Gene therapy for rhesus monkeys heterozygous for LDL receptor deficiency by balloon catheter hepatic delivery of helper-dependent adenoviral vector

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Autosomal dominant familial hypercholesterolemia (FH) is a monogenic life-threatening disease. We tested the efficacy of low-density lipoprotein receptor (LDLR) gene therapy using helper-dependent adenoviral vector (HDAd) in a nonhuman primate model of FH, comparing intravenous injection versus intrahepatic arterial injection in the presence of balloon catheter-based hepatic venous occlusion. Rhesus monkeys heterozygous for mutant LDLR gene (LDLR+/−) developed hypercholesterolemia while on a high-cholesterol diet. We treated them with HDAd-LDLR either by intravenous delivery or by catheter-based intrahepatic artery injection. Intravenous injection of ≤1.1 × 10¹² viral particles (vp) kg⁻¹ failed to have any effect on plasma cholesterol. Increasing the dose to 5 × 10¹² vp kg⁻¹ led to a 59% lowering of the plasma cholesterol that lasted for 30 days before it returned to pre-treatment levels by day 40. A further increase in dose to 8.4 × 10¹² vp kg⁻¹ resulted in severe lethal toxicity. In contrast, direct hepatic artery injection following catheter-based hepatic venous occlusion enabled the use of a reduced HDAd-LDLR dose of 1 × 10¹² vp kg⁻¹ that lowered plasma cholesterol within a week, and reached a nadir of 59% pre-treatment level on days 20–48 after injection. Serum alanine aminotransferase remained normal until day 48 when it went up slightly and stayed mildly elevated on day 72 before it returned to normal on day 90. In this monkey, the HDAd-LDLR-induced trough of hypocholesterolemia started trending upward on day 72 and returned to pre-treatment levels on day 120. We measured the LDL apolipoprotein B turnover rate at 10 days before, and again 79 days after, HDAd-LDLR treatment in two monkeys that exhibited a cholesterol-lowering response. HDAd-LDLR therapy increased the LDL fractional catabolic rate by 78 and 50% in the two monkeys, coincident with an increase in hepatic LDLR mRNA expression. In conclusion, HDAd-mediated LDLR gene delivery to the liver using a balloon catheter occlusion procedure is effective in reversing hypercholesterolemia in a nonhuman primate FH model; however, the unsustainability of the hypocholesterolemic response during 3–4 months of follow up and heterogeneous response to the treatment remains a challenge.

INTRODUCTION

Autosomal dominant familial hypercholesterolemia (FH) is caused by mutations in the low-density lipoprotein receptor (LDLR).¹ Homozygous FH patients present with massively elevated LDL cholesterol (LDL-C) and cardiovascular disease. They have severe atherosclerosis and die of ischemic heart disease usually in their third decade of life. The majority of homozygous and a substantial proportion of heterozygous patients are refractory to conventional pharmacological therapy. Therapeutic options for these resistant patients are limited to LDL apheresis, portacaval anastomosis or liver transplantation.² Gene therapy has been explored as an alternative treatment. Liver is the main target organ for FH gene therapy because of its capacity to dispose excess cholesterol by diverting it into bile acids; it is also accessible to gene delivery via the intravenous (i.v.) route or the hepatic artery. A number of studies have shown that hepatic reconstitution of LDLR expression ex vivo can reverse hypercholesterolemia, including promising results in a rabbit model of FH.³ In the only clinical gene therapy trial for FH to date, Grossman et al.⁴ isolated hepatocytes from FH patients, transduced them ex vivo with retroviral vector expressing LDLR and reimplanted them into the liver of the patients. Only marginal therapeutic benefit was achieved in this study. It was difficult to determine whether the reduction in LDL-C level was the direct result of the gene transfer or other factors were involved. Plasma LDL level is determined by LDL production and removal. For example, the decline of LDL-C after portacaval anastomosis is caused by a decreased secretion of very-low-density lipoprotein, a precursor of LDL, not by an enhanced LDL removal.⁶ In this clinical trial, LDL turnover was not measured, which led to the comment ‘a modest 17% fall in plasma cholesterol after 25% hepatectomy and re-infusion of hepatocytes infected with a retrovirus might have been due to either diminished lipoprotein production or to enhanced activity of the patient’s own receptor’.⁷ The focus has shifted to in vivo gene therapy thereafter. Helper-dependent adenoviral vector (HDAd) is devoid of all viral protein genes and has shown considerable promise for liver-directed gene transfer with long-term transgene expression, which lasted a lifetime in mice.⁸ In a previous study in LDLR−/− mice, we showed that a single injection of HDAd expressing monkey LDLR reduced plasma cholesterol over 2 years and attenuated atherosclerotic lesion progression.⁹ We also demonstrated that LDLR gene therapy induces the regression of...
established atherosclerosis in LDLR−/− mice.10 Despite promising results of gene therapy in small animal models, its efficacy in large animal models has not been tested; there are important differences in physiology and in immune responses between rodents and humans. This issue is particularly relevant in gene therapy for lipid disorders.11

