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Liver-directed gene therapy of chronic hepadnavirus infection using interferon alpha tethered to apolipoprotein A-I

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Abstract

Background & Aims—Current HBV management is challenging as treatment with nucleos(t)ide analogs needs to be maintained indefinitely and because IFN α therapy is associated with considerable toxicity. Previously we showed that linking IFN α to apolipoprotein A-I generates a molecule (IA) with distinct antiviral and immunostimulatory activities which lacks the hematological toxicity of IFN α .

Methods—Here, we analyze the antiviral potential of an adeno-associated vector encoding interferon alpha fused to apolipoprotein A-I (AAV-IA) in comparison to a vector encoding only IFN α (AAV-IFN) in two animal models of chronic hepadnavirus infection.

Results—In HBV transgenic mice, we found that both vectors induced marked reductions in serum and liver HBV DNA and in hepatic HBV RNA but AAV-IFN caused lethal pancytopenia. Woodchucks with chronic WHV infection that were treated by intrahepatic injection of vectors

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Author's contributions:

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encoding the woodchuck sequences (AAV-wIFN or AAV-wIA) experienced only a slight reduction of viremia which was associated with hematological toxicity and high mortality when using AAV-wIFN while AAV-wIA was well tolerated. However, when we tested AAV-wIA or a control vector encoding woodchuck apolipoprotein A-I (AAV-wApo) in combination with entecavir, we found that AAV-wApo-treated animals exhibited an immediate rebound of viral load upon entecavir withdrawal while, in AAV-wIA-treated woodchucks, viremia and antigenemia remained at low levels for several weeks following entecavir interruption.

Conclusions—Treatment with AAV-IA is safe and elicits antiviral effects in animal models with difficult-to-treat chronic hepadnavirus infection. AAV-IA in combination with nucleos(t)ide analogs represents a promising approach for the treatment of HBV infection in highly viremic patients.

Keywords

Chronic hepatitis B; IFN- α hematological toxicity; Antivirals; Genetic transfer; Woodchuck

INTRODUCTION

Chronic hepatitis B (CHB) is a global health problem, with more than 350 million individuals worldwide infected with hepatitis B virus (HBV) [1]. Available treatment options include nucleoside/nucleotide analogues (NAs) that are highly effective in suppressing hepatitis B virus (HBV) replication and preventing liver disease progression. However, NAs do not affect HBV RNA transcription from the covalently-closed circular DNA molecule of HBV within infected hepatocytes and, therefore, have only limited impact on HBV surface antigen serum levels. Since NAs do not directly stimulate immune clearance of HBV-infected hepatocytes, in most cases viral replication rebounds upon cessation of therapy. As a consequence, prolonged or even life-long treatment of HBV is needed. This long-lasting therapy is associated with high costs and the risk of emergence of drug resistant HBV variants [2]. Pegylated interferon alpha (IFN α) is an alternative therapy for CHB. This cytokine is endowed with immunostimulatory properties and targets both HBV transcription and replication. Antiviral response to pegylated interferon alpha treatment is often durable but the use of this drug is limited due to low response rates and frequent side effects that are sometimes severe [3]. Therefore, new and alternative treatment approaches for the control of CHB are warranted.

We have previously shown that the linkage of IFN α to apolipoprotein A-I (apoA-I) generates a fusion molecule termed InterApo (IA) which maintains the antiviral and immunostimulatory activities of IFN α but is devoid of the hematological and central nervous system toxicities that are mediated by this cytokine [4, 5]. In the present study, we aimed at evaluating if liver transduction with an adeno-associated virus (AAV) vector encoding IFN α (AAV-IFN) or IA (AAV-IA) is an effective therapeutic approach for chronic HBV infection. We tested this gene therapy approach in HBV transgenic (HBVTg) mice and in woodchucks with chronic woodchuck hepatitis virus (WHV) infection. The latter is a model which closely reproduces the virological and histopathological features of CHB [6]. An important difference between CHB and chronic WHV infection is that woodchucks frequently exhibit serum viral loads in excess of 10^{10} viral genomes (vg)/ml while in

humans HBV viremia rarely exceeds 10^7 vg/ml. The extremely high levels of circulating WHV virions cause exhaustion of immunological effectors thus rendering chronic WHV infection into a condition that is exceedingly difficult to treat. In an attempt to facilitate the response to IA, in one of our treatment protocols, we treated WHV-infected woodchucks with entecavir (ETV) during 4 weeks to see whether a transient decrease of viremia could make viral replication easier to control. Overall, our studies demonstrate that treatment with AAV-IA is safe and exerts antiviral effects both in HBVTg mice and in chronic WHV carrier woodchucks and suggest that combination of IA with nucleos(t)ide analogues is a promising approach to achieve sustained control of HBV replication.

