Gene Therapy (2013) **20**, 1124–1130

www.nature.com/gt

ORIGINAL ARTICLE

PEGylated helper-dependent adenoviral vector expressing human Apo A-I for gene therapy in LDLR-deficient mice

This article has been corrected since online publication and a corrigendum is also printed in this issue

E Leggiero[1,2], D Astone[1,2], V Cerullo[1,2,3], B Lombardo[1,2], C Mazzaccara[2], G Labruna[4], L Sacchetti[1,2], F Salvatore[1,4], M Croyle[5,6] and L Pastore[1,2]

Helper-dependent adenoviral (HD-Ad) vectors have great potential for gene therapy applications; however, their administration induces acute toxicity that impairs safe clinical applications. We previously observed that PEGylation of HD-Ad vectors strongly reduces the acute response in murine and primate models. To evaluate whether PEGylated HD-Ad vectors combine reduced toxicity with the correction of pathological phenotypes, we administered an HD-Ad vector expressing the human apolipoprotein A-I (hApoA-I) to low-density lipoprotein (LDL)-receptor-deficient mice (a model for familial hypercholesterolemia) fed a high-cholesterol diet. Mice were treated with high doses of HD-Ad-expressing apo A-I or its PEGylated version. Twelve weeks later, LDL levels were lower and high-density lipoprotein (HDL) levels higher in mice treated with either of the vectors than in untreated mice. After terminal killing, the areas of atherosclerotic plaques were much smaller in the vector-treated mice than in the control animals. Moreover, the increase in pro-inflammatory cytokines was lower and consequently the toxicity profile better in mice treated with PEGylated vector than in mice treated with the unmodified vector. This finding indicates that the reduction in toxicity resulting from PEGylation of HD-Ad vectors does not impair the correction of pathological phenotypes. It also supports the clinical potential of these vectors for the correction of genetic diseases.

Gene Therapy (2013) **20,** 1124–1130; doi:10.1038/gt.2013.38; published online 25 July 2013

Keywords: helper-dependent adenoviral vectors; PEGylation; atherosclerosis

INTRODUCTION

Atherosclerosis is a complex multifactorial disorder ultimately leading to coronary artery disease. Atherosclerosis is characterized by the accumulation of inflammatory cells, lipoproteins and fibrous tissues in the wall of large arteries.[1] The etiology of this disorder is highly heterogeneous, with numerous known and unknown genetic and environmental factors influencing both lipoprotein metabolism and inflammation.[2] One of the major predisposing factors is hypercholesterolemia. In fact, elevated low-density lipoprotein cholesterol (LDL-C), due to environmental as well as genetic factors, is frequently associated with the development of atherosclerosis and a higher frequency of coronary artery disease. Mutations in the LDL receptor gene cause familial hypercholesterolemia (FH), an inherited metabolic disorder characterized by an increase in LDL-C plasma levels and a consequent increased risk of premature atherosclerosis and coronary artery disease.[3]

Reduced levels of high-density lipoprotein cholesterol (HDL-C) are also associated with a higher incidence of coronary artery disease. Moreover, in some human disorders, premature atherosclerosis is associated with markedly reduced levels of HDL-C (for example, Tangier disease and mutations in the human apolipoprotein A-I (*hApoA-I*) gene).[4,5] HDL particles transport excess cholesterol from the periphery to the liver with a mechanism known as 'reverse cholesterol transport' that mediates their atheroprotective roles.[6] In fact, cardiovascular risk can also be reduced by increasing HDL-C levels.[7] HDL and its components (in particular, antioxidant enzymes) can reduce oxidized lipid species in LDL particles, thus reducing their atherogenic potential.[8] Apolipoprotein A-I (ApoA-I) constitutes ~70% of the apolipoprotein content of HDL particles, and there is a strong correlation between plasma ApoA-I and HDL-C levels.[9] In addition, the finding that ApoA-I has intrinsic antioxidant and anti-inflammatory properties led to the development of ApoA-I mimetic peptides and several other drugs that are currently being tested for their potential to reduce atherosclerosis.[10]

A variety of viral vectors have been tested in the attempt to elicit the overexpression of anti-atherogenic proteins.[11] First-generation adenoviral vectors are capable of inducing high levels of ApoA-I;[12] however, the duration of transgene expression induced by these vectors is very short, hence they are not suitable for clinical applications.[13] Adeno-associated vectors have been used to express ApoA-I; although these vectors have a more favorable toxicity profile than the first-generation adenoviral vectors, levels of expression were not sufficient to affect aortic atherosclerosis development.[14] Differently, helper-dependent adenoviral (HD-Ad) vectors can induce prolonged high levels of transgene expression that have a more favorable toxicity

[1]CEINGE-Biotecnologie Avanzate, Napoli, Italy; [2]Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università di Napoli 'Federico II', Napoli, Italy; [3]Division of Biopharmaceutics and Pharmacokinetics, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland; [4]Fondazione IRCCS SDN, Istituto di Ricerca Diagnostica e Nucleare, Napoli, Italy; [5]Division of Pharmaceutics, College of Pharmacy, The University of Texas at Austin, Austin, TX, USA and [6]Institute of Cellular and Molecular Biology, The University of Texas at Austin, TX, Austin, USA. Correspondence: Professor L Pastore, Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università di Napoli 'Federico II', Via Gaetano Salvatore 486 CEINGE-Biotecnologie Avanzate, Napoli 80145, Italy.
E-mail: lucio.pastore@unina.it
Received 18 January 2013; revised 23 May 2013; accepted 29 May 2013; published online 25 July 2013

profile.[13,15–18] We previously overexpressed human ApoA-I (hApoA-I) in two different mouse models using HD-Ad vectors and obtained long-term corrective levels of this transgene and a consequent reduction of aortic atherosclerosis.[13,17] Even though HD-Ad vectors induce a milder liver toxicity compared with the first-generation adenoviral vectors,[19,20] the viral capsid responsible for triggering the acute inflammatory response in a dose-dependent manner is identical for both types of vectors.[21,22] The possibility of mitigating the host response associated with adenoviral vector administration was widely investigated mainly after the death of a patient treated in a clinical trial with a high dose of a recombinant adenoviral vector containing a functional gene for ornithine transcarbamylase.[23] HD-Ad vectors reduce the long-term toxicity resulting from the accumulation of viral proteins;[13,19] however, innate immunity, with consequent cytokine secretion, is still present due to the interaction of Ad particles with Toll-like receptors at the plasma membrane[24] and at endosome level, where Toll-like receptor 9 interacts with the vector genome.[25]

