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Frédéric Vigant, Delphyne Descamps, Betsy Jullienne, Stéphanie Esselin, Elisabeth Connault, et al.. Substitution of hexon hypervariable region 5 of adenovirus serotype 5 abrogates blood factor binding and limits gene transfer to liver.. Molecular Therapy, 2008, 16 (8), pp.1474-80. 10.1038/mt.2008.132 . hal-00315859

HAL Id: hal-00315859

<https://hal.science/hal-00315859v1>

Submitted on 30 May 2020

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Substitution of Hexon Hypervariable Region 5 of Adenovirus Serotype 5 Abrogates Blood Factor Binding and Limits Gene Transfer to Liver

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Liver tropism potentially leading to massive hepatocyte transduction and hepatotoxicity still represents a major drawback to adenovirus (Ad)-based gene therapy. We previously demonstrated that substitution of the hexon hypervariable region 5 (HVR5), the most abundant capsid protein, constituted a valuable platform for efficient Ad retargeting. The use of different mouse strains revealed that HVR5 substitution also led to dramatically less adenovirus liver transduction and associated toxicity, whereas HVR5-modified Ad were still able to transduce different cell lines efficiently, including primary hepatocytes. We showed that HVR5 modification did not significantly change Ad blood clearance or liver uptake at early times. However, we were able to link the lower liver transduction to enhanced HVR5-modified Ad liver clearance and impaired use of blood factors. Most importantly, HVR5-modified vectors continued to transduce tumors *in vivo* as efficiently as their wild-type counterparts. Taken together, our data provide a rationale for future design of retargeted vectors with a safer profile.

Received 13 April 2008; accepted 14 May 2008; published online 17 June 2008. doi:10.1038/mt.2008.132

INTRODUCTION

Vectors derived from adenovirus serotype 5 (Ad) are extensively used in many clinical trials aimed at evaluating possible therapies for genetic diseases and cancer. However, Ad native tropism raises several concerns. Ad capsid-fiber protein interacts with coxsackievirus and Ad receptor (CAR) leading to cell attachment, then penton-base binding to α_v -integrins triggers Ad endocytosis.^{1,2} Many potential target cells appear to be resistant to Ad infection because of low (or no) CAR expression.³ Conversely, different untargeted tissues may capture virus particles (vp) and/or be transduced after systemic Ad administration. Notably, intravenous (IV)⁴⁻⁶ but also local⁷ administrations result in massive liver uptake of virus posing a high risk of toxicity.

To enhance Ad entry into refractory cells, targeting motifs were incorporated into capsid proteins displaying regions of

low structural constraint, such as fiber HI loops,⁸ penton-base RGD loops,⁹ and hexon hypervariable region 5 (HVR5).¹⁰ Most of these genetic modifications maintained liver Ad uptake and toxicity. Because liver uptake was first attributed to high CAR expression,¹ different strategies were developed to abrogate Ad binding to CAR. This can be achieved by modifying the fiber-shaft length,^{11,12} replacing the fiber with the one from other non-CAR-binding Ad serotypes¹¹ or even xenotypes.¹³ Unfortunately, the surrogate fiber endows Ad with another entry pathway, which could prove dangerous if the new receptor is not well characterized or ubiquitous in humans (e.g., CD46 for Ad subgroup B).¹⁴ More accurate detargeting of Ad from CAR was obtained following mutagenesis of residues in the fiber-knob domain interacting with CAR.^{5,15} Also, deletion of the RGD motif within the penton-base detargeted Ad from integrins. Although successful *in vitro*, elimination of interaction with CAR, integrins, or both failed to reduce liver transduction,^{5,16} highlighting the involvement of other interactions for Ad liver entry. A small motif lying within the fiber-shaft has been thought to be responsible for liver Ad entry through potential interactions with heparan sulfate proteoglycans.¹⁷ However, recent data demonstrated that mutation of this motif did not affect liver uptake but impaired intracellular trafficking, thus leading to inefficient liver-transgene expression.^{18,19}

Pertinently, Ad interactions with different plasma proteins were shown to be particularly important for liver Ad entry. For instance, liver-transgene expression was dramatically lower in complement C3-deficient mice, emphasizing a role of C3 in liver entry.²⁰ Ad fiber was also shown to bind complement C4-binding protein and vitamin K-dependent coagulation factor IX (FIX), thereby leading to gene transfer into liver, probably through low-density lipoprotein receptor-related protein or heparan sulfate proteoglycan.²¹ Coagulation factors FX, protein C, and FVII were also shown to promote Ad entry into hepatocytes and refractory cells.^{22,23} The role of coagulation factors in promoting Ad cell transduction and liver entry was also extended to Ad5 pseudotyped with subgroup D fibers.^{24,25} Altogether, these observations highlight major interplay between some yet uncharacterized capsid motifs and plasma components.

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Our earlier experiments showed that replacing the hexon HVR5 by an integrin-targeting ligand promoted Ad5 entry into non-CAR-expressing cells.¹⁰ Herein, we report that HVR5 substitution also led to dramatically less transgene expression in liver, due to abrogation of blood factor-mediated cell entry, whereas HVR5-modified vectors were fully able to transduce tumors.

