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Efficient targeting of adenoviral vectors to integrin positive vascular cells utilizing a CAR-cyclic RGD linker protein ☆

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Abstract

Vascular smooth muscle (VSMC) and endothelial cells (EC) are particularly resistant to infection by type 5 adenovirus (Ad) vectors. To overcome this limitation and target Ad vectors to ubiquitously expressed $\alpha_V\beta_{3/5}$ integrins, we have generated a linker protein consisting of the extracellular domain of the coxsackie adenovirus receptor (CAR) connected via avidin to a biotinylated cyclic (c) RGD peptide. After optimization of CAR to cRGD and to Ad coupling, infection of mouse heart endothelial cells (H5V) could be augmented significantly, as demonstrated by 600-fold increased transgene expression levels. In EOMAs, a hemangioendothelioma-derived cell line, the fraction of infected cells was enhanced 4- to 6-fold. Furthermore, the fraction of infected primary mouse VSMC was increased from virtually 0% to 25%. Finally, in human umbilical vein endothelial cells, the number of GFP positive cells was enhanced from 2% to 75%. In conclusion, CAR-cRGD is a versatile and highly efficient construct to target Ad vectors to both transformed and primary VSMC and EC.

Keywords: Adenovirus; Targeting; Cyclic RGD peptide; Endothelial cells; Vascular smooth muscle cells

Recombinant type 5 adenovirus (Ad) vectors are extensively used to modulate gene expression in a wide variety of cells and organs, both in vitro and in vivo. Part of this popularity can be ascribed to their relatively straightforward generation and amplification to high titers [1]. Ad entry and infection of cells requires at least two distinct interactions. First, attachment of the virus particle occurs via interaction of its fiber knob with the Coxsackie adenovirus receptor (CAR) present on the cell surface [2–5]. Second, the Arg-Gly-Asp (RGD) motifs present in the viral penton base will bind to $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins on the target cell surface and trigger internalization via receptor-mediated endocytosis [6–8]. In addition, recent data have shown the involvement of heparan sulfate glycosaminoglycans (HSGs) in adenoviral entry in vivo [9].

Recombinant Ad vectors encoding numerous wild type and mutant genes, as well as short hairpin RNA molecules have been generated. However, the application of Ad vectors in CAR negative cell lines, such as vascular smooth muscle cells (VSMC) and endothelial cells (EC) [10–12], is hampered by low infection efficiencies at low multiplicity of infection (MOI) and Ad associated cytotoxicity at high MOI.

☆ *Abbreviations:* Ad, type 5 adenovirus; cRGD-Ad, cRGD targeted Ad-vector; Bio-cRGD, biotinylated cyclic RGD peptide; CAR, coxsackie adenovirus receptor; CHO, Chinese hamster ovary; EC, endothelial cells; GFP, green fluorescent protein; HUVECs, human umbilical vein endothelial cells, LacZ, β-galactosidase; Luc, luciferase; MOI, multiplicity of infection; VSMC, vascular smooth muscle cells.

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To expand the applicability of Ad-mediated gene transfer, various strategies to modify Ad tropism have been undertaken. In the genetic modification approach, peptide ligands have been incorporated into the HI-loop of the Ad fiber knob [13–16], added to the C-terminus of the fiber knob [17] or inserted into the hexon protein [18]. However, it is not possible to predict which peptide or protein ligands will be tolerated and do not disturb fiber trimerization and/or capsid function. In addition, for each specific targeting application, rederivatization of the original recombinant Ad vectors is obligatory. Alternatively, bifunctional targeting proteins have been generated consisting of an Ad-binding domain coupled to a peptide or protein that confers a novel specificity [19]. This strategy enables the utilization of existing recombinant Ad vectors, but the generation of the bifunctional targeting protein may require chemical linkage and subsequent purification steps. In addition, Parrot and co-workers have introduced a novel approach to target viral vectors. They launched the concept of metabolically biotinylated vectors [20,21] and demonstrated the utility of the avidin–biotin based system for vector targeting.

Recently we have combined the advantages of the latter two targeting strategies, by developing a bi-functional linker protein that exploited the avidin–biotin concept (Gras, personal communication). This linker protein consists of the extracellular domain of the CAR fused to chicken avidin, which functions as a universal docking site for biotinylated ligands. It was demonstrated that a biotinylated dA_6G_{10} oligonucleotide coupled to the CAR-Avidin linker confers macrophage specificity (Gras, personal communication). In this study, the CAR-Avidin linker protein is coupled to a biotinylated cyclic RGD peptide (bio-cRGD) to increase infection efficiency of EC and VSMC. This cRGD peptide has a high affinity for $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins [22], which are expressed ubiquitously on transformed cell lines and most primary cells. It is demonstrated that linking of Ad to the CAR-cRGD targeting construct resulted in a highly significant improvement of infection efficiencies of transformed and primary VSMC and EC at all MOI used.

