Novel Adenovirus Vectors ‘Capsid-Displaying’ a Human Complement Inhibitor

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Introduction

The complement system is one of the first lines of innate defense against invading pathogens. Pathogen interactions with preexisting antibodies and/or circulating complement proteins result in the activation of one or more of the three major complement pathways: the classical (antibody-dependent), alternative (antibody-independent), and the lectin (antibody-independent) complement pathways. Activation of each of the pathways results in the rapid production of C3 convertases. This activation eventuates in the direct production of potent anaphylatoxins (i.e. C3a and C5a), indirectly promotes the high-level production of several cytokines and chemokines, and thereby initiates the recruitment of cellular elements of the innate and adaptive immune systems to the site of infection [1–4].

Inappropriate or excessive complement activation can, however, also cause thrombocytopenia, anaphylactoid reactions, systemic inflammatory responses, the adult respiratory distress syndrome, and/or death. Many of these same toxicities have been observed after high-dose administrations of adenoviruses (Ads) into rodents and non-human primates, as well in a trial in humans [1, 5–12]. Ad capsids are now known to interact with, and activate complement proteins in vitro and in vivo, inter-
actions that induce several complement-dependent toxicities in several species. Importantly, Ad capsids are non-enveloped and therefore are not thought to be directly lysed by terminal complement C6–C9, membrane attack complexes, however, it has been confirmed that Ads become opsonized by complement components, mainly C3b and C4b, both in vitro [8, 13] and in vivo [14]. Complement opsonization facilitates phagocytosis of Ad virions by cells of macrophage origin, if the latter contain proper complement receptors (CR1 [15]) on their membranes. These important interactions with the complement system lead to Ad vector-triggered, complement-dependent release of pro-inflammatory cytokines and chemokines. These responses also augment activation of the cellular part of the innate immune system, resulting in the recruitment of macrophages and granulocytes, and activation of endothelial cells. Importantly, many of these toxicities are avoided when Ad vectors are administered into complement-deficient (C3-KO) mice [8–12]. Based upon these observations, we have attempted to provide the Ad capsid with an inherent complement-inhibitory activity, in an effort to mitigate Ad capsid activation of human complement, and/or complement-dependent toxicities.

Materials and Methods

A 13-amino acid, synthetic peptide with complement-inhibitory activity (COMPinh) was initially identified by others in a high-throughput, phage-based screening effort [16–18]. Importantly, COMPinh (amino acid sequence: ICVWQDWGAHRCT) is an optimized version of the originally described peptide [17]. COMPinh is able to bind and inhibit complement component C3 of human and non-human primate origin, but not C3 from other species [16–18]. In this report, we confirm that we can successfully construct and isolate high titers of these novel Ad vectors ‘displaying’ COMPinh directly on the Ad capsid surface as a fusion protein with several Ad capsid proteins. Based upon this observation, we have performed relevant testing of the novel Ads utilizing several human model assays of Ad-dependent complement activation.

We constructed two Ad5-based vectors capsid-displaying a 13-amino acid sequence with known complement-inhibitory properties (COMPinh). The COMPinh DNA sequence was inserted in-frame into two sites of the Ad genome, forcing expression of the COMPinh peptides as (1) a carboxy-terminal fusion displayed from the cement capsule protein IX (Ad5-LacZ-IX-dCOMPinh), and (2) embedded within the HI loop of the Ad fiber protein (Ad5-LacZ-fiber-COMPinh) as diagrammed in online supplementary figure 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000284368). The viability and infectivity of all Ad vectors utilized in our studies were confirmed by electron microscopy of purified Ads (online suppl. fig. 2), and infectious units (TCID50) and transducing units titer assays. Viral particle (VP) titers were determined by spectrophotometry and validated by SDS-PAGE electrophoresis of purified Ads followed by silver staining and/or Western blotting. COMPinh-displaying Ads preserve VP/TCID ratios typically observed for conventional Ad vectors (online suppl. table 1). Direct sequencing of DNA derived from all CsCl-purified Ad vectors and capsid thermostability assays further confirmed the integrity of the constructs (data not shown).

