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Interactions of adenovirus vectors with blood: Implications for intravascular gene therapy applications

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Despite various obstacles the promise of gene therapy has begun to be realized, as demonstrated by the successful phenotypic correction of X-linked SCID in infants. Although ex vivo gene therapy is advantageous, many diseases, for example, disseminated cancers, require intravascular administration of the gene therapy vector in vivo. In this scenario, the development of sophisticated vectors suitable for targeted intravascular gene delivery is required to both improve efficacy and minimize toxicity. Vectors based on adenovirus (Ad) show immense promise because they are highly efficient in transducing non-dividing cells, can tolerate substantial genetic manipulation (eg, the incorporation of targeting agents), can be produced to high titer, do not integrate into the genome, and have undergone significant investigation in the clinic. However, the use of Ad-based vectors is limited by the inherent hepatic tropism of intravascularly administered Ad, which precludes targeted delivery to alternative organs or disease sites, and by the associated host inflammatory responses to the vector. An improved knowledge of the complex series of interactions is of fundamental importance to the field. This review discusses the current understanding of Ad vector and host interactions, as well as suitable technologies for optimizing delivery to target cells in vivo.

Keywords Adenovirus, blood, coagulation factor, gene therapy, virus

Introduction

The complex interactions between vector and host following intravascular delivery define not only the biodistribution and transduction profiles of the vector, but also the host inflammatory response and the corresponding implications for dose-limiting toxicities. Vectors based on adenovirus (Ad), including those based on the commonly utilized human Ad serotype 5 (Ad5), are highly appealing candidates for intravascular gene delivery as they are extremely efficient in transducing a wide range of cell types (including non-dividing cells), can tolerate substantial genetic manipulation (for example the incorporation of targeting agents), can be produced to high titer, are non-integrating and have undergone significant investigation in the clinic. However, the use of Ad-based vectors is limited by the inherent hepatic tropism of intravascularly administered Ad, which precludes targeted delivery to alternative organs or sites of disease, and by the associated inflammatory responses to the vector [1]. The adverse immunological responses associated with intravascularly administered Ad vectors were demonstrated in 1999 by the tragic death of a young volunteer patient, Jesse Gelsinger. Jesse died from acute respiratory distress syndrome and activation of systemic inflammation, which was complicated by multi-organ failure in response to high-dose Ad (3.8×10^{13} particles) [2,3]. This case highlighted the requirement for improved knowledge of the virological and biological characteristics of Ad infectivity and toxicity *in vivo* following exposure to the bloodstream before vectors based on Ad could be effectively utilized in the clinic.

Attempts to engineer Ad vectors to provide organ- or disease-specific gene expression have shown substantial promise *in vitro*, but have yielded little success *in vivo*. Despite some success in retargeting Ad-mediated gene expression with bispecific antibodies that simultaneously neutralize coxsackie and adenovirus receptor (CAR)-mediated binding while retargeting to alternative receptors (eg, the FAb-9B9 antibody conjugate that retargets via the pulmonary endothelial marker angiotensin-converting enzyme) [4], engineering Ad to incorporate organ- or disease-specific targeting peptides has shown a disappointing inability to retarget gene delivery away from the liver and to its intended site of action [5,6]. It is therefore apparent that Ad utilizes alternative pathways *in vivo* to mediate efficient transduction to the liver. Emerging evidence suggests that interactions between Ad and blood may not only dictate the immunological responses to Ad, but also provide a pivotal role in defining the pattern of infectivity *in vivo* [7,8,9,10] (reviewed in reference [11]). This is perhaps not surprising when the complex nature and composition of blood is considered. Rather than providing an inert substance for Ad vectors to circulate in until they reach their intended site of action, the blood comprises multiple cell types, as well as a plethora of proteins involved in coagulation, immunity and cell signaling. In this review known interactions that occur between Ad and blood following intravascular delivery

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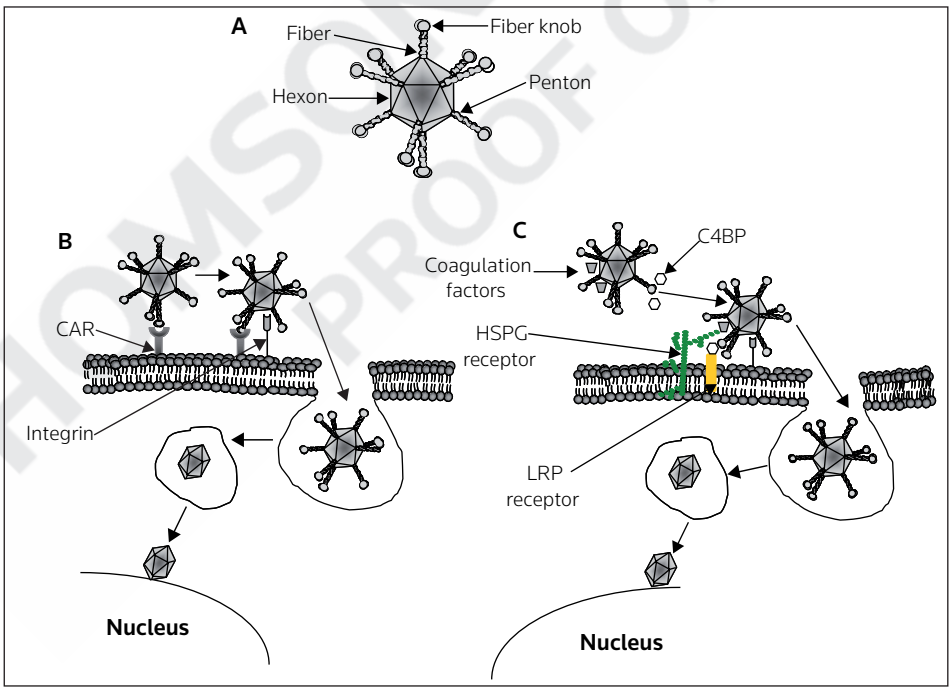
and their effects are discussed. In addition, potential methodologies currently in development that are designed to circumvent such deleterious interactions and that might improve systemic transductional selectivity of Ad-based vectors are discussed.