Materials and methods

Cell culture. Chinese hamster ovary (CHO) cells, H5V (mouse endothelial cell line derived from heart) and EOMA (mouse hemangioma-derived micro vascular cell line), were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL). Ramos cells (Burkitt lymphoma cells) and K-562 cells (chronic myelogenous leukemia cells from blast crisis) were cultured in RPMI 1640 medium. All media were supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and glutamax (Invitrogen). Human umbilical vein endothelial cells (HUVECs) were a generous gift from E. Pieterman (TNO Prevention and Health, Leiden, The Netherlands) and were isolated as previously described [23,24] and grown in medium 199 with 10% human serum. Mouse VSMC were isolated from aorta from male C57Bl6 mice as previously described [25] and cultured in DMEM with 10% newborn calf serum (NCS). All cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Production of recombinant Ad vectors. Recombinant E1, E3-deleted Ad-vectors expressing β-galactosidase gene (Ad.LacZ) and firefly luciferase (Ad.Luc) under the control of the cytomegalovirus promoter (CMV) were kindly provided by, respectively, Dr. Willnow (Houston, USA) and Dr. Hoeben (LUMC, Leiden, The Netherlands). Recombinant adenovirus vector carrying the green fluorescent protein under control of CMV (Ad.GFP) was constructed using the Ad-Easy-1 system as previously described by [26]. Additionally, the viruses were propagated in PERC6 cells as described [27]. The purification process involved two rounds of CsCl ultracentrifugation and dialysis against dialysis buffer (25 mmol/L Tris, 137 mmol/L NaCl, 5 mmol/L KCl, 0.73 mmol/L NaH_2PO_4, 0.9 mmol/L $CaCl_2$, and 0.5 mmol/L $MgCl_2$, pH 7.45) followed by dialysis against the same buffer supplemented with sucrose (50 g/L). Plaque titration was performed on 911 cells according to standard techniques [28]. Aliquots of 50 μl virus were stored at −80 °C. Generally, virus titers of the stocks varied from 1×10^{10} to 1×10^{11} pfu/ml.

Generation CAR-Avidin linker protein. The CAR-Avidin linker protein was generated by joining a series of PCR-generated fragments. In short, the extracellular domain of the coxsackie adenovirus receptor (CAR) was obtained by PCR using the plasmid pCAR (kind gift of Prof. R. Hoeben, LUMC, Leiden) as template (oligos: 5′-GCG GCC GCG GGT ACC CAC GGC ACG GCA G-3′ and 5′-CTA GCT AGC AGC TTT ATT TGA AGG AGG GAC-3′). The avidin fragment was obtained by RT-PCR on total RNA from chicken fibroblasts with random hexamer oligonucleotides and subsequent PCR using primers 5′-CGC GGA TCC GCC AGA AAG TGC TCG CTG -3′ and 5′-CCA TCG ATG GTC ACT CCT TCT GTG TGC G -3′. The CAR fragment was cloned into the pSG8 vector (generous gift of Prof. Henk Stunnenberg, Nijmegen, The Netherlands), in front of the VSV and His6 tag. Avidin was cloned in-frame into pSG8CAR behind the VSV and His6 tag. All constructs were sequence verified.

Production and purification CAR-Avidin linker protein. For production Cos-1 cells were transfected with pSG8CAR-Avidin using Fugene6 (Roche, Basel, Switzerland). Forty hours after transfection (serum-free, biotin-free culture medium), the supernatant, containing the linker proteins, was harvested. Linker proteins were purified from the supernatant by immobilized metal affinity chromatography using Talon metal affinity resin (Clontech, Palo Alto, USA). Equilibrated culture supernatant (300 mM NaCl, pH 7.0, and 20% glycerol) was incubated with Talon, 20 min at room temperature. After extensive rinsing (50 mM $NaPO_4$, 300 mM NaCl, and 20% glycerol, pH 7.0), resin was pre-eluted (4 volumes; 50 mM $NaPO_4$, 300 mM NaCl, 2,5 mM imidazole, and 20% glycerol) prior to its elution (10 volumes; 50 mM $NaPO_4$, 300 mM NaCl, 150 mM imidazole, and 20% glycerol). Presence of linker protein in the purified samples was detected by SDS–PAGE and Western blotting analysis using Hybond ECL nitro cellulose membranes (Amersham Biosciences, Buckinghamshire, UK) and antibodies P5D4 (α-VSV) or α-Avidin (Abcam, Cambridge, UK). Elution fractions 3–5 contained the linker protein and were dialyzed against PBS.

Quantification of CAR-Avidin linker protein. The linker protein was quantified by a biotin binding assay. Ten microliters of the linker protein elution fraction or an avidin calibration range of 0.3–10 pM avidin was incubated with 0.2 μl [^{3}H]Biotin (Du Pont NEN Research Products, Boston, MA, USA) for 1 h. The total reaction mixture was applied on a Sephadex G-50 column to separate CAR-Avidin bound biotin from the free biotin. The elution fractions were counted for [^{3}H]biotin radioactivity using 5 ml of Hionic fluor scintillation cocktail (Packard Instrument, Perkin Elmer, Boston, MA, USA) in a Packard 1500 TriCarb liquid scintillation analyzer. The summed radioactivity in peak fractions 3–5 correlated with the amount of avidin present in the sample ($R^2 = 0.997$). Elution fraction 3, which had the highest concentrations, was used for experiments and stored at −80 °C. A yield of the order of 900–1000 μg was typical.

Biotin binding assay. CAR-Avidin (5 μl of 30 nM) was incubated for 1 h at RT with bio-cRGD (cdFK(ε-C6-biotin)RGD), from Asynth Service BV (Roosendaal, Netherlands), at molar ratios ranging from 1:0.001 to 1:3, after which 2 μl of [^{3}H]biotin (NEN) was added and the mixture was incubated again for 1 h. To separate the CAR-Avidin-(^{3}H- or cRGD-)