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# AAV serotype-dependent apolipoprotein A-I$_{Milano}$ gene expression

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**Abstract**

Recent evidence from a double-blind, randomized study showed that treatment with apolipoprotein A-I$_{Milano}$ (ApoA-I$_{Milano}$) in a complex with phospholipids produced significant regression of the coronary atheroma burden in patients with acute coronary syndromes. We previously showed similar regression of atherosclerosis in an animal model. Here, we examined a viral vector-based gene delivery system as a basis for ApoA-I$_{Milano}$ gene therapy. Comparing levels of expression using combinations of the cytomegalovirus (CMV) promoter in a recombinant serotype 2 adeno-associated virus (rAAV2) linked to ApoA-I$_{Milano}$ or the enhanced green fluorescent protein (EGFP) genes, we found that a promoter construct of two CMV core promoters sharing a CMV enhancer was more active than other combinations or a single CMV promoter. In vivo assessment of this optimal CMV construct using rAAV2 virus particles for intravenous (IV) or intramuscular (IM) routes of delivery produced high circulating levels of ApoA-I$_{Milano}$ protein for extended periods (up to 220 ng/ml at 22 weeks p.i.) by IV delivery while the IM route resulted in a relatively short period of very low-level ApoA-I$_{Milano}$ expression. Since there was no difference in the immune response between the two routes of delivery, we reasoned that tissue tropism might be responsible for this differential gene expression. To explore this possibility, we investigated the effect of different AAV serotypes on ApoA-I$_{Milano}$ gene expression in vivo. It found that rAAV1-mediated expression of ApoA-I$_{Milano}$ was approximately 15- and 9-fold higher than rAAV2 and rAAV5, respectively when IM injection routes were compared while all three AAV serotypes produced substantial levels of ApoA-I$_{Milano}$ expression from IV injection. These studies demonstrate that by modifying the promoter and serotype, increases in the efficiency of AAV-directed transgene expression could be achieved and support the potential of AAV-mediated gene therapy.


*Keywords:* Adeno-associated virus; Apolipoprotein A-I; Atherosclerosis; Gene therapy; Serotype

## 1. Introduction

Apolipoprotein A-I$_{Milano}$ (ApoA-I$_{Milano}$) is a mutant form of ApoA-I with a single amino acid substitution (Arg$_{173}$ to Cys$_{173}$) [1]. This mutation appears to confer greater resistance to atherosclerosis in individuals with this genotype [2]. The structural alteration of ApoA-I$_{Milano}$ is associated with a higher kinetic affinity for lipids and an easier dissociation from lipid protein complexes, which contributes to its accelerated catabolism and increased uptake of tissue lipids [3]. The exact mechanism by which ApoA-I$_{Milano}$ confers its resistance to atherosclerosis remains to be elucidated.

We have previously reported that intravenous injection of 40–80 mg/kg dose of recombinant ApoA-I$_{Milano}$ over a 5-week period induces a significant reduction in aortic atherosclerosis in ApoE-deficient mice compared with untreated controls [4]. In a more recent double-blind, randomized, placebo-controlled study, Nissen et al. [5] demonstrated that infusion of recombinant ApoA-I$_{Milano}$–phospholipid complexes in patients with acute coronary syndromes with five doses at weekly intervals produced significant regression of the coronary atheroma burden. These finding suggests that therapy based on ApoA-I$_{Milano}$ could have potential as an effective anti-atherogenic strategy. However, frequent in-

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travenous injections and expenses related to the production of recombinant proteins limit the practical and widespread applicability of this approach in humans. An alternative approach would be a gene therapy strategy to deliver and express ApoA-$I_{Milano}$. This requires a system for gene delivery and expression, which would result in constitutive and persistent levels of circulating ApoA-$I_{Milano}$ protein. We now report development of a potential gene therapy approach for the prolong expression of circulating ApoA-$I_{Milano}$.

## 2. Materials and methods

### 2.1. Construction of recombinant adeno-associated virus vectors

A series of serotype 2 rAAV vectors (rAAV2) were constructed based on vectors previously constructed and utilized in our laboratory for the purpose of ApoA-$I_{Milano}$ expression. The changes that were made included the addition of a fully functional promoter, 582-nucleotide CMV promoter (nucleotide −574 to +8 in reference to the +1 transcription start site) along with the previous "short" length 308-nucleotide CMV promoter (−300 to +8). Various arrangements of these two forms of the CMV promoter were constructed and positioned upstream to the coding sequence of ApoA-$I_{Milano}$. In addition, the enhanced green fluorescent protein (EGFP) marker gene was also included in the constructs to simplify the monitoring procedure for transgene detection.

### 2.2. Cultured cells

Human embryo kidney (HEK) 293 cells were grown and maintained in MEM culture medium (Invitrogen, Carlsbad, CA) containing 5% donor horse serum, 5% fetal bovine serum, 0.1 mM MEM non-essential amino acids solution, 1 mM MEM sodium pyruvate solution, 100 units/ml–100 mg/ml penicillin–streptomycin, and 2 mM L-glutamine in 5% $CO_2$ at 37 °C.