A nonhuman primate model of FH has been described in rhesus monkeys,12,13 which carried a heterozygous nonsense mutation involving codon Trp28314 of the LDLR. Extensive cross-breeding of the affected monkeys failed to yield any homozygotes, indicating that the mutation may be linked to a lethal mutation. With the availability only of the heterozygous (LDLR+/−) rhesus monkey, we will be modeling heterozygous FH in humans, a relatively common genetic disorder that affects about 1 in 500 people in most ethnic groups.15 Heterozygous LDLR-deficient monkeys displayed elevated plasma cholesterol (5.17–6.47 mmol l−1 or 200–250 mg dl−1) compared with unaffected monkeys (2.59–3.36 mmol l−1 or 100–130 mg dl−1); the plasma cholesterol level further increased to 12.93–20.69 mmol l−1 (500–800 mg dl−1) when the animals were fed a high-cholesterol diet.16 In this study, we tested the efficacy of HDAd-based monkey LDLR gene therapy in high-cholesterol diet-fed LDLR+/− rhesus monkeys. We compared the effect of i.v. injection of HDAd-LDLR with that of a balloon catheter-based procedure developed by Brunetti-Pierri et al.17 We found that a single i.v. injection of HDAd-LDLR into LDLR+/− monkeys produced a >50% lowering of plasma cholesterol that lasted about a month. We next tested a modified percutaneous catheter-based gene delivery strategy also developed by Brunetti-Pierri et al.18 In this refinement, the HDAd-LDLR was injected directly into the hepatic artery in the presence of increased intrahepatic pressure induced by transient blockage of hepatic venous drainage by a balloon catheter positioned in the inferior vena cava (IVC). The optimized gene delivery strategy was highly efficacious in reducing the vector dose while substantially prolonging the therapeutic hypcholesterolemic response to the treatment regimen.

RESULTS

I.v. injection of HDAd-LDLR

We treated four LDLR+/− monkeys as study subjects with a single i.v. injection of escalating doses of HDAd-LDLR.9 We first treated monkey #8796 with 20 ml of saline and found no significant changes in plasma cholesterol levels after treatment (Figure 1). As expected, we also failed to detect any change in plasma cholesterol when we treated another LDLR+/− monkey #9908 with an empty vector HDAd-D0 (0.8 × 1012 viral particles (vp) kg). We next infected i.v. HDAd-LDLR into a third LDLR+/− monkey #7139 at a dose of 1.1 × 1012 vp kg−1, an HDAd dose that is 10-fold higher than the dose of HDAd-α-fetoprotein that stimulated significant elevation in α-fetoprotein secretion in serum in baboons,17 and again failed to observe any change in plasma cholesterol level. We then treated a fourth monkey #13090 at an even higher i.v. dose of 5 × 1012 vp kg−1 of HDAd-LDLR. The treatment was well tolerated by the monkey and led to a 60% reduction in plasma cholesterol from a baseline of 14.95 mmol l−1 (578 mg dl−1) to 5.90 mmol l−1 (229 mg dl−1) on day 7. The plasma cholesterol lowering persisted until day 21, when it went up to 10.70 mmol l−1 (413 mg dl−1) on day 28, and toward pre-treatment levels on day 42. These results indicate that a dose higher than 1.1 × 1012 vp kg−1 was needed to reverse hypercholesterolemia in LDLR+/− monkeys, and a dose of 5 × 1012 vp kg−1 significantly restored normal plasma cholesterol in a heterozygous FH monkey, an effect that lasted for about a month. We next treated a fifth monkey #11226 with an even higher dose of 8.4 × 1012 vp kg−1, which was modestly below a dose that had previously proven to be lethal,19 and observed severe acute toxicity and lethality within a day of treatment. The clinical picture and necropsy revealed hemorrhagic shock syndrome likely resulting from the high dose of HDAd vector used.

Balloon occlusion-based HDAd delivery into hepatic artery

To improve on i.v. vector injection as a delivery method, Brunetti-Pierri et al. developed a protocol15,16 to deliver the vector via an intrahepatic arterial catheter. Simultaneously, under fluoroscopic guidance, they inserted a balloon catheter into the IVC via the femoral vein and positioned it over the hepatic venous outflow (Figure 2a). Intrahepatic arterial HDAd injection when the balloon was inflated led to a 10-fold increase in efficiency in transgene expression (Figures 2b and c). The IVC occlusion was also monitored by the venous pressure (Figure 2d). We performed the same procedure in rhesus monkeys and injected the HDAd vector (2 ml) within a minute via a hepatic artery catheter immediately after the balloon was inflated.