Ad Vector Construction

Incorporation of COMPinh in the HI Loop of Fiber Protein. All novel Ad vectors were constructed utilizing the pAdEasy-based system [19] with modifications. pAdEasy plasmid was digested with SpeI and PacI restriction enzymes and the 6.20-kb fragment containing the fiber gene was gel purified and subcloned into the pBSX plasmid, giving rise to pBSX-fiberHI. The fiber HI loop was flanked with in-frame NotI and Xbal restriction sites, using an approach similar to that described by Fontana et al. [20] generating pBSX-fiberHI+Not/Xba. Next, 45-mer complementary oligonucleotides, encoding the 13-mer COMPinh nucleotide sequence, were synthesized. When hybridized together, these oligomers yielded NotI- and XbaI-compatible overhangs, allowing in-frame subcloning into the HI fiber loop of pBSX-fiberHI+Not/Xba. The plasmid obtained, pBSX-fiberHI+Not/Xba-COMPinh, was digested with SpeI and PacI and the 6.25-kb fragment was cloned back into the AdEasy backbone, giving rise to pAdfiber-COMPinh. Bacterial homologous recombination of pAd-fiber-COMPinh with Pmel-linearized pShuttle-CMV-LacZ yielded the pAd-CMV-LacZ-fiber-COMPinh plasmid. Further manipulations with this plasmid are identical to the ones described below.

Incorporation of COMPinh in the C-termius of Protein IX. Oligonucleotides encoding COMPinh with Nhel-compatible ends were subcloned in-frame into the C-terminus of viral protein IX into pShuttle-IX/Nhel, the latter contains an Nhel site just upstream of the IX stop codon [21]. A LacZ expression cassette was inserted into the MCS of the pShuttle-IX-COMPinh as previously described [9, 22], pShuttle-LacZ-IX-COMPinh was linearized with Pmel restriction enzyme and homologously recombined with the plasmid pAdEasyI [19], yielding pAd-LacZ-IX-COMPinh plasmid. HEK293 cells were transfected with PacI linearized Ad5-LacZ-IX-COMPinh or Ad5-LacZ-fiber-COMPinh plasmids. Recombinant viable viruses were isolated, amplified, and purified in CsCl gradients as previously described [23, 24] (a complete list of primer sequences utilized for construction and validation studies is shown in online suppl. table 2). All viruses were designed to be [E1-/-E3-] and found to be free of replication-competent adenovirus [25].

Reagents. Adult normal human serum (NHS; pooled from 30 individuals) was purchased from Complement Technology (Tyler, Tex., USA); theses monkey serum was purchased from Innovative Research (Novi, Mich., USA), and human peripheral blood mononuclear cells (PBMCs) were obtained from Astarte Biologics (Redmond, Wash., USA).

Complement Activation AP50 Serum-Based Assay. Alternative pathway complement activation assay (AP50) was performed as described previously [11] with modifications, as detailed in online suppl. material.

C3a-desArg ELISA and CH50 Assay. 200 μl of NHS or non-human primate serum (NHP) was mixed with 1 × 10^10 VP of each of the respective Ad vectors (final concentration equal to
Fig. 1. Novel COMPinh-displaying Ads minimize Ad-mediated complement activation in HS (a, b) and NHPS (c, d) assays. The error bars represent SD. Statistical analysis was completed using one-way ANOVA with a Student-Newman-Keuls post hoc test. Data from one representative experiment are shown. 

**a** Activation of the complement alternative pathway (AP50) mediated by control and COMPinh-displaying Ads was performed as described. Residual complement activity was normalized to HS, incubated with media (no virus control). $^a$ $p < 0.05$ vs. Ad5_WT and Ad5-LacZ; $^d$ $p < 0.001$, Ad5_WT and Ad5-IX-dGFP, and Ad5-LacZ.

**b** Overall complement activation mediated by control and COMPinh-displaying Ads in HS was determined by C3a-desArg-specific ELISA as described. Negative control was HS incubated with PBS prior to ELISA. $^a$ $p < 0.05$, $^b$ $p < 0.001$, vs. no virus controls, $^c$ $p < 0.05$, $^d$ $p < 0.001$, vs. Ad5_WT, Ad5-IX-dGFP and Ad5-LacZ, respectively.

