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Pharmacological Interventions for Improving Adenovirus Usage in Gene Therapy

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Abstract: Gene therapy may be an innovative and promising new treatment strategy for cancer but is limited due to a low efficiency and specificity of gene delivery to the target cells. Adenovirus is the preferred gene therapy vector for systemic delivery because of its unparalleled *in vivo* transduction efficiency. Intravenous administration of low doses of adenovirus results in adenovirus sequestration in the liver due to binding to the scavenger receptor present on Kupffer cells. When the amount of adenovirus surpasses the binding capacity of Kupffer cells, hepatocytes absorb adenovirus particles in a blood factor-dependent manner. Increasing the Ad dose even more will saturate both the Kupffer cells and hepatocytes. Then sinusoid endothelial cells bind adenovirus particles in an RGD motif-dependent manner. Strategies to eradicate the binding to liver cells include drugs to interfere or eliminate binding to specific cell types, adenovirus capsid protein mutations and chemical modifications of adenovirus to shield the capsid proteins from cellular receptors. The combined use of these approaches should ultimately lead to successful systemic application of adenovirus in humans.

Keywords: Adenoviruses; Kupffer cells; gene therapy; systemic administration

1. Introduction

Gene therapy involves the delivery of genes to specific cells of interest in order to treat a disease. In this manner, gene therapy can theoretically be used to deliver toxin or corrective genes to tumor cells specifically, which would be an innovative and promising treatment strategy for cancer. This is limited in practice however, due to a low efficiency and specificity of gene delivery to target cells. Improved delivery vehicles are therefore required for specific gene delivery and expression in target cells, without harming healthy cells. This is consistent with the NIH (Orkin−Motulsky Report, 1995), which notes, "To confront the major outstanding obstacles to successful somatic gene therapy, greater focus on basic aspects of gene transfer and gene expression within the context of gene transfer approaches is required. Such efforts need to be applied to improving vectors for gene delivery, achieving tissue-specific and regulated expression of translated genes, and directing gene transfer to specific cell types."

Delivery by viral vectors is the most common systemic delivery strategy currently being investigated in gene therapy. Viral vectors are used in almost 70% of clinical trials in gene therapy, approximately 25% being adenoviral vectors (Ads).[1] Advantages of the Ads are their unparalleled *in vivo* transduction efficiency, high expression of transgenes, large DNA payload capacity, high stability and low pathogenicity *in vivo* and ability to infect quiescent as well as dividing cells.

2. Ad Binding and Receptors

The basic Ad structure is an icosahedral capsid formed by hexon proteins. A penton assembly is attached to the

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vertices, formed by the penton base and fiber proteins protruding from it.[2]

Human Ad species of subgroup B [species 3, 7, 16, 21, 50 11, 14, 34, 35] use CD46 as a primary attachment receptor.[3] CD46 is a member of a family of proteins that regulate complement activation and is expressed on all human cells with the exception of erythrocytes.[4]

Binding of Ad species of subgroups A, D, E and F is mediated by the coxsackie and adenovirus receptor (CAR) on the cell surface, comprising the first step in cell entry of the virus.[3,5,6] The immunoglobulin-like D1 domain of this CAR receptor is able to bind to the carboxyterminal knob domain of serotype 5 fiber,[7] followed by cell-surface $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin mediated virus internalization through the arginine-glycine aspartic acid [RGD] sequence of the adenoviral penton base protein.[8] The viral particle is internalized into a clathrin-coated endosome, from which it can be released into the cytoplasm through acidification of the endosome.[9] After release the particle is being translocated to the nuclear pore, through which the genome gains entry for subsequent replication.