### 2.3. Preparation and purification of recombinant adeno-associated virus

Subcultured actively growing 293 cells were placed in 25 mm culture dishes with MEM medium and incubated in 5% $CO_2$ at 37 °C overnight. The medium was changed the next day and used for transfection 2–4 h. A plasmid mixture consisting of 5 mg of rAAV vector (individual constructs), 5 mg of AAV helper plasmid XX2, and 15 mg of adenovirus helper plasmid XX6-80 were mixed with EC buffer (Qiagen Inc., Valencia, CA) to a final volume of 750 ml. Enhancer (200 ml; Qiagen Inc.) was added and vortexed immediately for 5 s. Effectene (250 ml; Qiagen Inc.) was added to each tube and vortexed immediately for 10 s. The tubes were placed at room temperature for 10–15 min. Fresh MEM culture medium (6 ml) was added to each individual tube and mixed by pipetting up and down three times. The medium was then laid drop-wise onto 293 cells while the dish was gently swirled. Transfected 293 cells were scraped with a cell lifter at 66 h post-transfection in medium. The cells from two dishes were combined in a 50 ml disposable centrifuge tube and collected by centrifugation in a Sorvall TC centrifuge at 1000 rpm for 5 min at room temperature. The media were discarded, and the cell pellets were stored at −80 °C for later use.

The cells were resuspended in 1.5 ml of 150 mM NaCl, 50 mM Tris–HCl, pH 8.5. Cells from five tubes were combined into a 15 ml screw cap conical centrifuge tube. Cells were subjected to four cycles of freezing (dry ice–ethanol bath) and thawing (37 °C water bath) with vortexing for 30 s after each thawing. The lysed cells were treated with 50 units/ml Benzonase (Sigma–Aldrich, St. Louis, MO) at 37 °C for 30 min. The lysate was clarified and recovered by centrifugation at 3700 × *g* at 4 °C for 20 min. Purification of rAAV particles was accomplished by iodixanol density gradient centrifugation method as previously described by Muzyczka and the coworkers [6]. Viral particles were by dialyzed and concentrated prior to use. Viral infectious titer was measured by transduction of 293 cells in the presence of adenovirus helper with MOI 1 followed by FACS.

### 2.4. Western blot analysis of ApoA-$I_{Milano}$ protein

HEK 293 cells were transfected with plasmid DNA using Effectene reagent (Qiagen Inc.) in MEM medium containing 10% FBS. The growth medium was switched to serum free medium 293 SFM II (Invitrogen, Carlsbad, CA) 16 h after transfection. Medium was collected at 48 h post transfection, and cells were lysed. Total protein of 10 μg of cell lysate and a 10 μl aliquot of medium were subjected to SDS–polyacrylamide gel electrophoresis and proteins transferred onto nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The membrane was incubated with 1:2000 diluted polyclonal rabbit anti-human ApoA-I antibody (Chemicon, Temecula, CA) for two hours at room temperature. The protein–primary antibody complexes were visualized by horseradish peroxidase-conjugated secondary antibodies and ECL reagents (Amersham Biosciences, Piscataway, NJ). Imaging was performed using Kodak BioMax X-ray film and protein band images scanned and quantitated using 1-D Advanced Image Analysis software (Advanced American Biotechnology, Fullerton, CA).

### 2.5. Enzyme-linked immunosorbent assay (ELISA) for ApoA-$I_{Milano}$ protein

Mouse anti-human ApoA-I monoclonal antibody (0.4 μg in 100 μl PBS; Calbiochem–Novabiochem Corp., San Diego, CA) was added to each well of a 96-well microtiter plate as the capture antibody. Diluted plasma samples (20- to 100-fold) were prepared with PBS containing 0.1% Tween-20

and 10 mg/ml of BSA and added to the wells. Rabbit anti-human ApoA-I polyclonal antibodies (Chemicon, Temecula, CA), 0.05 μl in 100 μl PBS containing 0.1% Tween-20 and 10 mg/ml BSA, was added to each well. Biotin-conjugated anti-rabbit IgG antibody (BD Biosciences Pharmingen, San Diego, CA) diluted 1:2000 with PBS containing 10 mg/ml of BSA was combined with avidin-conjugated horseradish peroxidase (HRP) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:1000 with TBST containing 10 mg/ml of BSA. A volume of 200 μl per well was used to visualize the antibody-ApoA-$I_{Milano}$ complex in the presence of substrate 3,3′,5,5′-tetramethylbenzidine (TMB). To stop the enzymatic reaction and color development, 1 M phosphoric acid was added. Absorbance was measured with an ELISA reader at 450 nm. Serial dilutions of commercial purified human ApoA-I protein (Chemicon, Temecula, CA) were applied as standards for quantification purposes.

### 2.6. Animal procedures

Eight- to 12-week-old ApoA-I-deficient mice (Jackson Laboratory, Bar Harbor, ME) were used for viral transduction. Mice were restrained for intravenous injection of rAAV into the tail-vein and intramuscular injection into the hind leg. Blood samples were obtained by retro-orbital bleeding while animals were under anesthesia with isoflurane. The use of experimental animals was in accordance with the guidelines of the CSHS Institutional Animal Care and Uses Committee.

