



A targeted adenovirus vector displaying a human fibronectin type III domain-based monobody in a fiber protein

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ABSTRACT

A major drawback of adenovirus (Ad) vectors is their nonspecific transduction into various types of cells or tissue after *in vivo* application, which might lead to unexpected toxicity and tissue damage. To overcome this problem, we developed a fiber-mutant Ad vector displaying a monobody specific for epidermal growth factor receptor (EGFR) or vascular endothelial growth factor receptor 2 (VEGFR2) in the C-terminus of the knobless fiber protein derived from T4 phage fibrin. A monobody, which is a single domain antibody mimic based on the tenth human fibronectin type III domain scaffold with a structure similar to the variable domains of antibodies, would be suitable as a targeting molecule for display on the Ad capsid proteins because of its highly stable structure even under reducing conditions and low molecular weight (approximately 10 kDa). Surface plasmon resonance (SPR) analysis revealed that the monobody-displaying Ad vector specifically bound to the targeted molecules, leading to significant increases in cellular binding and transduction efficiencies in the targeted cells. Transduction with the monobody-displaying Ad vectors was significantly inhibited in the presence of the Fc-chimera protein of EGFR and VEGFR2. This monobody-displaying Ad vector would be a crucial resource for targeted gene therapy.

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1. Introduction

Among the currently available transduction vectors, replication-incompetent adenovirus (Ad) vectors achieve the highest level of transduction efficiency in a variety of cells, and thus have been widely used as gene delivery vehicles for not only gene therapy but also basic research, including gene function analysis [1,2]. However, systemic administration of an Ad vector results in nonspecific transduction in a variety of cells [3,4], and transduction into nontarget cells or tissue might cause unexpected toxicity and tissue damage [5,6]. To overcome this problem, fiber-modified Ad vectors, in which a foreign peptide is inserted to modify the tropism, have been developed. Representative foreign peptides used for insertion into Ad vectors include the RGD (Arg-Gly-Asp) peptides [7,8] and

poly-lysine peptides [9,10]. The RGD peptides bind with high affinity to cell surface integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) [11] and the poly-lysine peptides bind with high affinity to cell-surface heparin sulfate [12]. Insertion of an RGD peptide or poly-lysine peptide into the fiber knob significantly increases the transduction efficiencies, especially in cells lacking coxsackievirus and adenovirus receptor (CAR), leading to expansion of the tropism of Ad vectors. However, truly targeted cell- or tissue-specific transduction is not successfully achieved by the fiber-modified Ad vectors containing an RGD peptide or poly-lysine peptide, because α_v -integrins and heparin sulfate are broadly expressed on normal tissues [9,10,13]. Insertion of a peptide which binds to the receptor specifically expressed on the targeted cells into the Ad vector is crucial; however, only a few foreign peptides have been demonstrated to mediate targeted cell-specific transduction with an Ad vector [14–16].

In order to achieve targeted tissue-specific transduction using an Ad vector, capsid-modified Ad vectors incorporating a single-chain antibody fragment (scFv), which specifically binds to target molecules on targeted cells, into the capsid proteins by genetic recombination have been developed [17–19]. However,

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incorporation of an scFv into Ad capsid proteins is not successfully tolerated because translation and folding of an scFv commonly occurs in the oxidizing environment of the endoplasmic reticulum, followed by trafficking to the cell surface via the Golgi system and subsequent secretion [20–22]; on the other hand, virion assembly of Ad capsid proteins occurs in the nucleus, which is under the reducing environment [23,24]. Under a reducing condition, the disulfide bond in the scFv is cleaved [22,25]. Therefore, when an scFv is incorporated into the Ad capsid protein, the disulfide bonds of the scFv are cleaved when the protein enters the nucleus, thereby inactivating the scFv [24].

To overcome this problem, we selected a monobody as a target molecule for incorporating into the Ad capsid protein in this study. Monobodies are single domain antibody mimics based on the tenth human fibronectin type III domain (10Fn3) scaffold with a folded structure similar to the variable domains of antibodies [26–28]. Monobodies are designed to bind to therapeutically relevant targets (e.g., EGFR, VEGFR2) with high affinity and specificity comparable to those of an scFv [29,30]. The most attractive characteristic of a monobody for use in modification of the Ad capsid protein is that a monobody does not contain a cysteine residue, and thus would allow stable folding even in a reducing environment, including the nucleus [30,31]. This characteristic allows a monobody to be well accommodated for stable incorporation into the Ad capsid proteins. In this study, a monobody specific for EGFR or VEGFR2 was genetically incorporated into the deknobbed, fibrin-foldon trimerized fibers (knobless fiber) [32,33] of an Ad vector. We examined the transduction properties of the monobody-displaying Ad vectors, including the binding properties to the targeted molecules, in western blotting analysis, SPR analysis, and *in vitro* transduction experiments.

