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Delivery of human apolipoprotein (apo) E to liver by an [E1⁻, E3⁻, polymerase⁻, pTP⁻] adenovirus vector containing a liver-specific promoter inhibits atherogenesis in immunocompetent apoE-deficient mice

Research Article

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Abbreviations: apoE-deficient, (apoE$^{-/-}$); apolipoprotein E, (apoE); ATP-binding cassette transporter A1, (ABCA1); CD8$^+$ T lymphocytes, (CTLs); cytomegalovirus, (CMV); fetal bovine serum, (FBS); glyceraldehydes-3-phosphate dehydrogenase, (GAPDH); hepatic nuclear factors, (HNF); liver-specific promoter, (LSP); multiplicity of infections, (MOI); recombinant adenovirus, (rAd); scavenger receptor-B1, (SR-B1); virus particle, (vp)

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Summary

Recombinant adenovirus (rAd)-mediated apoE gene transfer to the liver of apoE$^{-/-}$ mice is anti-atherogenic. However, first generation rAd vectors were associated with immune clearance of transduced hepatocytes, while an improved [E1$^-$, E3$^-$ polymerase$^-$] adenovirus vector that persisted in the liver, had transient effects due to cellular shutdown of the cytomegalovirus (CMV) promoter (Ad-CMV-apoE). Here, we utilise an improved class of rAd vector with multiple deletions in the E1, E3, polymerase and pTP (pre-terminal protein) genes, which contains a modular synthetic liver-specific promoter (LSP) to drive expression of the human apoE cDNA (Ad-LSP-apoE) for hepatic gene transfer. Approximately 1 year old apoE$^{-/-}$ mice were injected intravenously with 4x10^{10} virus particles of either Ad-LSP-apoE or Ad-CMV-apoE. Animals were monitored for plasma apoE, total plasma cholesterol and plasma lipoprotein distribution. The effect of Ad-LSP-apoE on atheroma progression was assessed in animals killed at 8 and 28 weeks after the injections. Ad-LSP-apoE vector administration gave sustained, though low, levels of plasma apoE throughout the study period without inducing a humoral immune response, but failed to reduce plasma cholesterol or normalize the adverse lipoprotein profile. Animals killed 8 weeks after the injections, demonstrated no significant retardation of atherosclerosis, whereas aortic lesions in those killed at 28 weeks were significantly reduced by 30% (P<0.006) compared to untreated animals. In summary, the combination of a multiply deleted rAd vector with a liver-specific promoter provided sustained low levels of plasma apoE, resulting in significant retardation of aortic atherosclerotic lesions.

I. Introduction

Apolipoprotein E (apoE) is a 34 kDa plasma glycoprotein and is a major component in lipoprotein homeostasis and in the protection against the development of atherosclerosis. It mediates the hepatic clearance of atherogenic remnant lipoproteins (Mahley and Rall, 2000) and is involved in reverse cholesterol transport, where excess cholesterol from arterial and other peripheral tissues is transported to the liver for excretion (Fielding and Fielding, 1995).

The apoE-deficient (apoE$^{-/-}$) mouse has severe hypercholesterolaemia and spontaneously develops the full

range of atherosclerotic lesions (Piedrahita et al, 1992; Nakashima et al, 1994). First generation rAd vectors for liver-directed apoE gene transfer, resulted in transient correction of the hypercholesterolaemia and protection against atherosclerosis in the apoE$^{-/-}$ mouse (Kashyap et al, 1995; Stevenson et al, 1995). However, low level expression of viral genes still present in the adenovirus vector (Yang et al, 1994a,b, 1995, 1996a; Dai et al, 1995) and expression of a foreign transgene (Tripathy et al, 1996; Morral et al, 1997; Song et al, 1997) resulted in an immune response against adenovirus-transduced hepatocytes, causing rapid loss of transgene expression. Improvements in rAd vector design, including removal of certain sequences in the vector genome, reduced hepatotoxicity and allowed longer apoE transgene expression in apoE$^{-/-}$ mice (Tsukamoto et al, 1997, 1999; Harris et al, 2002a). This work included our own study on intravenous (liver-directed) injections of a [E1$^-$, E3$^-$, polymerase$^-$] rAd vector containing the CMV promoter driving expression of human apoE, resulting in acute regression of advanced and retardation of early aortic atheroma with normalization of the hyperlipidaemic phenotype (Harris et al, 2002a). However, apoE transgene expression was transient due to CMV promoter shutdown reflected in the rebound of plasma cholesterol and the re-accumulation of atherogenic remnant lipoprotein particles to pretreatment levels. CMV promoter shutdown is a recognised feature of transgene expression driven by virus-derived promoters such as CMV or Rous sarcoma virus (RSV) promoters, particularly in the liver (Kay et al, 1992; Guo et al, 1996; Qin et al, 1997; Loser et al, 1998).

To avoid promoter shutdown, we have constructed a [E1$^-$, E3$^-$, polymerase$^-$, pTP$^-$] rAd vector, containing a modular liver-specific promoter (LSP) (Ill et al, 1997; Wang et al, 1999, 2000), driving expression of human apoE and have performed intravenous (liver-directed) injections into ~1 year old apoE$^{-/-}$ mice. Despite low levels of plasma apoE that were unable to correct the hypercholesterolaemia, animals sacrificed 7 months after treatment demonstrated significant retardation of atherosclerosis (30%, P <0.006), compared to untreated endpoint control animals.

II. Materials and Methods

A. Recombinant adenovirus construction

Construction of pShuttle-LSP-pA has been described (Ding et al, 2002). The *Hind*III and *Eco*RV subfragment containing the apoE cDNA from pShuttle-CMV-apoE (Harris et al, 2002a) was ligated into the *Sal*1 (blunt-end filled)-*Hind*III digested pShuttle-LSP-pA to yield pShuttle-LSP-apoE-pA. The [E1$^-$, E3$^-$, polymerase$^-$] Ad-CMV-apoE vector was generated as previously described (Harris et al, 2002a). The pShuttle-LSP-apoE-pA construct was linearised with *Pme*1 and homologously recombined with the adenoviral plasmid containing deletions in the E1, E3, polymerase and pTP genes (Everett et al, 2003), to generate the recombinant [E1$^-$, E3$^-$, polymerase$^-$, pTP$^-$] adenoviral plasmid pAd-LSP-pA. The rAd plasmids were linearised with *Pac*1 and transfected into C7 cells (a derivative of human 293 cells that have been engineered to express both the adenovirus polymerase and pre-terminal protein genes), to generate the rAd vectors and high titre virus stocks were prepared using routine procedures (Graham and Prevec, 1995).