**c** Overall complement activation mediated by control and COMPinh-displaying Ads in NHPS was determined by C3a-desArg-specific ELISA as described. Negative control was NHPS incubated with PBS prior to ELISA. $^b$ $p < 0.01$ vs. PBS control; $^c$ $p < 0.05$ vs. Ad5-LacZ.

**d** Activation of the classical complement pathway mediated by control and COMPinh-displaying Ads was performed as described. After NHPS/Ad incubations, residual CH50 activity was measured and graphed in CH50 unit equivalents (U Eq) per milliliter. Negative control was NHPS incubated with PBS prior to ELISA. $^a$ $p < 0.05$, $^b$ $p < 0.01$, vs. PBS control, $^c$ $p < 0.05$, vs. Ad5-LacZ and Ad5-IX-dGFP.
5 × 10^{10}) and incubated at 37°C for 90 min. The reaction was then stopped by adding EDTA to a final concentration of 10 mM. C3a-desArg was then quantified for each vector by scanning densitometry (table). Note, that VP titer determined by spectrophotometry fall within a ~1.13-fold window confirming that capsid-displaying Ad5 vectors did not contain less virions compared to conventional first-generation Ad5 vectors.

Results and Discussion

To investigate the potential of COMPinh-displaying Ads to diminish complement activation, we utilized two different NHS-based assays: the AP50 and C3a-desArg ELISA. For example, identical amounts of control or COMPinh-displaying Ads were incubated with NHS (+EGTA) and the residual complement activity remaining in the human serum (HS) was then measured in the AP50 assay. NHS pre-incubated with Ad5_WT, Ad5-LacZ, and Ad5-IX-dGFP control viruses revealed significant complement consumption (~80%) compared to NHS samples pre-incubated with PBS, confirming that conventional Ad vectors significantly activate the alternative complement pathway. Note that the Ad5-IX-dGFP virus displays a non-specific peptide sequence (GFP) from pIX, confirming that random display of foreign peptides on the Ad capsid does not result in complement-inhibitory activity in this assay. Complement consumption was dramatically reduced in NHS incubated with similar particle numbers of the COMPinh-displaying Ads (fig. 1a) relative to conventional Ad vectors. Specifically, when NHS was incubated with Ad5-LacZ-IX-

Fig. 2. Silver staining of purified Ad vectors revealed marginal differences in spectrophotometry-determined VP titer. In total, 10^{10} VP of purified virions of each Ad vector were separated by 10% SDS-PAGE and subsequently stained with silver nitrate as described (online suppl. material). The amount of hexon protein (>98 kDa) was quantified for each vector by scanning densitometry (table). Note, that VP titer determined by spectrophotometry fall within a ~1.13-fold window confirming that capsid-displaying Ad5 vectors did not contain less virions compared to conventional first-generation Ad5 vectors.

Cytokine Quantification in Human PBMCs. Human PBMCs were plated as previously described [26]. Briefly, PBMCs were resuspended in RPMI-1640 with 10% FBS, 1% PSF and plated into 24-well tissue culture plate at a concentration of 10^6 PBMC/ml. Upon 24-hour incubation, cells were washed two times with HBSS and exposed to the following Ad5 vectors at a multiplicity of infection of 5,000 (5 × 10^9 VP/well): Ad5-LacZ, Ad5-LacZ-IX-dCOMPinh, and Ad5-LacZ-fiber-dCOMPinh. Media were collected 6, 24 and 48 h after infection, stored at –20°C until use, and levels of human IL-6, IL-8, RANTES, G-CSF, IL-10, MCP-1, and MIP-1β were measured utilizing a multiplex bead array system exactly as previously described [12, 27]. Only levels of IL-6, IL-8, and RANTES are reported, since levels of other analytes were not induced over mock (IL-10 and G-CSF) or were not significantly different between Ad-injected groups (MCP-1 and MIP-1β).