The monkeys used for this procedure are summarized in Table 1. We first performed the procedure in a chow-fed (Purina LabDietSLEO, St. Louis, MO, USA) normal LDLR+/+ (#19254) and a heterozygous LDLR+/− (#19499) monkey. The injection was done immediately after the balloon was deflated but while hepatic venous pressure remained high. As reported previously,17,18 systemic blood pressure fell significantly when the balloon was inflated. We found that serum interleukin (IL)-6 level increased 30 min after injection and peaked at 2 h (Figure 3a) but decreased to non-detectable levels by 72 h. The procedure also led to transient and inconsistent changes in plasma liver enzymes (Figures 3b and c). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels peaked at about 24 h; the increase was mild and resolved by day 5. Plasma total cholesterol levels in the LDLR+/− (#19499) monkey decreased from a baseline of 5.70 mmol l−1 (219 mg dl−1) to 3.90 mmol l−1 (150 mg dl−1) within 24 h. It gradually went back up over the next few days returning to baseline by day 5. The plasma cholesterol level did not change in the non-FH (LDLR+/+) (#19254) monkey (Figure 3d).

We next fed monkeys with a rhesus Western diet (Texas Biomedical Research Institute, San Antonio, TX, USA) for 7 weeks before treatment and were kept on the diet afterward. We injected HDAd-LDLR (2 × 1012 vp kg−1) into four monkeys immediately after the balloon was deflated. The plasma cholesterol did not change in two wild-type LDLR+/+ monkeys (#19360 and.
#21588) suggesting that the gene delivery does not have an effect on the cholesterol dynamics in monkeys that express normal amounts of LDLR. Of the two heterozygous LDLR+/− monkeys, one (#19251) showed no change in plasma cholesterol (Figure 4a, green line), whereas another LDLR+/− monkey (#19498) exhibited a 57% drop in plasma cholesterol level from 8.15 mmol l\(^{-1}\) (315 mg dl\(^{-1}\)) to 3.25 mmol l\(^{-1}\) (126 mg dl\(^{-1}\)) at day 7 (Figure 4a, red line). So there was a heterogeneous response in heterozygous FH monkeys treated at this dose of HDAd-LDLR. The cholesterol-lowering effect of HDAd-LDLR in the LDLR+/− (#19498) monkey that responded to the treatment was sustained for about 100 days. The plasma-lowering effect reached its nadir 7 days, and stayed at or near the nadir for another 3 weeks. Afterward, it gradually rose to 5.09 mmol l\(^{-1}\) (197 mg dl\(^{-1}\)) at day 78, and then to above the pre-treatment level (9.30 mmol l\(^{-1}\) or 361 mg dl\(^{-1}\)) by day 105 (Figure 4a, red line). The two wild-type LDLR+/+ monkeys maintained normal serum ALT throughout the observation period of 120 days. The LDLR+/− monkey (#19251) that did not show a hypocholesterolemic response also maintained normal ALT levels for 67 days, end of the observation period for this monkey. In contrast, the serum ALT of the LDLR+/− monkey (#19498) that showed a hypocholesterolemic response maintained a normal ALT level during the first 3 weeks of treatment when the plasma cholesterol showed an excellent response (Figure 4a, red line). ALT began to edge above normal to 70 U l\(^{-1}\) on day 36, and continued to go up to peak at 144 U l\(^{-1}\) on day 72, before it started trending down, eventually returning to normal on day 120 (Figure 4b, red line). It is noteworthy that this monkey that had responded to the treatment developed liver enzyme elevation late, and the delayed increase in serum ALT coincided with the onset of loss of the

**Table 1.** Summary of rhesus monkeys used for balloon catheter occlusion

| ID     | Sex | Age (years) | Bodyweight (kg) | Genotype | Dose (vp kg\(^{-1}\)) |
|--------|-----|-------------|-----------------|----------|-----------------------|
| #19254 | F   | 3           | 3.9             | N        | Saline                |
| #19499 | M   | 3           | 3.4             | H        | Saline                |
| #21588 | F   | 2           | 2.6             | N        | 2.0 × 10\(^{12}\)     |
| #19360 | F   | 3           | 4.0             | N        | 2.0 × 10\(^{12}\)     |
| #19251 | F   | 3           | 4.2             | H        | 2.0 × 10\(^{12}\)     |
| #19498 | M   | 3           | 5.0             | H        | 2.0 × 10\(^{12}\)     |
| #18340 | M   | 5           | 5.7             | H        | Saline                |
| #19269 | F   | 4           | 5.0             | H        | 1.0 × 10\(^{12}\)     |
| #19255 | M   | 4           | 5.7             | H        | Saline                |
| #19536 | F   | 4           | 4.4             | H        | 0.3 × 10\(^{12}\)     |
| #20031 | F   | 3           | 3.4             | N        | —                     |

Abbreviations: H, heterozygous low-density lipoprotein receptor deficient; N, normal. *Monkeys were used as donors for low-density lipoprotein turnover study.

