IFN-α Gene Therapy for Woodchuck Hepatitis with Adeno-associated Virus: Differences in Duration of Gene Expression and Antiviral Activity Using Intraportal or Intramuscular Routes

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Gene delivery of IFN-α to the liver may represent an interesting strategy to maximize its antiviral efficacy and reduce side effects. We used a recombinant adeno-associated virus (AAV) encoding woodchuck IFN-α (AAV-IFN) to treat animals with chronic woodchuck hepatitis virus infection. The vector was given by intraportal or intramuscular route. Long-term transgene expression was detected after intraportal administration of an AAV encoding luciferase. In contrast, in the majority of the animals that received AAV-IFN through the portal vein, the expression of IFN-α was transient (30–40 days) and was associated with a significant but transient decrease in viral load. One animal, in which hepatic production of IFN-α persisted at high levels, died because of bone marrow toxicity. The disappearance of IFN-α expression correlated with the disappearance of AAV genomes from the liver. Intramuscular administration of AAV-IFN resulted in prolonged but fluctuating expression of the cytokine with no significant antiviral effect. In summary, this report shows that long-term expression of IFN-α in muscle is feasible but higher interferon levels might be needed to control viral replication. On the other hand, IFN-α gene delivery to the liver using an AAV vector induces a significant but transient antiviral effect in the woodchuck model.

Key Words: gene therapy, interferon-α, adeno-associated viruses, chronic hepatitis B, woodchuck, drug delivery systems

INTRODUCTION

Hepatitis B virus (HBV) and hepatitis C virus (HCV) constitute the main etiologic factors of chronic viral hepatitis, a condition affecting more than 550 million people worldwide [1,2]. Interferon-α (IFN-α) is an accepted therapy for these two viral infections. When used as monotherapy, the response rate is about 25% in chronic hepatitis C, around 35% in HBe-positive chronic hepatitis B, and near 25% in anti-HBe chronic hepatitis B [3]. Pegylation of IFN-α prolongs the half-life of the drug, allowing weekly administration of the cytokine and increased antiviral efficacy. However, in a recent study, only 28% of patients with HBe-positive chronic hepatitis B experienced sustained HBV DNA suppression after treatment with pegylated IFN-α [4]. In the case of chronic hepatitis C, half of the treated patients with HCV genotype 1 infection failed to respond to the combination of pegylated IFN-α and ribavirin [5]. Therefore new strategies to increase the response rate to IFN-α in patients chronically infected with HBV or HCV are greatly needed.

In the present paper, we assess the potential of IFN-α gene therapy in chronic viral hepatitis. Gene therapy allows a continuous in vivo expression of the transgene at the desired site. To achieve long-term expression we have used recombinant adeno-associated virus (AAV) encoding IFN-α (AAV-IFN). AAV vectors lack pathogenicity in
humans, infect dividing and nondividing cells, show a broad range of infectivity, and have demonstrated prolonged expression with numerous genes, in several tissues, and in different animal models with intact immune systems [6-10]. Preclinical data demonstrating safety, efficiency, and efficacy of these vectors enabled the initiation of phase I and II clinical trials, which showed that AAV serotype 2 is a safe vector in humans [11-13]. In our study, two main strategies were pursued: (i) delivery of IFN-α gene to hepatocytes to allow higher levels of the transgene in liver than in systemic circulation and (ii) expression of IFN-α gene in the muscle to maintain sustained levels of IFN-α in blood. AAV-IFN was tested in woodchucks (Marmota monax) chronically infected with woodchuck hepatitis virus (WHV). The genomic organization and replicative cycle of WHV and the proteins encoded by WHV DNA are very similar to those of HBV [14]. Woodchucks infected perinatally with WHV develop chronic hepatitis, which may evolve to hepatocellular carcinoma and is considered to be the best model to study the pathogenesis, prevention, and treatment of HBV infection [15]. It should be noted that it is a difficult-to-treat model of hepatitis, with high viral load, low transaminases levels, and immune tolerance to viral antigens [16]. Thus, any sign of efficacy of gene therapy strategies might be predictive of success in the human setting. We have previously cloned and analyzed the woodchuck IFN-α gene family and characterized the different subtypes present in the woodchuck liver [17]. We found that wIFN-α5 combines a potent antiviral effect with the property of being the predominant IFN-α subtype expressed in the liver. We developed an AAV vector encoding the wIFN-α5 gene under the control of the elongation factor 1α promoter (AAV-IFN). Our results showed that intramuscular administration of AAV-IFN allows long-term transgene expression but poor antiviral activity. Intraperitoneal administration of a high dose of AAV-IFN induced a partial and transient antiviral effect in parallel with short-lived transgene expression. Interestingly, while AAV-Luc persisted in the liver for 6 months after vector administration, at this time point AAV-IFN was no longer present in the liver or was detectable only in trace amounts. These data underscore the difficulties of IFN-α-based gene therapy and provide information that could be useful to optimize this therapeutic modality for chronic viral hepatitis.