## 3. Results

In our initial approach to increase ApoA-$I_{Milano}$ gene expression, we reasoned that multiple CMV promoters may generate high levels of expression. To explore this possibility, we constructed a series of double CMV promoter constructs and compared their transcriptional efficiency to that of a single CMV promoter. In addition to ApoA-$I_{Milano}$, our constructs express a reporter protein, the enhanced green fluorescent protein (EGFP), which facilitates evaluation of promoter activity. The expression of EGFP was determined by FACS and expression of ApoA-$I_{Milano}$ was measured by Western blotting of cell associated proteins and secreted proteins into media using transient transfection of 293 cells (Fig. 1a). We found that the two CMV core promoter construct sharing one CMV enhancer (construct 4) expressed higher ApoA-$I_{Milano}$ protein levels of 213% (media) and 244% (cells) relative to the single CMV core promoter construct (construct 2) (Fig. 1b). These levels of ApoA-$I_{Milano}$ protein were also greater than that of a single CMV core promoter/enhancer construct (construct 3). Construct 5 consisting of two complete CMV core promoter/enhancer regions also produced elevated levels of ApoA-$I_{Milano}$ protein (251% and 183% in media and cells, respectively) that were comparable to using construct 4. Construct 5 appears to be slightly better than construct 4 in ApoA-$I_{Milano}$ protein production possibly due to the presence of the extra CMV enhancer element. However, the preparation of the plasmid vector of construct 5 produced minor plasmid bands of other sizes while the preparation of construct 4 produced a homogenous plasmid band (data not shown). Therefore, it was decided to use construct 4 for subsequent experiments.

To confirm the high expression level of the double CMV promoter construct, EGFP activity of construct 4 was compared to that of construct 2 containing a single CMV core promoter. FACS analysis of transfected 293 cells revealed that the double CMV promoter of construct 4 produced nearly three times the level of EGFP expression as compared to the single CMV promoter of construct 2 (data not shown). Interestingly, reversing the orientation of the CMV core/enhancer-EGFP reporter gene cassette (constructs 6 and 7) led to a marked reduction in ApoA-$I_{Milano}$ expression (17% and 21% in media and cells, respectively; Fig. 1b). Nonetheless, the expression levels of the different promoter constructs shown in Fig. 1 demonstrate that the optimal promoter construct for high level of ApoA-$I_{Milano}$ expression consists of two CMV core promoters in opposite orientation sharing one CMV enhancer (construct 4).

To determine whether our optimal double CMV promoter construct remains active in other cell types, we assessed the expression level of EGFP under the control of this specific promoter in human endothelial and smooth muscle cells, two cell types relevant to the development of atherosclerosis that may be cell targets from in vivo gene delivery. FACS analysis of transiently transfected cells showed that, relative to transfected 293 cells, infected cultured smooth muscle and endothelial cells had considerable levels of EGFP expression ranging from 49% to 56% (data not shown), suggesting that the optimal CMV promoter construct remains active in vascular cells.

Based on these in vitro experiments, we hypothesized that the double CMV promoter construct would also exhibit in vivo activity. To test this hypothesis, we used AAV serotype 2 virus (AAV2), a widely used AAV serotype, for transfer of the ApoA-$I_{Milano}$ transgene. We choose the AAV vector rather than an adenoviral (AV) vector for gene delivery due to its prolong gene expression in vivo and its reduced immunological reactivity [7]. For these in vivo studies, we used ApoA-I knockout mice in order to prevent complication due to the presence of endogenous ApoA-I protein in the determination of circulating ApoA-$I_{Milano}$ protein levels since commercially available anti-human ApoA-I antibodies cross-react with mouse ApoA-I. Recombinant AAV2 viral particles containing the ApoA-$I_{Milano}$ expression construct were prepared and injected intramuscularly (IM) or intravenously (IV) into ApoA-I-deficient mice. We used one dose ($4 \times 10^8$ T.U.) for IV delivery and two different doses of virus ($1 \times 10^8$ and $6 \times 10^8$ T.U.) for IM delivery in single injections into mice. The highest dose of $6 \times 10^8$ T.U. of virus was the maximum concentration of viral preparation. ELISA determination for ApoA-I was used to monitor systemic circulating level of ApoA-$I_{Milano}$ gene product (Fig. 2). In IM-