B. Ad-LSP-apoE transduction of hepatic and non-hepatic cell lines

The human hepatic carcinoma cell line HepG2 and the non-hepatic cell lines (NIH3T3, HeLa, C2C12) were maintained in DMEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B at 37°C and 8% CO_2. The HepG2, NIH3T3, HeLa and C2C12 cell lines were seeded into 6-well plates at densities of $2x10^6$, $1.5x10^5$, $3x10^5$ and $1.5x10^5$ per well, respectively. The following day the near-confluent monolayers were infected with the same dilutions of Ad-LSP-apoE at the virus particle (vp) multiplicity of infections (MOI) indicated in **Figure 2**, as previously described (Harris et al, 2002a). Two days after the infections the medium was harvested and analysed by Western blotting for secreted apoE (Harris et al, 2002a).

C. Intravenous administration of adenovirus vectors

Female C57BL/6 apoE$^{-/-}$ mice (Piedrahita et al, 1992) were provided by GlaxoSmithKline (Stevenage, UK) and were maintained on a normal chow diet. The Ad-LSP-apoE and Ad-CMV-apoE vector stocks were diluted appropriately for intravenous injection using diluent containing 10 mM Tris HCl pH 8.0, 2 mM $MgCl_2$ and 0.9% (w/v) NaCl. Blood (~50 μl) was taken from the tail-vein following 4 h fasts, anti-coagulated with sodium citrate and the plasma stored at -80 °C.

To determine the effect of liver-restricted *apoE* expression upon the hyperlipidaemic phenotype and atheroma progression over a 2 month study period, animals at 15 months of age (n=6) were each administered intravenously with Ad-LSP-apoE at $4x10^{10}$ vp in 200 μl. Blood (~50 μl) was taken from the tail-vein following 4 h fasts at 1, 6 and 8 weeks after the injections when animals were killed to examine the effect of *apoE* gene transfer upon aortic atherosclerotic lesion progression. Groups of untreated baseline (n=7) and endpoint (n=6) animals were also included.

To assess whether the Ad-LSP-apoE vector is capable of sustained apoE expression over an extended period, providing continual protection against the development of atherosclerosis, animals at 12 months of age were injected intravenously with either Ad-LSP-apoE (n=5) or Ad-CMV-apoE (n=6) at $4x10^{10}$ vp in 200 μl and bloods taken at 7 weeks after the injections then all animals were sacrificed at 28 weeks to examine the extent of atheroma progression. Untreated baseline (n=8) and endpoint (n=7) mice were also included. As to the difference in the age of the animals in the 2 month and 7 month studies at the time of vector administration, apoE$^{-/-}$ mice at 12 months of age have established advanced/complex atherosclerotic lesions which do not differ significantly in animals at 15 months of age (Reddick et al, 1994).

D. RT-PCR of apoE transcription

To determine the presence of human apoE gene transcription in apoE$^{-/-}$ mice injected intravenously with the adenovirus vector Ad-LSP/apoE, total RNA was isolated from the livers of animals sacrificed at 8 weeks (n=6) and 28 weeks (n=5) after the injections. Total RNAs were isolated from livers using Trizol Reagent (Invitrogen, Paisley, UK), according to the manufacturer's instructions. DNaseI-treated RNAs (5 μg) were reverse transcribed in a 50 μl reaction containing 62.5 U GeneScript Reverse Transcriptase, 2.5 μM oligo dT(15)-Y-N primer, 0.4 U/μl RNase inhibitor, 0.5 mM dNTPs, 5mM $MgCl_2$ and 1x Excite™ reaction buffer (GeneSys Ltd, Farnborough, UK). The reaction conditions were 25°C for 10 min, 48°C for 40 min, then 95°C for 5 min. The derived cDNAs were used to

amplify a 155 bp product using a primer set specific for the human apoE sequence (apoE primer 1, 5'-CTGGGAACTGGCACTGGG-3'; apoE primer 2, 5'-CCGATTTGTAGGCCTTCAAC-3'). Also, amplification of a 68 bp product derived from the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) using a published primer set was performed (DeGeest et al, 2001), to demonstrate the presence of equivalent copies of GAPDH transcripts between the RNA preparations. For the apoE and GAPDH amplifications, each 50 µl reaction contained 45 µl Accurase PCR Master Mix (GeneSys Ltd) and the remaining 5 µl contained 3 µl cDNA and 10 pmol of each primer of the appropriate primer set. The reaction conditions were 92°C for 2 min, then 25 cycles of 92°C for 20 s, 62°C (for apoE) or 60°C (for GADPH) for 20 s, then 72°C for 20s, followed by a final extension of 72°C for 10 min. The PCR reactions were subjected to 2% agarose gel electrophoresis and the products visualised by ethidium bromide staining.

E. Quantification of plasma apoE and cholesterol and analysis of lipoprotein distribution

Levels of human apoE in mouse plasma were monitored by Western blotting as previously described (Harris et al, 2002a) and quantified by a two-antibody sandwich ELISA kit (Stratech Scientific Ltd, Cambridge, UK). Plasma samples for ELISA were diluted 1/10 in assay diluent solution and apoE concentrations were determined according to the manufacturer's instructions; the sensitivity for detection of apoE was 4 ng/ml. Total cholesterol was measured in plasma diluted 1/10 in PBS (10 µl) using a commercial enzymatic kit (Infinity™ cholesterol reagent, Sigma-Aldrich, Poole, UK) and microtitre plates. Lipoprotein profiles were evaluated by electrophoresis of individual plasma samples (10 µl) on pre-cast alkaline-buffered (pH 8.8) 0.8% agarose gels (YSI, Farnborough, UK), followed by staining with Sudan black, as previously described (Harris et al, 2002a).

F. Dissection and examination of the aortic arch for atherosclerotic lesions

Animals in the short-term (8 week) and long-term (28 week) studies of rAd-mediated *apoE* gene transfer were killed and their aortae were removed, pinned out *en face* onto cork beds and stained with Oil-Red-O. Images of the aortae were captured with a Nikon digital camera fitted to a stereoscopic zoom microscope with fibre optic light illumination, and the lesion areas analysed as previously described (Harris et al, 2002a).

G. Detection of anti-human apoE and anti-adenovirus antibodies by Western blotting analysis

For the detection of anti-human apoE and anti-adenovirus antibodies, mouse plasmas were pooled in each of the animal groups and 3 µl was diluted in 600 µl of incubation buffer (Tris-buffered saline containing 2.5% (w/v) milk powder, 0.05% (v/v) Tween-20 and 0.2% (w/v) 2-chloracetamide). Hybond-PVDF Western blots of either partially purified human apoE (~1µg) or Ad-LSP-apoE virus stock ($1.88x10^{10}$ vp) were used to screen the plasmas for the respective anti-human apoE and anti-adenovirus capsid antibodies, using the Mini-Protean® II Multi-Screen apparatus as described previously (Harris et al, 2002a).

H. Statistical analysis

Results are expressed as the mean ± S.E. Student's unpaired 2-tailed *t*-test was used to compare total plasma cholesterol levels and aortic lesion areas in mice treated with Ad-LSP-apoE or Ad-CMV-apoE, against the untreated baseline and endpoint control groups; $P<0.05$ was considered significant.

