International Journal of Pharmaceutics 391 (2010) 155–161

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Selective targeting of adenovirus to $\alpha_v\beta_3$ integrins, VEGFR2 and Tie2 endothelial receptors by angio-adenobodies

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ARTICLE INFO

Article history:
Received 8 January 2010
Received in revised form 23 February 2010
Accepted 28 February 2010
Available online 6 March 2010

Keywords:
Adenovirus
Angiogenesis
$\alpha_v\beta_3$ Integrins
VEGFR2
Tie2

ABSTRACT

Tumor angiogenesis is a prominent mechanism, driving the development and progression of solid tumors and the formation of cancer cell metastasis. Newly formed tumor vessels represent an elective target for the activity and the delivery of cancer therapeutics. We targeted adenovirus (Ad5) to endothelial receptors which are up-regulated during the formation of new blood vessels, to enhance the efficiency of anticancer gene therapy applications.

Bifunctional angio-adenobodies were constructed by the fusion of a single chain antibody directed against the adenoviral fiber knob, to different peptides recognizing the $\alpha_v\beta_3$ integrins, VEGFR2 and Tie2 receptors on endothelial cells. The angio-adenobodies were coupled to the adenoviral vector, containing luciferase and GFP as reporter genes.

In vitro data showed selective targeting of the Ad5 to the endothelial receptors both in mouse (H5V) and human cell lines (HUVEC). H5V cells, refractory to Ad5 infection, showed high level of luciferase expression when cells were infected with targeted virus. Viral transgene expression increased in HUVEC cells when cells were infected with Ad5 conjugated with angio-adenobody thereby demonstrating the affinity of the peptides for human endothelial cells also. *In vivo* data obtained from mice bearing a C26 colon carcinoma subcutaneously show viral transgene expression only in tumors infected with angio-adenobodies retargeted adenovirus.

The results of the present study demonstrate that endothelial targeted angio-adenobodies represent a versatile tool to direct adenovirus from its native receptors to the integrins $\alpha_v\beta_3$, VEGFR2 and Tie2 receptors that are fundamental in many angiogenesis related diseases such as cancer.

1. Introduction

The formation of new blood vessels (angiogenesis) is a process that accompanies the development of tumors and the formation of metastasis (Carmeliet, 2005a). Since Folkman hypothesized in 1971 (Folkman, 1971) that tumor progression could be inhibited by blocking the formation of tumor blood vessels, many researches have been done to discover the players and their roles in blood vessel formation in physiological and tumor conditions (Carmeliet, 2005b). The tumor blood vessels appear structurally immature, leaky and hemorrhagic. They display a specific profile in protein expression and/or up-regulation when compared to those occurring in physiological conditions. In these terms, these proteins are hallmarks that are used as optimal targets to selectively deliver cancer therapeutics (Ferrara and Kerbel, 2005).

Viral vectors, such as the Ad5, have been extensively used in anticancer therapies (Alemany, 2007; Kanerva and Hemminki, 2005). Injection of the Ad5 virus directly in the tumor mass however has some limitations. Not all the tumors are easily accessible and the virus localizes mainly close to the needle tract. Targeting of the adenovirus to up-regulated receptors in tumor blood vessels would facilitate the arrival of the virus in tumors located in non-accessible positions for injection, would allow the diffusion of the virus in the tumor mass via the blood vessels and would permit the virus to reach the sites of metastasis formation. On the other hand, systemic administration of Ad5 results in the infection of organs/cells expressing the Ad5 native receptors, such as the liver, which is responsible for its clearance. Different receptors such as the coxsackie B virus and adenovirus receptor (CAR) (Bergelson et al., 1997), the heparan sulfate glycosaminoglycans

Abbreviations: Ad, adenovirus; CAR, oxsackie B virus and adenovirus receptor; HS-GAG, heparan sulfate glycosaminoglycans; SR-A, scavenger receptor A; scFv, single chain variable antibody fragment; VEGF, vascular endothelial growth factor; RGD, Arg-Gly-Asp.

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doi:10.1016/j.ijpharm.2010.02.032

(HS-GAGs) (Dechecchi et al., 2001), and the scavenger receptor A (SR-A) (Haisma et al., 2008a) have been described to be relevant in viral uptake and infection.

Several strategies have been employed to detarget the Ad5 from its native receptors and to direct it to specific cell surface molecules (Campos and Barry, 2007a). Adenoviral targeting has been performed by genetic alterations of the viral capsid proteins, such as the fiber, penton base, hexon and pIX, which are responsible for adenoviral attachment to cell surface receptors and/or internalization (Noureddini and Curiel, 2005; Mizuguchi and Hayakawa, 2004; Baker et al., 2005; Campos and Barry, 2007b). Non-genetic targeting has been achieved, either by coating Ad with a polymer linked to a targeting peptide or antibody (Ogawara et al., 2004), or by the use of bispecific antibodies or adenobodies, which consist of a single chain Fv antibody fragment (scFv) directed against the fiber knob on one side and of respectively a single chain antibody or a peptide directed against specific cell receptors (Korn et al., 2004; Nettelbeck et al., 2004; Wickham et al., 1996; Campos and Barry, 2007b; Haisma et al., 2000) on the other side.

