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Barriers to systemic application of virus-based vectors in gene therapy: lessons from adenovirus type 5

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Abstract Currently, virus-based vectors, namely derivatives of the adenovirus, are frequently used in a wide variety of ex vivo or local gene therapeutic applications. However, the efficacy of virus-based vectors in systemic applications is presently still extremely limited. Complex interactions of the various vector types with the patient's organism hinder successful vector deployment. Exemplary, here we summarize barriers to systemic application of Adenovirus-based vectors leading either to acute toxic effects or rapid vector neutralization and discuss strategies to overcome these barriers aiming to develop more efficient vector types.

Keywords Adenovirus · Virus-based vector · Vector-host interaction · Vector neutralization · Shielding

Introduction

During the last decades major progress has been achieved in the development of various safe and efficient virus-based vector systems for a wide variety of therapeutic applications ranging from the treatment of genetic diseases and malignant tumors to disease prevention by genetic vaccination. Unfortunately, however, despite the admission and availability of a few drugs based on nucleic acids in the Western hemisphere, up to date the clinical success of virus-based gene transfer vectors in humans has clearly been limited.

Clinical successes have mainly been achieved in ex vivo treatment strategies, which transduce patient-derived cells in culture dishes and, thus, circumvent direct contact between virus vector and patient (for example [1–4]). In addition, local in vivo application of vectors in immune privileged tissues was successful (for example [5–7]).

While by now virus-based vectors have proven to show efficient and safe transduction of various cell types in many ex vivo or in local treatments, the use of such vectors for systemic delivery is still unsatisfactory. Of note, the treatment of a wide variety of diseases affecting large organ systems like skeletal muscle or liver and the treatment of solid tumors and disseminated metastases requires vector delivery through the blood stream. Various mechanisms prevent pathogens to invade the human organism and impose barriers severely hindering systemic, clinical use of virus-based vectors. The human body's defense mechanisms often trigger acute toxicity, a significant problem for successful in vivo gene delivery. The acute toxicity narrows the therapeutic window of the vectors and the underlying mechanisms such as opsonization, scavenging and mistargeting mediate unwanted vector sequestration. Only in genetic vaccination, immunogenicity of the vector and involvement of immune cells is favorable to some extent, as co-stimulant of immune responses to the specific antigen product after vaccination.

Virus-based vector systems rely on virus surface proteins that bind to cell surface receptors and often trigger uptake and cellular reprogramming for intracellular transport. In systemic applications, these virus proteins often identify the vector as foreign substance to its host organism, leading to rapid clearance of the vector particles from

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the patient's blood system. To increase chances of vector particles to reach their target cells, vectors therefore need to be engineered to either circumvent the host's barriers or to be shielded against attacks of the host's defense mechanisms prior to systemic delivery.

Furthermore, while natural viruses exhibit a more or less specific tropism for defined cells and tissues, the vectors derived from them often need to be targeted to different cells for the treatment of genetic or malignant diseases. Therefore, the virus-specific tropism has to be ablated and replaced by new functions ensuring highly specific and efficient delivery.

Overall, to increase efficiency of the different virus-based vectors in systemic applications, it is paramount to study the complex molecular interactions of each vector type with the host's organism in great detail. This includes but is not limited to the analysis of very early interactions between vector and various blood components, tissue penetration mechanisms and intracellular trafficking of vectors in the target cell. As paradigm for the complexity of early interactions of virus-based vectors within a patient's organism, we summarize here the current knowledge on barriers to adenovirus (Ad)-based vectors in systemic applications. Ad vectors are the most commonly used vectors in gene therapeutic applications (see http://www.abedia.com/wiley/vectors.php) and human adenovirus type 5, concerning its interactions with host blood and tissues, is probably the best described vector system up to date. Understanding of barriers imposed by Ad5-host interactions, and in particular the understanding of early interactions between vector and blood, can certainly contribute to engineer safe and efficacious virus-derived vectors in general.