Several types of pretreatment, for example, corticosteroid administration[26] and tumor necrosis factor (TNF)-alpha blockade,[27] have been proposed to overcome innate immunity. In this context, we have focused on the modification of HD-Ad vector particles. Specifically, we chemically modified HD-Ad vectors by PEGylation.[28] PEGylated HD-Ad vectors have a better toxicity profile than native vectors in both mice[28] and non-human primates.[29] PEGylation with low-molecular weight PEG (mw 5000) does not significantly influence transduction efficiency in murine hepatocytes reducing Kupffer cell transduction and increasing vector half-life in the systemic circulation;[28,30,31] on the other hand, PEGylation significantly reduces vector-mediated production of inflammatory cytokines and protects vectors from inactivation by complement and neutralizing antibodies.[28] A reduction in transduction has been observed in non-human primates;[29] however, differences in transgenes and their determinations together with alternative methods for vector titration as well as possible species-specific responses may account for this observation. In addition, non-human primate data had been obtained by a single animal per dose and cannot be considered representative.[29] Adenoviral vectors PEGylated with low-molecular weight PEG (5000) retain the ability to transduce hepatocytes after binding with coagulation factor X;[32] on the other hand, higher-molecular weight PEG strongly reduces hepatocyte expression, enabling vector retargeting.[32] PEGylated vectors show a more favorable toxicity profile because capsid shielding avoids adenovirus hexon interaction with Kupffer-cell-scavenger receptors and its subsequent capture; therefore, using a low-molecular weight PEG modification makes it possible to express transgenes at levels comparable to unmodified vectors in the absence of innate response.[33,34] Liver pathology after PEGylated HD-Ad vectors administration has also been extensively studied and is comparable to that observed using unmodified HD-Ad vectors, presenting a favorable toxicity profile compared to first-generation adenoviral vectors.[28] PEGylated HD-Ad vectors have also been previously used for a short-term rescue of a model of propionic acidemia[35] and as cancer vaccines;[36] however, at the moment, there is no proof-of-concept for long-term *in vivo* efficacy of these vectors for therapeutic purposes.

In the attempt to evaluate whether the expression of different transgenes could be influenced by vector PEGylation and to determine the efficacy and safety of these vectors in a disease model, we have evaluated a PEGylated HD-Ad vector expressing hApoA-I in LDL receptor-deficient mice, which is the mouse model of FH.[37] These mice have only a modest hypercholesterolemia when fed a normal diet, whereas they develop extensive atherosclerotic lesions throughout the aorta when fed a high-fat diet.[38] This model has been previously shown to respond to an HD-Ad-mediated increase in ApoA-I levels associated with a significant reduction in aortic atherosclerosis.[17] We therefore evaluated whether we could reduce aortic atherosclerosis in LDLR-deficient mice without eliciting an innate host response by using a PEGylated HD-Ad vector expressing hApoA-I. To this aim, we administered a high dose of PEGylated vector to LDLR-deficient mice that were fed a high-fat diet and evaluated the effect of this treatment on both lipoprotein profile and aortic atherosclerosis; the data obtained were compared with those obtained in mice treated with the unmodified form of the vector. Our results support the clinical potential of PEGylated HD-Ad vectors for the correction of genetic diseases.

RESULTS

PEG-HD-Ad vectors induce high-level persistent expression of ApoA-I

To determine whether the PEGylated HD-Ad vector containing the entire *hApoA-I* gene was able to transduce cells in culture, we infected $1 \times 10E6$ 293 cells and $1 \times 10E6$ W20-17 cells with 10 vp per cell of the native (HD-Ad-AI) and 10 vp per cell of the PEGylated (PEG-HD-Ad-AI) vectors, and determined ApoA-I levels in the medium. As shown in Figures 1a and b, both 293 and W20-17 cells constitutively secrete a small amount of ApoA-I (17 ± 3.5 and 13 ± 0.3 mg dl^{-1} in untransfected media, respectively). Infection with HD-Ad-AI led to a four-fold increase in Apo A-I in 293 (77 ± 28 mg dl^{-1}) and two-fold increase in W20-17 (27 ± 6 mg dl^{-1}) cells. There was a three-fold (54 ± 8 mg dl^{-1}) and a two-fold increase (32 ± 6 mg dl^{-1}) in these cells, respectively, when they were infected with the PEGylated version of HD-Ad-AI. The lower levels of ApoA-I observed in W20-17 cells may be either due to a resistance of these cells to Ad5 infection or to a lower expression of the transgene driven by the endogenous ApoA-I promoter. Importantly, there were no significant differences in the secretion of ApoA-I after infection with either PEGylated or native vectors. This finding confirms the efficiency of

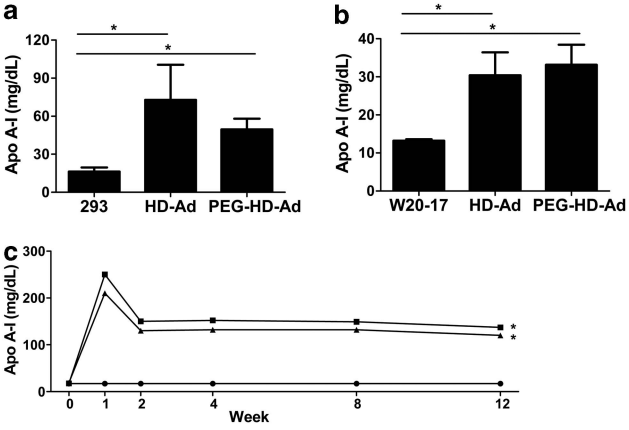

Figure 1. PEG-HD-Ad-AI and HD-Ad-AI vector treatment induce Apo A-I expression *in vitro* and *in vivo*. 293 (**a**) and W20-17 (**b**) cells were infected with HD-Ad-AI (10 vp per cell) and PEG-HD-Ad-AI (10 vp per cell), and human Apo A-I secreted in the medium was determined. Uninfected 293 and W20-17 cells served as negative controls. Infection with both vectors led to a significant increase ($^*P < 0.01$) in human Apo A-I levels in the medium in both cell lines. Groups of five LDLR-deficient mice were treated systemically with $1 \times 10E13$ vp kg^{-1} of HD-Ad-AI (■) or PEG-HD-Ad-AI (▲); a third group of five mice was treated with the same volume of PBS as control (●, **c**). Human Apo A-I levels were determined in blood samples 1, 2, 4, 8 and 12 weeks after treatment. Groups of five LDLR-deficient mice treated with HD-Ad-AI or PEG-HD-Ad-AI showed significantly higher levels of human Apo A-I expression that lasted throughout the experiment significantly different from untreated mice ($^*P < 0.01$). No significant differences were observed between treatments with the different vectors. Data are expressed as mean ± s.d.