III. Results

A. Ad-LSP-apoE transduction of hepatic and non-hepatic cell lines

To assess the liver specificity of LSP driving expression of human apoE, serial dilutions of the [E1⁻, E3⁻, polymerase⁻, pTP⁻] rAd vector Ad-LSP-apoE (**Figure 1**) were used to infect and engineer human hepatoblastoma HepG2 cells and the non-hepatic cell lines NIH3T3, HeLa and C2C12 to secrete human apoE into the culture supernatant. Two days after the infection culture supernatants from the infected HepG2 cells contained apoE above endogenous levels (as indicated by culture supernatants from the mock-infected and untreated HepG2 cultures) in a virus dose-dependent manner (**Figure 2**). Furthermore, apoE was absent in culture supernatants from the Ad-LSP-apoE-infected cultures of the non-hepatic cell lines NIH3T3, HeLa and C2C12 cells.

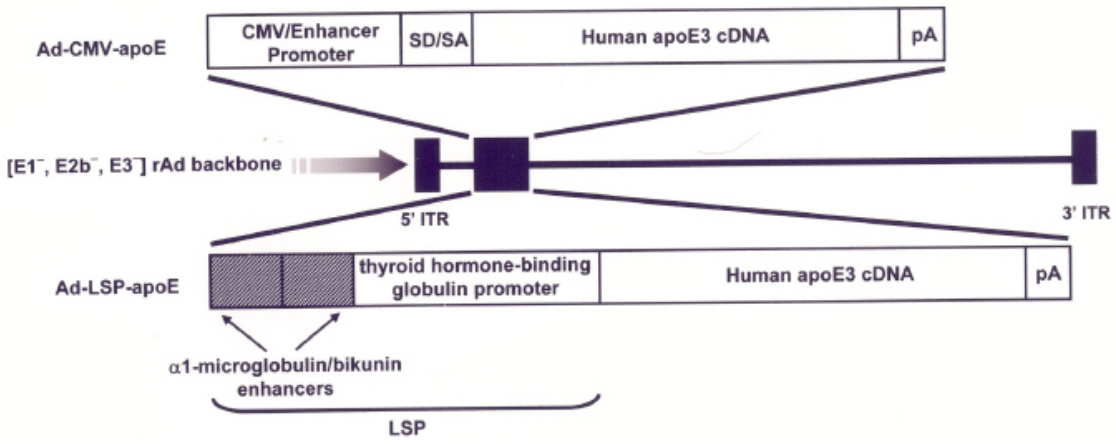

Figure 1. Schematic representation of the adenovirus vectors Ad-CMV-apoE and Ad-LSP-apoE. Both transgene cassettes drive expression of the human *apoE3* cDNA and are located at the 5' end of the [E1⁻, E2b⁻, E3⁻] Ad vector genome replacing the E1-deleted region. Ad-CMV-apoE contains the full human CMV early enhancer/promoter, whereas Ad-LSP-apoE contains the modular synthetic liver-specific promoter (LSP) comprised of two copies of the α1-microglobulin/bikunin enhancer and the thyroid hormone-binding globulin promoter. pA, SV40 polyadenylation signal; ITR, inverted terminal repeat. SD/SA, SV40 intron.

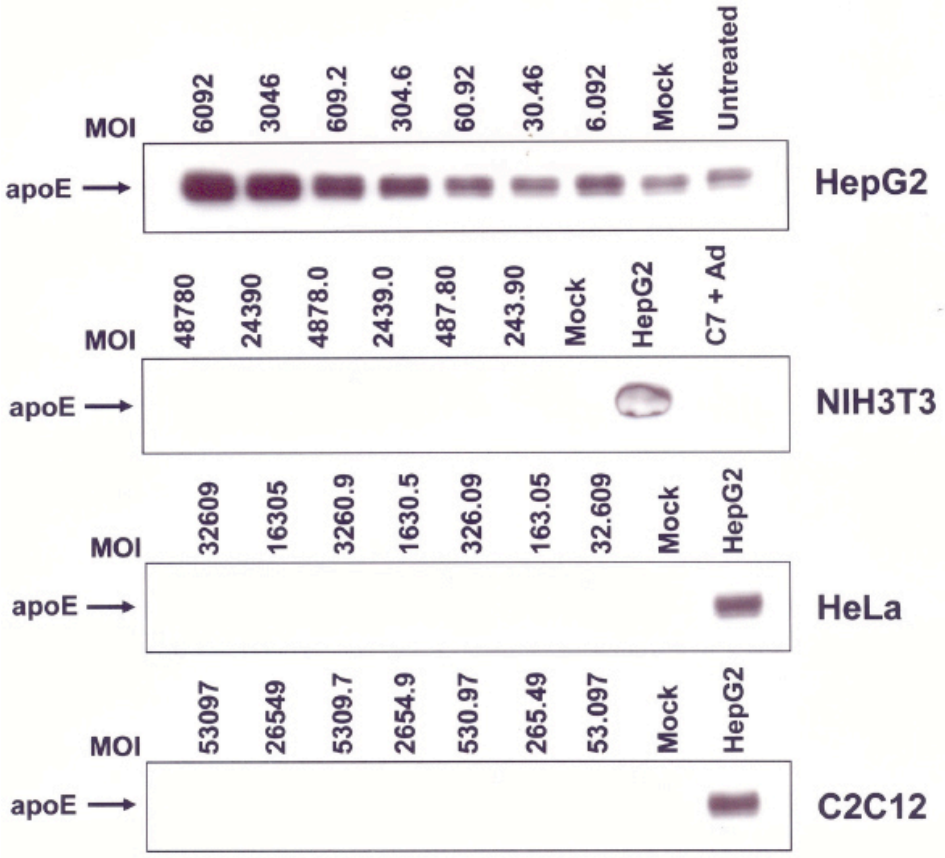

Figure 2. Ad-LSP-apoE infection of hepatic (HepG2) and non-hepatic (NIH3T3, HeLa, C2C12) cell lines followed by analysis for secretion of recombinant human apoE (34 kDa). The cell lines were infected at the indicated MOIs with the same virus dilutions. and analysed by Western blotting to detect secreted human apoE. Two days after the infections culture medium was harvested and analysed by Western blotting for the presence of apoE. HepG2 cells show apoE secretion above endogenous levels in a virus dose-dependent manner. Infection of HeLa, NIH3T3 and C2C12 cells resulted in no secretion of apoE following the Ad-LSP-apoE infections. HepG2, culture medium harvested from a HepG2 culture was used as a positive control for the NIH3T3, HeLa and C2C12 Western blots. C7 + Ad, culture supernatant harvested during propagation of Ad-LSP-apoE vector in C7 cells demonstrating 100% cytopathic effect.

B. Secretion of plasma apoE following intravenous injection of Ad-LSP-apoE and the effect on total plasma cholesterol and lipoprotein distribution

Over the course of the study, human apoE transcription was readily detectable in all animals sacrificed at 8 weeks and 4 out of 5 animals sacrificed at 28 weeks after administration of Ad-LSP-apoE (**Figure 3**). In contrast, our previous studies involving intravenous administration of Ad-CMV-apoE readily detected apoE-specific transcripts at 8 and 16 days post-injection, with a marked decline in apoE cDNAs at 70 days after vector administration (Harris et al, 2002a).