RESULTS

Recombinant AAV Vectors and Transgene Expression in AAV-Transduced Cells

We constructed two recombinant AAV vectors: one that encoded woodchuck IFN-α5 (AAV-IFN) and another containing the luciferase reporter gene (AAV-Luc), using in both cases the ubiquitous elongation factor 1α (EF1α) promoter and the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) (Fig. 1A).

To examine the ability of AAV-IFN and AAV-Luc to induce the production of biologically active IFN-α or luciferase, respectively, we infected HeLa cells, woodchuck fibroblasts, and rabbit fibroblasts with these vectors. After 48 h, we tested supernatants for IFN-α activity and cell lysates for luciferase activity. As shown in Fig. 1B, we found high values of luciferase activity in all cell types infected with AAV-Luc. Similarly, we detected IFN-α activity at high levels in the supernatant obtained from all cell types infected with AAV-IFN (Fig. 1C).

AAV Vectors Efficiently Transduce Woodchuck Liver

To analyze whether AAV could transduce woodchuck liver cells in vivo, we injected two woodchucks through the portal vein with 2.5 × 10^11 virus particles (vp) of AAV-Luc. To test if WHV infection affects AAV liver transduction, one of the animals used in the study was an uninfected woodchuck (WHV-negative), while the other woodchuck was chronically infected with WHV (WHV-positive). One month after vector administration, we
obtained a liver biopsy from both woodchucks and 6 months later sacrificed the animals. We tested luciferase expression in liver biopsies obtained 1 month after injection and in different organs including liver, kidney, heart, spleen, and lung obtained when the animals were sacrificed. We analyzed the presence of AAV genomes in liver samples by quantitative PCR. Results are summarized in Table 1. We found that luciferase activity was detected in the liver of both animals at 1 month and 6 months after AAV-Luc injection, the levels being higher at month 6. Luciferase activity could not be detected in any organ examined other than the liver (data not shown). The analysis of AAV DNA in liver biopsies showed a higher number of copies of viral DNA 1 month after injection than at month 6 (Table 1). This pattern of AAV DNA reduction over time associated with an increase of transgene expression has already been reported in mice after AAV injection [18].

We further confirmed data regarding AAV infectivity of WHV-infected livers by analysis of AAV DNA and luciferase activity in the livers of four additional WHV-infected woodchucks (wLucL1, wLucL2, wLucH1, and wLucH2), which we used as controls in experiments described below (data not shown). Thus, rAAV is able to transduce the liver of healthy woodchucks as well as that of animals with chronic WHV infection.