In this study we constructed angio-adenobodies composed of two fragments: a single chain antibody and a vascular targeting peptide specific for proteins that are up-regulated in tumor angiogenesis. The scFv (S11) (Schoemaker et al., 2008) provides a mechanic link between the virus and targeting peptide and physiologically inhibits viral uptake by native receptors such as CAR (Schoemaker et al., 2008) and SR-A (Haisma et al., 2008b). The peptides have been selected to target respectively the vascular endothelial growth factor receptor 2 (VEGFR2), the tyrosine kinase receptor Tie2, and the cell adhesion receptor $\alpha_v\beta_3$ integrins. The VEGFR2 is a kinase receptor that during angiogenesis upon activation via angiogenic factors such as vascular endothelial growth factor (VEGF), stimulates proliferation, migration and survival of endothelial cells (Kowanetz and Ferrara, 2006; Ferrara, 2005a; Carmeliet, 2005c). Moreover VEGF and VEGFR2 were found to be expressed in many types of tumors acting as an autocrine growth factor for tumor cells (Kerbel, 2008). VEGF signaling can be inhibited by antibodies and small molecules including VEGFR2 soluble forms that inactivate VEGF or its receptor (Ferrara, 2005b; Shojaei and Ferrara, 2007; Timar and Dome, 2008). The Tie2 receptors originally shown to be over-expressed in tumor vessels (Lewis et al., 2007; Kobayashi and Lin, 2005; Martin et al., 2008), more recently have been found to be expressed in several types of cancers including leukemia and solid neoplasm (Martin et al., 2008). Tie2 has different effects on malignancies ranging from angiogenesis and inflammation to vascular extravasion (Martin et al., 2008; Li et al., 2005). The role of Tie2 in angiogenesis during tumor development has been demonstrated successfully by blocking the activity of the Tie2 receptor with a soluble form of the Tie2 extracellular domain (Martin et al., 2008). The $\alpha_v\beta_3$ integrins, over-expressed in blood vessels of tumor, have a key role in endothelial cell survival and migration during angiogenesis. Antagonists of $\alpha_v\beta_3$ successfully inhibited angiogenesis and tumor growth in a variety of animal cancer models (Brooks et al., 1994a,b; Erdreich-Epstein et al., 2000; Avraamides et al., 2008).

In this study, the ATWLPPR, RLVAYEGWV and RGD peptides have been selected to target respectively VEGFR2, Tie2 and $\alpha_v\beta_3$ integrins. The ATWLPPR targeting peptide was isolated by a phage epitope library by affinity for membrane-expressing VEGFR2. The peptide was able to block interaction between the vascular endothelial growth factor and its cognate receptor VEGFR2 thereby inhibiting VEGF-mediated proliferation of human vascular endothelial cells (Binetruy-Tournaire et al., 2000). The RLVAYEGWV peptide was identified by a phage display approach as a substrate to inhibit the kinase activity of the Tie2 receptor (Deng et al., 2001). The RGD (Arg-Gly-Asp) motif recognized by the $\alpha_v\beta_3$ integrin (Koivunen et al., 1995) has been used to develop cyclic peptides to target $\alpha_v\beta_3$ on angiogenic endothelium (Kok et al., 2002). Both the ATWLPPR and the cyclic RGD peptides have been conjugated to the surface of PEG-liposomes resulting in binding of these liposomes to endothelial cells *in vitro* (Janssen et al., 2003).

In this study we demonstrated that all three angio-adenobodies could detarget the virus from native receptors and could target selectively mouse and human endothelial cells *in vitro*. Moreover, systemically injected Ad5 coupled to angio-adenobodies, selectively infected C26 colon carcinoma in mice.

Angio-adenobodies represent a versatile tool to be used to target Ad5 carrying anti-tumor therapeutics to tumor endothelium.

2. Materials and methods

2.1. Cell culture

The adenoviral transformed human embryonic kidney cell line 293 was cultured in DMEM-F12 (Gibco BRL) containing 10% heat-inactivated FBS (Invitrogen), 2 mM L-glutamine (Gibco BRL) and penicillin (100 U/ml)/streptomycin (μg/ml) (Gibco BRL). Human umbilical vein endothelial cells (HUVEC) were obtained from Endothelial Cell Facility RuG/UMCG (Groningen, The Netherlands). The H5V mouse endothelioma cell line was kindly provided by Dr. A. Vecchi (Mario Negri, Institute for Pharmacological Research, Milan, Italy). These cells were grown in a Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine (Gibco BRL), and 300 μg/ml gentamycin (Gibco BRL). The human hepatocellular carcinoma (HepG2) cell line and the murine colon carcinoma (C26) cell line, kindly provided by Dr. C.A.A.H. Daemen (Medical Microbiology: Molecular Virology, University Center Groningen, The Netherlands) were cultured in DMEM medium supplemented with 10% heat-inactivated FBS and penicillin/streptomycin. All cells were cultured in tissue culture flasks at 37 °C in humidified 5% CO_2/95% air. Upon confluency, the cells were detached from the surface by trypsin/ethylenediaminetetraacetic acid (EDTA; 0.5/0.2 mg/ml in PBS) and split at a 1:5 ratio.

