GENETIC RE-TARGETING AND DE-TARGETING OF ADENOVIRUS TYPE 5 IN ORDER TO CREATE VECTORS FOR GENE THERAPY

SUSANNA MYHRE

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY,
THE SAHLGRENSKA ACADEMY AT GÖTEBORG UNIVERSITY,
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CONTENTS

ABSTRACT

Gene therapy has been considered to be a revolutionary development in medicine, whereby the cause and not the symptoms of the disease would be treated. These expectations have as yet not been realized, mainly due to lack of suitable vectors. Adenoviruses type 5 (Ad5) are the most commonly used vectors for gene therapy and have a great potential in this field.
The main aim of this thesis was to generate genetically re-targeted and de-targeted Ad5 in order to create suitable vectors for gene therapy. To re-target the Ad5 vector ligands have been incorporated into the C-terminus at the pIX protein and at different positions in the fiber protein. Ligands that have been used are affibody molecules, with specificity for Taq DNA polymerase and for the tumour antigen HER2/neu, and a hyperstable single chain antibody, scFv, directed against β-galactosidase. In order to generate a virus with double specificity and determine which position is best suited for ligand–target cell interaction a re-targeted Ad5 vector with two different affibody molecules in the same genome was constructed. The re-targeted vectors were evaluated for growth, infectivity and specificity. For de-targeting assessments the neutralizing antibody reactivity in blood donor sera have been tested against a recombinant Ad5 vector with a shortened knobless fiber and a new cell binding ligand and a re-targeted vector with three different de-targeting steps have been evaluated for vector characterizations, tissue distribution and interaction with blood cells.

Ligands that are to be used for re-targeting of Ad must be able to fold correctly and stably in the reducing milieu of the eukaryotic cytoplasm which is not conducive to the formation of disulphide bonds. Both the affibody molecules and the scFv did fulfill these criteria and could be rescued into functional Ad. Incorporation of ligands in the HI-loop of the fiber knob was shown to be superior to ligand insertions into truncated knobless fibers in terms of growth characteristics. It was possible to incorporate the scFv at the pIX protein with retained antigen binding when loaded on Ad5 virions; however transduction experiments could not be performed because a suitable cell line was not available. It was shown that generation of a vector with dual specificity was feasible, on the other hand it was important to evaluate which positions is best suited for efficient binding to target cell. The virus with a truncated knobless fiber and a new cellular ligand showed a much-reduced sensitivity to human pre-formed antibodies compared to wild type (WT) Ad5. The re- and de-targeted Ad vectors did not bind to normal tissues in mice as much as WT Ad5 and the association with human blood cells was much decreased for the recombinant vectors when compared to WT.

In conclusion, both the affibody molecules and the scFv evaluated in this thesis can be used for genetic re-targeting of Ad5. Re-targeted viruses with ligands in the fiber often suffer from low growth rate, high infectivity indexes (PP/pfu) and low fiber content. However virus with ligand incorporation in the HI-loop does largely overcome those obstacles and the virus with ligand specificity for the tumour antigen HER2/neu may have relevance as a clinical vector. The adenovirus minor capsid protein IX can function as an anchor protein for relatively large ligand insertions, which is promising for future de-targeting purposes. Generation of recombinant viruses with double specificity may exploit the possibility to target several tumour antigens. The reduced neutralizing activity against the short knobless fiber and the reduced binding to normal tissues and interaction with blood cells of the re-targeted viruses with additional de-targeting steps represents, to this author, an important step towards the construction of “stealth” adenoviruses for gene therapy.

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA (SWEDISH SUMMARY)

Genterapi är en av de nyare teknologierna för att behandla sjukdomar och innebär man för in nytt genetiskt material till en patiens celler för att döda cellen, ersätta en defekt gen eller att tillföra en ny funktion till cellen. Forskningen inom genterapi riktar sig främst mot cancer, vaccin och vaskulära sjukdomar. Generna förs in med s.k. vektorer som oftast är modifierade virus.

Syftet med denna avhandling var att ta fram förbättrade vektorer för genterapi genom att målstyra vektorn till specifika tumörceller samtidigt som den ska undgå att binda till normal vävnad och blodceller samt undvika att inaktiveras av neutraliserande antikroppar. En begränsning med många virusvektorer är att de kan infektera många typer av celler och således inte är specifika. Genom att ta bort den del av viruset som normalt binder till cellytan och ersätta den med en ny cellbindande ligand som finns på tumörceller kan man konstruera målstyrda vektorer.

Vi har genetiskt modifierat ett adenovirus (serotyp 5) som normalt är ett harmlöst virus och orsakar övre luftvägsinfektioner. Normalt sett så binder adenoviruset till receptorer på cellernas yta via fiberproteinet som sticker ut från virusets skal. För att utvärdera i vilken position den nya liganden binder bäst till målcellen har vi genetiskt satt in den på två olika sätt i fibern, först i en kort fiber där liganden har ersatt den yttre delen av fibern och sedan i yttre delen på en fullängdsfiber. Resultaten visar att målstyrda virus med ligander insatta i en lång fiber är bättre på att infektera celler samt att det går att producera dem i hög koncentration jämfört med virus där liganden är insatt i en kort fiber. Vi har även testat att uttrycka liganderna ihop med pIX proteinet som också sitter i virusets skal, men pIX verkar inte lämpligt att använda som förankrings protein för målstyrning av adenoviruset.

Vi har utvärderat lämpligheten hos två olika typer av ligander att uttryckas ihop med adenovirus vektorn och analyserat hur de påverkar virusets förmåga att infektera celler. De olika liganderna är affibody molekyler och ett hyperstabilt fragment av en antikropp s.k. hyperstabil single chain antikropp. Våra resultat visar att båda liganderna kan inkorporeras i adenovirusgenomet och bilda funktionella virus.

För att bli en effektiv genterapi vektor måste den kunna injiceras i blodbanan och själv hitta sitt mål i kroppen. I och med att adenovirus typ 5 är ett mycket vanligt förkylningsvirus, innebär det att de flesta av oss redan har antikroppar som kan oskadliggöra (neutralisera) viruset. I neutralisationsförsök har vi blandat ett målstyrt virus med serum från blodgivare och visat på att det modifierade viruset inte inaktiveras i samma grad som det normala viruset. För att öka circulationstiden i blodbanan är det också viktigt att viruset inte inaktiveras pga. bindning till blodceller och normalvävnad. Genom att förändra viruset på tre olika positioner har vi visat att det är möjligt att konstruera ett virus som binder i mycket lägre grad till blodceller och till normal musvävnad jämfört ett omodifierat normalt adenovirus.

Sammantaget kan sägas att vi har identifierat vilket protein i adenovirusets skal som lämpar sig bäst att uttryckas ihop med nya cellbindande ligander och var i proteinet de ska sättas in för att producera ett effektivt virus med specifik bindning till målcellen. Både en single chain antikropp och affibody molekyler kan användas för målstyrning av adenovirus. Vi har även kommit ett steg på vägen för att öka circulationstiden för våra målstyrda virus i blodbanan och undgå att de inaktiveras av antikroppar eller binder till oönskade celler.

ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-V):

I. Myhre S, Henning P, Granio O, Tylo AS, Nygren PA, Olofsson S, Boulanger P, Lindholm L and Hong SS.
Decreased immune reactivity towards a knobless, affibody-targeted Adenovirus type 5 vector.
Gene Therapy 2007, Feb 14 (4):376-81.

II. Vellinga J, de Vrij J, Myhre S, Uil T, Martineau P, Lindholm L and Hoeben R.C.
Efficient incorporation of a functional hyper-stable single-chain antibody fragment protein-IX fusion in the adenovirus capsid.
Gene Therapy 2007 Apr;14(8):664-70.

III. Magnusson MK, Henning P, Myhre S, Wikman M, Uil TG, Friedman M, Andersson KM, Hong SS, Hoeben RC, Habib NA, Ståhl S, Boulanger P and Lindholm L.
An Adenovirus 5 vector genetically re-targeted by an Affibody® molecule with specificity for tumor antigen HER2/neu.
Cancer Gene Therapy 2007 May;14(5):468-79.

IV. Myhre S, Henning P, Lindholm L and Magnusson M.K.
In vitro and *in vivo* evaluation of HER2/neu re-targeted and CAR, α_v integrin- and HSPG-binding de-targeted Adenovirus vectors.
Manuscript.

V. Myhre S, Magnusson M.K, Friedholm M, Henning P, Ståhl S and Lindholm L.
A re-targeted Adenovirus with dual specificity; binding specificities for two different Affibody® molecules at differing positions in the HI-loop of the fiber knob.
Manuscript.

LIST OF ABBREVATIONS

aa	amino acid(s)
Ab	antibody
Ad	Adenovirus
bp	base pair(s)
CAR	Coxackie and Adenovirus receptor
CMV	cytomegalovirus
cpe	cytopathogen effect
Fab	fragment antibody binding
FACS	fluorescent activated cell sorting
Fv	fragment variable
GFP	green fluorescent protein
GON	group of nine
HER2/neu	human epidermal growth factor 2
HSPG	heparan sulphate proteoglycan
IF	immunofluorescent
ITR	inverted terminal repeats
kb	kilo base pairs
kDa	kilo Dalton
mAb	monoclonal antibody
MLP	major late promoter
moi	multiplicity of infection
mu	map unit
Nabs	neutralizing antibodies
nt	nucleotide(s)
PCR	polymerase chain reaction
pfu	plaque forming units
pi	post infection
PP	physical particles
Q-PCR	quantitative polymerase chain reaction
scFv	single chain fragment variable
SOE	splicing by overlap extension
SpA	staphylococcal protein A
WT	wild type
Å	Ångström, 1Å=0,1 nm

AIMS OF THE THESIS

This thesis was performed within a project with the aim to develop adenovirus vectors for gene therapy of human tumours. Such vectors have to be re-targeted to redirect the specificity of the virus to desired target cells and de-targeted to avoid neutralizing antibodies, transduction to normal tissues and interaction with blood cells and complement.

The specific aims were:

- To study the sensitivity of a recombinant virus with a truncated knobless fiber to human pre-formed neutralizing antibodies.

- To investigate the possibility to incorporate a scFv at the C-terminus of the pIX protein.

- To study the possibility to re-target an Ad vector with the affibody® molecule Z_{H} specific for the tumour antigen HER2/neu.

- To evaluate which positions (of three investigated) in the Ad5 fiber is best suited for ligand incorporation.

- To study the functionality of a HER2/neu re-targted vector with different de-targeting modifications.

- To evaluate the binding properties of two different affibody® molecules incorporated as a tandem repeat into the same adenovirus.

INTRODUCTION

ADENOVIRUS

History

Adenovirus constitutes a large group of DNA viruses that infect humans among other species, (Pereira *et al.*, 1963). The virus was first discovered 1953 by Rowe and colleagues (Rowe *et al.*, 1953) where they reported on a viral agent causing degeneration of epithelial-like cells from tonsils and adenoids surgically removed from children. Next year Hilleman and co-workers isolated related agents from military personnel with respiratory illness in the United States (Hilleman and Werner, 1954). The viruses were first called adenoid degeneration (AD), adenoid-pharyngeal conjunctival (APC) and acute respiratory disease (ARD) agents, but in 1956 they were named adenoviruses (Enders *et al.*, 1956). Adenoviruses were then associated with respiratory disease, acute hemorrhagic cystitis, gastroenteritis and epidemic keratoconjunctivitis (EKC). However, in 1962 it was demonstrated for the first time that adenovirus type 12 could induce malignant tumors in rodents (Trentin *et al.*, 1962). Other serotypes (including Ad5) were found to be non-oncogenic.

Classification

All Adenoviruses belongs to the family *Adenoviridae,* which is divided into four groups (genera): *Mastadenoviruses* infecting mammals, *Aviadenovirus* infecting birds, *Siadenovirus* infects birds and frogs and finally *Atadenovirus* infects a broad range of hosts including avian, reptile and marsupial host (Benko *et al.*, 2000). A possible fifth genera infecting fishes has been proposed to be called *Ichtadenovirus* (Benko *et al.*, 2002). The genera are separated primarily on the basis of difference in immunological properties of the virions (Norrby *et al.*, 1976). There are fifty-one human adenovirus serotypes and they have been distinguished on their basis of their resistance to neutralizing by antiserum to other known adenoviruses (De Jong *et al.*, 1999). The various serotypes are divided into six subgroups (species) A-F (table 1) based on their ability to agglutinate red blood cells (Rosen, 1960). Ad5 belongs to the subgenus C, together with Ad1, Ad2 and Ad6 which all are responsible for respiratory infections.

Pathogenicity

Human adenoviruses cause infections of various severities. They are associated with different clinical syndromes which are caused by cytopathogenic effect on cells in the infected organs (table 1). Subgroup C which includes Ad type 5 is associated with respiratory illness (Horwitz, 2007) and it is suggested that the disease is transmitted via aerosol. Other subgroups are associated with pharyngoconjunctival fever (PCF), epidemic keratoconjuncitvitis (EKC) and gastroenteritis. Those infections are suggested to be transmitted via poorly chlorinated pools (Bell *et al.*, 1955), eye-to-hand-hand-to-eye contact (Azar *et al.*, 1996) and fecal-oral route (Wadell, 1988; Horwitz, 2007).

Trials with anti-viral agents have been focused on ocular infections, but no treatment is yet in general use (Horwitz, 2007). The only vaccine existing is directed against Ad 4 and Ad 7 for the prevention of respiratory illness. The vaccine contains live virus, is taken orally to obtain attenuation (Top *et al.*, 1971), and is used by the US military.

Table 1. Properties of human adenovirus serotypes of subtype A to F.

Subgenus	Ad type	Haemagglutination pattern[a]	Tropism
A	12, 18, 31	IV	Intestine
B:1	3, 7, 16, 21	I	Respiratory tract, eye
B:2	11, 14, 34, 35, 50	I	Kidney, eye
C	1, 2, 5, 6	III	Respiratory tract
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51	II	Eye, intestine
E	4	III	Respiratory tract, eye
F	40, 41	IV	Intestine

Modified from (Wadell, 1988) and (Arnberg, 2001) with permission. [a]I = complete agglutination of monkey erythrocytes; II = complete agglutination of rat erythrocytes. Some members agglutinate human erythrocytes; III = partial agglutination of rat erythrocytes (fewer receptors); IV = agglutination of rat erythrocytes discernible only after addition of heterotypic antisera.

Structure

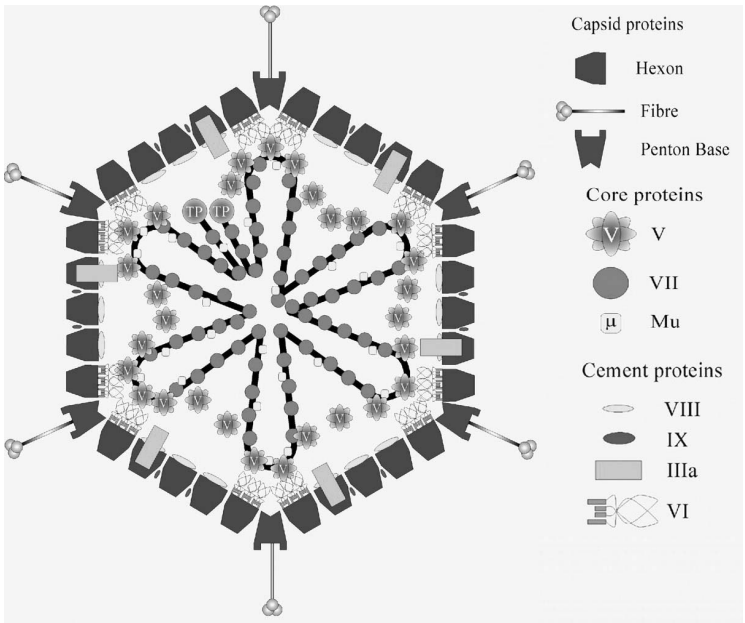


Fig. 2. Adenovirus structure. Modified from (Russell, 2000) with permission from W.C. Russell and the Journal of General Virology. The locations of the capsid and cement components are reasonably well defined. In contrast, the disposition of the core components and the virus DNA is mainly hypothetical.

