ApoE Gene Therapy: An Overview and Update

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Abstract

Atherosclerosis remains the leading cause of death in industrialized societies. Apolipoprotein E (ApoE) is an attractive candidate to treat hypercholesterolemia and coronary heart disease, as it is a circulating protein with pleiotropic atheroprotective actions. Here, we describe several ***"gene addition"*** approaches and on-going developments to achieve efficient delivery and long-term expression. The use of recombinant viruses is discussed, including adeno-associated viral vectors (AAV) where technological advances now allow the cross-packaging of different AAV serotypes. Nonviral delivery systems are also described, including plasmids and cell-based therapy. Finally, a radical, alternative technology to gene addition, which has the potential for permanent cure in many genetic diseases, is reviewed: ***"targeted gene repair"***, which aims to correct underlying point mutations in-situ. Synthetic oligonucleotides are designed to bind specifically to defective DNA, enabling the cell's own mismatch machinery to recognize and repair the faulty DNA. Although such gene editing technology has great potential it remains inconsistent and difficult to reproduce.

Introduction

Atherosclerosis remains the leading cause of death in industrialized societies. A significant risk factor is raised plasma LDL cholesterol. This is now effectively treated by statins. But statin monotherapy has limitations, particularly if levels of atheroprotective HDL are low. Hence, alternative strategies are needed to combat occlusive coronary heart disease (CHD) [1].

Somatic gene therapy promises cure of CHD where a gene is defective or effective treatment where its heterologous overexpression is therapeutic. Circulating proteins are attractive targets for genetic manipulation. Here, we focus on apolipoprotein E (ApoE) as a candidate to treat hyperlipidaemia and atherosclerosis, describing both gene addition and gene editing. Our commentary is also a paradigm of gene therapy for alternative atheroprotective molecules which circulate, including ApoAI-Milano which shows promise in clinical protein therapeutics [2].

ApoE is a polymorphic 34-kDa glycoprotein that mediates hepatic clearance of atherogenic remnant lipoproteins. This action is endorsed by the gross hypercholesterolemia and premature atheroma seen in ApoE-deficient [ApoE(-/-)] mice, or in transgenic mice expressing ApoE mutants. Plasma ApoE is largely secreted by liver (~90%), but other tissues including macrophages contribute. The rarest isoform, ApoE2 differs from wild-type ApoE3 by an Arg158Cys substitution and causes Type III hyperlipidemia [3], while ApoE4 (Cys112Arg) produces a dominant hyperlipidemia and is a significant risk factor for CHD as verified by meta-analysis [4].

Importantly, ApoE has multiple additional atheroprotective actions independent of cholesterol transport [4]. These include antioxidant potential [5], inhibition of vascular smooth muscle cell proliferation and migration [6], and our finding that ApoE has anti-platelet and anti-inflammatory activities by stimulating cellular release of nitric oxide (NO) [7,8]. This suggests that ApoE acts locally to restrict atherosclerotic lesion development; indeed, there is compelling evidence that independently of lowering serum cholesterol, ApoE can halt atherosclerosis progression and induce lesion regression [9].

The Case for ApoE Gene Therapy

Bolus infusion of plasma-purified or recombinant ApoE markedly reduces plasma cholesterol levels in rabbits with genetic or diet-induced hypercholesterolaemia, while a longer-term study prevents progression of atherosclerosis [10]. Similarly, synthetic peptide mimics of the ApoE binding region clear cholesterol-rich lipoproteins in ApoE-deficient mice [11]. Studies in transgenic mice overexpressing ApoE confirm its atheroprotective potential, protecting the animals from diet-induced or diabetic hyperlipidemia [12,13] - though paradoxically supraphysiological levels promote hypertriglyceridemia [14,15], albeit reversible if the C-terminal domain of ApoE is modified [16]. Finally, macrophage-restricted expression of ApoE in transgenic mice [17], or in ApoE(-/-) mice following bone marrow transplantation [18], is also able to inhibit atherogenesis.

In the 1990s, the majority of preclinical and clinical gene therapy trials used retrovirus-based vectors, since their ability to integrate into genomic DNA would ensure long-term expression. These included a pilot clinical trial of familial hypercholesterolemia by *ex-vivo* transduction of autologous hepatocytes and subsequent transplantation [19]. However, its invasive, costly and time-consuming nature, inherent inefficiency, and safety concerns — transgenes might insert into a critical part of the genome, as found during retroviral therapy for X-linked severe combined immunodeficiency (X-SCID) [20] — have emphasized the need to develop strategies for direct *in-vivo* delivery of therapeutic genes and for long-term expression.