**Effect of AAV-IFN on Viral Load**

We separated woodchucks chronically infected with WHV into four treatment groups according to the route of vector administration (through the portal vein or by intramuscular injection) and the dose of the vector (high or low dose). In two treatment groups the vector was given through the portal vein (ip): one of these groups received a single low (L) dose of 2.5 $\times$ 10$^{11}$ vp of AAV-IFN (wIFNipL1, wIFNipL2, wIFNipL3, wIFNipL4, wIFNipL5) and another group received a single high (H) dose of 2.5 $\times$ 10$^{12}$ vp of AAV-IFN (wIFNipH1, wIFNipH2, wIFNipH3, wIFNipH4). We treated two additional groups of woodchucks with a single intramuscular (im) dose of AAV-IFN: one of these groups received a single low dose of 2.5 $\times$ 10$^{11}$ vp (wIFNimL1, wIFNimL2) and the other group was given a single high dose of 8 $\times$ 10$^{11}$ vp (wIFNimH1, wIFNimH2, wIFNimH3). Control groups received 2.5 $\times$ 10$^{11}$ and 2.5 $\times$ 10$^{12}$ vp of AAV-Luc through the portal vein (low dose, wLucipL1 and wLucipL2, and high dose, wLucipH1 and wLucipH2). The characteristics of the woodchucks used in this study are summarized in Table A (supplemental information).

To evaluate the antiviral effect of AAV-IFN therapy, we obtained serum samples from the animals every 15 days. We purified WHV DNA from serum and determined the number of viral copies by quantitative PCR. During natural infection, the variation of serum viral load is less than 1 order of magnitude [19]. In fact, in the group of woodchucks that received AAV-Luc (control group), the most pronounced viremia descent ranged between 0.00 and 0.84 logs compared with the baseline value (maximal log decrease of viremia or MLDV) (Table A, supplemental information, and Fig. 2A). Moreover, the 99% confidence interval for the MLDV mean of four untreated woodchucks and four AAV-Luc-injected woodchucks was 1.24.

The analysis of serum WHV DNA in woodchucks that received the low dose of AAV-IFN through the portal vein showed that two of five animals reached MLDV values higher than 2.7 (Fig. 3). In one of these animals, the decrease in viremia was long-lasting (wIFNipL1), while in the other it was short-lived (wIFNipL4) (Fig. 2C). Woodchucks that received a high dose of AAV-IFN through the portal vein exhibited MLDV ranging between 1.48 and 2.61 (Table A, supplemental information, and Fig. 2D). In these animals, serum viral load fluctuated over the 28 weeks of the study (Fig. 2D) and in two of them, wIFNipH1 and wIFNipH2, viremia returned to the initial values 180 days after AAV administration. In woodchuck wIFNipH3 the viral load was below baseline values at the end of the study and woodchuck wIFNipH4 showed a tendency for a continuous decrease of the viral load after the first 6 weeks of therapy. Unfortunately this animal had to be sacrificed 3 months after treatment, due to the development of severe pancytopenia (Fig. 2D).

The statistical analysis showed that the MLDV in woodchucks treated with a high intraportal dose of AAV-IFN was significantly higher than the MLDV in control animals (Fig. 3). In contrast, only one woodchuck from those given AAV-IFN intramuscularly showed a decrease of viremia greater than 1.24 log during the 6 months of study (Fig. 2B).

**Analysis of IFN-α Expression in Woodchucks After AAV-IFN Administration**

We quantified the presence of IFN-α in serum by a cytopathic effect (CPE) reduction assay after CMV infection of a woodchuck cell line. First, we determined the mean value of the CPE reduction assay for serum samples taken before treatment from all woodchucks employed in this study. This value was equivalent to

