Journal of Controlled Release 150 (2011) 196–203

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Targeting adenovirus gene delivery to activated tumour-associated vasculature via endothelial selectins

Houria Bachtarzi [a], Mark Stevenson [a,*], Vladimir Šubr [b], Karel Ulbrich [b], Leonard W. Seymour [a,*], Kerry D. Fisher [a]

[a] Department of Clinical Pharmacology, University of Oxford, Old Road Campus, Headington, Oxford, OX3 7DQ, UK
[b] Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Heyrovsky Sq. 2, 162 06 Prague 6, Czech Republic

ARTICLE INFO

Article history:
Received 14 May 2010
Accepted 8 October 2010
Available online 18 October 2010

Keywords:
E-selectin
pHPMA
Adenovirus
Vascular targeting
Cancer

ABSTRACT

Clinical experience with adenovirus vectors has highlighted the need for improved delivery and targeting. Tumour-associated endothelium offers an additional mechanism for enhanced viral uptake into tumours which is accessible for systemic gene delivery. Building on expertise in using polymer 'stealthed' viruses for targeting *in vivo*, adenovirus expressing luciferase (Adluc) was coated with an amino-reactive polymer based on poly [N-(2-hydroxypropyl) methacrylamide] to ablate normal infection pathways. Direct linkage of a monoclonal antibody against E-selectin (MHES) demonstrated E-selectin-specific transduction of tumour necrosis factor-α (TNF-α)-activated endothelial cells. A two-component targeting system using protein G was developed, to provide optimal antibody orientation. We report an enhancement in transduction of TNF-α-activated endothelium *in vitro* and *ex vivo* in a human umbilical vein cord model using the MHES antibody. Similarly a virus retargeted using a chimeric P-selectin Glycoprotein Ligand-1-Fc fusion (PSGL-1) protein showed better circulation kinetics and significant uptake into HepG2 xenografts following systemic administration in mice, with 36-fold higher genome copies, compared with non-modified virus. Immunohistochemistry staining of tumour sections from mice treated with PSGL-1-retargeted virus showed a co-localisation of firefly luciferase with CD31 suggesting selective endothelial targeting. Employment of optimal viral modification using protein G will enable exploration and comparison of alternative targeting ligands targeting tumour-associated endothelium.

1. Introduction

Gene therapy as a concept provides an appealing alternative to small drugs for the treatment of complex diseases like cancer, allowing expression of specific therapeutic proteins rather than being limited to inhibition of tumour-associated targets. In addition, gene therapy offers the advantages of power through amplification of gene products, specificity through the use of tissue-specific promoters and, versatility, delivering agents with different mechanisms of action such as: suicide genes (virus-directed enzyme prodrug therapy) and immuno-modulatory proteins [1]. To date, most cancer gene therapy approaches have targeted tumour cells by local administration protocols, with few having evaluated targeting tumour-associated endothelium [2,3].

Therapeutic strategies that target tumour-associated vasculature have several advantages compared with direct targeting of tumour parenchymal cells. Firstly, the endothelium represents a primary point of contact with blood-borne molecules and is easily accessible from the bloodstream. Secondly, tumour-associated endothelium displays many similar properties regardless of tumour type that distinguish it from normal endothelium, giving rise to a broad class of therapy. Lastly, destruction of a relatively small number of endothelial cells has the potential to deprive many tumour cells of oxygen and nutrients, leading to an avalanche of tumour cell death [4].

Unlike normal endothelium, tumour-associated endothelial cells often display an inflamed phenotype due to activation by cytokines emanating from the tumour including tumour necrosis factor-α (TNF-α) and interferon-γ (INF-γ) [5]. This pro-inflammatory activation promotes faster endothelial proliferation and results in up-regulated levels of cell surface markers. αvβ3 integrins, vascular endothelial growth factor receptors and E-selectin have all been reported to be over-expressed on tumour-associated endothelium [6,7].