2.2. Constructions of angio-adenobodies

For the construction of angio-adenobodies, pUC119-pelB-S11-myc-His vector was used (a generous gift of Dr. R. Hawkins, Bristol University, UK; SJ Watkins). The peptide coding sequences were inserted downstream the Pel B leader sequence and upstream of the single chain antibody fragment (scFv) against adenoviral fiber knob (S11), the *myc* and histidine tag (6His) as depicted in Fig. 1a.

The three peptides ACDCRGDCFCG (I), ATWLPPR (V), RLVAYEGWV (T) were obtained by PCR (94 °C for 1 min, 46 °C for 30 s, 72 °C for 1 min) using the primers described in Table 1. The PCR amplified regions of DNA were gel purified and cloned as NcoI/BamHI fragments into the pUC119-pelB-S11-myc-His vector. A glycine linker is present between the peptide and the S11. Correct insertion of the DNA fragments was verified by restriction analysis. Each primer was designed to introduce a unique restriction site.

2.3. Expression of angio-adenobodies

BL21 *E. coli*, containing plasmids encoding ACDCRGDCFCG-S11 (I-S11) ATWLPPR-S11 (V-S11) and RLVAYEGWV-S11 (T-S11) angio-adenobodies, were grown overnight at 37 °C in 2TY medium (Invitrogen), which included 100 μg/ml ampicillin (Calbiogen) and 1% glucose (Merk). The overnight culture was diluted 1:100 in 2 l of 2TY medium containing 100 μg/ml ampicillin and 0.1% glucose. Bacterial suspensions were grown at 37 °C (200 rpm) until an OD_{600} of 0.8–1.0 was reached. Protein expression was induced by addition of 1 mM isopropyl B-D-thiogalactoside (IPTG) (Invitrogen)

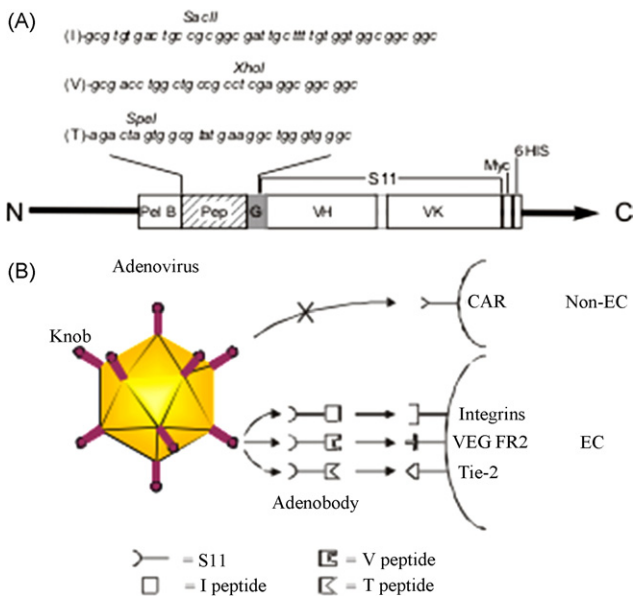

Fig. 1. a Schematic representation of angio-adenobody construction and targeting properties. (A) Three peptides ATWLPPR (V), RLVAYEGWV (T) and ACDCRGDCFCG (I), targeting respectively the VEGFR2, Tie2 and $\alpha_v\beta_3$ integrins receptors were obtained by PCR. By cloning, these were inserted into the pUC119-pelB-S11-myc-His bacterial vector downstream of the pelB region and upstream of the single chain antibody S11 directed against the adenoviral knob. Expression was driven from the upstream LacZ promoter. (B) The angio-adenobodies form a bridge between the virus and the endothelial receptors. The single chain antibody of the fusion protein S11 binds to the viral knob inhibiting Ad5 attachment to the viral natural receptors. The peptides direct adenovirus to the integrins $\alpha_v\beta_3$, VEGFR2 and Tie2 endothelial receptors.

followed by an incubation for 4 h at 30 °C (200 rpm). Bacteria were harvested by centrifugation at 6000 × g (4 °C) for 10 min, resuspended in 200 ml TS buffer (0.2 M Tris–HCl (Sigma), 0.5 M Sucrose (Merk), pH 8.0) and supplemented with 4 ml of 50 mM EDTA (Gibco BRL). After 10 min of shaking at room temperature, the cells were harvested by centrifugation at 8000 × g (4 °C) for 20 min and the supernatant containing the periplasmic content was collected.

2.4. Purification of adenobodies by FPLC

Purification of the His-tagged angio-adenobodies was performed by metal-affinity chromatography. A HiTrap Chelating HP column (Amersham) was charged with 0.1 M $NiSO_4$. The column was equilibrated with binding buffer (20 mM sodium phosphate buffer, 0.5 M NaCl (Merk), pH 7.4) containing 5% elution buffer (20 mM sodium phosphate buffer, 0.5 M NaCl, 0.5 M Imidazole (Fluka), pH 7.4). The periplasmic fraction containing the angio-adenobody was, after dialysis against binding buffer, loaded onto the column with a flow rate of 1 ml per minute, using a FPLC. The proteins were eluted with elution buffer containing 250 mM Imidazole. Peak fractions were collected and analyzed by ELISA, SDS page and Western blot. The fractions with high amounts of active fusion proteins were dialyzed against phosphate buffered saline (PBS) at 4 °C.