Gene Addition Studies - Adenoviruses (Ad)

The first ApoE gene transfer studies were reported 10 years ago using recombinant adenoviruses (rAd). They lowered plasma cholesterol in ApoE-deficient mice and after 1-month aortic atherogenesis had slowed [21]. Adenoviruses are nonenveloped, double-stranded DNA. They exist and propagate only as episomes, but do deliver foreign DNA sequences into mammalian liver with an efficiency approaching 100%. Unfortunately, the therapeutic effect of these 1st generation vectors was transient because of cytotoxicity and/or host immunological responses against co-expressed viral proteins. This led to clearance of transduced hepatocytes and precluded repeat vector administration. Nevertheless, the potential to ***regress*** advanced atherosclerosis was established by cross-breeding ApoE(-/-) and immunodeficient mice and then injecting rAd.ApoE3; plaque development was halted and was completely reversed at 6 months [22]. Recent improvements in rAd constructs, and the introduction of efficient liver-specific promoters, now offer hope of long-term benefits. For ApoE expression, these include a temperature-sensitive 2nd generation rAd [23], our application of polymerase-deleted vectors [24,25], and the development of helper-dependent adenovirus (HD-Ad) [26]. Notwithstanding these significant advances, the unexpected and tragic death of a patient undergoing clinical trial for a rAd therapeutic [20], has led many groups to critically examine alternative gene transfer systems.

Non-Viral Gene Transfer - Plasmids

Plasmids have low immunogenicity, can be manufactured in quantity and to high purity, and are expressed episomally, thereby making an insertional mutagenesis highly unlikely. Two laboratories have reported *in-vivo* injection of ApoE plasmids into ApoE(-/-) mice, both choosing skeletal muscle as the secretory platform. Muscle represents an alternative gene therapeutic target to liver, being accessible and a stable tissue with little nuclear turnover. It is also highly vascularized and actively secretory and, although it does not normally secrete ApoE, nonhepatic, nonmacrophage-derived ApoE is known to be atheroprotective [27]. One group reported a sustained lowering of plasma cholesterol [28], despite finding only very low levels of secreted ApoE (presumably reflecting the inefficient uptake of plasmids by cells), while our own study showed clear inhibition of xanthoma and atherosclerotic plaque formation [29].

Cell Therapy

Transplantation of bone marrow from wild-type mice into recipient ApoE(-/-) mice reverses their hypercholesterolemia and prevents development of atherosclerosis [18]. Mixing ApoE(-/-) bone marrow in increasing amounts with ApoE(+/+) marrow prior to transplanting suggested that only 400 ng ApoE/ml was needed to fully normalize plasma cholesterol [30]. This has encouraged cell-based therapeutic approaches. These include transducing ApoE(-/-) hematopoietic stem cells (HSCs) *ex-vivo* with a self-inactivating retroviral vector to express high levels of murine ApoE. Transplantation of these cells reversed both hypercholesterolemia and atherosclerosis [31].

A different strategy employs *in-vivo* ApoE-secreting mini-organs. One study embedded recombinant endothelial cells in Matrigel for intradermal injection into ApoE(-/-) mice; plasma cholesterol was halved for over 3 months and atherosclerotic plaque reduced [32]. In another investigation, we encapsulated engineered Chinese hamster ovary (CHO) cells expressing human ApoE3 into alginate-based microspheres. After confirming *in-vitro* that ApoE3 protein was secreted and biologically active, the capsules were implanted into the peritoneum of ApoE(-/-) mice. ApoE was readily detected in plasma and reduced total cholesterol levels, whilst increasing atheroprotective HDL [33]. Though we were encouraged by these findings, it was clear that the technology must be improved and refined before routine use, particularly for long-term treatment to retard or regress atherosclerosis.

AAV - A Safe and Effective Viral Vector?

Adeno-associated virus (AAV) contains a linear single-stranded DNA genome and represents a family of replication-defective parvoviruses. They have several advantages for gene transfer: they cause no disease in humans and, though wild-type AAV integrates into a 4kb region of chromosome 19q using Rep68/78 protein nicking activity [34], provide long-term expression as nearly all rAAV persist in target cells as transcriptionally active extrachromosomal (episomal) monomeric and multimeric double-stranded forms [35,36]. Indeed, in muscle high and stable transgene expression is reported without an immune response. However, when we injected rAAV-ApoE3 into leg muscles of ApoE(-/-) mice, only traces of plasma ApoE were detected and the hyperlipidemia was unaffected - though as with plasmid injections, the atherosclerotic plaque was significantly reduced [37].

This disappointing outcome can now be explained. The rAAV-ApoE3 we had employed was derived from serotype 2 and, though very effective *in-vitro*, it is now clear that the different serotypes have very variable transduction efficiencies *in-vivo*. Thus, for muscle-based expression rAAV serotype-1 produces 100-1000-times more secreted plasma Factor IX compared to serotype-2 [38]. There have been other important advances in rAAV technology. The cloning of additional serotypes has enabled the tropism of rAVV vectors for different target tissues to be critically evaluated, while the ability to cross-package rAAV-2 genomes with capsid proteins of different serotypes permits considerable experimental flexibility [39]. Of note, is emerging evidence that AAV2/1, AAV2/7 and AAV2/8 are highly efficient at transducing muscle fibres of immunocompetent mice compared to AAV2/2 [40], while AAV2/8 shows great promise for gene transfer to liver [41,42]. Such improvements in expression are suggested to reflect a much more rapid uncoating of the vector genome [43].