Adenoviruses are non-enveloped, regular icosahedrons (20 triangular surfaces and 12 vertices) that are approximately 70-100nm in diameter (Horne *et al.*, 1959) (fig. 2). The virus particles are composed of 252 capsomers, of which 240 are trimeric hexons and 12 are pentons (Ginsberg *et al.*, 1966). The hexon capsomere is composed of three tightly associated hexon molecules while the penton (or penton base) consist of five copies of the penton subunit. The fiber is anchored to the penton base at the corner of the icosahedron and the fiber and penton together form the penton capsomere (Everitt *et al.*, 1973). The capsid also contains polypeptides VI, VIII and IX, which are associated with the hexon (Everitt *et al.*, 1973), thought to be involved in stabilization and/or assembly of the particle.

Capsid proteins

Fiber

The viral fiber was first demonstrated in the Ad2 capsid in 1965 (Valentine and Pereira, 1965). The fiber is composed of a trimeric protein, and can be divided into a tail, a shaft and a head (knob) (Chroboczek *et al.*, 1995). Most mammalian adenoviruses have 12 fiber trimers, one at each vertex of the virion. Only subgroup F viruses (Ad 40 and Ad 41) encode two fiber proteins. There are two well-understood functions, which can be directly attributed to the fiber protein. The first is the structural role in the viral capsid and the second is the interactions with cellular adenovirus receptors. The N-terminal tail is preserved among the different adenoviruses and there are two regions which are highly conserved (2-KRλR, λ indicates G, A, T, S, V or L, and 11-FNPVYPYD/E) and thought to mediate nuclear localization (Hong and Engler, 1991) and binding to the penton base (Caillet-Boudin, 1989), respectively. The central shaft of Ad5 consists of 21.5 pseudo-repeats that contain 15-residues each, and which have hydrophobic residues at positions 1, 3, 9 and 11 and a conserved proline or glycine at position 8. It has been shown that the fiber is folded as a triple β-spiral (van Raaij *et al.*, 1999).

The C-terminal knob domain was first cloned in 1988 (McGrory *et al.*, 1988). The knob domain is arranged as a trimer and viewed as three-blade propeller when visualized along the three-fold symmetry axis. It contains both conserved and variable domains (loops) (fig. 3). High-resolution structures of the knob region of Ad2 and Ad5 showed that the knob forms an eight-stranded, anti-parallel β-sandwich structure (van Raaij *et al.*, 1999). The intrinsic trimerization domain of the fiber protein was found to lie within the shaft-knob junction and within the knob domain (Novelli and Boulanger, 1991; Hong and Engler, 1996).

The knob mediates binding to cellular receptors and has other biological properties which are involved in intracellular trafficking, endosomal release and virus maturation (Legrand *et al.*, 1999; Miyazawa *et al.*, 1999; Leissner *et al.*, 2001; Gaden *et al.*, 2004). It has also been shown that the knob domain has a positive control effect on the fiber protein translation (Henning *et al.*, 2006).

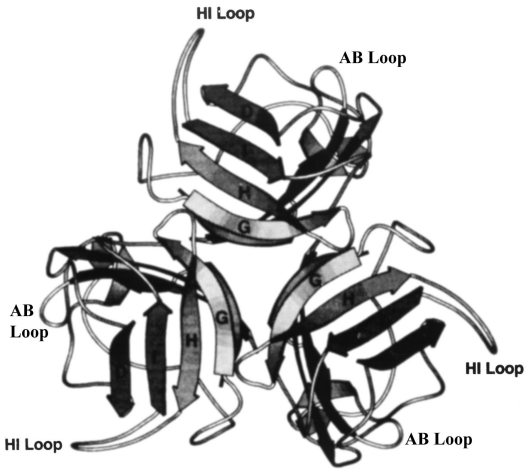


Fig. 3. Ribbon diagram of Adenovirus 5 knob viewed down the three-fold symmetry axis with the R-sheet β-strands D, I, H and G faces the cellular receptor. Reprinted from (Xia *et al.*, 1995) with permission.

Minor capsid protein IX

The adenovirus protein IX (pIX) is a small polypeptide of 140 residues (14.3kDa) that is incorporated into the mature viral capsid (Boulanger *et al.*, 1979). pIX associates with the hexon to form a group of nine (GON) that makes up the central region of each facet of the icosahedron. The stoichiometry of this assembly has revealed that there are 12 molecules of pIX organized as four trimers per GON and therefore 240 molecules per virion (van Oostrum and Burnett, 1985; van Oostrum *et al.*, 1987). pIX is expressed at an intermediate time during infection (after initiation of early genes but before expression of late genes). The protein acts as capsid cement and thereby enhances the thermal stability of the virions (Colby and Shenk, 1981; Furcinitti *et al.*, 1989) and is needed for packaging of the full-length Ad DNA (Ghosh-Choudhury *et al.*, 1987). It is also suggested that the pIX protein serves additional functions during the infection cycle (Lutz *et al.*, 1997). It is the only structural protein-coding gene, which is uncoupled from the major late promoter (MLP). The expression pattern follows a different time course and begins at intermediate times post infection, much earlier than the structural proteins.

Penton capsomere

The penton is a heteromeric association of a pentameric base and a trimeric fiber. Three-dimensional image reconstruction clearly indicated that the penton is composed by five subunits (Stewart *et al.*, 1991). The penton base protein sequence is highly conserved between Ads of the same subgroup, 98.6% homology has been found between Ad2 and Ad5 (Neumann *et al.*, 1988). The features common to the penton base are two moieties of high homology separated by a non-homologous spacer region of variable length which includes the conserved RGD motif that is responsible for virus internalization (Belin and Boulanger, 1993; Wickham *et al.*, 1993; Wickham *et al.*, 1994).

Hexon

The hexon is the largest capsid protein. The molecular mass is approximately 360kDa consisting of three identical subunits of app. 120kDa each (Cornick *et al.*, 1971). The overall shape of the trimeric hexon molecule is unusual and may be divided into a pseudo-hexagonal base rich in β-structure, and a triangular top formed from three long loops containing secondary structure (Athappilly *et al.*, 1994). The hexon surface loops display the highest variability between adenovirus serotypes and contain most of the type specific epitopes (Toogood *et al.*, 1989).

The genome

The human adenoviruses genome is made up of double-stranded DNA molecules, which have ITRs at both ends. The genomes vary in size between 34.1kbp up to 35.9kbp and are divided into early (E) and late (L) genes (fig. 4). During the early phase proteins are expressed from six different transcription units, E1A, E1B, E2A, E2B, E3 and E4. Unlike the early genes the late genes are transcribed from the same promoter, MLP (Ziff and Evans, 1978). The E-genes are involved in regulation and virus replication E1; (Flint and Shenk, 1989), replication E2; (Hay et al., 1995), escape from the immune system E3; (Wold et al., 1995) and cell cycle control E4 (Tauber and Dobner, 2001). The L-genes are transcribed later than the E-genes and encode the structural proteins mentioned above.

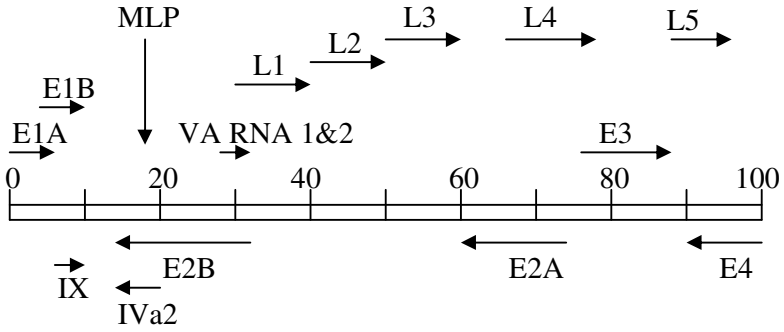


Fig. 4. The adenovirus genome showing the early (E1 to E4), late (L1 to L5), IX, IVa2, and VA RNA 1 and 2 transcripts. Arrows indicate the direction of transcription. Modified from Russell (2000).

Replication cycle

The life cycle of human adenovirus has been widely studied especially for serotype 2 and 5. The life cycle is divided into early and late phases just like the transcription units in the genome. The early stage includes cell targeting and attachment, internalization, nuclear transport and expression of early genes. The late phase is characterized by expression of late genes, virus assembly and cellular release (fig. 5).

Cell targeting and attachment

For any virus to infect a cell an initial attachment between the virus and the target cell is required. Adenovirus uses the knob domain of the fiber protein to mediate cell attachment and the main receptor for all subgenera (except subgenus B) is CAR (Bergelson *et al.*, 1997). CAR is a 46kDa protein, member of the immunoglobulin super

family, is widely expressed in humans, primary on epithelial cells and functions as a cell-cell adhesion molecule. For review see (Philipson and Pettersson, 2004). Sialic acid has been shown to be the cellular receptor for Ad 8, 19a and 37 of species D (Arnberg *et al.*, 2000; Arnberg, 2001) and Ad of species B use CD46 as cellular receptor (Gaggar *et al.*, 2003; Segerman *et al.*, 2003).

Internalization of virions into cells

Adenoviruses are internalized into host cells via a mechanism referred to as receptor mediated endocytosis into clathrin-coated vesicles (Varga *et al.*, 1991; Wickham *et al.*, 1994). First; the fiber protein binds to the primary receptor and second; the virus entry is mediated by binding of the RGD motif in the penton base to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Varga *et al.*, 1991; Wickham *et al.*, 1993; Wickham *et al.*, 1994).

Endosomal release and nuclear transport

The next step in the adenoviral life cycle is the release of virions from the endosomes. Analysis has shown that the penetration step requires a tyrosin-valin-aspartate motif in the cytoplasmic domain of the β_5 subunit. From the endosome the virus escapes to the cytosol with a half time of 5 min. Endosome disruption coincides with a drop in pH (Seth *et al.*, 1984). The penton base, $\alpha_v\beta_3$ integrins and the fiber have been suggested to be important for efficient endosomal escape (Seth *et al.*, 1984). Particles are thereafter transported to the nucleus though a process involving microtubules (Leopold *et al.*, 2000).

Transcription and DNA replication

E1A is the first viral transcription unit to be expressed after the viral chromosome reaches the nucleus. It is followed by transcription of E1B, E2, E3 and E4. These genes support an optimal environment for virus replication, protect the cell from the host immune response and promote synthesis of proteins that are needed for viral replication. Once they are completed the DNA synthesis begins at the inverted terminal repeats. The synthesis proceeds in two stages: (i) replication of the duplex templates to produce duplex daughter and parental strand plus a displaced single strand of DNA; and (ii) replication of the displaced single strand (Berk, 2007). At the start of replication, the late genes are transcribed that encodes for structural proteins and proteins needed for viral assembly (Berk, 2007).

Assembly

The virus assembly starts with the formation of the hexon, penton and fiber capsomers. The capsid structure is generated from the capsomeres by the addition of precursors of the IIIa, VI, and VIII proteins to form light intermediates. These particles lack DNA and core proteins. In the next step, the DNA linked to pre terminal protein is inserted creating heavy intermediates. DNA encapsidation is mediated via a specific sequence (the packaging sequence), which is located at the left end of the genome. The subsequent intermediate forms are called young virions

and although they contain all of the viral proteins and DNA, the precursor proteins are not processed. After activation of the viral protease p23, the precursor proteins are cleaved and the mature virion is produced (D'Halluin, 1995).

Cellular release

Ad are released from infected cells by cellular lysis, however the mechanism of adenovirus release is unclear but the release of adenoviral particles is facilitated by the adenovirus death protein, which seems to be required for efficient lysis (Tollefson *et al.*, 1996).

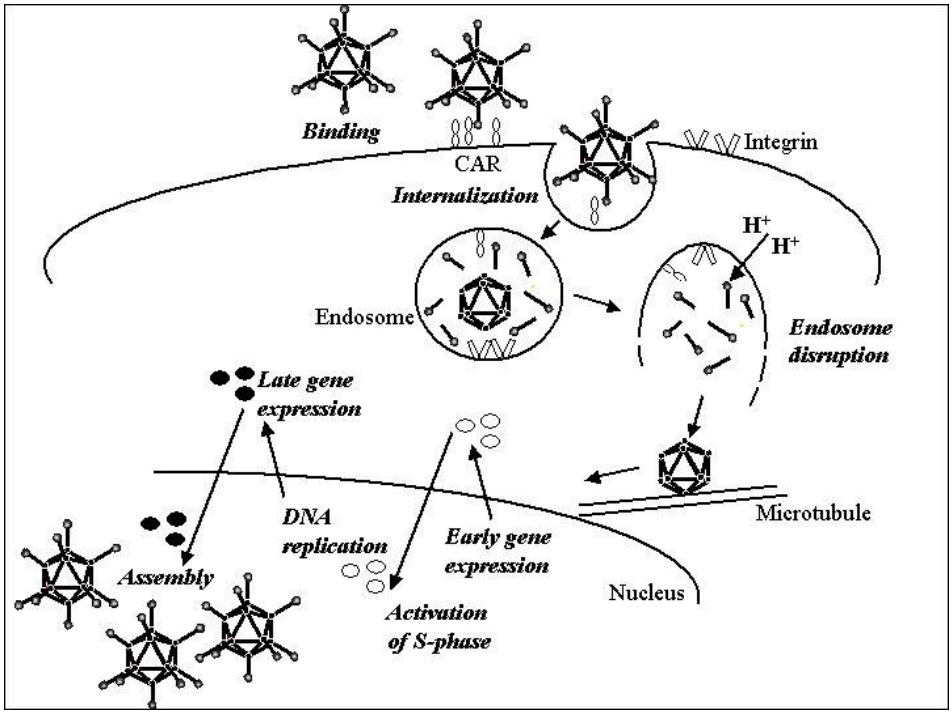


Fig. 5. Adenovirus infection cycle. Modified from (Barnett et al., 2002) with permission from J.T. Douglas.

Host Immune response

Cellular immune response

Cellular immunity is mediated by T-cells and provides an effective defense via both $CD8^+$ cytotoxic (CTLs) and $CD4^+$ helper cells. CTLs function by recognizing a virus antigen in a complex with class I proteins of the MHC on the cell surface. This event releases perforin, resulting in cell lysis, thereby eliminating infected cells at an early stage before any virus is assembled and released.

Adenoviruses can combat this cellular strategy by utilizing E3 gp19K to retain the MHC antigens in the ER and thereby disrupting the antigen recognition process (Kvist *et al.*, 1978). The E4 gene has also been demonstrated to function in the inhibition of T cell cytolysis (Kaplan *et al.*, 1999). The $CD4^+$ helper cells are important in mounting a proliferative response to infection. The $CD4^+$ response is mediated by recognition of a

virus target antigen associated with class II MHC. These T cells can stimulate proliferation of B cells to produce specific antibodies for the humoral response, see below.