MATERIALS AND METHODS

Animals

Female, 6 weeks old, C57BL/6 mice were purchased from Harlan (Barcelona, Spain). The HBVTg mouse lineage 1.3.32 (inbred C57BL/6, H-2b) was kindly provided by Professor Frank Chisari (The Scripps Research Institute, La Jolla, California, USA). These HBVTg mice encode a 1.3-overlength copy of the HBV genome (serotype ayw), replicate HBV at high levels within the liver and express all of the HBV antigens [7]. HBVTg mice were matched for age (6–10 weeks), sex (male), and serum HBV DNA and HBV surface antigen levels, as determined by real-time quantitative PCR or an enzyme-linked immunosorbent assay (ELISA) (Bioelisa HBsAg 3.0, Biokit), respectively. The mice were treated in accordance with the guidelines of the University of Navarra (Pamplona, Spain).

AAV vectors were inoculated in mice *via* retro-orbital injection in a total volume of 200µl. For viral challenge, a lethal dose of encephalomyocarditis virus (EMCV) was intraperitoneally injected. Blood samples were drawn by retro-orbital bleeding at the indicated time points. Serum samples were derived from two consecutive centrifugations of blood samples at 9100g for 5 minutes and stored at -20°C . Liver samples were stored at -80°C until assayed.

Captive-born, 12 months old Eastern woodchucks (*Marmota monax*), neonatally infected with WHV were purchased from Northeastern Wildlife Inc. (Harris, ID). Animal maintenance and handling were performed according to the regulations of the local Animal Care and Use Committee of the University of Navarra. Laparotomy was performed to inject intrahepatically 2.5×10^{11} or 1.25×10^{12} viral genomes of the AAV vectors. The second woodchuck study with additional ETV treatment was performed under an animal protocol approved by the institutional Animal Care and Use Committee of Georgetown University (Washington, DC). ETV (Selleck Chemicals) was mixed with woodchuck liquid diet (Dyets Inc., Bethlehem, PA) and orally administered by dose syringe. Daily, oral ETV dosing at a concentration of 0.5 mg/kg started at day 0 and continued thereafter for 4 weeks. A single dose of 5×10^{12} viral genomes of the AAV vectors was given at day 0 to woodchucks by intravenous injection. In both studies, blood was collected from the saphena vein or other approved sites using isoflurane or ketamine/xylazine anesthesia for sedation of woodchucks.

AAV Vectors

Experiments were performed using AAV serotype 8 vectors encoding luciferase (AAV-Luc), mouse IFN α 1 (AAV-IFN), the IA fusion protein generated by linking mouse IFN α 1 to mouse apoA-I (AAV-IA), woodchuck IFN α 5 [8, 9] (AAV-wIFN), woodchuck apoA-I [10] (AAV-wApo) or the IA fusion protein formed by linking wIFN α 5 to woodchuck apoA-I (AAV-wIA). All transgenes were placed under the transcriptional control of the elongation factor 1 α promoter. The above AAVs were produced by co-transfection of pDP8.ape and AAV plasmids into 293T cells [11]. Two days later, AAVs were purified from cell lysates by ultracentrifugation in Optiprep Density Gradient Medium-Iodixanol (Sigma-Aldrich, St Louis MO). For AAVs titration, viral DNA was isolated using the High Pure Viral Nucleic Acid kit (Roche Applied Science, Mannheim, Germany). The concentration of viral particles was subsequently determined by real-time quantitative PCR using primers specific to the elongation factor 1 α promoter (EF-Forward (Fw): 5'-ggtgagtcacccacacaaaagg-3' and EF-Reverse (Rv): 5'-cgtggagtcacatgaagcga-3').

ELISA for murine IFN α determination

IFN α levels in serum were measured using the VeriKineTM Mouse Interferon Alpha ELISA kit (PBL, NJ, USA) by following the manufacturer's recommendations.