both vectors *in vitro* and that PEGylation does not significantly influence vector transduction efficiency and transgene expression in cell lines.

To assess whether PEG-HD-Ad-AI administration leads to the persistent expression of hApoA-I *in vivo*, we administered $1 \times 10E13$ vp kg^{-1} of either PEG-HD-Ad-AI or HD-Ad-AI to two groups of LDLR-deficient mice ($n = 5$ per treatment group) fed a high-fat diet; a third group was treated with phosphate-buffered saline (PBS) as control ($n = 5$). Administration of PEG-HD-Ad-AI or HD-Ad-AI vectors led to the expression of human ApoA-I for the entire duration of the experiment (12 weeks, Figure 1c). One week after treatment, hApoA-I reached its highest levels in both groups (250 ± 3 and 210 ± 2 mg dl^{-1}). Thereafter, hApoA-I levels slowly decreased in vector-treated animals, although they remained within levels able to give therapeutic benefits according to our previous work.

PEGylation of HD-Ad vectors reduces host response in the presence of high transgene expression

To evaluate host response to vector administration, we assessed cytokine activation profiles in LDLR-deficient mice treated with HD-Ad-AI, PEG-HD-Ad-AI or PBS. Eight-week-old LDLR-deficient mice ($n = 5$) were fed a high-cholesterol diet for 4 weeks and then treated with $1 \times 10E13$ vp kg^{-1} of either HD-Ad-AI or PEG-HD-Ad-AI; control animals ($n = 5$) were treated with an equal volume of PBS. Six hours after treatment, blood samples were collected to determine interleukin (IL) 12p40, IL-12p70, IL-6 (one of the main markers of adenovirus-induced innate response), TNF-alpha, monocyte chemotactic protein-1 (MCP-1) and keratinocyte-derived cytokine (KC) levels. IL-6, IL-12p40, IL-12p70 and TNF-α are markers commonly associated with the activation of the innate immune response against recombinant adenoviral vectors.[39] Moreover, a large body of evidence indicates that administration of adenoviral vectors induces the secretion not only of cytokines but also of chemokines (MCP-1 and KC); the levels of both proteins increase upon the activation of Kupffer cells after vector transduction.[24,40–42] We determined cytokines levels 6 h after vector administration (Figure 2), as prior data[28,41] showed that activation of the cytokine response is highest at this time, and usually returns to baseline within 24–48 h.

Levels of IL-12 p70, KC, MCP-1 and TNF-alpha were significantly lower in mice treated with the PEGylated vector compared with those observed in mice treated with the unmodified vector (Figure 2a, d–f); only IL-12p40 levels did not differ significantly between mice treated with PEGylated vectors and those treated with native vectors (Figure 2b). Mice treated with HD-Ad-AI showed a significantly larger increase in serum levels of IL-6 compared with animals receiving PEG-HD-AI (1300 ± 300 and 400 ± 61 pg ml^{-1}, respectively, Figure 2c). Thus, PEGylated vectors induced a milder inflammatory response; in fact, levels of all the cytokines were significantly lower in mice treated with PEGylated vectors than in mice treated with native vectors. Taken together, these data further confirm that PEGylation of HD-Ad vectors reduces the innate host response in the presence of high levels of transgene expression.

Overexpression of hApo A-I after PEGylated-vector administration modifies the lipid profile and reduces aortic atherosclerosis

To evaluate the effects of overexpression of hApoA-I on cholesterol metabolism, we measured the levels of triglycerides, total cholesterol (TC), LDL-cholesterol (LDL-C) and HDL-cholesterol (HDL-C) at different time points (0, 1, 2, 4, 8 and 12 weeks) in the three groups of mice treated with $1 \times 10E13$ vp kg^{-1} of HD-Ad-AI, PEG-HD-Ad-AI or PBS and fed an atherogenic diet for 12 weeks ($n = 5$ per treatment). As shown in Figure 3a, baseline levels of plasma triglycerides were 85 ± 5 and 89.5 ± 2 mg dl^{-1} in HD-Ad-AI-treated mice, and 84.6 ± 1 mg dl^{-1} in the control, HD-Ad-AI and