The ability of liver-directed (intravenous) injection of Ad-LSP-apoE to achieve sustained hepatic secretion of human apoE into the circulation with subsequent alteration of total plasma cholesterol levels and lipoprotein distribution, was assessed over a 8 week study period. Following intravenous vector administration, tail-vein bleeds were taken at 1, 6 and 8 weeks after the injections, with all animals being killed at 8 weeks. At all time points the plasma levels of apoE were below the sensitivity limit of the apoE ELISA (<4 ng/ml). However, Western blotting analysis, which has a detection limit of ≤1pg of antigen, demonstrated that plasma apoE was present throughout the study period in individual animals (**Figure 4a**). Additionally, a slight increase in the accumulation of plasma apoE was observed between tail bleeds at 1 week and those at 6 and 8 weeks after the injections. In order for apoE to impact on the hyperlipidaemia in the apoE$^{-/-}$ mouse, the plasma levels of apoE need to exceed 0.4 μg/ml (Hasty et al, 1999a). Therefore, in animals treated with Ad-LSP-apoE, no alteration in total plasma cholesterol levels or lipoprotein distributions were observed (**Figure 5**).

To investigate liver-specific apoE expression over an extended study period, animals were treated with either Ad-LSP-apoE (*n*=5) or Ad-CMV-apoE (*n*=6) and tail vein bleeds taken at 7 and 28 weeks post-injection, with all animals being killed at 28 weeks. Following treatment with Ad-LSP-apoE plasma apoE levels were below the detection limit of the apoE ELISA, whereas following treatment with Ad-CMV-apoE, plasma from one animal at 7 weeks and another animal at 28 weeks contained plasma apoE levels of 47 ng/ml and 69 ng/ml, respectively. Upon Western blotting analysis plasma from animals treated with Ad-LSP-apoE, demonstrated sustained but low levels

of apoE (**Figure 4b**). In the case of Ad-CMV/apoE-treated animals, residual apoE levels were still evident at 28 weeks after the injections, compared to the higher systemic levels observed in plasmas harvested 7 weeks post-injection (**Figure 4b**).

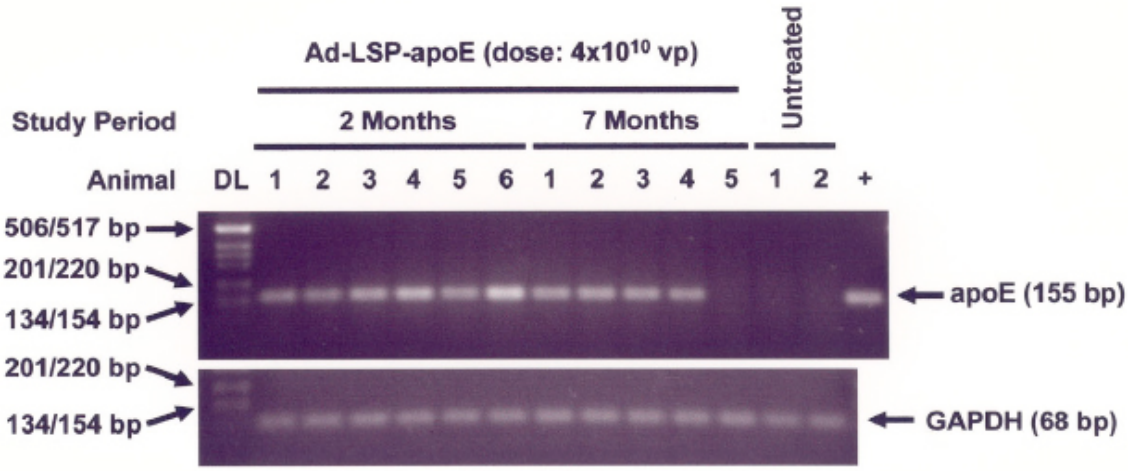

Figure 3. RT-PCR analysis of human apoE transcription in the livers of apoE$^{-/-}$ mice following intravenous injection of Ad-LSP/apoE (dose, 4x10^{10} vp). Total RNAs were isolated from the livers of animals at 2 months (*n*=6) and 7 months (*n*=5) after intravenous administration. The RNAs were subjected to reverse transcription followed by PCR amplification using primer sets specific for either the human apoE cDNA (155 bp product) or the housekeeping gene GAPDH (68 bp product). The positive control (+) is the plasmid pAd-CMV/apoE. DL, 1 kb DNA ladder with fragment sizes indicated on the left-hand side of the gel.

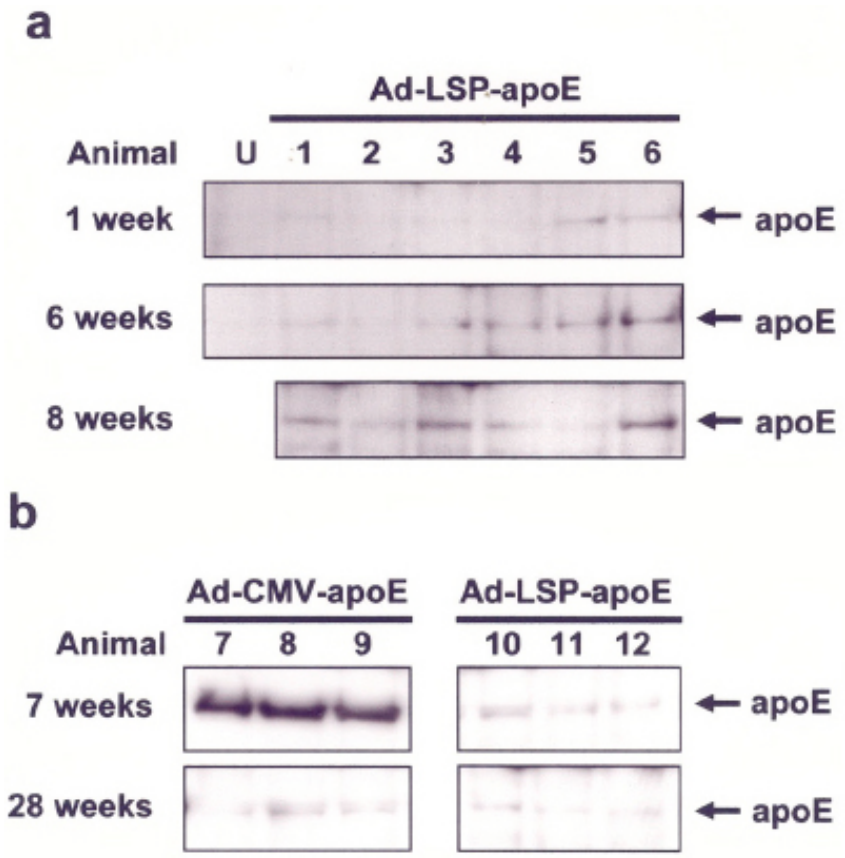

Figure 4. Immunodetection of human apoE (34 kDa) in the plasmas of apoE$^{-/-}$ mice treated with either Ad-LSP-apoE or Ad-CMV-apoE. Mice were injected intravenously with 4x10^{10} vp of rAd vector and tail-vein bleeds were taken as indicated and the presence of human apoE in plasmas samples (4 μl) was determined for individual animals by Western blotting analysis. A. Animals were treated with Ad-LSP-apoE (*n*=6) and tail-vein bleeds were taken 1 and 6 weeks, with all animals sacrificed at 8 weeks. B. Animals were treated with either Ad-LSP-apoE (*n*=5) or Ad-CMV-apoE (*n*=6) and tail vein bleeds were taken at 7 weeks, with all animals sacrificed at 28 weeks after the injections. Three representative plasmas from each group are shown. U, plasma from an untreated apoE$^{-/-}$ mouse.