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**Figure 2.** Balloon catheter-based hepatic artery injection. (a) Schematic diagram of hepatic artery injection. Liver circulation is isolated by inserting a balloon catheter via the femoral vein and placing it in the IVC. A second intra-arterial catheter is inserted into the hepatic artery through the contralateral femoral artery. The placement of the catheter is visualized using fluoroscopy. Once occlusion of the hepatic circulation has been established via the balloon catheter in the IVC, the vector is injected via the arterial catheter. The occlusion is confirmed by monitoring hepatic venous pressure through the third catheter inserted into the femoral vein. BD, bile duct; HA, hepatic artery; HV, hepatic vein; PV, portal vein. (b) Fluoroscopy image to confirm the position of a balloon catheter. (c) Fluoroscopy after the balloon inflated. Contrast reagent was injected to confirm that the catheter was placed at the IVC. (d) Venous pressure. Occlusion was monitored by venous pressure.

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Figure 3. Acute toxicity measurements associated with balloon catheter-based hepatic artery injection. One normal LDLR+/+ (#19254) and one heterozygous LDLR+/− (#19499) monkeys on normal chow were treated by an injection of saline and a complete blood test and IL-6 measurement were performed. (a) Plasma IL-6 levels. (b) Serum ALT levels. (c) Serum aspartate aminotransferase (AST) levels. (d) Plasma cholesterol levels.

Figure 4. Plasma cholesterol level of rhesus monkeys after balloon catheter-based hepatic artery delivery. HDAd-LDLR was injected into monkeys in a volume of 10 ml kg⁻¹ at a dose of 2 x 10¹² vp kg⁻¹. The vector was injected after the balloon was deflated. #19360 and #21588 were normal LDLR+/+ monkeys and #19251 and #19498 were heterozygous LDLR+/− monkeys. (a) Changes in plasma cholesterol levels. Pretreatment plasma cholesterol levels were 7.55 mmol l⁻¹ (292 mg dl⁻¹) in #19360, 7.25 mmol l⁻¹ (281 mg dl⁻¹) in #21588, 10.30 mmol l⁻¹ (399 mg dl⁻¹) in #19251 and 8.15 mmol l⁻¹ (315 mg dl⁻¹) in #19498. (b) Plasma ALT levels. Normal ALT range (5–61 IU l⁻¹) is shown by filled area. #19251 had a low hematocrit level at day 78 and blood analyses were not performed. Because this animal did not show any effects of gene therapy, further follow up was deemed unnecessary and #19251 was removed from the study.
cholesterol-lowering effect of the treatment. Although the significance of the timing is unclear, we note that a similar pattern is evident in an experiment involving another LDLR+/- monkey (#19269, see below).

Optimized balloon occlusion protocol increases efficacy of HDAd-LDLR therapy

The HDAd-LDLR-mediated hypocholesterolemic response was encouraging in monkey #19498. However, the dose used (2.2 × 10^{12} vp kg^{-1}) was only approximately fourfold lower than a lethal dose (8.4 × 10^{12} vp kg^{-1}, lethal for monkey #11226). In an attempt to increase the efficiency of HDAd-LDLR treatment so as to obtain comparable results with a lower dose of HDAd, we decided to modify the protocol by keeping the balloon inflated (and thus intrahepatic pressure maintained at a high level) throughout the vector injection. We applied the modified protocol on LDLR+-/- monkey (#19269, fed a rhesus Western diet) at half the dose used in the last group of monkeys, that is, at 1 × 10^{12} vp kg^{-1}, immediately after the balloon was inflated. The injection line was flushed with 20 ml of saline and the IVC balloon was kept inflated for an additional 5 min before it was deflated (Figure 2d).

Despite the use of a lower dose, the plasma cholesterol of this monkey (#19269) decreased from 10.40 mmol l^{-1} (402 mg dl^{-1}) to 5.75 mmol l^{-1} (222 mg dl^{-1}) at day 7; at day 20, it decreased further to 4.30 mmol l^{-1} (165 mg dl^{-1}), constituting a 59% reduction from the pre-treatment level. The plasma cholesterol level stayed at the same level (4.30 mmol l^{-1}) until day 48 (Figure 5a), when ALT level went up modestly to 81 U l^{-1} and stayed mildly elevated on day 72 (89 U l^{-1}; Figure 5b). At day 72, plasma cholesterol level started trending upward to 6.00 mmol l^{-1} (232 mg dl^{-1}) and returned to the pre-treatment level by day 120.