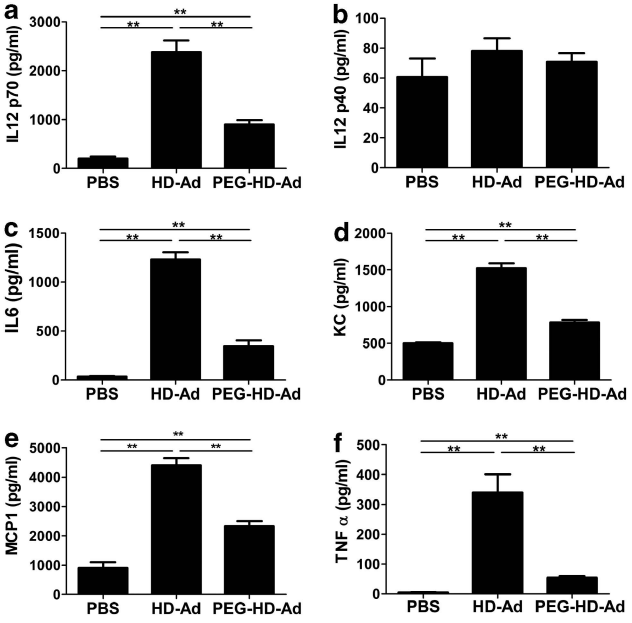

Figure 2. Administration of PEG-HD-Ad-AI in LDLR $-/-$ mice is associated to a lower toxicity compared with unmodified vector. Groups of five LDLR-deficient mice were treated systemically with $1 \times 10E13$ vp kg^{-1} of PEG-HD-Ad-AI or HD-Ad-AI; a third group of mice ($n = 5$) was treated with the same volume of PBS as negative control. Six hours after the treatment, blood samples were collected and cytokine levels were determined. Levels of IL-12p70 (**a**), IL-12p40 (**b**), IL-6 (**c**), KC (**d**), MCP-1 (**e**) and TNF-alpha (**f**) were compared in the three groups to evaluate vector toxicity. All the cytokines evaluated except IL-12p40 differed significantly between mice treated with PEGylated vectors and those treated with native vector. Statistically significant differences are expressed as $^{**}P < 0.01$. Data are expressed as mean $\pm$ s.d.

PEG-HD-Ad-AI groups of mice, respectively, and remained essentially unchanged after treatment. Basal levels of TC were 549.4 ± 1, 569.06 ± 1 and 554 ± 1 mg dl^{-1} in the control, HD-Ad-AI and PEG-Hd-Ad-AI groups, respectively. However, already 1 week after administration, the two vectors induced a significant decrease in TC levels: 500.78 ± 1 and 512.94 ± 1 mg dl^{-1} in HD-Ad-AI-treated- and PEG-Hd-Ad-AI-treated mice, respectively, versus 565.46 ± 1 mg d^{-1} in control mice (Figure 3b). TC levels were significantly different between mice treated with either native or PEGylated vector and untreated animals. Mice treated with the PEGylated vector had slightly higher levels of TC, but the difference with the animals treated with the unmodified vector was not statistically significant.

To evaluate the effect of hApoA-I on reverse transport of cholesterol, we also evaluated serum levels of LDL-C and HDL-C. Basal levels of HDL-C were also similar in the three groups (112.2 ± 1, 115.2 ± 1 and 113.6 ± 2 mg dl^{-1} in the PBS, HD-Ad-AI and PEG-HD-Ad-AI groups, respectively). However, 1 week after treatment, HDL-C values increased in the mice treated with HD-Ad-AI or PEG-HD-Ad-AI (Figure 3c) and remained significantly higher than in control mice for the entire duration of the experiment. The basal LDL-C level was similar in the three groups (420 ± 2, 436 ± 2 and 423.5 ± 1 mg dl^{-1} in the PBS, HD-Ad-AI and PEG-HD-Ad-AI groups, respectively). In HD-Ad-AI- and PEG-HD-Ad-AI-treated mice, LDL-C started to decrease 1 week after vector administration and levels remained significantly lower for the entire duration of the experiment (Figure 3d). Taken together, these data suggest that treatment with the PEG-HD-Ad-AI vector induces persistent modifications of lipid metabolism.

Lastly, we evaluated whether changes in lipid metabolism affected the development of atherosclerotic lesions. We killed the

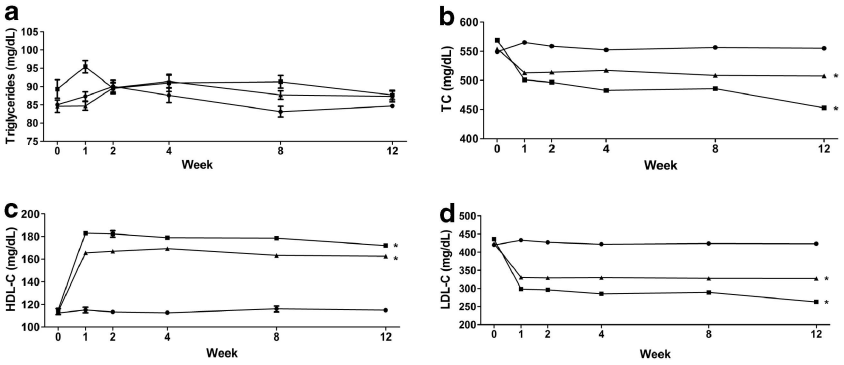

Figure 3. Administration of PEG-HD-Ad-AI increases Apo A-I levels and modifies cholesterol metabolism in LDLR −/− mice. Two groups of five LDLR-deficient mice were treated systemically with 1×10 E13 vp kg^{-1} of HD-Ad-AI (■) or PEG-HD-Ad-AI (▲). A third group of mice ($n = 5$) was treated with the same volume of PBS as control (●). Samples were collected before treatment and 1, 2, 4, 8 and 12 weeks after treatment. Triglyceride (**a**), TC (**b**), HDL-C (**c**) and LDL-C (**d**) levels were determined in the three groups of mice. TC, HDL-C and LDL-C levels differed significantly between HD-Ad-AI- or PEG-HD-Ad-AI-treated mice and the control group ($^*P < 0.05$). Data are expressed as mean ± s.d.

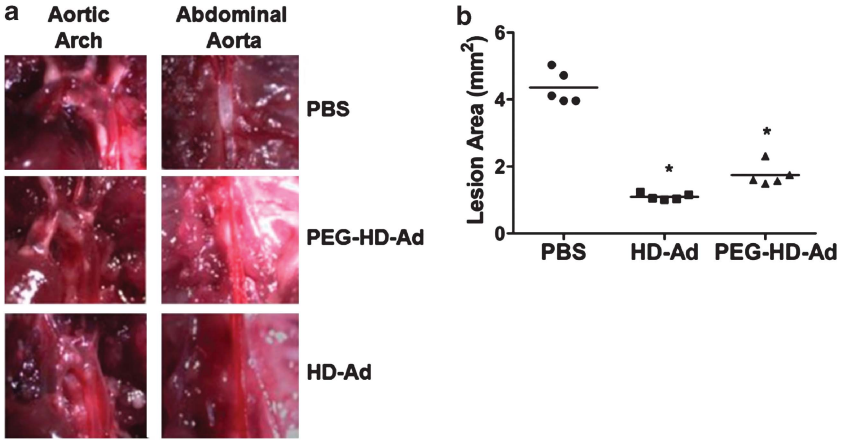

Figure 4. PEG-HD-Ad-A-I treatment reduces aortic atherosclerosis development. Two groups of five LDLR-deficient mice were treated systemically with 1×10E13 vp kg^{-1} of HD-Ad-AI or PEG-HD-Ad-AI; a third group ($n = 5$) of mice was treated with the same volume of PBS as control. Twelve weeks after treatment, mice were killed and their aortas were dissected. Fat deposits in the aortic arch and abdominal aorta were lower in mice treated with PEG-HD-Ad Apo AI or HD-Ad Apo AI than in the control group (**a**). Aortas were then stained with Oil Red-O to identify fat deposits in LDLR-deficient mice treated with HD-Ad-AI (■), PEG-HD-Ad-AI (▲) or PBS (●). Stained areas were measured and compared with the area of the entire aorta as index of atherosclerotic lesions (**b**); statistically significant differences are expressed as $^*P < 0.01$.