Humoral immune response

Humoral immunity is mediated by antibodies and can be transferred to un-immunized individuals by cell-free fractions of the blood, i.e., plasma or serum.
The humoral response is a major component of the defense strategy of the host and depends on the ability of B cells expressing surface immunoglobulins to recognize a specific epitope on a foreign antigen. Following activation of the specific B cells, immunoglobulins are released into the plasma as antibodies that interact with the antigens. The immunoglobulins can neutralize virus infection very efficiently. Adenovirus–neutralizing antibodies are directed against epitopes primarily on the fiber, penton base and hexon in the adenoviral genome (Willcox and Mautner, 1976; Gahery-Segard *et al.*, 1997; Gahery-Segard *et al.*, 1998; Myhre *et al.*, 2007). The neutralizing antibody response is responsible for blocking successful re-administration of the current generation of adenovirus gene transfer vectors, which primarily are based on Ad5 (Kass-Eisler *et al.*, 1996).

GENE THERAPY

History

Gene therapy has been described as "the treatment or prevention of disease by gene transfer", and was considered to be a revolutionary development in medicine, whereby the cause and not the symptoms of the disease would be treated.
The aim of gene therapy is to introduce a defined DNA sequence into specific cells of a patient either to replace a defective gene, or to import a new function to the cell. This means that almost any disease, at least in theory, can be a candidate for gene therapy. App. 85% of gene therapy clinical trails have addressed cancer, vascular diseases and inherited monogenic disease (www.wiley.co.uk/genmed/clinical/).

The first human gene therapy clinical trial was performed in 1989 by Rosenberg and co-workers (Rosenberg *et al.*, 1990). These investigators used a retrovirus to introduce the bacterial gene coding for resistance to neomycin into human tumour-infiltrating lymphocytes before infusing them to five patients with advanced melanoma. This pioneer study demonstrated that gene transduction for human gene therapy was feasible and sparked hopes that the technique could be used to treat serious diseases for which there is no conventional cure. From then until 1999, the number of clinical gene therapy trials initiated raised rapidly. The first major negative effects on recruitment of patients in gene therapy clinical trials came in 1999, when a 18-year old boy died from an unexpected inflammatory reaction due to an adenoviral vector administration (Raper *et al.*, 2003) and in 2002 there was a report of development of a leukemia-like illness due to retroviral insertion in patients (Hacein-Bey-Abina *et al.*, 2003). These obstacles had a sobering effect on the entire field of gene therapy and slowed

the clinical development in this area. However, despite the drawbacks, gene therapy continues to harbor potential for an entirely new approach to many clinical conditions and recent clinical studies have shown therapeutic benefits (Khuri *et al.*, 2000). At present Adenovirus type 5 (Ad5) is the most popular choice for early preclinical and clinical development following the initial development by ONYX Pharmaceutical (McCormick, 2003). The ONYX-015 vector was mutated in the gene encoding E1B-55K. This gene binds and inactivates p53 which results in an inhibition of apoptosis in infected cells. The thought was that infection with ONYX-015 should drive normal cells to apoptosis while tumour cells that have lost p53 function would promote replication and result in cell lysis and virus spread within the tumour. It was on the other hand shown that E1B-55K was not limited to p53 deficient cells and ONYX-015 was capable of replication also in normal cells. However, it has been shown that i.v. infusions of ONYX-015 at doses of 2×10^{13} particles alone or in conjunction with chemotherapy demonstrate safety and feasibility (Nemunaitis *et al.*, 2001; Nemunaitis *et al.*, 2003) but with limited clinical effectiveness. A possible explanation of this lack of effectiveness is an accelerated viral clearance mediated by the patient's immune response.

The first virotherapy product licence is now placed in China, using a genetically modified Ad5 to treat head and neck cancer. The approach has shown to be efficient, but at present all successful clinical treatment involves direct intra tumoural injection, for review see (Peng, 2005). For reviews on gene therapy see (Mountain, 2000; Vile *et al.*, 2000; Edelstein *et al.*, 2007).

Gene transfer systems

There are two main types of gene-delivery vectors, viral and non-viral. Viral vectors have been used in about 70% of the trials performed (www.wiley.co.uk/genmed/clinical/) and the most commonly used are adenoviruses (Ad) and retroviruses. Other viruses that have been less widely used are vaccinia viruses, pox viruses, herpes simplex viruses and adeno-associated viruses. Retrovirus has been the second most commonly used vector in clinical gene therapy protocols so far (22.8% of all trials) (www.wiley.co.uk/genmed/clinical/). However, the transduction efficiency of retroviruses is low and limited to proliferating cells. Retroviruses integrate the transgene into the target cell DNA, are less immunogenic than Ad, are difficult to produce in high titers and the safety of retrovirus has moreover been in doubt after the report of the development of a leukaemia-like illness due to retroviral insertion, mentioned above (Hacein-Bey-Abina *et al.*, 2003). Examples of non-viral approaches include the delivery of naked DNA or DNA that is complexes with cationic lipids. The major benefits with non-viral vectors are their simple manufacturing, low immunogenicity and unlimited insert size. The major drawbacks are low *in vivo* transduction efficiency and poor targeting capacity. Many studies have been done with this type of vectors and a recent publication has showed the combination of lipopolyplex formulations and multivalent cationic lipids are promising tools for *in vitro* and possibly also *in vivo* gene transfer to colorectal cancer cells (Pelisek *et al.*, 2006). All of the delivery

systems have both advantages and disadvantages, and an ideal vector that is suitable for all applications has, to the knowledge of the author, not yet emerged.

Adenovirus vectors for gene therapy

Adenoviruses are the most commonly used vectors (24.7% of all trials) (www.wiley.co.uk/genmed/clinical/). There are numerous serotypes of adenovirus known to infect humans and the Ad vector used in gene therapy is primary based on serotype 5. Adenoviruses have great potential as gene therapy vectors for human diseases and there are several reasons for the use of Ad: they show a high transduction efficiency *ex vivo* and *in vivo*, they infect both proliferating and non-proliferating cells, in contrast to other vectors that are limited to proliferating cells, they are easy to grow to high titers and they are biological safe compared to other vectors since they are mostly responsible for benign infections in immune competent individuals. The disadvantages are: pre-existing neutralizing antibodies and induction of strong immune responses which reduces the effectiveness of repeat dosing, short circulation time *in vivo* and an insert-size limit of app. 7.5kb for incorporation of foreign DNA sequences into the genome (Russell, 2000; Volpers and Kochanek, 2004).

In first-generation adenovirus vectors, the E1 and/or the E3 genes were removed to make the virus replication-incompetent and to allow the introduction of up to 6.5-8 kb of foreign DNA sequences (Russell, 2000; Volpers and Kochanek, 2004). In second-generation vectors some or all of the E2 and E4 genes have been deleted to eliminate the production of replication-competent adenovirus (RCA) due to homologous recombination of E1 gene in 293 cells. The third-generation vectors have removed of all (or almost all) of the virus genes. These "gutless" vectors retain only the ITRs (inverted terminal repeats) and the packaging sequence and require helper virus and complementing cell lines for production (Imperiale and Kochanek, 2004; Volpers and Kochanek, 2004).

Targeting of adenovirus type 5 vectors

The coxsackie and adenovirus receptor (CAR) is widely expressed in human tissue causing unwanted infection with wild type (WT) Ad. In addition, CAR is often down regulated in many tumor cells (Hunt and Vorburger, 2002; Kim *et al.*, 2002b). An ideal Ad vector should therefore be de-targeted from its natural receptor and re-targeted to a new selected receptor in order to decrease the transduction of Ad to undesired cells and to increase the transduction to target cells.

Transcriptional re-targeting

In transcriptional re-targeting the vector is modified to replicate or express the transgene only in certain cells. For replication incompetent vectors tissue specific or tumour specific promoters (TSPs) are used to restrict the expression of the transgene, for review see (Bauerschmitz *et al.*, 2002). Conditionally replicative adenovirus vectors (CRAds) are genetically modified to replicate in specific cells either by deletion of viral genes important for virus replication that is compensated for in tumour cells or

by replacing viral promoters with TSPs (Gomez-Navarro and Curiel, 2000; Bauerschmitz *et al.*, 2002). However, all transcriptional re-targeting presuppose that the virus can deliver its DNA to the target cell nucleus.

Transductional re-targeting

The two general approaches for allowing targeting to other cellular receptors than CAR are the physical (two-component) and the genetic (one-component). In physical or two-component systems bi-specific conjugates are used that binds to both the virus and the targeting receptor. They can be either polymers conjugated to targeting ligands (PEGylation), chemically cross linked targeting ligands, bi-specific fusion proteins or bi-specific peptides, for reviews see (Barnett *et al.*, 2002; Bauerschmitz *et al.*, 2002; Volpers and Kochanek, 2004). For genetic re-targeting (one-component) ligands are ligated into various capsid genes of the adenoviral genome. Targeting ligands have mainly been inserted into the fiber protein, but other capsid proteins such as the penton base, hexon and pIX have also been evaluated as an anchoring structure (Barnett *et al.*, 2002; Curiel, 2002; Wickham, 2002; Vellinga *et al.*, 2007). It is also possible to change tropism of the adenoviral vector by combining the capsid with a fiber from another serotype to make chimeric viruses (Miyazawa *et al.*, 1999).

De-targeting

In order to achieve useful *in vivo* gene therapy adenovirus vectors have to be de-targeted from normal tissue receptors of Ad5. Earlier it was assumed that de-targeting required: i) ablation of the natural binding of the fiber knob to the CAR receptor, ii) mutation of a motif in the 3rd shaft repeat which seems to be responsible for the strong liver tropism of WT Ad5 (Smith *et al.*, 2003a; Smith *et al.*, 2003b) and finally iii) ablation of the integrin-binding motifs in the penton base which otherwise leads to unwanted uptake in normal tissues (Akiyama *et al.*, 2004). Recent results have however shown that Ad can infect liver cells via binding to blood proteins and platelets and also interact with blood cells for review see (Baker *et al.*, 2007). For efficient de-targeting the binding sites need to be mutated or shielded.

Another problem for adenoviral gene therapy is the virus-neutralization antibodies, which are a common result of adenoviral infections and a major obstacle to the systemic administration of adenoviral vectors for therapeutic purposes (Gahery-Segard *et al.*, 1997; Gahery-Segard *et al.*, 1998; Russell, 2000). Human antibodies against adenovirus are directed against various virus capsid proteins (Russell, 2000). The antibody response against adenovirus in patients receiving Ad vectors mainly occurs as an anamnestic response. In order to create an adenoviral vector that reaches its target before being eliminated by the immune system, the immunogenic epitopes in the adenoviral genome have to be replaced by mutation or hidden by non-immunogenic proteins. It has also been demonstrated that anti-Ad antibodies that neutralize binding to CAR can mediate Ad infection via Fc receptors on antigen presenting cells, which might explain the success of Ad based vaccines in the presence of neutralizing antibodies (Ab) (Leopold *et al.*, 2006).

Box 1. Adenovirus as gene therapy vector, pros and cons

Advantages

- High transduction efficiency
- Infect both dividing and non-dividing cells
- Easy to grow to high titers
- Biologically safe compared to other vectors

Disadvantages

- Pre-existing antibodies which results in neutralization of the vector
- Vector-induced immune response
- Do not infect all cell types, e.g. tumour cells
- Broad tropism – infect unwanted cells, due to the widely expressed CAR receptorInefficient uptake into tumour tissue following systemic administration of vector due to high intra-tumoural fluid pressure and insufficient passage of vector over neovasculature

LIGANDS FOR ADENOVIRUS RE-TARGETING

Background

The rate limiting step for tumour cell transduction of Ad5 is the binding to the primary receptor CAR (Kim *et al.*, 2002a). Since CAR is down-regulated in most tumour cells the transduction can be greatly improved by removing the CAR binding site and inserting new ligands that allow targeting to other cellular receptors. There are several types of targeting ligands that have potential for re-targeting of adenovirus vectors i.e. peptides, scFvs and affibodies. There have also been initial attempts to incorporate a single chain T-cell receptor and epidermal growth factor into truncated knobless fibers, but these have met with failure (Magnusson *et al.*, 2002). However, it has recently been shown that a single chain T cell receptor could be introduced into a knobless fiber carrying just one shaft repeat, rescued into functional virus and shown to be able to re-target the virus to cells carrying MHC-I/peptide complexes recognized by the T cell receptor (Sebestyen, 2007). In addition, incorporation of a single chain antibody fragment in the same way was not effective (Willemsen, personal communication).
The ligands used in this thesis are scFv antibody and affibodies.

ScFv

Antibody molecules and their fragments have a large number of applications in research, diagnosis and therapy (Carter and Merchant, 1997; Hudson, 1998). The antigen recognizing paratopes on antibodies are located in hyper variable segments of the so-called Fv fragment, a heterodimer compromising a variable domain of the light chain (V_L) and the heavy chain (V_H) (Glockshuber *et al.*, 1990). Single-chain Fv (scFv) are antibodies in which the V_L and V_H sequences are joined by a short, flexible linker peptide. Antibodies expressed in the cytoplasm of eukaryotic cells are often not

folded correctly resulting in low yields of soluble protein. The aggregation of antibodies in the cell cytoplasm is mainly due to the reduction of the two-intrachain disulphide bonds. Most single-chain antibodies use disulphide bounds for their folding stability. However, a few antibodies, so called hyperstable antibodies that can be expressed in the cytoplasm of eukaryotic cells with retained specificity have been described (Martineau *et al.*, 1998; Ohage *et al.*, 1999; Tavladoraki *et al.*, 1999). One example is scFv 13R4, reactive against β-galactosidase. ScFv 13R4 was originally isolated from a phage display library (Vaughan *et al.*, 1996) based on naïve human scFv sequences by selection for cytoplasmic antibody stability in E.coli. The selected scFv was subsequently subjected to random mutation by error-prone polymerase chain reaction to further increase its cytoplasmic solubility, yielding the scFv 13R4 (Martineau *et al.*, 1998) (fig. 6).

Affibody® molecules

Affibody molecules are based on a disulphide bond independent 58 aa three-helix bundle structure from the Ig binding domain B of staphylococcal protein A. In the origin (named Z_{wt}) two aa are substituted in order to generate a domain that is more stable to chemical treatment and with less affinity for IgG Fab than the wild type B domain (Nilsson *et al.*, 1987; Nord *et al.*, 1995; Jansson *et al.*, 1998) (fig. 6). Phagemid libraries have been constructed, based on mutagenesis of 13 surface aa in the Z_{wt} domain, from which novel affibody molecules have been selected to desired targets using phage display technology (Nord *et al.*, 1997; Hansson *et al.*, 1999). It has been shown by us that affibody molecules can efficiently be used for genetic re-targeting of adenovirus (Henning *et al.*, 2002).

