Vasoactive Intestinal Peptide Increases Hepatic Transduction and Reduces Innate Immune Response Following Administration of Helper-dependent Ad

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Helper-dependent adenoviral vectors (HDAd) are effective tools for liver-directed gene therapy because they can mediate long-term transgene expression in the absence of chronic toxicity. However, high vector doses required for efficient hepatocyte transduction by intravascular delivery result in systemic vector dissemination and dose-dependent activation of the innate immunity. Therefore, strategies to achieve high-efficiency hepatocyte transduction using low vector doses and/or to reduce the acute elevations of proinflammatory cytokines and chemokines may have significant clinical potential. Vasoactive intestinal peptide (VIP) is an endogenous neuropeptide involved in the regulation of hepatic blood flow and plays an important role as modulator of immune functions. Here, we show that VIP pretreatment in mice is able to increase hepatocyte transduction by HDAd, decrease vector uptake by the spleen, reduce elevation of proinflammatory serum cytokines interleukin (IL)-6 and IL-12, and reduce serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) following intravenous HDAd injection. VIP pretreatment also resulted in a reduction in the expression of the chemokines macrophage-inflammatory protein 2 (MIP-2), monocyte chemotactic protein 1 (MCP-1), and regulated on activation normal T-cell expressed and secreted (RANTES) in the livers of mice injected with HDAd. These results suggest that VIP can improve the therapeutic index of HDAd by increasing hepatocyte transduction efficiency while reducing cytokine and chemokine expression following intravascular delivery of HDAd.

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INTRODUCTION

Helper-dependent adenoviral vectors (HDAd), which are devoid of all viral coding sequences, have demonstrated a tremendous potential for liver-directed gene therapy because they can transduce hepatocytes to direct long-term transgene expression in the absence of chronic toxicity.[1,2] However, the high doses required for efficient hepatocyte transduction result in widespread vector dissemination as well as dose-dependent activation of the innate immune response resulting in acute toxicity with potentially severe and lethal consequences.[3–5] This acute activation of the innate immune response is characterized by high levels of serum inflammatory cytokines and chemokines within a few hours postinjection.[3] In order to overcome this major obstacle toward clinical implementation of HDAd gene therapy, new strategies are needed to achieve high-efficient hepatocyte transduction using low vector doses.

It has been shown that the size of liver sinusoidal endothelial fenestrae (SEF) (≤100 nm) is a critical determinant of hepatocyte transduction by adenoviral vectors with a virion diameter of ≥100 nm.[6] The size of the SEF can be enlarged by means of drugs such as Na-decanoate[6] or N-acetylcysteine combined with transient liver ischemia[7] or by increasing the intrahepatic pressure by hydrodynamic injection.[8] However, further studies are necessary to determine the real clinical potential of these drugs, and hydrodynamic injection as performed in rodents is not suitable for human application due to the rapid injection of a large volume.

Vasoactive intestinal peptide (VIP) is a 28-amino acid peptide originally described in the lung and small intestine that exerts a broad spectrum of actions including the control of liver, spleen, and intestine microcirculation and modulation of the innate and adaptive immunity.[9–11] Previous studies have demonstrated that systemic VIP administration in rodents results in the enlargement of SEF mediated by an increase in liver blood flow and a concomitant reduction in the splenic microcirculation.[12–16] Regarding its role in the innate immunity, VIP is released from immune cells during inflammatory reactions and inhibits the production and release of several proinflammatory cytokines and chemokines including tumor necrosis factor–α, interleukin (IL)-6, IL-12, regulated on activation normal T-cell expressed and secreted (RANTES), monocyte chemotactic protein 1 (MCP-1), and macrophage-inflammatory protein (MIP-2) from activated

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macrophages as well as negatively regulating the expression of Toll-like receptors.[9,17–21] VIP exerts therapeutic effects by acting as a potent anti-inflammatory factor in many different disease models, including septic shock, rheumatoid arthritis, and inflammatory bowel disease.[21–24] The mechanisms by which VIP exerts these immunological actions are mediated by the interaction with three different G-coupled receptors: VPAC1, VPAC2, and PAC1. Using a cyclic adenosine monophosphate-dependent pathway, VIP acts as a potent vasodilator whereas through cyclic adenosine monophosphate-dependent or independent pathways VIP negatively regulates downstream players involved in the innate and adaptive immune responses, including nuclear factor-κB, Jak1-2/STAT1/IRFs, ERK1/2, and p38/MAP kinases.[9,17,18,24,25] It is well known that the ERK1/2, p38/mitogen-activated protein kinase pathways and nuclear factor-κB activation play a pivotal role in the adenovirus induction of the innate immune response.[26–28] For these reasons we choose to investigate VIP pretreatment as a pharmacological approach to improve the therapeutic index of HDAd by increasing hepatocyte transduction efficiency and reducing the innate immune response.