mice 12 weeks after treatment and dissected the aortas from the heart to iliac branching. In a macroscopic analysis, we observed fat deposits as expected at the level of three branches of the aorta: in the intimal layer of brachiocephalic trunk, left common carotid artery and left subclavian artery (Figure 4a). Other lesions were present throughout the abdominal aorta, upstream the bifurcation of kidney arteries. Fat deposits were more evident in mice treated with PBS than in mice treated with either HD-Ad-AI or PEG-HD-Ad-AI. In fact, fat deposits in PBS-treated animals were larger at the level of both the aortic arch and abdominal aorta than in the other two groups of mice. PBS-treated mice showed an occlusion of the aorta associated with dilatation of the vessel at the sites of larger fat deposits. This pattern was not observed in mice treated with HD-Ad-AI or PEG-HD-Ad-AI vector.

To quantify the atherosclerotic lesions that were directly correlated with fat deposits, we stained the aortas *en-face* with Oil Red-O after the removal of external fat and residual tissues. The area of fat deposits was quantified in the experimental animals to determine the efficacy of vector treatment. As shown in Figure 4b, lesion areas were significantly smaller ($P < 0.01$) in mice treated with either HD-Ad-AI or PEG-HD-Ad-AI (1.09 ± 0.48 and 1.74 ± 0.67 mm^2, respectively) than in the PBS-treated mice (4.22 ± 0.53 mm^2). These results strongly suggest that PEGylated HD-Ad-induced overexpression of hApo A-I leads to persistent transgene expression and modification of cholesterol metabolism, which ultimately reduces aortic atherosclerosis development.

DISCUSSION

The aim of our study was to evaluate whether PEG-HD-Ad vectors could be used to correct a disease in an animal model. In fact, very few data are available about the expression of transgenes other than LacZ after transduction with PEGylated HD-Ad vectors, as previously mentioned in the Introduction. In addition, to the best of our knowledge, there is no proof-of-concept that PEGylated HD-Ad vectors are able to achieve long-term correction of a pathological phenotype. In the present work, we tested this hypothesis in a mouse model of FH for a number of reasons: firstly, LDLR-deficient mice have been extensively studied and are known to develop high basal levels of LDL-C and extensive atherosclerotic lesions under a regimen of a high-cholesterol diet;[17] in addition, we have previously found that the administration of HD-Ad vector expressing hApoA-I reduced atherosclerosis development in this model.[13] We also found that the delay in

atherosclerosis progression and remodeling of the lesions was due to the overexpression of hApoA-I and the consequent increase in HDL and, therefore, in reverse transport of cholesterol. In the present study, we treated LDLR-deficient mice with high doses of the same hApoA-I-expressing HD-Ad vector and its PEGylated version, and monitored, for 12 weeks, changes in cholesterol metabolism in terms of TC, LDL-C and HDL-C to determine whether the PEGylated vector was also able to affect the lipid profile and aortic atherosclerosis. In addition, we evaluated whether the extremely favorable toxicity profile observed with the LacZ-expressing PEGylated HD-Ad vectors[28,29] were confirmed in this vector.

We found that the hApoA-I-expressing PEGylated HD-Ad vector induced a smaller increase in pro-inflammatory cytokines than the native version. IL-6 and TNF-alpha are associated with a severe inflammatory reaction to Ad vectors and are therefore direct markers of toxicity. Six hours after the administration of PEG-HD-AI, the increase in both cytokines was significantly lower than after administration of the native form. Moreover, the levels of the other cytokines and chemokines that we assessed (IL-12 p70, KC and MCP-1) were significantly lower than those observed with the native version; only IL-12 p40 was moderately increased after treatment with both PEGylated and native vectors versus mice treated with PBS. These data demonstrate that treatment with a high dose of a PEGylated HD-Ad vector does not induce a significant host response, thereby increasing the therapeutic window for these vectors.

The favorable safety profile of the PEGylated vector is even more surprising, as the very high dose administered is usually toxic in mice; indeed, in our previous study, $1 \times 10E13$ vp kg^{-1} of HD-Ad-AI was the only dose that significantly altered the lipid profile and reduced aortic atherosclerosis in LDLR-deficient mice.[17] In the present study, the more favorable safety profile of the PEGylated vector compared with the unmodified version was relevant only if phenotype correction was maintained; in mice treated with either HD-Ad-AI or PEG-HD-Ad-AI, levels of TC, LDL-C and HDL-C were significantly different compared with mice treated with PBS in a similar manner. This observation is mirrored by the analysis of the accumulation of fat deposits in the aortic arch and abdominal aorta. In fact, fat deposits were larger in PBS-treated mice than in HD-Ad-AI- or PEG-HD-Ad-AI-treated mice. This is the first demonstration that vector PEGylation reduces host response irrespective of the transgene used and that this modification does not affect the expression or efficacy of the expressed transgene.