Barriers

Upon injection of Ad5 into the blood system, germline encoded natural IgM antibodies of the innate immune system can recognize and bind to highly repetitive structures of the virus surface [8, 9]. Subsequently, the immune complexes of antibodies and the virions activate both the classical and non-classical pathway of the complement system. Thus, the opsonization of the virions leads to rapid complement-mediated neutralization [8]. For Ad5 it has been shown that after intravenous delivery, macrophages, in particular Kupffer cells residing in the liver, rapidly remove a very large portion of the injected virions from the blood system [10]. Scavenger receptors have been identified, which are involved in the phagocytic uptake of Ad5 vectors into these cells [11]. This sequestration mechanism is mainly responsible for the very low efficacy of Ad-based vectors in systemic applications [12]. Importantly, the interaction of the virions with macrophages in blood, spleen, and liver is associated with acute toxic and hemodynamic side effects [13, 14]. In addition to macrophage cells, liver sinusoidal endothelial cells (LSECs) [15] express specific scavenger receptors and appear to contribute to vector elimination [16]. The extent, however, to which this mechanism contributes to vector elimination, is still under evaluation.

A further hurdle in systemic application of Ad-based vectors is the ability of several adenoviruses to efficiently bind to human erythrocytes [17] either via the Coxsackie and Adenovirus Receptor (CAR) or the complement receptor CR1 which both are expressed on the surface of human erythrocytes [18]. This molecular interaction leads to a very efficient sequestration of Ad-based vectors and needs to be prevented to enable delivery through the blood. Interestingly, this is a paradigm example for the importance to study virus-host interactions in appropriate model systems: mouse erythrocytes do not express CAR and studies with mouse blood consistently fail to predict the human situation. It has to be kept in mind that the mouse is a suitable model organism to study important aspects of vector delivery, yet can only be used to answer a limited set of questions. All neutralization pathways described so far, work independent of specific antibodies and therefore independent of prior exposure to Ad antigens.

A new barrier to successful systemic application of virus-based vectors is raised after the first vector delivery or already exists due to previous infection with the natural virus: anti-vector antibodies generated by the adaptive immune system [19, 20]. Preexisting antibodies against adenovirus 5 are carried by a large percentage of the world's population [21, 22]. In sub-saharan Africa, e.g., up to 90% of the population is seropositive for Ad5. In addition, it is likely that most applications of adenovirus-based vectors will require repeated vector delivery, and, therefore, robust strategies to escape from Ad-specific antibodies need to be developed.

Surprisingly, the mechanisms establishing the tropism of Ad5 are only partially understood up to date. It has been known since long that Ad5 exhibits a strong hepatocyte tropism in various model organisms, but only in 2008 one important molecular determinant for this hepatotropism has been revealed. It is based on a high affinity interaction of the virus particle with blood coagulation factor X (FX) [23–25]. FX interacts with the hexon protein of Ad5 and bridges the virus to heparin sulfate proteoglycans (HSPGs) [23, 26–28]. HSPGs in fact are expressed on many different cell types, but the extent of N- or O-sulfation on HSPGs in the liver seems to be decisive for FX-mediated hepatocyte transduction [27]. In this scenario no participation of the primary CAR receptor is involved in transduction of hepatocytes. Additionally, recent results hint to further

unknown pathways, independent of FX or CAR interaction, that mediate hepatocyte transduction [8, 29, 30]. FX-binding not only plays a role for the transduction of hepatocytes, but furthermore FX-binding recently has been shown to also shield the vector against attacks by complement, thus leaving the vector less vulnerable to complement-mediated neutralization [8]. Therefore, impeding FX-binding in order to reduce transduction of hepatocytes can increase complement-mediated clearance of the vector by the innate immune system. Taken together, these findings, summarized in Fig. 1, give a glimpse into the complexity of the network of vector-host interactions and show that modification of vectors could affect not only one pathway but could interfere as well with further vector-host interactions leading to unwanted side effects.

For effective systemic application of Ad-based vectors, it is mandatory to overcome all of the barriers mentioned above. A single barrier like the sequestration by erythrocytes is probably sufficient to completely prevent systemic vector delivery—independent of the genius with which the other barriers have been overcome before. Therefore, comprehensive strategies addressing all barriers at once need to be developed and employed to create potent and efficacious vectors based on adenovirus and other viruses. At the same time suitable model systems should be chosen to generate viable results.

Strategies

During the past years a number of different strategies have been developed that can be used to circumvent the barriers mentioned above. These strategies are genetic capsid modifications, the use of different human and non-human adenovirus types and chimeric vectors, as well as chemical modification strategies. Barriers and strategies are summarized in Table 1.