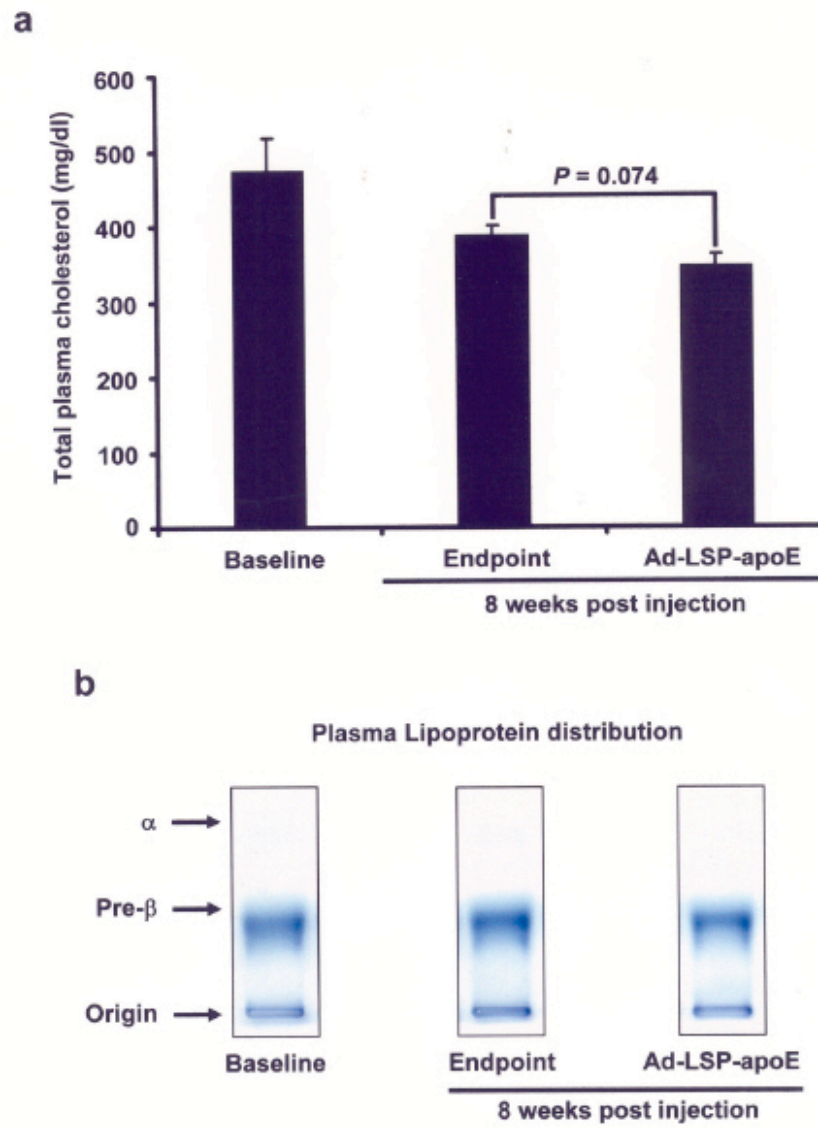

Figure 5. Analysis of total plasma cholesterol and lipoprotein distribution of 15 month old apoE$^{-/-}$ mice 8 weeks after intravenous administration with 4x10^{10} vp of Ad-LSP-apoE. (**A**) Total plasma cholesterol levels were determined for individual animals following tail-vein injections of Ad-LSP-apoE (*n*=6). Values are means ± S.E. and data from untreated baseline (*n*=7) and endpoint (*n*=5) animal groups are also shown. (**B**) Lipoprotein distribution in representative plasmas from the Ad-LSP-apoE-treated and untreated baseline and endpoint control groups. Plasma samples (10 μl) were separated by 0.8% native agarose gel electrophoresis, followed by staining with Sudan black to reveal lipoprotein mobilities and estimate relative amounts.

C. Analysis of atherosclerotic lesion progression following intravenous injection of Ad-LSP-apoE

The effect of Ad-LSP-apoE on atherosclerotic lesion progression was assessed in animals sacrificed at 8 and 28 weeks after the injections. The aortae were removed and the percentage of luminal area containing atheroma from the aortic arch down to the diaphragm which stained with Oil-Red-O was measured. Upon examination of the aortae from the animal group treated at 15 months of age with Ad-LSP-apoE and sacrificed 8 weeks later, a mean aortic lesion area of 39.3±3.3% (*n*=6) was observed, whereas untreated baseline and endpoint control groups had lesion areas of 40.4±2.7% (*n*=7) and 47.6±5.4% (*n*=5), respectively (**Figure 6a and 6b**). Although the reduced aortic lesion area of the Ad-LSP-apoE-treated animals compared to the untreated control groups was insignificant (baseline, *P*=0.8. endpoint, *P*=0.2), two of the Ad-LSP-apoE-treated animals had strikingly reduced lesion areas of 33.3% and 27.1% compared to lesion areas of the remaining treated animals (i.e. 47.0%, 40.2%, 48.0% and 40.3%).

Analysis of aortic lesions in animals treated at 12 months of age with either Ad-LSP-apoE or Ad-CMV-apoE and sacrificed 28 weeks later, had mean aortic lesion areas of 34.3±4.3% (*n*=5) and 29.7±3.3% (*n*=6), respectively (**Figure 7a and 7b**). Moreover, compared to the endpoint untreated control group (*n*=7), treatment with Ad-LSP/apoE or Ad-CMV/apoE resulted in clear retardation of atherosclerosis of 30% (*P*<0.006) and 39% (*P*<0.0002), respectively.