2. Materials and methods

2.1. Cells

MDA-MB-231 (a human breast carcinoma cell line) and A431 (a human epithelial carcinoma cell line) cells were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and L-glutamine. NIH3T3 (a mouse embryo fibroblast cell line) and KDR-3T3 (NIH3T3 cells stably expressing human VEGFR2) [34] were cultured with minimum essential medium (MEM) supplemented with 10% FCS. KDR-3T3 cells were kindly provided by Dr. Masabumi Shibuya (Jobu University, Gunma, Japan). K562 (a human erythromyeloblastoid leukemia cell line) cells were cultured with RPMI1640 supplemented with 10% FCS. CHO (a Chinese hamster ovary cell line) and CHO-EGFR (CHO cells stably expressing human EGFR) cells were cultured with minimum essential medium α -modification (MEM- α) supplemented with 10% FCS.

2.2. Construction of an Ad vector displaying a monobody on the C-terminus of knobless fiber protein

The conventional Ad vector expressing firefly luciferase, Ad-L2, was constructed as described previously [35]. An Ad vector containing the knobless fibers was constructed as described below. Briefly, a 776 bp fragment (T4 phage genome, bp 685–1461) of the T4 phage fibrin gene was amplified by PCR using the following primers: T4-fib-for primer (5'-CACGGCATGCTTCGCTCAAAATGGGCAACGGCTCTCTG-GACGAGGCCGGAACCTTACCTACAGAACGTTTATAGTCG-3') and T4-fib-rev (5'-ATC-GATGATCTCTGCTGCTGATAAAAAGGTAGAAAGGAATACCATTCGCC-3'). This PCR fragment was cloned into the *PmeI* site of pcDNA3.1/Zeo(–) (Invitrogen, Carlsbad, CA), resulting in pcDNA-fib. Next, pcDNA-fib was digested with *BamHI*/*Clal* and ligated with the following oligonucleotides: knobless-G4Sx2-F (5'-GATCTGG CGGGGGCGGCTCCGAGGCGGAGGCAGCAT-3') and knobless-G4Sx2-R (5'-CGATGCT GCCTCCGCTCCGAGCGGCCCCCGCA-3'), resulting in pcDNA-fib-G4S. pHM14-Eco2 [36] was digested with *SphI*/*Clal* and ligated with the *SphI*/*Clal* fragment of pcDNA-fib-G4S, resulting in pHM14-Eco25. pAdHM41-4, which includes the fragment of the Ad vector genome in AdHM41 [36] (bp 1–32,787), was digested with *XbaI*/*SrfI* and ligated with the *SpeI*/*SrfI* fragment of pHM14-Eco25, resulting in pAdHM72. For insertion of a firefly luciferase expression cassette into the Ad vector plasmid, pAdHM72 was digested by *I-CeuI*/*Pi-SceI* and ligated with *I-CeuI*/*Pi-SceI*-digested pCMVL1a [37], which contains a cytomegalovirus (CMV) promoter-driven firefly luciferase expression cassette, resulting in pAdHM72-L2.

The vector plasmids for Ad vectors containing VEGFR2-specific monobody (pAdHM72- α V-L2) or EGFR-specific monobody (pAdHM72- α E-L2) were constructed as described below. First, to insert the gene encoding VEGFR2-specific monobody into the C-terminus region of the knobless fiber, a shuttle plasmid pHM17 was constructed as described below. pHM5 [35] was digested with *Acc65I*/*I-CeuI* and ligated with the oligonucleotides oligo-1 (5'-TCTAGAATCGATGCTAGCTTCGAA CCTAGGGCCAGCCGGCCATGG-3') and oligo-2 (5'-GTACCCATGGCCGGCTGGGCCC-TAGGTTCCGAAGCTAGCATCGATTCTAGATTAG-3') to create pHM16. Then, pHM16 was digested with *Acc65I*/*Pi-SceI* and ligated with the oligonucleotides oligo-3 (5'-GTACCCGGCCGCTGTAATGAATAGCTAGGTTCCGAAGCTAGCATCGATTCTAGAGTGC-3') and oligo-4 (5'-TCTAGAATCGATGCTAGCTTCGAACCTAGGCTATTTCATTACAGC GGCCGCG-3'), resulting in pHM17. The *Csp45I* recognition sites in the primers are underlined. pHM17 was then digested by *Csp45I*, treated by alkaline phosphatase, and ligated with *Csp45I*-digested pUC57-VEGFR2-monobody (purchased from GenScript, Piscataway, NJ), resulting in pHM17-VEGFR2-monobody. Oligonucleotides encoding the NLS sequence derived from the SV40 large T-antigen was inserted into the region encoding the C-terminus of the VEGFR2-specific monobody. *Clal*-digested pAdHM72-L2 was ligated with *Csp45I*-digested pHM17-VEGFR2-monobody, resulting in pAdHM72- α V-L2.