<table>
<thead>
<tr>
<th>TABLE 1: Expression of AAV-Luc in the liver of woodchucks</th>
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<tbody>
<tr>
<td>WHV positive</td>
</tr>
<tr>
<td>Viral DNA (copies Luc/copies β-actin)</td>
</tr>
<tr>
<td>Luciferase activity (RLU/μg)</td>
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45.21 pg/ml of the recombinant wIFN-α protein, and we determined the 99% confidence interval for the mean of the baseline serum samples to be 65.25 pg/ml. Thus, we considered only values above this level to be induced by the therapy. Starting 2 weeks after AAV-IFN administration, we monitored serum IFN-α levels every 2 weeks. We could not find detectable levels of IFN-α in sera from woodchucks treated with AAV-Luc or woodchucks that received the lowest dose of AAV-IFN intraportally (wIFNipL) (data not shown). Serum IFN-α values in woodchucks treated with 2.5 × 10^11 vp AAV-IFN intraportally are shown in Figs. 4A and 4B. We set apart data from males and females due to the marked sex differences in hepatic AAV expression that have been previously reported in mice [20]. We observed that in all cases except for wIFNipH4, expression of IFN-α peaked around day 30 and then went back to pretreatment levels. However, in wIFNipH4, IFN-α concentration increased to very high values (around 4000 pg/ml) and remained persistently elevated. As mentioned above, we sacrificed this woodchuck due to the development of severe pancytopenia. Woodchucks treated with intramuscular injection of AAV-IFN showed a completely different pattern of IFN-α expression. In these two groups serum IFN-α concentration showed a fluctuating pattern, which persisted along the 28 weeks of the study (Figs. 4C and 4D). The mean values were higher in animals treated intramuscularly with high doses of AAV-IFN than in those receiving the lower dose (159.5 (SD = 73.7) vs. 115.7 (SD = 64.6); P = 0.035). Thus, when the AAV-IFN vector was injected into the muscle we did not observe the disappearance of IFN-α expression that occurred when the vector was directed to the liver.
To confirm further that the inhibition of ECMV-mediated CPE was due to IFN-α, we incubated sera from AAV-IFN-treated woodchucks obtained at the peak time point of IFN-α expression with wIFN-α neutralizing antibodies (P. Berraondo et al., manuscript in preparation) previous to the CPE reduction assay. The inhibitory activity present in those sera was completely neutralized (data not shown).

**Immune Response against the Transgene**

It has been previously described that the administration of AAV vectors could elicit antibodies against the transgene, which could neutralize the activity of the expressed protein [21,22]. Furthermore, chronic hepatitis C patients treated with the recombinant protein have been shown to develop anti-IFN-α antibodies. We analyzed the presence of anti-IFN-α antibodies or molecules with IFN-α inhibitory activity in the sera of the woodchucks injected via portal vein in which the expression of IFN-α had disappeared. For this purpose, we performed a CPE assay using recombinant woodchuck IFN-α protein previously incubated with woodchuck sera for 1 h at 37°C. We could not detect neutralizing anti-IFN-α antibodies in any case. Similarly, the analysis by ELISA of total antibodies against IFN-α also failed to show the presence of anti-IFN-α antibodies. These data indicated that gene silencing or the elimination of AAV-IFN-transduced hepatocytes, rather than the development of anti-IFN-α antibodies, was responsible for the transient IFN-α expression observed when the vector was directed at the liver.

**Analysis of AAV Genome in Woodchuck Liver**

To investigate further the mechanisms of the disappearance of IFN-α expression in animals treated through the portal vein, we analyzed the presence of AAV viral genomes in the liver of woodchucks given AAV-IFN or AAV-Luc by intraportal injection at month 6 after vector administration (the exception was wIFNipH4, sacrificed at month 3 as mentioned above). Results are summarized in Table 2. Viral DNA was detected in all animals that received AAV-Luc but was absent or present in only trace amounts in all but one of the woodchucks treated with AAV-IFN. This woodchuck was the same one (wIFNipH4) that showed high sustained levels of IFN-α in serum and had to be sacrificed (Table 2). Therefore, the disappearance of IFN-α expression seems to be due to the elimination of AAV-IFN-transduced cells.