Although intrahepatic delivery of HDAd-LDLR at a dose of 1 × 10^{12} vp kg^{-1} was effective in reversing hypercholesterolemia, the beneficial effect of the treatment did not last beyond ~100 days. Contrary to the transient nature (lasting up to 3–4 months only) of the cholesterol-lowering effect of HDAd-LDLR in LDLR+-/- rhesus monkeys, wild-type baboons that had been treated with a low-dose (3 × 10^{10} vp kg^{-1}) HDAd-a-fetoprotein vector were reported to exhibit a much more prolonged expression of α-fetoprotein.\textsuperscript{17} One possible explanation is that there is heightened host immune responses to HDAd vector because the dose we used in monkeys was 30 times higher than that in baboons. Interestingly, observations similar to the current study have been reported in hemophilia B patients treated with adeno-associated virus (AAV) expressing factor IX in which transgene factor IX expression dropped precipitously at days 50–60 after treatment, an effect attributed to pre-existing immunity against AAV vector.\textsuperscript{20} To examine the possibility of pre-existing memory T cells against Ad by HDAd administration, we tested the effect of a dose of 0.3 × 10^{12} vp kg^{-1}, which is 10-fold higher than the effective dose reported in baboons\textsuperscript{17} but threefold lower than the dose that induced the increase in ALT in our study. Two LDLR+/- monkeys were treated with either 0.3 × 10^{12} vp kg^{-1} of vector (#19536) or saline (#19255). HDAd administration at this dose did not lower plasma cholesterol or increase liver enzymes (data not shown). We collected peripheral blood mononuclear cells at days –21, +34 and +70 and measured cytotoxic T lymphocyte activity as reflected by interferon-gamma (IFN-γ) secretion by lymphocytes on stimulation with Ad peptides.\textsuperscript{21} There was no significant increase in cytotoxic T lymphocyte activity in either monkey (Supplementary Figure S1), suggesting that hepatic arterial injection of HDAd at a dose of 0.3 × 10^{12} vp kg^{-1} did not stimulate pre-existing memory T cells. We also measured neutralizing antibodies before and after vector injection. Plasma collected from monkey #19522 treated with saline did not have any significant neutralizing antibodies at any time of sampling, whereas plasma from monkey #19536 treated with HDAd-LDLR inhibited the infection of 116 cells with HDAd-EGFP at 1:80 dilution at day +34 and +70 but not at day –21.

Efficacy of LDLR gene therapy

In addition to evaluating the plasma cholesterol as the downstream response to LDLR gene therapy, we also monitored the functional activity of the LDLR gene transfer 10 days before, and 79 days after, HDAd-mediated LDLR gene transfer. Hepatic LDLR gene delivery in LDLR+-/- monkeys, #19498 (Figure 4, red line) and #19269 (Figure 5), increased LDLR mRNA levels by 10- and 27-fold, respectively (Table 2; Figure 6). It markedly lowered LDL-C

\begin{figure}[h]
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\caption{Plasma cholesterol and serum ALT levels in heterozygous LDLR-deficient monkey treated by an optimal procedure. Monkey #19269 was treated with a single injection of HDAd-LDLR at a dose of 1 × 10^{12} vp kg^{-1} while the balloon was inflated. (a) Plasma cholesterol level. Plasma cholesterol level was 10.40 mmol l^{-1} (402 mg dl^{-1}) before gene transfer. (b) Serum ALT levels. Normal range (5–61 IU l^{-1}) is shown by filled area.}
\end{figure}
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DISCUSSION

FH is the most common and severe form of monogenic lipid disorder. Because of its severity and limited availability of conventional therapeutic options, homozygous FH has been an important candidate disease for gene therapy. We and others reported that in a mouse model of homozygous FH, hepatic delivery of LDLR gene by i.v. HDAd or AAV inhibits atherosclerotic lesion progression and may effect lesion regression.9,10,22,23 As a nonhuman primate model of LDLR deficiency exists only in the heterozygous form, we used the LDLR+/− monkeys as a model to test the feasibility of LDLR gene therapy in heterozygous FH, a much more common but serious disorder. Initially, we delivered the HDAd via i.v. injection, a minimally invasive and preferred route of administration. However, i.v. injection requires a high dose, approximately half the lethal dose in baboons,19 to be effective (Figure 1). At 5 × 10^{12} vp kg^{-1}, i.v. HDAd-LDLR led to a 60% reduction in plasma cholesterol on day 7. However, the hypocholesterolemic effect was short lived and by day 42 plasma cholesterol level had returned to pre-injection levels. A higher dose (8.4 × 10^{12} vp kg^{-1}) caused severe acute toxicity, as reported in a baboon that received a similar lethal dose.19

