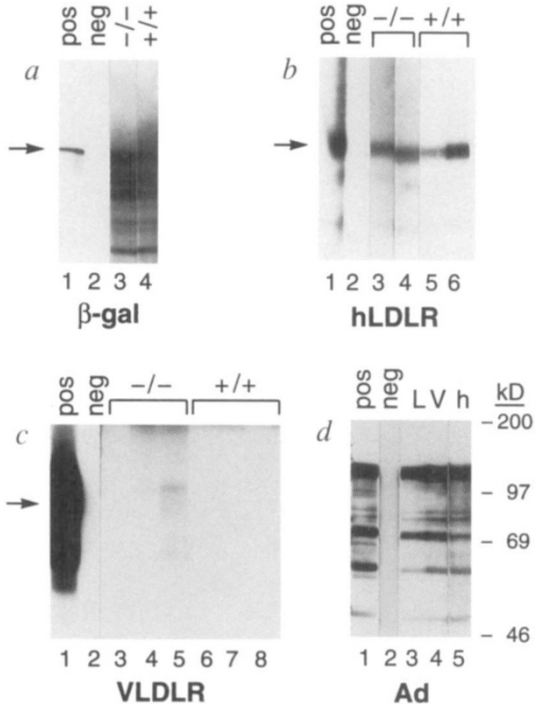

Fig. 5 Western blot to detect antibodies to the transgene product. Sera obtained from normal (+/+) or LDL receptor knockout (−/−) mice between 3 and 4 weeks post-infusion of recombinant adenovirus were used to probe western blots. Mice were infused with H5.010CMV*lacZ* (*a*), H5.010CB*hLDLR* (*b*) or H5.010CMV*VLDLR* (*c*). The protein preparations subjected to western blotting were β-galactosidase (*a*), lysates prepared from cells expressing the human LDL receptor (*b*), lysates prepared from cells expressing the VLDL receptor (*c*) or purified adenovirus (*d*). In (*b*), the lanes corresponding to C57BL/6 mice (+/+) were from a longer exposure. In (*d*), sera were obtained from mice infused with the *lacZ* adenovirus (L), VLDL receptor adenovirus (V), or the human LDL receptor adenovirus (h). pos, positive control antibody; neg, negative control antibody; −/−, LDL receptor knockout mice; +/+, C57BL/6 mice.

of *lacZ* adenovirus (Fig. 7*a*), consistent with a previous study in LDL receptor knockout mice[8]. Similarly, VLDL clearance was accelerated in LDL receptor treated animals as compared to *lacZ* infused mice (Fig. 7*b*). LDL turnover in VLDL receptor-infused mice was indistinguishable from *lacZ* infused mice (Fig. 7*a*), consistent with *in vitro* data which indicates that LDL is not a ligand for the VLDL receptor[19,22]. VLDL clearance in VLDL receptor infused mice was slightly faster than in *lacZ* infused mice, but significantly slower than in LDL receptor infused mice (Fig. 7*b*).

VLDL receptor expression

FH mice were analysed directly for expression of recombinant derived VLDL receptor by immunocytochemical analysis of liver tissue (Fig. 3, right column). Analysis of liver harvested 3 days post-infusion of VLDL receptor virus revealed VLDL receptor protein in >80% of hepatocytes; the bright fluorescent signal, which localized to the perimeter of the cell, was not present before gene transfer or in tissues of animals infected with *lacZ* (data not shown) or LDL receptor containing adenovirus (Fig. 3, top right panel). Expression of VLDL receptor protein was remarkably stable with recombinant protein detected in approximately 5 to 10% of hepatocytes from tissue harvested 105 days post-infusion of virus. This is in contrast to the results obtained with *lacZ* and LDL receptor virus where expression of the transgene extinguished to undetectable levels within 3 weeks of gene transfer. VLDL receptor protein was detected through the duration of the experiment (22 weeks).

