Biomaterials 32 (2011) 4185–4193

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Enhanced *in vivo* gene transfer into the placenta using RGD fiber-mutant adenovirus vector

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ARTICLE INFO

Article history:
Received 10 February 2011
Accepted 19 February 2011
Available online 15 March 2011

Keywords:
Gene expression
Gene transfer
Adenovirus
RGD peptide
Drug delivery

ABSTRACT

Among viral vectors, the fiber-mutant adenovirus vector carrying the Arg-Gly-Asp (RGD) peptide sequence (Ad-RGD) seems to have potential for both clinical gene therapy and basic research. As a part of a thorough evaluation of Ad-RGD in preclinical studies, we designed an experiment to investigate in detail the distribution of Ad-RGD compared with conventional adenovirus vector (WT-Ad) in pregnant mice. Surprisingly, Ad-RGD had substantial placental tropism, at 10–100 times that of WT-Ad. Transgene expression was sustained for at least 7 days, and Ad-RGD expressing firefly luciferase or red fluorescent protein has so far caused no placental dysfunction leading to fetal death. Ad-RGD showed high levels of transduction efficiency in *in vitro*-differentiated trophoblast stem cells, in which higher expression of αvβ3 integrin than in undifferentiated cells was observed. Our results suggest that the use of Ad-RGD or another RGD-mediated targeting strategy holds promise for drug delivery to the placenta.

1. Introduction

Gene therapy holds promise for treating inherited disorders caused by single gene defects, such as cystic fibrosis, hemophilia, and sickle cell disease. Moreover, recent advances in bioscience and biotechnology are expanding the promise of gene therapy for treating a wide range of diseases, such as polygenic or infectious diseases. A key factor in the success of gene therapy is the development of gene delivery systems that are capable of efficient gene transfer in a broad variety of tissues or targeting certain cell types, without having pathogenic effects. Several kinds of viruses, including retrovirus, adenovirus, adeno-associated virus, and herpes simplex virus, have been manipulated for use in gene transfer and gene therapy applications. As different viral vector systems have their own unique advantages and disadvantages, they each have applications for which they are best suited.

Recombinant adenovirus (Ad) vectors are widely used in preclinical and clinical gene therapy [1]. This vector system provides numerous advantages over other viral vector candidates, including (1) the ability to deliver the gene of interest *in vivo* because the Ad vectors can infect a wide range of dividing and nondividing cells and can generate large amounts of protein, and (2) lack of insertion into the host DNA and, therefore, absence of risk of insertional mutagenesis [2,3]. Although Ad vectors are widely available for gene therapy, some limitations in the use of the vectors *in vivo* have been noted previously. These limitations include unresolved issues related to tissue tropism, cell specificity, the size of the transgene, and antigenic properties. To overcome the limitations of Ad vectors, our colleagues have been developing a wide variety of genetically modified Ad vectors [2,4–8].

Conventional Ad type-5 vector (Ad5), which is widely used but requires the binding of coxsackievirus and adenovirus receptor (CAR) on the cell surface via the fiber protein, has limited tropism *in vitro* and *in vivo*. Studies by Mizuguchi and others demonstrated that a fiber-mutant Ad vector carrying the Arg-Gly-Asp (RGD)

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doi:10.1016/j.biomaterials.2011.02.038

sequence in the HI loop of the fiber knob (Ad-RGD) could transduce the gene of interest into various cells more efficiently than conventional Ad vector (WT-Ad), and we reasoned that this fiber-mutant Ad might target αv-integrins during the initial attachment to the target cells [7,9–11]. Furthermore, the ability of the vector to attach to the target cells via RGD-fiber protein results in increased tissue retention of Ad-RGD at tissue sites where Ad-RGD is locally injected, suggesting that the use of Ad-RGD is advantageous in gene therapy [12]. Consequently, we believe that Ad-RGD can be a very useful and powerful tool for future basic research and clinical gene therapy.