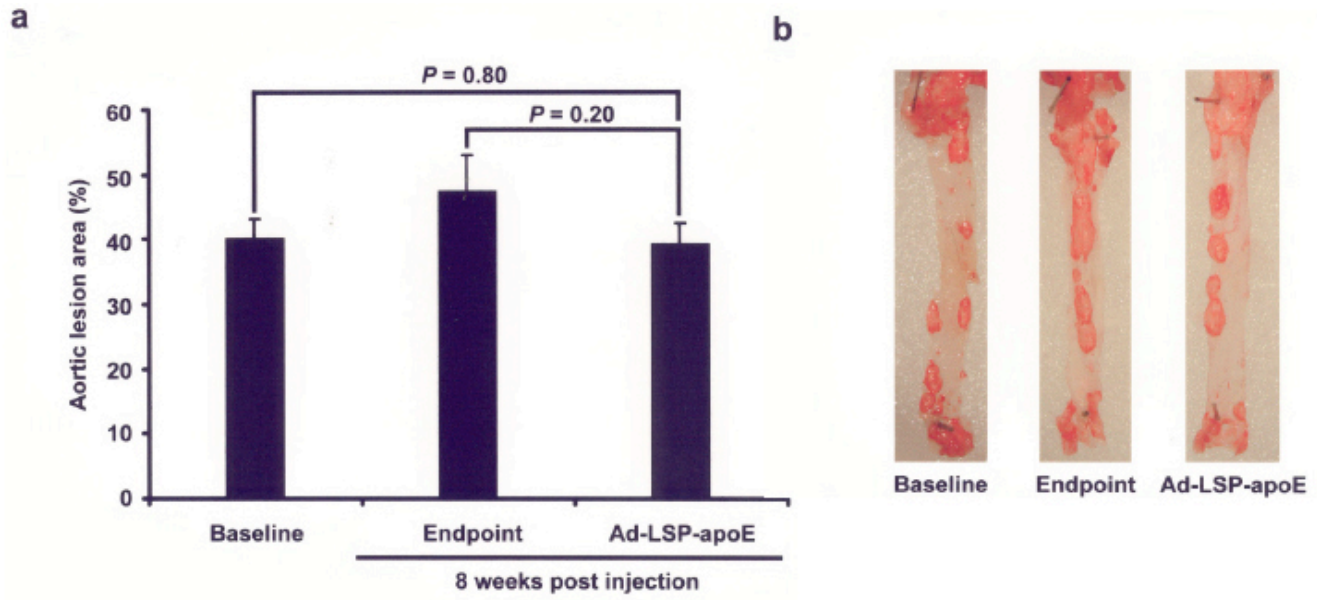

Figure 6. Quantification of atherosclerotic lesion areas in the aortae of apoE$^{-/-}$ mice 8 weeks after intravenous administration with 4x10^{10} vp of Ad-LSP-apoE. Animals were treated at 15 months of age and sacrificed 8 weeks later. The aortae were removed, dissected *en face* onto cork beds and stained with Oil-Red-O. (**A**) Mean percentage aortic lesion areas of the Ad-LSP-apoE (n=6) and the untreated baseline (n=7) and endpoint (n=5) animal groups. Values are shown as mean ± S.E. P values, 2-tailed unpaired t-test. (**B**) Representative aortae from each of the animal groups stained with Oil-Red-O.

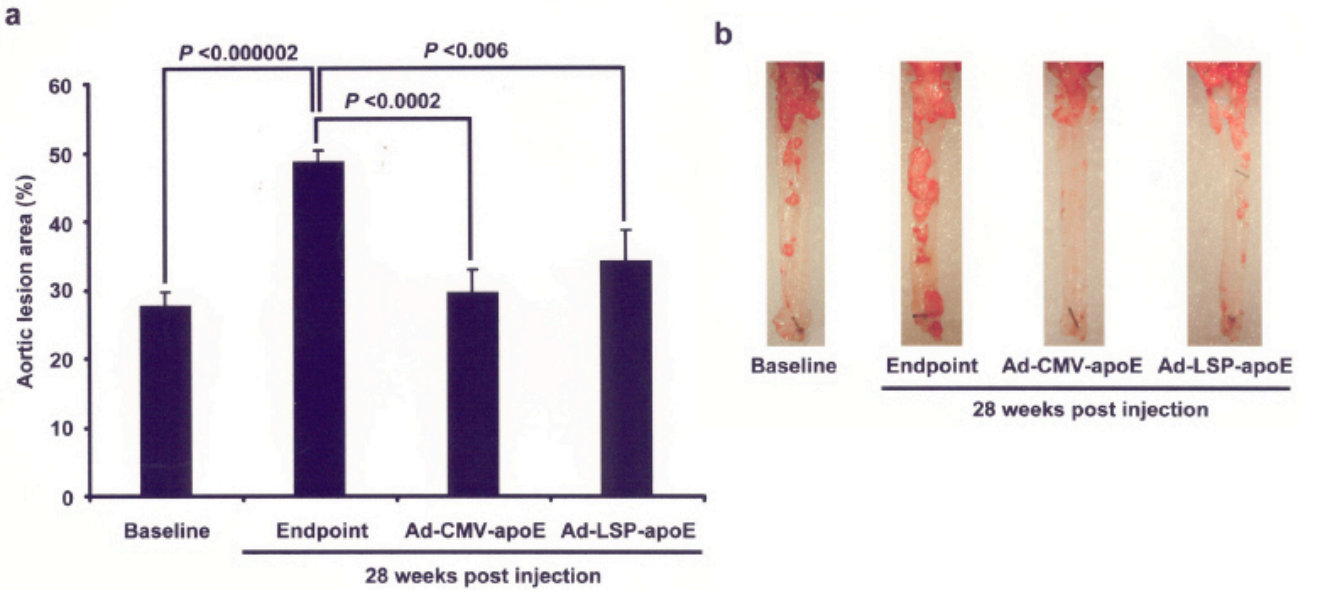

Figure 7. Retardation of advanced atherosclerotic aortic lesions in apoE$^{-/-}$ mice 28 weeks after intravenous administration with either Ad-LSP-apoE or Ad-CMV-apoE. Animals at 12 months of age were injected intravenously with either Ad-LSP-apoE or Ad-CMV-apoE at a dose of 4x10^{10} vp. Animals were sacrificed 28 weeks later and the aortae removed, dissected *en face* onto cork beds and stained with Oil-Red-O. (**A**) Mean percentage aortic lesion areas of the Ad-LSP-apoE- (n=5) and Ad-CMV-apoE- (n=6) treated groups and the untreated baseline (n=8) and endpoint (n=7) control groups. Values are shown as ± S.E. P values, 2-tailed unpaired t-test. (**B**) Representative aortae from each of the animal groups.

D. Humoral immune responses against the apoE transgene product and adenovirus vector

Throughout the course of the study, intravenous injection of either Ad-LSP-apoE or Ad-CMV-apoE did not result in the stimulation of a humoral immune response against the transgene product. However, administration of both vectors resulted in the appearance of antibodies against the adenovirus hexon capsid protein at weeks 6 and 7 respectively and persisted throughout the study period (**Figure 8**).