The affibody molecules used in this thesis were Z_{taq} and Z_H. Z_{taq} is reactive with Taq DNA polymerase (Gunneriusson *et al.*, 1999) and Z_H is specific for the extra cellular domain of epidermal growth factor 2 (HER2/neu, also called ErbB2) (Wikman *et al.*, 2004). HER2/neu is a transmembrane protein belonging to the tyrosine kinase receptor family (Codony-Servat *et al.*, 1999) and is associated with increased proliferation and decreased apoptotic capacity (Menard *et al.*, 2003). HER2/neu is over-expressed in about 25-30% of all diagnosed breast cancer and the current treatment is conventional chemotherapy and surgery combined with a specific anti-HER2 monoclonal antibody (Trastuzumab) (Nahta and Esteva, 2003; Piccart-Gebhart *et al.*, 2005). As Trastuzumab treatment is, however, very cost consuming an alternative treatment is in demand. A re-targeted adenovirus with an affibody molecule directed to the same structure as Trastuzumab is therefore very promising from a therapeutic point of view.

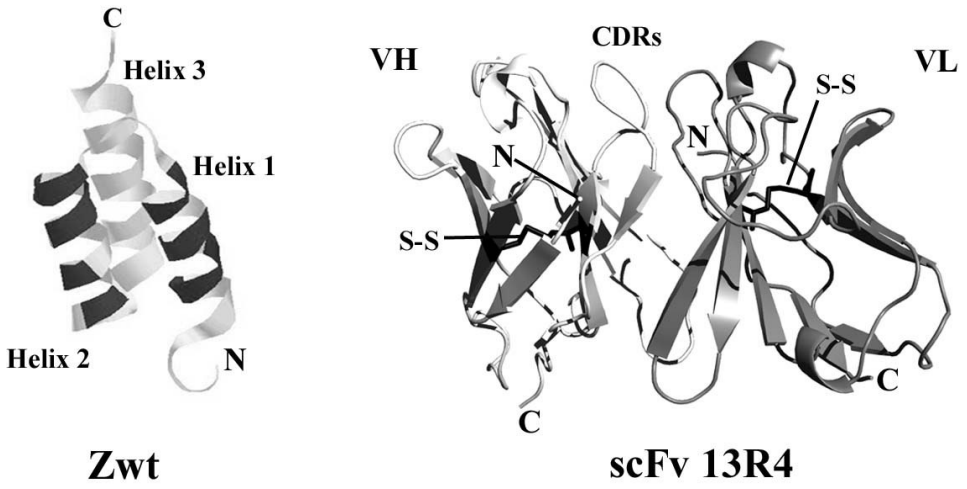
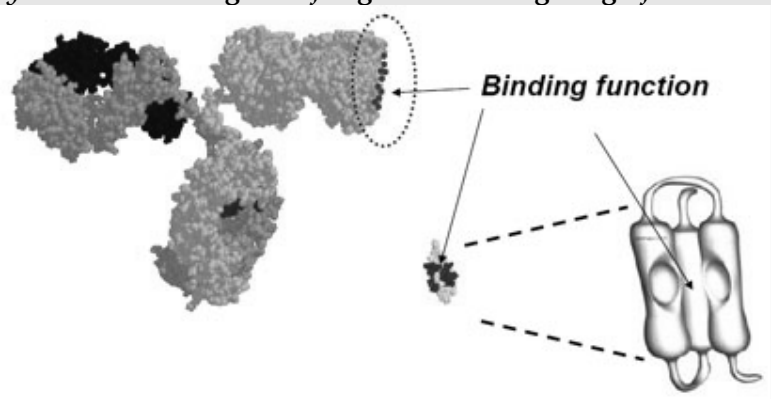


Fig. 6. A diagram of Z_{wt} and scFv 13R4. The relative sizes are NOT proportional. Zwt picture provided by Per-Åke Nygren, Royal Institute of Technology, Stockholm, Sweden. Sequences randomized in affibody molecule libraries are shown in black. ScFv 13R4 provided by Pierre Martineau, CNRS UMR5094, Faculté de Pharmacie, Montpellier, France.

Box 2. Affibody molecules as ligands for genetic re-targeting of Ad vectors



Comparison of binding sites of an antibody (left) and an affibody molecule (right). Despite its small size, an affibody molecule has a binding size roughly the same as an antibody Fab. Image courtesy of Affibody AB.

Affibody molecules for genetic re-targeting of Ad

- High affinity binders to most protein targets possible.
- Stable in the cytoplasm – not dependent on disulphide bonds.
- Can be genetically incorporated into the HI-loop of Ad to produce virus with nearly WT growth characteristics.
- Affinity can be manipulated to create binders suitable for cells expressing varying amounts of the receptor in question. This is important for e.g. Ad vectors where the total binding avidity of the vector would be important.
- Flat binding surface roughly 600Å^2.
- Binders to small epitopes like carbohydrates difficult to obtain.

Peptides

Small peptides binding to variety of targets e.g. cancer cells and neovasculature have been identified by phage display (Arap *et al.*, 1998; Ruoslahti and Rajotte, 2000). Identification of peptides with this method is relatively fast and efficient, but it has been shown that many ligands loose their folding/binding abilities when incorporated into Ad (Wickham, 2002). So far smaller peptides have only been used to set up the model and as to date no re-targeted Ad vector with a tumour specific peptide that binds to its aimed target *in vivo* has been published.

DE-TARGETING OF ADENOVIRUS VECTORS

Systemic administration of Ad5 derived gene therapy vectors result in vast amount of transduction to unwanted cells and tissues. In addition adenovirus is quickly inactivated by components in the blood stream, including neutralizing antibodies, complement and blood cells. Hence, it is equally important to de-target the Ad vector as it is to re-target to new receptors. The *in vitro* and *in vivo* situation however differs and de-targeting by CAR, integrin and heparan sulphate proteoglycan (HSPG) binding-ablated vectors reduce the transduction to hepatocytes *in vitro* but not *in vivo* (Martin *et al.*, 2003).

Results in vitro

At the cellular level Ad5 infection is well characterized and initiated by virus binding, through the fiber knob, to CAR on the cell surface. The second step is binding of the viral penton base to integrins and followed by receptor-mediated endocytosis.

For *in vitro* de-targeting interactions with CAR, integrins and HSPG, which receptors are widely expressed on many cell types, have to be ablated. It has been shown that such double or triple de-targeted adenovirus vectors results in a decreased transduction to liver cells *in vitro* (Koizumi *et al.*, 2006; Rittner *et al.*, 2007).

Interactions with CAR can be ablated by different approaches: first, the entire fiber can be removed to generate fiberless virus particles, although these viruses show decreased infectivity and maturation (Legrand *et al.*, 1999; Von Seggern *et al.*, 1999) or to substitute the Ad5 fiber with a fiber from another non CAR-binding serotype (Smith *et al.*, 2003b). In the second approach the fiber can be replaced by a truncated fiber with an extrinsic trimerization signal and a new cell binding ligand (Magnusson *et al.*, 2001) these viruses however often suffer from low growth rate, high infectivity indexes (PP/pfu) and low fiber content (Henning *et al.*, 2006). The third way is to mutate the amino acids in the knob domain responsible for CAR interactions (Roelvink *et al.*, 1999; Kirby *et al.*, 2000). The RGD motif in the penton base is responsible for binding to integrins and internalisation of the virus into the cell. Integrins is expressed on a variety of cells which also contribute to unwanted tissue uptake. For deletion of α_v integrin binding the RGD motif in the penton base has been either deleted or mutated (Akiyama *et al.*, 2004; Henning *et al.*, 2005).

The KKTK motif in the third shaft repeat of the fiber is involved in HSPG binding and was thought to be responsible for direct Ad attachment to HSPG and thus enabling efficient Ad liver cell transduction *in vivo* (Smith *et al.*, 2003a; Smith *et al.*, 2003b; Bayo-Puxan *et al.*, 2006). The motif has been changed in different ways to try to avoid transduction to hepatocytes, which has been successfull *in vitro* but not *in vivo.* (Koizumi *et al.*, 2006; Di Paolo *et al.*, 2007; Rittner *et al.*, 2007).

Results in vivo

Although the early steps of Ad5 infection *in vitro* are known in great detail there is still a poor understanding of the mechanisms responsible for the efficient natural Ad5 infection *in vivo*. In all animals models analysed the liver is the main organ transduced by Ad5-based vectors (Smith *et al.*, 2002; Nicol *et al.*, 2004). It was suggested that the CAR binding and the binding to HSPG via the KKTK motif were responsible of Ad liver cell infection. It has however been shown that when vectors with ablated CAR-binding were injected intravenously, they transduced liver cells as efficient as WT Ad5 (Alemany and Curiel, 2001; Smith *et al.*, 2002). Although, it is worth noting that transduction via the CAR-dependent pathway can occur *in vivo* since in a blood free perfusion model of the liver the CAR-ablated vectors showed less transduction then WT Ad5 (Shayakhmetov *et al.*, 2005). The involvement of the KKTK motif in liver infection is discussed and there is still no clear evidence that the motif actually binds to hepatic HSPG and mediates liver transduction. A recent publication suggested that the KKTK motif is not the major device for liver transduction *in vivo* and is thought to be more important for post-internalization steps of virus infection rather than for initial cell binding (Di Paolo *et al.*, 2007). Shayakhmetov and co-workers also propose that there is a complex interplay between Ad and the host system that may influence *in vivo* virus bio-distribution and cell transduction (Shayakhmetov *et al.*, 2005).

The interaction of Ad5 with blood cells greatly affects the bio-distribution *in vivo* and over 90% of applied Ad5 virus associate with blood cells *ex vivo* and hence decrease the virus access to extravascular target cells (Lyons *et al.*, 2006). On the other hand some coagulation factors (factors VII, IX, X and protein C) have the capability to enhance the Ad transduction of hepatoma cells *in vivo* (Parker *et al.*, 2006).

To achieve a de-targeted effect of recombinant virus *in vivo* ablation of CAR, integrin and HSPG binding described in the *in vitro* section is not enough. The most efficient way is probably to physically shield the vector through coating with polymers such as polyethylene glycol or poly-(N-hydroxypropyl) methacrylamide. Masking the surface of the adenovirus vector by coating is very efficient in terms of avoiding neutralizing antibodies and binding to blood cells and increase the circulation time of the vector *in vivo* (O'Riordan *et al.*, 1999; Green *et al.*, 2004; Fisher *et al.*, 2007).

Box 3. The ideal Ad vector

- De-targeted and de-sensitized to avoid unwanted tissue uptake and interactions with blood components and to avoid neutralisation by pre-formed antibodies preferably by coating the vector.
- Genetic transductional, transcriptional re-targeted to change the specificity to relevant tumour cell.
- Use of by-stander effects of certain suicide genes or toxins to increase tumour killing.
- Use of CRAds to obtain oncolysis of tumours.
- Replication competent – increased gene delivery, cell-to-cell spread.
- Should be able to cross from the blood stream into tumour tissues.

METHODOLOGICAL CONSIDERATIONS

All methods are thoroughly described in the manuscripts and are only briefly covered in this section to provide an overview of the cloning procedures and methods used in this thesis.

Cells

The recombinant viruses that were used are replication incompetent (ΔE1) and require cells stably transfected with the E1 region for propagation. The cells used for this purpose were HEK-293 (Graham *et al.*, 1977) and 911 cells (Fallaux et al., 1996). 293 cells were generated by transformation of human embryonic kidney cells with fragments of Ad5 DNA and shown to contain nucleotides (nt) 1 to 4344 (E1 region) of the Ad genome (Graham *et al.*, 1977). 911 cells (human embryonic retinoblasts (HER) were produced by transformation of a plasmid containing base pairs 79-5789 of the Ad5 genome (Fallaux et al., 1996). For production of Ad vectors targeted to other cellular receptors, that are not present on 293 or 911 cells, new packaging cell lines were generated. We produced 293 cells with surface bound anti-idiotypic Z_{taq} affibody here designated Z_{Ztaq} (Eklund *et al.*, 2002). 293_{Ztaq} cells are HEK-293 cells stably transfected for surface expression of a single chain tandem repeat version of Z_{Ztaq}. $293_{HER2/neu}$ cells are HEK-293 cells stably transfected with membrane bound HER2/neu, amplified from human breast tumor cDNA (Magnusson *et al.*, 2007).
SKOV-3 cells (from human carcinoma), SKBR-3 cells (from human breast cancer) and RD (rhabdomyosarcoma) cells were used for fiber and virus binding and Spodoptera frugiperda (Sf9) cells were used for phenotypic analysis of fiber proteins.
MM-39 cells was originally isolated from the tracheal mucosa of a young healthy adult (Merten *et al.*, 1996) and the HepG2 cell line was isolated from human hepatoma cells.

Targeting ligands

Two different types of ligands were incorporated in the Adenoviral genome, to allow targeting to other cellular receptors than CAR. These ligands have been described in detail in the introduction. Affibody molecules were used in four papers. The affibody molecule Z_{taq} reactive with taq polymerase (Gunneriusson *et al.*, 1999) was incorporated as a dimer into a truncated fiber in paper I and as a monomer together with Z_H in the HI-loop of the fiber knob in paper V. In paper III Z_H, specific for the extra cellular domain of epidermal growth factor receptor 2 (Wikman *et al.*, 2004) was incorporated as a monomeric version and as a tandem repeat into truncated fibers with different shaft repeats and in the HI-loop of the fiber knob, in paper IV as a tandem repeat in the HI-loop of the fiber knob and in paper V as a monomer before and following Z_{taq} in the HI-loop of the fiber knob.
ScFv 13R4, reactive against β-galactosidase (Martineau *et al.*, 1998) was used in paper two and inserted on the pIX protein. The genes for the new ligands were generally amplified by PCR to introduce flanking restriction sites followed by sequence

analysis. They were fused to the capsid genes by ligation or splicing by overlap extension (SOE) (Horton and Pease, 1991).

Antibodies

The antibodies used for phenotypic characterization of recombinant fiber proteins, neutralization assay, detection of recombinant pIX and blocking of gene transfer of recombinant Ad vectors are listed in table 2.

Table 2.

Antibody	Binding specificity	Origin	Reference/Manufacture
4D2.5	Fiber tail	Mouse	(Hong and Engler, 1996)
2A6	Fiber trimer	Mouse	(Hong and Engler, 1996)
GAL-13	β-galactosidase	Mouse	Sigma-Aldrich
α-CAR	CAR receptor	Mouse	ATCC
α-FLAG M2	FLAG epitope	Mouse	Sigma-Aldrich
α-Her2/neu Herceptin	Her2/neu receptor	Humanized mIgG1	Roche
α-Her2/neu	Her2/neu-ECD	Rabbit Polyclonal	(Adams et al., 2001)
α-Hexon	Hexon	Rabbit Polyclonal	Generous gift from Saw-See Hong and Pierre Boulanger
α-Penton base	Penton Base	Rabbit Polyclonal	Generous gift from Saw-See Hong and Pierre Boulanger
Penta-HIS	HIS epitope	Mouse	Qiagen

GENERATION OF RECOMBINANT ADENOVIRUS, PRODUCTION AND CHARACTERISATION PAPER I, III, IV AND V

Construction of recombinant adenoviral genomes

Recombinant fibers

Two principal types of recombinant fibers were constructed: truncated de-knobbed fibers (R7 and R13) and fibers with the new ligand inserted into the HI-loop of the knob. The basic structures of the two types of fibers are shown in fig 7.