3. The Reticuloendothelial System (RES)

Upon systemic administration, the majority of the Ad rapidly accumulates in the liver. This is accompanied by the induction of acute phase responses including elevation of cytokines (TNF alpha, IL-6 etc.) and chemokines (Mip-2, Ip-10 etc) and expression of genes involved in leukocyte trafficking.[10−14] Hepatic Kupffer cells (KCs) have been shown to contribute to the inflammatory response, and to be responsible for the short half-life of the circulating viral particles and, indirectly, for the lower transgene expression in other cell types.[15−21] Elimination of KCs from the liver by treatment of mice with either clodronate, liposomes or gadolinium chloride results in a marked increase in the levels of hepatocyte-specific Ad-mediated gene transfer, suggesting that significant amounts of infectious virus particles might be trapped by KCs.[17,20,22,23]

In the past it was believed that the CAR receptor was the major player in liver uptake of virus. Various attempts, largely unsuccessful, have been made to avoid Ad liver sequestration by ablating CAR- or integrin-binding motifs

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in the adenoviral capsid[24−28] In more recent studies, it has been shown that the uptake of Ad5 by liver is CAR-independent and rather involves either a direct or indirect interaction with liver cells.[15,26,29]

Direct Interaction. Recently, it was shown by us, both *in vitro* and *in vivo,* that polyinosinic acid (poly(I)) administration into mice prior to Ad injection drastically reduced the capacity of KCs to trap adenoviral particles and to reduce KCs necrosis[21,30] which normally occurs after adenoviral infection.[31] Since poly(I) is a selective inhibitor of scavenger receptor A (SR-A), we investigated whether this receptor was involved in Ad uptake. In *in vitro* experiments we demonstrated that CHO cells expressing SR-A had increased viral transgene expression when compared with wild type cells. Moreover, preincubation of J774 macrophage cells with SR-A ligands significantly decreased Ad uptake. Finally, infection of mice with Ad resulted in a substantial decrease of the virus in liver macrophages when SR-A was blocked.[32] Based on this, we concluded that SR-A is a direct route of Ad to enter macrophages such as KCs.

Indirect Interaction. Ad has been shown to bind to different blood cells such as human erythrocytes, neutrophils, and PBMC (mixed lymphocytes and monocytes) and circulating proteins.[33]

Circulating Blood Proteins. Shayakhmetov et al.[34] showed that Ad5 and type 35 fiber knob domain bind to coagulation factor IX (FIX) and complement component C4-binding protein (C4BP). Virus uptake is than mediated through cell surface heparan sulfate proteoglycans (HSPGs) and/or low-density lipoprotein receptor-related protein (LRP). Mutations in the fiber knob, that abolish interaction of Ad5 with FIX and C4BP, were able to prevent Ad uptake by KCs.[34] In contrast, mutations in the fiber shaft, which have been shown to be essential for binding of Ad to HSPG,[26] did not influence Ad uptake by KCs.[35]

According to Parker et al. hepatocyte transduction with Ad *in vitro* can be enhanced by the vitamin K-dependent factors such as factor X (FX), protein C, and factor VII (FVII) in addition to FIX but not by prothrombin (FII), factor XI (FXI), and factor XII (FXII).[36] In an *ex vivo* liver perfusion model, they showed that human FX enhanced hepatocyte transduction by CAR-permissive and mutated viruses.[36] *In vivo*, downregulation of vitamin K-dependent zymogens by warfarin diminished liver uptake of CAR-binding ablated Ads. This phenomenon, however, was fully rescued by the addition of human FX, indicating that Ad5 can also bind directly to the coagulation factor X.[37] They later demonstrated that FX binds to the Ad5 hexon, via interaction of the FX Gla domain and the hypervariable regions (HVR) of the hexon surface. Liver infection by the FX-Ad5 complex is than mediated through a heparin-binding exosite in the FX serine protease domain.[38] Mutations in the HVR 5 and 7 at specific amino acids were able to prevent *in vitro* and *in vivo* gene transfer.[39]

These results are in agreement with those of Kalyuzhniy et al. who demonstrated that the binding affinity of the Ad5 capsid protein, hexon, for the human coagulation factor X was 40-fold stronger than the affinity of Ad5 fiber for the cellular receptor CAR. They also showed, using cryoelectron microscopy and single-particle image reconstruction, that the

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FX attachment site is localized to the central depression at the top of the hexon trimer.[40]