Hepatocyte transduction *in vivo* is mediated through interactions of adenovirus with blood coagulation factors

The mechanisms utilized by systemic Ad that lead to the efficient transduction of hepatocytes are a controversial area of research. Infectivity, at least *in vitro*, is known to be mediated (for Ad5) via a primary cell tethering interaction between the Ad5 fiber knob domain and CAR [12], followed by a secondary, endocytosis-stimulating interaction between Arg-Gly-Asp (RGD) tri-peptide motifs in the Ad penton-base protein and $\alpha_v\beta_3$ and $\alpha_v\beta_5$ cellular integrins (Figure 1A and 1B) [13]. The expression of CAR is limited to tight junctions [14], which are continuous circumferential intracellular contacts at the apical poles of lateral cell membranes that function as a barrier to regulate the transit of water, solutes and immune cells across an epithelium and serve to establish cell polarity by separating the apical and basolateral domains of polarized endothelial cells. As tight junctions are found ubiquitously in all organs, the considerable levels of hepatocyte transduction observed following intravascular delivery of Ad5 is challenging to reconcile with the expression of CAR alone. Furthermore, the introduction of point mutations in the Ad5 fiber knob domain to abrogate CAR binding (eg, the KO1 mutation [15]) has a profound impact on transduction efficiency *in vitro* [16], but no apparent effect on levels of liver transduction when introduced systemically *in vivo* [15-19]. These results have fuelled additional research into non-CAR-mediated pathways of cell entry *in vivo* following intravascular Ad5 administration.

Cellular heparan sulfate proteoglycans (HSPGs) are implicated as potential alternative receptors to facilitate Ad-mediated cell entry *in vitro* [20]; studies have demonstrated that the S* mutation (mutation of the Lys-Lys-Thr-Lys motif in the Ad5 fiber to either Gly-Ala-Gly-Ala or Gly-Ala-Thr-Lys) of a putative heparin binding motif in the Ad fiber shaft led to substantially decreased transduction of hepatocytes *in vivo* in mice, rats and non-human primates, suggesting that the critical determinant in hepatic detargeting *in vivo* requires the ablation of cellular HSPG and Ad interactions [18,19]. However, retargeting Ads to alternative receptors by the incorporation of targeting peptides with this mutation has proved futile [21,22]. Data suggest that the S* mutation, which is located proximal to a pivotal hinge region in

Figure 1. Adenovirus serotype 5 infection.



(**A**) The icosahedral adenovirus (Ad) capsid is composed of three main proteins: the trimeric fiber protein with a globular knob domain that protrudes from the capsid surface at the 12 vertices of the virus, the pentameric penton base protein at the base of the fiber shaft, and the trimeric hexon protein that makes up the remaining 240 capsid proteins. (**B**) *In vitro* infection of cells is mediated via an interaction between the Ad fiber knob domain and the coxsackie Ad receptor (**CAR**), which tethers the virus to the cell surface [12]. The flexible fiber shaft enables the penton base at the bottom of the fiber shaft to bind and activate $\alpha_v\beta_{3/5}$ integrins and leads to internalization of the virus [13]. (**C**) *In vivo* infection following intravascular delivery of Ad is mediated via the interaction of proteins in the blood, such as complement component C4-binding protein (**C4BP**) and Factor (F)IX [9] or FX [8], with the virus capsid and subsequent bridging to LDL receptor-related protein (**LRP**) or heparan sulfate proteoglycans (**HSPGs**) on the hepatocyte surface.

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the fiber shaft, may render the fiber inflexible and lead to a lack of virion infectivity at either the internalization or intracellular trafficking steps [22].