2.5. Biochemical methods

Purified proteins were analyzed on 12.5% SDS-PAGE stained with Coomassie Brilliant Blue. Western blotting was performed by established procedures. Proteins were detected with primary anti-*myc* monoclonal antibody at a dilution 1:10. This antibody was collected from supernatant of hybridoma cells 1729 (ATCC) culture medium after centrifugation at 200 × g. Rabbit anti-mouse antibody horseradish peroxidase (DAKO A/S) was used as secondary antibody at a dilution 1:3000. For detection AEC staining kit (Sigma) was used according to the manufacturer's instructions. For *in vitro* targeting experiments, cells were seeded in 96-well culture plates. After 24 h, cells were combined with Ad previously incubated with the angio-adenobodies. One hour after infection, the infection medium was replaced by normal culture medium and cells were incubated for 48 h before performing the luciferase assay and GFP observations. To this end, the cells were lysed with cell culture lysis buffer (Promega) and the lysates analyzed with the Luciferase Assay System (Promega) on a Lumicount luminometer (Packard, Groningen, The Netherlands). All data are expressed as relative light units (RLU).

2.6. ELISA

The S11 binding activity of the angio-adenobodies was analyzed by enzyme-linked immunosorbent assay (ELISA). Fusion proteins were added to a 96 wells plate (Nunc) pre-coated with 1 μg adenoviral type 5 knob per well. Elisa was performed with anti-*myc* monoclonal antibody (1:10). Secondary antibody, rabbit anti-mouse HRP conjugate (1:3000) was added and incubated for 1 h at 37 °C, followed by addition of 1,2 orthophenylenediamine dihydrochloride (OPD) substrate (DAKO). After washing the wells, binding of the adenobodies was determined by measuring the absorbance at 490 nm.

2.7. Adenoviral vector

AdTL is an E1- and E3-deleted recombinant serotype 5 adenovirus. It contains a green fluorescent protein (GFP) and luciferase gene expression cassette, each under control of a cytomegalovirus (CMV) promoter (Alemany and Curiel, 2001). The vector was grown on 293 cells and purified in HEPES/sucrose buffer, pH 8.0 according to conventional double CsCl gradient centrifugation method (Becker et al., 1994), and the number of viral particles was calculated from the optical density at 260 nm (OD 260). The number of plaque forming units (PFU) was determined by plaque forming assay.

Table 1
Primer sequences for the construction of fusion proteins.

Peptides	Sequences	Receptors
	Forward primers	
ACDCRGDCFCG (I)	TTA A CC ATG GCC **GCG TGT GAC TGC *CGC GGC* GAT TGC TTT TGT GGT** GGC GGC GGC AGT TCT ATG GCC CAG GTG CAA CTG	Integrins $\alpha_v\beta_3$
ATWLPPR (V)	TTA A CC ATG GCC **GCG ACC TGG CTG CCG *CCT CGA*** GGC GGC GGC AGT TCT ATG GCC CAG GTG CAA CTG	VEGFR2
RLVAYEGWV (V)	TTA ACC ATG GCC***AGA CTA GTG*** **GCG TAT GAA GGC TGG GTG** GGC GGC GGC AGT TCT ATG GCC CAG GTG CAA	TIE2
	Reverse primer	
	TTA ATC TAG ATT ATT AAT GGT GAT GAT GG	

The region in bold represent the coding sequence for the peptide, the underlined sequence indicates the unique restriction sites NcoI, XhoI and SpeI. The gray-shadowed box indicates the glycine linker.

2.8. Targeting assay

H5V, HUVEC and HepG2 cells were cultivated in 96-well tissue culture plates at a density of 10,000 cells per well and incubated overnight. Infections were performed with AdTL alone or AdTL pre-incubated with angio-adenobodies, 30 min at 37 °C, with a multiplicity of infection (MOI) of 5000 for H5V and HUVEC cells, and 1000 for HepG2. The various viral vectors were incubated with the cells for 1 h at 37 °C. The incubation medium was replaced by normal culture medium and cells were incubated for 48 h before performing the luciferase assay. In the case of competition experiments, cells were pre-incubated with recombinant knob (20 μg/ml). All data are expressed as relative light units (RLU).

2.9. Animal infection studies

Anaesthetized (isoflurane/N_2O/O_2 inhalation) balb/6 mice (Harlan CPB), were injected at day one subcutaneously on the right flank with 100 μl PBS containing 1×10^6 C26 colon carcinoma cells. After 15 days, mice that develop a tumor of about 7 mm were anaesthetized and injected via the orbital plexus with adenoviral particles, diluted in HEPES/sucrose buffer (pH 8.0). The virus was injected alone or conjugated to angio-adenobodies. One group of animals was sacrificed 30 min after viral injection to perform viral localization studies. A second group of animals was sacrificed 2 days after viral injection to measure viral transgene expression. Tumor and organs were excised and frozen in liquid N_2. The experiments were performed according to the European ethical board statement.