Recently, it was described that, in addition to the CAR-integrin pathway, blood-borne Ad infects hepatocytes through an indirect pathway that involves blood coagulation factors[34,36,38] which may bridge to cellular heparan sulfate glycosaminoglycans [HSG]. The HSG putative binding site KKTK of the Ad5 fiber shaft domain has been shown to be involved in Ad5 liver transduction in mice, rats and nonhuman primates.[26,41] Liu et al.[42] showed that preinjection of snake venom factor X-binding protein [X-bp] reduces hepatocyte transduction and increases the circulation time in blood of an intravenously injected, fiber-chimeric Ad5/35 vector. X-bp pretreatment resulted in improved Ad5/35 transduction of liver metastases and increased the antitumor efficacy of an Ad5/35-based oncolytic Ad.

Lately Jonsson et al.[43] demonstrated that sinusoid endothelial cells can take up Ad particles in an RGD motif-dependent manner. They proposed a mechanism by which small amounts of Ad particles present in the blood are taken up by KCs as a first dominant mechanism. When the Ad dose exceeds the viral uptake capacity of KCs, the virus uptake becomes hepatocyte-mediated in a blood factor-dependent manner. Finally, when the Ad dose is high and both the KCs and blood factor pathways are saturated, sinusoid endothelial cells and the anatomical architecture of liver sinusoids become the third line of defense mechanism.

Interaction with Blood Cells. Recent studies demonstrate that Ad5 can activate platelets and promotes the formation of platelet−leukocyte aggregates both *in vitro* and *in vivo* through an interaction with the major P-selectin ligand, PSGL-1, on leukocytes.[44] Stone et al. reported that these aggregates are trapped in the hepatic reticuloendothelial system and are subsequently degraded by Kupffer cells.[45] According to Shimony, attachment of Ad to platelets is RGD dependent. Moreover, adhesion to platelets is enhanced in modified Ad where RGD is inserted in the HI fiber knob loop.[46]

The presence of CAR on erythrocytes leads to a prolonged *in vivo* blood half-life and significantly reduced liver infection when a CAR-tropic Ad was injected intravenously.[47]

4. Adaptor Modifications of Ad

Complex binding ligands, including antibodies, have been successfully employed in two-component targeting strategies, where they were bound to the Ad fiber indirectly via a second protein moiety such as anti-fiber knob antibodies or soluble CAR.[48−56] This leads to increased target specificity by ablating CAR-mediated entry, since the viral fiber knob is blocked by the adaptor protein. Administration of adapter-

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modified Ad which included a single chain anti-fiber knob antibody resulted in very low gene expression in the liver.[57] The antibody covering the knob of the virus is thus able to prevent interaction of the virus with native receptors *in vivo*. In this regard, we have recently shown that anti-Ad fiber knob antibodies are able to prevent Ad uptake via SR-A in liver macrophages.[32] Systemic injection of antiangiogenesis peptide-targeted Ad in mice bearing a subcutaneous carcinoma resulted in viral gene expression in tumor in contrast to nontargeted virus or to virus coupled to a nontargeted peptide.[57]

5. Molecular Modifications of Ad

Detargeting Ad from normal tissue upon intravenous injection may require ablation of CAR, αv integrin or heparan sulfate glycosaminoglycan binding sites. Mutations that abolish CAR and integrin binding in the genome should produce an Ad lacking CAR- and αv integrin-binding sites.[58]

Alemany et al.[24] constructed a mutant adenoviral vector unable to bind CAR. The infectivity of a CAR-ablated vector was greatly reduced and not susceptible to inhibition with wild-type knob. Biodistribution and hepatotoxicity were, however, not affected by CAR-binding ablation, possibly related to an increased blood persistence of the CAR-ablated vectors combined with their residual infectivity through other receptors.[59] Mutations in the fiber knob, that abolished the interaction of Ad5 with FIX and C4BP, were able to prevent Ad uptake by KCs.[34]

In contrast, mutations in the fiber shaft, which have been shown to be essential for binding of Ad to HSPG, did not influence Ad uptake by KCs.[35] However, shaft mutation that ablated HSG binding on the background of a normal capsid was sufficient to abrogate liver transduction *in vivo*.[59]