Shayakhmetov *et al* were the first to demonstrate interactions between plasma components and Ad that potentially act as molecular 'bridges' to retarget the virus to alternative receptors [9]. Their study identified the association of several proteins, notably coagulation Factor (F)IX and the complement component C4-binding protein, but not FX, to the Ad5 and Ad35 fiber knob domain; these interactions might provide the bridge between Ad and cellular HSPG and LDL receptor-related protein receptors, respectively (Figure 1C). Their study also developed a significant new methodology to study transduction of hepatocytes in the absence of blood [9]. An *in situ* perfusion procedure was used to remove blood prior to delivery of Ad via the hepatic artery and in the presence or absence of other factors that may enhance hepatic uptake. Using this system, they were able to demonstrate enhanced levels of transduction in the presence of FIX, thereby confirming the potential involvement of FIX in hepatocyte transduction [9]. Subsequently, the homologous vitamin K-dependent serine proteases FVII, FIX, FX and protein C were all shown to be capable of interacting with the Ad capsid *in vitro*, thereby enhancing gene delivery to hepatocytes through interactions with cellular HSPGs [8]. These coagulation factors arose by gene duplication and, therefore, share a defined conserved structure comprising γ-carboxyglutamic acid (Gla)-EGF1-EGF2-serine protease domains. Surface plasmon resonance (SPR) was used to detect a calcium-dependent interaction between FX and Ad5 [8]. In addition, an important new *in vivo* methodology was developed for depleting Gla-containing coagulation zymogens using warfarin (a widely utilized anticoagulant drug that prevents maturation and secretion of the vitamin K-dependent coagulation zymogens by blocking γ-carboxylation) to examine the effects of these proteins on hepatic transduction [8]. Hepatic transduction mediated by infusion of CAR-binding ablated virus (AdKO1) was dramatically reduced in warfarin-treated mice (> 300-fold reduction) compared with control (untreated) mice [8]. Crucially, hepatic transduction could be 'rescued' in warfarin-treated mice to levels identical to untreated mice by injection of physiological levels of human FX prior to virus delivery, suggesting a pivotal role for virus:FX interactions in mediating hepatocyte transduction *in vivo* [8]. Furthermore, identical effects were demonstrated with Ad5 vectors capable of binding CAR, which confirm the lack of CAR-mediated hepatocyte transduction *in vivo* and underline the critical role for coagulation zymogens in mediating liver infectivity [10]. Additionally, hepatocyte transduction of Ad5-based vectors pseudotyped with fibers from species D Ads (ie, Ad5 capsids possessing fibers [f] derived from alternate serotypes) were greatly enhanced by the presence of FX *in vitro* [7] and (at least for Ad5/f47) hepatocyte infectivity was dependent on the presence of functional coagulation zymogens *in vivo* [10].

Using a combination of cryo-electron microscopy-based reconstruction of Ad5 in complex with FX and SPR in a series of Ad mutants and Ad proteins in isolation, Waddington *et al* observed high-affinity binding of the FX Gla domain to the hypervariable regions (HVRs) of the Ad5 hexon protein at a stoichiometry of one FX molecule per hexon trimer (Figure 2) [23]. This observation was not anticipated because the hexon protein was considered to play a purely structural role in the Ad capsid, and the fiber knob domain was pivotal for coagulation factor binding [9]. Replacement of the HVRs of the Ad5 hexon with those of Ad48 (which did not bind to FX) ablated FX-binding as measured by SPR, suggesting that the HVRs are key to coagulation factor binding [23]. This vector, Ad5HVR48, was devoid of FX-enhanced transduction of cell lines *in vitro* and showed greatly reduced liver transduction when introduced intravascularly in mice [23]. Furthermore, a potent biological inhibitor of the Ad5 hexon:FX interaction was described that could be utilized to inhibit gene transfer, both *in vitro* and *in vivo* [23]. FX binding protein (X-bp), a 29-kDa protein isolated from the venom of the snake *Deinagkistrodon acutus* (commonly known as the "100 pace snake") was observed to bind with high affinity (0.4 nm) to the FX Gla domain [24]. By pre-injecting untreated mice with X-bp prior to administering Ad5 intravascularly, X-bp substantially reduced Ad5-mediated liver gene transfer [23]. Additionally, both nematode anticoagulant protein c2 [25], an 85-amino-acid polypeptide, and ixolaris, a 12 kDa two-Kunitz domain protein isolated from *Ixodes scapularis* (tick) salivary glands [26], inhibited FX-mediated liver gene transfer, both *in vitro* and *in vivo*, by interfering with the FX serine protease domain and cellular receptor interactions [23]. Kalyuzhniy *et al* also observed similar effects [27]. Utilizing an Ad5 vector that contained a large biotin-acceptor-peptide insertion within the HVR5 of the Ad5 hexon (Ad5BAP), SPR analysis demonstrated that Ad5BAP was unable to bind to FX, with a corresponding reduction in hepatic gene transfer observed following systemic administration [27]. Extensive SPR on a variety of Ad proteins and coagulation factors demonstrated that FX bound to the Ad5 hexon protein with picomolar affinity and that this interaction was fundamental to liver gene transfer *in vivo* [27]. The fiber knob:FIX interaction, which had been observed previously [9], was not detectable, suggesting this interaction was not important for hepatic gene transfer *in vivo* [27]. Vigant *et al* described a number of Ad vectors, each mutated within the hexon HVR5 and which all reduced hepatic gene transfer (to greater or lesser extents) when introduced systemically [28]. This study also demonstrated that these mutations reduced hepatic gene transfer because of abrogated interactions with blood coagulation factors [28]. These studies together represent a paradigm shift in Ad targeting, since it had previously been assumed that the hexon protein played only a structural role in the Ad capsid, while tropism was dictated through fiber and penton interactions. It is clear that to achieve specific targeting following systemic delivery, ablation of FX binding through site-directed mutagenesis is required in addition to the incorporation of elements to Ads to retarget the vector to sites of disease.