A gene encoding EGFR-specific monobody was amplified by PCR using pUC57-VEGFR2-monobody as a template. First, fragment 1 was amplified by PCR with the following primers: EGFR-10FN3-Csp45I-for (5'-GATCTTCGAAATGGTGTCT-GATGTTCCCGGGACCTGG-3', the *Csp45I* site is underlined) and EGFR-10FN3-BC-rev (5'-GGTTTACCCGTATGTATCCGGTAATCTGCTAGGAGCCCTGCCGCTGTCCAGCTGATCAG-CAGCTAG-3'). Second, fragment 2 was amplified by PCR using fragment 1 and the primer EGFR-10FN3-DE-rev (5'-CAGGCCGCTGATGCTAGCAGTATGACGGGGCCAGGGA-CAGTGAATTCCTGAACC-3'). Next, fragment 3 was amplified by PCR using the following primers: EGFR-10FN3-FG-for (5'-CTATACCATCACTGTGCTGCTGTACTGACCA-CAAGCCCGCAGCGGCGCCCTCACACCTACACGAGAGCCCATCTCCATTAACTACCGCACC GC-3') and EGFR-10FN3-Csp45I-rev (5'-CTAGTTCGAAGGGTCTCCACCTTCCGCTTT TTCTTAGG-3', a *Csp45I* site is underlined). Finally, a gene encoding the EGFR-specific monobody was amplified by PCR using the fragments 2 and 3. pHM17 was digested with *Csp45I*, treated with alkaline phosphatase, and ligated with the *Csp45I*-digested PCR fragment encoding the EGFR-specific monobody, resulting in the pHM17-EGFR-monobody. *Clal*-digested pAdHM72-L2 was ligated with the *Csp45I*-digested pHM17-EGFR-monobody, resulting in pAdHM72- α E-L2.

The preparation of Ad-KL-L2, Ad- α V-L2, and Ad- α E-L2 was performed as described below. *PacI*-digested pAdHM72-L2, pAdHM72- α E-L2, and pAdHM72- α V-L2 were transfected into Fiber-293 cells, which stably express Ad5 fiber protein [38], because the viruses were not generated in conventional 293 cells. At the final stage of virus amplification, the viruses were allowed to infect conventional 293 cells to avoid the inclusion of the Ad5 fiber protein in the capsid. Viruses were purified by CsCl step gradient ultra-centrifugation followed by CsCl linear gradient ultra-centrifugation. Ad-L2 was prepared as described previously [35]. Virus particle (VP) titers were measured using the method of Maizel et al. [39].

2.3. Western blotting for Ad fiber protein

Ad-L2, Ad-KL-L2, Ad- α E-L2, and Ad- α V-L2 were lysed in SDS-PAGE loading buffer (62 mM Tris [pH 6.8], 2% SDS, 10% glycerol, 0.001% bromophenol blue). The virus lysates were loaded onto each gel lane of 5–20% SDS-polyacrylamide gels with or without prior denaturation of the lysates by boiling. Then, the proteins were electro-transferred onto a PVDF membrane. The Western blot assay was carried out as previously described [40].

2.4. Surface plasmon resonance analysis

Surface plasmon resonance experiments were carried out on a Biacore 3000 instrument (Biacore, Stevenage, UK). Recombinant human EGFR-Fc chimera protein or VEGFR2-Fc chimera protein was covalently immobilized onto a CM3 biosensor chip by amine coupling according to the manufacturer's instructions. Virus in 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.005% Tween 20 (HBS-EP) was passed over the chip at a flow rate of 20 μ l/min. Sensor chips were regenerated after virus injections by injection of 10 mM Glycine-HCl buffer.

2.5. Ad vector-mediated transduction into cultured cells

CHO, CHO-EGFR, A431, MDA-MB-231, KDR-3T3, and K562 cells were seeded at 1×10^4 cells/well onto a 96-well plate. On the following day, the cells were transduced with each Ad vector at 3000 VP/cell for 1.5 h. After a 48 h-incubation, luciferase production in the cells was determined using a luciferase assay system (PicaGene LT2.0; Toyo Inki, Tokyo, Japan).

In the transduction experiment in the presence of recombinant EGFR Fc-chimera protein (R&D Systems, Minneapolis, MN), A431 and CHO-EGFR cells were seeded at 1×10^4 cells/well onto a 96-well plate. On the following day, the Ad vectors were incubated with 100 μ g/ml EGFR Fc-chimera protein for 60 min at room temperature, and the mixtures were added to the cells at 3000 VP/cell. After a 1.5 h-incubation, the medium was replaced with fresh medium. Following a total 48 h-incubation, luciferase expression was measured as described above. The transduction

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