2.10. Statistical analysis

Results are presented as the mean of at least three independent experiments ± standard deviation. Differences in gene expression were analyzed using a two-tailed paired student's *t*-test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Angio-adenobodies construction and production

In order to selectively target adenoviral vectors to receptors on activated endothelium (Fig. 1A), three angio-adenobodies (S11-V, S11-T and S11-I) were constructed (Fig. 1B and Table 1). The angio-adenobody consists of a single chain antibody (S11) directed against the viral fiber knob and a peptide recognizing endothelium receptors. The peptide sequences selected for affinity with the *V*EGFR2, *T*ie2 and $\alpha_v\beta_3$ *i*ntegrins receptors (V, T and I) were obtained by PCR and were fused by cloning to a bacterial expression plasmid between the pelB sequence, necessary to drive the production of the proteins to the periplasmic space, and the S11 single chain antibody. A glycine linker separates the peptides and S11 to give more flexibility to the molecule. Angio-adenobody productions were performed in the Bl21 *E. coli* strain. Correct protein expression was detected by Western blot analysis (data not shown), at the expected size of 30 kDa using an antibody against the *myc* tag (Fig. 1). The angio-adenobodies were isolated using the 6His tag, present in the construct at the C terminus, by FPLC. An angio-adenobody with a *n*on-*t*arget peptide (S11-NT) was produced and isolated as described above, and was used as a negative control (Schoemaker et al., 2008).

3.2. Binding capacity of S11-angio-adenobodies to the fiber knob

The binding capacity of the S11-angio-adenobodies to the fiber knob was tested by ELISA. The ELISA plates were first pre-coated with purified recombinant Ad5 fiber knob and subsequently by the same volumes of the angio-adenobodies. ELISA was performed with anti-*myc* monoclonal antibody. As shown in Fig. 2, all angio-adenobodies bound to the fiber knob, demonstrating the functionality of S11.

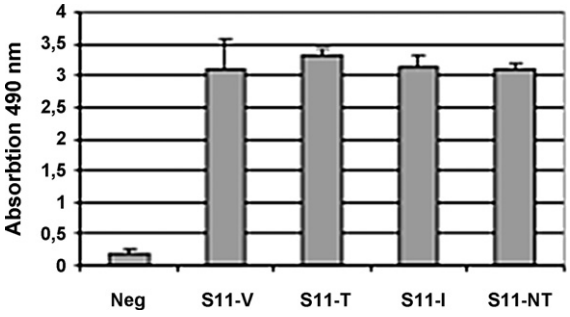

Fig. 2. S11 binds to recombinant fiber knob. Binding activity of the S11 single chain antibody fragment against adenoviral fiber knob using ELISA. ELISA was performed using the three angio-adenobodies targeting the VEGFR2 receptor (S11-V), the Tie2 receptor (S11-T), the $\alpha_v\beta_3$ integrins (S11-I) and the non-targeted angio-adenobody (S11-NT). PBS was used as a negative control (Neg).

3.3. S11 inhibits adenovirus binding to CAR positive cell line

To demonstrate that the S11 single chain Fv antibody fragments, specific for the fiber knob, are able to prevent CAR-dependent viral infection, HepG2 cells were infected with AdTL, coupled with increasing amounts of the S11-T angio-adenobody. After 1 h infection, the medium containing virus was replaced by cellular growth medium. The activity of the luciferase reporter gene was measured 48 h post-infection. As shown in Fig. 3 the levels of luciferase are very low (about 14%) in cells infected with adenovirus coupled with angio-adenobody when compared to those infected with AdTL alone.

By binding to the viral knob the single chain antibody prevents attachment of the virus to the CAR receptor, which is highly expressed on HepG2 cells, and subsequent internalization (Liu and Deisseroth, 2006). Similar results were obtained for AdTL coupled with the other angio-adenobodies (data not shown).

3.4. Selective infection of endothelial cells by targeted adenovirus

The angio-adenobodies were tested for the ability to target adenovirus to VEGFR2, Tie2 and $\alpha_v\beta_3$ integrins receptors on murine endothelial cells (H5V), which are CAR negative and VEGFR2, Tie2 and $\alpha_v\beta_3$ integrins positive. Cells were infected with AdTL alone or with AdTL coupled with increasing concentrations of angio-adenobodies. Luciferase expression was measured 48 h post-

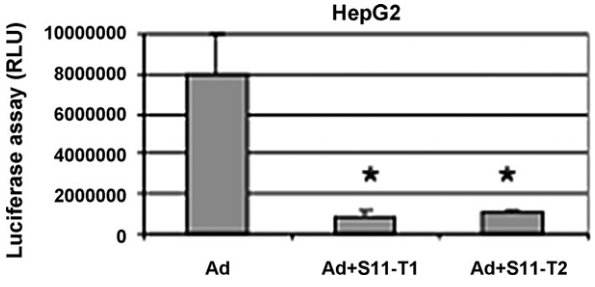

Fig. 3. S11 conjugates inhibit adenovirus binding to CAR positive cell line. HepG2 cells were infected with 1000 viral particles/cell AdTL (Ad) alone or coupled to S11-T. A ratio of 5 μg (Ad + V1) or 10 μg (Ad + V2) angio-adenobody per 10^9 viral particles was used for conjugation. The luciferase activity of the marker gene is expressed in RLU. $^*p < 0.5$ for Ad + V1 and Ad + V2 vs Ad.