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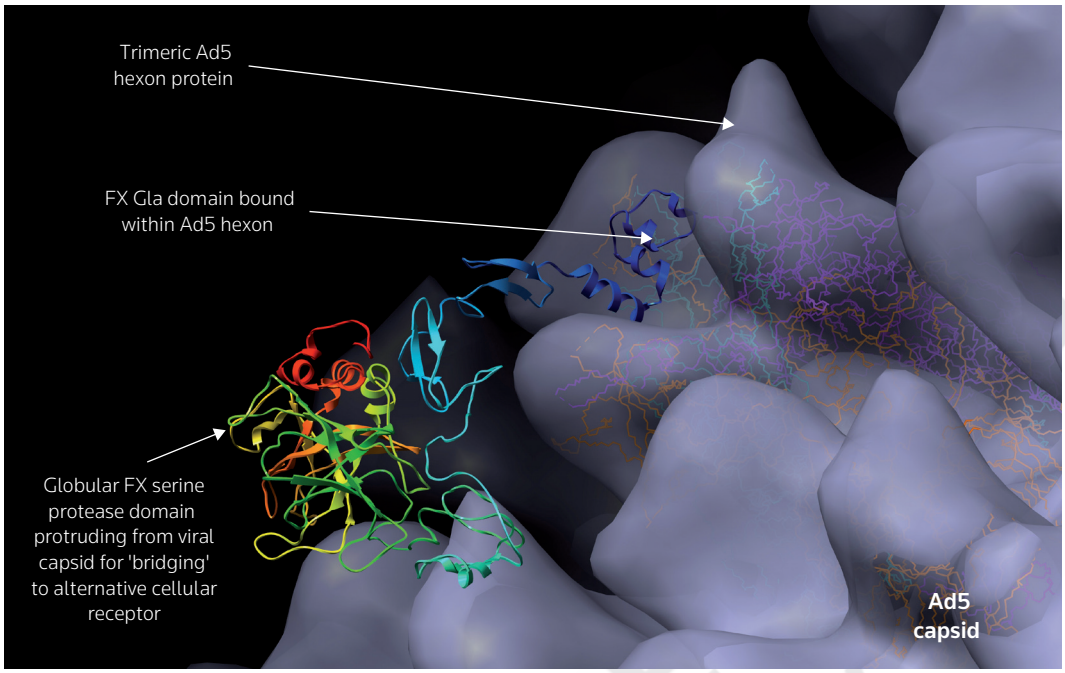
Figure 2. The adenovirus serotype 5 hexon:Factor X interaction.



Computational reconstruction depicting the high-affinity interaction between the Factor (**F**)X molecule and the adenovirus serotype 5 (**Ad5**) hexon molecule. The FX γ-carboxyglutamic acid (**Gla**) domain interacts with the hypervariable regions of the trimeric Ad5 hexon protein at a stoichiometry of one FX molecule per hexon trimer. The Gla domain locates within the trimeric hexon 'cup', with the globular serine protease domain protruding out. The interaction of the Ad5 hexon:FX complex with cellular receptors is mediated through an exosite within the serine protease domain of FX, which can be blocked using either nematode anticoagulant protein C2 or ixolaris.

In addition to Ad interactions with blood coagulation factors, complement component C3 has also been implicated in hepatic transduction [29]. The association of C3 with the Ad5 capsid primes the virion for Kupffer cell-mediated degradation (see following section) [30]. Intriguingly, however, studies in C3 knockout mice suggest that liver transduction was reduced 99-fold compared with wild-type mice [29], although this effect was only apparent at low viral doses and has not been reproduced at higher doses [31].

Other interactions between adenovirus and blood

In addition to Ad interactions with coagulation factors and complement-related proteins, there is significant evidence suggesting that Ads are able to interact directly with blood cells. Lyons *et al* demonstrated that in *ex vivo* culture > 90% of administered Ad vector bound directly to human erythrocytes, thereby restricting Ad bioavailability and reducing the transduction of target cells (Figure 3) [32]. Interestingly, this effect was not observed when murine blood was used in the *ex vivo* model, suggesting a species-specific interaction of Ad with erythrocytes. Moreover, these data correlate with data from a study by Nicol *et al*, in which Ad was observed to stimulate hemagglutination of human and rat red blood cells, but not those of mice [18]. A reduction in blood platelet counts that leads to thrombocytopenia is a side effect following intravascular administration of Ad [33]. Stone *et al* suggested that a direct interaction between Ad5 and circulating platelets led to uptake of Ad5:platelet complex within liver sinusoids with subsequent degradation via Kupffer cells [34]. The transduction profiles and immunological responses to a range of Ad serotypes from species B, C, E and F were evaluated and, while levels of toxicity did not appear to correlate directly with the degree of sequestration in the lung, liver and spleen, all serotypes activated coagulation, which was potentially mediated through an interaction with platelets [34]. Othman *et al* proposed that the Ad:platelet interaction could be primed by an interaction of Ad5 with von Willebrand factor or P-selectin, or both, and co-recruitment of leukocytes, and observed that thrombocytopenia was markedly reduced in von Willebrand factor knockout mice [35].