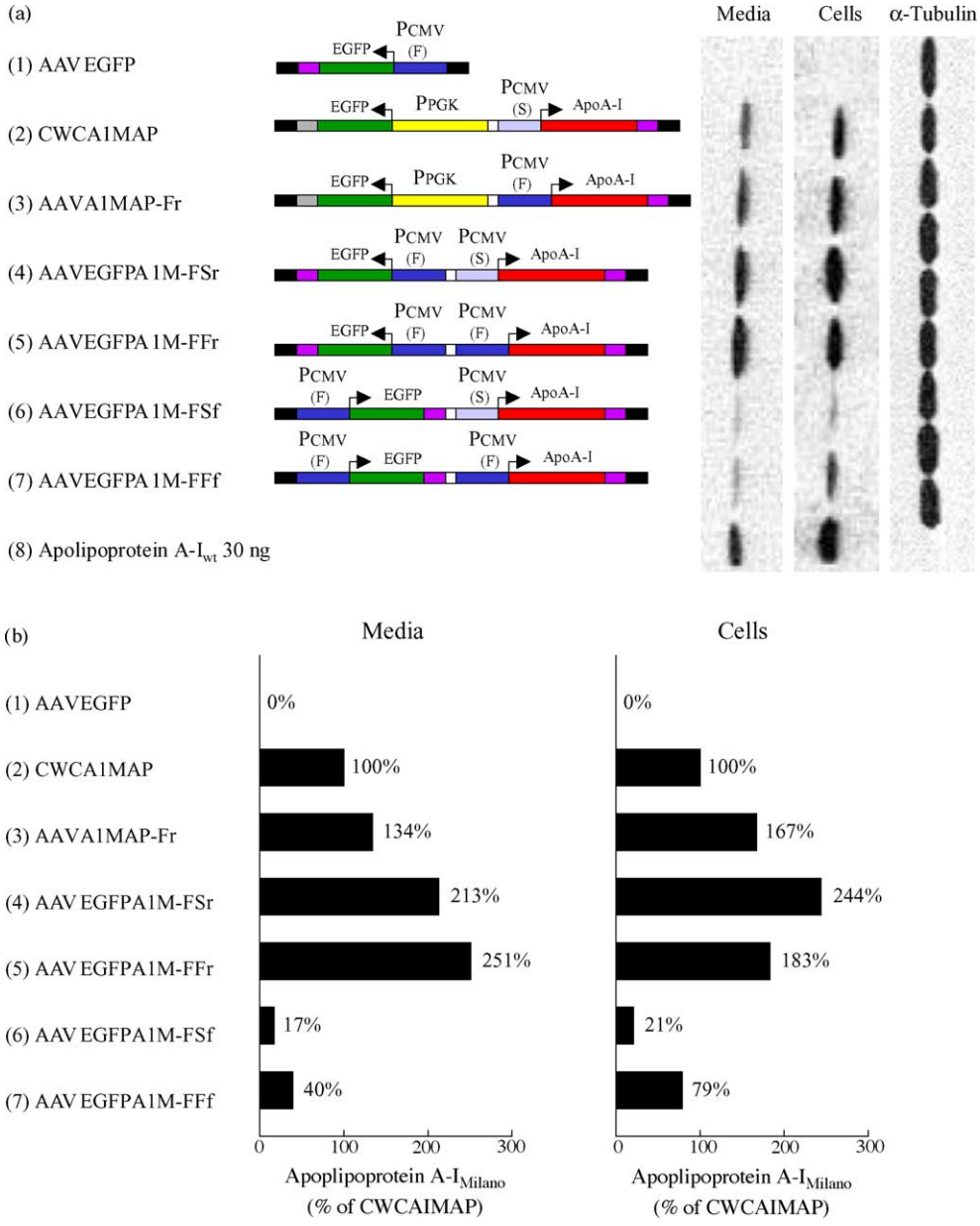


Fig. 1. Expression of ApoA-I$_{Milano}$ in rAAV vectors with various CMV promoter domains. A series of serotype 2 rAAV vectors (rAAV2) were constructed using a "core" 308-nucleotide CMV promoter segment (nucleotides −300 to +8 relative to the +1 transcription start site) and a "core + enhancer" 582-nucleotide CMV promoter segment (nucleotides −574 to +8). Various arrangements of the CMV core promoter and enhancer were positioned upstream to EGFP and ApoA-I$_{Milano}$. (a) The various ApoA-I$_{Milano}$ expression vectors are shown using the following domains: ApoA-I$_{Milano}$ mutant (ApoA-I), EGFP, human phosphoglycerate kinase promoter (PPGK), CMV core promoter (C), and CMV core + enhancer promoter (C + E). Also included is a negative control vector (construct 1) for comparative purposes. ApoA-I$_{Milano}$ expression in transfected 293 cells was determined in media and cells by Western blot analysis. Medium from cells was collected at 48 h post transfection and cells were lysed. Total protein of 10 μg of cell lysate and a 10 μl aliquot of medium were subjected to SDS–polyacrylamide gel electrophoresis and proteins transferred onto nitrocellulose membrane followed by incubation with 1:2000 diluted polyclonal rabbit anti-human ApoA-I antibody. Visualization was accomplished with by horseradish peroxidase-conjugated secondary antibodies and ECL reagents (Amersham Biosciences). (b) Exposed X-ray film images were scanned and densitometric measurement of the protein bands from the blots was accomplished using A.A.B. 1-D Advanced Imaging Analysis software. All values were normalized to α-tubulin levels and expressed as a percentage of construct 2, which contains the single CMV core promoter sequence. The bottom lane of the Western blot contains commercial ApoA-I$_{wt}$ protein as a standard.

injected mice, ApoA-I$_{Milano}$ protein was detected at week 10 post-injection with maximal expression of 1.2 and 23.6 ng/ml at week 14 for the low and high doses of rAAV2 particles, respectively. By week 22, serum levels of ApoA-I$_{Milano}$ protein started to decline in the IM-injected mice. In contrast, IV delivery resulted in much higher levels of circulating ApoA-I$_{Milano}$ protein of 61.6 ng/ml appearing early at week 3 post-injection which increased continuously to 220 ng/ml by week 22. These data demonstrate that the double CMV promoter construct remains active for a prolonged period in vivo. In