In summary, this study demonstrates that administration of a PEGylated HD-Ad vector expressing hApoA-I induces a milder host response than unmodified vectors, without affecting efficacy. PEGylation should be considered as an improvement of HD-Ad vectors, which, together with pretreatment[26] and safer administration routes,[43,44] can support their clinical application. Reduced hepatocyte transduction has been observed in non-human primates and may reduce the utility of this approach;[29] however, primate experiments have been performed on a single animal per dose and may not be representative. In addition, differences in transgenes and vector titration methods may also contribute to this observation. In this study, we used a very high dose of vector in order to obtain ApoA-I levels that would significantly reduce aortic atherosclerosis; however, lower doses, which are more readily applicable to clinical settings, may be used when PEGylated vectors are administered to treat other inherited errors of metabolism that require correction of a lower number of hepatocytes.[45] As also demonstrated previously, ApoA-I overexpression has the advantage of reducing aortic atherosclerosis development regardless of the underlying genetic cause.[13,17] Therefore, PEG-HD-Ad-mediated overexpression of hApoA-I may become a valid therapeutic alternative, especially in subgroups of FH patients in whom other therapies are poorly effective and the genetic causes are poorly characterized. In these patients, a single administration of PEG-HD-Ad may exert a long-lasting favorable effect on lipid metabolism.

MATERIALS AND METHODS

Production of HD-Ad vectors

The HD-Ad adenoviral vector (HD-Ad-AI) used in this study contains 10 kb of the *hApo A-I* gene, including the promoter region.[17] Rescue and amplification of the vector were performed using the HV-Ad-NG163R-2 helper virus as described elsewhere.[46,47] Briefly, a 60-mm dish of 116 cells at 80% confluence was transfected with 20 μg of *Pme*I-digested parental plasmid. Next day, the cells were infected with AdNG163R-2 at a multiplicity of infection of 1000 vp per cell. The vector was amplified by serial co-infections of 60-mm dishes of 116 cells at 90% confluence with 10% of the crude lysate from the previous passage and AdNG163R-2 at a multiplicity of infection of 200 vp per cell. After three infections in a 60-mm dish (P1, P2, P3), for P4 one 150-mm dish of 116 cells at 90% confluence was co-infected with 10% of the crude serial passage 2 lysate and Ad-NG163R-2 at an multiplicity of infection of 200 vp per cell. Large-scale HD-Ad production was performed in 3 l of 116 cells ($3–4 \times 10E5$ cells ml^{-1}) co-infected with 100% of the crude lysate from the 150-mm dish of serial passage 3 and Ad-NG163R-2; 48 h later, co-infected cells were harvested and resuspended in TM solution (10 mM Tris-HCl pH 8.0 and 2 mM $MgCl_2$). The harvested cells were lysed by using three freeze-thaw cycles and incubated with 2 M $MgCl_2$ and DNaseI for 1 h at 37 °C. After incubation, the cellular debris was spun down and the lysate was subjected to ultracentrifugation, as described elsewhere.[48] Vector concentration was measured as particle number and determined using the absorbance at 260 nm. Helper virus contamination and vector characterization were obtained as described previously.[46]

PEGylation of HD-Ad vectors

The aliquots of vectors were desalted on Econo-Pac 10DG disposable chromatography columns (Bio-Rad, Hercules, CA, USA) and equilibrated with 0.2 M sodium phosphate (pH 7.2) buffer for optimal conjugation. Viral concentrations were determined using the absorbance at 260 nm. The protein content of each viral preparation was determined with Bio-Rad DC Protein assay reagents using bovine serum albumin as standard. A total of 10 mg of monomethoxypoly (ethylene) glycol activated with succinimidyl succinate (SSPEG, mw 5000) was added for each microgram of proteins present in each preparation. Conjugation reactions were performed at 25 °C with gentle agitation for 2 h. Reactions were stopped by addition of a 10-fold excess of L-lysine with respect to the amount of PEG added. Unreacted PEG, excess lysine and reaction products were eliminated using buffer exchange over a second Econo-Pac 10DG disposable chromatography column equilibrated with 100 mM KPBS (pH 7.4). A separate aliquot of virus was treated and processed in the same manner as the conjugated virus in the absence of SSPEG and served as unPEGylated control. PEGylated and unPEGylated adenoviral vectors were characterized using capillary zone electrophoresis as previously described.[28]

Immunoturbidimetric assay

hApo A-I levels were determined using immunoturbidimetric analysis. 293 and W20-17 cells were infected with 200 vp per cell of HD-Ad-AI and PEG-HD-Ad-AI. Uninfected cells were used as negative controls. Forty-eight hours after infection, the medium was collected and a detergent solution was added at 37 °C for 2 min. Absorbance was read at 610 nm (Abs1) and goat serum containing anti-human Apo A-I was added. The absorbance at 610 nm was read again (Abs2) and the concentration of human Apo A-I was calculated as $\Delta Abs = Abs2 - Abs1$ on the calibration curve. Human sera with high and low concentrations of hApoA-I were used as controls.

Animal studies

All experimental procedures were conducted in accordance with institutional guidelines for animal care and use. Food and water were provided *ad libitum*. The mice used in the toxicity experiment were 8-week-old female LDL receptor-deficient (LDLR $-/-$) mice on a C57BL/6 background. Mice were fed a diet supplemented with 0.2% (wt/wt) cholesterol and 10% coconut oil (vol/wt) for 4 weeks. HD-Ad was diluted in sterile PBS,

prewarmed at 37 °C and injected into the tail vein as described.[13] Injections were performed in a total volume of 200 μl.

Mice were anesthetized with Avertine before blood was collected from the retroorbital plexus. For cytokine analyses, blood was collected 6 h after injection. For triglycerides, TC, LDL-C and HDL-C analyses, blood was collected at 0, 1, 2, 4, 8 and 12 weeks after vector administration from 12-h fasting mice. Serum was frozen immediately and stored at − 20 °C until further processing. Mice were terminally killed using carbon dioxide inhalation 12 weeks after treatment and aortas were taken for the assessment of atherosclerotic lesions.

Evaluation of acute toxicity

Mouse IL-6, IL-12p40, IL-12p70, TNF-alpha, MCP-1 and KC levels were determined using the Bio-Rad Bioplex cytokine multiplex and analyzed using a Bioplex instrument, according to the manufacturer's instructions (Bio-Rad).[49] Sera were tested on an Ortho Clinical Vitros 250 Chemistry System (Ortho Clinical Diagnostics, Johnson & Johnson Co, Rochester, NY, USA) using a dry chemistry approach. Samples were run at Bio-Plex Readers and data were analyzed using the Bioplex Software (Bio-Rad). Briefly, beads coated with antibodies against the selected cytokines were mixed with 10 μl of serum and incubated at room temperature for 1 h. After incubation with streptavidin-PE detection reagent for 30 min and subsequent bead resuspension, the plate was read on the Bioplex instruments.