RESULTS

VIP increases hepatic transduction and reduces splenic vector uptake

It has been reported that supraphysiologic doses of systemically injected VIP in rodents is able to enlarge SEF and increase the liver blood flow.[14,15] Therefore, given the critical role of SEF in the efficiency of adenovirus-mediated liver transduction,[6–8] we hypothesized that VIP might improve the efficiency of hepatocyte transduction by HDAd as a result of SEF enlargement. To test this hypothesis, various doses of VIP (0, 5, 10, 50 nmol/mouse) were administered to wild-type C57BL/6 mice by intraperitoneal injection. HDΔ28E4LacZ, a HDAd bearing a murine cytomegalovirus-LacZ expression cassette,[3] at a dose of 1×10^{12} vector particles (vp)/kg, was administered by retro-orbital injection at the same time as VIP administration, or at 30 or 120 minutes after VIP administration. To determine hepatic transduction, livers were harvested 48 hours postvector, total proteins were extracted and the amount of β-galactosidase activity was determined. The results indicated that VIP increases the efficiency of hepatic transduction with the highest level of β-galactosidase activity achieved at the dose of 10 nmol/mouse given 30 minutes before HDΔ28E4LacZ administration; a 2.9-fold increase over no VIP ($P = 0.01$) (**Figure 1**). Therefore, for all subsequent experiments, 10 nmol VIP was given to each mouse by intraperitoneal injection 30 minutes before HDAd administration.

We next determined the effect of vector dose on hepatic transduction in mice pretreated with VIP. To accomplish this, saline or 10 nmol of VIP was administered to the mice and then, 30 minutes later, various doses of HDΔ28E4LacZ (1×10^{11}, 3×10^{11}, 1×10^{12}, and 5×10^{12} vp/kg) were administered by retro-orbital injection and the livers were harvested 48 hours later for analyses. At 1×10^{11} and 3×10^{11} vp/kg, saline pretreatment (**Figure 2a,c**) yielded qualitatively less β-galactosidase positive cells than VIP pretreatment (**Figure 2b,d**) as determined by X-gal histochemistry. At the higher doses of 1×10^{12} and 5×10^{12} vp/kg, qualitative differences in the number of β-galactosidase positive cells were not

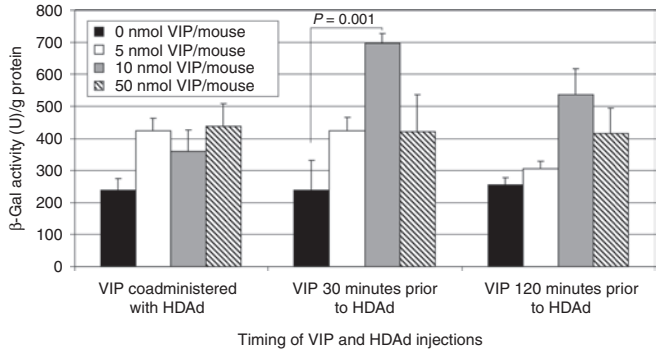

Figure 1 Effect of timing and dose of VIP on liver transduction efficiency by HDΔ28E4LacZ. Hepatic β-galactosidase activity was determined 48 hours postinjection of 1×10^{12} vp/kg HDΔ28E4LacZ into C57BL/6 mice treated with VIP at various doses and times by intraperitoneal injection. Means ± SD shown ($n = 5$). HDAd, helper-dependent adenoviral vectors; VIP, vasoactive intestinal peptide; vp, vector particles.

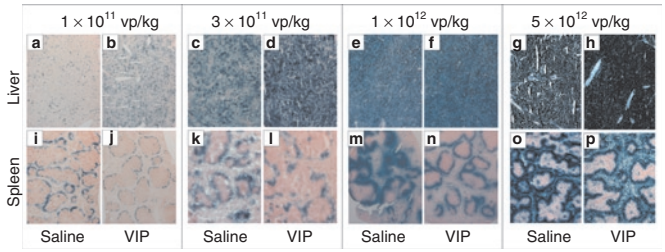

Figure 2 Liver and spleen transduction efficiency. X-gal histochemistry of the (**a**–**h**) liver or (**i**–**p**) spleen 48 hours following injection of various doses of HDΔ28E4LacZ into mice pretreated 30 minutes prior with either saline or VIP (10 nmol/mouse) by intraperitoneal injection. The results from one representative mouse from each treatment group is shown ($n \geq 5$ per treatment). The liver and spleen shown for each treatment condition are from the same mouse. VIP, vasoactive intestinal peptide; vp, vector particles.