Genetic capsid modifications

Small genetic modifications of the adenovirus capsid can be employed to insert ligands (often derived from phage display screening) into the virus surface (for overview see [41]). It has to be noted that such genetic modifications are

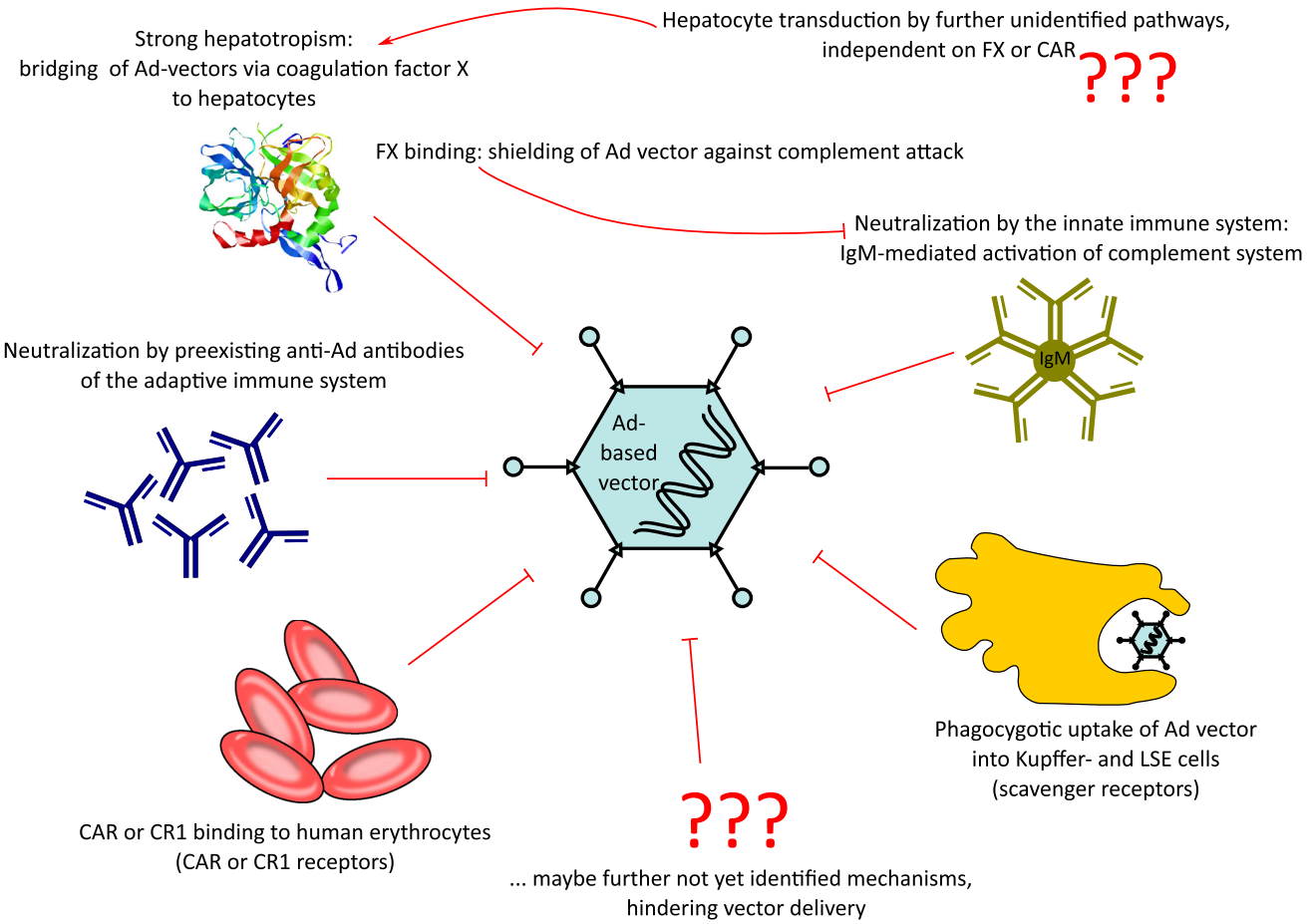

Fig. 1 Molecular interactions within the patient's organism hinder efficient deployment of Ad-based vectors in systemic application