Selectins are a family of structurally-related, Ca^{2+}-dependent, type-I transmembrane carbohydrate-binding proteins which include: E-selectin (CD62E), P-selectin (CD62P) and L-selectin (CD62L). E-selectin and P-selectin are of particular interest in inflammation and cancer, as they are functionally used by circulating macrophages to home towards the regions of disease [8,9]. Both E- and P-selectins are

* Corresponding authors. Stevenson is to be contacted at Tel.: +44 1865 617041; fax: +44 1865 617028. Seymour, Tel.: +44 1865 617040; fax: +44 1865 617028.
E-mail addresses: houria.bachtarzi@clinpharm.ox.ac.uk (H. Bachtarzi), mark.stevenson@clinpharm.ox.ac.uk (M. Stevenson), len.seymour@clinpharm.ox.ac.uk (L.W. Seymour), kerry.fisher@clinpharm.ox.ac.uk (K.D. Fisher).

0168-3659/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2010.10.011

expressed on activated endothelial lumen, are rapidly internalised via an endosomal pathway, and have successfully been exploited to target tumour-associated vasculature *in vivo* [10,11]. Redirecting viral tropism via endothelial selectins is therefore an attractive option worth further study. Ogawara et al. examined redirecting adenoviral tropism using bifunctional PEG attached to the surface of the virus capsid inhibiting fibre knob/CAR interactions. Subsequent introduction of E-selectin antibodies to functional groups of the PEG molecule allowed retargeting to activated endothelial cells *in vitro* and *in vivo* [12]. Despite the conceptual advantages of targeting therapeutics using viruses to endothelium, this strategy remains fraught with difficulties due to complement [13,14], antibodies [14], blood cell binding [15–17], clotting factor interactions [18,19] and liver capture [20,21]. In an effort to improve virus bloodstream circulation and survivability for a sufficient time to allow vascular targeting, we have used a polymer encapsulation technique based on poly-[N-(2-hydroxypropyl) methacrylamide] (pHPMA) to de-target viruses and protect against unwanted vector–host interactions [17]. Several ligands have previously been successfully incorporated ranging from peptides to monoclonal antibodies [22–24]. One drawback of this approach for using antibodies as targeting ligands is the random nature of antibody orientation on the polymer due to the presence of multiple reactive amino groups. Krasnykh and co-workers have previously shown that genetic modification of the Ad fibre protein incorporating the immunoglobulin (Ig)-binding domain of *Staphylococcus aureus* protein A, created a vector capable of binding targeting ligands incorporating the Fc domain of immunoglobulin. Targeting ligands incorporating CD40 single chain antibodies or CD40L mediated a significant increase in the transduction of CD40-positive target cells [25].

In order to identify receptors and ligands that may be useful for endothelial targeting we chose to use *Streptococcus aureus* protein G (StrepG) as a platform targeting system. The advantage of this approach is that it allows a correctly orientated linkage of Fc-bearing ligands to tropism-ablated polymer coated virus particles. Accordingly in this study we have explored the use of StrepG-based retargeting of polymer coated adenovirus to endothelial selectins *in vitro, ex vivo* and *in vivo*.

2. Materials and methods

2.1. Cell lines

Human Umbilical Vein Endothelial cells (HUVECs; PromoCell, Heidelberg, Germany) were maintained in EGM™-2-Endothelial Cell Medium-2 (Cambrex Bio Science, Walkersville, USA), supplemented with hydrocortisone, h-FGF-B, VEGF, R3-IGF-1, ascorbic acid, heparin, FBS, hEGF, and GA-1000 (Cambrex Bio Science, Walkersville, USA). Brain endothelioma cell line (bEnd-3; ATCC, Manassas, USA) was grown in Dulbecco's modified Eagle's medium (DMEM) containing 20 mM HEPES, 10% foetal calf serum (FCS) and 2 mM glutamine (PAA laboratories, Yeovil, UK). HepG2 human hepatocyte carcinoma were maintained in Minimal Essential Medium with Earle's salts (EMEM), 2 mM glutamine (PAA Laboratories GmbH, UK), supplemented with 10% FCS, and 0.1 mM non-essential amino-acids. The H18/7 (mouse IgG2a anti-human E-selectin) monoclonal antibody-producing hybridoma (ATCC, Manassas, USA) was grown in RPMI 1640 medium (PAA Laboratories, Yeovil, UK) supplemented with 10% horse serum (Invitrogen, Paisley, UK) with NEAA (PAA laboratories, Yeovil, UK) and 1 mM sodium pyruvate (Sigma-Aldrich, Gillingham, UK).

