A novel technology to target adenovirus vectors : application in cells involved in atherosclerosis

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A novel technology to target adenovirus vectors

Application in cells involved in atherosclerosis

Proefschrift

ter verkrijging van de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof.mr. P.F. van der Heijden,

volgens besluit van het College voor Promoties te verdedigen op

donderdag 15 november 2007 klokke 13.45 uur

door

Jan Cornelius Emile Gras

geboren te Utrecht in 1974

Promotie commissie

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The studies described in this thesis were performed at the Center for Human and Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands

The studies described in this thesis were supported by a grant of the Netherlands Heart Foundation (grant number 1999B149)

Financial support by the Netherlands Heart foundation for the publication of this thesis is gratefully acknowledged.

zing, vecht, huil, bid, lach, werk en be(ver)wonder

Ramses Shaffy

Voor Marty en Stijn

Cover from top to bottom:
EM photo of adenoviridae: Veterinary Sciences Division (1994), Prof. Stewart McNulty
Schematic representation of an adenovirus: Structure and composition of the adenovirus type 2 core, Brown DT, Westphal M, Burlingham BT et al., J. Virol. 16:366, 1975
Artistic impression of the utilization of an adenovirus as gene therapy vector: United States National Library of Medicine

Printing Febo Druk B.V., Enschede

ISBN 978-90-9022355-1

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Chapter 1

General introduction

1. Introduction

This thesis describes the development and characterization of a novel linker protein designed to target Ad vectors to cells involved in atherosclerosis. The introduction first gives an outline on atherosclerosis. Subsequently, gene therapy and one of its powerful tools, namely adenovirus vectors, are introduced. Next, the current state on gene therapeutic approaches in atherosclerosis are described indicating the need for targeting of vectors. Finally, an overview on targeting strategies for adenovirus vectors is given.

1.1 Atherosclerosis

1.1.1 Pathogenesis

Atherosclerosis is a disease characterized by localized, chronic inflammation of the vessel wall of the large elastic arteries and the slightly smaller muscular arteries. At predisposed sites, lesions (plaques) in different stages of severity are present. Although the disease manifests itself usually in the middle aged/ elderly population, early stages of the atherogenic process already arise in young individuals ([1-3]). Based on morphological characteristics of the plaque during development, plaques have been classified into six distinct stages (fig. 1, ([4;5])). The first stage is characterized by the local infiltration of lipoproteins and the presence of lipid laden macrophages in the intima. From stages II to VI severity of the atherosclerotic lesion increases, and lesions are characterized by smooth muscle cell (SMC) migration from the media into the intima, lipid deposition and necrosis in the core of the plaque and synthesis of large amounts of extra cellular matrix, resulting in loss of lumen diameter. More complicated lesions may give rise to intra-plaque hemorrhage and thrombosis ([6]). Rupture of plaques and subsequent thrombosis caused by the exposure of the plaque content to the circulation may result in thromboembolic events and arterial occlusion exemplified by myocardial infarction or stroke.

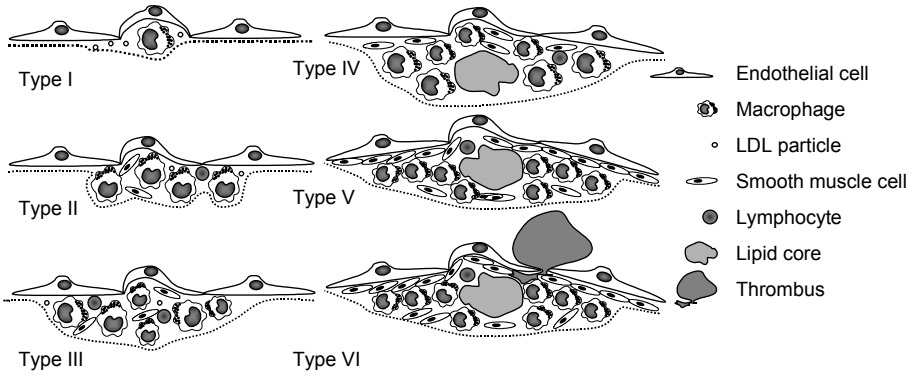

Figure 1. *Lesion progression and classification according to the American Heart Association*

1.1.2 Etiology

Atherosclerosis has a complex multi-factorial etiology. A wide range of genetic and environmental factors are known to be involved including elevated low-density-lipoprotein (LDL) cholesterol levels, hypertension, smoking and diabetes mellitus. Atherosclerosis is commonly described as the consequence of an exaggerated response of the endothelium to injury ([7-10]). Upon injury of the endothelium (caused by a range of stimuli, such as oxidized LDL ([11]), low, turbulent shear stress ([12]), infectious agents ([13]), or homocysteine ([14]), the endothelial lining of the vessel becomes activated and more permeable to circulating monocytes, T lymphocytes and lipoproteins. The resulting vicious circle of monocyte adhesion, differentiation, the secretion of chemo-attractants and subsequent SMC proliferation results in a chronically inflamed vessel wall and plaque development as mentioned above.

1.1.2.1 Shear stress

Hematological blood flow is not uniform along the circulatory tree. At branching points in the arterial system, blood flow is not lamellar resulting in differences in shear stress ([15;16]); shear stress being the force of passing blood exerted parallel to the vessel wall. Atherosclerotic lesions are not evenly distributed throughout the aorta and muscular arteries but are found at sites where shear stress is low or oscillating, early lesions are almost exclusively found at these specific predisposed sites such as the aortic arch, the carotid and iliac bifurcations, and the branching points in

the coronary circulation ([15;16]). Gimbrone and colleagues found that expression of certain extra cellular molecules on the endothelium is regulated by differences in shear stress ([17]). Turbulent blood flow, found at atherosclerosis-prone sites, upregulates the expression of pro-atherogenic molecules such as vascular cell adhesion molecule 1 (VCAM-1), E-Selectin, P-Selectin, Endothelin-1 ([18-20]). In contrast, laminar blood flow down-regulates VCAM-1 expression ([21]). Causal involvement of VCAM-1 in atherosclerosis has been demonstrated by Cybulsky, showing that VCAM-1 deficiency fully protects against diet induced atherosclerosis formation in LDLr$^{-/-}$ mice ([22]). Laminar flow also stimulates the production of athero-protective factors such as the enzymes endothelial nitric oxide synthase (eNOS) and cyclooxygenase-2 (COX-2), whereas eNOS production is attenuated by oscillatory shear stress ([23;24]). Much of the molecular and signal transduction pathways linking shear stress to protein expression remain to be identified but do involve the pro-inflammatory transcription factor nuclear factor κ B (NF-κB). NF-κB was found to be upregulated in endothelial cells subject to disturbances in laminar sheer stress (reviewed by Lehoux ([25])). Therapeutic intervention in these pathways may be beneficial. However, a complete absence of NF-κB has been shown to result in apoptosis *in vitro* ([26;27]), absence of RelA *in vivo* results in embryonic lethality and liver degeneration ([28]), indicating the importance of the NF-κB signaling pathway. This indicates that the NF-κB regulated response is a delicate balance between survival, activation and apoptosis that must be fully understood before intervention may be considered.

1.1.2.2 Inflammation

In atherosclerosis, the immune system responds to injury to the endothelial cells. Different proinflammatory stimuli such as tumor necrosis factor-α (TNF-α), interleukin-1, modified LDL, advanced glycation end products and bacterial lipopolysaccharides induce activation of intracellular transduction pathways. The nuclear factor-κB (NF-κB) is thought to be central to these pathways in endothelial cells ([29;30]). NF-κB is responsible for the expression of endothelial adhesion molecules such as E-selectin, ICAM-1, and VCAM-1 as well as for the expression of chemokines such as MCP-1 and IL-8 ([31]). Although many stimuli can activate the NF-κB pathway, there are multiple levels of control and diversification. Because NF-κB-mediated responses are both cell and stimulus specific, it is likely that not all activators utilize the same signaling components and cascades.

The inflammatory response is aimed towards elimination of the initiating stimulus, damage control and subsequent repair of the site of injury. Leukocytes play a key role throughout the inflammatory response. Since intervention in the first stage of the immune response, extravasation of leukocytes, might be beneficial to prevent or interrupt the continuous circle resulting in chronically activated endothelium, this introduction only focuses on the extravasation. Five sequential steps characterize this extravasation: capture, rolling, adhesion, transmigration and chemotaxis ([32]). To facilitate the leukocyte response, the endothelium undergoes several changes including vasodilation, increase in vascular permeability and the upregulation of the expression of several adhesion molecules to enhance adhesiveness of the endothelium for leukocytes. Adhesion molecules involved in leukocyte extravasation can be divided into three classes: selectins, integrines and glycoproteins of the immunoglobulin family ([33]). Three different selectins are involved in endothelium – leukocyte adhesion. 1) L-selectin is constitutively expressed by leukocytes ([34]). 2) P-selectin is expressed by activated platelets and endothelial cells that are involved in the initial rolling of the leukocytes on the endothelium ([35;36]). 3) E-selectin is expressed by the activated endothelium, acts at a later stage in the immune response and is involved in decreasing rolling velocity to allow firm adhesion ([37;38]). Studies in mice have shown that P-selectin is a predominant player in endothelium-leukocyte adhesion ([39;40]). Transmigration through the endothelial barrier is mediated by integrins, expression of VLA-4 and MAC-1 (CD11b/ CD18) by leukocytes and VCAM-1 and, to a lesser extent, ICAM-1 expression by endothelial cells ([41]), and reviewed by Jackson ([42]). Finally, chemotaxis is mediated by factors in the plaques i.e. invaded lipoproteins, or factors secreted by cells in the lesion i.e. IL-8 and MCP-1 reviewed by Charo ([43]).

1.1.2.3 Plasma components

Plasma components are in constant interaction with the endothelial cells lining the vessels and therefore have a major influence on the condition of these endothelial cells. A strong correlation exists between the incidence of ischemic heart disease and plasma levels of components of the coagulation cascade, plasma glucose and lipid levels ([44]). High levels of LDL cholesterol, lipoprotein (a) and triglycerides are associated with an increased risk of ischemic heart disease ([45]), whereas high levels of high density lipoprotein (HDL) cholesterol have a protective effect ([46]).

Lipids (triglycerides and cholesterol) are insoluble in water and their transport is mediated via specialized lipoproteins. These particles have a

hydrophobic core containing the a-polar cholesterol esters and triglycerides. The surface of lipoprotein particles is composed of polar phospholipids and proteins. These so-called apolipoproteins are involved in the production, distribution and uptake throughout the body of the lipoprotein particles. Lipoproteins are divided into five categories: Chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) depending on their density and their lipid and apolipoprotein (apo) composition ([47]). Lipid and cholesterol transport can be divided into three different pathways: the exogenous pathway, dealing with dietary lipids and cholesterol, the endogenous pathway dealing with cholesterol and lipids produced by the liver and the reverse cholesterol transport pathway, dealing with the cholesterol transport from the periphery back to the liver.

In the exogenous pathway, chylomicrons play an important role. These particles are based on a single ApoB48 molecule, formed intra-cellularly in the enterocyte and contain mostly triglycerides ([48]). In the bloodstream they acquire several apolipoproteins including apoC-II and apoE. ApoC-II is a co-activator of the enzyme lipoprotein lipase (LPL), present on the luminal surface of capillaries and arteries. LPL hydrolyses the triglycerides from the chylomicron core into free fatty acids to enable their uptake by the cells. After delipidation of the triglycerides and the phospholipids chylomicron remnants remain, which are efficiently cleared from the circulation by the liver through apoE-LDL receptor interaction ([49;50]).