Gene Editing

A radical, alternative technology to gene transfer, which has the potential for permanent cure in many genetic diseases, has been proposed: ***targeted gene repair***. This elegant strategy aims to correct underlying point mutations *in-situ* and initially used synthetic RNA-DNA oligonucleotides, termed RDOs or chimeraplasts. These were designed to bind specifically to defective DNA, enabling the cell's own mismatch machinery to recognize and repair the faulty DNA. Using 68-mer RDOs we have successfully converted the defective ε2 allele (C→T) to the fully-functional ε3 allele at a level of ~35% in recombinant CHO cells. The correction was verified by gene sequencing and by isoelectric focusing analysis of secreted protein, while prolonged passaging and cloning of cells showed that the corrected allele was stably maintained [44]. A preliminary attempt to target human *APOE2* in the liver of

transgenic mice was also promising.

However, our attempts to extend the work and correct the dysfunctional human ε4 allele in recombinant CHO cells and in EBV-transformed lymphocytes from patients have disappointed. Conversions were noted by PCR-RFLP analysis, but were unstable and corrected clones could not be isolated [45]. Although we ruled out PCR artifacts for the initial repair [45], we cannot yet explain our failure though, as noted elsewhere [46], gene editing technology remains inconsistent and difficult to reproduce. Our initial conclusion was that manufacturing changes had produced poorer quality reagents [45,47]; in turn, this meant we used higher amounts of RDO and delivery vehicle to effect repair, opening the possibility of cytotoxic/ apoptotic actions. An intriguing alternative, or co-possibility, is that cells have effective defense mechanisms that counteract targeted genome sequence alterations [48].

Concluding Remarks and Future Perspectives

The design of safe and effective gene therapeutic strategies is the goal of many laboratories, albeit often a distant one. In the cardiovascular field its potential clinical significance is profound: the prevention and regression of atherosclerotic plaques i.e. reduction in the clinical risk of atherosclerosis and treatment of pre-existing disease. But which vector will emerge to clinically deliver ApoE? - a complex question to answer as each has inherent characteristics which confer both advantages and disadvantages.

Of the viral vectors, the new AAV isolates appear promising for safe and efficient gene transfer, particularly AAV-2/1 for muscle and AAV-2/8 to transduce liver or muscle [49]. As the rate-limiting step in AAV-directed transgene expression is thought to be second-strand DNA synthesis, the introduction of self-complementary AAV vectors may further improve transduction efficiency [50]. However, one concern prior to widespread clinical trials is rAAV chromosomal integration. This occurs at low frequency compared to episomal retention, but in liver rAAV preferentially integrates into active genes [51] — though interestingly rAAV integrants could not be detected following transduction of muscle [52].

Despite the setback of a fatality, adenovirus-based therapies remain attractive. The helper-dependent 'gutless' rAd vectors have much potential [26], provided current limitations of inefficient production and helper-virus contamination can be overcome. Moreover, production of effective high-capacity hybrid vectors is underway, coupling the efficient cell-entry and nuclear targeting properties of Ad to the site-specific integration ability of wild-type AAV [53]. Lentiviral vectors are also receiving attention for direct *in-vivo* delivery, as unlike other retroviruses, they can transduce a high percentage of nondividing cells, such as hepatocytes. Indeed, recombinant lentiviruses have delivered human ApoE2, E3 & E4 to a mouse model of Alzheimer's disease using direct intracerebral administration [54].

There is renewed interest in non-viral DNA ('naked' DNA) delivery, driven by technological advances, as well as safety considerations. These include the use of minicircle DNA vectors devoid of bacterial sequences [55], the design of plasmids which carry scaffold/matrix attached region (S/MAR) for long-term episomal gene expression [56], and the use of transposon systems or phage integrase to direct naked DNA integration [57,58]. In addition, there is improved delivery efficiency, including to liver by rapid, high volume injection (hydrodynamic transfection, by intravenous or regional delivery [59]) or to muscle by electric pulsing of injection sites (electroporation) [60].

Finally, there is the exciting new technology of gene editing, which unfortunately has underperformed since the encouraging early reports. We, and others, now use single-stranded all-DNA oligonucleotides (SSOs), which are end-protected to minimize nuclease degradation. Early data on targeting the *APOE4* locus are promising, as conversion of both transcribed and non-transcribed strands has been detected. Whether this can develop into a viable therapeutic remains open, though this will become clear once we understand the mechanisms by which cells accomplish mismatch repair, an area about which we remain largely ignorant.

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