2.2. Antibodies

MHES (mouse IgG2a anti-human E-selectin) monoclonal antibody was purified from the H18/7 culture medium by protein A affinity columns (nProtein A sepharose 4 fast flow, GE Healthcare). The mouse IgG2a isotype control monoclonal antibody was kindly provided by Prof P.E. Thorpe (University of Texas, Southwestern Medical Center, Dallas). RMES (mouse IgG2a anti-rat E-selectin) monoclonal antibody was purchased from BD Pharmingen (Oxford, UK). Recombinant human P-selectin Glycoprotein Ligand-1 (PSGL-1)-/IgG1 Fc fusion protein was purchased from R&D systems (Abingdon, UK). Ad5 fibre was a gift from Dr. R. Carlisle (University of Oxford, UK).

2.3. Viruses

E1, E3-deleted Ad5 expressing cytomegalovirus immediate-early (CMV IE) promoter-driven luciferase (Adluc) was grown in HEK293 cells until a cytopathic effect was observed. Virus was released from cells by freeze/thawing, extracted with *N*-butanol and purified by double banding on a CsCl gradient with an intermediate benzonase (Merck Biosciences, Nottingham, UK) step to remove contaminating DNA. The virus was dialysed into 50 mM HEPES/phosphate-buffered saline (PBS), 10% glycerol pH 7.8 (storage buffer) and stored in small aliquots. Particle numbers were estimated using the PicoGreen assay (Molecular Probes, Paisley, UK) assuming 1 μg/ml DNA = 2.7×10^{10} particles/ml [26]. Typical particle:infectivity ratios of 10:1 were obtained.

2.4. Virus modification

2.4.1. Direct conjugation of antibody

Amino-reactive multivalent hydrophilic polymer based on poly[N-(2-hydroxypropyl)methacrylamide] [pHPMA-gly-gly-TT] [27] supplied by Institute of Macromolecular Chemistry (Prague, Czech Republic) was resuspended in water to make a 100 mg/ml polymer stock solution. Ten μl was then added to 90 μl of virus for 40 min at room temperature (pcAdluc). Free un-reacted polymer was removed from pcAdluc using S400 micro-spin columns (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions. Mouse anti-human E-selectin monoclonal antibody (MHES) was conjugated to pcAdluc by adding 0.5 mg/ml antibody to the virus initially for 1 h at room temperature and then overnight at 4 °C.

2.4.2. Indirect conjugation through protein G

pcAdluc was generated by incubating 1 ml of Adluc ($1–3 \times 10^{12}$ particles/ml) in storage buffer with the pHPMA-gly-gly-TT polymer at a final concentration of 10 mg/ml for 40 min at room temperature. pcAdluc was purified away from the unincorporated polymer by caesium chloride banding and subsequently mixed with *S. aureus* protein G (StrepG) (Sigma-Aldrich, Gillingham, UK), at a final concentration of 1 mg/ml for 1 h at room temperature before incubation overnight at 4 °C. Protein G-reacted polymer coated virus (StrepGpcAdluc) was purified again by caesium chloride banding to remove unincorporated StrepG proteins. Mouse anti-human E-selectin monoclonal antibody (MHES) (10 μg/ml), rat anti-mouse E-selectin monoclonal antibody (RMES) (1-100 μg/ml) or chimeric P-selectin Glycoprotein Ligand-1 (PSGL-1)-Fc fusion protein (1-50 μg/ml), were linked via their Fc regions to StrepG-modified polymer coated virus through affinity interaction following a 1 h-incubation at room temperature.

2.5. Dot blot analysis

The amount of protein G associated with each virus particle was determined by dot blot analysis. A standard curve of protein G ranging from 78 to 5000 pg was prepared by serial dilution and application to a nitrocellulose membrane. Purified StrepGpcAdluc was serially diluted and added to the nitrocellulose membrane (ranging from 1.5×10^6 to 1×10^8 virus particles). The nitrocellulose was then probed with polyclonal anti-protein G antibodies (Abcam, Cambridge, UK) diluted 1:2000, followed by goat anti-rabbit-HRP antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:2500. Signals were visualised using ECL Western blotting detection reagent (GE Healthcare Biosciences, Chalfont St. Giles, UK).