RESULTS

Hexon HVR5 modification impaired Ad-transgene expression in liver

We previously demonstrated that RGD inclusion in HVR5 (AdHRGD) provided Ad with a new entry pathway into smooth muscle cells.¹⁰ To further characterize this vector, we performed biodistribution studies after IV injection into BALB/c mice. Unexpectedly, a 97.6% decrease of liver-transgene expression of AdHRGD was observed 48 hours after injection, when compared to its counterpart containing an RGD motif in the fiber-protein HI loop (AdFRGD) and control Ad (AdHwt), both of which triggering similar transgene expressions (**Supplementary Figure S1**). In accordance with these findings, liver Ad-genome content was markedly lower in AdHRGD- than AdFRGD- or AdHwt-injected mice (**Supplementary Figure S1**).

To prevent potential retargeting to α_v -integrins of AdHRGD, although expression in other organs did not differ (data not shown), we constructed two other Ad in which HVR5 was replaced by nontargeting peptides containing 8 (AdH[GA]8) or 24 (AdH[GA]24) glycine-alanine (GA) residues. Like AdHRGD, GA-containing Ad were produced at titers comparable to AdHwt (**Table 1**). Sodium dodecyl sulfate polyacrylamide gel electrophoresis and transmission electron microscopy analyses confirmed no differences in virus composition and integrity (data not shown). Also, all HVR5-modified vectors showed similar thermostability to AdHwt (**Figure 1a**). Thus, consistent with unmodified virus production (**Table 1**), HVR5 substitution by different peptides did not markedly affect Ad capsid assembly and stability. *In vitro*, AdH[GA]8 and AdH[GA]24 transductions were comparable to those of AdHwt and AdHRGD in CAR-overexpressing CHO cells (CHO-CAR), hepatoma cell-line Hepa 1-6 (**Supplementary Materials and Methods** and **Supplementary Figure S2**), and primary hepatocytes (**Figure 1b**), whereas a previously described Ad5 pseudotyped with a non-CAR-binding Ad3 fiber (AdF3)¹¹

engendered much less efficient transduction. Thus, HVR5 modification *per se* did not impair Ad entry nor transgene expression into CAR-expressing cells, including hepatocytes.

We next assessed HVR5-modified Ad-mediated liver transduction *in vivo*. Three different mouse strains (BALB/c, C57BL/6, and C3H) were injected IV with AdHwt, AdF3 or HVR5-modified Ad (AdHRGD, AdH[GA]8, and AdH[GA]24). Transduction efficiencies were assessed after 48 hours by β -Gal-activity determination in liver lysates. Transgene expression decreased by up to 99.9% according to mouse strain and virus construct used, compared to AdHwt-injected mice (**Figure 2a-c**). In accordance with these findings, Ad-genome content in total liver DNA was up to 97.3% lower, depending on the mouse strain and virus construct used (**Figure 2d-f**). These observations emphasize that the absence of transgene expression in hepatocytes after injection of HVR5-modified Ad reflected less Ad-genome transfer. It should be noted that the AdHRGD pattern was unique; markedly less gene transfer into BALB/c liver and, to a lesser extent, in C57BL/6 mice, whereas AdHRGD achieved gene transfer similar to AdHwt in C3H mice.

We also analyzed whether switching the Ad5 HVR5 to Ad subgroup C (Ad2) or D (Ad19 and Ad30) HVR5 could rescue transgene expression in liver. AdHAD2, AdHAD19, and AdHAD30 were produced at yields similar to AdHwt or other HVR5-modified Ad (**Table 1**), and efficiently transduced CHO-CAR cells *in vitro* (data not shown). The abilities of these HVR5-modified Ad to drive transgene expression in C57BL/6 liver were severely impaired (**Supplementary Figure S3**). Notably, insertion of HVR5 derived from Ad2, which has strong liver tropism when

Table 1 Features of wild-type and HVR5-modified Ad

Virus	Upstream sequence	Inserted peptide	Downstream sequence	Titer ($\times 10^{12}$ vp/ml)
AdHwt	FFS ₂₆₈	TTEAAAGNGDNLIT	P ₂₈₂ KV	12.2 \pm 6.6
AdHRGD	FFS ₂₆₈	GSDCRGDCFGS	P ₂₈₂ KV	5.2 \pm 2.0
AdH[GA]8	FFS ₂₆₈	GGGAGAGAGLGG	P ₂₈₂ KV	8.5 \pm 0.5
AdH[GA]24	FFS ₂₆₈	GGGGAGAGGA GGAGGAGAGGAGA GALGG	P ₂₈₂ KV	12.8 \pm 3.4
AdHAD2	FFS ₂₆₈	NTTSLNDRQGNATK	P ₂₈₂ KV	8.3
AdHAD19	FFS ₂₆₈	TPGANPPAGGSG NEEYK	P ₂₈₂ KV	11.6
AdHAD30	FFS ₂₆₈	STTINI	P ₂₈₂ KV	7.3