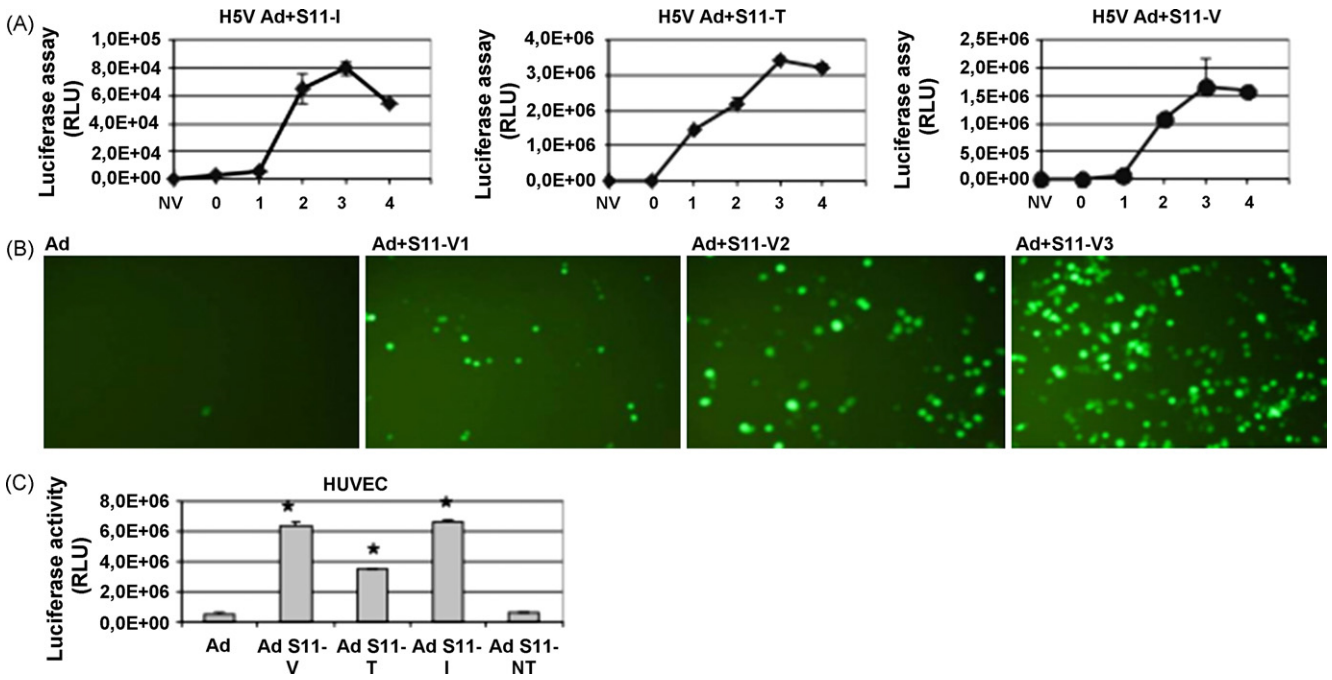

Fig. 4. Selective infection of endothelial cells by targeted adenovirus. (A and B) Luciferase activity and GFP expression of H5V cells infected with AdTL (Ad) non-coupled or coupled to increasing amount of the angio-adenobodies. The amount of angio-adenobodies (μg) per 1×10^9 viral particles was 0 μg (0); 0.5 μg (1);1.5 μg (2); 2.5 μg (3); 5 μg (4). NV = non-infected cells (C) HUVEC cells were infected with AdTL (Ad) or AdTL conjugated to angio-adenobodies in a ratio of 2.5 μg per 1×10^9 viral particles. All the conjugations virus/angio-adenobodies were performed for 30 min at 37 °C. Infections were performed with 5000 viral particles per cells. Luciferase activity was measured 48 h post-infection and is expressed in RLU. $p < 0.05$ for Ad S11-V, Ad S11-T, Ad S11-I vs Ad and Ad S11-NT.

infection. After exposure to non-coupled adenovirus or to the non-targeted angio-adenobody H5V cells did not exhibit luciferase expression (Fig. 4A). On the contrary, cells infected with targeted adenoviruses showed an increase of luciferase up to 10^4 in cells when infected with AdTL coupled to S11-I and up to 10^6 when AdTL was conjugated with S11-V or S11-T. Upon reaching a plateau, luciferase expression starts to decrease. Excess of angio-adenobodies most possibly saturate the endothelial receptors present in the cells thereby inhibiting uptake of the virus. Adenoviral GFP expression was monitored in H5V cells infected with targeted virus in an angio-adenobody concentration dependent way (Fig. 4B).

In competition experiments, adenoviral transgene expression was not affected by pre-incubation of cells with recombinant knob (data not shown). This indicates that H5V infection of targeted adenovirus is not CAR dependent. Targeted adenoviruses were tested on HUVEC to verify whether the peptides were able to interact with human endothelial receptors. As shown in Fig. 4C cells infected with AdTL conjugated with targeted angio-adenobodies had higher levels of luciferase expression. With S11-V and S11-I angio-adenobody, the luciferase expression was enhanced about 11 times compared with cells infected with non-targeted viruses (AdTL and AdTL + S11-NT) (Fig. 4C).