Hemogram

Blood samples were collected in Microvette 500 Blood Collection tubes with EDTA (Sarstedt, Landskrona, Sweden). The hemogram was analysed using the Drew Scientific HemaVet Hematology Analyzer according to the manufacturer's recommendations.

RNA extraction and cDNA synthesis

Total RNA was extracted from liver tissue using the Maxwell[®] 16 Total RNA Purification kit (Promega, Madison, Wisconsin, USA). Concentration and purity of RNA was determined by absorbance at 260 and 280 nm in a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA). One microgram of RNA was transcribed to cDNA with Moloney Murine Leukemia Virus reverse transcriptase from Promega by following the manufacturer's assay instructions. The reaction was incubated for 1 hour at 37°C, denatured for 1 min at 95°C and cooled at 4°C. Samples were immediately used for PCR or stored at -20°C until assayed.

Quantitative PCR analysis of interferon-stimulated genes

Mouse interferon-stimulated-gene 15 (ISG15), ubiquitin-specific peptidase 18 (USP18), 2'-5'-oligoadenylate synthetase (2'-5' OAS) and β -actin expression was determined by quantitative real-time PCR using specific primers for each gene (ISG15-Fw: 5'-gattgccagaagattggtg-3' and ISG15-Rv: 5'-tctgcgtcagaaagacctca-3'; USP18-Fw: 5'-ccaaaccttgaccattcacc-3' and USP18-Rv: 5'-atgaccaagtcagcccatcc-3'; 2'-5'OAS-Fv: 5'-actgtctgaagcagattgcg-3' and 2'-5'OAS-Fv: 5'-tggaactgttgaagcagtc-3') and SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). As β -actin transcript levels remain unchanged across different experimental conditions, the expression of this house-keeping gene was used to standardize gene expression (ACT-Fw: 5'-cctggtgcctaggcg-3' and ACT-

Rv: 5'-cgcggtccacccgcgag-3'). For determination of HBV DNA or RNA following reverse transcription into cDNA, the following primers were used: HBcAg-Fw: 5'-TTCGCACTCCTCCAGCTTAT-3' and HBcAg-Rv 5'-GGCGAGGGAGTTCTTCTTCTA-3'. Murine GAPDH served as a reference and the following primers were used: GAPDH-Fw 5'-TGCACCACCAACTGCTTA-3' and GAPDH-Rv 5'-CAGAAGACTGTGGATGGCCCCTC-3'.

The amount of each transcript was expressed by the formula 2^{-Ct} (2ct(housekeeping gene) – ct(gene)) where ct is the point at which gene fluorescence rises significantly above background fluorescence. Real-time PCR reactions were performed using BioRad reagents in accordance with the manufacturer's recommended protocol.

Virological determinations

Serum WHV and HBV DNA was isolated using the High Pure Viral Nucleic Acid kit (Roche Diagnostics, Lewes, United Kingdom) or the QIAmp DNA Mini kit (Qiagen, Valencia, CA), respectively, and were quantified by real-time PCR as described previously [9, 12].

Serum WHV surface antigen (WHsAg) was assayed quantitatively against woodchuck serum with a known concentration of WHsAg by using a monoclonal antibody-based ELISA as described [13]. Serum HBsAg was analyzed using Bioelisa HBsAg 3.0 (Biokit, Barcelona, Spain) following manufacturer's instructions.

Determination of wIFN α bioactivity

The antiviral activity mediated by wIFN α present in serum was assayed in a cytopathic effect reduction assay using woodchuck WCH17 cells following challenge with EMCV in a 96-well microtiter plate format as described previously [8].

Statistical analysis

Statistical analyses were performed using the Prism 5 computer program (GraphPad Software Inc, San Diego, CA, USA). The survival data were represented in Kaplan-Meier graphs and analysed by the log-rank test. The remaining parameters were analyzed by one-way ANOVA followed by the Tukey's multiple comparisons test. The comparisons of WHV viremia and WHsAg relapse after ETV withdrawal were performed by fitting the data to a dose-response stimulation curve followed by the extra sum-of-squares F test. $P < 0.05$ values were considered significant.