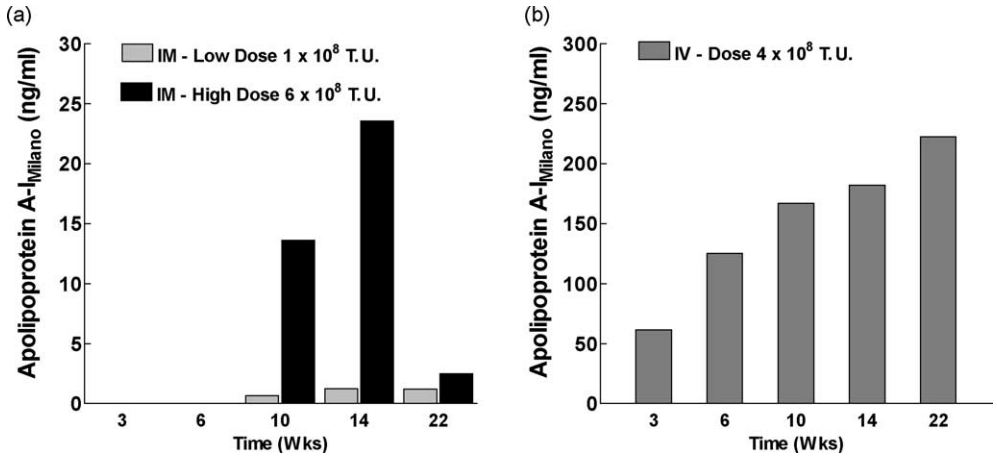


Fig. 2. ApoA-$I_{Milano}$ transgene expression in mice from IM and IV delivery with rAAV2. For preparation and purification of recombinant adeno-associated virus, actively growing 293 cells were transfected with a plasmid mixture consisting of rAAV2 vector, AAV helper plasmid XX2 and adenovirus helper plasmid XX6-80. After 66 h, isolated cells were lysed by freeze thawing and treated with 50 units/ml Benzonase at 37 °C for 30 min. Purification of rAAV particles was accomplished by the iodixanol density gradient centrifugation method as previously described by Muzyczka and coworkers [6]. Viral infectious titer was measured by transduction of 293 cells in the presence of adenovirus helper with multiplicity of infection (MOI) of 1 followed by FACS. Eight- to 12-week-old ApoA-I-deficient mice were used for viral transduction of ApoA-I protein since the absence of ApoA-I facilitates the measurement of ApoA-$I_{Milano}$ protein using available anti-ApoA-I antibody capable of recognizing both wild-type and Milano isoforms of the protein. Infectious rAAV particles were delivered by (a) a single IM injection into the hind leg (low dose, $1 \times 10^8$ T.U. or high dose, $6 \times 10^8$ T.U.) or (b) a single IV injection into the tail-vein ($4 \times 10^8$ T.U.). Serum samples from retro-orbital bleeding were measured for ApoA-$I_{Milano}$ levels by ELISA. Mouse anti-human ApoA-I monoclonal antibody (0.4 μg in 100 μl PBS; Calbiochem–Novabiochem Corp.) was used as the capture antibody in a 96-well plate. Each data point represents pooled serum samples from four mice. Note the scale difference of the *y*-axis of the two plots.

addition, we found that IV delivery of the vector resulted in ApoA-$I_{Milano}$ protein levels in the serum that were nearly 10-fold higher than those observed after IM delivery.

The exact reason for the difference in transgene expression between IM and IV routes of AAV2-mediated transgene delivery is not immediately apparent. We speculated that the ectopic expression of ApoA-$I_{Milano}$, a liver gene, in muscle tissue may generate a protein with different immunological properties than the natural protein expressed in liver tissue. In addition, since we used ApoA-I knockout mice, it is likely that the ApoA-$I_{Milano}$ protein produced would be immunogenic in these mice. Therefore, we examined the production of antibodies to the ApoA-I protein in pooled serum samples from IM- and IV-injected mice. Detectable levels of antibodies against ApoA-I appeared in both groups of mice as early as 10 weeks after injection and continued to be present up to 26 weeks after injection ($79 \pm 0.3$ ng/ml for IV and $33 \pm 6.7$ ng/ml for IM; Fig. 3). Despite variability in the levels of anti-ApoA-I antibody over the course of time, overall production of antibody from IV delivery was not less than that from IM injection. Thus, the higher level of ApoA-$I_{Milano}$ expression from IV versus IM injections does not appear to be related to the level of antibody response to the transgene product.

In addition, we assessed the production of antibodies against AAV2 coat proteins. Pooled serum samples from AAV2/ApoA-$I_{Milano}$-injected mice were analyzed by an antibody neutralization assay. rAAV2 particles harboring the EGFP reporter gene were incubated with the serum samples from the IM- and IV-injected mice and the treated viral particles were assessed for their capacity to transduce 293 cells. The level of suppression of in vitro transduction by the serum was quantified by FACS. Within 4–6 weeks, suppressive activity of serum from both IV- and IM-injected mice achieved

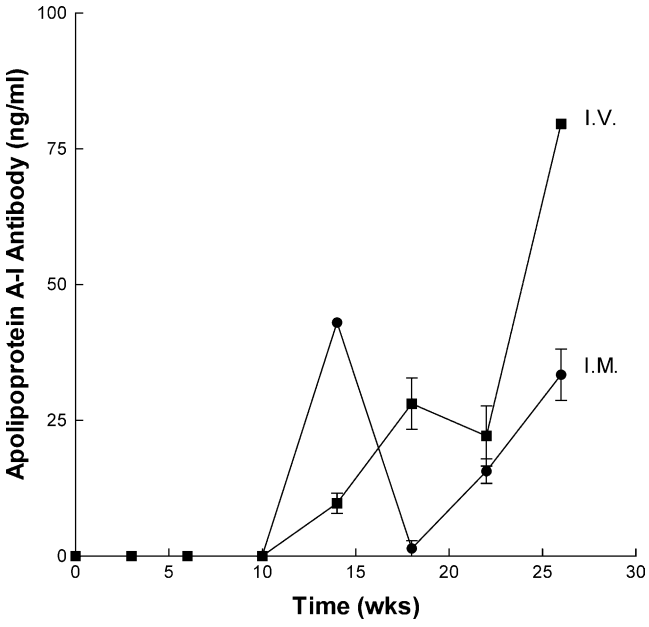


Fig. 3. Production of anti-ApoA-I antibody in rAAV transduced mice. Serum samples from IM- and IV-injected mice were assayed for production of antibodies against ApoA-I protein by using commercial ApoA-I as the capturing antigen on an ELISA plate and detection of IgG binding with an anti-mouse IgG antibody. Values are expressed as concentration of IgG in the serum sample (ng/ml).