R7 and R13 fibers

In order to construct Ad fibers with new specificity, the knob which mediate trimerization and binding to cellular receptors was removed and replaced with an

external trimerization signal, a linker and a new cell binding ligand. The trimerization motif was the neck region peptide (NRP) from the lung surfactant protein D (hSP-D) (Hoppe *et al.*, 1994). For extra flexibility a linker sequence from staphylococcal protein A (SpA) was added. The original R7 fiber was created by using splicing by overlap extension resulting in a product containing the SpA linker, NRP and the new ligand: NheI-NRP-SpA-ClaI-ligand-STOP-XhoI (Magnusson *et al.*, 2001). The recombinant gene was then ligated to a WT fiber gene using the NheI site after repeat seven and an XhoI site inserted at the end of the fiber gene, at the same time deleting repeat 8 to 22 and the knob. To generate a recombinant R13 fiber shaft, repeat 8-13 were amplified by PCR and ligated into R7-knob fiber using the NheI site at the 7th shaft repeat. Ligands were changed using the ClaI and XhoI sites (Magnusson *et al.*, 2007).

HI-loop

To construct fibers with a cell binding ligand in the HI-loop a ClaI restriction site was inserted into the HI-loop of the Ad5 wt fiber between the GGA (amino acid G541) and GAC (amino acid D542) using splicing by overlap extension (SOE). The same sequence was introduced at the cell binding ligand by PCR. The ligands were then cloned into the new ClaI site in the HI-loop of the fiber. To introduce flexible linkers an adapter containing aa Gly and Ser where introduced on either side of the ligand before cloning it into the ClaI site as described above (Magnusson *et al.*, 2007).

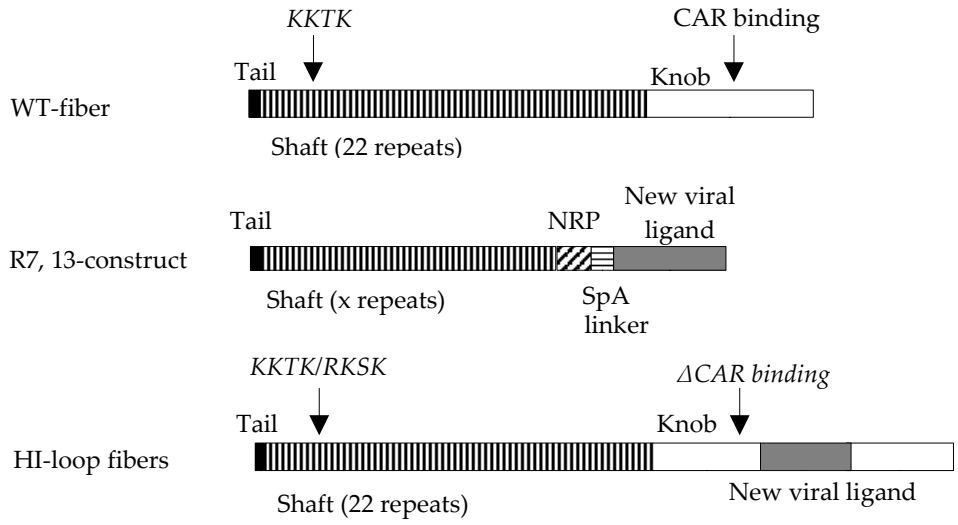


Fig. 7. Schematic representation of the different types of fiber constructs. The sizes are not proportional.

Ablation of CAR

Ablation of the CAR binding site was constructed through deletion of amino acids Leu 485 and Thr 486 (Kirby *et al.*, 1999) of the β-strand in the fiber knob.

Mutation in the 3rd shaft repeat of the fiber

The KKTK motif in the third shaft repeat was changed to amino acids RKSK to avoid HSPG binding to the liver (Dechecchi *et al.*, 2001). The rationale for choosing the RKSK motif was the resemblance of the K/R and T/S amino acids. An adaptor containing restriction sites NaeI and BsgI was used to substitute WT repeat 3 with the mutated form containing RKSK.

Recombinant penton base

An Ad5 with ablated integrin binding was constructed by mutation of the RGD motif in the penton base to EGD originated from the Ad2 penton base mutant R340E (Karayan *et al.*, 1997).

Phenotypic analysis of fiber proteins

The recombinant fibers were analyzed phenotypically for solubility, trimerization, cell binding and by biosensor analysis. To carry out these analyses the baculovirus-insect Sf9 cells expression system was chosen. Recombinant fibers were cloned into the pBacPak9 vector (Clontech, Palo Alto, CA). Recombinant baculoviruses (*Autographa californica* Nuclear Polyhedrosis Virus, AcNPV) were generated by homologous recombination in Sf9 cells using the BacVector-1000 DNA Kit, as described by the manufacturer (Novagen, Madison, WI). Baculovirus-infected Sf9 cells were harvested 48 h post infection, lysed in hypotonic buffer at 0°C and adjusted to isotonic conditions.

Solubility

For analysis of solubility the cell lysates were divided into two aliquots. One aliquot was analyzed as whole cell lysate, the other aliquot was centrifuged at 15 000 *g* for 10 min. Supernatants, pellets and whole cell lysate were then analyzed by conventional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, using anti-tail 4D2.5 mAb as primary antibody and anti-mouse-AP as secondary antibody (DAKO).

Trimerization

For studies of trimerization the supernatant was analyzed by NDS-PAGE gel electrophoresis, (NDS differs from SDS in that samples were not boiled prior the electrophoresis) using the same antibodies as for the solubility assay.

Cell binding

For cell binding assay the fiber containing supernatants were applied to cells in suspension pre-washed in Phosphate Buffered Saline (PBS). After washing the cells they were incubated with antibody 4D2.5 and washed in PBS, followed by incubation with FITC-conjugated rabbit anti-mouse Igs. FITC-labelled cells were counted using fluorescent-activated cell sorter (FACS) (FACSsort, Becton Dickinson, Heidelberg, Germany).

BIAcore (paper III)

To further evaluate if the recombinant fiber bound to the aimed target, receptor biosensor analyses of fiber proteins were carried out on a BIAcore® 2000 instrument by real-time biospecific interaction analysis (BIA). The extra cellular domain of HER2/neu (HER2-ECD) (diluted in 10 mM NaAc, pH 4.5) was immobilized (~ 2600 RU) on the carboxylated dextran layer of one flow-cell surface of a CM5 sensor chip (research grade) (BR-1000-14, Biacore) by amine coupling, according to the manufacturer's instructions. Another flow-cell surface was activated and deactivated for a reference surface. Human IgG (Amersham Biosciences), and HIV-1 gp120 (2003-MN, Protein Sciences Corp., Meriden, CT) were immobilized on separate flow-cell surfaces on the CM5 sensor chip, to serve as negative controls. The recombinant fiber sample was diluted in HBS-EP (5 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4) and the samples were thereafter filtrated (0.45 μm; Millipore, Billerica, MA). Binding analyses were performed at 25 °C, and HBS-EP was used as running buffer. The FibΔCAR-HI-link-Z_HZ_H fiber sample was injected over all surfaces with a flow rate of 20 μl/min and run in duplicates. As non-binding controls, two fibers liganded by unrelated affibody molecules were also injected.

Rescue of recombinant fiber and penton base genes into the Ad genome

Fiber

A three-step rescue system has been developed to generate recombinant Ad5 genomes. The Ad5/WT-fiber gene was replaced by recombinant fibers in the Ad5 genome using a previously described procedure based on homologous recombination and cosmid cloning (Magnusson et al., 2001). Briefly, fiber constructs were first ligated into a shuttle vector pGAG3 using the NdeI-XhoI sites (step1, fig. 8A) and further rescued into pGAG9 (step2, fig. 8A) using homologous recombination in *E.coli* BJ5183. The third step (fig. 8A) was to join the recombinant pGAG9 to the Ad5 genome by cosmid cloning, which is applied for ligations with fragments between 30-42kb in size (SuperCos 1 cosmid vector kit manual; Stratagene).

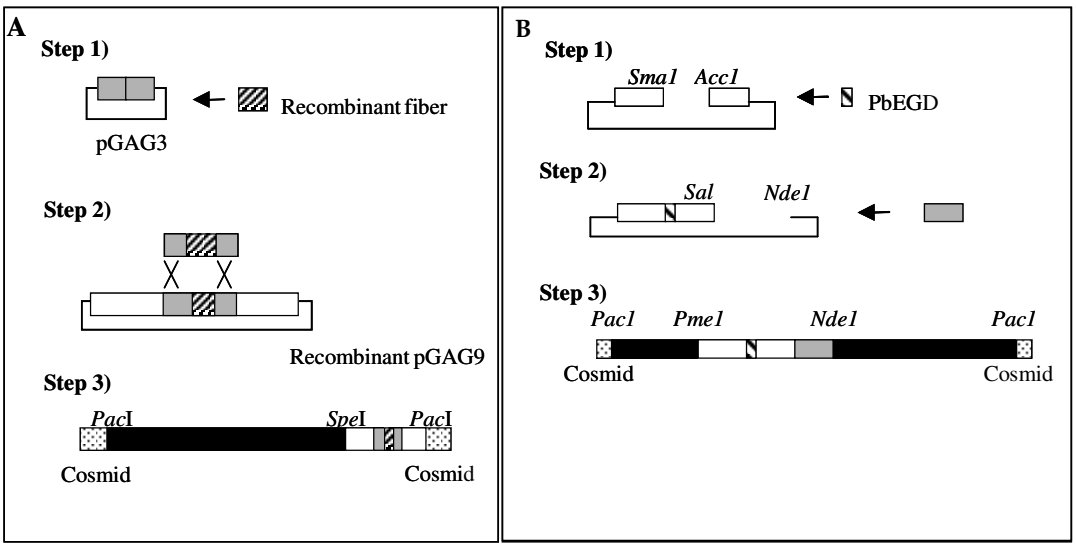


Fig: 8. Cloning strategies for fiber (A) and penton base (B) as described in the text.

Penton Base

WT penton base was replaced by a RGD binding mutant made in the Ad2 Pb (Karayan *et al.*, 1997). Pb-mutant R340E was inserted into Sma1-Asc1 of a shuttle plasmid containing the Ad5 Pme1-Sa1 fragment from mu 36.9-46.6 (step 1, fig 8B). The Ad5 fragment from Sal I to Nde I (mu 46.6-54.4) was ligated to this vector (step 2, fig 8B), resulting in a 36.9-54.4 mu R340E Ad2/Ad5 mutant which was joined to the rest of the Ad5 genome by cosmid cloning together with the Pac I-Pme I (mu 1-36.9) and Nde I-Pac I (mu 54.4-100) fragments (step 3, fig 8B) (Henning *et al.*, 2005). There is a 98.6% homology between the Pb from Ad2 and Ad5 (Neumann *et al.*, 1988)

Production and characterisation of recombinant adenovirus

In the recombinant Ad5 genomes, the E1 region was replaced with the green fluorescent protein (GFP) gene under a CMV promoter. PCR amplification and DNA sequencing was used to verify the presence of the correct gene. The genome was also controlled by cleavage with HindIII and SpeI. Recombinant Ad5 genomes were restricted with PacI and used to transfect desired cells using the FuGene technology and the manufacturer's protocol (Roche). Cells were evaluated for cytopathogen effect (cpe) and GFP expression for up to three weeks. Presence of recombinant Ad5 genomes was verified by PCR and the recombinant viruses were purified from infected cells by freeze thawing followed by cesium chloride (CsCl) gradient centrifugation. Briefly, cells were infected with culture medium from virus-lysed cells and followed until prominent cpe was observed. Cells were pelleted by centrifugation and resuspended in a small volume of culture medium followed by four rapid cycles of freeze-thawing in liquid N_2/37ºC water bath to lyse the cells and release virions. Cellular debris was removed by centrifugation and the cleared virus containing supernatant mixed with 0.6 volumes of saturated CsCl in 0.01 M Tris-HCl pH 8, 0.001 M EDTA and ultra centrifuged at 176000 g, 4ºC for 14 h. The virus band was collected and in paper I and III diluted with 5 volumes of viral storage medium (50 % glycerol, 10 mM Tris-HCl pH 8, 100 mM NaCl and 0.1 % bovine serum albumin). In paper IV and V the virus band after CsCl centrifugation was collected and dialyzed against NNKS buffer (140mM NaCl, 5mM $Na_2HPO_4.2H_2O$, 1.5mM KH_2PO_4, 5% sucrose, pH 7,8). Virus preparations were aliquoted and stored at -80ºC until use.

Determination of titers and physical particles

Plaque-forming units (pfu) and physical particles (pp) were determined for all the recombinant viruses. Viral titer was analyzed by end-point dilution (O'Reilly *et al.*, 1994), which is performed by inoculation of multiple cultures with different dilutions of the virus, and estimation of the largest virus dilution that infected 50% of the cultures. This value was converted into pfu/ml.

The number of viral particles per milliliter was determined by measurement of optical absorbance (Mittereder *et al.*, 1996). The sample was diluted 1:20 in a lysis buffer, incubated at 56°C for 10min and measured at OD_{260}. The concentration of Adenovirus

vector virions was determined by multiplying the absorbance by the dilution factor and then dividing with a coefficient determined for WT Adenovirus ($9.09 * 10^{-13}$) corresponding to $1.1 * 10^{12}$ virions per OD_{260}.

Fiber content of recombinant Ad5 virions

The fiber content in CsCl-purified Ad5 virions was determined using SDS-PAGE and Western Blot analysis. The virus loads in SDS-PAGE were normalized to equal amounts of viral particles. A blot with the anti penton base polyclonal antibody was used as a control. Fiber was detected with anti-Ad5 fiber tail mAb 4D2.5 (Hong and Engler, 1996). Rabbit polyclonal sera were used for detection of penton base (serum made by P. Boulanger and S.S. Hong).

Growth rate of recombinant Ad

Growth rate was measured by the yield of pfu per infected cell at 24, 48 and 72 h pi. First the cells were infected with equal amount of pfu, for each recombinant virus, for one hour. Cells and medium were harvested after different time points and following cell lysis by freeze thawing the number of infectious virions in the lysates was determined on appropriate cell line by end-point dilution as described above.

Viability of cells infected with replication competent virus (paper IV)

The viability of cells infected with replication competent virus was performed in 96-well plate using different infection doses. Virus was added to cells and incubated for 8 days. The viability of the cells was measured using the XTT cell proliferation test II (Roche) at 450nm. Wells with known percentage of viable cells were used to create a standard curve.

Re-targeting of adenovirus

Re-targeting and binding specificity of recombinant virus was evaluated by gene transfer to cells expressing different surface receptors.

The re-targeting of recombinant Ad was studied by gene transfer of GFP to different cell lines in order to demonstrate the novel cellular specificities. Infections were performed in 24 well plates for 1 h at 37°C. The cells were harvested approximately 15-20 h pi, and GFP-expressing cells were counted using FACS (FACSsort, Becton Dickinson). Blocking of infection was performed by adding Ad5 knob, a fiber lysate or an affibody to the cells and incubated at 37 or at 4°C for one hour before adding the virus to the cells.

Studies with coagulation factors IX, X, Xa and Protein C were performed by adding 1U/ml of each factor, or 4µg/ml Protein C, to the medium prior addition of 1000 PP of the recombinant viruses. Infections were performed in 24 well plates for 3h at 37°C and analyzed as above.