often limited by structural constraints of the ligand motif and the vector surface proteins. Therefore, typically, small peptide stretches are inserted into flexible loops of different capsid proteins [41]. Depending on the site of insertion, motifs of up to 83 amino acids were successfully incorporated. Adenovirus vectors have been targeted, for example, by inserting an integrin-binding RGD motif in the flexible HI-loop of the fiber protein [42]. By inserting this ligand peptide into the HI-loop of the fiber protein, gene transfer to various CAR-deficient cell lines was significantly improved in vitro. However, the overall number of functional ligand motifs that were successfully inserted into the Ad capsids is rather low, presumably due to the size limitations. To circumvent these limitations so-called adapter strategies have been developed. Here, fusion proteins comprised of the soluble ectodomain (sCAR) of the adenovirus receptor CAR and a ligand protein are produced and incubated with the vector. Since the sCAR ectodomain is capable of binding to the adenovirus fiber knob, the virus in this way is non-covalently equipped with the ligand-sCAR-fusion protein. Using a sCAR-EGF fusion protein the virus could for example be targeted to the EGF receptor [43, 44] and more recently to polysialic acid [45]. Although not tested up to date, by blocking the fiber knob domain with the sCAR-fusion protein, this strategy can presumably prevent binding of the vectors to CAR on erythrocytes. Alternatively and importantly, small genetic modifications can also be used to ablate CAR- [46] and FX-binding [23]. However, small genetic modifications or adapter strategies so far do not allow circumventing recognition by the complement system and natural IgMs or preexisting IgGs. In order to achieve this, modifications of larger areas of the capsids have to be employed.

Different Ad types and chimeric vectors

Nature offers a wide variety of different human adenoviruses types (> 70 types of human adenoviruses) and also non-human adenoviruses of different origins. Thus, one promising approach to potentially circumvent the

Table 1 Summary of barriers to systemic application of Ad-based vectors and strategies to develop efficient vectors overcoming these barriers

Barriers to systemic application of Ad-based vectors	Strategies to overcome barriers
Recognition of Ad-based vectors by natural IgM antibodies of the innate immune system [8, 9]	Chemical shielding [31–33]
	Geneti-chemical shielding [34]
	Use of different Ad types [35]
	Chimeric vectors [35]
Complement-mediated neutralization of Ad-based vectors [8]	Chemical shielding [31–33]
	Geneti-chemical shielding [34]
Receptor-mediated phagocytic uptake of Ad-based vectors into Kupffer as well as LSECs [10, 11]	Chemical shielding [31–33]
	Geneti-chemical shielding [34]
	Use of different Ad types [35]
CAR- or CR1-mediated binding of Ad-based vectors to human erythrocytes [11, 18]	Chemical shielding [31–33]
	Geneti-chemical shielding [34]
	Genetic modification [18]
Neutralization of Ad-based vectors by preexisting humoral immunity [19, 20]	Chemical shielding [31–33]
	Geneti-chemical shielding [34]
	Ad vectors based on different Ad serotypes
Prevention of FX-mediated hepatotropism [23]	Chemical shielding [31–33]
	Geneti-chemical shielding [34]
	Generation of genetically modified FX binding-ablated Ad vectors [23, 36, 37]
Targeting to specific cells and tissues	Genetic modification [38–40]
	Chimeric vectors [40]
	Directed evolution [35]
	Chemical shielding [31–33]
	Geneti-chemical shielding [34]

described barriers is to exploit nature's diversity and thoroughly analyze and describe the different adenoviruses. As one example, chimpanzee adenoviruses have successfully been used as vectored vaccines [47–49]. Rare types with a low seroprevalence can be delivered to patients at least once to circumvent the barrier of preexisting immunity to types with higher seroprevalence like Ad5. However, a systematic and comparative analysis of early virus-blood interactions has not been performed up to date and for many adenoviruses. Despite their successful vectorization, very little is known, in fact, about their biology. Research on novel adenovirus types is an urgent matter and greatly facilitated by innovative high-throughput direct cloning and adenovirus genome engineering technologies [50]. Therefore, it can be expected that rare adenovirus types will play an important role for local and systemic delivery in the future.

Currently, the use of chimeric vectors is an option to combine advantageous features of the rare types with the knowledge on adenovirus type 5. Roberts et al. demonstrated that hexon chimeric Ad5-based vectors that had the seven short hypervariable regions (HVRs) on the surface of the Ad5 hexon protein replaced with the corresponding HVRs from the rare adenovirus serotype Ad48, escaped from preexisting immunity [51]. However, the extensive modification was correlated with difficulties in production of these vectors, a phenomenon that can often be observed, when large genetic modifications of Ad5 capsids are performed. The use of chimeric vectors can also be combined with small genetic modifications for targeting. Behr et al. targeted Ad5-based chimeric vectors bearing the fiber knob domain of the rare type 41 by inserting a small peptide ligand for the EphA2 receptor into different positions of the chimeric fiber protein [38]. An extensive analysis on the interaction of such vectors with blood still has to be performed.