In addition to interactions involving red blood cells, Cotter *et al* demonstrated that Ad5 particles can be efficiently internalized by neutrophils [36]. Furthermore, this interaction occurred independently of both CAR- and integrin-mediated pathways, and provided convincing evidence for uptake via complement receptor 1 and fragment crystallizable γ receptors.

Additional knowledge on the mechanistic basis of these interactions is required to develop vectors with improved pharmacokinetics and safety profiles *in vivo*.

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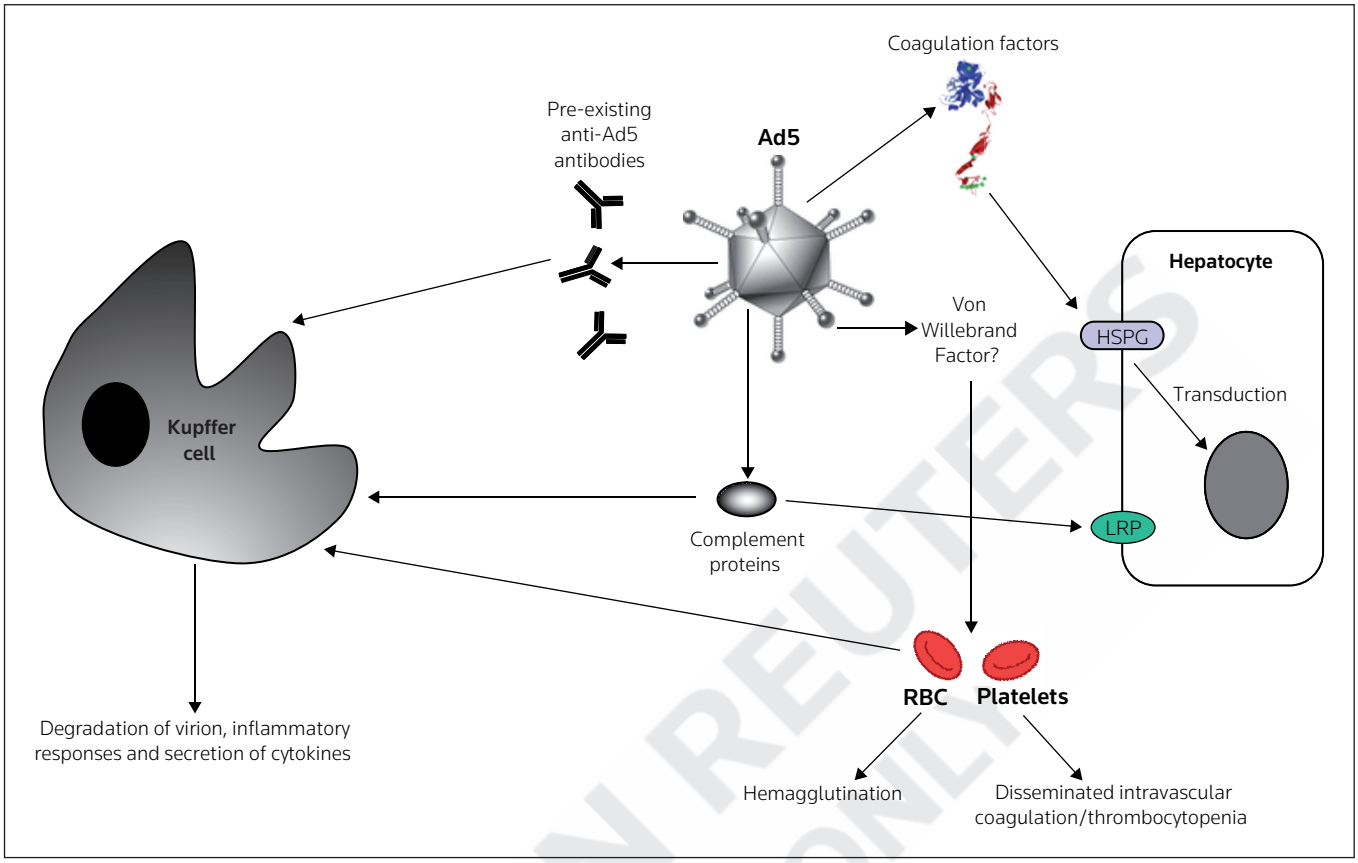
Figure 3. Adenovirus-host interactions.



Once in contact with the bloodstream, adenovirus (**Ad**) serotype 5 interacts with various proteins and cells. Binding of pre-existing neutralizing antibodies can lead to opsonization and uptake of the virus by components of the reticuloendothelial system, for example, Kupffer cells, which eliminate the virus from the circulation as well as activating the inflammatory response. Interaction with complement proteins, for example, complement component C4-binding protein [9] or vitamin K-dependent coagulation Factor (F)IX [9] or FX [8] can lead to transduction of liver hepatocytes. Ad can directly interact with blood cells, such as red blood cells (**RBCs**) to initiate hemagglutination or platelets to stimulate thrombocytopenia or disseminated intravascular coagulation. **HSPG** heparan sulfate proteoglycan, **LRP** LDL receptor-related protein.