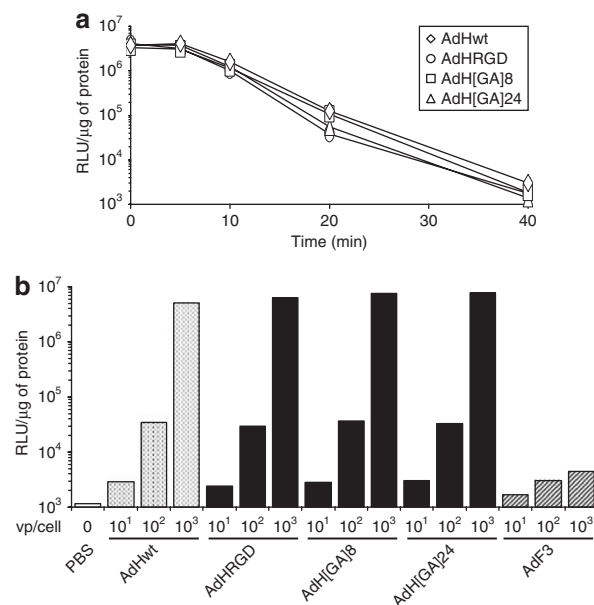


Figure 1 Thermostability of and *in vitro* gene transfer by HVR5-modified Ad vectors. **(a)** AdHwt, AdHRGD, AdH[GA]8, or AdH[GA]24 were incubated at 45°C for different times before infecting CHO-CAR cells at the multiplicity of infection (MOI) of 10^3 vp/cell. **(b)** Freshly isolated rat primary hepatocytes were mock-infected [(phosphate buffered saline (PBS)) or infected with increasing MOI of AdHwt, AdHRGD, AdH[GA]8, AdH[GA]24, or AdF3, and β -Gal activity was measured in cell lysates 24 hours later. Experiments run in duplicate were performed twice and representative results are shown. Each symbol represents the mean \pm SD of duplicate determinations.

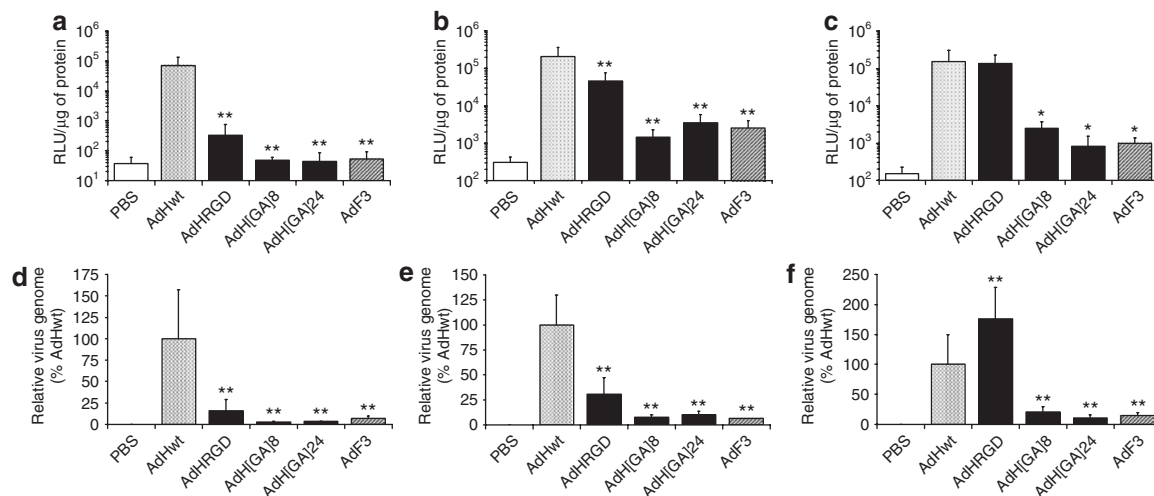


Figure 2 Transgene expression and Ad-genome content in liver after systemic administration of HVR5-modified Ad. **(a, b, c)** β -Gal activity measured in liver lysates. **(d, e, f)** Relative Ad-genome content in total liver DNA expressed as a percentage of that measured in AdHwt-injected mice. **(a, d)** BALB/c, **(b, e)** C57BL/6, and **(c, f)** C3H mice were injected IV with PBS or 10^{11} vp of AdHwt, AdHRGD, AdH[GA]8, AdH[GA]24, or AdF3, and livers were collected 48 hours later. One of two experiments is shown, $n = 4$ –5 mice; means + SD. * $P < 0.01$ and ** $P < 0.001$ versus AdHwt-injected mice.

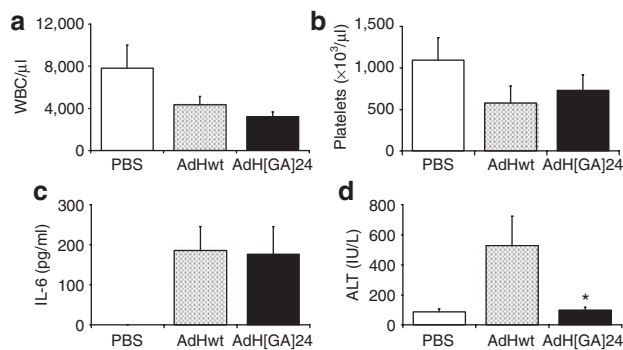


Figure 3 Toxicity profile of HVR5-modified Ad. C57BL/6 mice were injected IV with phosphate buffered saline (PBS) or 10^{11} vp of AdHwt or HVR5-modified AdH[GA]24. **(a)** White blood cells (WBC) and **(b)** platelets were counted 48 hours later. **(c)** Serum interleukin-6 (IL-6) and **(d)** serum ALT levels were determined 6 hours and 4 days later, respectively. One of two experiments is shown, $n = 4$ –6 mice; means + SD. * $P < 0.001$ versus AdHwt-injected mice.

injected IV, did not rescue the defective liver-transgene expression of HVR5-modified Ad5. Thus, lower transgene expression in liver was independent of mouse strain and the incorporated motif.