Quantification of atherosclerotic lesions

After killing, aortas were dissected from the heart to iliac branching, with particular attention to the external fat in order to stain exclusively sub-intimal aortic fat. Aorta staining was performed as previously described.[17] Images were acquired using Nikon Coolscope (Nikon, Tokyo, Japan), and the fat amount was quantified with NIS elements program (Nikon).

Statistics

Results were statistically analyzed using GraphPad Prism (GraphPad Software) and $P<0.05$ was considered statistically significant. Differences among groups were analyzed using analysis of variance with Bonferroni correction. All data are expressed as mean ± s.d.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the Telethon grant GGP04039, and from MIUR, project PRIN protocol number 2004069479_004. We thank Jean Ann Gilder (Scientific Communication srl, Naples, Italy) for revising and editing the text.

REFERENCES

1 Fitzgerald ML, Mujawar Z, Tamehiro N. ABC transporters, atherosclerosis and inflammation. *Atherosclerosis* 2010; **211**: 361–370.
2 Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F *et al.* Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet* 2004; **364**: 937–952.
3 Civeira F. Guidelines for the diagnosis and management of heterozygous familial hypercholesterolemia. *Atherosclerosis* 2004; **173**: 55–68.
4 Rashid S, Marcil M, Ruel I, Genest J. Identification of a novel human cellular HDL biosynthesis defect. *Eur Heart J* 2009; **30**: 2204–2212.
5 Oram JF, Vaughan AM. ATP-Binding cassette cholesterol transporters and cardiovascular disease. *Circ Res* 2006; **99**: 1031–1043.
6 Lewis GF, Rader DJ. New insights into the regulation of HDL metabolism and reverse cholesterol transport. *Circ Res* 2005; **96**: 1221–1232.
7 Robinson JG, Bakris G, Torner J, Stone NJ, Wallace R. Is it time for a cardiovascular primary prevention trial in the elderly? *Stroke* 2007; **38**: 441–450.
8 Assmann G, Gotto Jr AM. HDL cholesterol and protective factors in atherosclerosis. *Circulation* 2004; **109**(23 Suppl 1): III8–14.
9 Van Craeyveld E, Gordts S, Jacobs F, De Geest B. Gene therapy to improve high-density lipoprotein metabolism and function. *Curr Pharm Des* 2010; **16**: 1531–1544.
10 Van Lenten BJ, Wagner AC, Anantharamaiah GM, Navab M, Reddy ST, Buga GM *et al.* Apolipoprotein A-I mimetic peptides. *Curr Atheroscler Rep* 2009; **11**: 52–57.
11 Harris JD, Evans V, Owen JS. ApoE gene therapy to treat hyperlipidemia and atherosclerosis. *Curr Opin Mol Ther* 2006; **8**: 275–287.
12 De Geest B, Van Linthout S, Collen D. Sustained expression of human apo A-I following adenoviral gene transfer in mice. *Gene Therapy* 2001; **8**: 121–127.
13 Pastore L, Belalcazar LM, Oka K, Cela R, Lee B, Chan L *et al.* Helper-dependent adenoviral vector-mediated long-term expression of human apolipoprotein A-I reduces atherosclerosis in apo E-deficient mice. *Gene* 2004; **327**: 153–160.
14 Kassim SH, Wilson JM, Rader DJ. Gene therapy for dyslipidemia: a review of gene replacement and gene inhibition strategies. *Clin Lipidol* 2010; **5**: 793–809.
15 Toietta G, Mane VP, Norona WS, Finegold MJ, Ng P, McDonagh AF *et al.* Lifelong elimination of hyperbilirubinemia in the Gunn rat with a single injection of helper-dependent adenoviral vector. *Proc Natl Acad Sci USA* 2005; **102**: 3930–3935.
16 Oka K, Pastore L, Kim IH, Merched A, Nomura S, Lee HJ *et al.* Long-term stable correction of low-density lipoprotein receptor-deficient mice with a helper-dependent adenoviral vector expressing the very low-density lipoprotein receptor. *Circulation* 2001; **103**: 1274–1281.
17 Belalcazar LM, Merched A, Carr B, Oka K, Chen KH, Pastore L *et al.* Long-term stable expression of human apolipoprotein A-I mediated by helper-dependent adenovirus gene transfer inhibits atherosclerosis progression and remodels atherosclerotic plaques in a mouse model of familial hypercholesterolemia. *Circulation* 2003; **107**: 2726–2732.
18 Cerreto M, Mehdawy B, Ombrone D, Nistico R, Ruoppolo M, Usiello A *et al.* Reversal of metabolic and neurological symptoms of phenylketonuric mice treated with a PAH containing helper-dependent adenoviral vector. *Curr Gene Ther* 2012; **12**: 48–56.
19 Morral N, Parks RJ, Zhou H, Langston C, Schiedner G, Quinones J *et al.* High doses of a helper-dependent adenoviral vector yield supraphysiological levels of alpha1-antitrypsin with negligible toxicity. *Hum Gene Ther* 1998; **9**: 2709–2716.
20 Schiedner G, Morral N, Parks RJ, Wu Y, Koopmans SC, Langston C *et al.* Genomic DNA transfer with a high-capacity adenovirus vector results in improved *in vivo* gene expression and decreased toxicity. *Nat Genet* 1998; **18**: 180–183.
21 Brunetti-Pierri N, Palmer DJ, Beaudet AL, Carey KD, Finegold M, Ng P. Acute toxicity after high-dose systemic injection of helper-dependent adenoviral vectors into nonhuman primates. *Hum Gene Ther* 2004; **15**: 35–46.
22 Muruve DA. The innate immune response to adenovirus vectors. *Hum Gene Ther* 2004; **15**: 1157–1166.
23 Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP *et al.* Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab* 2003; **80**: 148–158.
24 Appledorn DM, Patial S, McBride A, Godbehere S, Van Rooijen N, Parameswaran N *et al.* Adenovirus vector-induced innate inflammatory mediators, MAPK signaling, as well as adaptive immune responses are dependent upon both TLR2 and TLR9 *in vivo*. *J Immunol* 2008; **181**: 2134–2144.
25 Cerullo V, Seiler MP, Mane V, Brunetti-Pierri N, Clarke C, Bertin TK *et al.* Toll-like receptor 9 triggers an innate immune response to helper-dependent adenoviral vectors. *Mol Ther* 2007; **15**: 378–385.
26 Seregin SS, Appledorn DM, McBride AJ, Schuldt NJ, Aldhamen YA, Voss T *et al.* Transient pretreatment with glucocorticoid ablates innate toxicity of systemically delivered adenoviral vectors without reducing efficacy. *Mol Ther* 2009; **17**: 685–696.
27 Mane VP, Toietta G, McCormack WM, Conde I, Clarke C, Palmer D *et al.* Modulation of TNFalpha, a determinant of acute toxicity associated with systemic delivery of first-generation and helper-dependent adenoviral vectors. *Gene Therapy* 2006; **13**: 1272–1280.
28 Croyle MA, Le HT, Linse KD, Cerullo V, Toietta G, Beaudet A *et al.* PEGylated helper-dependent adenoviral vectors: highly efficient vectors with an enhanced safety profile. *Gene Therapy* 2005; **12**: 579–587.
29 Wonganan P, Clemens CC, Brasky K, Pastore L, Croyle MA. Species differences in the pharmacology and toxicology of PEGylated helper-dependent adenovirus. *Mol Pharm* 2011; **8**: 78–92.
30 Alemany R, Suzuki K, Curiel DT. Blood clearance rates of adenovirus type 5 in mice. *J Gen Virol* 2000; **81**(Pt 11): 2605–2609.
31 Prill JM, Espenlaub S, Samen U, Engler T, Schmidt E, Vetrini F *et al.* Modifications of adenovirus hexon allow for either hepatocyte detargeting or targeting with potential evasion from Kupffer cells. *Mol Ther* 2011; **19**: 83–92.
32 Hofherr SE, Shashkova EV, Weaver EA, Khare R, Barry MA. Modification of adenoviral vectors with polyethylene glycol modulates *in vivo* tissue tropism and gene expression. *Mol Ther* 2008; **16**: 1276–1282.
33 Weaver EA, Barry MA. Effects of shielding adenoviral vectors with polyethylene glycol on vector-specific and vaccine-mediated immune responses. *Hum Gene Ther* 2008; **19**: 1369–1382.