Histopathologic analysis of liver tissue from mice infused with the VLDL receptor virus revealed inflammation and apoptotic cells at early time points that diminished within one month. The timing and extent of the pathologic findings were indistinguishable from liver tissues of mice infused with *lacZ* and LDL receptor viruses (Figure 4*b* and data not shown). At 15 and 22 weeks post-infusion, however, liver tissue from VLDL receptor-infused mice displayed discernible accumulations of neutral lipids, as demonstrated by haematoxylin and eosin as well as oil red O staining (Figure 4*d* and data not shown). Similar changes were observed infrequently in LDL receptor knockout mice infused with PBS, or with LDL receptor and *lacZ* viruses (Fig. 4*a*, *c*, and data not shown). No lipid accumulations were observed in livers of normal C57BL/6 mice infused with the VLDL receptor virus, despite long-term transgene expression indistinguishable from that observed in the LDL receptor knockout mice (data not shown). This indicates that VLDL receptor expression alone is not sufficient for the changes observed in LDL receptor knockout mice; instead, there is some lipid accumulation in the LDL

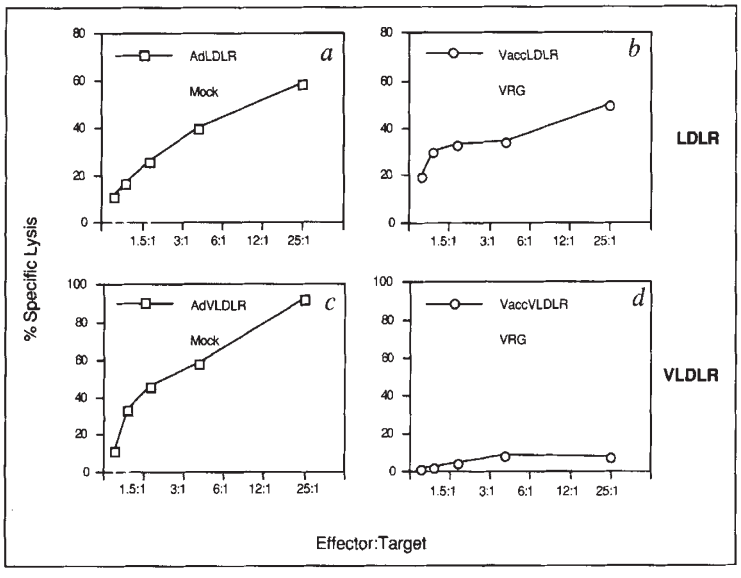

Fig. 6 CTL assays. Mice were infused with recombinant adenovirus, splenocytes isolated and restimulated with the same recombinant adenovirus for 5 days. CTL activity was measured using targets cells infected with either recombinant adenovirus or recombinant vaccinia. Top row (*a*, *b*) CTL activity in mice infused with the LDL receptor adenovirus. *a*, Target cells were infected without (Mock) or with the LDL receptor adenovirus (AdLDLR). *b*, Target cells were infected with vaccinia expressing an irrelevant gene (VRG) or vaccinia expressing the LDL receptor (VaccLDLR). Bottom row (*c*, *d*) CTL activity in mice infused with the VLDL receptor adenovirus. *c*, Target cells were infected without (Mock) or with the VLDL receptor adenovirus (AdVLDLR). *d*, Target cells were infected with vaccinia expressing an irrelevant gene (VRG) or vaccinia expressing the VLDL receptor (VaccVLDLR). These findings were reproduced in at least four independent experiments.

receptor knockout mice which have been maintained on a high cholesterol diet for ≥ 18 weeks, that is accelerated by prolonged VLDL receptor expression.

Plasma samples from mice infused with VLDL receptor adenovirus were analysed for the presence of antibodies to the VLDL receptor protein. Only one mouse out of twelve generated antibodies to the VLDL receptor (Fig. 5*c*, and data not shown) despite the presence of high level antibodies to adenovirus capsid proteins (Fig. 5*d*, lane V). Animals infused with the VLDL receptor adenovirus mounted a CTL response to adenoviral proteins indistinguishable to that obtained from animals infused with either *lacZ* or LDL receptor adenoviruses (compare Fig. 6*c* to 6*a*). These mice, however, did not mount a CTL response to the VLDL receptor protein (Fig. 6*d*). Thus, the development of a CTL response to the transgene following infusion of recombinant adenovirus is dependent on the antigenicity of the transgene.