Toxicity profiles of HVR5-modified Ad

To determine whether hexon modification modulates the acute toxicity observed following systemic Ad administration,²⁶ different hematological parameters were analyzed. In C57BL/6 mice, control AdHwt or AdH[GA]24 triggered 48 hours after IV injection similar decreases of white blood cell (Figure 3a) and platelet (Figure 3b) counts, compared to phosphate buffered saline (PBS)-injected mice. Serum interleukin-6 levels were also comparable 6 hours after injecting AdHwt or AdH[GA]24 (Figure 3c). Conversely, serum alanine aminotransferase (ALT) levels 4 days after injection were significantly higher in AdHwt-injected mice, than the comparable levels in AdH[GA]24- or PBS-injected mice

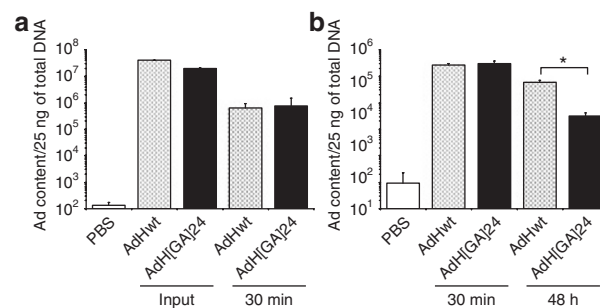


Figure 4 Ad clearance from blood and liver. C57BL/6 mice were injected IV with 10^{11} vp of control AdHwt or AdH[GA]24. **(a)** Ad input and Ad-genome content in blood 30 minutes later. **(b)** Ad-genome content in liver 30 minutes and 48 hours after injection. One of two experiments is shown, $n = 4$ –6 mice; means + SD. * $P < 0.001$.

(Figure 3d). This limited hepatocyte injury in mice injected with HVR5-modified Ad is consistent with the lower transgene expression and Ad-genome content in liver (Figure 2).

Mechanisms involved in the lower liver-transgene expression

To obtain insight into liver detargeting, we monitored Ad-genome contents in blood and liver 30 minutes after virus injection. As previously described,²⁷ mice rapidly cleared AdHwt and AdH[GA]24 genomes from blood (Figure 4a), consistent with extensive Ad trapping in liver (Figure 4b). However, as mentioned earlier (Figure 2), Ad-genome content in liver was more dramatically reduced 48 hours after AdH[GA]24 than AdHwt injection (Figure 4b), thereby demonstrating more rapid AdH[GA]24 clearance from liver.

Because Kupffer cells (KCs) are involved in the trapping of large amounts of IV-injected Ad, their potential role in accelerated liver clearance of AdH[GA]24 was assessed. As previously described,²⁸ mouse pretreatment with clodronate-liposomes to deplete KCs

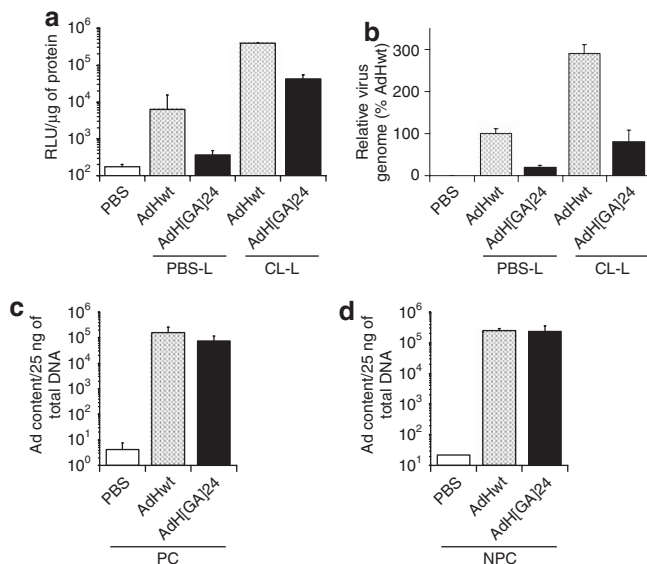


Figure 5 Ad distribution in liver cell subpopulations. **(a, b)** C57BL/6 mice were pretreated with clodronate (CL-L)- or phosphate buffered saline-liposomes (PBS-L) 24 hours before IV injection of 10¹¹ vp of AdHwt or AdH[GA]24 and livers were collected 48 hours later. **(a)** β-Gal activity measured in liver lysates. **(b)** Relative Ad-genome content in total liver DNA expressed as a percentage of that measured in PBS-L pretreated AdHwt-injected mice. **(a)** and **(b)** are representative of two independent experiments, *n* = 4 mice, PBS consisted of PBS-L- and CL-L-pretreated mice (*n* = 2 × 2 mice); means + SD. **(c)** Ad-genome content in liver parenchymal (PC) and **(d)** nonparenchymal cells (NPC) after systemic administration of HVR5-modified Ad. C57BL/6 mice were injected IV with 1,011 vp of AdHwt or AdH[GA]24; collagenase was perfused 30 minutes later to separate PC and NPC. *n* = 4–6 mice; means + SD.