In the endogenous pathway, VLDL produced by the liver plays the predominant role. VLDL is formed in the hepatocyte by the addition of triglycerides to apoB100 via the enzyme MTP ([51]). Release of the triglycerides from VLDL in the periphery is mediated by LPL in the same way as occurs with chylomicrons. The remaining particles, called VLDL remnants or intermediate density lipoproteins (IDL), are cleared from the circulation by the liver via interaction of apoB100 and the LDL receptor. A fraction of VLDL is further converted to cholesterol-rich LDL, which is poor in triglycerides. In humans approximately 70% off all cholesterol in the plasma is present in LDL particles.

In the reverse cholesterol transport pathway, HDL transports cholesterol from the periphery back to the liver ([52]). This is one of the potential mechanisms by which HDL confers its positive effect on the incidence of atherosclerosis ([53]). HDL particles are relatively lipid poor and have therefore a high density. Cholesterol efflux from the cells is mediated via members of the ATP binding cassette (ABC) family ([54-57]). Cholesterol in HDL is rapidly converted into cholesterylesters by the enzyme Lecithin

Cholesterol Acyltransferase (LCAT) ([58]). HDL contains apoAI and apoE. The cholesteryl esters can be transferred to triglyceride-rich-lipoprotein particles via cholesteryl ester transfer protein (CETP) reviewed by Stein ([59]). HDL particles are removed from the circulation via interaction with the LDL receptor and scavenger receptor BI ([60]).

LDL is the primary source for cholesterol present in the plaques ([61;62]). The LDL uptake and retention in the intima is affected by LDL concentrations in blood, blood pressure and endothelial permeability. Macrophage derived foam cells have a relatively low level of LDL receptor expression and they internalize modified and oxidized LDL (OxLDL) very efficiently via members of the scavenger receptor family ([63]). In the plaque, endothelial cells, macrophages and smooth muscle cells are able to oxidize lipoproteins. Thus formed oxidized fatty acids act as chemoattractant to monocytes present in the circulation and induce monocyte adhesion to the endothelial cells([64]). Furthermore, oxLDL is responsible for the shift in gene expression profiles of endothelial cells and for instance the upregulation of monocyte chemoattractant protein 1 (MCP-1) and intra cellular adhesion molecule 1 (ICAM-1) ([65-67]). OxLDL is toxic and can trigger the release of cytosolic enzymes and cause necrosis, aggravating the inflammation in the vessel wall.

1.2 Gene therapy

Current therapies for atherosclerosis are aimed at prevention by lowering plasma cholesterol levels through inhibition of HMG-CoA-reductase ([68;69]) or at treatment of stenosis with coronary artery bypass grafting (CABG) or percutanious transluminal coronary angioplasty (PTCA) ([70]). However, both CABG and PTCA are hampered by the occurrence of neo-intima formation in over 25% of the treated patients ([71;72]). Prevention of atherosclerosis or neo-intima formation by subtle modulation of the various molecular pathways involved would be an attractive alternative. Modulation of rate-limiting steps in the lipoprotein metabolism could reduce the “triglyceride and cholesterol stress” on the vessel wall ([68;69]). Patients suffering from familial hypercholesterolemia might be helped by introduction of the LDL receptor in the liver. Proof of concept of this approach was shown by dichek and colleagues ([73]). Locally, the immune response can be modulated by intervention in the NF-κB mediated signaling pathway ([74;75]) or vasodilation can be induced through stimulation of the eNOS expression ([76;77]).
For all of these options, functional vectors capable of modulating gene expression in specific tissues are required. The promise of gene therapy

lies in the possibility to intervene genetically in a specific cell or tissue in a well-defined process at a specific time-point. Because this intervention alters expression of endogenous proteins, it is capable of subtle intervention and minimizing toxic, adverse effects.

Below, these requirements and the current tools to achieve these goals are reviewed, with specific emphasis on the adenoviral vector system. Subsequently the approaches for gene therapy and adenoviral vector targeting with relevance to atherosclerosis are reviewed.

1.2.1 Requirements and Tools

Gene therapy can be defined as the introduction of genetic material into a cell to exert certain therapeutic effects. This genetic material is aimed at altering the expression level of endogenous genes or the introduction of exogenous genes. Modulating expression levels of endogenous genes may be beneficial in a variety of pathological processes. Introduction of exogenous genes encoding for the correct version of defective proteins has long been regarded as the solution in treating monogenetic disorders (Reviewed by Griesenbach ([78]), D'Azzo ([79]) and Kizana ([80])). Alternatively, novel genes could be introduced designed to counteract or compensate the effect of endogenous genes. For gene therapy to be safe and effective Anderson has formulated three criteria ([81]):

- The vector used for the introduction of the genetic material should be targeted to the tissue and/ or cells where the gene-product is to exert its effect
- The expression of the genetic material should be at the right level and for the right duration
- Since uncontrolled (germ line) transmission of genetic material is undesirable, therapy has to occur within acceptable safety limits.

The sequence of events ultimately resulting in therapeutic gene expression requires several, sometimes mutually conflicting properties from the vectors. (I) They must contain the genetic material in such a way that it can be transported through the organism to the tissue/ cells of interest, ideally escaping the host immune system and specifically target the tissue/ cells of interest. (II) The amount of genetic material (promoter, gene, enhancer etc) that can be carried should be sufficient to enable the desired expression level. Expression cassette sizes differ among different applications: down regulation of endogenous genes requires relatively small expression cassettes whereas insertion of complete genes (including enhancers and promoter) into host cells requires the accommodation of up to hundreds of kilobases of DNA. (III) Once arrived

at the tissue of interest, the genetic material must be transported over the plasma membrane or facilitate the uptake of the genetic material by the host cell and ensure delivery to the appropriate cellular compartment to prevent degradation and assist expression. Expression of the inserted material needs to be at the right time, at the right expression level and for the right duration in order to be effective.

Taken all these requirements into account, it becomes clear that a universal tool that meets all of them is currently not available; different tissues, expression levels and/ or duration will require different tools. Viruses are natural tools that fulfill some of these requirements. Viral vectors differ in their properties, so specific applications may demand specific vectors. Retrovirus vectors are able to integrate their genome into that of the host cell resulting in sustained expression. Unfortunately, they can only infect dividing cells. An extensive discussion on the biology and applications of retroviral vectors is beyond the scope of this introduction but complete overviews are given by Sinn and colleagues ([82]) and Anderson and colleagues ([83]). Related to the adenovirus vectors are the lenti or adeno associated viruses (AAV). AAV are non-enveloped icosahedral particles of approximately 22 nm containing a 4.7 kb single stranded DNA genome. In contrast to adenovirus vectors AAV are able to integrate their genetic payload in the genome of the host cell, ensuring a longer expression as compared to adenovirus mediated expression. As for the retrovirus vectors, an extensive introduction on AAV is beyond the scope of this introduction. Ample overviews on AAV have been written by Grierger and Samulski ([84]) and Gonçalves ([85]). In contrast to retroviral vectors, AAV and adenovirus vectors are capable of infecting both mitotic and quiescent cells. Adenoviral vectors do not integrate into the host genome and consequently the expression time is shorter but the expression levels are higher. Other advantages are the extensive knowledge of adenovirus biology, the capability of high titer production and the broad range of available vectors with different transgenes, promoters and combinations hereof.

1.2.2 Adenovirus vectors

1.2.2.1 Introduction

Since their introduction in biological research in the early 80's adenovirus vectors (Ad) have become powerful tools to influence gene expression in mammalian cells. Ad vectors are non-enveloped icosahedral particles encapsulating a 36 kB double stranded DNA genome, leaving ample room to accommodate a biological payload. Ads have been classified into six

distinct subgroups (A to F), containing at least 51 serotypes ([86-88]), on the basis of their genetic variability, oncogenic potential, and G+C content of their DNA ([89-91]). Ads are able to infect a wide variety of tissues. Detailed phylogenetic analysis of the different Ad serotypes has yielded several phenotypic clusters; the gastrointestinal cluster, with subgroups A and F ([89-91]) and references therein, ([92]), the respiratory cluster, with subgroups B, C and E ([89-91;93]). Phylogenetic relations between all different serotypes are depicted in figure 2.

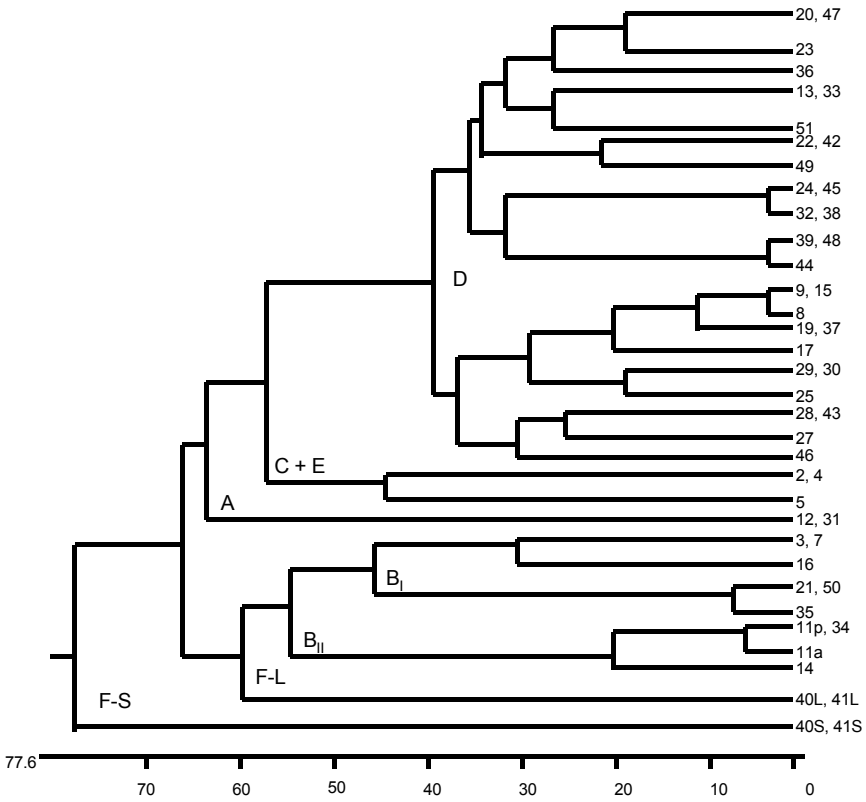

Figure 2. *Phylogenetic tree generated by parsimony analysis of fiber knob amino acid sequences of fiber derived from human adenovirus serotypes. The fiber's origin from the wild-type serotype is indicated by the number to the right. Designations A, B_I, B_{II}, C, D, E, F_L (long fiber of subgroup F virus), and F_S (short fiber of subgroup F virus) correspond to subgroup determination based on classical subtyping assays i.e., HA inhibition assays and cross-neutralization ([94])*

The majority of vectors in research use today are of serotype 5 origin (Ad5). The capsid of the virion consists of 3 major proteins (Fig. 3): (i) The hexon, this structural protein is the most abundant (720 copies per virion) but does not play a role in the uptake of the particle. (ii) The penton, this protein is of vital importance as it anchors the fiber firmly into the virion and it plays a crucial role in the uptake of the particle and (iii) the fiber (figure 3).