To increase hepatic transduction efficiency and reduce dose-dependent toxicity, Brunetti-Pierri et al.17,18,24 developed a catheter-based balloon occlusion method in baboons. We applied this approach to our rhesus monkey model. We first tested the procedure by injecting saline into two monkeys (one normal LDLR+/+ and one LDLR+/− monkeys) to determine their response to the procedure. The animals tolerated the procedure well. An acute increase in IL-6 was found in a LDLR+/+ monkey as previously documented in baboons17 but not in a LDLR+/− monkey, suggesting mild but variable responses of animals to the procedure itself. We then treated two LDLR+/+ and two LDLR+/− monkeys that had been fed a rhesus Western (high-cholesterol) diet for 2.5 months. Only one monkey showed a therapeutic response. In this monkey, when we injected the HDAd immediately after the IVC balloon was deflated, the plasma cholesterol went down by 57% at day 6. Therefore, balloon catheter occlusion method appears to be ~ 2.5-fold more effective than peripheral i.v. injection (2 × 10^{12} vp kg^{-1} vs 5 × 10^{12} vp kg^{-1}) in reducing plasma cholesterol (Figure 4). Unexpectedly, the LDLR+−/− monkey (#19498) that showed a cholesterol-lowering response developed a delayed rise in plasma ALT level at day 45 after the gene therapy, which lasted until day ~ 100. This transient rise in ALT was followed by the attenuation of the HDAd-LDLR-induced plasma cholesterol normalization and return of hypercholesterolemia. Contrary to our observations in rhesus monkeys, the previous study in baboons using a similar balloon procedure led to prolonged transgene expression that was detectable for at least 963 days at a dose of 3 × 10^{10} vp kg^{-1} that is 70-fold lower than the dose used here in rhesus monkeys. We considered the unforeseen side effects of the high-dose HDAd and heterogeneous response possibly resulting from the intrahepatic venous pressure not being maintained during vector injection, and reduced the dose to 1 × 10^{12} vp kg^{-1} but kept the balloon inflated to maintain elevated intrahepatic venous pressure during vector injection. This modification in protocol led to a slightly more intense cholesterol-lowering effect using half the HDAd-LDLR dose (Figure 5). Again, however, we observed a mild transient serum ALT elevation from day 50 to day 70, followed by a gradual return of the previously normalized plasma cholesterol to pre-treatment (elevated) levels by day 120.

It is not clear what causes the increase of liver enzymes and subsequent loss of LDLR gene transfer effects. We induced overexpression of the monkey LDLR gene in LDLR+−/− monkeys so that humoral immunity to the expressed LDLR would not be an issue, although it is possible that there are individual variations in antigen (or transgene product) processing and presentation. Interestingly, Brunetti-Pierri et al.18 reported that rhesus monkeys treated with HDAd expressing human factor IX at a dose of 1 × 10^{12} vp kg^{-1} expressed human factor IX for up to 1029 days despite development of neutralizing antibodies. There is a fundamental difference in the nature of therapeutic proteins and levels required to reverse phenotype between FH and hemophilia B individuals. LDLR is a membrane protein and the LDLR activity in vivo (Table 2; Supplementary Figure S2).

Table 2. Effects of gene therapy on plasma cholesterol and LDL metabolism

| Pre-treatment | Post-treatment |
|---------------|---------------|
| #19498        |               |
| Plasma cholesterol (mmol l^{-1}) | 7.60 | 4.75 |
| LDL cholesterol (mmol l^{-1}) | 5.90 | 3.10 |
| HDL cholesterol (mmol l^{-1}) | 1.75 | 1.75 |
| Relative LDLR mRNA expression | 1.37 | 14.32 |
| LDL apoB FCR (pool per day) | 0.230 | 0.410 |
| LDL apoB production rate (mg kg^{-1} per day) | 11.28 | 11.52 |
| #19269        |               |
| Plasma cholesterol (mmol l^{-1}) | 9.60 | 4.50 |
| LDL cholesterol (mmol l^{-1}) | 6.80 | 2.25 |
| HDL cholesterol (mmol l^{-1}) | 2.75 | 2.30 |
| Relative LDLR mRNA expression | 0.47 | 12.64 |
| LDL apoB FCR (pool per day) | 0.276 | 0.415 |
| LDL apoB production rate (mg kg^{-1} per day) | 10.32 | 10.80 |

Abbreviations: FCR, fractional catabolic rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LDLR, LDL receptor. The pre-treatment studies were done 10 days before gene therapy and the post-treatment studies were performed 79 days after gene transfer. Both were heterozygous LDLR+/− monkeys. #19498 was injected by 2 × 10^{12} vp kg^{-1} HDAd-LDLR after balloon was deflated, whereas #19269 was treated by 1 × 10^{12} vp kg^{-1} while balloon was inflated.

(by 47 and 67%, respectively; Table 2) and raised the LDL apoB fractional catabolic rate in both monkeys by 78 and 50%, respectively, indicating that the hypocholesterolemic response to the gene therapy was the result of markedly increased LDLR activity in vivo (Table 2; Supplementary Figure S2).
to therapeutic proteins are most likely not relevant to our findings, we cannot completely exclude such a possibility.