GENERATION OF LENTIVIRUS VECTORS AND INCORPORATION OF RECOMBINANT pIX PROTEIN IN THE ADENOVIRUS CAPSID, PAPER II

Lentiviral production system

For incorporation of the 13R4 scFv in the adenovirus genome a fusion gene was constructed in which the coding region of pIX was fused via a flag epitope with the codons for a 75-Angstrom spacer, and with the codons for 13R4 scFv. The fusion gene was then incorporated into a lentivirus expression vector. The lentiviral vectors are so called self inactivating (SIN) vectors (Zufferey *et al.*, 1998) and contain the Rev-responsive element sequence, the central polypurine tract (cPPT) and the human hepatitis B derived post-transcriptional regulatory element. The lentiviral vectors were derived from the plasmid pRRL-cPPT-CMV-eGFP-PREsense-SIN (Seppen *et al.*, 2002) and named pLV-CMV-eGFP. The gene for pIX.flag.75 was obtained from the pCDNA3.1-derived construct pAd5pIX.MYC.flag.75.MYC (Vellinga *et al.*, 2004) and the gene encoding the scFv 13R4 was subcloned from the plasmid pPM163R4 (Martineau *et al.*, 1998) The lentiviral vectors were produced by three "helper" plasmids encoding the HIV-1 gag-pol, HIV-1 rev, and the VSV-G envelope. The plasmids were co-transfected together with the pLV-CMV-pIX.MYC.flag.75.13R4.MYC.HIS-eGFP construct into 293 cells. After overnight incubation the cells were washed and cultured. The medium was harvested 48 and 72h post transfection and passed through a 0.45μm filter and stored at -80°C. The viruses were quantified by antigen-capture ELISA measuring HIV p24 amounts (Back *et al.*, 1996). Approximately 1 ng p24 is equal to 2 x 10^3 transducing units (Barry *et al.*, 2001).

Transduction of lentiviral vectors

For generating a 911 cell line that expresses pIX.MYC.flag.75.13R4.MYC, cells were transduced by adding the lentiviral supernatant together with Polybrene (Sigma-Aldrich). After overnight incubation the medium was replaced and cells were cultured in medium containing G418.

Adenovirus vector

The Ad5ΔpIX.CMV.GFP/Luc vector was produced by a previously described protocol (Vellinga *et al.*, 2004). Briefly; the pIX gene in the shuttle plasmid pTrackCMV-GFP was deleted through introduction of a ScaI site at the start codon and a SpeI site at the C-terminus and blunt-end self ligation after ScaI and SpeI digestion and Klenow treatment. The vector was then generated by transfection to 911 cells following CsCl purification (Fallaux *et al.*, 1996).

Western Blot

Western analysis were performed to study the pIX.MYC.flag.75.13R4.MYC incorporation into the 911 cells, the loading of pIX.MYC.flag.75.13R4.MYC at Ad5 virus particles and to study β-galactosidase binding of pIX.MYC.flag.75.13R4.MYC at the surface of the Ad5ΔpIX.CMV.GFP/Luc-pIX.MYC.flag.75.13R4.MYC virus.

Sodium dodecyl sulfate (SDS)-denatured proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% acrylamide gels using a discontinuous buffer system.

Immunohistochemistry assay

Immunohistochemistry was used for analysing the amount of the pIX fusion genes in transduced 911 cells. 911-pIX.flag.75.13R4 cells were grown on glass cover slips in a six well plate, fixed and washed and incubated with anti-FLAG M2 antibody followed by a FITC labelled goat anti-mouse serum. The nuclei of the cells were stained with propidium iodide.

β-galactosidase binding

To study the functionality of the scFv in the Ad capsid, the β-galactosidase-binding capability of 13R4, on the surface of the adenoviral vector, was assayed. The viruses were mixed with β-galactosidase and purified by CsCl gradient centrifugation in order to separate free β-galactosidase from β-galactosidase bound to 13R4 of the adenoviral particles. To examine the binding to β-galactosidase the viruses were analyzed by Western Blot and immunoelectron microscopy.

Another approach to measure the β-galactosidase binding capability was to trapp the loaded virus particles in DAKO IDEA microwells and incubated with β-galactosidase. The DAKO kit was developed for demonstration of Ad5 in clinical specimens and the microwell strips contain anti-Ad5 antibody. After blocking and washing the particles were exposed to the substrate (ONPG) and compared to WT Ad5.

DE-TARGETING ASSAYS PAPER I AND IV

Neutralization assays (paper I)

Endpoint

To evaluate if a recombinant Ad 5 vector with the fiber knob domain replaced by a new cell binding ligand would have decreased sensitivity to pre-formed neutralizing antibodies, the neutralizing activity in sera from fifty blood donors were assayed against recombinant virus and compared to the activity against Ad5 with WT fiber. The endpoint neutralization assay is described in (Losman *et al.*, 2001). Briefly: Adenovirus endpoint ID50 titers were determined in microplates using the Reed-Muench method (Reed, 1938). Twofold serial dilutions of serum specimens were performed. Pre-titrated virus suspensions were added to serum dilutions and the virus antibody mixtures were allowed to interact at 37°C for 4 hours. Suitable target cells were added, and the cultures were incubated at 37°C for 4 days. All specimens were analyzed in triplicate and the titer was defined as the reciprocal of the serum dilution inhibiting cytopathic effects in at least two of the three wells.

Pre-absorption

The neutralization activity carried out by knob and hexon antibodies was assayed by pre-absorption of sera onto purified knob or hexon proteins or both and subsequently assayed for their neutralizing activity (NA) against Ad5Luc3. Pre-absorption of sera were performed by over night incubation at 4°C on 96-well microplate coated with purified protein consisting of knob, hexon or both knob and hexon. The sera were then serially diluted and incubated with Ad5Luc3 virus suspension. The mixture was then added to 293 cells and after 24h collected and assayed for luciferace activity.

Ad-binding antibodies (paper I)

The presence of antibodies and their isotypes, in blood donor sera, against adenovirus antigens was determined by immunohistochemistry using secondary antibodies specific for IgG, IgA and IgM. Cells were incubated with virus for 1h at 37°C washed once and incubated over night. Cells were detached and centrifuged onto microscope slides in a Cytospin2 cytocentrifuge, fixed and permeabilized. The slides were incubated with human sera as primary antibody and with anti-IgG, anti-IgA and anti-IgM as secondary antibodies. Preparations were mounted in PBS with 50% glycerol and viewed in a Zeiss Axophot microscope.

Antibody reactivity to Ad capsid proteins (paper I)

Determination of antibody reactivity to Ad capsid proteins was performed by Western Blotting. Sodium dodecyl sulfate (SDS)-denatured proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% acrylamide gels using a discontinuous buffer system. Native proteins were separated in 8% polyacrylamide gels in the same discontinuous buffer system but without SDS and twice the normal buffer concentration. Blots were reacted with patient sera, followed by phosphatase-labelled anti-human IgG conjugate (Sigma-Aldrich).

Virus particles associated with blood cells (paper IV)

To test the association of recombinant Ad vectors with human blood cells aliquots of whole blood were mixed with equal amount of PP for 30min. Unbound particles were removed by three washes in PBS and DNA which had bound to blood cells was purified using QIAampDNA kit (Qiagen) and analysed by Q-PCR. In comparison, DNA was purified from blood mixed with virus without washing to give the value of 100% association.

Tissue distribution (paper IV)

To examine the tissue distribution of different Ad vectors in mice, viruses (1×10^{11} PP/mouse in NNKS buffer) were injected intra venously (iv) in the lateral tail vein and tissues were collected after 24h. DNA from liver, kidney, spleen, lung and heart was purified with QIAampDNA kit (Qiagen) and Q-PCR was used to determine the amount of DNA from each tissue.

Quantitative Polymerase Chain Reaction (Q-PCR) (paper IV)

Real-time PCR was carried out using Power SYBR Green (Applied Biosystems, Warrington UK) in an Applied Biosystems 7500 real-time PCR system detector. Primers were designed using the Primer Express software (Applied Biosystems). The final reaction consisted of 10µl Power SYBR Green Master Mix, 1µl each primer and up to 8µl sample. All samples were amplified in triplicates under the following conditions: 95°C for 10min and 40 cycles of 95°C for 15s and 60°C for 1min.

RESULTS AND COMMENTS

This section summarises the result from the five papers. The figures can be seen in the individual papers.

PAPER I

Decreased immune reactivity towards a knobless, affibody-targeted Adenovirus type 5 vector.

Aim: To investigate if an Ad5 vector, which has the fiber knob domain replaced by a new cell ligand, would have decreased sensitivity to human pre-existing Ad Nabs.

Results

- Recombinant adenovirus with a truncated, knobless fiber and with the affibody molecule Z_{taq} as a new cellular ligand was generated and successfully rescued into the adenoviral genome and subsequently into functional virions. The recombinant virus was named Ad5/R7-$Z_{taq}Z_{taq.}$
- A panel of fifty blood donor sera was used to test the presence of neutralizing antibodies in a normal population against WT Ad5 and the recombinant virus Ad5/R7-$Z_{taq}Z_{taq.}$
- The recombinant virus showed a decreased sensitivity to human pre-formed antibodies in all sera except for no 36 and 49, when compared to Ad5 WT.
- The neutralizing activity against Ad5/R7-$Z_{taq}Z_{taq}$ in sera no 36 and 49 was likely due to hexon antibodies.
- Antibody neutralization due to the fiber was mainly localized to the knob.
- All sera contained antibodies to Ad antigens. There where mainly IgA and IgG antibodies indicating that the response was of the mucosal type.
- All sera examined were rich in penton-base- and fiber antibodies.

Comments

The neutralization assay showed a strong reduction of titers against the recombinant virus when compared to WT Ad. The sera examined were not reactive with the R7-$Z_{taq}Z_{taq}$ fiber indicating that high-titer neutralizing activity is dependent on antibodies against the penton base or the fiber knob, except for sera no 36 and 49 were the neutralizing activity was due to hexon antibodies. The low neutralizing activity against the affibody liganded virus implied that there was a very low immunity detected by the neutralization assays to the scaffold portion of the affibody molecule which is derived from the B domain of Staphylococcal protein A.

PAPER II

Efficient incorporation of a functional hyper-stable single-chain antibody fragment protein-IX fusion in the adenovirus capsid.

Aim: To evaluate the efficiency of incorporation of a single-chain antibody fused with the minor capsid protein pIX in adenovirus vectors.

Results

- A fusion gene was constructed where the coding region of pIX was fused to a flag epitope, the codons for a 75-Angstrom spacer and the sequence of the scFv13R4. The resulting fusion gene, pIX.flag75.13R4.MYC.HIS, was inserted into a lentiviral expression vector.
- The pIX.flag75.13R4.MYC.HIS containing lentiviral vector was transduced to 911 cells, in order to generate a cell line expressing the recombinant pIX.
- The pIX.flag75.13R4.MYC.HIS amounts produced by the transduced 911 cells were similar to the pIX level produced by 911 cells during an Ad5 infection.
- It was possible to incorporate the recombinant pIX protein pIX.flag75.13R4.MYC.HIS into the adenoviral genome which had a deleted pIX gene.
- The amount of pIX in pIX.flag75.13R4.MYC.HIS loaded Ad5ΔpIX.CMV.GFP/LUC particles was similar to the pIX amounts in WT Ad5.pIX.flag75.13R4.MYC.HIS was accessible on the outside of the Ad5 virion.
- The Ad5 particles loaded with pIX.flag75.13R4.MYC.HIS protein were able to bind to β-galactosidase.

Comments

New ligands that will be incorporated and expressed on the adenoviral capsid must be able to fold correctly in the reducing environment in the cell cytoplasm. This excludes the use of most ligands dependent on disulphide bond formation for proper folding. The 13R4 however, is an example of a hyperstable single chain antibody that is independent on disulphide bridges for correct folding. Tumour specific hyperstable scFv can most likely be developed by construction of scFv libraries based on a stable scFv framework. In this study we demonstrated that it is possible to incorporate a scFv fused to the pIX protein into the Ad5 viral capsid with retained binding to β-galactosidase. However, re-targeting of Ad5 loaded with pIX.flag75.13R4.MYC.HIS protein could not be performed since β-galactosidase cannot be used as receptor on cell surfaces. It is therefore important to evaluate and compare the re-targeting efficiency with a scFv fused with different capsid proteins.

PAPER III

Adenovirus 5 vector genetically re-targeted by an Affibody molecule with specificity for tumour antigen HER2/neu.

Aim: To construct a re-targeted Ad5 vector using the HER2/neu reactive affibody molecule (Z_H) specific for human epidermal growth factor receptor 2 that is over-expressed in different cancers, above all breast and ovarian and to evaluate the best position of the ligand in the fiber.

Results

- The affibody molecule (Z_H) was incorporated in two different ways in the adenoviral genome: First, Z_H was incorporated into truncated fibers,

containing 7 or 13 shaft repeats, which have the fiber knob domain removed and replaced by an external trimerization signal and the ligand. Second, Z_H was incorporated in the HI-loop of a CAR ablated fiber with or without flexible linker. In all fiber constructs Z_H was inserted both as a monomer and a dimer.

- In the phenotypic analysis all fibers showed the same pattern, they were all soluble, able to form trimers and did bind to the ovarian cancer cell line SKOV-3, when expressed in the cytoplasm of Sf9 cells.
- BIAcore measurement further demonstrated that the fiber FibΔCAR-HI-Link-Z_HZ_H bound to the aimed target HER2-ECD (the extra cellular domain of HER2/neu).
- All fibers were able to be rescued into the adenoviral genome and FibΔCAR-HI-Link-Z_HZ_H was also cloned to an Ad genome with an aa substitution in the penton base (RGD to EGD).
- Ad5/FibΔCAR-HI-Link-Z_HZ_H was shown to be better in terms of generating functional viruses when compared to the other recombinant Ad5 constructs.
- The fiber content for Ad5/FibΔCAR-HI-Link-Z_HZ_H was 22% compared to WT Ad5 and for Ad5/**EGD**/FibΔCAR-HI-Link-Z_HZ_H 12% and the growth rate suggested that the recombinant viruses were slower than WT Ad5.
- The FibΔCAR-HI-Link-Z_HZ_H viruses showed specificity for $293_{HER2/neu}$ cells in transducing experiments and the binding was shown to be mediated by Z_H.
- Both the Ad5/FibΔCAR-HI-Link-Z_HZ_H and Ad5/**EGD**/FibΔCAR-HI-Link-Z_HZ_H viruses did target the breast cancer cell line (SKBR-3) and the ovarian cancer cell line (SKOV-3).

Comments

Affibody molecules have a small robust framework and are independent of disulphide bounds which make them suitable for Ad re-targeting. The affibody molecule presented here has specificity towards the tumour antigen HER2/neu and is the first affibody with tumour specificity that is used for Ad re-targeting.

The results showed that Z_H inserted with flexible linkers in the HI-loop of the fiber knob was superior to fibers with Z_H inserted without linkers in the HI-loop or in knobless truncated fibers. Ad5/FibΔCAR-HI-Link-Z_HZ_H was close to WT Ad5 in terms of growth characteristics and the virus with the Z_HZ_H tandem repeat was more effective in virus production and infectivity than the virus with the Z_H monomer variant. Recombinant virus with mutation in the RGD motif in the penton base (Ad5/**EGD**/FibΔCAR-HI-Link-Z_HZ_H) was a virus with a higher infectivity index compared to Ad5/FibΔCAR-HI-Link-Z_HZ_H, demonstrated that the mutation had a hampering effect on virus growth. On the other hand the RGD mutated virus did infect cells equally well as the recombinant virus with WT RGD (using the same pfu/cell), indicating that the Z_H re-targeted viruses do not need RGD binding to integrins for internalization.