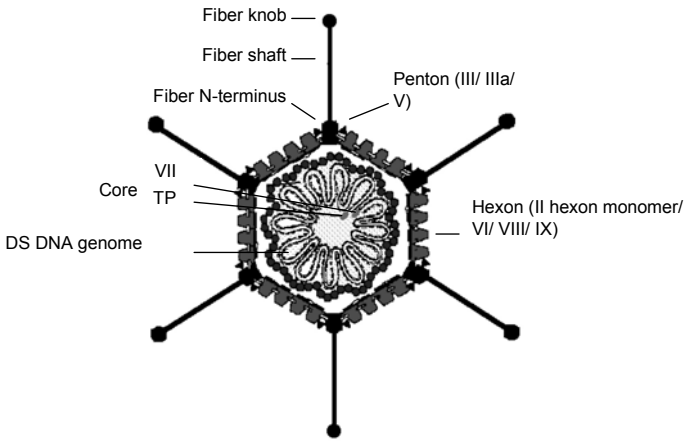

Figure 3. *Schematic representation of an Adenovirus*

The fiber protein consists of three parts: the amino terminus, responsible for anchoring in the capsid, the shaft, a rod like shaped structure variable in length between the several serotypes (16 nm in the case of Ad3, up to 37.3 nm in the case of Ad2 and 5) and the carboxy terminal knob. This knob domain is the major component in host cell binding and subsequent uptake of the particle. The other function of the knob is the initiation of trimerisation of the fiber, a key process for normal function of the fiber. X-ray crystallography studies with *E. coli* produced fiber proteins have revealed the trimerised knob as a structure similar to a three bladed propeller ([95]). Each blade consists of two anti parallel β-sheets and several connecting loops ([96;97]). Besides these three major proteins several other proteins are present in the capsid including pIIIa and pIX which serve as glue between the hexon proteins.

1.2.2.2 Infection pathways

Since the vector needs to be taken up by specific cells or tissues to exert its effect, it is essential to understand the pathway of cell entry and

infection. Infection of the host cell resulting in the expression of the viral genome, depicted in figure 4, is initiated via the interaction between the viral capsid proteins and cell surface components of the host cell (fig. 4, step 1).

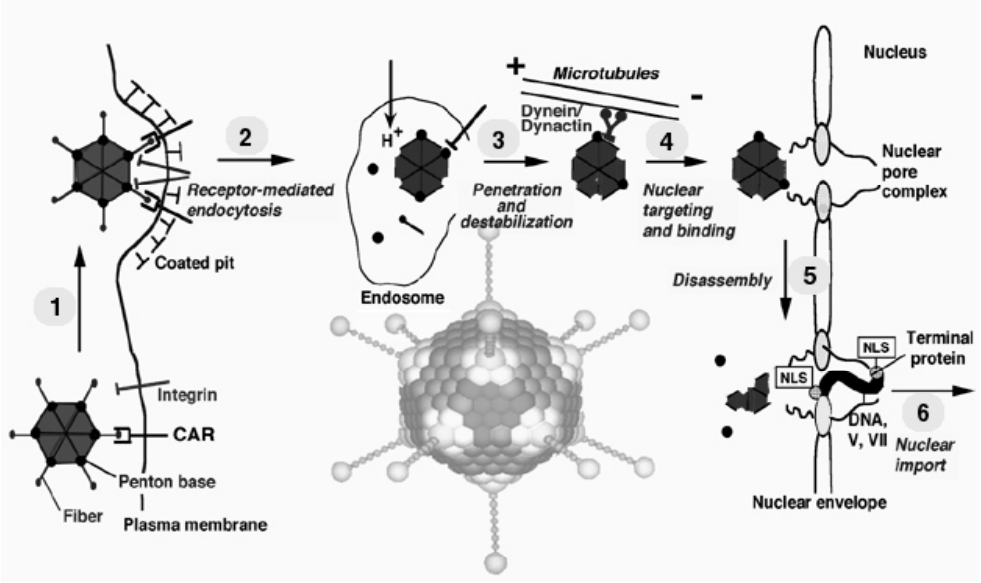

Figure 4. *Sequential steps resulting in viral genome expression*

All serotypes except serotype B use the Coxsackie Adenovirus Receptor (CAR) on the host cell surface as docking protein ([98;99]) and references therein). Therefore the tropism of Ad5 vectors is determined by the tissue specific expression pattern of CAR. This receptor is highly, but not exclusively, expressed on parenchymal cells in the liver, thus explaining the broad tropism of the vector *in vitro* and the high preference for infection of the liver after systemic application of Ad5 vectors ([100;101]). Sequence analysis suggested that the 46 kD glycoprotein CAR is composed of two immunoglobulin like domains (IgV and IgC2), followed by a transmembrane domain and an intracellular C terminus ([98]). The physiological function of this receptor, other then docking of Ad particles, is not exactly known. Experiments in heterozygous knockout mice (homozygous knockouts are lethal ([102])) however, suggest a function for CAR in the GAP-junctions ([103]) in several organs including the heart ([104]) and kidney ([105]). Interaction between the C-terminal part of the fiber protein of the virion and the Ig like domains located in the N-terminal part of the CAR is the fist step in the high affinity binding and uptake of the virion.

In addition to CAR, Fibronectin type III (FNIII) and MHC-I α2 are involved in binding of serotypes 2 and 5 to the cellular surface. When expressed in fusion with GST, both FNIII- and MHC-I α2-derived motifs showed a serotype 5 and 2 (but not 3) fiber knob-binding capacity *in vitro*. In *in vivo* assays, peptides representing the MHC-$I_{\alpha}2$ segment efficiently neutralized serotype 5 and 2 (but not 3), whereas peptides representing the FNIII motif, significantly enhanced Ad5 cellular attachment. These data suggest that a segment of MHC-I α2, which is conserved between all HLA alleles, is involved in the primary binding of Ad5 to cells, and that FNIII modules carried by other membrane components could act as auxiliary receptors or binding co-factors ([106]).

Next, in a process that has been shown to be independent of fiber-cell recognition ([107-109]), the penton base proteins of the virus particles bind to αV-integrins present on the cell surface through the Arg-Gly-Asp (RGD) motive, a tripeptide motif that protrudes from the tertiary peptide structure of the penton base, resulting in rapid endocytosis of the virus particle through clathrin-coated vesicles ([90;109-113]), depicted in fig. 4, step 2.

After internalization the viral capsid is dismantled in a stepwise process starting with the removal of the fiber proteins followed by the release of capsid proteins IIIa and VIII (fig. 4, step 3). The reactivation of cysteine protease p23 by penton base-integrin interactions and the reducing environment in endosomes or the cytosol, present in the capsid, facilitates the degradation of protein VI ([114;115]). Import of the viral DNA into the nucleus, a process for which the nuclear calcium levels are very important, requires contact between the now weakened capsid and elements of the nuclear pore complex ([115]) (fig. 4, step 5 and 6). Transcription of the Adenovirus genome is regulated by virus-encoded trans-acting regulatory factors.

1.2.3 Gene therapeutic approaches in cardiovascular disease

Pathological mechanisms often involve the (deregulated) expression, or absence of a limited set of key regulators. Restoring expression levels or inducing expression of novel therapeutic genes might have beneficial effects. The therapeutic gene might encode an intracellular protein, in which case the therapeutic effect is predominantly autocrine. Alternatively, the therapeutic protein could be secreted and exert physiological effects in a paracrine or endocrine fashion. This latter approach has as a major advantage that not all cells have to be transduced to exert an effect in a larger number of cells. “Gain-of-function” strategies have been used successfully for the overexpression of for instance pro-angiogenic genes

in animal models and in patients with vascular and myocardial disease ([116-118]).

An alternative approach is the “loss of function“ strategy. Silencing of certain pathogenic genes might slow down disease progression. Transcriptional or translational silencing of can be achieved by treatment with short single-stranded antisense oligodeoxynucleotides serving as decoys ([119;120]), ribozymes, RNA interference ([121;122]), hybridizing to specific mRNA target sequences. Alternatively, silencing can be achieved by the expression of dominant negative proteins. Transcription factors can be inhibited by binding of double-stranded decoy oligonucleotides to cis-elements in DNA consensus binding sequences ([119]). This latter approach has been validated by Wang and colleagues who disrupted NF-κB mediated expression of tissue factor ([123]) in HUVEC. Abnormal tissue factor (TF) expression on vascular endothelial cells may account for thrombotic events associated with cardiovascular disease ([124;125]). In humans, several clinical trails have shown proof of principle in gene therapeutic approaches towards cardiovascular disease (next paragraphs).

1.2.3.1 Vascular tone control

A balance between vasodilator (i.e. nitric oxide) and vasoconstrictor (i.e. angiotensin, adrenalin) elements controls vascular tone. Overexpression of vasodilator genes, including NO-synthase has proven to reduce blood pressure in hypertensive animals. Induction of endothelial nitric oxide synthase (eNOS) expression by intravenous delivery of a plasmid has resulted in a sustained hypotensive effect in spontaneously hypertensive rats (SHRs) ([126]). Systemic delivery of adenoviral vectors encoding atrial natriuretic factor ([127]), adrenomedullin ([128]) or kallikrein ([129]) decreased blood pressure and attenuated renal and myocardial damage in salt-fed Dahl salt-sensitive and in deoxycorticostersone acetate fed rats.

Intervention in the vasoconstrictor side of the balance also has beneficial effects on blood pressure. Tang and colleagues have shown that delivery of angiotensinogen antisense cDNA by AAV dose-dependently decreases arterial blood pressure in adult SHRs combined with reduced angiotensinogen levels ([130]). Inhibition of other components of the renin– angiotensin signaling system, including the antisense inhibition of angiotensinogen-converting enzyme (ACE) ([131]) and the angiotensinogen II type 1 (AT1) receptor ([132]), gave comparable results. Inhibition of the β1-adrenoceptors via antisense oligonucleotides also reduces blood pressure ([133]), suggesting that gene therapeutic intervention could be used as an alternative to pharmacological β-blockade.

1.2.3.2 Atherosclerosis and thrombosis

Since plaque stability is directly linked to acute coronary events, gene therapy aimed at increasing thromboresistance and plaque strength resulting in long-term plaque stabilization could potentially offer great benefits for the patient ([134]).

For example, adenovirus mediated overexpression of apolipoprotein ApoA1 in mice was shown to increase serum high-density lipoprotein levels, and thus stimulating the transport of cholesterol back to the liver. ([135]). Inhibition of monocyte chemoattractant protein-1 (MCP-1) receptor results in the blockade of monocyte infiltration and activation in the arterial wall and was shown to retard the onset and limit the progression and destabilization of established atherosclerotic lesions in ApoE knockout mice ([136]). eNOS has vasoprotective functions in addition to controlling vascular tone, including inhibition of vascular smooth muscle cell proliferation and migration and inhibition of platelet activation and adhesion ([137]). Gene therapy aimed at increasing NO activity and thus enhancing its anti-atherogenic properties of the vessel wall could be beneficial ([138]). For example, local administration of adenovirus vectors encoding eNOS to the carotid arteries of cholesterol-fed rabbits reduces inflammatory cell infiltration and lipid deposition ([139]). Gene transfer of cytoprotective genes, such as heme oxygenase-1 (HO-1) and superoxide dismutase (SOD), can also result in vasoprotective effects ([140]). Adenovirus-mediated local delivery of HO-1 decreased iron depositions and attenuated the development of aortic lesions in ApoE-deficient mice ([141]), whereas gene therapy inducing manganese SOD expression in pre-atherosclerotic carotid arteries improved endothelium-dependent vasorelaxation in hypercholesterolemic rabbits ([142]). De Nooijer and colleagues have shown that local infection of advanced atherosclerotic lesions with adenovirus mediated expression of TIMP-1 in mice reduced intra-plaque hemorrhage as compared to adenovirus mediated MMP-9 expression ([143]). These data indicate that inhibition of MMP-9 might have a stabilizing effect on advanced lesions.