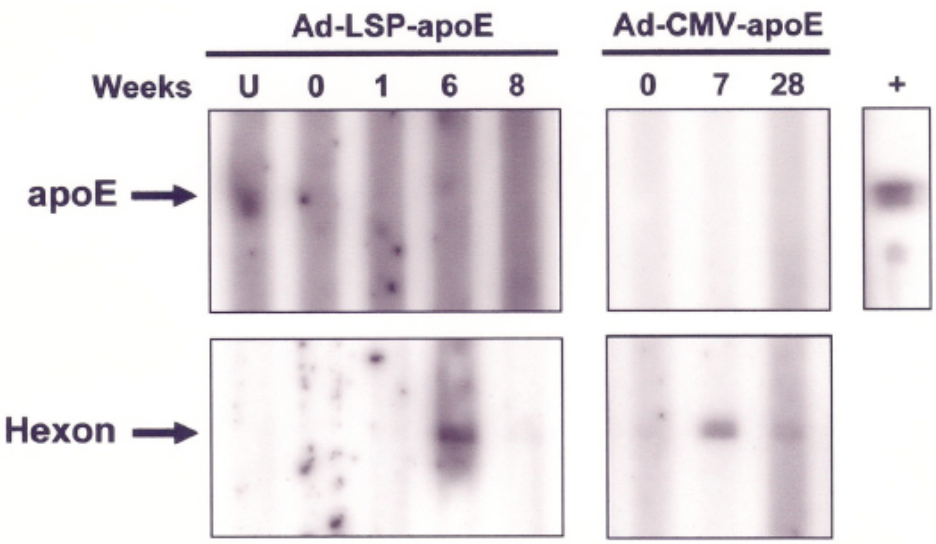

Figure 8. Analysis of humoral immune responses against the apoE (34 kDa) transgene product and the hexon (105 kDa) capsid protein of the rAd particle in transduced apoE$^{-/-}$ mice. Western blots of partially purified human apoE or adenovirus protein preparations were used to screen plasmas from animals injected intravenously with $4x10^{10}$ vp of either Ad-LSP-apoE or Ad-CMV-apoE. U, plasma from an untreated apoE$^{-/-}$ mouse. +, mouse monoclonal anti-human apoE antibody.

IV. Discussion

Nearly all apoE circulating in plasma is derived from the liver (Mahley, 1988) and therefore this organ is the natural target for apoE gene transfer in the apoE$^{-/-}$ mouse. Indeed, the liver synthesises a great variety of essential proteins, where many undergo post-translational modifications necessary for full functional activity. The human *apoE* gene is a member of the 44 kb apoE gene cluster located on chromosome 19 that also includes *apoCI*, *apoCII* and *apoCIV* and two distinct hepatic control regions (HCR1 and HCR2), that are responsible for the hepatocyte-restricted expression of these genes (Allen et al, 1997). This indicates that there are regulatory elements that provide liver-restricted expression of genes such as *apoE*. In this study, liver-restricted production of human apoE in apoE$^{-/-}$ mice was achieved by intravenous injection of a [E1^{-}, E3^{-}, polymerase^{-}, pTP^{-}] rAd vector containing the LSP driving expression of the human apoE cDNA. The LSP is a modular synthetic promoter composed of two copies of the enhancer sequence from the α_1-microglobulin/bikunin gene (Rouet et al, 1992) and the promoter sequence from the thyroxine-binding globulin gene (Hayashi et al, 1993). The α_1-microglobulin/bikunin gene contains a weak promoter with the potential for ubiquitous expression and the presence of the upstream enhancer, which contains a cluster of liver-specific elements for hepatic nuclear factors (HNF), confers full hepatocyte-restricted α_1-microglobulin/bikunin expression (Rouet et al, 1992). Indeed, such multiple *cis*-acting regulatory DNA sequences that bind these transcription factors are a common feature of genes that demonstrate strong hepatocyte-restricted expression, such as albumin and α1-antitrypsin (Lai et al, 1991). The promoter from the thyroxine-binding globulin gene was chosen as it has been fully characterised and contains a HNF-1 binding motif that imparts strong liver-specific transcriptional activity (Hayashi et al, 1993).

In this study, intravenous administration of Ad-LSP-apoE into apoE$^{-/-}$ mice resulted in sustained, albeit low, levels of plasma apoE throughout the study period (7 months) and resulted in significant retardation of aortic atherosclerotic lesion progression (30%, P<0.006). This is consistent with our previous investigations whereby intramuscular injection of apoE$^{-/-}$ mice, with either a plasmid or recombinant adeno-associated virus vector expressing human apoE, gave low levels of plasma apoE that were atheroprotective without correcting the hypercholesterolaemia (Athanasopoulos et al, 2000; Harris et al, 2002b). Similarly, transgenic mice in which apoE expression is restricted to adrenal glands or macrophages have low plasma apoE (<1-7% of wild-type levels), but are atheroprotected whilst remaining hyperlipidaemic (Thorngate et al, 2000; Hasty et al, 1999b; Bellosta et al, 1995). Intriguingly, studies in which apoE$^{+/+}$ bone marrow was mixed with apoE$^{-/-}$ marrow in increasing amounts and transplanted into apoE$^{-/-}$ recipient mice, indicated that a threshold of 0.4 μg apoE/ml plasma is required for the hepatic clearance of remnant lipoproteins (Hasty et al, 1999a). These investigations suggest that apoE can be atheroprotective by mechanisms other than its ability to lower plasma cholesterol levels.

Recombinant apoE is known to localise to the arterial intima following rAd-mediated liver transduction (Tsukamoto et al, 1999; Tangirala et al, 2001). This may explain why treatment with Ad-LSP-apoE inhibits atherosclerotic lesion progression in the presence of high levels of atherogenic lipoproteins, since atheroprotection is also seen when apoE expression in transgenic apoE$^{-/-}$ mice is restricted to the artery wall or to macrophages (Bellosta et al, 1995; Shimano et al, 1995; Hasty et al, 1999b). One proposed mechanism is that apoE promotes cholesterol efflux from the arterial wall and its transport to the liver

for excretion (Bellosta et al, 1995; Shimano et al, 1995; Hasty et al, 1999b). In support, there is evidence that macrophage-derived apoE can restore the capacity of apoE-deficient plasma to efflux cholesterol from cultured fibroblasts (Zhu et al, 1998) and that apoE facilitates interaction of apoA1 in HDL particles with scavenger receptor-B1 (SR-B1), which selectively extracts cholesteryl esters from HDL into liver (Arai et al, 1999; Owen and Mulcahy, 2002). The ATP-binding cassette transporter A1 (ABCA1) mediates the first step in reverse cholesterol transport where free cholesterol is released from peripheral cells to lipid-poor apolipoproteins (Owen and Mulcahy, 2002). However, inhibiting ABCA1 activity does not attenuate apoE-mediated cholesterol efflux (Huang et al, 2001), while ABCA1-mediated cholesterol efflux in apoE$^{-/-}$ and apoA-I$^{-/-}$/apoE$^{-/-}$ mice is not restored by low level expression of apoE (Thorngate et al, 2003). These findings suggest that apoE can be atheroprotective at the arterial wall by mechanisms that are independent of its involvement in reverse cholesterol transport. These may include apoE inhibition of platelet aggregation (Riddell et al, 1997) and smooth muscle cell migration and proliferation (Ishigami et al, 1998), the prevention of LDL retention in the subendothelial matrix (Saxena et al, 1993), and antioxidant (Mabile et al, 2003) and anti-inflammatory activities (Stannard et al, 2001). Recently, Raffai et al, (2005) demonstrated for the first time that apoE promotes regression of atherosclerosis independently of lowering plasma cholesterol. They utilized hypomorphic apoE mice that express an apoE4-like variant of mouse apoE at plasma levels that are ~2% to 5% of normal and carry the inducible Mx1-Cre apoE transgene, which allows for induction of physiological levels of apoE. After 18 weeks on a hypercholesterolaemic diet to promote a high atherosclerotic burden, animals were placed on a normal chow diet for 16 weeks, with half the animals induced to express physiological levels of apoE. Although cholesterol levels between the non-induced and induced animal groups were insignificant, the induced animals demonstrated an enhanced regression of aortic atheroma (Raffai et al, 2005).