**TABLE 2: Presence of AAV-Luc and AAV-IFN genomes in the liver at 6 months after vector administration**

<table>
<thead>
<tr>
<th>DNA</th>
<th>wLucH1</th>
<th>wLucH2</th>
<th>wIFNipL1</th>
<th>wIFNipL2</th>
<th>wIFNipL4</th>
<th>wIFNipH1</th>
<th>wIFNipH2</th>
<th>wIFNipH3</th>
<th>wIFNipH4</th>
</tr>
</thead>
<tbody>
<tr>
<td>(viral DNA/β-actin DNA) × 10^7</td>
<td>319,688</td>
<td>16712.2</td>
<td>0.0</td>
<td>1.4</td>
<td>19.8</td>
<td>0.0</td>
<td>1.5</td>
<td>8.6</td>
<td>2283.5</td>
</tr>
</tbody>
</table>

**FIG. 4.** IFN-α expression in AAV-IFN-treated woodchucks. Serum IFN-α was measured by CPE assay. Recombinant IFN-α was used as standard. Serum concentration of IFN-α (pg/ml) is presented as a function of time after vector administration. The following groups are presented: (A) female, high dose (2.5 × 10^12 vp) intraportal AAV-IFN; (B) male, high dose (2.5 × 10^12 vp) intraportal AAV-IFN; (C) low dose (2.5 × 10^11 vp) intramuscular AAV-IFN; (D) high dose (8 × 10^11 vp) intramuscular AAV-IFN. Gray lines indicate the 99% confidence interval for the mean baseline serum IFN-α levels.
We observed that the site of injection of AAV-IFN was a key determinant in the pattern of transgene expression. Animals that received the vector by the intramuscular route showed persistent elevation of IFN-α in serum for several months, although with a fluctuating pattern. The oscillatory pattern of IFN-α expression observed in animals treated with AAV-IFN by the intramuscular route is intriguing. We have no clear explanation for this interesting phenomenon. It could be hypothesized that high levels of IFN-α might act autocrinely on the transduced muscle cell, inhibiting transgene expression, since several interferon-stimulated genes are inhibitors of protein synthesis [23]. This inhibition would cease upon decreasing IFN-α concentration, leading to a reactivation of transgene expression with initiation of a new cycle. A remarkable and unexpected finding of the present study was the transient expression of the transgene in woodchucks that received via the portal vein a high dose of AAV-IFN. Thus, despite claims of efficacy of IFN-α gene therapy to fight viral hepatitis [24,25], our results show important limitations of this therapeutic strategy. Data shown in Table 2 illuminate the mechanisms responsible for the disappearance of transgene expression. AAV vector encoding luciferase can be found in the liver 6 months after its injection into the portal vein. In sharp contrast, in all animals treated with low or high doses of AAV-IFN via the portal vein, AAV DNA was absent, or present only in trace amounts. The only remarkable exception was woodchuck wIFNipH4 who had to be sacrificed due to severe toxicity resulting from a very high production of interferon. Our experimental data suggest that the AAV-IFN-transduced cells were eliminated from the liver. The disappearance of AAV-IFN-infected hepatocytes could not be explained by the turnover of hepatocytes since AAV-Luc is present in the liver 6 months after injection into the portal vein. In sharp contrast, all animals treated with low or high doses of AAV-IFN via the portal vein, AAV DNA was absent, or present only in trace amounts. The only remarkable exception was woodchuck wIFNipH4 who had to be sacrificed due to severe toxicity resulting from a very high production of interferon. Our experimental data suggest that the AAV-IFN-transduced cells were eliminated from the liver. The disappearance of AAV-IFN-infected hepatocytes could not be explained by the turnover of hepatocytes since AAV-Luc is present in the liver 6 months after injection. On the other hand, AAV vectors do not contain any viral open reading frames, leaving the transgene product and the virus capsids as the only source of non-self antigen [18]. In our study, no immune response against the transgene was observed. Thus, an immune response against viral capsids (or against WHV antigens), stimulated by the expression of IFN-α, could be the reason for the disappearance of the AAV vector. The increase in serum liver enzymes observed between 3 and 4 months after AAV-IFN administration, although it may be ascribed simply to the progression of the disease, might most likely reflect an immune response against viral antigens expressed by infected hepatocytes (Fig. 5). A similar pattern of delayed liver enzyme elevation in serum has been reported to occur after interferon therapy in patients with chronic hepatitis B in relation to an activation of anti-HBV immune responses [26]. The analysis of the antiviral efficacy of AAV-IFN after intramuscular administration showed nonsignificant changes in viremia, even though the levels of IFN-α in serum were around 100 pg/ml, which is the concentra-
tion achieved in the blood of HCV-infected patients after the administration of a therapeutic dose of IFN-α [27]. This lack of antiviral efficacy might be due to the marked resistance to IFN-α therapy exhibited by WHV-chronically infected woodchucks. Thus, the levels of IFN-α attained in this group of animals appear to be insufficient to control viral replication. Alternatively, the fluctuating pattern, with periods of low IFN-α gene expression, may hamper the efficacy of the therapy. On the other hand, the animals that received a high dose of AAV-IFN via portal vein experienced a significant reduction of viremia (Fig. 3). Interestingly, we could also find a noticeable reduction of viremia in two woodchucks, wIFNipL1 and wIFNipL4, treated via the portal vein with low dose of AAV-IFN. In the first of these animals, the viremia showed a continuous decrease, reaching values near 3 logs below the baseline level. This woodchuck had the lowest viral load of all animals included in this study, suggesting that the efficacy of IFN-α gene therapy could be increased by combination with other therapies that might reduce the viral load. The presence of an antiviral effect in this group of animals in the absence of detectable IFN-α in serum indicates that gene therapy may allow one to reach therapeutic levels of IFN-α in the transduced liver tissue without increasing the systemic concentration of this cytokine. This is a favorable situation when attempting to control viral replication in the liver while avoiding the toxicity associated with systemic administration of IFN-α.