Host immune responses to the HDAd vector itself could be another factor in the delayed failure of treatment. A similar silencing of transgene expression following an asymptomatic increase of transaminases has been reported in clinical trials of factor IX gene therapy using AAV. It was attributed to pre-existing T cells to AAV capsids, which were reactivated on AAV-mediated gene transfer, eliminating the transduced cells. This response appears to be dose dependent. We studied the responses of peripheral blood mononuclear cells against immunogenic Ad hexon peptides before and after HDAd administration. We did not find any significant T-cell responses. The dose used in this experiment did not influence plasma cholesterol levels, which suggests that this dose is below the minimum effective dose for LDLR gene therapy. At such a low dose, HDAd vector does not re-stimulate pre-existing memory T cells despite elevated neutralizing antibodies. In support of this explanation, the frequent presence of memory T cells against human adenovirus has been reported in humans, whereas nonhuman primates have very low frequency of adenovirus-specific T cells in peripheral blood mononuclear cells. However, high frequency of pre-existing adenovirus-specific T cells in livers has been reported after active immunization. Therefore, the responses of T cells isolated from peripheral blood mononuclear cells may not be sufficient to detect pre-existing cellular immunity against Ad in rhesus monkeys. However, we exhausted available animals after the last experiment and there are no more LDLR+/− monkeys available to further evaluate the possibility of cellular immunity by treating LDLR+/− monkeys at a dose of 1 × 10^{12} vp kg^{-1} or higher, which could replicate the increase of liver enzymes preced ing diminished effects of LDLR gene therapy. Therefore, the cause of the transient nature of the efficacy of LDLR gene therapy in this study remains speculative. Nonetheless, if the cause of our findings is related to the pre-existing cellular immunity against HDAd vector, a possible solution is suggested by a recent clinical trial of AAV-mediated transfer of factor IX for hemophilia B, where short-term glucocorticoid administration normalized liver enzymes and maintained factor IX level.

Ad vectors are recognized by the host innate immune system during viral entry and replication in host cells. We did not measure cytokine levels in the two monkeys (#19498 and #19269) when they showed asymptomatic increase of ALT. Although the innate immune response reactions have been reported in early phase but not in late-phase toxicity associated with Ad, we cannot completely exclude such a possibility.

Despite the disappointment from the unexpected transient elevation of ALT followed by the loss of efficacy of LDLR gene therapy, we showed that HDAd-mediated LDLR gene therapy works in a nonhuman primate model of FH. We performed functional assay for LDLR activity 10 days before and 79 days after gene transfer. The two monkeys that showed a good cholesterol-lowering response displayed markedly higher hepatic LDLR mRNA expression concomitant with an accelerated LDL fractional catabolic rate. This result supports a substantial functional enhancement. It is important to note that we took advantage of a natural nonhuman primate model of heterozygous FH after our initial experiments in FH mouse models. Not only are there differences in immune responses between rodents and humans, there are also major differences in lipoprotein physiology between these species. Critically important are hemodynamic forces, which cause vascular site-specific effects on atherosclerosis. Thus, it is difficult to extrapolate the effect of gene therapy on atherosclerosis development in rodent models to that in nonhuman primates and humans.

Proprotein convertase subtilisin-kexin type 9 (PCSK9), a secreted protease that mediates degradation of LDLR, has attracted much attention as a therapeutic target for treating hypercholesterolemia. Both monoclonal antibodies and small interfering RNA (siRNA) have been reported to reduce LDL-C. Recently, the phase 2 trial targeting PCSK9 using a monoclonal antibody was reported to have achieved substantial further LDL-C reduction in patients with heterozygous FH who were treated with high-dose statins and raised the question whether inhibition of PCSK9 will be a treatment of choice for FH. ApoB100, the major protein component of LDL, is another potential therapeutic target. The use of lipid-encapsulated siRNA targeting apoB100 was found to silence apoB mRNA in rodents and nonhuman primates. Alternatively, AAV expressing apoB mRNA-specific shRNA produced long-term apoB silencing and LDL-C lowering in mice. It is unclear which of these therapeutic approaches will turn out to be the most safe and efficacious therapies to lower plasma lipids in FH patients.

In summary, we have found that a single intrahepatic arterial injection of HDAd expressing LDLR accompanied by balloon catheter-based hepatic venous occlusion method corrects hypercholesterolemia in nonhuman primate model of heterozygous FH. Nevertheless, an invasive nature of the procedure, the narrow margin between the effective and the toxic dose and the delayed immune response that could be associated with delayed treatment failure remain a significant challenge.