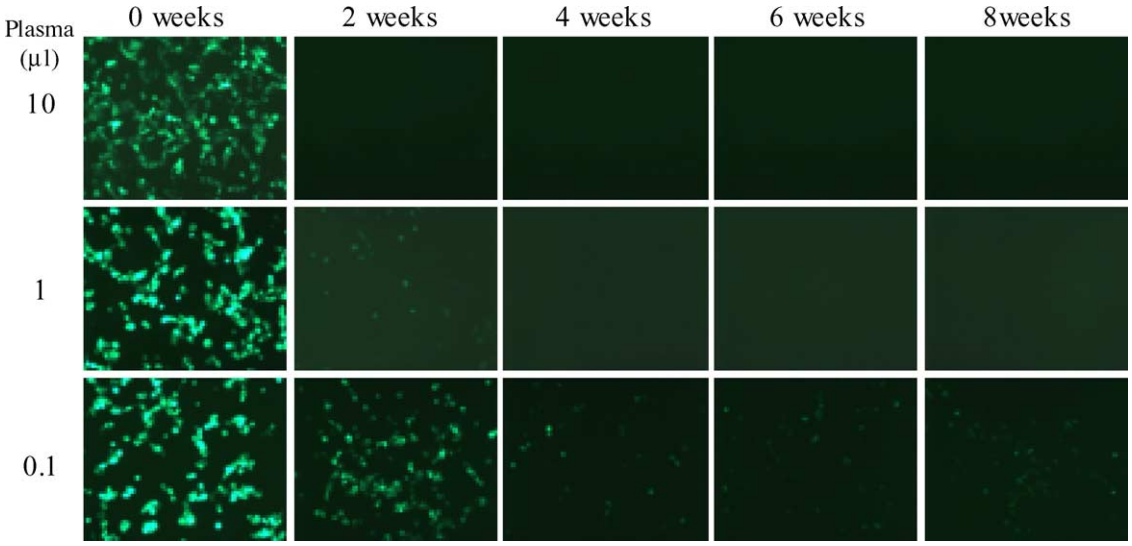


Fig. 4. Neutralization of rAAV2 transduction by anti-viral coat protein antibodies. Serum from mice IM- or IV-injected with rAAV infectious particles containing the ApoA-$I_{Milano}$ transgene was assayed for the production of anti-rAAV coat protein antibodies. Varying concentrations of serum was added to rAAV2 particles containing the reporter EGFP transgene. Treated rAAV2 particles were then assessed for their capacity to transduce 293 cells with quantitation of suppressive activity performed by FACS. A 100-fold dilution of serum (0.1 ml into 10 ml) from both types of injected mice suppressed viral transduction greater than 80% by 4–6 weeks post-injection (bottom row of panels). Higher dilutions from both groups of mice lowered suppression of transduction below the 80% threshold (data not shown). Shown are the fluorescent micrographs of transduction suppression with serum from IV-injected mice. IM-injected mice showed similar results (data not shown).

their maximum capacity (Fig. 4). During this period, a 100-fold dilution of serum from both types of injected mice suppressed viral transduction in 293 cells by greater than 80%; higher dilutions of serum from both groups of mice lowered suppression of transduction below the 80% threshold. These data demonstrate that IM and IV routes of delivery resulted in comparable immune responses to rAAV2 viral particles. Taken together, the elevated and prolonged expression of ApoA-$I_{Milano}$ from IV delivery over that of IM delivery does not appear to be related to differences in the immune response to either the transgene or the virus.

Past studies have shown that the natural tropism of viruses is the fundamental limitation to efficient gene transfer. It has been previously shown that a hierarchy for efficient serotype-specific AAV vector transduction depends on the target tissue [8]. Therefore, we reasoned that the lower level of ApoA-$I_{Milano}$ gene expression in muscle tissue might be related to the AAV2 serotype. To explore this possibility, we packaged the same AAV2 replication vector encoding the ApoA-$I_{Milano}$ gene into AAV1 and AAV5 serotype virions essentially as previously described [8]. An equal number of viral particles ($2 \times 10^{11}$) of each AAV serotype was injected either IV or IM into ApoA-I knockout mice and production of circulating ApoA-$I_{Milano}$ was monitored for 24 weeks by ELISA (Fig. 5). Consistent with our earlier experiment, levels of circulating ApoA-$I_{Milano}$ using AAV2 serotype particles were markedly higher in IV-injected mice than in IM-injected mice (Fig. 5b and e). IM delivery of AAV1 serotype resulted in significantly elevated and prolonged serum levels of ApoA-$I_{Milano}$ achieving a level of $108 \pm 8.8$ ng/ml (Fig. 5d) at 24 weeks after injection. However, IM injection of AAV5 serotype produced a maximum ApoA-$I_{Milano}$ serum level of $11.3 \pm 2.3$ ng/ml at 12 weeks post-injection (Fig. 5f) while AAV-2 produced a maximum of $7.2 \pm 0.9$ ng/ml at 18 weeks p.i. (Fig. 5e) with both serotypes displaying decreased ApoA-$I_{Milano}$ serum levels by week 24. In comparison to the IM delivery routes, all three AAV serotypes resulted in higher levels of ApoA-$I_{Milano}$ expression from IV injection (Fig. 5a–c). Notably, IV injection of AAV1 resulted in ApoA-$I_{Milano}$ serum levels that were over two-fold higher than using AAV2 ($253 \pm 114$ ng/ml versus $93 \pm 19$ ng/ml, respectively). These data demonstrate that the expression of ApoA-$I_{Milano}$ by AAV following IM injection is exquisitely dependent on the virus serotype and that AAV1 is more efficient than AAV-5 and AAV2.