distinguishable between saline (**Figure 2e,g**) or VIP (**Figure 2f,h**) pretreatment because all cells appeared β-galactosidase positive as determined by X-gal histochemistry, likely because these high doses were saturating. In contrast to the liver, VIP pretreatment at all doses appeared to yield qualitatively less β-galactosidase positive cells than saline pretreatment in the spleen as determined by X-gal histochemistry (**Figure 2i–p**).

To quantitate the amount of transgene expression, total protein was extracted from the livers and the amount of β-galactosidase activity was determined by enzymatic assay. The results revealed that VIP pretreatment yielded higher activities than saline pretreatment in the liver at all doses (**Figure 3**); 1.8-fold higher at 1×10^{11} vp/kg ($P = 0.04$), 2.9-fold higher at 3×10^{11} vp/kg ($P = 0.002$), 2.3-fold higher at 1×10^{12} vp/kg ($P = 0.00006$), and 1.7-fold higher at 5×10^{12} vp/kg ($P = 0.006$). In contrast to the liver, saline pretreatment yielded higher activities than VIP pretreatment in the spleen at all doses (**Figure 3**); 2.6-fold higher at 1×10^{11} vp/kg ($P = 0.04$), 1.7-fold higher at 3×10^{11} vp/kg ($P = 0.01$), twofold higher at 1×10^{12} vp/kg ($P = 0.000008$), and 1.6-fold higher at 5×10^{12} vp/kg ($P = 0.01$). To determine the amount of vector genomes taken up by the liver, total DNA was extracted and subjected to quantitative real-time PCR to determine HDAd genome copies. The results revealed that VIP pretreatment yielded greater number of vector