Another successful approach to generate chimeric vectors was established by Kuhn et al. and termed "directed evolution" [35]. The group pooled an array of seven adenovirus types and passaged the pools under conditions that favored recombination between the types. Stringent, directed selection on cancer cell lines was performed with the aim to generate a potent oncolytic virus. In fact, a promising oncolytic virus termed ColoAd1 was obtained by this strategy. This group B virus (with a capsid based on Ad11p) was shown to have a favorable haemocompatibility profile with significant evasion from innate and adaptive defense mechanisms in human blood compared to Ad5.

Chemical modifications

A very promising approach to overcome barriers for the systemic delivery of adenovirus-based vectors is provided by shielding the vector surface with synthetic or natural polymers [52]. To achieve this, polymers like polyethylene glycol (PEG) or poly-N-hydroxypropylmethacrylamide (pHMPA) are chemically coupled to the surface of the vector capsid [52]. This chemical reaction generates stable covalent bonds typically between the surface amine groups of the virus capsid proteins and a reactive group of the respective polymer. Importantly, the chemical reaction leaves the capsid intact and, thus, can maintain the natural virus functions to some degree. The polymers are hydrophilic and non-toxic and generate a shield around the virus surface that can prevent interactions with cellular and non-cellular blood components. The density of the shield is controlled by the stoichiometry during the coupling reaction and determines the extent of shielding effect [53]. Even more, by a chemical modification of the polymer it is possible to incorporate ligands into the shield [31, 54]. Of note, in contrast to genetic approaches, here full length proteins [31, 54], carbohydrates [55], or peptides can be used. Polymer-modified vectors have, in fact, been shown to exhibit an altered tropism compared to adenovirus vectors with unmodified capsids. For example, the liver tropism can, depending on the size of the polymer, be ablated and neutralization by antibodies can be prevented to a large degree [31, 53, 56, 57]. At the same time, acute toxicity is significantly dampened. Advanced polymer modifications with charged polymers allow for evasion from binding to FX, binding to erythrocytes [58], and can enable systemic delivery to solid tumors in mouse xenograft models [59].

One disadvantage of chemical modifications is that a delicate balance between sufficient shielding and maintaining the virus' natural potency to transduce/infect cells has to be kept. Furthermore, it has been shown that the position to which a ligand is attached on the virus surface can impact on intracellular trafficking and gene transfer efficiency [60, 61]. As a consequence, instead of cloaking the whole virus particle with polymers, it can be advantageous to couple only a few polymers to carefully selected sites on the capsid. This can be achieved by a genetic introduction of cysteine residues at selected positions of the capsid. In a combined genetic and chemical approach (the so-called geneti-chemical approach [54]), the cysteine residues provide thiol groups for a specific chemical modification with polymers or ligands [29, 34, 54, 62]. It has been shown that this site-specific shielding significantly improves the pharmacokinetics of the vectors, enables evasion from macrophage scavenging and can prevent binding of FX. In combination with additional minimal genetic mutations that do not affect vector production (e.g. CAR-binding ablation) potent vectors can be generated [34]. Furthermore, strategies have been developed to couple polymers in a bio reversible form, releasing the polymers upon entry of the vector into the cytoplasm [63, 64].

Concluding remarks

Adenovirus type 5 belongs to the best characterized viruses. Its extensive use as a vector in various model systems and in the clinics has revealed a number of barriers that hinder systemic delivery. A successful development of adenovirus and other virus-based vectors that are suitable for systemic application can only be achieved after a careful molecular analysis of virus-host blood interactions. Since such analysis is advanced for adenovirus type 5, this virus can be considered a paradigm. A multitude of different techniques and technologies has been developed to improve adenovirus-based vectors. Interestingly, almost all of these technologies have already been applied to other vectors. Future developments may be guided by the knowledge obtained with adenovirus type 5 and may lead to efficacious oncolytic viruses, genetic vaccines, and gene transfer vectors.

Compliance with ethical standards

Disclosure The authors do not have to disclose any conflict of interest. Research involving human participants or animals was not performed for this manuscript.

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