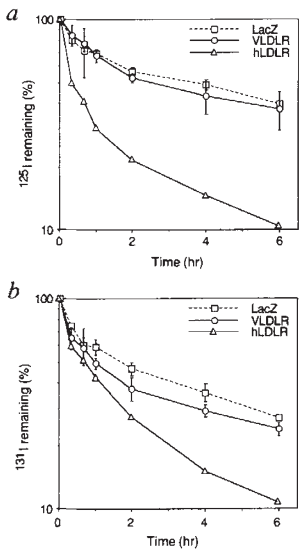

Fig. 7 LDL and VLDL turnover in LDL receptor knockout mice. Five days following infusion of recombinant adenovirus, mice were infused with a mixture of ^{125}I-labelled LDL (*a*) and ^{131}I-labelled VLDL (*b*). Results are expressed as the average percentage (± standard deviation) of label remaining in the plasma for mice infused with the *lacZ* virus (squares, $n = 3$), and the VLDL receptor virus (circles, $n = 3$). Results from a single mouse are shown for mice infused with the human LDL receptor virus (triangles).

DNA was isolated from liver and subjected to DNA hybridization studies to determine the presence of adenoviral DNA sequences. Previous studies have shown that in immunodeficient mice, sustained transgene expression is accompanied by retention of adenovirus DNA[9]. In C57BL/6 mice infused with the *lacZ* adenovirus, viral DNA diminished rapidly with time, plateauing at barely detectable levels (~0.05 copies/cell) through day 70 post-infusion (Fig. 8, top panel). Mice infused with VLDL receptor had slightly higher initial levels of DNA, but a similar time course of loss of adenovirus sequences (Fig. 8, lower panel). Additional DNA hybridization studies showed that the majority of adenovirus DNA initially delivered to the liver is not integrated into the mouse genome (data not shown).

Discussion

Cellular immune responses to the LDL receptor subverts gene therapy. One strategy for gene therapy of metabolic diseases such as FH is adenovirus-mediated gene transfer to liver. *In vivo* administration of the virus in rabbit and mouse models of FH leads to high level recombinant LDL receptor expression in liver and complete correction of the dyslipoproteinaemia[6–8]. The transient nature of the therapy and the associated hepatic inflammation preclude the use of E1-deleted recombinant adenoviruses in FH patients. In this study, we have explored mechanisms responsible for these limitations, with a focus on immune responses of the recipient to the therapeutic gene product, the LDL receptor protein.

Previous studies by multiple groups have suggested that immune responses to the virus and genetically engineered cells subvert the successful application of adenovirus-based therapy to liver and other organs[11–13,20,21]. Experiments in mice suggest that antigen-specific responses to the infected hepatocytes contribute to the loss of transgene expression in a way that requires activation of both T helper cells of the T_{H1} subset and cytotoxic T lymphocytes (CTLs[13]. Antigenic targets of these cellular immune responses have been shown to reside on viral proteins and in the case of experiments with adenoviruses containing *lacZ*, the E. coli β-galactosidase protein[23]. It is difficult to quantify the relative contribution of viral versus transgene derived epitopes in the activation of T helper and CTL pathways required for reducing transgene expression. In some mouse models, cellular immune responses to viral derived epitopes are sufficient to mediate the elimination of transgene expression[24].

Our experiments with adenovirus containing human LDL receptor in both C57BL/6 and the FH mouse demonstrated transient expression of the transgene in the setting of hepatitis and a full complement of T helper and CTL responses to adenovirus proteins and the recombinant derived human LDL receptor protein. The development of antibodies to both viral proteins and human LDL receptor confirms processing of both viral and transgene-derived proteins by antigen presenting cells as well as the activation of T helper cells. CTLs to viral antigens and LDL receptor proteins were documented in ^{51}Cr release assays of bulk cultured splenocytes prestimulated *in vitro* with adenovirus infected splenic macrophages. These experiments implicate T cell acti-

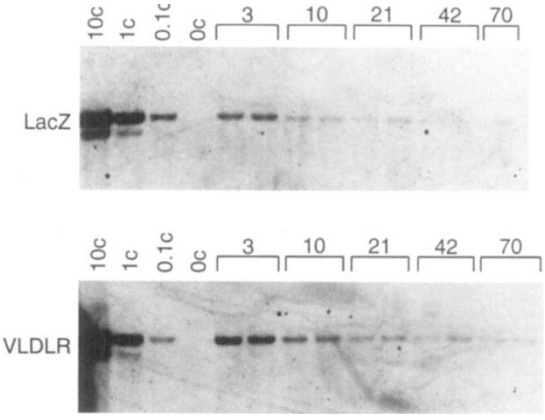

Fig. 8 Southern blot of liver DNA to determine the persistence of adenovirus sequences. DNA was isolated from liver tissues obtained from duplicate C57BL/6 mice killed on the days indicated and subjected to Southern blotting using a probe specific for adenovirus sequences. Control lanes used untreated mouse liver DNA spiked with purified adenovirus: 10c, 1c and 0.1c, 10 copies, 1 copy and 0.1 copies/haploid genome, respectively. 0c, no adenovirus DNA added.