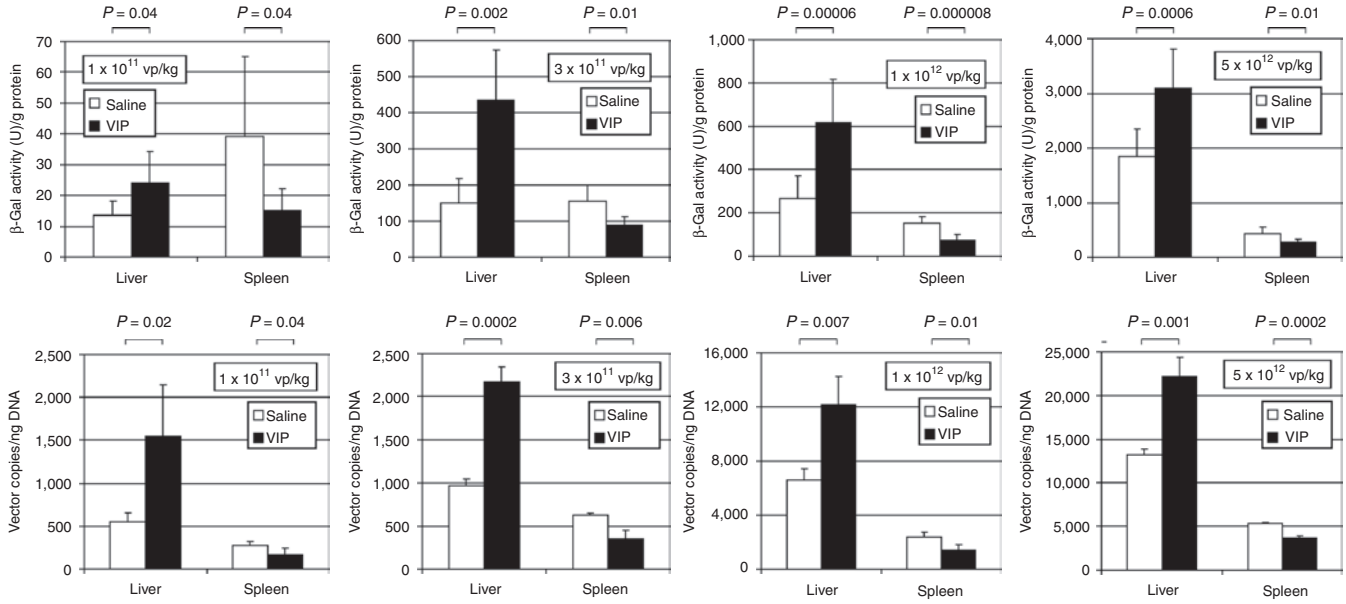

Figure 3 β-Galactosidase activity and vector genome copy number in the livers and spleens of mice 48 hours following injection of various doses of HDΔ28E4LacZ into mice pretreated 30 minutes prior with either saline or VIP (10 nmol/mouse) by intraperitoneal injection. Means ± SD shown ($n \geq 5$). Background β-galactosidase activity (no vector control) was 12.5 ± 2.5 U/g protein for the liver and 9.3 ± 0.5 U/g protein for the spleen. Vector genome was undetectable in no vector control animals. VIP, vasoactive intestinal peptide; vp, vector particles.

genomes per ng of liver DNA than saline pretreatment at all doses (Figure 3); 2.8-fold at 1×10^{11} vp/kg ($P = 0.02$), 2.2-fold at 3×10^{11} vp/kg ($P = 0.0002$), 1.8-fold at 1×10^{12} vp/kg ($P = 0.007$), and 1.7-fold at 5×10^{12} vp/kg ($P = 0.001$). In contrast to the liver, saline pretreatment yielded greater number of vector genomes per ng of spleen DNA than VIP pretreatment at all doses (Figure 3); 1.6-fold at 1×10^{11} vp/kg ($P = 0.04$), 1.8-fold at 3×10^{11} vp/kg ($P = 0.006$), 1.7-fold at 1×10^{12} vp/kg ($P = 0.01$), and 1.4-fold at 5×10^{12} vp/kg ($P = 0.0002$).

Taken together, these data show that VIP pretreatment improves liver transduction efficiency by HDAd while reducing splenic uptake of the vector.

VIP reduces HDAd-mediated acute cytokine activation and liver toxicity

Systemic intravascular HDAd injection results in activation of the innate inflammatory response, marked by elevations of proinflammatory cytokines, the magnitude of which is dose dependent.[3,26,29,30] Therefore, we next compared the serum levels of proinflammatory cytokines IL-6 and IL-12 in the mice injected with either 1×10^{12} or 5×10^{12} vp/kg presented above at 6 hours postinjection. For IL-6, the results revealed that saline pretreatment yielded higher levels than VIP pretreatment at both vector doses; 2.5-fold at 1×10^{12} vp/kg ($P = 9 \times 10^{-5}$) and 1.7-fold at 5×10^{12} vp/kg ($P = 0.002$) (Figure 4). Likewise, for IL-12, saline pretreatment yielded higher levels than VIP pretreatment at both vector doses; 2.4-fold at 1×10^{12} vp/kg ($P = 8 \times 10^{-6}$) and 2.9-fold at 5×10^{12} vp/kg ($P = 3 \times 10^{-5}$) (Figure 4). The two lower HDAd doses, 1×10^{11} and 3×10^{11} vp/kg, did not result in elevation of serum IL-6 and IL-12 over baseline values regardless of saline or VIP pretreatment (not shown).

Systemic intravascular injection of HDAd also results in acute but transient hepatoxicity, the severity of which is also

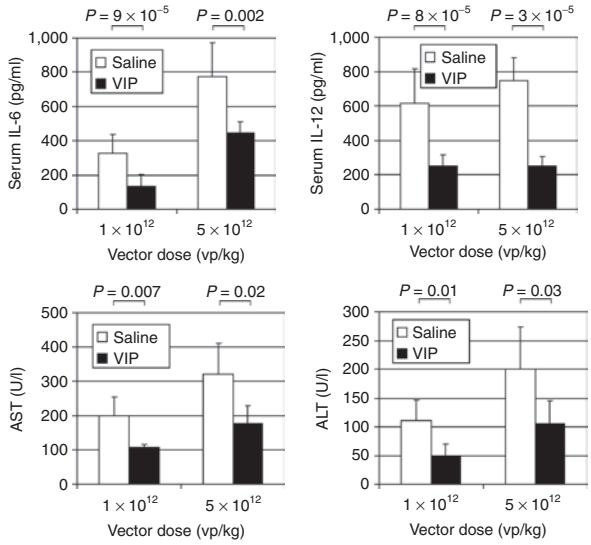

Figure 4 Serum levels of IL-6, IL-12, AST, and ALT from mice injected with either 1×10^{12} or 5×10^{12} vp/kg of HDΔ28E4LacZ pretreated 30 minutes prior with either saline or VIP (10 nmol/mouse) by intraperitoneal injection. IL-6 and IL-12 levels were measured 6 hours postvector. AST and ALT levels were measured 24 hours postvector. Means ± SD shown ($n \geq 5$). Background levels were 11.2 ± 8.5 pg/ml for IL-6, 24.8 ± 2.8 pg/ml for IL-12, 43 ± 14.3 U/l for AST, and 29 ± 4.2 U/l for ALT. ALT, alanine aminotransferase; AST, aspartate aminotransferase; IL, interleukin; VIP, vasoactive intestinal peptide.