Monogenic disorders, such as hemophilia and cystic fibrosis in humans were among the first diseases that could potentially be cured with gene therapy (Reviewed by Griesenbach ([78]), D'Azzo ([79]) and Kizana ([80])). Because inherited lipid metabolism disorders such as familial hypercholesterolemia (FH) and ApoE deficiency do not or hardly respond to medical treatment, gene therapy aimed at lowering lipid levels is likely to be useful. In the case of FH, gene therapy has been applied in a small phase I feasibility trial ([144]). A moderate reduction of 6-23 % in plasma LDL

levels was found in 3 out of 5 patients after administration of a retroviral vector expressing the wild-type LDL receptor. The effect was temporary likely due to silencing of the exogenous gene.

1.2.3.3 Neointima formation

Success of percutanious transluminal coronary angioplasty (PTCA) and coronary artery bypass grafting (CABG) is limited by the (re)occurrence of intimal hyperplasia following the procedures ([71;72]). Hyperplasia is either due to migration of SMC from the media, proliferation of VSMC or a combination of both. Prevention of neointima formation is a likely target for gene therapeutic intervention since it is initiated by local damage due to the PTCA procedure or altered flow conditions and increases stress in the graft in the case of CABG and specific processes are involved. Gene therapeutic intervention in cell-cycle mediators of endothelial and SMC provides the opportunity to render these cells resistant to atherosclerosis and neointimal formation after PTCA or CABG ([133;145]). Delivering anti-proliferative genes, such as those coding for the NOS isoforms, inhibits the intimal hyperplasia ([138]).

Gene transfer of eNOS in balloon-injured rat carotid arteries using fusigenic liposomes has proven to be vasculoprotective and antiproliferative ([146]). Both endothelial and inducible NOS gene transfer was efficacious in reducing neointimal proliferation after PTCA ([138;147]). Direct inhibition of the cell cycle via the inhibition of key regulatory proteins controlling progression is another approach to intervene in neointima hyperplasia ([148;149]). Jugular veins of New Zealand rabbits were treated with hemagglutinating virus of Japan (HVJ)–liposome complexes containing antisense oligonucleotide against the cell-cycle regulators proliferating- cell nuclear antigen (PCNA) and cdc2 kinase *in vivo* before carotid-artery interpositional grafting ([150]). Adaptive remodeling and induction of medial hypertrophy combined with inhibition of intimal hyperplasia of the graft was observed after the gene therapy, resulting in vessels resembling normal arteries ([150]). Likewise, treatment of grafts prior to implantation with a decoy deoxyoligonucleotide containing the consensus binding sequence for E2F-1, a transcriptional factor involved in cell-cycle progression, resulted in prolonged resistance to neointima hyperplasia of the graft after transplantation in rats ([151]).

Another interesting target for intervention is the plasminogen activation system, which plays a central role in cell migration ([152;153]). Lamfers and colleagues have constructed a fusion protein consisting of the amino-terminal fragment (ATF) of human urokinase plasminogen activator linked to bovine pancreas trypsin inhibitor, a very potent inhibitor of plasmin, (BPTI) ([154]). It was demonstrated that ATF.BPTI strongly

reduces SMC migration and neointima formation in both human and murine blood vessels ([155]).

In humans, intervention in cell cycle progression as a means to inhibit neointima formation and vein-graft failure has been evaluated in early-phase clinical trials. Mann and colleagues have undertaken the PREVENT-I trial, a prospective randomized doubleblind trial in which human saphenous vein-grafts were treated with E2F decoy oligonucleotides in high-risk patients suffering from peripheral arterial occlusion ([156]). This phase I trial demonstrated that E2F decoy oligonucleotides can be administered safely to the graft *ex vivo*, prior to implantation. In the recent follow-up phase II trial (PREVENT-IV) the study design was expanded with a placebo arm ([157]). Interim results of this study confirmed the results from PREVENT-I. Analysis of the secondary endpoints, using quantitative coronary angiography and three-dimensional intravascular ultrasound, demonstrated increased vessel patency, adaptive vessel remodeling and reduction in neointima size. Critical stenosis was reduced by 40% in the treated group one year after treatment (yet unpublished data, but commented in ([158])). These results await conformation and validation by an appropriately powered phase III trial to demonstrate the feasibility of this approach. A separate phase I trial is evaluating the efficacy of preventing restenosis of coronary arteries after PTCA by catheter-based iNOS gene delivery (REGENT-I) ([77]).

1.2.3.4 Inflammation and apoptosis

Overexpression of anti-apoptotic genes and the inhibition of proinflammatory and cell-adhesion molecules might have therapeutic potential for the treatment of apoptosis and inflammation of the vessel wall in atherosclerosis ([159;160]).

In vitro, adenovirus mediated expression of IκBα and a dominant negative form of IKK-2 inhibited TNFα-induced expression of E-selectin, VCAM-1 and ICAM-1 in human umbilical vein endothelial cells ([161]). *In vivo,* acute myocardial ischemia during cardiac transplantation could be reduced by the inhibition of proinflammatory genes. For example, treating the myocardium of rabbits *ex vivo*, before transplantation, with Ad expressing the immunosuppressive cytokine IL-10 prolonged cardiac allograft tolerance and longterm survival ([162]).

Von der Thusen and colleagues showed that IL-10 is able to attenuate atherosclerosis after systemic or local adenovirus mediated IL-10 expression ([163]), systemically because of monocyte deactivation and lowering of serum cholesterol levels, and locally because of reduction in

stenosis. Adenovirus mediated IκBα expression in a rabbit iliac artery restenosis model reduced ICAM-1 and MCP-1 expression and reduced the recruitment of macrophages and lumen narrowing ([164]).

Overexpression of anti-apoptotic genes such as Bcl-2 and Akt ([165;166]), immunosuppressive cytokines i.e. Il-10 ([162]), adenosine A1 and A3 receptors ([167]) and hepatocyte growth factor (HGF) ([168-170]) protectes the myocardium from reperfusion damage and ischemia. Likewise, a promising strategy for acute protection against these kinds of injury could be the inhibition of proinflammatory genes. Morishita and colleagues have shown that pretreatment of the myocardium with a decoy oligonucleotide capable of inhibiting the transactivating activity of NF-κB reduced infarct size after coronary artery ligation in rats ([171]). Hepatic Growth Factor (HGF), an organotrophic and angiogenic factor, has also been reported to exert cardio protective properties. Intracoronary delivery of HGF in rat hearts reduced myocardial enzyme leakage significantly and enhanced cardiac recovery after global ischemia in isolated hearts ([169]). Intramyocardial, adenoviral HGF delivery in mice attenuated the remodeling of the left ventricle and progression to heart failure after myocardial infarction together with enhanced angiogenesis and reduced myocyte apoptosis ([170]).

Despite the preclinical evidence demonstrating the therapeutic potential of over-expression of protective genes in or near the myocardium, the efficacy of these therapies for patients with coronary artery disease remains to be determined.

1.2.3.5 Angiogenesis

For patients suffering from occlusive coronary or peripheral vascular diseases, gene therapy mediated stimulation of angiogenesis might be beneficial. Angiogenesis is initiated by a shifted balance between pro and anti-angiogenic factors. Subsequent degradation of the vascular basement membrane and endothelial cell migration and proliferation results in the formation of new capillary tubes ([172]).

Banai and colleagues have shown that intracoronary vascular endothelial growth factor (VEGF) protein delivery enhances the development of small coronary arteries supplying ischemic myocardium, resulting in marked improvement of maximal collateral blood flow delivery ([173]). In rabbits with operatively induced hindlimb ischemia plasmid DNA encoding each of the three principal human VEGF isoforms resulted in augmented collateral vessel development demonstrated by serial angiography, and improvement in calf blood pressure ratio (ischemic to normal limb), resting and maximum blood flow, and capillary to myocyte

ratio ([174]). Treatment of rabbits with an adenovirus expressing acidic fibroblast growth factor (aFGF) prior to coronary artery occlusion resulted in a 50% reduction of the risk region for myocardial infarction and an increase in length density of intramural coronary arterioles ([175]). Intramuscular administration of Ad mediated VEGF into rats and rabbits stimulates neovascularization in nonischemic skeletal muscle ([176]). Direct administration of a comparable vector to ischemic myocardium in pigs resulted in the formation of collateral vessels and a significant improvement in both myocardial perfusion and function. ([177])

In humans, two relatively small placebo controlled trials for coronary disease are reported. In the first trial, plasmids mediating VEGF-2 expression are applied intramyocardially ([178]), leading to a reduction in angina as well as to a significant improvement in cardiac perfusion and function. In the second trial, adenovirus vectors are used to mediate FGF expression intracoronary ([179]). This study also shows promising results. In peripheral artery disease, two phase-1 studies have been reported. The first study used adenoviral NV1FGF (FGF type 4 with the excretion signal of FGF type 1) and reported a decrease in rest pain, ulcer size and an improved the ankle: brachial index after gene transfer. However, no placebo group was present in this trial ([180]). The second study used VEGF and showed significant improvement in endothelial function (measured by acetlycholine infusion), and increased ankle: brachial index after VEGF gene transfer ([181]), indicating an improved blood flow to the foot. A larger phase 2 trial by Makinen and colleagues in patients with lower limb ischemia with both adenoviral and plasmid VEGF gene transfer at angioplasty showed increased vascularity ([182]). A complete overview of human gene therapy trial for atherosclerosis has been given by Freedman ([183]).

1.3 Targeting in gene therapy

1.3.1 Targeting of adenovirus vectors

As illustrated in the previous paragraphs gene therapeutic approaches in cardiovascular disease are promising. However, gene therapy is hampered by the relative lack of vectors capable of directing tissue specific expression of the therapeutic transgene. Targeting of the vectors provides a means to direct the expression towards the desired cells and thus enhance the efficacy of gene therapeutic approaches.

Understanding the CAR mediated and integrin assisted infection pathways of Ad vectors has led to different targeting strategies to redirect the vector

to specific cell types. Regardless of the strategy chosen to target the virus, two critical problems have to be solved; 1) the native tropism for the CAR should be eliminated to prevent uptake of the vector by the liver and 2) a novel tropism for an alternative cellular receptor should be generated. Sequestration of the virions by the liver, via CAR mediated uptake by parenchymal cells severely compromises the efficacy of the therapy. Moreover many target cells relevant to gene therapeutic intervention including tumors macrophages or endothelial cells do not, or hardly express CAR rendering them resistant to Ad infection. To overcome these limitations, several targeting strategies have been developed, which can be divided into two distinct categories: (I) genetic modification of the virus vector or (II) the use of adapter proteins facilitating the binding of the virus to specific cell types. Both approaches require a ligand-receptor interaction with the receptor present on the target cell and the ligand fused to the Ad virus.

1.3.1.1 Genetic targeting

In genetic targeting, the viral capsid proteins responsible for binding to target cells are modified in such a way that affinity is induced for other cells. In principal all of the viral capsid proteins (hexon, penton, pIX, or fiber) can be used for the insertion/ addition of ligands. The choice of the particular protein or peptide and the position of the ligand in the capsid protein should be dictated by the following considerations: Incorporation of the ligand should not interfere with Ad assembly and only minimally affect the Ad protein in which it is inserted, not hampering its function. The ligand should be presented in such a way that it adopts its correct configuration and therefore is able to bind its target receptor. These modifications have been achieved via pseudotyping of the fibers or via cloning of ligands for novel receptors into the HI-loop or the C-terminal domain of the fiber knob, as will be discussed below.

