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Redirecting adenoviruses to tumour cells using therapeutic antibodies: Generation of a versatile human bispecific adaptor

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ABSTRACT

Effective use of adenovirus-5 (Ad5) in cancer therapy is heavily dependent on the degree to which the virus's natural tropism can be subverted to one that favours tumour cells. This is normally achieved through either engineering of the viral fiber knob or the use of bispecific adaptors that display both adenovirus and tumour antigen receptors. One of the main limitations of these strategies is the need to tailor each engineering event to any given tumour antigen. Here, we explore bispecific adaptors that can utilise established anti-cancer therapeutic antibodies. Conjugates containing bacterially derived antibody binding motifs are efficient at retargeting virus to antibody targets. Here, we develop a humanized strategy whereby we synthesise a re-targeting adaptor based on a chimeric Ad5 ligand/antibody receptor construct. This adaptor acts as a molecular bridge analogous to therapeutic antibody mediated cross-linking of cytotoxic effector and tumour cells during immunotherapy. As a proof or principle, we demonstrate how this adaptor allows efficient viral recognition and entry into carcinoma cells through the therapeutic monoclonal antibodies Herceptin/trastuzumab and bavituximab. We show that targeting can be augmented by use of contemporary antibody enhancement strategies such as the selective elimination of competing serum IgG using "receptor refocusing" enzymes and we envisage that further improvements are achievable by enhancing the affinities between the adaptor and its ligands. Humanized bispecific adaptors offer the promise of a versatile retargeting technology that can exploit both clinically approved adenovirus and therapeutic antibodies.

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1. Introduction

Adenoviruses are increasingly being exploited as a means of therapeutic intervention in human disease (Danthinne and Imperiale, 2000) and adenoviral therapy is now a realistic prospect for the treatment of many forms of cancer (Arnberg, 2012). The anti-tumour activity of adenoviral therapy relies on coupling their innate host-cell lytic ability of the virus with an effective means of altering viral tropism away from its natural targets to those associated with tumour cells.

Adenoviruses offer several significant advantages to tumour cell therapy. They can be replicated to very high titre, transduce a wide variety of cell types, lytically kill their host cell as part of their natural infective cycle, do not integrate into host genomes and possess a viral genome capable of accepting a significant degree of molecular engineering. This latter trait has led to a variety of innovative developments including oncolytic strains that selectively kill tumour

Abbreviations: Ad5, adenovirus-5; CAR, coxsackievirus and adenovirus receptor; DMEM, Dulbecco's Modified Eagle's Medium; Dox, doxycycline; ELISA, enzyme-linked immunosorbent assay; Endo S, endoglycosidase S; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; Fuc, fucose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GST, glutathione *S*-transferase; HEK, human embryonic kidney; HER2, human epidermal growth factor receptor 2; HPLC, high-performance liquid chromatography; IgG, immunoglobulin G; IPTG, isopropyl β-D-1-thiogalactopyranoside; Man, mannose; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline pH 7.4; PEI, polyethyleneimine; PS, phosphatidylserine; P/S, penicillin/streptomycin; PNGase F, peptide N-glycosidase F; SA, sialic acid; SDS, sodium dodecyl sulfate; TAA, tumour associated antigen.

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[1] This article is dedicated to Chris Scanlan who passed away on the 4th May 2013 after a short battle with cancer.

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cells such as the p53 null specific strains H101 and Onyx-015 that have reached phase III clinical trials (Garber, 2006).

Adenoviruses have a broad range of natural targets. Therefore, selectively modifying viral tropism, also known as transductional retargeting, is clearly a key step in the use of adenoviruses in killing any tumour cell *in vivo*. Adenoviruses must be engineered in such a manner that they ignore their natural ligand in favour of a marker presented by tumour cells. A second consideration is that prolonged adenoviral exposure in humans results in not only their effective hepatic clearance, with accompanying liver toxicity, but also immunogenic toxicity as a result of neutralising antibody production. This latter issue can be overcome by engineering out the dominant antigenic determinants of the virus such as the generation of the so-called 'gutless' series of Ad5 (Alba et al., 2005) or masking them by direct modifications such as PEGylation (O'Riordan et al., 1999).

Engineering targeting molecules directly into the viral fiber knob has shown promise in altering tropism in favour of tumour cells. These insertions together with selective mutations to the fiber knob designed to ablate natural receptor recognition have been shown to work against several tumour-associated antigens (TAA). However, a significant drawback is the need to genetically re-engineer the virus for each TAA targeted. Moreover, given the necessity for correct cytoplasmic folding of viral capsid proteins prior to nuclear transport and virion assembly, many preferred TAA ligands, particularly those dependent on disulphide bonds and glycosylation, are unsuitable for this methodology (Magnusson et al., 2002). Nevertheless, relatively large high affinity peptide ligands, such as affibody antibody mimics (Magnusson et al., 2012), can be incorporated into the fiber H-loop that successfully re-target adenoviruses.