dose dependent. Therefore, we also compared the levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in mice injected with 1×10^{12} or 5×10^{12} vp/kg presented above at 24 hours postinjection. For AST, the results revealed that saline pretreatment yielded higher levels than VIP pretreatment at both vector doses despite lower efficiencies of hepatic transduction; 1.8-fold at both 1×10^{12} vp/kg ($P = 0.007$) and 5×10^{12} vp/kg

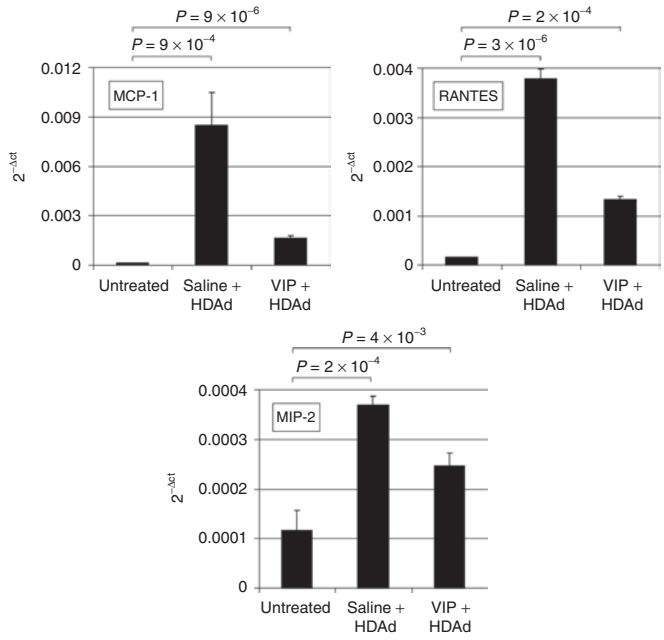

Figure 5 Expression levels of MCP-1, MIP-2, and RANTES in the livers of mice 3 hours after injection of 1 × 10^{12} vp/kg of HDΔ28E4LacZ into mice pretreated 30 minutes prior with either saline or VIP (10 nmol/mouse) by intraperitoneal injection. Shown as mean ± SD of three to four independent experiments, each performed in duplicate. HDAd, helper-dependent adenoviral vectors; MCP-1; monocyte chemotactic protein 1; MIP-2, macrophage-inflammatory protein 2; VIP, vasoactive intestinal peptide.

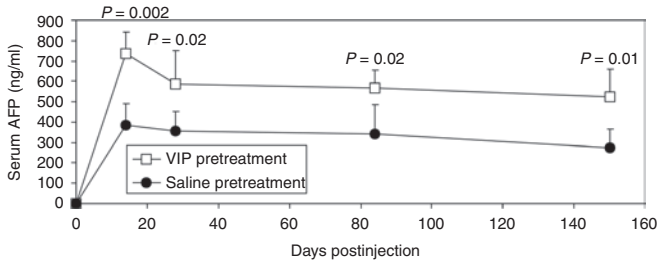

Figure 6 The mean ± SD levels of serum AFP from C57BL/6 mice (n = 5) following injection of 1 × 10^{11} vp/kg of HDΔ21.7-PEPCK-bAFP-WL in VIP (10 nmol/mouse) or saline pretreated mice. AFP, α-fetoprotein; VIP, vasoactive intestinal peptide.

(P = 0.02) (**Figure 4**). For ALT, the results also showed that saline pretreatment yielded higher levels than VIP pretreatment at both vector doses despite lower efficiencies of hepatic transduction; 2.3-fold (P = 0.01) and 1.9-fold (P = 0.03) at 1 × 10^{12} and 5 × 10^{12} vp/kg, respectively (**Figure 4**). Taken together, these results indicate that VIP pretreatment reduces HDAd-mediated activation of the innate immune response and hepatotoxicity.