## 4. Discussion

Transgene delivery systems have frequently included the use of the CMV immediate early promoter [9–13] since it is one of the most active promoters among viral and eukaryotic species without a specific host cell type requirement. Past studies have demonstrated that multiple copies of promoter/enhancer domain regions can improve gene expression [14–16]. In this current study, we were able to optimize ApoA-$I_{Milano}$ expression using two CMV core promoters in opposite orientation sharing one CMV enhancer (Fig. 1, construct 4). As mentioned in Section 3, reversing the direction of the upstream CMV core/enhancer-EGFP reporter gene cassette (Fig. 1, constructs 6 and 7) produced a significant decrease in ApoA-$I_{Milano}$ expression. This latter observation is not without precedent since enhancers stimulate transcription

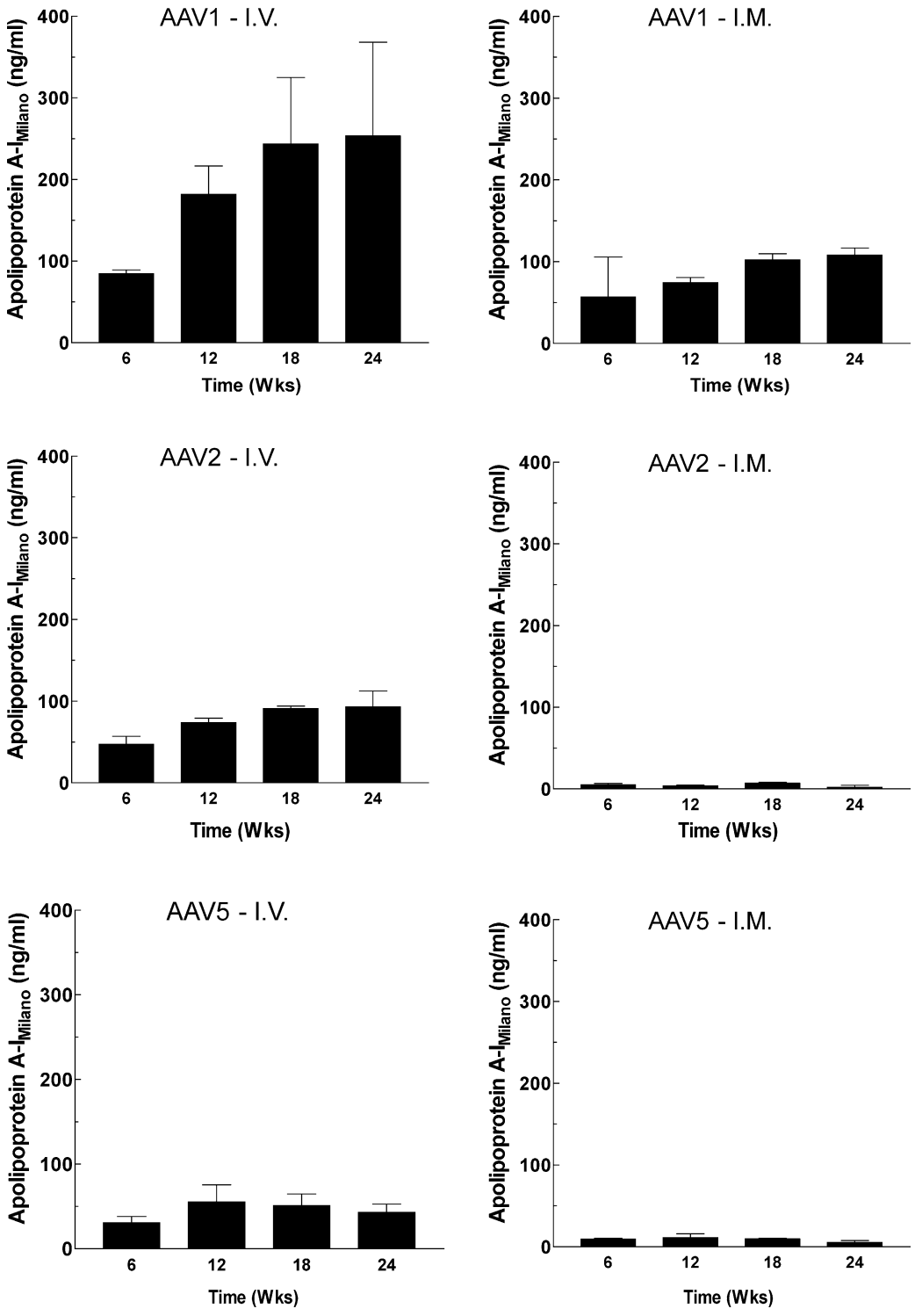


Fig. 5. Levels of ApoA-I protein from AAV1-, AAV2- and AAV5-transduced mice. Generation of recombinant adeno-associated virus type 2 (rAAV2) was essentially accomplished by the protocol detailed in Fig. 2. The production of rAAV1 and rAAV5 utilized the same procedures except that the plasmid mixture contained AAV helper plasmid XX1 and XX5, respectively, in place of plasmid XX2. Infectious rAAV virions ($2 \times 10^{11}$ particles) were delivered to ApoA-I-deficient mice red by (a–c) IV injection or (d–f) IM injection. Serum ApoA-I$_{Milano}$ levels were measured by ELISA as described in Section 2.

in a distance- and orientation-independent manner [17–20], it has been reported that reversing the orientation of an upstream regulatory region not only abolishes its enhancer capability but also can inhibit promoter activity [21].