As a part of a thorough evaluation of Ad-RGD-mediated gene transfer in preclinical toxicology studies, we investigated in detail the distribution of Ad-RGD in pregnant mice compared with that of WT-Ad. We examined Ad tissue tropism and subsequent gene expression by detecting the Ad genome with quantitative real-time PCR and using a luciferase reporter gene assay. We also used a well-established cell-differentiation model of trophoblast stem cells *in vitro* to characterize the properties of Ad-RGD-mediated gene transfer. Our findings, reported in this paper, redraw our attention to the need for thorough preclinical evaluation of improved viral vectors with modified tropism and are of interest not only to clinical researchers in gene therapy but also to scientists conducting fundamental research into placentation, placental pathology, or fetal development.

2. Materials and methods

2.1. Preparation of recombinant adenoviruses

A type-5, E1/E3-deleted Ad containing the RGD amino acid sequence in its fiber knob (Ad-RGD), or an Ad with a wild-type fiber knob (WT-Ad), was generated as previously reported in Refs. [7,13,14]. Briefly, the reporter gene sequence of firefly luciferase gene (GL3, Promega, Madison, WI) or a red fluorescent protein (DsRed2, Takara Bio, Otsu, Japan) was cloned into a multi-cloning site of a shuttle plasmid (pHMCMV6) that expresses the inserted gene under the control of the cytomegalovirus promoter. An I-CeuI/PI-SceI-digested fragment of the shuttle plasmid was inserted into the corresponding site of pAdHM4 or pAdHM15RGD in accordance with the *in vitro* ligation method.

To generate Ad vector particles, each vector plasmid was digested with *Pac*I to release the recombinant viral genome, and the Ad vector genome was transfected into 293 cells plated on 60-mm dishes with SuperFect (Qiagen, Valencia, CA) transfection reagent according to the manufacturer's instructions. All Ad vectors were propagated in 293 cells, purified by two rounds of cesium chloride-gradient ultracentrifugation, dialyzed, and stored at −80 °C [7,13,14]. The concentrations of plaque-forming units of individual stocks were determined from the $TCID_{50}$ titers, as previously described in Ref. [15].

2.2. Adenovirus-mediated in vivo gene transfer into pregnant mice

Specific-pathogen-free pregnant ddY and female C57BL/6J mice were purchased from SLC Japan Inc. (Shizuoka, Japan) and CLEA Japan Inc. (Tokyo, Japan), respectively. A green fluorescent protein (GFP)-transgenic mouse (green mouse) line (C57BL/6-Tg(CAG-EGFP)C14-Y01-FM131Osb) which expresses GFP in most tissues including placenta [16] was kindly provided by Dr. M. Okabe of the Research Institute for Microbial Diseases, Osaka University. To generate pregnant C57BL/6J mice that had GFP-transgenic green placentas and fetuses, females were mated overnight with males of the green mouse line. The females were checked for vaginal plugs the next morning and only those that showed a plug were considered pregnant; noon on the day of discovery of the plug was considered as day 0.5 dpc. Pregnant females were given Ad vectors by tail vein injection as a suspension in phosphate-buffered saline (PBS) (injection volume, 200 μl). Forty-eight hours later (except where otherwise noted), maternal tissues and conceptuses were collected for luciferase assay or histological examination.

When Ad vectors expressing the luciferase gene were used, the collected samples were homogenized in PBS containing protease inhibitors (2 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM PMSF). The mixture was then centrifuged at 20,000 *g*. The luciferase activity of the supernatants was measured with Luciferase Assay Reagent (Promega).

Placentas of pregnant mice injected with Ad-RGD-DsRed2 were fixed in 4% paraformaldehyde for 6 h, cryoprotected in 30% sucrose overnight at 4 °C, and frozen in Tissue-Tek O.C.T. compound (Sakura Fine-technical, Tokyo, Japan) on an aluminum block chilled by liquid nitrogen. Cryostat sections (6 μm) were observed by fluorescent microscopy.

All animal experiments were approved by the institutional animal care and use committee of Osaka University.