1.3.1.1.1 Pseudotyping

Different adenovirus serotypes have different tropisms due to variations in the structure of the fiber and knob of the virus capsid ([94]). Exchange of CAR-binding fibers with fibers of serotypes not having affinity for CAR, a process called pseudotyping, alters the tropism of the vectors. For instance, improved infection of airway epithelial cells was obtained with pseudotyping serotype 2 vectors with serotype 17 fibers ([184]). Gene

delivery to umbilical vein endothelial cells, saphenous vein segments and smooth muscle cells was improved by pseudotyping Ad5 with fibers originated from Ad16, a subgroup B virus. ([185]). Improvement of infection of several cell lines from hematopoietic origin with Ad5 was observed when replacing the endogenous fibers with subgroup B fibers ([186;187]). Switching Ad5 fibers with serotype 30 fibers enhances the affinity for human endothelial cells in culture ([188]). Serotype 4 and 11 enhance the infectivity of Ads to vascular endothelial cells ([189]). Caution should be taken when shifting serotypes as fibers are also important for the intracellular trafficking of the virus as was demonstrated with fibers from Ad35. The latter fibers are able to enhance the delivery of the virion to the nuclear membrane but also mediated trafficking back to the cell surface ([190]). From the above, it will be obvious that targeting via pseudotyping is limited to naturally occurring fiber polymorphisms.

1.3.1.1.2 Capsid and fiber modification

The genetic approach in targeting consists of the insertion of ligands for cellular receptors in the capsid of the virion. Effective targeting should ideally abolish the affinity for CAR and induce affinity for a novel receptor. The fibers of the virion normally involved in cellular entry are therefore the most obvious candidates although the insertion of FLAG epitopes in the penton bases of the fiber and subsequent addition of ligand-αFLAG antibody fusion-proteins strategies has also been successful ([191]), as well as modifications of capsid protein pIX ([191;192]). As for the fiber, ligands can either be inserted in the HI-loop, a lose structure protruding from the fiber knob, or they can be attached to the C-terminus of the fiber. Both strategies have resulted in successful targeting of the virus. Introduction of peptides, including RGD, in the HI-loop ([108;193-199]) or the C-terminus ([200;201]) conferred a high affinity for several receptor including αV-integrins. Affinity for heparan sulfate protyoglycans was induced via a lysine stretch linked to the C-terminus ([196]). As the ligands are inserted during virion synthesis, they should be compatible with the structure of the fiber, not changing its configuration as this is important for its function and proper virion assembly. Insertion of large peptides to the C terminus was shown to disturb fiber trimerization preventing proper virus assembly ([202]). Because Ad particles are assembled in the nucleus of the host cell, all capsid proteins are transported to the nucleus directly after translation. Therefore the ligands should not require any posttranslational modifications not available in the cytosolic or nuclear compartment. Polypeptides of up to 63 residues have been inserted into the HI-loop without hampering virion assembly demonstrating the great flexibility of

this insertion site. Since generation and amplification of Ad5 vectors relies on cell lines expressing CAR, it should be recognized that a successfully retargeted Ad vector, one that has lost affinity for CAR and has acquired a novel specificity, may require a specially adapted cell line for generation and amplification.

A novel approach replaces the entire fiber of the virus with a virion-anchoring domain of the endogenous fiber and the oligomerization domain of reovirus attachment protein sigma1. This approach ablates the native adenovirus tropism as demonstrated by a 35-fold reduced infection on 293 cells. The His tag incorporated into the chimeric attachment protein conferred His-tag-dependent tropism to the AdV, which resulted in a 12- to 40-fold greater infection efficiency on two different cell lines expressing a His-tag-binding receptor. ([203])

1.3.1.2 Conjugate based targeting

1.3.1.2.1 Covalent cross linking systems

Conjugate-based targeting approaches involve the use of bifunctional linker proteins, which on the one hand bind to the adenovirus and on the other hand bind to the target cell.

Several approaches have proven to be successful in providing a means to bind the adenovirus. One of the approaches involves the use of antigen binding fragments (Fab) or single chain antigen binding fragments (scFv) of virus binding antibodies. Antibodies can be selected for high affinities to Ad and the neutralization of infection, which likely aids to minimize the endogenous uptake by the liver ([107]), reviewed by ([204]). The use of monoclonal antibodies, however, is associated with specialized chemical conjugation of the moieties conferring target cell specificity. An alternative approach uses a CAR fragment to bind the virus. This CAR receptor has an affinity in the low nanomolar range ([205]) and after binding to the adenovirus reduces the endogenous tropism by competition, making it suitable for targeting strategies ([206;207]). Furthermore, CAR fragments can be produced by *Escherichia coli* (*E. coli*) (([208]) relatively easily and in large amounts.

Introduction of a ligand for a cell specific receptor in the targeting protein results in tropism for the target cell. In antibody based approaches several strategies have proven to be successful i.e.: (I) A virus neutralizing scFv antibody fragment was isolated from a phage library and a C-terminal fusion protein with epidermal growth factor (EGF) constructed. The efficiency of viral infection could be markedly enhanced by using this fusion protein to target the virus to the EGF receptor. ([209]). (II) A variation

on the previous strategy is the fusion of two antibodies. Haisma and colleagues generated a bifunctional antibody containing two epitopes, one directed against the adenovirus fiber to one directed against the epidermal growth factor receptor ([210]). This fusion antibody markedly enhanced the infection efficiency of adenoviral vectors in epidermal growth factor receptor expressing cell lines. In cultured endothelial cells, an anti-E-selectin monoclonal antibody conjugated to an anti-FLAG antibody enhanced the uptake of an adenovirus vector expressing the FLAG peptide in its capsid cultured 20-fold ([211]). A bispecific antibody synthesized by covalent linkage of an adenovirus fiber protein antibody to a CD70 antibody enhanced infection of Epstein-Barr virus-transformed lymphoblastoid cell lines 10- to 20-fold ([212]).

In CAR based fusion proteins, generation of an epidermal growth factor-CAR fusion protein has proven to be successful in mediating Ad infection of EGF-receptor positive, CAR negative cells ([206]). Pereboev and colleagues constructed a fusion protein of CAR and a CD40 ligand via a trimerization motif. This fusion protein enhanced gene transfer to bone marrow derived dendritic cells to over 70% infection efficiency, compared to undetectable infection using untargeted Ad5 ([207]). A CAR- scFv antibody against c-erbB-2 oncoprotein constructed by Kashentseva and colleagues proved efficient in infecting c-erbB-2 overexpressing cells up to 130-fold increase in comparison with untargeted Ad complexed with sCARf control protein ([213]). An extensive overview of conjugate based targeting strategies has been given by Krasnykh ([214]).

One of the challenges of the above approaches is to overcome the complicated chemistry associated with the conjugation step and subsequent purification. In addition, a number of CAR based fusion proteins are produced as single proteins, meaning that for each novel specificity a new targeting protein needs to be designed and produced.

1.3.1.2.2 Non-covalent cross linking systems

An alternative approach within the conjugate based targeting strategies is the use of non-covalent cross-linking systems to target virus particles. In non-covalent cross linking systems, the virus binding moiety and the receptor binding ligand are not covalently linked. An advantage of this approach is that the chemistry is often easier as compared to the chemistry involved in direct coupling. This approach results in a relatively flexible system to equip Ad with a variety of ligands. For example, Rogers used an anti-Ad Fab fragment coupled to phenylboronic acid (PBA) and attached FGF2 -dihydroxybenzohydroxamic acid (DHBHA) thus exploiting the binding of PBA to DHBHA to redirect Ad to FGF-receptor positive cells

([215]). Smith and Parrott applied the avidin-biotin interaction to retarget adenovirus vectors ([216;217]). Smith has chemically biotinylated adenovirus particles and targeted them to biotinylated cells via an avidin bridge. When equipping the virus with biotinylated monoclonal antibodies anti-CD117, -CD34 or –CD44, via the avidin bridge various kinds hematopoietic cell subsets could be infected ([216]). Parrott et al. introduced a biotin acceptor peptide (BAP) in the C-terminus of the fiber of Ad5 ([217]). After metabolic biotinylation of the virus particle, it could be equipped with a variety of ligands via an avidin bridge. Using this system, it was shown that Ad5 could be equipped with biotinylated monoclonal antibodies, biotinylated manose and biotinylated -oligonucleotides and targeted to primary dendritic cells ([217]). Introducing this BAP into capsid protein IX and the subsequent equipment with transferrin (via avidin) allowed for the targeting to CD71 ([218]). Li and colleagues followed a different approach in Ad targeting. They developed a fusion ligand protein consisting of CAR and the antibody Fc-binding domain from protein A ([219]). Because the Fc-binding domain in protein A is capable of binding to any immunoglobulin, this strategy can be adapted to target a wide variety of tissues or cells, as long as an antibody recognizing a membrane marker on the target tissue or cell is available.

1.4 Outline of the thesis

The studies in this thesis describe a novel technology to target adenovirus vectors to novel receptors. In **chapter 2,** the characterization of an oligonucleotide dA_2G_{10} ligand capable of mediating drug delivery through interaction with scavenger receptor A present on macrophages is described. This ligand was shown capable of mediating the uptake of a liposomal drug carrier particle both *in vitro* and *in vivo*. In **chapter 3,** a novel linker protein, CAR-Avidin, to equip Ad vectors with novel ligands is designed and characterized. This linker protein consists of the virus-binding moiety of CAR, genetically fused to avidin. As a proof of principle, the ligand developed in chapter 2 is applied in a slightly modified form, and used to aid the infection of primary and transformed macrophages by adenovirus vectors. In **chapter 4,** the versatility of the CAR-Avidin linker protein is demonstrated as it is used to equip Ad vectors with a cRGD peptide ligand specific for $\alpha_V\beta_{3/5}$ integrins and subsequently aid the Ad infection of human umbilical cord endothelial cells, bovine and murine endothelial cell lines and primary murine vascular smooth muscle cells. **Chapter 5** describes the challenges associated with the targeting of Ad vectors in vivo. Ad vectors are, via the linker protein, equipped with

several different ligands and liver uptake and target organ uptake are investigated.

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Chapter 2

Selective targeting of liposomes to macrophages using a ligand with high affinity for the macrophage scavenger receptor class A

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Curr Drug Discov Technol. 2006 Jun;3(2):135-44

Abstract

Macrophages play an important role in inflammatory processes and are crucially involved in the onset and progression of atherosclerosis and tumorigenesis. Therefore, macrophages are regarded as an excellent target for therapeutic intervention. Since the scavenger receptor class A (SRA) is highly expressed on macrophages, we developed in the present study an SRA-specific particulate drug carrier by providing phosphatidylcholine liposomes with a targeting ligand for SRA. To enable firm association with liposomes, the high-affinity SRA ligand decadeoxyguanine was covalently attached via a linker to lithocholic oleate (LCO-dA_2dG_{10}). Incorporation of LCO-dA_2dG_{10} into liposomes resulted in an increased electronegative surface charge and a dramatically enhanced serum clearance (t½ < 2 min versus > 5 h). The LCO-dA_2dG_{10}-induced liposome clearance was fully dependent on SRA, as the clearance could be efficiently inhibited by the SRA competitor polyinosinic acid. LCO-dA_2dG_{10} enhanced the affinity of liposomes for SRA *in vivo* selectively, since introduction of overall or clustered negative charges by other modifications (*e.g.* oxidation, inclusion of phosphatidylserine, or exposure of glutamic acid residues) did not affect their serum clearance substantially, albeit that these modifications resulted in an at least equally high negative surface charge. LCO-dA_2dG_{10} also increased the association of liposomes with RAW264.7 cells, resulting in an enhanced intracellular delivery and bioactivity of encapsulated dexamethasone-phosphate. Therefore, the SRA-specificity of LCO-dA_2dG_{10}-liposomes may be applied for the specific delivery of drugs to macrophages, which may be of therapeutic benefit in general inflammatory disorders, atherosclerosis, and tumorigenesis.