Another important finding of our study was the toxicity associated with the intraportal administration of a high dose of AAV-IFN that occurred in one of the woodchucks (wIFNipH4). In this animal, the production of IFN-α continued at very high levels, leading to a progressive reduction of viremia but also to catastrophic toxic effects consisting of severe pancytopenia and marked liver steatosis that motivated the sacrifice of the animal. This case illustrates the severe toxicity that may occur from unregulated synthesis of IFN-α in the liver when employing constitutive promoters that do not allow the fine modulation of transgene expression that could be obtained by the use of inducible promoters.

In summary, our data show the promises and limitations of IFN-α-based gene therapy for chronic viral hepatitis. Muscle transduction with AAV vectors allows the rise of IFN-α serum levels for a long period of time. This approach has demonstrated few antiviral effects in WHV chronic infection probably due to the fact that the systemic levels of IFN-α were not high enough for this “difficult-to-treat” chronic hepatitis model. On the other hand, although IFN-α gene expression in the liver was able to induce a significant reduction of viral load in this animal model, the effect was transient due to elimination of cells expressing the transgene. In cases in which interferon continues to be produced in the liver at high levels, severe toxicity may ensue. Therefore, controlling the potential toxicity of IFN-α with inducible promoters, and the development of strategies to avoid elimination of transduced hepatocytes, might contribute to the safety and the efficacy of this treatment.

**Materials and Methods**

**AAV vector construction.** All the AAV vectors used in the present study were AAV serotype 2. For construction of AAV-IFN (Fig. 1A), a fragment containing the wIFNα-s cDNA was obtained by digestion of the plasmid pCDNA3.1wIFN [17] with EcoRI and cloned into an EcoRI site of plasmid pGTC1100 (kindly provided by Dr. C. Qian, CIMA, Navarre, Spain), which carries the human EFlα promoter and a polyadenylation signal. To improve transgene expression, the WPRE was included in the expression cassette (Fig. 1A) [17]. The expression cassette from this plasmid was excised by digestion with SacI and SalI and cloned into the plasmid that contained the ITRs described in [28]. For construction of AAV-Luc (Fig. 1A), a fragment containing the luciferase cDNA was obtained by PCR using the plasmid pCDNA2.1-Luc as template and cloned into the plasmid containing the human EFlα promoter, the polyadenylation signal, and the WPRE, to obtain plasmid pGTC-Luc-WPRE. The expression cassette was excised from this plasmid and cloned into the plasmid that contained the AAV ITRs [28].