2.3. Real-time RT-PCR analysis for adenovirus receptors

Real-time RT-PCRs for the adenovirus receptors CAR (mCAR), integrin αv, and integrin β3 were performed as previously described, with slight modification [17]. In brief, total RNA extracted from each homogenized tissue or culture cell was reverse transcribed using SuperScript II reagent (Invitrogen, Carlsbad, CA) and oligo-dT primer. Aliquots of diluted cDNA were amplified with reagents of the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) and 0.5 mM of each primer by using LightCycler (Roche Diagnostics, Mannheim, Germany). The primers used were as follows: mCAR, 5′-ACGCAGAGAAGAGAAGTACGAGAA-3′ and 5′-GGGAG-GAATGGTTGCTGCCAATATA-3′; integrin αv, 5′-CCAGCCTGGGATTGTAGAAG-3′ and 5′-ACTCCAGTGGGTCATCTTTG-3′; and integrin β3, 5′-TCTGGCTGTGAGTCCTGTGT-3′ and 5′-GCCTCACTGACTGGGAACTC-3′.

2.4. Real-time PCR analysis for quantitative detection of adenovirus copy number

Quantitation of adenovirus copy number in tissues after i.v. injection of Ad-RGD-Luc or WT-Ad-Luc was conducted by using a real-time TaqMan RT-PCR system. DNA was purified from the collected tissue by using a QIAamp tissue kit (Qiagen). The purified DNA (10 ng) was mixed with 25 μl of Real-Time PCR Master Mix (Toyobo, Osaka, Japan), 800 nM of the primer pair (primer: 5′-caccacctcccggtaccata-3′/5′-ccgcacctggttttgctt-3′, TaqMan Probe (Hokkaido System Science, Sapporo, Japan): FAM-aacctgcccgccggctatacactg-TAMRA), and 200 nM of TaqMan Probe (total volume: 50 μl). The mixture was amplified in an automated fluorometer (ABI Prism 7000 Sequence Detection System, Applied Biosystems, Foster City, CA) under the following conditions: one cycle of 50 °C for 2 min and 95 °C for 10 min, and 45 cycles of 95 °C for 20 s and 62 °C for 90 s Ad5 vector plasmid pAdHM4 was used as a standard. The PCR conditions showed a strong linear response ($R^2 = 0.997$) and efficiency (slope = −3.29).

2.5. Culture of trophoblast stem cells and induction of differentiation

Mouse TS cells (kindly provided by Dr. Satoshi Tanaka, University of Tokyo, Japan) were maintained in a proliferative state in a medium consisting of 70% primary mouse embryonic fibroblast-conditioned medium (EMFI-CM), 30% TS cell medium, 25 ng/ml FGF4 (PeproTech, Rocky Hill, NJ) and 1 μg/ml heparin (Sigma, St. Louis, MO); this state was denoted as "stem-cell conditions," as previously described in Refs. [18,19]. To induce differentiation of TS cells, FGF4, heparin, and EMFI-CM were removed from the medium to give "differentiation conditions." To induce differentiation of TS cells into giant cells, all-*trans*-retinoic acid (RA) (Sigma) was added to a final concentration of 5 μM to give "giant cell conditions." The TS cell medium consisted of RPMI1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 20% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate (Invitrogen), 100 μM β-mercaptoethanol (Invitrogen), 2 mM L-glutamine (Invitrogen), streptomycin (100 μg/ml), and penicillin (100 U/ml).

2.6. Statistical analysis

All results are expressed as means ± SEM or means ± SD. Statistical comparisons were made by using Student's *t*-test or Scheffé's method after analysis of variance (ANOVA). Results were considered significantly different at $P < 0.05$.

3. Results

3.1. Ad-RGD-mediated gene transfer in pregnant mice

To determine the distribution of Ad-RGD in pregnant mice, Ad-RGD carrying the firefly luciferase gene (Ad-RGD-Luc) was intravenously (i.v.) injected and the level of luciferase gene expression was observed after 48 h (Fig. 1A). As Mizuguchi and others have previously reported in Refs. [6,8,20], the highest gene expression level was observed in the liver. Interestingly, the second-highest luciferase activity was found in the placenta. Comparison of Ad-RGD-Luc and WT-Ad carrying the firefly luciferase gene (WT-Ad-Luc) revealed that the high level of gene transfer into the placenta by Ad-RGD-Luc was related to the presence of the RGD amino acid sequence in the fiber knob of Ad-RGD (Fig. 1B). The enhanced gene expression in the placenta following i.v. injection of Ad-RGD was not affected by the time of i.v. injection or the amount of vector (Fig. 1C and D). Transgene expression was sustained for at least 7