vation to LDL receptor protein in immune responses of the host that underlie inflammation and transient expression. The production of antibodies to LDL receptor, which has a substantial extracellular domain, suggests other antigen specific processes such as antibody-dependent cell-mediated cytotoxicity (ADCC) may contribute to the apparent diminution of transgene function. The development of antibodies to LDL receptor following adenovirus-mediated gene transfer differs from the results with *ex vivo* gene therapy in WHHL rabbits and FH patients where a serologic response to the retroviral encoded LDL receptor protein was not detected[2,4,5]. This may be explained by one of several factors, including the nature of the LDL receptor gene defect, an adjuvant effect of the adenovirus, the higher level of expression obtained with adenoviruses versus retroviruses, or the likely infection of antigen presenting cells that occurs following infusion of recombinant adenoviruses but not in the *ex vivo* approach.

Hepatic expression of VLDL receptor corrects dyslipidaemia. Concerns regarding potential host immune responses to LDL receptor in FH homozygotes undergoing adenovirus-mediated gene therapy led us to consider strategies whereby an alternative pathway for LDL metabolism is modified by ectopic and/or overexpression of another gene. Recombinant adenoviruses were used to achieve high level expression of the VLDL receptor in livers of FH mice. This protein, which is homologous to but distinct from the LDL receptor, is normally expressed in nonhepatic tissues[14–17]. The endogenous VLDL receptor is not believed to substantially regulate serum levels of lipoproteins[18,25] although it may play a role in cholesterol and/or triglyceride uptake in the tissues in which it is expressed. In our studies, hepatic expression of recombinant VLDL receptor resulted in sustained decreases in plasma cholesterol.

The original hypothesis for using hepatic VLDL receptor expression to regulate serum LDL was based on enhancing catabolic degradation of VLDL and/or LDL. *In vitro* assays have suggested that the VLDL receptor does not bind LDL efficiently[14,19]. This was confirmed in our system by measuring LDL turnover directly in adenoviral infected FH mice where recombinant derived, hepatic expression of VLDL receptor had no detectable effect on LDL clearance.

VLDL turnover in mice infused with the VLDL receptor adenovirus was faster than in *lacZ* infused mice although the magnitude of this effect was far less than that seen in animals treated with LDL receptor virus. This suggests that mechanisms other than VLDL receptor-mediated clearance of circulating VLDL, such as diminished VLDL production, may contribute to diminished serum VLDL. One potential mechanism is secretion-recapture, which occurs with hepatic uptake of chylomicron remnants[26,27], and would result in decreased secretion of VLDL and reduced levels of plasma VLDL. A second mechanism may involve the interaction of the VLDL receptor with receptor-associated protein (RAP)[22,28] which interacts with a variety of receptors inside the cell, apparently to prevent ligand binding before the receptor reaches the cell surface[29]. It is possible that the high levels of VLDL receptor expressed in the livers of adenovirus-infused mice overwhelms the available RAP, so that VLDL receptor is binding to newly synthesized ligand (apoE, either free or in association with lipid) within the cell, and preventing its secretion into the plasma. The effects of hepatic VLDL receptor expression on total plasma cholesterol as well as on lipoprotein cholesterol levels demonstrate that the VLDL receptor can play a major role in lipoprotein metabolism *in vivo*. Further clarification of the relative contributions of these mechanisms will require more detailed metabolic studies.