3.5. Stability in serum of angio-adenobody coupled to adenovirus

The stability of the complex adenovirus/angio-adenobody in blood is a prerequisite to guarantee that the virus infects only target cells *in vivo*. For this reason AdTL was incubated with S11-I angio-adenobody in mouse serum at 37 °C for a period of 3 h before infection of H5V cells. As shown in Fig. 5, the expression of luciferase did not change significantly in time indicating the blood factors such as pH and/or proteins did not influence the conjugation of the virus with the angio-adenobody.

3.6. Angio-adenobodies target in vivo Ad5 to tumor and detarget it from liver

C26 colon carcinoma cells were injected subcutaneously in a flank of Balb 6 mice. After 15 days mice that developed a tumor were injected with 5×10^{10} of AdTL conjugated with angio-adenobody. A first group of mice was sacrificed 30 min after viral injection to perform viral localization studies, a second group of animals was sacrificed after 2 days to evaluate viral transgene expression. Immunohistochemistry performed with an antibody directed against the viral hexon protein shows staining in tumor sections of animals infected with targeted adenovirus but not of animals infected with AdTL alone or AdTL conjugated with the non-targeted angio-adenobody (data not shown). Sections of tumor analyzed at the fluorescent microscope shows the expression of GFP in samples of mice injected with targeted viruses (Fig. 6A). No GFP was observed in sections of tumors of animals injected with not targeted virus or conjugated with the non-targeted angio-adenobody (Fig. 6A). On the contrary, livers of mice injected with AdTL alone shows high level of GFP when compared with livers of mice injected with targeted virus (Fig. 6B) indicating that angio-adenobodies were not only able to target the virus to tumor but also to detarget viral uptake by liver.

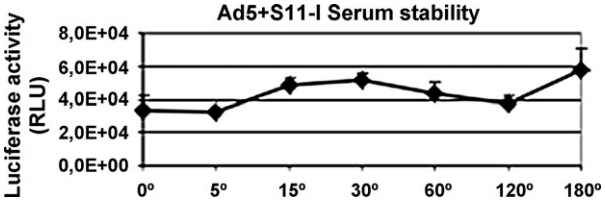

Fig. 5. *In vitro* stability of conjugated adenovirus. AdTL was conjugated to S11-I angio-adenobody in mouse serum at 37 °C. H5V cells were infected with 5000 vp/cell after different incubation times. The luciferase activity of the marker gene is expressed in RLU.

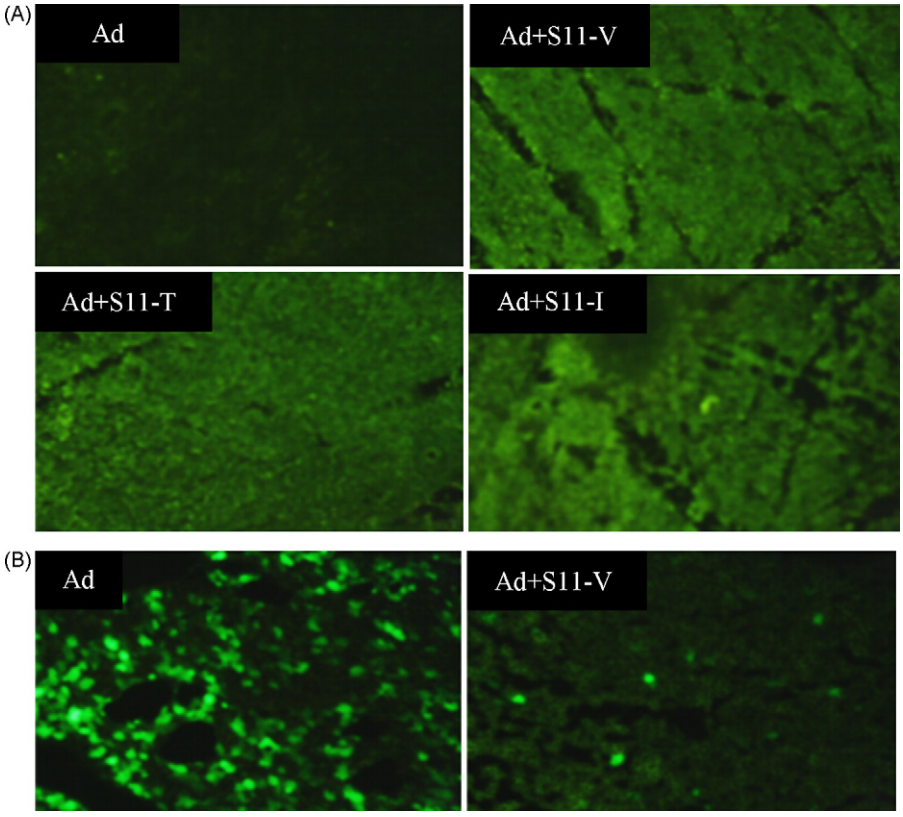

Fig. 6. Homing adenovirus conjugated to angio-adenobodies to tumor. GFP trangene expression in tumor (A) and liver (B) sections of mice injected with AdTL alone (Ad) or conjugated to angio-adenobodies (Ad + S11-V, Ad + S11-T, Ad + S11-I). Pictures of random areas were done at the Zeiss Axiovert 25 CFL fluorescence microscope.