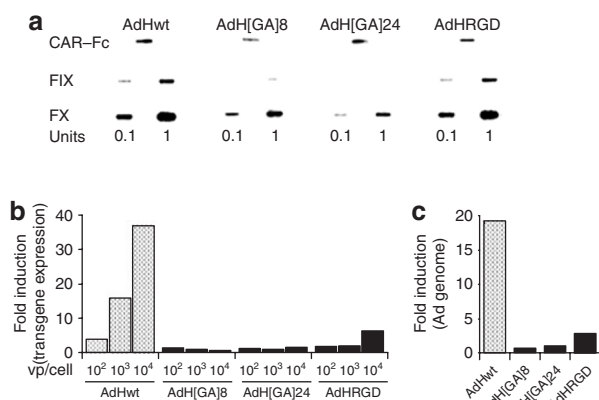


Figure 6 Ad interaction *in vitro* with coagulation factors FIX and FX. **(a)** Binding of AdHwt, AdH[GA]8, AdH[GA]24, or AdHRGD to immobilized CAR-Fc, FIX, or FX. Fold-inductions of **(b)** Ad-mediated transgene expression and **(c)** Ad-genome content, 24 hours and 1 hour after CHO cells infection, respectively, in the presence of physiological levels (1 U/ml) of FX in comparison with transgene expression or genome content in the absence of FX. This figure is representative of two independent experiments.

led to increased transgene expression (Figure 5a) and Ad-genome content (Figure 5b) of AdHwt and AdH[GA]24 compared to PBS-liposomes-injected mice. However, levels remained lower in AdH[GA]24- than AdHwt-injected mice, thereby excluding a major role of KCs in faster liver clearance.

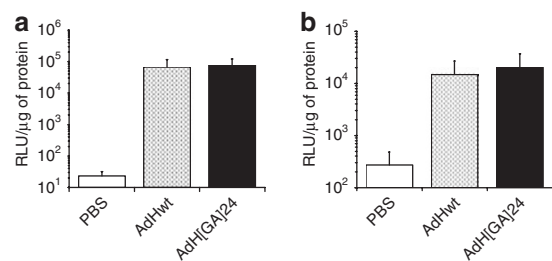


Figure 7 *In vivo* transgene expression in tumors after *in situ* administration of HVR5-modified Ad. PBS or 10¹¹ vp of AdHwt or AdH[GA]24 was injected directly into tumors, which were collected 48 hours later. β-Gal activity measured in lysates of **(a)** Lewis lung carcinoma tumors pre-implanted in nude mice or **(b)** B16F10 tumors pre-implanted in C57BL/6 mice. *n* = 4–7 mice; means + SD. This figure is representative of two independent experiments.

To confirm those observations, the cellular distributions of AdH[GA]24 and AdHwt in liver were examined. Thirty minutes after IV Ad injection, parenchymal cells (PC: hepatocytes) and nonparenchymal cells (NPC: KCs and endothelial cells) were isolated and their DNA extracted. AdHwt- and AdH[GA]24-genome contents did not differ significantly in PC and NPC liver subpopulations (Figure 5c,d), thereby confirming that AdH[GA]24 uptake by NPC was not enhanced.

Hexon HVR5 modification impaired Ad interaction with blood factors

HVR5-modified Ad still interacted with hepatocytes as efficiently as AdHwt, despite their genomes being cleared more rapidly from liver (Figure 4b). Thus, HVR5 substitution might have modified a step between Ad attachment to the cell surface and its transcription in the nucleus. Because some blood factors are known to enhance cell transduction *in vitro* and *in vivo*,^{21–25,29–31} we wondered whether HVR5 modifications could have altered these features. Although all Ad bound to immobilized CAR, only AdHRGD and AdHad2 interacted as efficiently as AdHwt with immobilized coagulation factors FIX and FX (Figure 6a and Supplementary Figure S4). Indeed, the abilities of AdH[GA]8, AdH[GA]24, AdHad19, and AdHad30 to bind FIX were strongly impaired, and their abilities to bind FX were also decreased but to a lesser extent.

We next examined the ability of physiological FIX and FX levels to enhance *in vitro* cell transduction by HVR5-modified Ad. Consistent with previous reports,^{24,25,30} FX coincubation with AdHwt at different multiplicities of infection triggered enhanced transgene expression in CAR-negative CHO cells than without FX. However, coincubation of FX with any HVR5-modified Ad did not significantly enhance transgene expression (Figure 6b and Supplementary Figure S4). Because 1 hour after infection Ad-genome levels were not increased in cells transduced with any HVR5-modified Ad (Figure 6c), the absence of FX-mediated increases in transgene expression was linked to unenhanced Ad entry into CHO cells, thus ruling out an HVR5-modified-Ad degradation. As reported by others,^{21,22,30} FIX coincubation with AdHwt also led to increased transgene expression in CHO cells, but to a lesser extent than with FX. Interestingly, no such enhancement was observed after FIX coincubation with any HVR5-modified Ad

(**Supplementary Figure S5**). Overall, HVR5 substitution strongly impaired the use of either FIX or FX for cell Ad entry.