VLDL receptor circumvents destructive cellular immune responses. The most striking result in this study was that expression of adenovirus encoded VLDL receptor is relatively stable leading to prolonged improvement in hypercholesterolaemia in FH mice. Mechanisms responsible for sustained expression of VLDL receptor have not been identified although the relative immunogenicity of the transgene product should be considered. Animals infused with VLDL receptor adenoviruses failed to produce transgene specific CTLs or antibodies, and by inference, did not activate T helper cells. These observations contrast with the experience with other recombinant adenoviruses such as those encoding LDL receptor or *lacZ* where transgene specific CTLs and antibodies are produced. Preliminary experiments indicate that in the hamster, as in the mouse, VLDL receptor expression is prolonged, and there is no detectable humoral immune response to the transgene product (unpublished data). The human VLDL receptor protein is >94% homologous to the mouse protein[15], in contrast to the human LDL receptor, which is approximately 77% homologous to the mouse protein[30,31] in C57BL/6 mice and truly a neoantigen in the FH mice, which express no endogenous LDL receptor protein. An alternative explanation is that the VLDL receptor protein may have a protective or stabilizing effect on the recombinant adenoviral genome. While VLDL receptor expression is prolonged, it eventually diminishes to subtherapeutic levels possibly due to immune responses to viral proteins expressed from these first generation viruses or inherent instability of the adenoviral genome. In addition, the use of VLDL receptor does not prevent the development of hepatitis that occurs following infusion of high dose virus which likely has significant non-antigen specific components.

Differences in persistence of transgene expression were not associated with differences in the time course of detection of adenovirus DNA between animals infused with the *lacZ*, human LDL receptor and the VLDL receptor virus. One possible explanation is that in mice given the VLDL receptor virus, the adenovirus DNA that is retained may be in a different subcellular compartment, or in a different conformation or state of methylation. In addition, the DNA hybridization studies do not distinguish DNA contained within parenchymal cells versus non-parenchymal cells such as Kupfer cells. The observation that adenovirus DNA was detected in mice (infused with *lacZ* or human LDL receptor viruses) at time points beyond detectable transgene expression suggests that not all of the cells initially infected with recombinant adenovirus were destroyed.

These experiments document for the first time the development of both humoral and cellular immune

responses to the therapeutic gene product following *in vivo* gene therapy. The implications of these host responses are unclear; however, their potential to subvert the therapy may explain, in part, the limitations we have encountered in replacing the LDL receptor protein by adenovirus-mediated gene transfer in liver of FH mice. We illustrate a strategy for circumventing potentially destructive immune responses to the therapeutic gene product in replacement therapy of deficiency states which is based on enhancing alternative metabolic pathways. This may be a strategy that needs to be employed in replacement gene therapy of other deficiency states, especially in patients completely void of residual protein expression.

Methods

Recombinant adenoviruses. All adenoviruses used were human Ad5 based, containing deletions of the E1a and part of E1b, and partial deletion of the E3 region. The construction of H5.010CMV*lacZ*, which contains the *lacZ* gene under the control of the CMV enhancer/promoter, and H5.010CB*hLDLR*, containing the human LDL receptor cDNA under the control of the CMV enhanced chicken β-actin promoter, have been described[6,32]. H5.010CMV*VLDLR*, the recombinant adenovirus encoding the VLDL receptor under the control of the CMV promoter/enhancer, was generated as follows. A plasmid containing the VLDL receptor cDNA in the polylinker region of pRC/CMV (Invitrogen) was digested with *Sna*BI and *Not*I, and the fragment containing the CMV promoter/VLDL receptor cDNA was isolated. This fragment was ligated to the plasmid pAdCMVlacZ[6] which had been digested with *Sna*BI and *Not*I to remove the CMV promoter and *lacZ* cDNA. The resulting plasmid was co-transfected into 293 cells with sub360 DNA as described[32] and H5.010CMV*VLDLR* was isolated using two rounds of plaque purification. Recombinant adenoviruses were grown and purified by CsCl density ultracentrifugation as described[33]. CsCl was removed by passing the adenovirus over a BioRad 10DG desalting column equilibrated with PBS. Glycerol was added to the preparation to a final concentration of 10% (v/v) and aliquots were stored at –70 °C.

Infusion of mice with adenovirus. Virus was thawed immediately before use and diluted with PBS to a concentration of 1×10^{12} particles/ml. Mice were injected via the tail vein with 0.1 ml of virus containing 1×10^{11} particles. LDL receptor knockout mice were obtained from Jackson Labs and maintained as a breeding colony. Following weaning at 3 weeks of age, mice were given unrestricted access to water and a 0.2% cholesterol diet consisting of Purina Mouse Chow (#5001) supplemented with 10% coconut oil, 0.2% cholesterol, and 0.05% cholic acid. On the days indicated, mice were anaesthetized with methoxyflurane and blood was collected into heparinized capillary tubes by puncture of the retro-orbital venous plexus.