34 Weaver EA, Nehete PN, Buchl SS, Senac JS, Palmer D, Ng P *et al.* Comparison of replication-competent, first generation, and helper-dependent adenoviral vaccines. *PLoS One* 2009; **4**: e5059.

35 Hofherr SE, Senac JS, Chen CY, Palmer DJ, Ng P, Barry MA. Short-term rescue of neonatal lethality in a mouse model of propionic acidemia by gene therapy. *Hum Gene Ther* 2009; **20**: 169–180.

36 Weaver EA, Chen CY, May SM, Barry ME, Barry MA. Comparison of adenoviruses as oncolytics and cancer vaccines in an immunocompetent B cell lymphoma model. *Hum Gene Ther* 2011; **22**: 1095–1100.

37 Ishibashi S, Brown MS, Goldstein JL, Gerard RD, Hammer RE, Herz J. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J Clin Invest* 1993; **92**: 883–893.

38 Tangirala RK, Rubin EM, Palinski W. Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. *J Lipid Res* 1995; **36**: 2320–2328.

39 Benihoud K, Esselin S, Descamps D, Jullienne B, Salone B, Bobe P *et al.* Respective roles of TNF-alpha and IL-6 in the immune response-elicited by adenovirus-mediated gene transfer in mice. *Gene Therapy* 2007; **14**: 533–544.

40 Aldhamen YA, Seregin SS, Amalfitano A. Immune recognition of gene transfer vectors: focus on adenovirus as a paradigm. *Front Immunol* 2011; **2**: 40.

41 Appledorn DM, Aldhamen YA, Depas W, Seregin SS, Liu CJ, Schuldt N *et al.* A new adenovirus based vaccine vector expressing an *Eimeria tenella* derived TLR agonist improves cellular immune responses to an antigenic target. *PLoS One* 2010; **5**: e9579.

42 Hartman ZC, Kiang A, Everett RS, Serra D, Yang XY, Clay TM *et al.* Adenovirus infection triggers a rapid, MyD88-regulated transcriptome response critical to acute-phase and adaptive immune responses *in vivo*. *J Virol* 2007; **81**: 1796–1812.

43 Brunetti-Pierri N, Liou A, Patel P, Palmer D, Grove N, Finegold M *et al.* Balloon catheter delivery of helper-dependent adenoviral vector results in sustained, therapeutic hFIX expression in rhesus macaques. *Mol Ther* 2012; **20**: 1863–1870.

44 Brunetti-Pierri N, Ng T, Iannitti DA, Palmer DJ, Beaudet AL, Finegold MJ *et al.* Improved hepatic transduction, reduced systemic vector dissemination, and long-term transgene expression by delivering helper-dependent adenoviral vectors into the surgically isolated liver of nonhuman primates. *Hum Gene Ther* 2006; **17**: 391–404.

45 Brunetti-Pierri N, Clarke C, Mane V, Palmer DJ, Lanpher B, Sun Q *et al.* Phenotypic correction of ornithine transcarbamylase deficiency using low dose helper-dependent adenoviral vectors. *J Gene Med* 2008; **10**: 890–896.

46 Toietta G, Pastore L, Cerullo V, Finegold M, Beaudet AL, Lee B. Generation of helper-dependent adenoviral vectors by homologous recombination. *Mol Ther* 2002; **5**: 204–210.

47 Zhou H, Pastore L, Beaudet AL. Helper-dependent adenoviral vectors. *Methods Enzymol* 2002; **346**: 177–198.

48 Palmer D, Ng P. Improved system for helper-dependent adenoviral vector production. *Mol Ther* 2003; **8**: 846–852.

49 Bolin LM, Zhaung A, Strychkarska-Orczyk I, Nelson E, Huang I, Malit M *et al.* Differential inflammatory activation of IL-6 (-/-) astrocytes. *Cytokine* 2005; **30**: 47–55.