6. Polymer Modifications of Ad

Polymer modifications of Ad offer the possibility to block the interaction of viral proteins with cellular receptors or blood components. To this end, Fisher et al. modified the surface of the Ad with a multivalent copolymer based on poly-[*N*-(2-hydroxypropyl) methacrylamide] (pHPMA), a hydrophilic polymer.[60] The incorporation of targeting ligands such as basic fibroblast growth factor and vascular endothelial growth factor on to the polymer-coated virus produced ligand-mediated, CAR-independent binding and uptake into cells bearing appropriate receptors. The retargeted virus was resistant to antibody neutralization and infected receptor-positive target cells selectively in xenografts *in vivo*.

Along the same line of research, Ogawara et al.[61] conjugated bifunctional polyethylene glycol (PEG) onto the viral capsid of Ad. For the retargeting of Ad, they introduced an RGD peptide or antibody against E-selectin to the other functional group of the PEG molecule. PEGylated, retargeted Ad showed longer persistence in the blood circulation with area under plasma concentration−time curve (AUC) values increasing 12-fold compared to unmodified virus. Anti-E-selectin antibody-PEG-Ad selectively homed to inflamed skin in mice with a delayed-type hypersensitivity (DTH) inflammation, resulting in local expression of the reporter transgene luciferase.[61]

7. Pharmacological Interventions

Hepatic KCs have been shown to be responsible for the short half-life of Ad and indirectly for the lower transgene expression in other cell types.[15−21] Elimination of KCs from the liver may be an effective way to increase transgene expression. This can be accomplished by treatment of mice with gadolinium chloride[17] or clodronate liposomes. Gadolinium chloride forms a colloidal precipitate in the bloodstream which is phagocytosed by KC, resulting in cell destruction. Similarly, clodronate liposomes are ingested by the macrophages, which are then killed following phospholipase-mediated disruption of the liposomal bilayers and release of the clodronate.[62] This results in significantly smaller amounts of infectious virus particles trapped by KCs and results in a marked increase in the levels of hepatocyte-specific Ad-mediated gene transfer.[17,20,22,23]

Systemic administration of different doses of Ad shows a lack of linearity in dose response suggesting that there was a viral dose threshold effect for efficient viral transduction.[19]

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This is not due to an inability to deliver the recombinant viral vectors to the liver but rather to sequestration, at least in part, of the viral vectors by the KCs. This lack of correlation between dose and expression levels can be resolved by supplementing the low dose of desired Ad with an unrelated adenoviral vector which saturates the binding to KCs.[19,23]

Polyinosinic acid [poly(I)], an SR-A ligand, was analyzed for its capability to inhibit Ad uptake specifically in macrophages. In *in vitro* studies, the addition of poly(I) before virus infection resulted in a specific inhibition of Ad-induced gene expression in a J774 macrophage cell line and in primary KCs. In *in vivo* experiments, preadministration of poly(I) caused a 10-fold transient increase in the number of Ad particles circulating in the blood. As a consequence, transgene expression levels measured in different tissues were enhanced (by 5- to 15-fold) compared with those in animals that did not receive poly(I). Finally, necrosis of KCs, which normally occurs as a consequence of systemic Ad administration, was prevented by the use of poly(I). No toxicity, as measured by liver-enzyme levels, was observed after poly(I) treatment. From our data, we conclude that poly(I) can prevent Ad sequestration by liver macrophages.

8. Conclusions

When a low dose of Ad is administered intravenously, the virus is trapped by KCs by binding to the scavenger receptor, mediating Ad sequestration in the liver.[32] When the amount of Ad exceeds the binding capacity of KCs, hepatocytes absorb Ad particles in a blood factor-dependent manner.[34,36,38] Increasing the Ad dose even further will saturate both the KCs and blood factor pathways. Then sinusoid endothelial cells sequester Ad particles in an RGD motif-dependent manner.[63] Strategies to eliminate the interactions with liver cells include Ad capsid protein mutations, chemical modifications to shield the proteins from receptors and drugs to interfere or eliminate specific cell types. The combined use of these approaches should ultimately lead to systemic application of Ad in human patients.

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