PAPER IV

In vitro and in vivo evaluation of HER2/neu re-targeted and CAR-, α_v integrin- and HSPG-binding de-targeted Adenovirus vectors.

Aim: To investigate the properties of a Z_H re-targeted Ad vector with additional de-targeting steps.

Results

- The FibΔCAR-HI-Link-Z_HZ_H construct from paper III was further evaluated by insertion of an additional de-targeting step, where the KKTK motif in the third shaft repeat was changed to RKSK.
- The fibers (deleted CAR binding or deleted HSPG-binding or both) re-targeted with Z_H were successfully cloned to Ad genome containing RGD or the mutated version EGD in the penton base. This gave rise to single, double and triple de-targeted recombinant Ad vectors.
- The vector characterizations showed that the infectivity ratio (PP-to-PFU) for the recombinant viruses ranged from 140-1020 compared to an average of 50 for WT Ad. The fiber content was approximately 25% of WT and the growth rate demonstrated that the recombinant viruses were slower than WT.
- Binding of Ad5/FibΔCAR-HI-Link-Z_HZ_H to MM39 cells could be blocked by heparin whereas the binding of Ad5/Fib**RKSK**ΔCAR-HI-Link-Z_HZ_H could not.
- The CPE study demonstrated that Ad5/**EGD**/Fib**RKSK**ΔCAR-HI-Link-Z_HZ_H virus was most efficient in terms of killing SKOV-3 cells, whereas Ad5/FibΔCAR-HI-Link-Z_HZ_H had a better infectivity.
- Tissue distribution in mice showed that the transduction levels in different tissues were lower for the recombinant vectors as compared to WT except in the lung and heart where Ad5/Fibwt and Ad5/FibΔCAR-HI-Link-Z_HZ_H showed similar levels. The only difference between the recombinant vectors were detected in lung and heart where Ad5/**EGD**/Fib**RKSK**ΔCAR-HI-Link-Z_HZ_H showed less transduction than Ad5/FibΔCAR-HI-Link-Z_HZ_H.
- The association with blood cells was much reduced for the recombinant vectors (less than 10%) when compared to WT (app. 90%).

Comments

The functionality of the Z_H re-targeted virus from paper three was further evaluated and another de-targeting step where the KKTK motif was altered. RKSK was chosen due to the similarity between K/R and T/S amino acids. K and R are basic and T and S are uncharged. The virus with RKSK modifications spread more rapidly in the cell monolayer and produced better growth curves than the Ad5/FibΔCAR-HI-Link-Z_HZ_H virus, suggesting that RKSK in some way gives a positive effect on the transduction. There were no differences in the association with blood cells between the recombinant viruses and in tissue distribution there was a minor discrepancy between single-, double- and triple de-targeted viruses indicating that the deleted CAR binding and/or insertions of Z_H are the reasons for the loss of binding.

PAPER V

A re-targeted Adenovirus with dual specificity; binding specificities for two different Affibody molecules at differing positions in the HI-loop of the fiber knob

Aim: To construct a re-targeted Ad5 vector with two different affibody molecules in the HI-loop of the fiber knob in order to generate a virus with double specificity and determine which position is best suited for ligand – target cell interaction.

Results

- Recombinant fiber constructs were generated with Z_H and Z_{taq} at altered positions surrounded by flexible linkers in the HI-loop of the fiber knob.
- Phenotypic analysis showed that the fibers with two different affibody molecules were soluble and formed homotrimeres.
- Cell binding assays showed that FibΔCAR-HI-Link-Z_HZ_{taq} unpredictably only bound to 293_{Zztaq} cells and not to $293_{HER2/neu}$ cells whereas FibΔCAR-HI-Link-$Z_{taq}Z_H$ was able to bind both cell types.
- Both FibΔCAR-HI-Link-Z_HZ_{taq} and FibΔCAR-HI-Link-$Z_{taq}Z_H$ were successfully rescued into the adenoviral genome.
- The recombinant viruses were transfected to both 293_{Zztaq} cells and $293_{HER2/neu}$ cells. Ad5/FibΔCAR-HI-Link-Z_HZ_{taq} was able to produce plaques only on 293_{Zztaq} cells whereas Ad5/FibΔCAR-HI-Link-$Z_{taq}Z_H$ generated plaques on both cell lines.
- The PP/PFU ratio was 680 for Ad5/FibΔCAR-HI-Link-$Z_HZ_{taq,}$ 667 for Ad5/FibΔCAR-HI-Link-$Z_{taq}Z_H$ and 150 for Ad5/FibΔCAR-HI-Link-Z_HZ_H.
- The gene transfer assay showed that Ad5/FibΔCAR-HI-Link-ZtaqZH infects 293_{Zztaq} and $293_{HER2/neu}$ cells at equal efficiency whereas Ad5/FibΔCAR-HI-Link-ZHZtaq has much higher specificity for 293_{Zztaq} cells than for $293_{HER/neu}$ cells. Ad5/FibΔCAR-HI-Link-ZHZH showed specificity for $293_{HER2/neu}$ cells, but bound only marginally to 293_{Zztaq} cells.

Comments

The results presented in paper V showed that is possible to incorporate two different affibody molecules into the HI-loop of the fiber knob and retain the cell binding specificity for both ligands. On the other hand the order, in which the affibody molecules should be placed to achieve the most efficient binding of ligand to target cells, is important and has to be evaluated. The affibody molecules used were Z_H which binds to the tumour antigen HER2/neu and has a clinical relevance for cancer gene therapy purposes and Z_{taq} which binds to taq polymerase and is a ligand used only to set up the model. The lack of binding of Z_H to HER2/neu when inserted in the first position was an unexpected result and is to the author not clear.

DISCUSSION

Ad5 vectors should ideally be used systemically and be able to target selected cells with minimal targeting of other tissues. To achieve this, the vectors need to be genetically re-targeted to a specific receptor, e.g. a tumour specific antigen. This is because the normal Ad5 receptor, CAR, is present on cells of many normal tissues and is at the same time often down regulated on tumour cells, making the cells unresponsive to transduction by Ad.

The role of de-targeting has also been clearly established. It has been demonstrated that tumour targeting with intraperitoneally administered Ad vectors is possible in mice if the survival time in the circulation is sufficiently prolonged. Such prolongation can be achieved by double-ablated (CAR and integrin binding) de-targeted vectors that are re-targeted to a tumour cell antigen (Akiyama *et al.*, 2004). In addition, there are data suggested that much of the un-wanted liver uptake in mice, rats and non-human primates of WT Ad is due to the KKTK motifs in the third fiber shaft repeat (Smith *et al.*, 2003a; Smith *et al.*, 2003b).

In addition, when vectors are to be used for human purposes, there are other problems like interaction with components in the blood stream such as blood cells, complement and neutralizing antibodies which hamper the vector to reach its target. Pre-formed neutralizing antibodies are the sequel of earlier infections with Ad. Such antibodies are present in a majority of individuals (Gahery-Segard *et al.*, 1997; Gahery-Segard *et al.*, 1998; Russell, 2000) and constitute a severe obstacle for systemic administration of Ad. Hence it is important to generate Ad vectors that are protected against the action of pre-existing neutralizing antibodies and association with blood cells and complement.

RE-TARGETING OF AD (PAPERS II, III AND V)

Choice of targeting ligands

There is a restriction on ligands to be used for genetic re-targeting of Ad; i.e. the ligands must be able to fold correctly and maintain their receptor binding in the reducing environment of mammalian cell cytoplasm (Magnusson *et al.*, 2001). It was previously shown that affibody molecules, that are based on a partially randomized three-α-helix bundle scaffold and are stable in the cytoplasm, could be successfully used to create genetically re-targeted adenoviruses (Henning *et al.*, 2002). In paper III we presented an affibody molecule (Z_{HER2}) specific for human epidermal growth factor 2 receptor, which is over-expressed in human breast cancer. Z_{HER2} is the first affibody molecule with a therapeutic potential that has been genetically incorporated into an Ad vector. In paper II we demonstrated that a hyperstable scFv, 13R4 that is essentially stable in the reducing milieu of the cytoplasm could indeed be incorporated into Ad virions with retained antigen binding.

Affibody molecules are small ligands compared to mAb´s and scFv´s, which is an advantage for genetic incorporation into the Adenovirus genome. We have also shown that it is possible to incorporate the sequences for two different affibody molecules in the same genome to achieve double specificity (Henning *et al.*, 2002; Myhre, 2007). Even though affibodies are small molecules, it has been shown that the antigen-binding surface is quite similar in size to the antigen-binding surface of antibodies (Hogbom *et al.*, 2003). Recently a new identified affibody molecule directed against epidermal growth factor receptor (EGFR) which is over expressed on many malignant tumours such as glioblastoma, head and neck carcinoma and urinary bladder carcinoma has been selected (Friedman *et al.*, 2007). However this affibody molecule has yet not been incorporated into Ad and tested for targeting purposes.
The field of affibody molecules is still not thoroughly investigated and at present we do not know how useful the system is in order to identify more different tumour specific ligands suitable for Ad re-targeting.

Unlike affibody molecules, mAb's have been selected towards a wide variety of targets and several are in clinical use for cancer therapy. This has shown the safety and specificity of antibodies *in vivo*. ScFvs are larger molecules than affibodies but this was not a problem when the scFv 13R4 was fused with the pIX protein and loaded on Ad5 particles. The stability of single chain antibodies has been a major dilemma for their expression in the cytoplasm of eukaryotic cells and unfortunately re-targeting of 13R4 inserted at the Ad capsid could not be performed since β-galactosidase can not be used as a cellular receptor. However the present demonstration that a hyperstable scFv can be rescued into functional Ad may open the way to use such single chain antibodies for clinical re-targeting of Ad. Just a few years ago there was a report on an antibody bacteriophage display library built on the scaffold of a single-chain variable fragment and with randomization in the CDRs that allowed isolation of new stable binding specificities for intracellular applications (Desiderio *et al.*, 2001). Furthermore, it should be possible to graft the specificity-determining CDR regions from e.g. tumour specific antibodies onto the framework of scFv 13R4 to create hyperstable, tumor specific antibodies. This principle has successfully been demonstrated (Donini *et al.*, 2003).

Most commonly used for re-targeting purposes of Ad are peptides, including RGD binding to integrins and polylysine binding to heparan sulphate proteoglycans (Wickham *et al.*, 1997; Dmitriev *et al.*, 1998; Ranki *et al.*, 2007). There are also studies showing that disulfide bond dependent proteins or peptides can be incorporated into viable Ad, however, *in vivo* studies for homing to a tumour have not been performed (Dmitriev *et al.*, 1998; Majhen *et al.*, 2006). Although identification of peptides binding to tumour antigens, by phage display, is relative fast and efficient the peptides often loose their folding and/or binding when incorporated into Ad. For example the RGD4C peptide was described to bind αvβ3 and αvβ5 integrins but the specificity is largely dependent of which cysteins that are combined in the disulfide bond

formation of the cyclic structure (Arap *et al.*, 1998; Assa-Munt *et al.*, 2001). New phage display designs where peptides are displayed on phages in the framework of the Ad fiber protein or fiber knob increase the possibility to select new binders that maintain the specificity when incorporated into Ad vectors (Pereboev *et al.*, 2001; Ghosh and Barry, 2005; Miura *et al.*, 2007).

To address the folding/binding issue of different peptides we have, in an unpublished study, tried to incorporate different tumour specific peptides into various parts of the affibody molecule Z_H and inserted the construct in the HI-loop of the fiber knob. This to make use of the extremely stable framework of the affibody molecule to ensure the correct loop formation of these peptides and try to generate a recombinant Ad vector re-targeted with a tumour specific peptide. Unfortunately none of these peptides bound to their cellular receptor when expressed in the cytoplasm of Sf9 cells indicating that i) the peptides have lost their folding even though they are expressed on the surface of the affibody molecules or ii) that the peptides do not bind to their target antigens with high affinity. This suggests that ligands like affibody molecules and scFv are superior to smaller peptides in terms of their targeting capacity, when genetically incorporated into an Ad vector.

Construction of recombinant viruses and choice of anchoring structure

The choice of anchoring capsid protein on the Ad vector is of great importance since the manipulations of virus capsid protein sequences connected with insertion of ligand sequences affects virus phenotype and it has been shown that adenovirus with modified capsid proteins often are difficult to produce to wild type titers (Magnusson *et al.*, 2001). In order to compare phenotypic effects we have therefore generated adenovirus vectors with ligand insertions at different positions: i) into a knobless, shortened fiber and ii) in the HI-loop of the fiber knob. We have also produced an Ad vector with a ligand inserted at the pIX protein.

Fiber (paper III)

i) In the truncated fiber, shaft repeats 8-22 or 14-22 were deleted and an external trimerization signal, a linker and a new cell binding ligand was inserted (Magnusson *et al.*, 2001; Magnusson *et al.*, 2002).

ii) Ligands were incorporated in the HI-loop of the fiber knob, with and without flexible linkers, through engineering of a new Cla I site. The reason for choosing the HI-loop for ligand insertions were due its properties; being exposed on the outside of the knob and demonstrating high flexibility. There have also been several publications showing that ligands can be inserted in the HI-loop (Krasnykh *et al.*, 1998; Belousova *et al.*, 2002). Flexible linkers were introduced upstream and downstream to allow independent folding of the ligand as well as flanking knob domains (Magnusson *et al.*, 2007).

The shortened knobless fibers are promising in respect to their ability to bind to cells expressing the corresponding receptor and there are several constructs that have

been rescued into functional virions (Magnusson *et al.*, 2001; Henning *et al.*, 2002; Magnusson *et al.*, 2002), which demonstrate the basic functionality of the system. Other advantages of the shortened knob-less fibers are that we have not been able to identify a size limit to the new ligands that can be added to the C-terminus and the demonstrated possibility to incorporate ligands with different receptor reactivity in the same fiber for targeting to different receptors. The disadvantages with the de-knobbed fibers are that the corresponding viruses all have a decreased fiber content, growth and infectivity, which make them less suitable for re-targeting *in vivo*.

Another approach was to re-target the virus via the fiber by incorporation of ligands into the HI-loop of the fiber knob, based on the fact that the knob is essential for normal production of re-targeted Ad (Henning *et al.*, 2006). Fibers with HI-loop insertions were demonstrated to fulfill our criteria of high solubility, trimerization and ligand reactivity, but only the fibers with a tandem repeat of Z_H surrounded by linkers were able to be rescued into functional viruses. Furthermore, these viruses showed an improved infectivity index (PP to PFU) and growth characteristics compared to knobless viruses from previous publications (Magnusson *et al.*, 2002; Henning *et al.*, 2005).

The HI-loop-linker constructs from paper III were demonstrated to be superior to short knobless fibers and seems to be more suitable for generation of re-targeted Ad-vectors.