The livers and spleens were obtained from mice 24 hours after injection of HDAd at 5 × 10^{12} vp/kg with and without VIP pretreatment for hematoxylin and eosin histology. In the livers of four mice injected with HDAd without VIP pretreatment, multiple small clusters of acute parenchymal necrosis with no special zonal distribution and small foci with microsteatosis were observed in two animals (**Supplementary Figure S1a**), whereas in the other two animals a few foci of sinusoidal inflammation with virtually no necrosis or steatosis were observed (data not shown). Variation was observed in the livers of four mice injected with HDAd with VIP pretreatment. One animal had extensive ductular proliferation and small foci of hepatocyte or duct cell necrosis at the interface, as well as focal lobular necrosis, and hepatic vein endothelialitis (**Supplementary Figure S1b**). Another animal had similar abnormalities which were less extensive (data not shown). Two other animals had only focal cell necrosis without ductular proliferation (**Supplementary Figure S1c**). Animals injected with VIP alone had lobular inflammation and very rare single cell necrosis with very little focal steatosis (**Supplementary Figure S1d**). The spleens of all animals injected with HDAd with or without VIP pretreatment presented with similar findings; depletion of lymphocytes from interfollicular regions and depletion of megakaryocytes, as well as an increased number of neutrophils (**Supplementary Figure S1e**). The white pulp was unaffected. Spleens from animals injected with VIP alone appeared normal (**Supplementary Figure S1f**). These data show that histological abnormalities in the liver and spleen are associated with HDAd, with or without VIP pretreatment.

VIP downregulates chemokine expression in the liver

The liver is the major site of adenovirus vector deposition following intravascular administration causing an increased expression of chemokines in that organ.[26] Therefore, we next analyzed the *in vivo* effect of VIP on hepatic chemokine expression following intravenous HDAd administration. To accomplish this, 1 × 10^{12} vp/kg of HDΔ28E4LacZ was administered into saline or VIP pretreated mice by retro-orbital injection. Three hours later, the livers were harvested and the expression levels of MCP-1, MIP-2, and RANTES were determined by quantitative real-time PCR. As shown in **Figure 5**, injection of HDΔ28E4LacZ into saline pretreated mice resulted in a 53-fold increase in hepatic MCP-1 expression compared to untreated mice (P = 9 × 10^{-4}). In contrast, injection of HDΔ28E4LacZ into VIP pretreated mice resulted in only a tenfold increase in MCP-1 expression in the liver compared to untreated mice (P = 9 × 10^{-6}) (**Figure 5**). Likewise, injection of HDΔ28E4LacZ into saline pretreated mice resulted in a 24-fold increase in hepatic RANTES expression compared to untreated mice (P = 3 × 10^{-6}). In contrast, injection of HDΔ28E4LacZ into VIP pretreated mice resulted in only an 8.6-fold increase in RANTES expression compared to untreated mice. In the case of MIP-2, injection of HDΔ28E4LacZ resulted in a threefold increase in expression (P = 0.0002) compared to untreated mice. In contrast, injection of HDΔ28E4LacZ into VIP pretreated mice resulted in only a twofold increase in MIP-2 expression compared to untreated mice (P = 0.004). These data indicated that VIP pretreatment down regulates cytokine and chemokine expression in the liver following administration of HDAd *in vivo*.

VIP pretreatment does not affect long-term transgene expression from HDAd

Long-term transgene expression is desirable feature of HDAds. Therefore, we next tested whether VIP pretreatment affects the duration of transgene expression from HDAd. To accomplish this, mice were first pretreated with VIP or saline and then 1 × 10^{11} vp/kg of HDΔ21.7-PEPCK-bAFP-WL was administered by

retro-orbital injection. This vector expresses the α-fetoprotein (AFP) from a liver-specific promoter.[31] As expected, VIP pretreatment yielded 1.7–1.9-fold higher levels of serum AFP than saline pretreated mice (*P* value 0.002–0.02) (**Figure 6**). Importantly, VIP pretreatment did not affect the duration of transgene expression because, like saline pretreatment, high levels of AFP were observed for the duration of the experiment of at least 150 days (**Figure 6**).