Statistical Analysis

Statistically significant differences were determined using one-way ANOVA with a Student-Newman-Keuls post hoc test (p value < 0.05). Two-way ANOVA with a Bonferroni post hoc test was used to analyze the levels of cytokines released by PBMCs 6, 24 and 48 h after infection to determine significant differences (p < 0.05) between groups. All graphs in this paper are presented as the mean of the average ± SD. GraphPad Prism software was utilized for statistical analysis.
dCOMPinh particles, only 20% of the available complement activity in the NHS was consumed. Notably, the display of COMPinh from the pIX protein facilitated a significantly improved ability to prevent complement activation, relative to the fiber protein display of COMPinh. This may be due to several reasons, inclusive of the simple fact that the pIX protein is present in 240 copies on each Ad capsid, versus 36 copies for the fiber protein.

To further investigate the properties of COMPinh-displaying Ads, we incubated NHS with the novel Ad vectors and measured C3a-desArg protein levels generated after the incubations. Control Ad vectors including Ad5-LacZ control first-generation Ad, Ad5-LacZ-IX-dCOMPinh or Ad5-LacZ-fiber-dCOMPinh at a multiplicity of infection of 5,000 VP/cell, n = 4 for all groups. At indicated time points, media were collected and cytokine concentrations were analyzed using a multiplexed bead array-based quantitative system. Statistical analysis was completed using two-way ANOVA with a Bonferroni post hoc test. Means ± SD. *p < 0.05, **p < 0.001, vs. mock samples at the same time point, †p < 0.05, ‡p < 0.001, vs. Ad5-LacZ group at the same time point.
the presence of pre-existing neutralizing antibodies to Ad in the NHS (as the latter was derived from numerous individuals), complicating the efficacy of capsid-displayed COMPinh to inhibit complement activation in this assay, in contrast to COMPinh-mediated inhibition of complement activation in the C3a-desArg assay or NHPS-based assays.

Since COMPinh has been proven to be effective in inhibiting complement activation not only in HS, but also in NHPS [16–18], we have evaluated the properties of COMPinh-displaying Ads upon incubation with rhesus monkey serum. High homology between human and rhesus monkey complement proteins allowed using human specific antibodies to detect the level of complement activation in NHPS [28]. We found that overall complement activation was significantly reduced when Ad5-LacZ-IX-dCOMPinh was incubated with NHPS as compared to Ad5-LacZ first-generation vector, as measured by C3a-desArg levels in NHPS exposed to the respective vectors (fig. 1c). Moreover, both pIX-dCOMPinh- and fiber-dCOMPinh capsid-displaying Ads caused reduced activation of the classical complement pathway compared to conventional Ad vectors, as evidenced by significantly higher CH50 unit equivalents being measured in NHPS exposed to COMPinh capsid-displaying Ads as compared to NHPS exposed to control Ads (fig. 1d). Finally, to determine if COMPinh-displaying Ads can minimize Ad-triggered cytokine release from human cells, we have infected human PBMCs with control or experimental Ad5 vectors, and quantified the cytokine/chemokine released from those cells into the media 6, 24 and 48 h after infection. Our results demonstrated that human PBMCs exposed to pIX-dCOMPinh- and fiber-dCOMPinh-displaying Ads triggered significantly reduced IL-6 (at all time points tested), IL-8 and RANTES (24 and 48 h after infection) levels compared to similar exposures of the PBMCs to the control Ad vector (fig. 3). These studies highlight previously published studies, demonstrating that membrane-localized complement components can mediate Ad-triggered innate inflammatory responses; this previously described mechanism may be responsible for our results utilizing COMPinh-displaying Ads in vitro [13].

Overall, our results confirm that the functional activity of a peptide to specifically inhibit portions of an important innate immune response can be retained when displayed from the Ad5 capsid. Specifically, the COMPinh peptide can retain its anti-complement activity when displayed as a genetic fusion peptide in several locations on the Ad capsid. As these vectors can be propagated to high titer without need for chemical modifications after purification (such as PEGylation or other potentially non-scalable manipulations), they may allow for more widespread and safer use of so-modified Ad vectors in gene therapy or vaccine applications. Furthermore, COMPinh-displaying Ads could be combined with other methods to further minimize the innate toxicity of Ad vector-mediated gene transfer (i.e. prophylactic glucocorticoid therapy or surgical bypass techniques) [27, 29]. Based upon these data, future, more-advanced studies are now justified.

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References