The scFv 13R4 has been incorporated alone or together with a tandem repeat of Z_{wt} into a short knobless fiber with seven shaft repeats and has been inserted into the HI-loop of a CAR binding ablated fiber knob (unpublished data). The phenotypic analysis of these fibers showed that they were soluble, able to trimerize and had a retained antigen binding capacity. Although phenotypic characteristic seemed promising only the R7-13R4-$Z_{wt}Z_{wt}$ fiber was able to be rescued into adenoviral virions. The other fibers were not able to generate functional virions when using 293 cells expressing the WT-fiber. The explanation could be that the recombinant fibers produced in the cells preoccupies the pentons and inhibit WT-fibers from being incorporated allowing for propagation of virus. An interesting finding was that the fiber content in Ad5/R7-13R4-$Z_{wt}Z_{wt}$ was higher than the fiber content of the Ad5/R7-$Z_{wt}Z_{wt}$ virus. Unfortunately the virus containing the 13R4 protein still had a subnormal infectivity suggesting that the phenotypic characteristics other than the fiber content are affected in virus with recombinant knobless fibers. The scFv 13R4 has further been inserted in the HI-loop surrounded with flexible linkers of a CAR containing fiber knob in order to rescue the recombinant fiber into Ad on 293 cells. However, the attempt fail which could be due to that the CAR binding site is blocked by the large 13R4 protein or that the ligand is too bulky which makes the fiber unable to be rescued into Ad and produce functional viruses. Phenotypic characteristics at fiber level including antigen binding to CAR and β-galactosidase are under investigation.

pIX (paper II)

The pIX protein is a minor capsid protein of the adenoviral genome and acts as capsid cement. The C-terminus is exposed on the outer surface of the viral capsid (Furcinitti *et al.*, 1989; Akalu *et al.*, 1999) and has been demonstrated to tolerate incorporation of targeting ligands (Rosa-Calatrava *et al.*, 2001; Vellinga *et al.*, 2004). The ligand insertion at the pIX protein was created through a fusion gene where the sequence of 13R4 was fused via a flag epitope and a 75Å spacer to the pIX protein. The pIX protein is located below the surface of the hexons and a spacer was needed to expose the ligand above the hexons to achieve efficient ligand – antigen binding (Vellinga *et al.*, 2004).

One advantage of using pIX as the anchoring protein is that one can keep the knob to increase growth, infectivity and fiber content to be able to produce recombinant re-targeted viruses to titers that can be used for *in vivo* experiments, on the other hand for *in vivo* purposes the CAR binding in the fiber knob have to be ablated. ScFv 13R4 fused to the pIX protein retained its antigen binding when incorporated into virions. However it remains to be shown whether a virus carrying scFv on pIX protein can use the ligand to infect cells. This could not be examined in the present system hence a producer cell line with surface bound β-galactosidase was not available. Since the re-targeting efficiency of the recombinant pIX virus could not be performed it is important to consider that re-targeting efficiency can be dependent on the capsid protein to which the scFv is added. Recent studies have shown that cell targeting via the fiber is more effective when compared to targeting via the pIX protein both with high affinity ligands (such as antibodies, EGF and transferrin) (Campos and Barry, 2006) and low affinity ligands (RGD) (Kurachi *et al.*, 2007). The reason for this is still unclear but they suggest that the receptor binding and uptake through the pIX protein result in an altered trafficking and poor transduction due the entrapment of targeted virions on their bound receptors in the endosomes or in cytoplasm of the target cell.

Fibers with dual specificity (paper V)

It has been shown that it is possible to incorporate the sequences for two different affibody molecules in the same knobless truncated fiber to achieve double specificity (Henning *et al.*, 2002). In paper V we evaluated if it was possible to accomplish a virus with double specificity with diverse affibody molecules in the HI-loop of the fiber knob. We also examined if the order in which the affibody molecules had been positioned was important. The affibodies Z_H and Z_{taq} were inserted together and placed before and after each other (Z_HZ_{taq} vs. $Z_{taq}Z_H$) surrounded by flexible linkers in the HI-loop of the fiber knob. The result showed that it was possible to generate virus with double specificity with different ligands in the HI-loop. On the other hand it is important to evaluate for each construct in which order the ligands should be placed due to the fact that Z_H only bound efficiently to its receptor when incorporated in the last downstream position. The reason for this is not clear because misfolding of the affibody molecule is not likely considering the very stable folding of these molecules and the long and flexible linkers ought to provide freedom for the affibody molecule

to move and bind to its epitope. From paper III we have also seen that the virus with a tandem repeat of Z_H had much better growth and binding properties than the virus with Z_H as a monomer. At the same time affinity measurements indicated that both constructs bound to HER2/neu in the same nM range suggesting that there is a possibility that only one of the two affibody molecules in the tandem repeat construct bound to the receptor. It could be that the dimeric version imposes less strain of the flanking knob sequence than the monomer.
Generation of Ad vectors with double specificity may make it possible to explore several possibilities and could for instance be used to target two different therapeutic cellular receptors or to target one tumour receptor and a neo-endothelial antigen for an assisted passage into the tumour from the blood stream.

DE-TARGETING OF AD (PAPER I AND IV)

Protection against pre-formed antibodies (paper I)

Virus-neutralizing antibodies are a regular consequence of Ad infections in humans. For adenovirus gene therapy it is very important that the vector reaches its target before being eliminated by the immune system. The presence of pre-formed antibodies is therefore one of the major problems for the systemic administration of Ad vectors. The results of the neutralization assay in paper I demonstrated that there is a strong reduction of titers against the recombinant knob-less virus compared to WT. This would be compatible with the finding that neutralizing antibodies against the fiber react with the trimeric knob (Wilcox and Ginsberg, 1963). The neutralizing activity in the two sera that had high titers against the recombinant knob-less virus was directed against the hexons.
There was no demonstrable antibody binding to affibody liganded fibers, implying that there is no prior immunity that can be detected by neutralization assay to the scaffold of the affibody molecules. However the finding does not exclude the possibility that the variable parts could be immunogenic in individual cases.
In the study we also evaluated antibody reactivity against different capsid proteins and found that there was a strong reactivity against the penton base and varied reactivity against hexon and fiber. It has been described that neutralization activity against WT virus can occur against the penton base alone (Akiyama *et al.*, 2004). It is possible that the RGD motif in the penton base, which is an integrin binding site, is a target for neutralizing antibodies. A further development step towards an adenovirus vector protected against pre-formed antibodies is to test the recombinant viruses with the RGD-EGD mutation for virus neutralization. Presence of different epitopes reactive with neutralizing antibodies in the Ad penton base has been described by Hong and co-workers (Hong *et al.*, 2003).
One important issue to be considered when choosing anchoring protein and targeting ligands is the immunogenicity of the final virus construct. The major advantage with truncated knobless fibers is the simultaneous ablation of the CAR

binding knob domain making further de-targeting of the knob unnecessary. In addition, neutralizing antibodies directed towards the knob are circumvented.

Evaluation of de-targeted vectors (paper IV)

To achieve a successful Ad vector for e.g. cancer gene therapy it is equal important to de-target the virus as it is to re-target to tumour antigens. For de-targeting purposes the virus needs to avoid binding and uptake to normal tissues, neutralizing by pre-formed antibodies and viral association with blood cells or blood factors. In paper IV we further investigated the Z_H targeted virus from paper III with an additional de-targeting step in order to evaluate the vector functionality and targeting properties possibilities for these viruses. The vectors were single ablated (CAR binding), double ablated (CAR and integrin binding) or triple ablated (CAR, integrin and HSPG binding). The modification in the fiber shaft where KKTK was changed to RKSK made the virus spread more efficiently in the cell layer and produced better growth curves indicating that the mutation had a positive effect on cell transduction. The reason for this is still unknown but may be due to binding of RKSK to other cellular ligands, altered intracellular trafficking or a possible change of the hinge in the fiber giving a positive effect on HER2/neu binding. The results from tissue distribution in mice showed that the binding to normal tissue was reduced for all recombinant vectors compared to WT, but the difference between single, double and triple ablated vectors were minor. The most interesting result was the much decreased binding of the recombinant viruses to human blood cells compared to WT Ad5, less than 10% in contrast to 90% of WT Ad5. There was no difference in association with blood cells between the recombinants so the loss of binding could be dependent on the mutation of the CAR binding site, imposed by a sterical hindrance of Z_HZ_H in the HI-loop or a consequence of both.

However for *in vivo* de-targeting ablation of CAR, integrin and heparan sulphate proteoglycan binding sites are not sufficient. This is due to the fact that the virus can interact and infect cells via other pathways *in vivo* that are not dependent of CAR and integrins. A solution to the problem can be to shield the vector by chemically coating with polymers such as polyethylene glycol or poly-(N-hydroxypropyl) methacrylamide, which has shown to be very effective in terms of avoiding neutralizing antibodies and interaction with blood cells and compliment (Fisher *et al.*, 2001; Green *et al.*, 2004; Turner *et al.*, 2007). To take in consideration when covalently attach polymers to the virus capsid is that the shield will be lost when the virus starts to replicate. On the other hand this may not be a problem if the vector already has target tumour and the released virions will be inactivated when enter the circulation from the lysed tumour cells. Another approach to mask the virus capsid is to introduce a shielding protein or to incorporate an albumin binding domain (ABD) preferably at the pIX protein. ABD will shield the antibody binding epitopes on the hexon by binding to serum albumin when administered *in vivo.* However, almost all modifications of Ad capsid proteins results in a virus with defect growth characteristics when compared to WT.

When novel gene therapy vectors are developed to treat human diseases studies in pre-clinical animal models must initially be conducted to estimate the vector performance *in vivo*. However, currently existing animal models for testing human Ad-based vectors have limitations which decrease the information value of vector behaviour *in vivo*. For example in used animal models there is no prevalence of pre-formed antibodies and Ad5 interaction with erythrocytes is species dependent (Cichon *et al.,* 2003). These complexed interactions must stay in mind for future development of Ad based vectors followed by intravascular applications. For review see (Baker *et al.,* 2007).

GENERAL CONCLUSIONS

- The recombinant knob-less virus showed a decreased sensitivity to human pre-formed
  antibodies when compared to Ad5 WT. There was no antibody reactivity of the sera tested against the Z_{taq} domain.
  This is an important first step towards the development of a stealth adenoviral vector, protected against the neutralizing activity of pre-existing human antibodies.

- The hyperstable scFv 13R4 has been identified to be suitable for incorporation into Ad and could successfully be incorporated at the C-terminus of the pIX protein with retained antigen binding.
  The discovery that the hyperstable scFv can be incorporated into Ad with retained antigen binding opens new possibilities to the development of efficient Ad vectors for cancer gene therapy.

- The affibody molecule Z_H is suitable for Ad insertions and was able to be incorporated into de-knobbed truncated fibers and the HI-loop of the fiber knob with preserved cell binding.
  Z_H reactive with HER2/neu is the first identified affibody molecule with therapeutic potential.

- Incorporation of new viral ligands into de-knobbed truncated fibers result in virus with changed tropism but with decreased fiber content, infectivity and growth. However, fibers with ligands enclosed by flexible linkers in the HI-loop showed promising phenotypic characteristic when evaluated in the cytosol of insect cells and the resulting virus demonstrated growth characteristics that were similar to WT Ad5.
  Vectors with ligands incorporated into the HI-loop surrounded by flexible linkers showed to be superior to vectors with ligand insertions into de-knobbed fibers and is suitable for in vivo studies.

- The re-targeted and de-targeted HER2/neu specific Ad vectors were shown to have good infectivity indexes, did not bind to normal tissues in mice as much as WT Ad5 and the association with human blood cells were greatly decreased for the recombinant vectors when compared to WT. However the difference between single-, double- and triple- ablated vectors was minor.

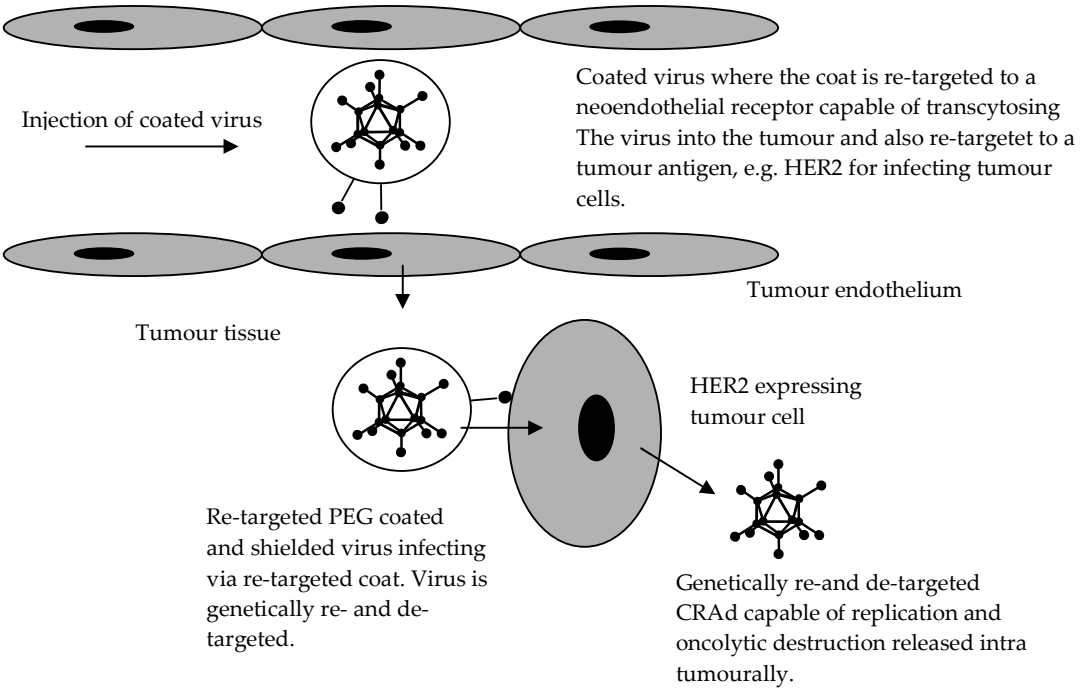
- It is possible to construct a vector with dual specificity by incorporation of different affibody molecules in the HI-loop of the fiber knob. It is however important to evaluate which positions is best suited for efficient binding to target cell.
  Generation of Ad vectors with double specificities exploit new possibilities, i.e. to construct a virus that can target two different tumour antigens. It remains to be shown whether it is

possible to incorporate more affibody molecules to generate vectors with several binding specificities.

FUTURE PERSPECTIVES

When constructing Ad vectors for systemic gene therapy it is important to consider how the vector will pass the anatomical barriers to enter the tumour. Few studies have addressed this issue and yet there are no known solutions. The first barrier is the high intestinal fluid pressure within the tumour which results in a flow of fluid from the tumour into the circulation and makes it difficult for the vectors to enter the tumour from the circulation (Fukumura and Jain, 2007). Newly formed tumour blood vessels have neoendothelium which has several features that makes it different from the normally vascular endothelium such as widened inter-endothelial junctions, lack of normal basement membrane and perivascular cells. By targeting the vector to both neoendothelium and tumour cells one could conceive spread to the underlying tumour cells. Tumour neoendothelial cells express surface proteins absent from normal endothelium and these can be used for targeting (Ruoslahti and Rajotte, 2000).
One way to circumvent the problem of getting the vector from the circulation and into the tumour could be to arrange for the viral vector to be trancytosed over the endothelial cells of the tumour (fig. 9). This could be achieved if a trancytosis receptor/mechanism was present in the tumour neoendothelial cells and the vector construct could be target to this receptor. The next anatomical barrier is inside the tumour and consists of the extracellular matrix (Sauthoff *et al.*, 2003). Expression of matrix-degradative proteins such as relaxin and metalloproteinase is one way to increase intra tumoural spread (Cheng *et al.*, 2007; Ganesh *et al.*, 2007).

Fig. 9. An idea how to solve the tumour entry problem for Ad based virus therapy for solid tumour diseases.



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