Cholesterol determinations and FPLC analysis of plasma. The cholesterol content of whole plasma was analysed using a Cholesterol HP kit (Boehringer Mannheim) and Preciset standards. FPLC analysis was performed on 50 µl of plasma from individual mice adjusted to a volume of 250 µl in FPLC column buffer (1 mM EDTA, 154 mM NaCl, pH 8.0). Diluted samples (200 µl) were loaded onto two Superose 6 columns (Pharmacia) in series at a flow rate of 0.4 ml/min, and 1 ml fractions were collected. Cholesterol content was analysed in a microplate assay on 100 µl samples. 100 µl of a freshly prepared solution containing 50 mM PIPES, pH 6.9, 7.8 g/l HDCBS, 0.51 g/l 4-AAT, 1.27 g/l cholic acid, 0.245% Triton X-100, 7.31 g/l KCl and supplemented with 1.22 U/ml cholesterol oxidase, 7.64 U/ml cholesterol esterase, and 245 U/ml peroxidase was added to samples, incubated overnight at room temperature, and the O.D. at 490 nm was determined.

Immunohistochemistry. Tissues were processed, stained with X-gal, or with haematoxylin and eosin as described[6]. The antibody used in immunofluorescence for the VLDL receptor was a rabbit polyclonal anti-peptide antibody (gift of D. Strickland, American Red Cross, Gaithersburg, MD). Staining for the LDL receptor utilized a rabbit polyclonal antibody which was generated by intravenous infusion of H5.010CB*hLDLR*[6].

Western blots. Western blots were performed as described[6,32]. To detect anti-adenovirus antibodies, purified adenovirus was used as the antigen, and a polyclonal rabbit antiserum isolated following intravenous infusion of purified recombinant adenovirus as a positive control. Western blots with β-galactosidase were performed using purified protein (Sigma), with a monoclonal antibody specific for β-galactosidase (Sigma) as a positive control. Antibodies to the human LDL receptor were detected using lysates prepared from 24–23 cells, a 3T3 cell line which was transduced with retrovirus encoding the human LDL receptor. For detection of anti-VLDL receptor antibodies, a lysate was prepared from HeLa cells two days following infection with H5.010CMV*VLDLR*.

Southern blots. Genomic DNA was isolated from mouse liver, digested with *Eco*RI, and subjected to Southern blotting as described[6]. Adenovirus sequences were detected using the Genius kit (Boehringer Mannheim), followed by chemiluminescent detection.

Turnover studies. LDL receptor knockout mice were infused with recombinant adenovirus after 3 weeks on the high cholesterol diet as described above. Three mice each were injected with the *lacZ* and VLDL receptor adenoviruses; once mouse was injected with the LDL receptor adenovirus. On day 5 post-infusion, mice were injected via the tail vein with approximately 8×10^6 cpm of ^{125}I-labelled human LDL, and 1.6×10^5 cpm of ^{131}I-labelled human VLDL in a total volume of 0.2 ml. A blood samples was obtained 1 min post-injection of radiolabel, and designated the 'time zero' sample. Blood was collected into heparinized capillary tubes at the indicated times, and radioactivity remaining was determined using a gamma counter.

Recombinant vaccinia. The VLDL receptor cDNA (in the pRC/CMV plasmid) was subcloned into the *Hin*dIII site of Bluescript KS+. The LDL receptor cDNA in the pUC19 vector[34] was excised with the restriction enzymes *Hin*dIII and *Sac*I and ligated into the *Hin*dIII and *Sac*I sites of Bluescript KS+. Each of the cDNAs was then excised using the enzymes *Sac*II and *Kpn*I and cloned into the *Sac*II and *Kpn*I sites of a modified form of the vaccinia expression vector pSCII[35] (kindly provided by I. Eisenlohr). The control recombinant vaccinia, VRG, expresses a rabies virus glycoprotein[36].

CTL assays. CTL assays were performed as described[13]. Target cells expressing recombinant vaccinia proteins were generated by infecting with recombinant vaccinia at a multiplicity of infection of 1 for one hour prior to ^{51}Cr labelling.

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