Immunological responses to adenovirus vectors

Intravascular delivery of Ad elicits both a primary innate immunological response to the vector, characterized by rapid increases in levels of serum cytokines and chemokines, and subsequent adaptive immunological responses that restrict repeated administration of the vector. While adenoviruses have evolved sophisticated strategies for overcoming cell-mediated adaptive immune responses to infection, systemic administration is often limited by innate inflammatory responses. This is reflected in the death of Jesse Gelsinger in 1999, which was directly attributed to innate immune responses initiated by high-dose Ad [3]. Molecular epitopes on the Ad capsid are recognized by macrophages and dendritic cells, the key cells of the reticuloendothelial system (RES), and trigger innate immune responses. These interactions have been visualized in mice [37,38], rats [18] and non-human primates [39], and lead to a rapid accumulation of Ad virions within hepatic macrophages (Kupffer cells), which represents the primary mechanism for Ad removal from the circulation [37].

The spleen is also responsible for clearing smaller quantities of vector, where the major site of uptake is in marginal zone phagocytes [38,39]. In some species, for example, in pigs [40,41] or animals with liver cirrhosis [42], where there are increased levels of pulmonary intravascular macrophages, vector clearance can be skewed toward the lung. Uptake via the RES leads to rapid virion degradation and minimal transduction and limits the bioavailability of the vector for systemic targeting [43]. The removal of vector from the circulation by the RES is extremely rapid and attempts to quantify the rate of removal have suggested up to 99% of virions can be removed from the circulation within 3 min of administration [44].

Uptake into Kupffer cells activates intracellular signaling pathways, such as NFκB and mitogen-activated protein kinase, which activate downstream signaling pathways

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and secretion of proinflammatory chemokines and cytokines (eg, including IL-1β, IL-6, IL-8 and IL-12, TNFα, RANTES, IFN-inducible protein 10, macrophage inflammatory protein [MIP]-1β and MIP-2 [38,43,45,46]). Upregulation of each of these cytokines occurs with markedly differing kinetics; serum concentrations of early markers, such as IL-1β and MIP-2, are maximized at 30 min post-infusion of Ad, while serum levels of IL-6 and TNFα peak 6 h post-infusion [47]. High levels of circulating alanine aminotransferase could also be detected in the blood 24 h after infusion of Ad, reflecting significant levels of systemic toxicity following intravascular administration [47]. Inflammatory responses to intravascularly administered Ad were significantly reduced in animals deficient for IL-1, suggesting that IL-1 is critical in initiating the anti-Ad host responses, and that signaling through IL-1RI is essential for establishing the inflammatory responses that cause the early hepatotoxic side effects following intravenous administration of Ad5 vector [47].

The association of complement proteins to Ad also contributes to Kupffer cell-mediated Ad clearance; for example, the complement protein C3b covalently coats pathogen surfaces and targets them for opsonization and degradation by macrophages [30]. C3 knockout mice show reduced activation of inflammatory markers following intravascular administration of Ad, including keratinocyte-derived chemokine, IL-5, G-CSF, GM-CSF and IL-6 [31]. Interestingly, levels of IFNγ, IL-1β, IL-12 and RANTES were unaffected, suggesting that multiple pathways may be responsible for eliciting the innate immune responses [31]. Levels of liver transduction were unaffected by C3 knockout status, suggesting that divergent pathways are responsible for mediating Kupffer cell-mediated clearance and transduction of hepatocytes [31].

In addition to the potent cytokine-mediated responses apparent following intravascular administration of Ad5, severe adverse effects on cardiovascular function have also been observed in several studies during the acute phase following administration. A study by Machemer *et al* characterized these adverse outcomes [48]. Telemetric cardiovascular monitoring demonstrated that intravascular infusion of Ad5 into BALB/c mice led to a blockade in the SA node 3 to 4 min following infusion, followed by secondary pacemaking initiated at the AV node. In addition, associated acute bradycardia, reduced blood pressure, and hypothermia were observed [48]. Such adverse events were not apparent in the presence of Ad-primed murine sera or following Kupffer cell depletion, suggesting that Ad5 interactions with cells of the RES play a pivotal role in the induction of cardiovascular responses to Ad5 as well as cytokine responses to the vector [48].

Intravascular delivery of Ad vectors also activates activated endothelial cells, as detected by enhanced expression of endothelial nitric oxide synthase, phosphorylated protein kinase B and nitrotyrosine [49]. Endothelial cell activation by the interaction of virions with Kupffer cells was dependent upon RGD penton motifs [49], and led to widespread injury to the vascular endothelium of liver sinusoids [50].