Key words: atherosclerosis; dexamethasone; drug targeting; liposome; macrophage; oligodeoxynucleotide; polyanion; scavenger receptor.

Introduction

Macrophages are crucially involved in inflammatory responses, and play an essential role in the body's innate defense system by protecting the host during the early phase of an infection. On the other hand, macrophages in the arterial wall contribute to atherosclerosis at all phases of disease progression (reviewed in ([1;2])). Upon differentiation of monocytes into intimal macrophages, scavenger receptors such as scavenger receptor class A (SRA) type I/II, CD68/macrosialin, and CD36 are substantially upregulated. These receptors recognize a range of (poly)anionic macromolecules including polyinosinic acid (polyI) ([3]), lipopolysaccharide (LPS) ([4]), oligodeoxynucleotides ([5]), anionic phospholipids such as phosphatidylserine (PS) ([6]), and modified lipoproteins such as oxidized LDL (oxLDL) and acetylated LDL (acLDL) ([7;8]). Although the capacity of macrophages to recognize and internalize LPS and PS may be beneficial for the clearance of pathogens and apoptotic cells ([9]), the uptake of oxLDL is thought to be deleterious as it results in foam cell formation due to massive accumulation of cholesterol ([10;11]). These foam cells secrete an array of mediators (*i.e.* pro-inflammatory cytokines, mitogens, and chemokines), thereby amplifying oxidative reactions and stimulating the proliferation of smooth muscle cells into the atherosclerotic plaque. Moreover, besides their adverse contribution to atherosclerosis, macrophages also play a major role in tumor growth ([12]).

The various scavenger receptors show different substrate specificities. SRA type I/II ([13;14]) is highly expressed on liver endothelial cells as well as on macrophages, and recognizes both acLDL and oxLDL ([7;8]). Both acLDL and oxLDL are mainly cleared by the liver, a process which can be effectively blocked by polyI ([15]). However, whereas the hepatic clearance of acLDL is mainly exerted by endothelial cells ([16]), the uptake of oxLDL is attributable to Kupffer cells ([15]) and mainly mediated by a specific oxLDL receptor ([17]), *i.e.* CD68/macrosialin ([18]). In addition, the class B scavenger receptor CD36 is also able to bind and internalize acLDL and oxLDL ([19;20]). CD36 is expressed on macrophages and adipose tissue, but not in the liver ([21]). Besides recognizing modified lipoproteins, CD36 also strongly binds PS-containing liposomes ([6;22-24]), but not the broad array of other polyanion ligands for SRA such as polyI and polyG ([20]). Peritoneal macrophages isolated from mice that lack both SRA and CD36 fail to accumulate modified LDL ([25]), indicating the importance of these two receptors in macrophage foam cell formation.

The crucial involvement of macrophages in atherogenesis has been illustrated by the observation that hypercholesterolemic mice that lack macrophages are extremely resistant to atherosclerosis ([26]). Therefore, macrophages are regarded as an excellent target for

therapeutic intervention in atherosclerosis ([2;27]). Macrophage-directed anti-atherogenic therapies could be aimed at inhibition of scavenger receptor activities to reduce the influx of cholesterol, lowering of inflammatory responses *e.g.* by dexamethasone ([28]), and increasing ABCA1/apoE-mediated cholesterol efflux *e.g.* by LXR activation ([29]). However, these goals require that drugs are selectively targeted to macrophages within atherosclerotic lesions. General inhibition of scavenger receptors or systemic attenuation of the inflammatory response will negatively interfere with innate immunity [9], and synthetic LXR agonists cause liver steatosis and hypertriglyceridemia by stimulating VLDL secretion by hepatocytes ([30]).

Therefore, the goal of this study was to develop a liposomal drug carrier with high affinity for macrophage SRA. For this purpose, we provided long circulating phosphatidylcholine liposomes ([31]) with a decadeoxyguanylic acid residue (dG_{10}) with high affinity for SRA ([32]). Hereto, dG_{10} was equipped with lithocholic oleate (LCO) that shows stable association with liposomes *in vivo* ([33;34]). We show that dG_{10} results in the efficient and highly specific targeting of liposomes to SRA in mice *in vivo*, and leads to increased intracellular delivery of encapsulated dexamethasone phosphate in a biologically active form into macrophages *in vitro*. Therefore, these SRA-specific liposomes are considered to be promising candidates for the development of novel macrophage-directed drug therapies.

Material and methods

Synthesis of LCO-dA_2dG_{10} and LCO-EE. LCO-dA_2dG_{10} was synthesized and purified essentially as described previously ([35]). In short, 5'-C12-NH_2-AAG GGG GGG GGG-3' (NH_2-dA_2dG_{10}, Eurogentec, Seraing, Belgium) was precipitated as a lithium salt with 10 volume equiv of 3% $LiClO_4$ in acetone. Next, the oligodeoxynucleotide was dissolved in H_2O and precipitated again with 10 volume equiv of acetone to remove residual traces of $LiClO_4$. NH_2-dA_2dG_{10} (100 nmol) was dissolved in 350 µl of H_2O/ N,N-dimethylformamide (DMF)/ 1,4-dioxane (1:4:2, v/v/v). Subsequently, 5β-cholanic acid-3α-ol-pentafluorophenyl ester (lithocholic oleate, LCO, Sigma, St. Louis, USA) (1 µmol) dissolved in 100 µl of dioxane, and 35 µmol of N,N-diisopropylethylamine (DiPEA) were added. The mixture was incubated for 48 h at 37°C. Solvents were removed in a speed-vac concentrator, and the residue was taken up in 200 µl of dichloromethane and 200 µl of H_2O. The layers were separated by centrifugation, and the organic layer was washed twice with 200 µl of H_2O. The H_2O fractions were combined and freeze-dried. LCO-dA_2dG_{10} was separated from nonconjugated dA_2dG_{10} by gel electrophoresis in a 1% LM-MP agarose

gel (Boehringer Mannheim, Mannheim, Germany) containing 0.1% Tween 20, in 0.5× TBE [45 mM Tris-borate and 0.1 mM EDTA (pH 8.4)]. The gel slice containing LCO-dA_2dG_{10} was melted for 5 min at 65°C. The agarose was digested with agarase (40 U/ml of gel) (Boehringer Mannheim, Mannheim, Germany) at 45°C in 30 mM Bis-Tris,10 mM EDTA (supplied with the enzyme) for 2 h. LCO-dA_2dG_{10} was precipitated with 10 volume equiv of acetone. To remove traces of undigested agarose, the precipitate was taken up in 200 µl of H_2O and passed over a filter paper (no. 589, Schleicher and Schüll). For the synthesis of LCO-EE, N-glutamyl glutamic acid (N-EE, 100 µmol) (Bachem, Bubendorf, Germany) was dissolved in 3 ml of H_2O/ DMF/ 1,4-dioxane (1:4:2, v:v:v), and 5β-cholanic acid-3α-ol-pentafluorophenyl ester (110 µmol) in 1.0 ml of H_2O/ 1, 4-dioxane/ DiPEA (330 µmol) was added. The mixture was incubated for 48 h at room temperature after which the solvents were removed under reduced pressure. The residue was extracted with dichloromethane/ acetic acid (0.1 M). The organic phase was washed twice with 0.1 M acetic acid. The oily residue was chromatographed over Kieselgel 600 (Merck, Darmstadt, Germany) using dichloromethane/ methanol/ acetic acid (100:5:1, v:v:v) as eluent. All chemicals were of analytical grade.

Preparation and characterization of liposomes. Liposomes were prepared as described ([31]). Phosphatidylcholine (PC) liposomes were prepared by mixing egg yolk phosphatidylcholine (EYPC) (Fluka, Buchs, Switzerland) and cholesteryl oleate (CO) (Janssen, Beerse, Belgium) in $CHCl_3$/CH_3OH (1:1, v:v) (25:1, w:w), and PC/phosphatidylserine (PS) liposomes from EYPC, 3-sn-phosphatidyl-L-serine (from bovine brain, Fluka), and CO (12.5:12.5:1, w:w:w). For preparation of radiolabeled liposomes, 50 µCi of [1α,2α-^{3}H]CO (Amersham Biosciences, Little Chalfont, UK) was included in the lipid mixture. Solvents were evaporated under N_2. For preparation of liposomes containing oxidized PC (oxPC), EYPC was exposed to air (72 h at 60°C) before addition of [^{3}H]CO. Lipids were hydrated in 10 ml of 0.1 M KCl, 10 mM Tris.HCl, pH 8.0, and sonicated for 1 h using a Soniprep 150 (output 10 µm) at 54°C under Argon to prevent (further) oxidation. PC and oxPC liposomes were purified and concentrated by density gradient ultracentrifugation and harvested by aspiration ([31]). PC/PS liposomes were dialyzed against PBS, pH 7.4, to prevent salt-induced particle aggregation. PC concentrations were determined using the Phospholipid enzymatic colorimetric test kit (Roche, Basel, Switzerland) with Precipath as internal standard. When indicated, PC liposomes were incubated (30 min at 37°C) with LCO-dA_2dG_{10} (50-500 molecules per particle) or LCO-EE (1000 molecules per particle). These calculations were based on $7.62x10^{13}$ liposomes per mg of phospholipid ([31]) and the molecular weights of LCO-dA_2dG_{10} (M_w = 4588) and LCO-EE (M_w = 927). Initial experiments using LCO-YE, of which the tyrosine (Y)

residue was radioiodinated, showed complete incorporation into the liposomes up to 3333 molecules per particle, as evidenced by colocalization of LCO-[^{125}I]YE with [^{3}H]CO-labeled liposomes (not shown). Assuming that LCO-dA_2dG_{10} and LCO-EE show a similar affinity for the liposomes as LCO-YE, it can thus be expected that complete incorporation of these compounds into liposomes will be achieved at only 1000 molecules per particle. The PC, oxPC, and PC/PS liposomes were homogenous particle populations with mean sizes of 30±2 (n=8), 32±2 (n=2), and 33±4 nm (n=2), respectively, as determined by photon correlation spectroscopy using a Malvern 4700 C system (Malvern Instruments, Malvern, UK) at 27°C and a 90° angle between laser and detector. Liposomes were stored under Argon and used within 2 weeks after preparation. When indicated, 50 mg dexamethasone 21-phosphate (dexaP, Sigma) was added to the buffer before sonication, and liposomes were concentrated and purified from non-encapsulated dexaP by density gradient ultracentrifugation as described above ([31]). Assuming an incorporation efficiency of 0.55 µl/mg of phosholipid for 30 nm-sized liposomes, as we have previously determined by [^{3}H]inulin incorporation studies ([31]), it can be calculated that 1 nM PC liposomes corresponds to 40 nM of loaded dexaP.