Using this double CMV promoter construct, we were able to achieve prolonged and relatively high levels of ApoA-I$_{Milano}$ expression by intravenous delivery of our AAV2 viral vector into ApoA-I-deficient mice. In contrast, intramuscular

delivery resulted in a short interim period of low ApoA-$I_{Milano}$ expression. The difference in the levels of ApoA-$I_{Milano}$ expression by the two routes of viral delivery could not explain by the any significant differences in the levels of antibodies to either the ApoA-I protein or to viral coat proteins. Rather, we were able to show that ApoA-$I_{Milano}$ gene expression in muscle tissue was likely related to the AAV2 serotype by demonstrating that IM delivery of ApoA-$I_{Milano}$ using an AAV1 serotype virion significantly increased expression levels of the transgene. These findings confirm and extend earlier studies of various AAV serotypes having a significant impact on tissue-specific gene expression. Hauck et al. showed that AAV1 is significantly more effective than AAV2 in transducing muscle tissue for the expression of factor IX (FIX) gene [22]. Chao et al. demonstrated that AAV serotypes 1, 3 and 5 produced 100- to 1000-fold more canine FIX than AAV2 after IM injection in NOD/SCID mice [23]. Xiao et al. also found that AAV1 transduced muscle more efficiently than AAV2 with gene transfer of the erythroprotein and α1-antitrypsin genes into mice [24]. Furthermore, it was previously shown that AAV1 was superior to AAV2 in the expression of human α1-antitrypsin and factor IX [8] in non-neuronal tissues. Several recent gene transfer studies have also shown that other AAV serotypes performed better than AAV2 [25–28]. Taken together, we suggest that the higher efficiency of transgene expression by AAV-1 in non-neuronal tissue may be a general property of this viral serotype.

The levels of circulating ApoA-$I_{Milano}$ protein achieved with adeno-associated viral vectors demonstrated in this current study are relatively low compared to endogenous levels or those using adenoviral gene transfer for the expression of wild-type human ApoA-I although it remains to determined if our recombinant AAV vectors could produce higher levels of ApoA-$I_{Milano}$ expression in wild-type mice. In an earlier study that utilized a recombinant human ApoA-I adenovirus, De Geest et al. demonstrated transient expression of wild-type human ApoA-I in ApoE-deficient mice of over 150 mg/dl peaking at 6 days [29]. In a similar study, Tsukamoto et al. showed that intravenous injection into ApoE-deficient, low density lipoprotein receptor-deficient and wild-type C57BL/6 mice resulted in mean peak plasma human ApoA-I concentrations of 235, 324 and 276 mg/dl, respectively, after 3 days post-injection and declined thereafter [30]. The overall decrease in levels of human ApoA-I transgene expression may be attributed to an inflammatory response to virally infected cells [31]. In another study, gene transfer with an adenovirus and the 256-bp ApoA-I promoter, the genomic ApoA-I DNA, and four ApoE enhancers resulted in ApoA-I expression above 20 mg/dl for up to 6 months [32]. However, there is no data with regards to the effective concentration of circulating ApoA-$I_{Milano}$. Rather, past studies have suggested that the circulating levels of serum proteins do not necessarily correlate with their effective biological concentration. For example, bone marrow transplantation studies using $ApoE^{+/+}$ donor and $ApoE^{-/-}$ recipient mice showed significant improvements in atherosclerotic lesions in recipient mice with 10% chimerism [33]. Other investigators have also examined the effect of macrophage-derived ApoE by bone marrow transplantation with wild-type marrow [34–36]. These studies demonstrated normalization of plasma cholesterol 4–5 weeks post-transplant and reduction of atherosclerosis 14–20 weeks post-transplant. The level of ApoE varied from 3.8% to 12.5% of normal levels in C57BL/6 mice [34,36,37]. Therefore, considerably lower levels of the circulating bioactive serum molecules may be sufficient for an effective biological dose.

In a recent study, Van Linthout et al. used a "gutted" helpervirus independent adenoviral vector and an expression cassette consisting of the human α1-antitrypsin promoter, the human genomic ApoA-I DNA and four copies of the human ApoE enhancer in an effort to improve duration of ApoA-I transgene expression [38]. The investigators were able to produced long-term and high levels of ApoA-I expression ($170 \pm 16$ mg/dl at 6 months) in C57BL/6 mice. However, there may be still some concerns of toxicity in using these adenoviral vectors in humans (see Ref. [39] for review). In the case of AAV vectors, since the parental wild-type AAV is non-pathogenic [40], it is likely that the use of rAAV vectors would be virtually harmless for in vivo gene transfer. Our data may offer a viable approach for elevated and sustained levels of ApoA-$I_{Milano}$ expression which could have important implications on the development of therapeutic gene transfer.

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