RESULTS

IA but not IFN α can be expressed in the liver for a prolonged period of time without significant hematological toxicity

We generated AAV vectors encoding IA or IFN α for evaluating the feasibility and safety of long-term expression of these molecules in rodents. As shown in Fig. 1A, immunoreactive IFN α was detected in serum of mice injected with a single dose of 5×10^{11} vg of AAV-IFN or AAV-IA, but IFN α levels were substantially higher with the latter vector. However,

while mice treated with AAV-IA remained alive during the study period (and beyond 6 months after vector administration; data not shown), all mice that received AAV-IFN died within 60 days (Fig. 1B). In these animals, a dramatic and significant decline in erythrocytes, leukocytes and platelets was observed while the red blood cell and platelet counts remained unchanged in AAV-IA treated mice (Fig. 1C). Neutrophil and monocyte counts decreased significantly in mice treated with AAV-IFN but not in those which received AAV-IA while lymphocytes showed a decline in mice treated with either vector (Supplementary Fig. 1).

The antiviral activity of AAV-IA is comparable to that of AAV-IFN

We then analysed if the antiviral activity of AAV-IA was similar to that of AAV-IFN. To this aim, mice were injected intravenously with 5×10^{11} vg/mouse of AAV-IA, AAV-IFN or the control vector AAV-Luc, and 1 month later we measured the hepatic expression of interferon-stimulated genes. We found that both, AAV-IFN and AAV-IA but not AAV-Luc, stimulated the expression of interferon-stimulated genes at similar levels (Fig. 2A). Next, we tested the antiviral effect of AAV-IA or AAV-IFN by injecting mice with a lethal dose of EMCV. We found that animals treated with either of the two IFN-vectors were equally protected against EMCV infection provoked one month after the vector administration (Fig. 2B). These data show that AAV-IFN and AAV-IA exhibit comparable antiviral activity but haematological toxicity is much attenuated with the latter vector. Also it should be noted that no histological changes were observed in the liver of animals 6 months after AAV-IA administration (Supplementary Fig. 2).

Antiviral effects and hematological toxicity of AAV-IA and AAV-IFN in HBVTg mice

For evaluating the therapeutic effects of AAV-IA in chronic HBV infection, we tested this vector in HBVTg mice, an animal model that exhibits levels of viremia comparable to those observed in patients with CHB (7). HBVTg mice received a single dose of 1.5×10^{11} vg of AAV-IA, AAV-IFN or AAV-Luc control vector *via* the tail vein. Blood samples were collected 7 days after the AAV injection, and animals were sacrificed thereafter.

Immunoreactive IFN α was not detected in the serum of control mice (AAV-Luc) while detectable amounts of this cytokine were present in the blood of mice that received AAV-IFN α or AAV-IA (Supplementary Fig. 3). IFN α serum levels were higher with AAV-IA than with AAV-IFN α and both groups demonstrated a marked and significant decline in serum viral loads and in serum HBsAg in comparison to control animals (Fig. 3A). The reduction in circulating HBV viral particles was associated with a decline in hepatic HBV-DNA abundance and disappearance of HBV-DNA replicative forms in the liver (Supplementary Fig 4) accompanied by a marked reduction of HBV core antigen mRNA levels in the liver (Fig. 3A). Similarly to our observations in normal mice, leukocyte, red cell and platelet counts dropped significantly in HBVTg mice that received AAV-IFN being the descent of leukocytes and platelets significantly less pronounced in AAV-IA treated mice (Fig. 3B).

AAV-IA mono therapy in woodchucks with chronic WHV infection

WHV is an hepadnavirus closely related to HBV, and woodchucks with chronic WHV infection accurately reproduce the virological and pathological features of CHB, thus representing a relevant preclinical model for testing novel anti-HBV therapies [6]. We generated AAV vectors encoding woodchuck IFN α (AAV-wIFN) and woodchuck IA (AAV-wIA) and groups of two animals were treated by intrahepatic injection of a single dose of either 1.25×10^{11} or 1.25×10^{12} vg of AAV-wIFN or AAV-wIA. In relation to animal weight, this dose was inferior to that given to mice. This is because of our previous observations (unpublished data) that the woodchuck liver is very easily transduced when the vector is given by intrahepatic injection.