**AAV vector production and purification.** These plasmids and the packaging/helper plasmid, pDG, coding for all AAV and adenovirus proteins required for amplification and packaging of AAV vector plasmids [29] were prepared using the EndoFree Plasmid Mega Kit (Qiagen, Valencia, CA, USA). A mixture of plasmid (20 μg of AAV plasmid and 55 μg pDG per plate) was transfected into 293 T cells using linear polyethylenimine 25 kDa (Polysciences, Warrington, PA, USA) as described in [30]. After 48 h, cells were harvested, resuspended in DMEM (2.5 ml/plate), and lysed by two freeze-thaw cycles. Cell extract was further digested with 40 μg/ml DNase and RNase (both from Roche Diagnostic GmbH, Mannheim, Germany) for 30 min at 37°C and centrifuged for 15 min at 4°C, 960 g. The supernatant was then treated with 0.5% deoxycholic acid (Sigma, St. Louis, MO, USA) for 30 min at 37°C and clarified by filtering through a 0.22-μm filter. Virus purification was done by affinity chromatography into HiTrap heparin columns as described [12] (Amersham Biosciences, Uppsala, Sweden). Once purified, the virus was concentrated using an Ultralite 15 centrifugal filter device (Millipore, Bedford, MA, USA) using PBS-MK (PBS containing 1 mM MgCl2 and 2.5 mM KCl). Virus titration was performed by real-time PCR as vp/ml. Primers and TaqMan probes were designed using the Primer-Express software (supplemental information). The reactions were performed as described [17]. The fluorescence signal delivered during PCR amplification was monitored using the LightCycler System (Roche Diagnostics, Basel, Switzerland).

**Cell culture and in vitro transduction with AAV vectors.** Woodchuck fibroblasts, rabbit fibroblasts, and HeLa cells were chosen for in vitro evaluation of AAV vectors. HeLa cells were obtained from the ATCC. Woodchuck and rabbit fibroblasts were obtained from the cornea of animals that had recently died. Cells were cultured and maintained in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum under standard conditions.

HeLa cells (1 × 10⁶) and woodchuck and rabbit fibroblasts (1 × 10⁵) were plated in 35-mm plates, and 24 h later cells were infected with AAV viruses at 100 vp/HeLa cell and 4000 vp/rabbit fibroblast. After 48 h, supernatants were harvested and tested to detect wIFN-α by a cytopathic effect reduction assay described below using a purified recombinant wIFNα as standard. Cells were collected in 1× Cell Culture Lysis Reagent (Promega, Madison, WI, USA). After centrifugation at 20,800 g for 1 min, 2 μl of sample was added to 18 μl of 1× Cell Culture Lysis Reagent and luciferase activity was determined using Luciferase Assay Reagent (Promega) in a luminometer. Luciferase activity was normalized by total protein measured using the Bradford method.
Animals. Wild-caught, chronically WHV-infected woodchucks (purchased from Northeastern Wildlife, Ithaca, NY, USA) were used. Anesthesia was induced with isoflurane, from 3 to 5%. Intramuscular injection of the gene therapy vector was carried out in the thigh muscle. Four injections of 75 μl of virus diluted in 150 mM NaCl were applied in the muscles of the two legs. Two different doses were used, a low dose of 2.5 × 10^{11} vp and a high dose of 8 × 10^{11} vp. Intraperitoneal injection was done by laparotomy. With the aim of increasing hepatocyte infection and to restrict unwanted extrahepatic dissemination of the recombinant virus, the vector was injected diluted in 2.5 ml of 150 mM NaCl into the portal vein, while both suprahepatic veins and portal pedicle (excluding bile duct) were clamped for a total time of 3 min. Blood was collected at regular intervals from the saphenous vein and serum was stored at −40 °C. Routine serum chemistry and hematological analysis were performed. Surgical liver biopsies were performed by laparotomy under general anesthesia and stored at −80 °C for DNA/RNA extraction, and for histological analysis a portion was embedded in Tissue Tek (Sakura, Zoeterwoude, The Netherlands) and stained with hematoxylin–eosin and fast red.