Following systemic administration of rAd vectors, the majority of virus is rapidly cleared from the circulation by the liver (Alemany et al, 2000; Jaffe et al, 1992; Herz et al, 1993; Kay et al, 1994). The virus (which has an average diameter of ~80 nm) enters the liver sinusoids via the portal vein and passes unhindered through the ~100 nm fenestrae of the sinusoidal endothelium into the space of Disse, which is in direct contact with hepatocytes (Fechner et al, 1999). Here, adenovirus is initially taken up by Kupffer cells, the resident macrophages of the liver, which act as a barrier to hepatocyte transduction, where the vector dose has to be sufficient to saturate this cell population before hepatocytes are efficiently transduced (Tao et al, 2001). Uptake of adenovirus by Kupffer cells activates these cells to release proinflammatory cytokines and chemokines that lead to leukocyte recruitment and acute hepatic inflammation, resulting in the elimination of >90% of vector genome within 24 h of vector administration (Lieber et al, 1997; Worgall et al, 1997; Muruve et al, 1999; Schnell et al, 2001; Zhang et al, 2001). This innate immune response is triggered by the viral capsid and in the absence of viral gene expression does not proceed beyond 24 h (Lieber et al, 1997; Muruve et al, 1999; Borgland et al, 2000; Liu et al, 2003). Hence, multiply deleted (Lieber et al, 1997) and helper-dependent (Muruve et al, 2004) adenovirus vectors cause this inflammatory response to similar levels to those observed with first generation adenovirus vectors, as they are packaged in a common intact viral particle. Following administration of first generation adenovirus vectors, the innate immune response is proceeded by an adaptive immune response 5 to 7 days after vector administration (Liu et al, 2003). This is directed against newly synthesised viral proteins and transgene product, where activated Kupffer cells present *de novo* synthesised viral proteins and/or transgene product to MHC class I-restricted CD8$^+$ T lymphocytes (CTLs). The adaptive immune response together with direct toxicity of the virus, go on to eliminate the remainder of transduced hepatocytes by 3 weeks after vector administration, thus resulting in the loss of transgene expression (Yang et al, 1994a,b, 1995, 1996a; Tripathy et al, 1996; Morral et al, 1997; Song et al, 1997; Worgall et al, 1997). Additionally, Kupffer cells express MHC class II molecules and therefore the presentation of input viral capsid proteins to CD4$^+$ T helper cells, may lead to the formation of neutralizing anti-Ad antibodies preventing successful readministration of Ad vector (Yang et al, 1996b). In relation to the distribution of CTL targets in an adaptive immune response against systemic Ad vector administration, the level of CTL response and whether it is directed against adenovirus antigens and/or the transgene product is dependent on the MHC haplotype of the host. Indeed, the variation in CTL response to Ad vector between mouse strains is due to differences in the *H-2* haplotype (Sparer et al, 1997; Jooss et al, 1998).

Kupffer cells are an integral component of the immune response against Ad vectors. Selective Kupffer cell depletion followed by injection of a low dose of Ad vector ($\leq 1x10^9$ pfu/mouse), results in decreased plasma cytokine levels, with increased and prolonged transgene expression (Kuzmin et al, 1997; Wolff et al, 1997; Worgall et al, 1997; Schiedner et al, 2003). Thus, hepatocyte-restricted expression of an adenoviral transgene may prevent immune clearance of transduced cells. Hepatocytes express MHC Class I molecules that are upregulated by activated CD8$^+$ T cells releasing interferon-γ, sensitizing the hepatocytes to CTL-mediated cytolysis (Yang et al, 1995). However, hepatocytes do not express co-stimulatory molecules and therefore MHC Class I-restricted presentation of antigenic peptides to naïve T cells does not result in T cell activation, but may lead to anergy or immune tolerance against the epitope (Guerder et al, 1995). Hence, in this study, the utilisation of a polymerase/pTP-deleted Ad vector, in combination with hepatocyte-specific transgene expression, may have contributed to the sustained expression of recombinant apoE. The removal of the polymerase and pTP genes from the Ad vector backbone virtually eliminates adenoviral late gene expression, which significantly reduces hepatotoxicity, as well as removing the trigger for the

adaptive immune response that would otherwise lead to the immune clearance of the remainder of adenovirus-transduced cells (Everett et al, 2003; Amalfitano et al, 1998; Hu et al, 1999). Indeed, rAd vectors devoid of all viral coding sequences, referred to as helper-dependent Ad vectors, and containing liver-specific promoters driving transgene expression, provide long lasting levels of transgene product (Morral et al, 1998; Aurisicchio et al, 2000). In support of our findings, is the observation that a humoral immune response against the transgene product was absent in animals treated with Ad-LSP-apoE, as well as Ad-CMV-apoE, which confirms our previous findings (Harris et al, 2002a).

Hepatocyte-restricted transgene expression may be preferable to selective Kupffer cell depletion, as the injection of higher vector doses, in combination with Kupffer cell depletion, caused a greater inflammatory response and decreased transgene expression in hepatocytes (Kuzmin et al, 1997; Lieber et al, 1997). In fact, the reticuloendothelial system in the liver is primarily composed of Kupffer cells and may prevent the development of a systemic inflammatory response by sequestering an infectious agent, thus limiting its dissemination to lymphoid organs such as draining lymph nodes and the spleen (Jooss et al, 2003).

In conclusion, we have demonstrated that liver-directed administration of a [$E1^-$, $E3^-$, $polymerase^-$, pTP^-] rAd vector containing the LSP driving human apoE expression in $apoE^{-/-}$ mice, resulted in sustained, albeit low, levels of circulating apoE throughout the course of the study (7 months) and a significant retardation of atherosclerosis (30%, $P<0.006$) without correcting the hypercholesterolaemia. As well as providing sustained transgene expression, rAd vectors containing liver-specific promoters provide a safer therapeutic profile in terms of minimising the immune response against the adenovirus and transgene product.

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