Methods for circumventing detrimental adenovirus interactions in the blood

The development of methodologies to extend the circulatory half-life of Ad by interfering with its interaction with the RES has been the focus of considerable research (Figure 4). A potential method involves transiently depleting Kupffer cells *in situ*, either by predosing with a microbial agent (eg, a virus [43]) or with a chemical treatment (eg, gadolinium chloride [51] or liposomes containing dichloro-methylene-bisphosphate [clodronic acid] [38]). Depletion of Kupffer cells transiently depletes macrophages, providing a window of opportunity for Ad delivery before macrophage repopulation in the liver [38]. Transient depletion of Kupffer cells increased hepatic transduction of Ad and reduced proinflammatory cytokine secretion, suggesting that Ad-mediated transduction of hepatocytes is independent of the RES [38]. However, while these agents transiently deplete macrophages, their clinical use as part of a gene therapy protocol is far from ideal because of their potential toxicity.

Studies have therefore focused on the chemical modification of the Ad capsid to mask immunogenic epitopes and prevent uptake into Kupffer cells. Research has centered on the monovalent hydrophilic polymer PEG to modify the Ad capsid through covalent linkage to reactive surface amines [52]. Viruses modified with PEG or its derivatives showed decreased innate immunity [53] and antibody association [54]. PEG also offers the possibility to couple alternative targeting ligands to Ad that modify viral tropism to successfully retarget gene transfer to EGF receptors [55], FGF receptors [56] and integrins [57]. An alternative polymer for covalent modification of Ad vectors is poly *N*-(2-hydroxypropyl) methacrylamide (pHPMA) [58]. Because this polymer is multivalent, each molecule reacts with multiple amino groups on the Ad capsid to produce a molecular 'cage' or 'cloak' cross-linked across the surface of the Ad capsid [59]. This cage provides 'lateral stabilization' in addition to the 'steric stabilization' conferred by PEGylation [59]. This hydrophilic shielding prevents the association of neutralizing antibodies or other bloodborne factors that might facilitate Kupffer cell-mediated virion degradation [60,61].

Adenoviruses covalently modified by pHPMA have demonstrated several advantageous and pharmacologically relevant outcomes when administered intravenously, including decreased antibody recognition [60], prolonged circulatory half-life [61] and decreased liver damage, as monitored by the circulatory levels of liver enzymes [61]. To provide an alternative means of cell entry, Ads were retargeted with a variety of alternative cell-targeting ligands, including growth factors, for example, basic FGF [60], galactose and mannose [62],

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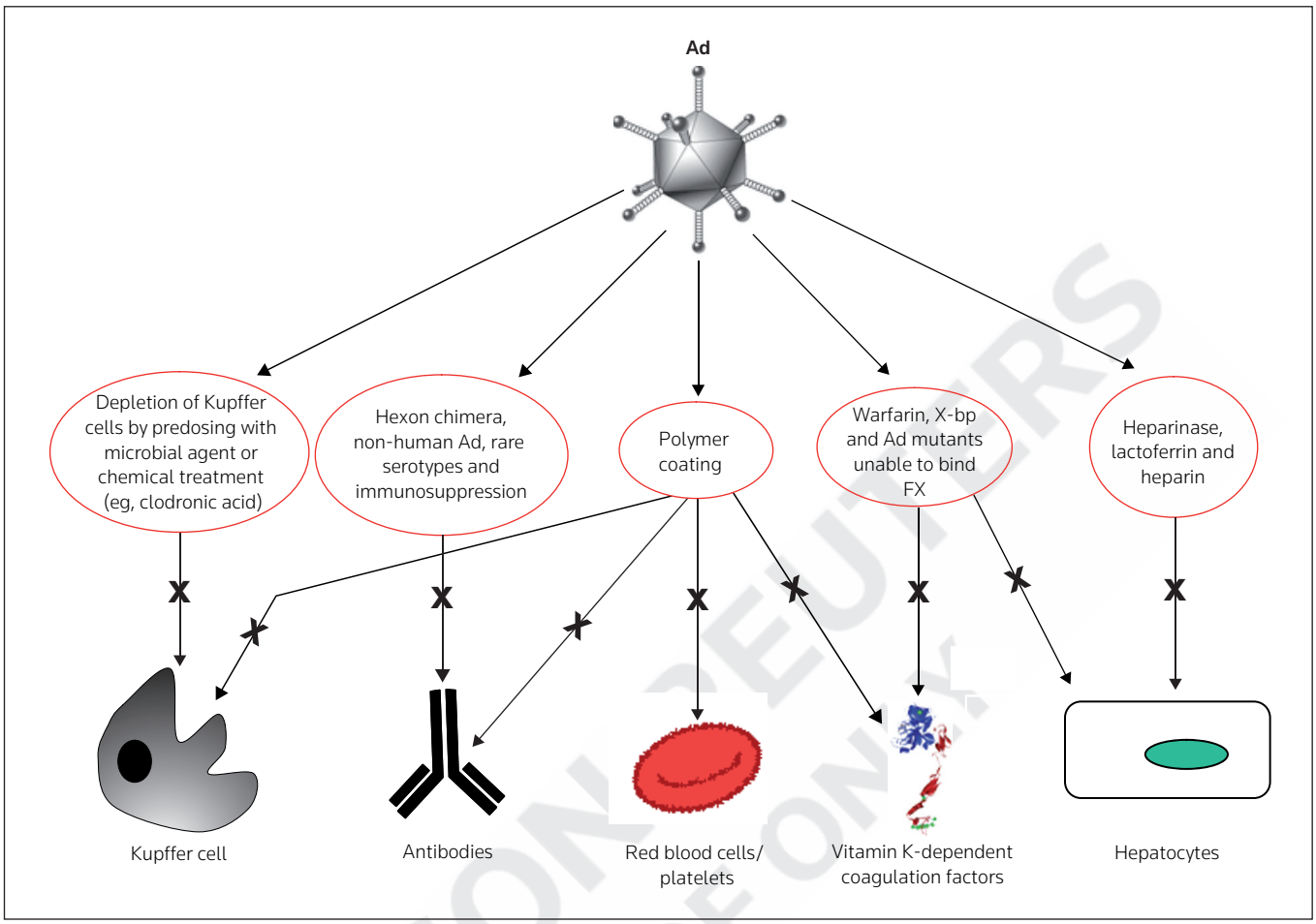
Figure 4. Strategies to avoid adenovirus:host interactions.