Luciferase assay in tissue samples. Livers were homogenized in 1× Cell Culture Lysis Reagent and frozen at −80°C for 5 min. After centrifugation at 20,800 g for 1 min, 2 μl of sample was added to 18 μl of 1× Cell Culture Lysis Reagent and luciferase activity was determined using Luciferase Assay Reagent (Promega) in a luminometer. Luciferase activity was normalized by total protein measured using the Bradford method.

**Determination of WHV DNA levels.** Serum WHV DNA was quantified by means of real-time quantitative PCR. WHV DNA was isolated from 50 μl of serum using the High Pure Viral Nucleic Acid Kit (Boehringer Mannheim GmbH, Mannheim, Germany), following the manufacturer’s instructions. Primers and TaqMan probes (Applied Biosystems, Foster City, CA, USA) were designed for the WHV core gene and a single copy gene sequence for the core gene using PrimerExpress software (supplemental information). The reaction was performed as described [17]. The detection limit of the assay was 100 virus genomes per microliter of serum. The largest decrease in viremia in logs compared with the baseline value (MLDV) was used as an indicator of the efficacy of treatment. The value that represents the 99% confidence interval for the mean MLDV values of four untreated woodchucks and four AAV-Luc-injected woodchucks analyzed every 2 weeks for 7 months was 1.24. Thus, we have considered as a significant decrease in viremia any MLDV value higher than 1.24. The statistical analysis was performed using the Kruskal–Wallis tests followed by comparison of the control group with the experimental groups applying the Mann–Whitney test with Bonferroni’s correction using SPSS software (SPSS, Inc., Chicago, IL, USA).

Liver DNA analysis. DNA was extracted using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). To detect AAV viral DNA, primers and probe were designed to amplify a fragment of the human EF1α promoter, which is a sequence common to AAV-Luc and AAV-IFN, using the PrimerExpress software. AAV DNA content was normalized by the β-actin copy number (supplemental information). The reactions were performed as described [17].

**Production of recombinant wIFN-α, biossay for wIFN-α, and detection of neutralization antibodies.** We produced recombinant woodchuck interferon by fusing wIFN-α cDNA to a polyhistidine tag using the pPrHeN1S TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and protein was expressed in Escherichia coli and purified under denaturing conditions by affinity chromatography using the ProBond purification system (Invitrogen) following the manufacturer’s instructions.

wIFNα concentration was determined by the CPE reduction assay described elsewhere [17]. wIFN-α concentration is expressed in pg/ml using a purified recombinant wIFN-α as standard. The levels of wIFN-α expression found between groups were compared using the Student t test. To detect the presence of IFN-α neutralizing antibodies, twofold serum dilutions in complete DMEM were done starting with 50 μl of serum and adding 10 pg of recombinant woodchuck IFN-α in each well. Then, 2 × 10^6 woodchuck hepatoma cells WCH-17 (ATCC No. CLR-2082) per well were added and IFN-α activity was determined following the standard protocol. The antibody wIFN-10A12 was developed in our laboratory by immunization of rats with wIFN-α recombinant protein and was used as positive control (P. Berraondo et al., manuscript in preparation).

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**APPENDIX A. SUPPLEMENTARY DATA**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymthe.2005.02.017.

**REFERENCES**