Following vector administration, IFN α bioactivity was measured by determining the ability of serial dilutions of serum to protect WHC17 cells, a woodchuck hepatic cell line, against the cytopathic effect of EMCV (Supplementary Fig. 5). As shown in Fig. 4A, we observed a dose-dependent increase in serum IFN α , with levels that were comparable between woodchucks treated with AAV-wIFN or AAV-wIA. However, all four woodchucks treated with AAV-wIFN, independently of the dose, needed to be euthanized because of severe pancytopenia (Fig. 4B, C and D). This was in contrast to the four woodchucks that received AAV-wIA that survived during the study, except for one animal that was euthanized because of the development of hepatocellular carcinoma, a frequent finding in woodchucks with chronic WHV infection. Notably, in AAV-wIA-treated animals, we observed no remarkable changes in red blood cell counts and only a moderate reduction of leukocytes and platelets in some woodchucks followed by posterior recovery in some of them. In all groups treated with AAV-wIA or AAV-wIFN, ALT levels did not show significant changes with respect to basal levels (data not shown).

All woodchucks in this study exhibited very high viremia, with levels between 10^{11} and 10^{13} vg/ml. In agreement with reports indicating that an elevated viral load is associated with poor response to IFN α therapy in the woodchuck model [14], we observed little changes in circulating WHV DNA in all treatment groups (Fig. 5). Only the woodchuck that was euthanized due to the development of hepatocellular carcinoma showed a drop in viral load of greater than 2 logs from pretreatment values.

Combination therapy with ETV and AAV-IA in woodchucks with chronic WHV infection

As high viral loads, such as those present in woodchucks with chronic WHV infection (Fig. 5), impair the response to IFN α [14], we tested the effect of AAV-IA in combination with 0.5 mg/kg ETV for 4 weeks in order to lower viral load during this period of time. In this study, animals (n=5 per group) received a single dose of 5×10^{12} vg of AAV-wIA or the control vector AAV-wApo (encoding woodchuck apoA-I) and ETV therapy was initiated immediately after vector injection. In this experiment, technical reasons obliged to use the intravenous route for vector administration and for this reason the dose administered was higher than in the previous study. Treated woodchucks were followed-up for 16 weeks. We found that ETV treatment resulted in a marked decline in viremia in all animals (between 4 and 6 log₁₀ from pretreatment), accompanied by a moderate reduction of circulating WHsAg (Fig. 6). Upon ETV discontinuation, two animals in the control group died

following a liver biopsy at week 6, but the other three animals showed a rapid relapse in viremia and antigenemia to pretreatment levels. In AAV-wIA treated woodchucks, however, the rebound of WHV-DNA and WHsAg was delayed for several weeks (Fig. 6). In two woodchucks (M15IVIA and F16IVIA), serum WHV DNA never reached pretreatment values and remained at reduced levels of 1.34 and 1.10 log₁₀, respectively, at the end of the follow-up period at week 16 (Fig. 6). The relapse in WHV DNA and WHsAg after ETV discontinuation was significantly lower in AAV-wIA treated woodchucks than in control animals ($P < 0.0001$). Notably, these antiviral effects were achieved with serum IFN α levels that were below the detection threshold of our assay (data not shown). Consonant with this observation, no significant changes in blood cell counts (leukocytes, erythrocytes, and platelets) nor in other biochemical alterations including renal and liver function parameters were found in treated or control animals (data not shown). Thus, combination treatment with ETV and AAV-wIA was safe and achieved significant antiviral effects in a very difficult-to-treated model of chronic hepadnavirus infection