Various strategies to reduce virus:host interactions have been investigated and include: depletion of Kupffer cells by either predosing with a microbial agent (eg, a virus [43]) or with a chemical treatment (eg, gadolinium chloride [51] or liposomes containing dichloro-methylene-bisphosphate [clodronic acid] [38]) to transiently deplete Kupffer cells; the use of rare or non-human adenoviral (**Ad**) serotypes instead of Ad5, for example, chimeric Ads; and the use of virus capsid polymer coating or immunosuppression, which can alleviate Ad neutralization via antibodies. Polymer coating can also avoid interactions with blood cells such as erythrocytes or platelets. Warfarin-mediated depletion of vitamin K-dependent coagulation factors or biological compounds, such as Factor (**F**)X binding protein (**X-bp**), blocks the FX interaction with Ad and consequently transduction of hepatocytes. Heparan sulfate proteoglycans or LDL receptor-related protein blocking agents can also prevent Ad hepatocyte delivery.

and peptides [63,64]. Such vectors have shown considerable promise for retargeting gene delivery to alternative receptors *in vitro* [60]. Efficacious retargeting following intraperitoneal delivery of Ad5 has been demonstrated via this strategy using murine EGF as a targeting ligand [65]. To date this technology has not successfully been utilized to retarget Ad5 gene expression via the intravascular route, but the development of optimized targeting ligands may ultimately enable targeting via this route. Continued advances in polymer chemistry and more powerful peptide or antibody targeting strategies are necessary before such technologies can be utilized clinically.

In addition to the use of hydrophilic polymers to evade neutralizing antibodies and affect biodistribution *in vivo*, biodegradable lipids have been utilized to encapsulate the vector [66]. Although such technologies show promise in their capacity to evade neutralizing antibodies and correspondingly demonstrate reduced vector-specific immunological responses [67], the formulations are often polydispersed and too large (5 to 10 μm) to evade capture by the RES.

More than 50 human Ad serotypes have been isolated to date and some rarely isolated alternative Ad serotypes have been utilized to overcome pre-existing immunity to the vector [68]. Ads derived from species B have undergone considerable development, especially for cancer gene therapy [69]. Several serotypes from this species utilize the complement-related receptor CD46 as an attachment receptor [70], which is upregulated within the tumor endothelium [71].

Since the majority of neutralizing antibodies to Ad are directed against the major capsid protein, hexon [72], antibody evasion can also be achieved by modulation of

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HVRs in the Ad5 hexon. The HVRs represent the molecular epitopes most exposed on the outer surface of the Ad capsid and therefore represent the major site of antibody neutralization [73]. The HVRs are markedly different between each of the 51 isolated serotypes of Ad and their differing structures are the major contributing factor for the limited cross-reactivity observed for antibodies obtained from patients previously exposed to Ad [72]. Using the HVRs from the rarely isolated serotype Ad48 engineered into the Ad5 hexon, Roberts *et al* demonstrated that the resulting particles showed significantly reduced immunogenicity in mice and primates pre-immunized with Ad5 [74]. Therefore, viral vectors can be engineered to overcome pre-existing anti-vector immunity by modification of immunodominant epitopes on the capsid surface. The development of adenovirus serotypes from non-human species provides a strategy to circumvent anti-vector immunity, and Ads of canine [75], bovine [76], ovine, [77] porcine [78] and chimpanzee [79] origins are under development for gene therapy applications (reviewed in reference [80]). While such serotypes exhibit decreased immunological recognition, levels of transgene expression are often markedly reduced compared with human Ads. This may be because of the incomplete knowledge of structural/genomic components of these vectors and/or the lack of high titer producer cell lines in comparison with the highly characterized human Ad5 vector.

Conclusion

The mechanism(s) that govern adenovirus infectivity *in vivo* remain poorly understood, especially following exposure of the virus to the bloodstream. Complex interactions between Ads and blood cells and plasma proteins are critical to viral transduction in the liver and spleen. Therefore, additional research into Ad bloodstream interactions for Ad5, as well as alternate human and non-human Ads, is expected to improve our knowledge of viral and host interactions as well as the development of viruses for human gene therapy.

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