Agarose gel electrophoresis. The electronegative surface charge of the liposomes was determined by electrophoresis (2 h at 60 mA) on agarose gel [0.75% agarose (Eurogentec) in 7.5 mM Tris, 8.0 mM hippuric acid, 6.5 mM EDTA, pH 8.8]. After fixation (75% ethanol, 5% acetic acid, 3% glycerol) and drying of the gel, liposomes were visualized using Sudan Black B [1.5 mg/ml Sudan black B, 50 mg/ml zinc acetate in ethanol/ water (1:1, v:v)]. Excess Sudan Black B was removed from the gel using 55% ethanol, 2% glycerol. All liposome preparations migrated as single spots, and their electrophoretic mobility was determined relative to the front marker bromophenol blue.

Distribution of liposomes in mice. 10-12-wk-old male C57Bl/6KH mice of mass 22-24 g from Broekman Instituut BV (Someren, The Netherlands), fed *ad libitum* with regular chow were used for the *in vivo* experiments. Mice were anesthetized by subcutaneous injection of a mixture of ketamine (120 mg/kg body weight, Nimatek, Eurovet, Bladel, the Netherlands), thalamonal (0.03 mg/kg fentanyl and 1.7 mg/kg droperidol, Janssen Pharmaceutica, Beerse, Belgium), and Hypnorm (1.2 mg/kg fluanisone and 0.04 mg/kg fentanyl citrate, Janssen Pharmaceutica), and the abdomens were opened. [^{3}H]CO-labeled PC, oxPC, or PC/PS (1:1; w/w) liposomes (50 µg of PL) were injected via the inferior vena cava. When indicated, PC liposomes were incubated (30 min at 37°C) with LCO-EE (1000 molecules per particle) or LCO-dA_2dG_{10} (50 or 500 molecules per particle) before injection. In some cases, mice received a preinjection

of polyinosinic acid (polyI) (20 mg/kg, Sigma) at 1 min before injection of the liposomes. At the indicated times, blood samples (<50 µl) and liver lobules were taken and processed as described in detail ([36]). At 30 min after injection, the mice were killed, and their organs (liver, spleen, and lungs) were excised and weighed. ^{3}H-radioactivity in 20 µl-serum samples was counted in 2.5 ml of Emulsifier Safe (Perkin Elmer, Boston, MA, USA). The total serum volumes of the mice were 1.068±0.066 ml, as previously determined ([36]). The tissue samples were solubilized in Soluene-350 (Packard) (approx. 150 mg tissue per mL of Soluene-350) for 5 h at 65°C. Subsequently, their radioactivity was counted in 15 ml of Hionic Fluor (Packard) and corrected for the serum radioactivity present in the tissues at the time of sampling ([36]).

Binding of liposomes to mouse macrophages. RAW264.7 cells were cultured at 37°C in RPMI 1640 (Biowhittaker, Verviers, Belgium) supplemented with 10% bovine calf serum (Biowhittaker), 100 units/ml penicilin, 100 µg/ml streptomycin (Biowhittaker) in a humidified 5% CO_2/95% air atmosphere. Prior to experiments, cells were plated in 24-well plates. When 70% confluency was achieved, cells were washed twice with RPMI/2% BSA (37°C) and pre-incubated (2 h) with RPMI/2% BSA after which cells were washed once with RPMI/2% BSA (4°C) and put on ice. Cells were incubated with indicated amounts of [^{3}H]CO-labeled liposomes with or without polyI (100 µg/ml) in RPMI for 3 h at 4°C. After extensive washing (0.15 M NaCl, 2.5 mM $CaCl_2$, 50 mM Tris, pH7.4), cells were lysed in 0.1 M NaOH. Protein concentrations were determined as described [37] using BSA as a standard. ^{3}H-activity was counted in Emulsifier Safe (Packard), and was used to calculate the binding of liposomes as normalized for cell protein.

Effect of liposomes on luciferase expression in mouse macrophages. RAW264.7 cells were seeded into 12-well plates in Dulbecco's modified eagle medium (DMEM, Invitrogen, Carlsbad, CA, USA) without phenol red supplemented with 2% charcoal-stripped bovine calf serum (Gibco). After 24 h, cells were cotransfected using lipofectamin (Roche) with a reporter gene construct encoding growth hormone responsive element-driven firefly luciferase (pTAT3-Luc), an expression vector containing the growth hormone receptor (pHR), and a construct encoding cytomegalovirus-driven renilla luciferase (pRL-CMV; Promega) as an internal control for transfection efficiency (pTAT3-Luc and pHR were generated and generously supplied by Dr. O.C. Meijer, LACDR, Leiden). The molar ratio of pTAT3-Luc: pHR: pRL-CMV was 100: 100: 1, and the total amount of DNA used for transfection was 1.5 µg. At 16 h after transfection, cells were incubated (3 h) with dexaP or the indicated amounts of dexaP-containing liposomes in the absence or presence of LCO-dA_2dG_{10} (500 molecules per particle), and thoroughly washed. Cells were harvested

after 16 h, lysed in Passive Lysis Buffer (Promega), and luciferase activity was determined using the Dual-Glo Luciferase Assay System (Promega) according to the protocol supplied by the manufacturer.

Results

To generate a particulate drug carrier with high affinity for SRA, we combined the advantageous properties of the previously described SRA ligand oligodeoxyguanylic acid (dG_n) ([32]) with PC liposomes that are stable under *in vivo* conditions and show a relatively long serum half-life of >5 h ([31]). Since a stretch of 10 deoxyguanylic acid residues appeared to be the shortest oligodeoxynucleotide sequence with maximum inhibiting effects on the cellular binding and uptake of AcLDL ([32]), we chose to couple dG_{10} via a dA_2 spacer to a highly lipophilic lithocholic oleate (LCO) structure, resulting in the amphiphilic compound LCO-dA_2dG_{10} (Fig. (1)).

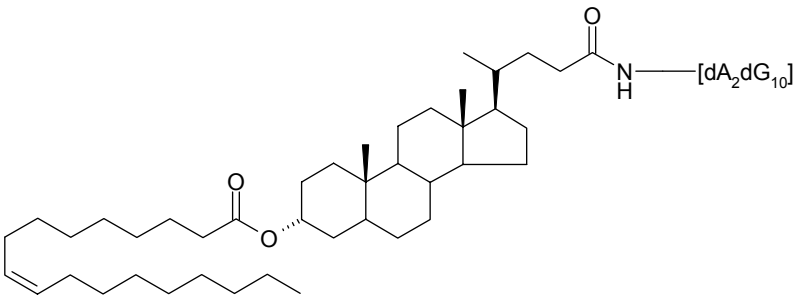

Figure 1. Chemical structure of LCO-dA_2dG_{10}. *The oligonucleotide deoxy(adenosine)$_2$ deoxy(guanosine)$_{10}$ (dA_2dG_{10}) is attached via an amide bond to 3α(oleoyloxy)-5ß-cholanic acid (LCO).*

Incorporation LCO-dA_2dG_{10} into PC liposomes. Previously, we showed by FPLC and agarose gel electrophoresis that the bile acid residue LCO mediates rapid and stable incorporation of antisense oligodeoxynucleotides ([35]) and cluster galactosides ([33;34]) into lipidic particles. To investigate the interaction of the presently derivatized oligodeoxynucleotide with liposomes, the effect LCO-dA_2dG_{10} on their electrophoretic pattern was examined (Fig. (2)). Incubation of PC liposomes with increasing concentrations of LCO-dA_2dG_{10} resulted in a dose-dependent increase of the liposome mobility on agarose gel, up to 3.2-fold upon addition of 500 molecules per particle, suggesting that LCO-dA_2dG_{10} is readily incorporated into the liposomal bilayer thereby increasing the electronegative liposomal surface charge.

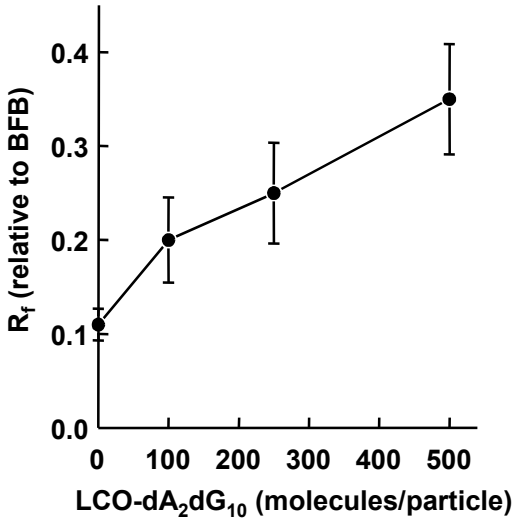

Figure 2. LCO-dA_2dG_{10} increases liposomal surface charge. *Liposomes (100 µg of PC) were incubated with LCO-dA_2dG_{10} (0, 6, 15, and 30 µg, corresponding to 0, 100, 250, and 500 molecules per particle, respectively) (30 min at 37°C) and subjected to 0.75% agarose gel electrophoresis at pH 8.8. Liposomes were visualized with Sudan Black, and their electrophoretic mobilities were determined relative to the front marker bromophenol blue (BPB). Values are means ± S.D. of three experiments.*

SRA-dependent distribution of LCO-dA_2dG_{10}-laden PC liposomes in mice. In earlier studies, we observed that liposomally incorporated LCO-derivatized cluster galactosides were retained on PC liposomes upon intravenous injection into mice, and induced efficient uptake of liposomes via asialoglycoprotein receptors on hepatocytes ([33;34]). To evaluate whether LCO-dA_2dG_{10} would lead to efficient uptake of liposomes by SRA *in vivo*, we first determined the effect of LCO-dA_2dG_{10} on the uptake of liposomes by the liver, which is the major organ responsible for the clearance of well-established SRA ligands (*i.e.* modified LDL) ([8]) (Fig. (3)). Upon injection into mice, the [^{3}H]CO-labeled liposomes showed a low uptake by the liver (7.7±0.4% of the injected dose at 30 min after injection) and a high residual fraction retained in the serum (81.3±2.1%), as a consequence of their low affinity for the reticuloendothelial system ([31;33]). Incubation of the liposomes with LCO-dA_2dG_{10} dose-dependently accelerated their serum clearance (Fig. (3), left panel), indicating that LCO-dA_2dG_{10} remains firmly associated with the particle *in vivo* because

of its highly hydrophobic moiety, and does not readily redistribute to serum lipoproteins. The increased serum clearance of the liposomes was mainly caused by liver uptake, which was enhanced via 12.8±0.2% (50 molecules per particle) to 63.2±1.9% (500 molecules per particle) of the injected dose at 30 min after injection (Fig. (3), right panel).