Efficient transduction of tumors mediated by HVR5-modified Ad

Because HVR5-modified Ad generated inefficient transgene expression in hepatocytes and less hepatotoxicity, they might prove to be valuable tools for gene transfer into tumors. Therefore, we examined the ability of HVR5-modified AdH[GA]24 to achieve gene transfer in two models of subcutaneously implanted tumors, Lewis lung carcinoma and B16F10 melanoma, after direct *in situ* injection. Transgene-expression analyses indicated that AdH[GA]24 was as effective as AdHwt in transducing Lewis lung carcinoma (**Figure 7a**) and B16F10 tumors (**Figure 7b**). Thus, although HVR5-modified Ad had impaired abilities to express transgenes in liver, they retained full capacity to deliver genes into tumors.

DISCUSSION

Massive liver uptake of Ad5-derived vectors and their associated hepatotoxicity have largely hampered their use in systemic injection schemes in clinical trials and still constitute drawbacks to local injections in which leakage can occur. Most of the studies designed to control liver entry through elimination of CAR-, integrin-, and heparan sulfate proteoglycan-binding were unsuccessful.⁵ Recent data highlighted the role of blood factors in liver entry,^{21–25,30} but the mechanism of this facilitation remain to be elucidated. During our manuscript preparation, two independent teams^{29,31} showed that modification of different hexon HVR resulted in dramatically less liver-transgene expression.

Herein, we observed that HVR5 substitution alone with different peptides impaired liver Ad-transgene expression after systemic injection into three mouse strains. Consistent with lower transgene expression, hepatotoxicity (ALT) was dramatically lower. However, other toxicity parameters were unmodified. Of note, similar interleukin-6 production was not surprising because levels of HVR5-modified vectors in the spleen, the major source of interleukin-6 production,^{32,33} were comparable to AdHwt.

In contrast to other capsid modifications, *e.g.*, deletion of the KKTK fiber-shaft motif,^{18,19} HVR5-modified Ad are clearly not disabled. First, HVR5 replacement did not impede vector production. Second, electron microscopy and sodium dodecyl sulfate polyacrylamide gel electrophoresis analyses of virions confirmed their integrity, and heat-inactivation experiments did not reveal any enhanced capsid instability. Third, *in vitro* studies showed comparable AdHwt and HVR5-modified Ad transduction of CAR-positive cells, including primary hepatocytes. Most importantly, like unmodified Ad, HVR5-modified Ad drove efficient transgene expression in tumors. Altogether, our data indicated that HVR5 substitution did not affect hexon role in Ad-genome translocation into the nucleus.³⁴

To analyze the HVR5 substitution effect on Ad behavior *in vivo*, we conducted experiments with AdH[GA]24. HVR5 modification did not significantly change Ad blood clearance or liver uptake at early times. Moreover, KCs depletion led to similarly increased AdHwt- and AdH[GA]24-mediated liver-transgene expression, excluding greater uptake by KCs, as previously described for some

fiber-modified Ad.³⁵ In accordance with these data, 30 minutes after vp injection Ad-genome levels associated with hepatocytes were similar in AdH[GA]24- and AdHwt-injected mice. This observation contradicted the markedly lower transgene expression in hepatocytes 48 hours after injection of any HVR5-modified Ad. The lower ALT release strengthens the hypothesis that, despite their binding to hepatocytes, HVR5-modified Ad were unable to transduce them.

To elucidate the biology of HVR5-modified Ad, we first assessed their interactions with FIX or FX. Some HVR5-modified Ad bound to immobilized FIX and FX as efficiently as AdHwt whereas others did not. However, none of the HVR5-modified Ad was able to use these blood factors to more efficiently transduce CAR-negative cells. Thus, parameters driving Ad binding to FIX or FX are different when these factors are in solution or immobilized. More importantly, HVR5 modification alone was sufficient to disable capsid-protein interactions with both factors. This feature may explain the lower liver-transgene expression triggered by AdH[GA]24, despite its efficient liver uptake at early times. Such discrepancy between transgene expression and early Ad accumulation in liver was previously reported in mice pretreated with warfarin, a drug reducing functional blood-factor levels.^{24,25} Inability to use blood factors could lead to either Ad degradation at the cell surface or impaired intracellular trafficking, but our *in vitro* studies did not support the latter hypothesis.

The inability of HVR5-modified Ad to use FIX or FX makes us wonder which capsid components are involved in Ad-blood factors interactions. As a first hypothesis, HVR5 might interact directly with both blood factors. Alternatively, HVR5 substitution might have modified the whole three-dimensional structure of the Ad capsid, thereby altering the previously described fiber-FIX interaction.²¹ Finally, blood factors might bridge fiber and hexon together to achieve efficient cell transduction. During the preparation of this manuscript, Waddington *et al.*²⁹ and Kalyuzhniy *et al.*³¹ reported a high-affinity interaction between purified hexon and FX. Furthermore, replacement of all Ad5 HVR with those from Ad48, a serotype unable to bind FX, lowered liver transduction *in vivo*.²⁹ Their observations did not preclude FX interaction with other capsid components. Our results using six different HVR5-modified Ad support their findings but also emphasize that HVR5 modification alone sufficed to lower FX- and FIX-mediated Ad entry into cells. Pertinently, replacing the Ad5 HVR5 with that from Ad2, an FX-binding Ad,²⁹ did not rescue the phenotype. Perhaps, FX binding to the hexon requires complex interplay among different serotype-specific HVR.