DISCUSSION

HDAds have shown to be a very promising vector for liver-directed gene therapy. Several studies indicate that a single injection of HDAd can result in therapeutic levels of transgene expression that can be maintained for several years.[1,31–35] However, the major limitation of these vectors is the acute toxicity occurring rapidly after systemic injection of high vector doses. This dose-dependent toxic response is due to the activation of the innate immunity, which may have lethal consequences.[3] In this study, we found that VIP, a potent anti-inflammatory factor and vasodilator, increases liver transduction efficiency by HDAd, and attenuates vector-mediated acute toxicity. The improvement in liver transduction is presumably a consequence of the effect of VIP on liver blood flow and the increase in the SEF diameter.[11,12,14,15] Oda *et al.* showed that the enlargement of the diameter of SEF can occur either as a secondary effect mediated by an increase in the portal blood flow or by a direct action on the liver sinusoidal endothelial cells.[15] There are several potential additional mechanisms that could explain the enlargement of SEF, including the activation of cyclic adenosine monophosphate and the Ca^{2+}-calmodulin–actomyosin system which is modulated by VIP.[14,15] Interestingly, we found that splenic uptake of HDAd was also reduced by VIP. This effect may be due to the increased liver transduction resulting in less available vector for splenic uptake or a result of a specific action on the splenic microcirculation. As suggested from a previous study,[12] it is possible that VIP reduces HDAd splenic uptake by decreasing the splenic blood flow through an active mechanism. According to previous studies, the spleen is a major organ involved in the release of some proinflammatory cytokines and chemokines after intravascular administration of adenovirus.[36,37] Therefore, we speculate that reducing splenic uptake of HDAd may reduce the systemic release of cytokines. Although we did not determine the source(s) of cytokines in the serum, it is possible that the change in the vector biodistribution might account, at least in part, for the reduction in the cytokine and chemokines observed. Moreover, it is well known that in pathologic stimulation such as presence of lipopolysaccharide, VIP is released *in vivo* by activated T cells and interacts with VPAC1 or VPAC2, two G-coupled receptors present on the macrophage plasma membrane.[19,38] The signaling cascade culminates in the repression of the mitogen-activated protein kinase/p38/ERK pathway and ultimately in the inhibition of nuclear factor-κB nuclear translocation, leading to reduction in promoter activation of several cytokines and chemokines.[19,25,38,39] Because the mitogen-activated protein kinase/p38/ERK and nuclear factor-κB are key pathways involved in the innate immune response to adenovirus, it is possible that VIP pretreatment might repress these molecular mediators and attenuate the early inflammatory response initiated by HDAds. Previous studies have shown that adenovirus-induced cytokine/chemokine recruitment of immune effector cells into the liver results in hepatotoxicity.[26,40] It is remarkable that although the liver transduction was increased by VIP, elevations in AST and ALT were concomitantly reduced. This finding appears inconsistent with previous studies.[41,42] However, it is possible that the VIP can counteract the proinflammatory effect of HDAd by preventing the expression and/or release of chemokines and cytokines by Kupffer cells and/or other parenchymal cells of the liver. In our *in vivo* experiments, we were not able to exactly identify the cell types involved in the anti-inflammatory effects of VIP.

The ability of VIP to attenuate some aspects of the HDAd immune response suggests a new and unexpected role for this neuropeptide in the context of adenovirus-host interaction. It has been proposed that VIP, through its ability to induce the synthesis of interferons and to modulate the function of important immune cells such as dendritic cells and natural killer cells, plays a protective role against viral infection.[43,44] VIP and other related neuroimmune mechanisms may therefore be involved in the modulation of immune responses to natural adenovirus infection by acting through these additional mechanisms. VIP is well tolerated in clinical studies in human volunteers[45] and currently, there are several studies being conducted involving VIP as an immunosuppressant/vasodilator in chronic obstructive pulmonary disease, sepsis, asthma among others with several ongoing clinical trials in different phases with active recruitment (ClinicalTrials.gov identifiers: NCT00272896, NCT00464932, NCT00004494, NCT00255320).

In conclusion, our study shows that VIP pretreatment (i) increases liver transduction by HDAd, (ii) reduces splenic uptake of HDAd, and (iii) attenuates the HDAd-mediated innate immune response as evident by reduction in cytokine and chemokine expression, and attenuates HDAd-mediated hepatotoxicity as evident by reductions in AST and ALT. Thus, VIP pretreatment could represent a potentially useful strategy to improve the therapeutic index of HDAds.

MATERIALS AND METHODS

Vectors. HDΔ28E4LacZ bears a murine cytomegalovirus-LacZ expression cassette[46] and HDΔ21.7E4PEPCK-bAFP-WL contains a liver-restricted baboon AFP (bAFP) expression cassette and has been described previously.[31] HDAd was produced in 116 cells[47] with the helper virus AdNG163 as described elsewhere.[48] Helper virus contamination levels were determined as described elsewhere[47] and were found to be <0.05%. DNA analyses of HDAd genomic structure was confirmed as described elsewhere.[47] All vector preparations were tested using Multi-test Limulus Amebocyte Lysate (Pyrogent; Biowhittaker, Walkersville, MD) for the presence of endotoxin and were found to be below the limit of detection (endotoxin <0.5 endotoxin units/ml).