4. Conclusions

We selectively targeted adenoviral vector to up-regulate endothelial receptors in tumor endothelium such as VEGFR2, Tie2 and $\alpha_v\beta_3$ integrins by the use of angio-adenobodies. These receptors and their conjugates regulate fundamental stages in the development of the tumor blood vessels from which is depending the growth of the primary tumor and the formation of tumor metastasis. The use of viral vectors as Ad5 for delivery of anti-angiogenic genes for anticancer treatment represents an effective strategy for different reasons: they are easy to produce and to purify at high titers, they are stable in time, they can be engineered to bind to markers on tumor endothelial cells, they can be modified to express anti-angiogenic genes to block and/or destroy the formation of tumor blood vessels and/or to express anticancer therapeutics (Liu and Deisseroth, 2006). Although the adenoviral vector represents one of the most popular vectors for gene therapy, it also has some disadvantages for its applications. Systemic administration of adenovirus results in uptake of the virus by the liver, which is responsible for the inflammatory and immune response and for the clearance of the virus from the blood (Haisma et al., 2008a). The use of angio-adenobodies in this respect offers the double advantage to detarget the virus from its native adenoviral receptors present in the liver and target the vector to the endothelial receptor. As shown in Fig. 6, injection of animals with AdTL alone resulted in transgene expression of GFP in the liver. On the contrary livers sections of mice injected with adenovirus coupled to angio-adenobody have very low GFP expression. It is well possible that the single chain antibody (S11) by covering the knob of the virus is able to prevent interaction of the virus with native receptors. We recently showed that S11 was able to prevent adenoviral uptake via scavenger receptor A in liver macrophages (Haisma et al., 2008b). However, more studies need to be performed in order to understand exactly how and which receptors are blocked by the S11 single chain antibody to prevent liver uptake.

Nicklin et al. (2000) showed that the SIGYPLP peptide fused upstream to the single chain Fv S11 was able to target endothelial cells in a cell-specific manner and increase the activity of a reporter protein when compared to a non-retargeted adenovirus. In this study we selected three peptides for their affinity to known endothelial receptors (Binetruy-Tournaire et al., 2000; Deng et al., 2001; Kok et al., 2002). Coupling of the angio-adenobodies to virus showed *in vitro* viral transgene expression in mouse endothelial cells (H5V) non-permissive to adenoviral infection. Luciferase expression increased from almost zero in cells infected with non-targeted virus to 10^6 in cells infected with targeted adenovirus (Fig. 4). Also the human endothelial cells showed an increase of luciferase expression when infected with targeted virus demonstrating the affinity of the peptides to the human endothelial receptors. Prior to test the targeted virus *in vivo* we checked the coupling stability of the virus with angio-adenobodies in conditions that mimic an *in vivo* situation. Targeted virus mixed with mouse serum for few hours did not affect the infection capability of adenovirus. It is possible that angio-adenobodies sterically inhibit interaction of the virus with blood components responsible for adenoviral uptake by liver cells (Shayakhmetov et al., 2005) and that may interfere with adenoviral infection of target cells. Systemic injection of targeted virus in mice bearing a subcutaneous carcinoma results in viral GFP expression in tumor in contrast to non-targeted virus or to virus coupled to a non-targeted angio-adenobody. The distribution of GFP was scattered through a large surface of the tumor section and not localized to endothelial areas. We speculate that the virus by escaping the leaky tumor vessel infected also tumor cells.

Because we injected the animals only ones, the effects of neutralizing antibodies on tumor targeting with the targeted angio-adenobodies remains to be determined.

In *in vitro* experiments it results that C26 cells used to stimulate tumor growth in mice, can be infected by targeted virus and not by non-targeted virus (data not shown). It has been shown recently that the $\alpha_v\beta_3$ integrins, VEGFR2 and Tie2 receptors are present not only in activated endothelial cells but also in some tumors.

In this study we demonstrated targeting of adenovirus selectively to the $\alpha_v\beta_3$ integrins, VEGFR2 and Tie2 receptors both *in vitro* and *in vivo* and contemporary detargeting of the viral vector from liver uptake. Angio-adenobodies offer the advantage to be conjugated easily to any adenoviral vector containing therapeutics without the need of genetic modifications. Currently angio-adenobody targeted adenovirus, containing genes to prevent angiogenesis, are tested in animals bearing tumor.

Funding sources

Grant support: this work has been supported by the European Community "Anti-tumor Targeting", Grant Number: LSHC-CT-2005-518178.

Conflict of interest

No conflict of interests to disclose.

Acknowledgements

We thank G. Molema (Endothelial Cell and Vascular Drug-targeting Research, University Medical Center Groningen, University of Groningen, Netherlands) for her helpful discussions on the peptide targeting and Zusanne Bohmova (University of Veterinary and Pharmaceutical Science Brno, Czech Republic) for her assistance in the experiments.

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