HVR5 substitution might trigger conformational changes that would abrogate effective binding of blood factors. The particular behavior of only HVR5-modified Ad could be explained by HVR5 proximity to the FX-binding site in the cup of hexon trimers.^{29,31} Further study of previously described HVR1-modified Ad³⁶ or HVR2-modified Ad,³⁷ and mutagenesis of other HVR such as HVR3 and 7, which were proposed to contain FX-binding sites,³¹ should help localize blood factor-binding sites. Finally, hexon and fiber involvement in binding to blood factors did not exclude blood-component interactions with other capsid proteins.

In addition to its roles as a structural protein and in Ad-genome translocation into the nucleus,³⁴ the hexon plays a prominent role in

Ad liver tropism through interactions with blood components. Most importantly, although HVR5-modified Ad yielded less liver-transgene expression and a better toxicity profile, they were still able to transduce very efficiently tumors. Perhaps further control of liver Ad entry can be achieved by combining HVR5 substitution and penton modifications aimed at preventing interactions with native receptors. Moreover, efficacy of HVR5-modified Ad might be further enhanced by incorporating ligands able to redirect them to other receptors, as observed for AdHRGD.¹⁰ However, at least in C3H mice, RGD-peptide incorporation seemed to overcome the inability of blood factors in promoting HVR5-modified Ad entry. Thus, attention must be paid to potential retargeting to liver when choosing targeting peptides. Finally, HVR5 modification could be combined with other retargeting strategies, while retaining lower liver-transgene expression and hepatotoxicity.

MATERIALS AND METHODS

Ad vectors. *LacZ* recombinant control AdHwt (described as AE18 in ref. 10) was derived from Ad5 with E1 and E3 regions deleted. All modified viruses (Table 1) were derived from AdHwt and were constructed using recombinational cloning in *Escherichia coli*.¹⁰ AdF3 (described as DB6 in ref. 11) contains a chimeric fiber, where the Ad5 shaft and knob were replaced with those of Ad3. AdHRGD (described as AE57 in ref. 10) and AdFRGD contain a DCRGDCF peptide inserted into hexon or fiber proteins, respectively. AdFRGD was derived from AdHwt by substitution of G₅₃₈TQETGDTTPS₅₄₈ residues from the fiber-knob HI loop with a DCRGDCF peptide flanked with GSS linkers.

All viruses were obtained using standard procedures as described,¹⁶ and stored at -80°C in PBS-7% glycerol. The concentrations of the viruses were quantified using HPLC.³⁸ Production yields of all viruses were about 50,000 vp/cell and viral particle-to-pfu ratio in the range of 30–50. All experiments were run with at least two independent preparations of each virus except for AdHAD2, AdHAD19, and AdHAD30.

In vivo experiments. All animal experiments were approved by the IGR Institutional Animal Care and Use Committee. C57BL/6, BALB/c, and C3H mice (8–12-week-old females) were purchased from Janvier (Le Genest-St-Isle, France). Viruses (10¹¹ vp) in PBS (200 µl) were injected IV into the retro-orbital plexus; mice were subsequently killed at the indicated times. Livers were harvested and snap-frozen in liquid nitrogen for subsequent determination of transgene expression and Ad-genome content.

For tumor experiments, 8-week-old athymic *nu/nu* from our animal facilities or C57BL/6 mice were injected subcutaneously with Lewis lung carcinoma (2 × 10⁶) or B16F10 (10⁶) cells, diluted in 100 µl of PBS, respectively. When tumor size exceeded 70 mm³, 10¹¹ vp diluted in PBS (50 µl) was injected intratumorally. Tumors were harvested 48 hours later and snap-frozen in liquid nitrogen for subsequent assessment of transgene expression.

Transgene expression in liver (50 mg) or whole tumors lysates was determined by measurement of β-Gal activity as described.¹⁶ Results are expressed as relative light units per microgram of protein.

Real-time Q-PCR. DNA was extracted from liver, blood, or isolated cells using Nucleospin tissue or blood kit (Macherey-Nagel, Hoerd, France) according to the manufacturer's instructions. To quantify the Ad-genome content, duplicate samples of total DNA (25 ng) were subjected to PCR using the ABI Prism 7900HT system and buffers provided by the manufacturer (Perkin-Elmer Applied Biosystems, Foster City, CA). The previously described primers (forward: 5'-CTT CGA TGA TGC CGC AGT G-3' and reverse 5'-GGG CTC AGG TAC TCC GAG G-3') and the fluorogenic probe (5'-FAM-TTA CAT GCA CAT CTC GGG CCA GGA C-TAMRA-3') correspond to the hexon gene.⁶ Amplification was conducted

in a reaction volume of 25 µl under the following conditions: 25 ng of DNA, 1X TaqMan Universal PCR Master Mix (Applied Biosystems), 0.8 µmol/l forward and reverse primers, and 0.2 µmol/l hexon probe. Thermal cycling conditions were: 2 minutes incubation at 50°C, 10 minutes at 95°C, followed by 40 cycles of successive incubations at 95°C for 15 seconds and 60°C for 1 minute with continuous monitoring of the fluorescence. Data were collected and analyzed using the 7900HT Sequence Detection Systems software (Applied Biosystems, Courtabeuf, France). Ad-genome copy number was quantified either using a standard curve consisting of log₁₀ serial dilutions of an AdHwt plasmid backbone, from 10⁷–10² copies on a background of nontreated mouse liver genomic DNA, or following the ΔCT method using 18S (Eukaryotic 18S rRNA, Applied Biosystems) as an external reference. In the latter case, results are expressed as percentages of the Ad-genome level measured in AdHwt-injected mouse liver.