DISCUSSION

The introduction of effective NAs possessing a high barrier to viral resistance such as ETV or tenofovir has undoubtedly represented a formidable progress in the fight against HBV infection. However, treatment of CHB is still challenging since these drugs do not affect transcription of viral proteins from covalently-closed circular DNA molecule of HBV [15] nor exert direct immunostimulatory effects to facilitate immune clearance of HBV-infected hepatocytes (15). Persistent antigenemia despite NAs therapy favors the maintenance of immune tolerance to the virus and, as a result, rebound of HBV replication is frequently observed following the cessation of therapy [3]. Thus, treatment needs to be maintained for an indefinite period of time to ensure sustained control of HBV infection. Pegylated IFN α is an effective drug for treatment of chronic HBV infection as it inhibits HBV replication as well as HBV transcription. In addition, IFN α possesses immunostimulatory properties that facilitate the activation of anti-HBV T cell immune responses. However, the use of IFN α is limited by severe toxicity in a majority of patients [16]. Thus, in the present work, we have tested a protein that is formed by the fusion of IFN α to apoA-I (termed IA) as a potential therapy for CHB. Previously, we have shown that IA displays immunostimulatory activities without inducing hematological toxicity [4]. Because of the favourable toxicology profile of IA, we now investigated if this protein could be administered to the liver by gene transfer. In this study we used a serotype 8 AAV vector. This is a hepatotropic long-term expression vector that has been used in the clinic for the treatment of hemophilia B with remarkable success and excellent tolerance [17]. We found that the AAV vector encoding IA (AAV-IA) was able to protect normal mice against a lethal challenge of EMCV as efficiently as an AAV vector encoding IFN α only (AAV-IFN). AAV-IA was also comparable to AAV-IFN in the control of HBV transcription and replication in HBVTg mice. Notably, AAV-IFN induced severe leukopenia and thrombocytopenia and moderate anemia in treated animals while AAV-IA therapy was associated with minor changes in the platelet count, slight anemia and moderate leukopenia. Because of these encouraging results, AAV-IA was compared to AAV-IFN in woodchucks with chronic WHV infection using the appropriate woodchuck apoA-I and IFN α gene sequences. In the first study, the vectors (AAV-wIA and

AAV-wIFN) were administered at two different doses by intrahepatic injection (a route which ensures high transduction efficiency) [18]. We could again confirm the hematological toxicity risk associated with the intrahepatic expression of IFN α at high levels that caused death of all animals. In contrast, AAV-wIA did not produce relevant hematological changes, and all animals survived suggesting that this transgene may be suitable for clinical application. However, neither AAV-wIFN nor AAV-wIA promoted any significant decline in viremia in the treated woodchucks. This was not surprising as these animals exhibited extremely high viral loads and it has been shown that the level of viremia is the main factor that negatively predicts the antiviral response to IFN [14]. As viremia is in the range of 10^{11} and 10^{13} vg/ml in woodchucks with chronic WHV infection while viral load in patients with CHB rarely exceeds 10^7 vg/ml [6], we designed a new protocol combining AAV-IA (or the control vector AAV-Apo) with 4 weeks of treatment with ETV. In this protocol, we selected the intravenous route for vector administration as this would be more acceptable for clinical use. Using this route, the transduction efficiency was lower compared to previous protocols, and in fact serum IFN α levels were below the detectable threshold. In this study, we did not observe any toxicity related to vector administration but we found a significant enhancement and prolongation of the antiviral effect of ETV in such a way that the decrease of viremia persisted during several weeks after ETV interruption in animals that received AAV-IA while viremia relapsed rapidly to basal values in animals treated with the control vector. It seems possible that by increasing liver transduction with AAV-IA (using a higher vector dose or a different administration route) or by prolonging ETV treatment, we may control chronic WHV infection in a more efficient and sustained manner.

To conclude, our results show in two different animal models that a non-toxic IFN α -based therapy can be administered in combination with NAs for more stringent control of hepadnavirus infection. In our study, we have used a gene therapy approach to ensure sustained intrahepatic expression of IA, but it seems possible that similar results could be obtained by combining ETV with repeated periodic injections of recombinant IA which is easier to use in clinical practice. However, the gene therapy option should not be ruled out for difficult-to-treat cases considering the excellent tolerance demonstrated by AAV vectors in different clinical trials [19, 20], the possibility of employing inducible promoters and the fact that liver-directed gene therapy would allow attaining high intrahepatic IA concentration without unduly increasing systemic levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

AAV	adeno-associated virus
AAV-IFN	adeno-associated vector encoding mouse interferon alpha
AAV-IA	adeno-associated vector encoding mouse interferon alpha fused to apolipoprotein A-I
AAV-Luc	adeno-associated vector encoding luciferase
AAV-wApo	adeno-associated vector encoding woodchuck apolipoprotein A-I
AAV-wIFN	adeno-associated vector encoding woodchuck interferon alpha
AAV-wIA	adeno-associated vector encoding woodchuck interferon alpha fused to apolipoprotein A-I
apoA-I	apolipoprotein A-I
HBV	hepatitis B virus
HBVTg	HBV transgenic mice
CHB	chronic hepatitis B
ELISA	enzyme-linked immunosorbent assay
EMCV	encephalomyocarditis virus
ETV	entecavir
IFNα	interferon- α
IA	interferon fused to apolipoprotein A-I
ISG15	interferon-stimulated-gene 15
NAs	nucleoside/nucleotide analogues
2'-5' OAS	2'-5'-oligoadenylate synthetase
USP18	ubiquitin-specific peptidase 18
vg	viral genomes
WHV	woodchuck hepatitis virus
WHsAg	woodchuck hepatitis virus surface antigen