MATERIALS AND METHODS

Recombinant helper-dependent adenoviral vector

Seed stock of HDAd expressing rhesus monkey LDLR was prepared as described and large-scale vector production was carried out using a suspension system. Helper virus contamination and potential rearrangement were determined by quantitative PCR using SYBR Green (Quanta Biosciences, WVR, Dallas, TX, USA) and Southern blot analysis. The infectious titer of HDAd was defined by relative infectivity to an Adenovirus Type 5 Reference Material (VR-1516, American Type Culture Collection (ATCC)) in competition to infect HEK293 cells, except quantification of vectors by quantitative PCR. Helper virus contamination measured by real-time PCR was 0.05–0.01% and the ratio of viral particles and infectious particles was ~15:1. Endotoxin levels tested by Limulus amebocyte lysate was < 0.05 EU ml^{-1}.

Nonhuman primates

Normal LDLR+/+ and heterozygous LDLR+/- rhesus monkeys were housed at the Southwest Foundation for Biomedical Research. Animals of both sex between 3 and 6 kg body weight (Table 1) were fed rhesus Western type diet (40% calories from saturated fat and 0.3 mg kcal^{-1} cholesterol) for 7 weeks before initiating the sampling schedule and maintained on the diet through the experiment. Animals used for balloon occlusion-based injection into hepatic artery are summarized in Table 1. All animal protocols were performed according to the guidelines of Institutional Animal Care and Use Committee at the Texas Biomedical Research Institute.

Direct vector delivery into hepatic artery

HDAd was directly delivered to hepatic artery after hepatic venous flow occlusion (Figure 2a) as described by Brunetti-Pierri et al. In brief, a 4 French sheath was placed in the right femoral vein, an 11 French sheath in the left femoral vein and a 4 French sheath in the left femoral artery by standard percutaneous technique. A 20-gauge arterial catheter was placed in the femoral artery for continuous monitoring of blood pressure. The custom-made 8 × 3 cm² balloon occlusion catheter (NuMED, Hopkinton, NY, USA) was introduced into the right femoral vein sheath and positioned in the IVC with the tip just within the IVC-right arterial junction. The placement of the balloon was visualized using fluoroscopy after inflating
the balloon catheter (Figures 2b and c). HDAd or saline in a volume of 2 ml was injected at a rate of 0.5 ml 15 s⁻¹ while balloon was inflated through a catheter placed in the hepatic artery. The catheter was flushed with 20 ml of saline; the balloon remained inflated for additional 5 min and then deflated. The occlusion was monitored by venous pressure (Figure 2d).

Assay for cytotoxic T lymphocytes
Cytotoxic T lymphocyte activity was measured by IN-F-γ secretion by lymphocytes on stimulation with adenoviral peptides. 40, 41 Blood (5 ml) was collected with preservative-free heparin at −21, 34 and 70 days post-treatment and lymphocytes and plasma were isolated using lymphocyte separation medium (Lymphoprep, Axis-Shield, Dundee, Scotland). Lymphocytes were frozen at −80 °C in freezing medium until use. Cells were thawed and incubated overnight. Pools of 188 overlapping 20-mer peptides derived from immunodominant virion protein, hexon (JPT Peptide Technologies, Berlin, Germany), 42 were added to the culture next day, and the secreted IFN-γ was captured by the immobilized antibody using a kit from R&D Systems, Inc. (Minneapolis, MN, USA; cat#EL961). After the formation of colored spots, the membrane was sent to ZellNet Consulting, Inc. (Fort Lee, NJ, USA) for the analysis.

Assay for neutralizing antibodies
The neutralizing antibody titer was determined by an in vitro transduction-inhibition assay. In brief, cells (116 cell line) were plated in a 96-well plate at the density of 1 x 10⁵ cells per well 2 days before infection. Plasma was heat inactivated at 55 °C for 30 min and serially diluted into a 96-well plate (0.1 ml per well). HDAd vector expressing EGFP (enhanced green fluorescent protein) under elongation factor-1 promoter (HDAd-EGFP) was diluted to 2 x 10⁶ vp ml⁻¹ and 0.1 ml of the diluted HDAd vector per well was added to the 96-well plate containing diluted plasma. The plate was incubated at 37 °C for 1 h and then 0.1 ml of plasma/HDAd-EGFP mixture was transferred to wells of a 96-well plate containing the 116 cells. After 30 min, 0.1 ml of growth medium was added and incubated in CO₂ incubator for 20 h and the fluorescence was measured by FLUO Star Omega microplate reader (BMG Labtech Inc., Durham, NC, USA).

Kinetic analysis
LDL (d= 1.019–1.063) was isolated from donor monkeys, iodinated and intravenously injected into vector-treated monkeys. 49 LDL apoB turnover data collected for plasma and urinary radioactivity at designated times were analyzed using a two-compartment model, which is characterized by a plasma compartment and an extravascular exchange compartment. 49

Other procedures
Serum IL-6 concentrations were determined by Specialty Laboratories (Santa Monica, CA, USA). LDLR mRNA levels in needle biopsies of liver were quantified by TaqMan RT-PCR and normalized to β-actin using human probes (Life Technologies, Grand Island, NY, USA).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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