Mice and injections. Nine- to twelve-week-old male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were used for all the experiments. Intravenous HDAd vector administrations were performed in saline solution by retro-orbital injection with a volume of 200 µl. VIP (Calbiochem; EMD Bioscience, La Jolla, CA), resuspended in saline, was administered by intraperitoneal injection in a volume of 200 µl. For mice injected with HDΔ28E4LacZ, blood was collected retro-orbitally for analyses at 6 hours postinjection and at the time of sacrifice at 3 or 48 hours postinjection. Upon sacrifice, the liver and spleen were harvested and kept on dry ice or at −80 °C until analyses. For mice injected with HDΔ21.7E4PEPCK-bAFP-WL, blood was collected retro-orbitally weekly for analyses. Serum was frozen immediately and stored at −80°C until analyses.

Analyses of tissue and blood. X-gal histochemistry was performed on liver and spleen and from mice as described previously.[8] Total protein was extracted from the liver and spleen and the β-galactosidase activity was determined using the β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega, Madison, WI) following quantification using the Micro BCA Protein Assay Kit (Pierce, Rockford, IL). Total DNA was extracted from liver and spleen using the QIAamp DNA extraction kit (Qiagen, Valencia, CA) and quantitated by absorbance at 260 nm. Quantitative real-time PCR was performed using the LightCycler FastStart DNA Master SYBR Green I (Roche, Indianapolis, IN) in a total volume of 20 µl with 2 µl of template DNA, 4 mmol/l $MgCl_2$, and 5 mol/l each HDAd-specific primer (5′-TCT GAA TAA TTT TGT GTT ACT CAT AGC GCG-3′ and 5′-CCC ATA AGC TCC TTT TAA CTT TTA AAG TC-3′). Cycling conditions consisted of 95 °C for 10 minutes followed by 45 cycles at 95 °C for 10 seconds, 60 °C for 7 seconds, and 72 °C for 20 seconds. Serial dilutions of a plasmid bearing the PCR target sequence were used as a control to determine the amounts of HDAd and results were analyzed with LightCycler software version 3.5 (Roche). Serum levels of IL-6 and IL-12 were determined by enzyme-linked immunosorbent assay (Biosource; Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Serum bAFP was measured by immunoassay, using antihuman AFP (ADVIA Centaur; Bayer Diagnostics, Tarrytown, NY). Total RNA from livers was isolated using RNeasy mini kit (Qiagen) according to manufacturer' instructions. Statistical analyses were performed with the *t*-test.

Reverse transcription and real-time quantitative PCR. Complementary DNA was synthesized from 1 µg of total RNA by reverse transcription using Superscript II according to the manufacturer's instruction (Invitrogen). Samples were then diluted 1:10 in nuclease free water and used as template for real-time quantitative PCR. Real-time quantitative PCR was performed using the Light Cycler Faststart DNA Master SYBR Green I (Roche) in a total volume of 20 µl with 2 µl of template DNA, 4 mmol/l $MgCl_2$, and 5 µmol/l of each specific primer. Sequences of the mouse primer used in real-time PCR were as follows: MCP-1: 5′-CGG AAC CAA TGA GAT CAG AAC CTA C-3′ and 5′-AAT TAA GGC ATC ACA GTC CGA GTC AC-3′. MIP-2: 5′-GCC CAG ACA GAA GTC ATA GCC A-3′ and 5′-GAC AGC GAG GCA CAT CAG GTA CGA-3′. RANTES: 5′-AGG ATA GAG GGT TTC TTG ATT CTG-3′ and 5′-CAT TTT CCC AGG ACC GAG T-3′. GAPDH: 5′-CGA CCC CTT CAT TGA CC TCA ACT-3′ and 5′-GGC CTC ACC CCA TTT GAT GTT AG-3′. Results were obtained using the $2^{-\Delta CT}$ method (normalized for GAPDH as housekeeping gene). Cycling conditions consisted of 95 °C for 10 seconds, 50–65 °C for 7 seconds, and 72 °C for 20 seconds. The results were analyzed with LightCycler software version 3.5 (Roche).

SUPPLEMENTARY MATERIAL

Figure S1. Hematoxylin and eosin histology of liver and spleen from mice 24 hours postinjection.

ACKNOWLEDGMENTS

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