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Fig. 1. Long-term expression of interferon alpha linked to apolipoprotein A-I is devoid of hematological toxicity

(A) IFN α serum levels in C57BL/6 mice after intravenous injection of a single dose of 5×10^{11} viral genomes (vg) of an AAV vector encoding IFN α (AAV-IFN) or IA (AAV-IA). n=6 mice per group. (B) Kaplan-Meier survival curves of mice treated with intravenous injection of AAV-IFN or AAV-IA. n=6 mice per group. (C) Peripheral blood platelet (PLT), leukocyte (WBC) and erythrocyte (RBC) counts were determined one month after the intravenous injection of AAV-IFN or AAV-IA and were compared to a control AAV vector encoding luciferase (AAV-Luc). n=4 mice per group. One-way ANOVA followed by Tukey's multiple comparisons test. **P < 0.01; ***P < 0.001.

Fig. 2. AAV-IFN and AAV-IA display similar antiviral activity

(A) C57BL/6 mice were treated by intravenous injection with a single dose of 5×10^{11} vg AAV-IFN, AAV-IA or AAV-Luc. Relative expression of interferon-stimulated-gene 15 (ISG15), ubiquitin specific peptidase 18 (USP18) and 2'-5'-oligoadenylate synthetase (2'-5' OAS) in the liver of mice one month after injection. $n=3$ mice per group. One-way ANOVA followed by Tukey's multiple comparisons test. $*P < 0.05$, $**P < 0.01$. (B) One month after the intravenous injection of AAV-IFN, AAV-IA or AAV-Luc, mice were challenged with a lethal dose of EMCV by intraperitoneal injection. Kaplan-Meier survival curves of mice following EMCV challenge are presented. $n=7$ mice per group. Log rank test. $*P < 0.05$.

Fig. 3. Antiviral activity and hematological toxicity of AAV-IFN and AAV-IA in HBV transgenic mice

HBV transgenic mice received intravenously a single dose of 1.5×10^{11} vg of AAV-Luc, AAV-IFN or AAV-IA. Seven days after vector administration, we determined the levels of HBV DNA and HBsAg in serum, and the levels of HBV DNA and RNA in the liver (A), and the number of circulating leukocytes (WBC), erythrocytes (RBC) and platelets (PLT) was determined (B). $n=3$ mice per group. One-way ANOVA followed by Tukey's multiple comparisons test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 4. IFN α serum levels and hematological parameters in woodchucks with chronic WHV infection treated with AAV-wIFN (left) or AAV-wIA (right)
 (A) IFN α serum levels in woodchucks treated with a single dose of 1.25×10^{11} (open symbols) or 1.25×10^{12} vg (closed symbols) of AAV-wIFN or AAV-wIA by direct intrahepatic injection. (B) Number of leukocytes (WBC), (C) erythrocytes (RBC) and (D) platelets in treated woodchucks. †, dead woodchucks.

Fig. 5. Antiviral effect of AAV vectors in woodchucks with chronic WHV infection

Viremia at different time points in woodchucks treated by intrahepatic injection of a single dose of 1.25×10^{11} (open symbols) or 1.25×10^{12} vg (closed symbols) of AAV-wIFN or AAV-wIA. †, dead woodchucks.

Fig. 6. Antiviral effect in chronic WHV carrier woodchucks treated with AAVs in combination with entecavir (ETV)

Woodchucks were orally treated with ETV for 4 weeks at a daily dose of 0.5 mg/kg ETV. ETV treatment started immediately after intravenous injection of a single dose of 5×10^{12} vg of AAV-wIA or control vector AAV-wApo. Animals were followed for 16 weeks and the values of serum WHV DNA (A) and of serum WHsAg (B) were determined. †, woodchuck which died following liver biopsy. Male woodchucks are represented with closed symbols and female animals with open symbols. Gray area indicates the period of entecavir administration.