The main alternative to recombinant viral capsids is the use of chimeric bispecific adaptor proteins that directly link viral binding to an appropriate retargeting entity. These adaptors fuse either an anti-fiber knob antibody or natural receptor fragment with a second TAA binding domain, such as antibody fragments (Beatty and Curiel, 2012; Kashentseva et al., 2002; Sapinoro et al., 2007; Haisma et al., 2000) or peptide ligands (Kim et al., 2002; Dmitriev et al., 2000; Watkins et al., 1997). An interesting variant on this theme is the effective use of bifunctional PEG in linking Ad5 with TAA antibody (Jung et al., 2007; Reetz et al., 2013). The use of such adaptors, whether chemical or protein, does not impact upon viral infective ability as binding is a sufficiently distinct mechanism from internalization (Wickham et al., 1993). Moreover, the lack of recombination within the viral genome ensures viral replicative fitness. Several such adaptors have been described based on Ad5's high affinity recognition of its natural primary receptor CAR (coxsackievirus and adenovirus receptor) (Hong et al., 1997; Bergelson et al., 1997). For example, Ad5 binding to trimeric CAR fused to a cluster of anti-HER2 scFv antibody fragments effectively retargets Ad5 to HER2 expressing breast carcinoma cells (Kashentseva et al., 2002).

Several groups have reported success coupling Ad5 to fragments of protein A, an immunomodulatory virulence protein that generically binds IgG Fc, either engineered directly into the fiber shaft (Korokhov et al., 2003; Volpers et al., 2003; Kawashima et al., 2011) or as part of a CAR fusion adaptor (Li et al., 2003). These strategies fulfil the criteria of a universal monoclonal antibody Ad5 adaptor that effectively refocuses Ad5 to the TAA dictated by the monoclonal specificity. However, a potential impediment to the use of such constructs in humans is that protein A, being bacterially derived, is immunogenic and individuals with prior exposure will have developed immunity. Nevertheless, minimising the amount of protein A to just the critical 33 amino acid Ig binding domain Z33 may circumvent this potential pitfall (Volpers et al., 2003; Kawashima et al., 2011; Tanaka et al., 2006).

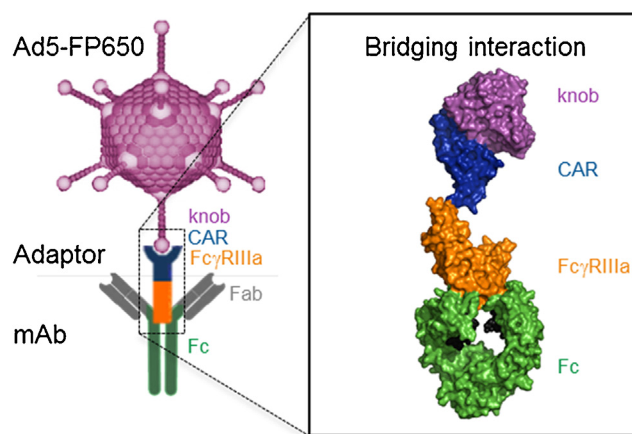

Fig. 1. Theory and construction of a chimeric CAR-FcγRIIIa Ad5 adaptor. Cartoon of proposed adaptor mechanism showing Ad5 bound to a monoclonal antibody *via* an intermediate CAR-FcγRIIIa adaptor protein. Colours used to identify specific components are Ad5: purple, CAR: blue, FcγRIIIa: orange and monoclonal antibody: green. The zoom box displays a putative molecular model generated using the deposited crystal structures of Ad12 knob-CAR D1 (PDB 1KAC) and the IgG1 Fc-FcγRIIIA (PDB accession codes 1KAC and 1T83, respectively) depicted using the same colour scheme. The model highlights the key binding events mediated by this adaptor, specifically Ad5 knob/CAR and FcγRIIIa/IgG Fc. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We now describe the logical next step in making a fully human bispecific adenoviral adaptor that can be used with any IgG based therapeutic monoclonal antibody (Fig. 1). We have fused CAR with the IgG Fc binding receptor FcγRIIIa (CD16a). Docking of this adaptor with two test therapeutic antibodies, the clinically licensed anti-HER2 monoclonal Herceptin and bavituximab a current phase III clinical trial anti-phosphatidylserine (PS) monoclonal (DeRose et al., 2011), allow effective Ad5 recognition and internalization into carcinoma cells expressing the relevant antigen. Finally, we also demonstrate a useful synergy between this methodology and a means of clearing non-specific serum antibody Fc receptor engagement using the enzyme, endoglycosidase S (EndoS).

2. Materials & methods

2.1. Adenovirus strain

All experiments were performed using E1A/E1bΔ replication incompetent Gateway® adapted adenoviral-5 vector (pAd/CMV/V5-DEST) (Invitrogen/Life technologies) recombined with pENTR/D. pTurbo FP650 gene encoding near-infrared fluorescent protein from the pTurbo FP650-C a mammalian expression vector (#FP731, emission wavelength 635 nm) was cloned into directional TOPO vector pENTR/D-TOPO (Invitrogen/Life technologies). The pENTR/D-TOPO is designed to facilitate cloning of blunt end PCR products and generate the entry clone into Gateway System. The entry of desired clone containing the FP650 gene is performed by LR recombination reaction between the pENTR/D-TOPO and pAd/CMV/V5-DEST. The *Pac I*–digested vector is used to transfect 293A cells (genetically modified to carry Ad5 sequences necessary for replication) to produce an adenovirus stock. After amplification in in 293A cells the adenovirus stock was titred and frozen at −80 °C in 200 μl aliquots at 2×10^8 particles/ml.

2.2. CAR-FcγRIIIa construct cloning

CAR cDNA was obtained from Source Bioscience (ORFeomeID 100,008,823/OCAA69 A07) and FcγRIIIa (Genebank accession: BC033678, CD16a) cDNA was isolated as previously described (Yu

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