Hematology, cytokines, and transaminases. Blood was collected by retro-orbital puncture at the indicated times after virus inoculation. Hematological parameters were measured in citrated blood using an automated counter calibrated for blood cells (MS9, Schloessing Melet, Cergy-Pontoise, France). Interleukin-6 in mouse sera was quantified using a duo-set enzyme-linked immunosorbent assay, according to the manufacturer's instructions (R&D, Abingdon, UK). Serum ALT level was determined using Enzyline ALT/GPT 20 Kits (bioMérieux, Marcy l'Étoile, France).

Ad blood clearance. Citrated blood samples were collected by retro-orbital bleeding 30 minutes after Ad injection. Total DNA, including Ad genome, was extracted from whole blood and Ad genome was evaluated using real-time Q-PCR as described earlier and expressed as Ad content/25 ng of total DNA. Ad-genome blood concentration at the time of injection (input) was estimated considering a blood volume of 2 ml.

Transient depletion of KCs. Transient depletion of macrophages was realized by IV administration of clodronate (gift from Roche Diagnostics, Mannheim, Germany) encapsulated in liposomes (clodronate-liposomes). PBS encapsulated in liposomes (PBS-L) was used as a control. Liposome complexes (200 µl) were injected 24 hours before Ad injection. Liver sections taken at the time of Ad injection and analyzed by immunolabeling with the monoclonal antibody F4/80 (BD Biosciences, Le Pont-De-Claix, France) confirmed that >90% of the liver macrophages were eliminated (data not shown).

Purification of liver parenchymal and nonparenchymal cells. Thirty minutes after Ad injection, mice were anesthetized by intraperitoneal administration of 10% urethane. Liver cells were separated into parenchymal (PC: hepatocytes) and nonparenchymal cells (NPC: KCs and endothelial cells) using a two-step collagenase retrograde perfusion,³⁹ before mincing the liver, and dispersed cells were separated into PC (pellet) and NPC (supernatant) fractions by differential centrifugation (50 g, 3 minutes). DNA extraction and real-time Q-PCR analysis of Ad-genome content were performed as described earlier.

In vitro Ad binding to coagulation factors. CAR-Fc, a fusion protein consisting of CAR extracellular domain fused to mouse IgG1-Fc, was purified by affinity chromatography on protein A-Sepharose from supernatants of AdCAR-Fc-infected HeLa cells. CAR-Fc (0.1 µg), human FIX, and FX (0.1 and 1 units, Innovative Research, Southfield, MI) were immobilized on a nitrocellulose membrane. After PBS-5% milk-0.05% IgePal (Sigma, France) blockage of free sites on the membrane, the latter was incubated with 10¹¹ vp of different Ad in PBS-0.5% milk-0.05% IgePal, for 4 hours at room temperature. The blot was probed with rabbit polyclonal anti-Ad antibody followed by goat anti-rabbit antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA).

In vitro analysis of coagulation factor-induced transduction. For transgene-expression analyses, CHO-pcDNA cells were infected with

increasing multiplicities of infection of different Ad with or without 1 U/ml of human FX (Innovative Research) in 400 µl of serum-free medium. For Ad-genome quantification, cells were infected at the multiplicity of infection of 10^3 vp/cell without or with FX (1 U/ml) in 400 µl of serum-free medium. In both cases, after 1 hour at 37°C, cells were harvested for real-time Q-PCR analysis or complete medium was added and 24 hours later, β-Gal activity or Ad-genome content was determined.

Statistics. A one-way ANOVA with Fisher's least-significant-difference test was applied.

ACKNOWLEDGMENTS

We are very grateful to Céline Ransy for Ad titrations and to all members of IGR animal facility for technical help. We thank Dmitry M. Shayakhmetov for helpful discussions. The work was funded in part by the Centre National de la Recherche Scientifique, the Association Française pour la Myopathie (AFM), and the Association pour la Recherche sur le Cancer (ARC). F.V., D.D., and B.J. received fellowships from the Ministère de la Recherche et de la Technologie. F.V. received other fellowships from the AFM and the Société Française d'Hématologie, D.D. received another fellowship from Vaincre la Mucoviscidose, and B.J. from the Société Française du Cancer and the ARC. The authors have no competing financial interests to declare.

SUPPLEMENTARY MATERIAL

Figure S1. Transgene expression and Ad-genome content in liver following administration of RGD-containing Ad.

Figure S2. *In vitro* gene transfer by HVR5-modified Ad.

Figure S3. Transgene expression and Ad-genome content in liver following administration of HVR5-modified Ad with sequences derived from other Ad serotypes.

Figure S4. Ad interactions *in vitro* with coagulation factors.

Figure S5. Ad interactions *in vitro* with coagulation factor FIX.

Materials and Methods.

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