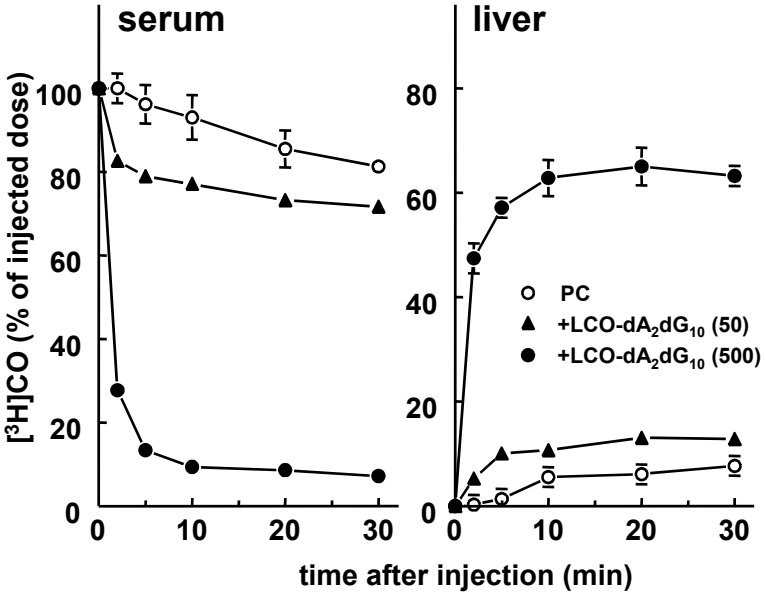

Figure 3. LCO-dA$_2$dG$_{10}$ induces SRA-mediated liver uptake of PC liposomes in mice. *[^{3}H]CO-labeled liposomes (50 µg of PC) were injected into anesthetized mice, without (white circles) or with previous incubation with 50 (black triangles) or 500 (black circles) molecules of LCO-dA$_2$dG$_{10}$ per particle. At the indicated times, the serum decay (left) and liver uptake (right) were determined. Liver values are corrected for serum radioactivity. Values are means ± variation of two experiments.*

To establish the involvement of SRA in the tissue distribution of the LCO-dA$_2$dG$_{10}$-laden PC liposomes, the effect of a preinjection of polyI on the liposome kinetics was evaluated, as polyI blocks the serum clearance of SRA ligands such as acLDL and oxLDL ([15]). Indeed, polyI almost completely prevented the LCO-dA$_2$dG$_{10}$-induced uptake of PC liposomes by both the liver and spleen, which is fully compatible with SRA-dependent clearance of the LCO-dA$_2$dG$_{10}$-laden PC liposomes (Fig. (4)).

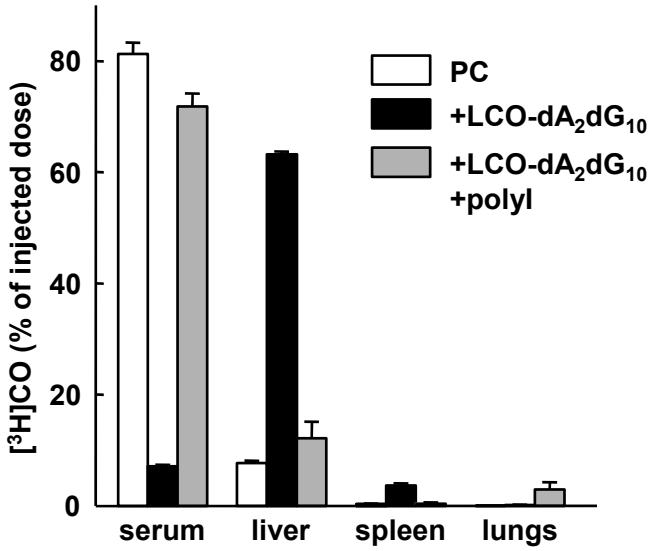

Figure 4. Effect of LCO-dA$_2$dG$_{10}$ on organ distribution of PC liposomes in mice. *[^{3}H]CO-labeled PC liposomes (white bars) or LCO-dA$_2$dG$_{10}$ (500 mol/mol)-laden PC liposomes (black bars) (50 µg of PC) were injected into anesthetized mice, without or with preinjection of polyI (20 mg/kg) at 1 min before injection of the LCO-dA$_2$dG$_{10}$-laden liposomes (shaded bars). At 30 min after injection, the amount of liposomes remaining in the serum and the amount of liposomes taken up by the indicated organs was determined. Organ values are corrected for serum radioactivity. Values represent means ± variation of two experiments*

Selectivity of LCO-dA$_2$dG$_{10}$-laden PC liposomes for SRA-mediated distribution in mice. Subsequently, we evaluated the selectivity of the presently developed LCO-dA$_2$dG$_{10}$-laden liposomes with respect to SRA affinity *in vivo* as compared to other modifications of the liposomes that may confer binding to macrophage receptors such as SRA and CD36. Since both receptors are known to recognize electronegatively charged particle surfaces, we first prepared liposomes from negatively charged phospholipids consisting of either extensively oxidized PC or a PC/PS mixture (50:50; w/w). Both liposomes displayed a substantially increased negative liposomal surface charge, as demonstrated by a 4.1-fold (R_f 0.53) and 5.5-fold (R_f 0.71) higher electrophoretic mobility on agarose gel as compared to PC liposomes, without affecting the particle size (32±2 and 33±4 nm vs. 30±2 nm, resp.). Despite the increased surface charge, oxidation of PC did not alter the *in vivo* distribution of liposomes, suggesting that oxPC liposomes had a low affinity for scavenger receptors

in vivo. Likewise, PS inclusion only marginally accelerated the serum clearance. Remarkably, whereas the uptake of the PC/PS liposomes by the liver was only 2-fold increased as compared to that of PC liposomes, the association with the spleen was enhanced to the same extent as for LCO-dA_2dG_{10}-exposing PC liposomes (Fig. (5)). This may indicate that the receptor recognition profile may differ between these particles and/or that two different scavenger receptor subtypes may be present in the spleen and liver. Finally, we evaluated whether the SRA-dependent liposome distribution as induced by LCO-dA_2dG_{10} may be mimicked by attaching structurally unrelated negatively charged amino acids (*i.e.* glutamic acid) to the LCO anchoring structure (LCO-EE), resulting in three negative charges per molecule at physiological pH. Again, although incorporation of 1000 molecules of LCO-EE per PC liposome did increase the negative liposomal surface charge to an even higher extent as LCO-dA_2dG_{10} (R_f 0.48 vs 0.35), the compound was unable to affect the serum decay of the liposomes, albeit that the affinity for the spleen was slightly enhanced (Fig. (5)).

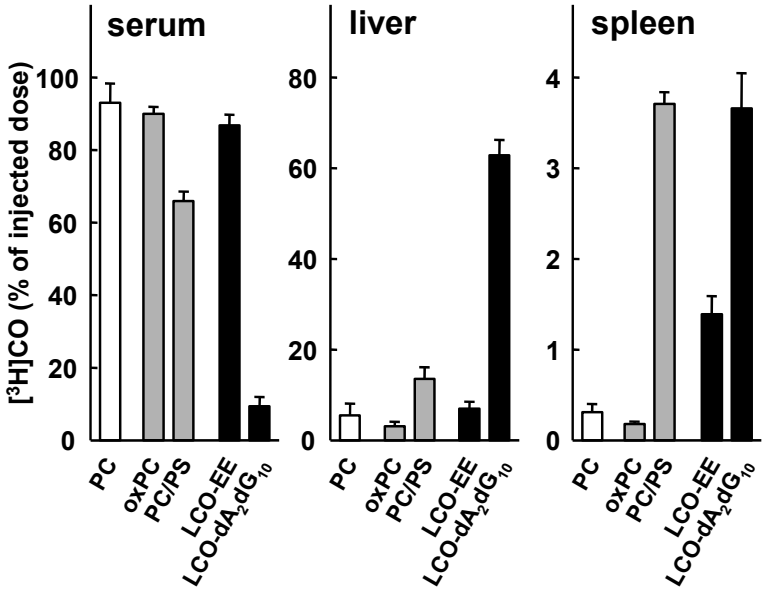

Figure 5. Selectivity of LCO-dA$_2$dG$_{10}$-laden PC liposomes for SRA-mediated distribution in mice. *[^{3}H]CO-labeled PC liposomes, oxPC liposomes, PC/PS (1:1; w/w) liposomes, LCO-EE (1000 molecules/particle)-laden PC liposomes and LCO-dA$_2$dG$_{10}$ (500 molecules/particle)-laden PC liposomes (50 µg of phospholipid) were injected into anesthetized mice. At 10 min after injection, the amount of liposomes remaining in the serum and the amount of liposomes taken up by the liver was determined. After 30 min, mice were killed, and the uptake of liposomes by the spleen was determined. Organ values are corrected for serum radioactivity. Values represent means ± variation of two experiments.*

SRA-mediated intracellular delivery of bioactive dexamethasone into macrophages. Since the LCO-dA$_2$dG$_{10}$-laden PC liposomes appeared superior over the other tested liposomes that bear overall (oxPC, PC/PS) or clustered (LCO-EE) negative surface charges with respect to *in vivo* SRA targeting, we next determined whether these liposomes may be effective in the SRA-mediated intracellular delivery of encapsulated agents, as would be aimed for in macrophage-directed therapies. First, an incubation study was performed with the mouse macrophage-like cell line RAW264.7 at 4°C, which permits receptor-mediated binding to the cell surface while cellular uptake activities are inhibited. This confirmed that LCO-dA$_2$dG$_{10}$ enhanced the binding of [^{3}H]CO-labeled liposomes to the

cells approx. 3.1-fold, whereas LCO-EE was hardly effective (Fig. (6)). From the binding curves, it can be calculated that the LCO-dA_2dG_{10}-laden PC liposomes show a high affinity for RAW264.7 cells, with a calculated dissociation constant (K_d) of 5.1±1.9 nM and a maximal binding (B_{max}) value of 27.8±5.1 µg PL/mg of cell protein, when normalized for the binding of PC liposomes (Fig. (6)).

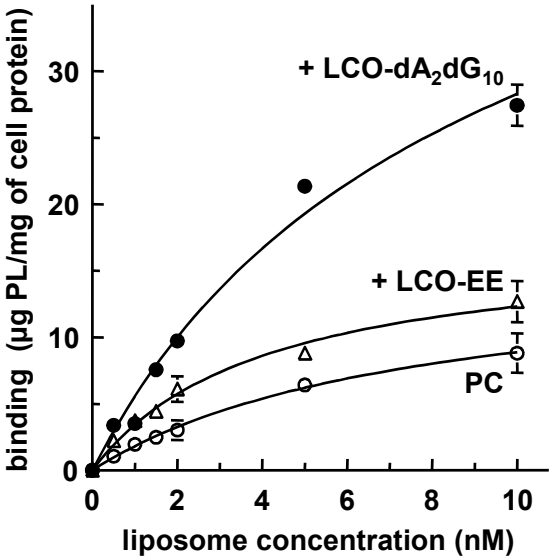

Figure 6. LCO-dA_2dG_{10} increases binding of PC liposomes to RAW 264.7 cells. *RAW264.7 cells were incubated (3 h at 4°C) with [^{3}H]CO-labeled PC liposomes without (white circles) and with previous association of LCO-EE (1000 molecules per particle, white triangles) or LCO-dA_2dG_{10} (500 molecules per particle, black circles). Subsequently, cells were washed, lysed, and cell protein and cell-associated radioactivities were determined. Values represent means ± S.D. of three experiments.*

As a read-out system for the LCO-dA_2dG_{10}-mediated intracellular delivery of liposomally encapsulated dexaP (a model drug with strong anti-inflammatory properties), we used a reporter gene analysis assay, in which dexaP binds to the transfected growth hormone receptor. After nuclear translocation of this complex it activates the growth hormone responsive element in the promotor that drives luciferase expression, which can be quantified (Fig. (7)). Liposomal dexaP was able to dose-dependently induce luciferase activity in transfected RAW264.7 cells. LCO-dA_2dG_{10} did not affect the luciferase activity as induced by 1 nM liposomes (~40 nM of dexaP), since the LCO-dA_2dG_{10}-liposome concentration is well below the calculated K_d value (*i.e.* 5.1 nM). In contrast, the compound did increase the luciferase activity-inducing effect

of 5 nM liposomes (~200 nM of dexaP) by 65%, thereby reaching an equal activity as induced by 1000 nM unencapsulated dexaP. These data indicate that after internalization and degradation of the liposomes, dexaP was able to diffuse into the cytoplasm and exert its biological effect. Moreover, specific uptake of dexaP liposomes by SRA as mediated by LCO-dA_2dG_{10} enhanced the intracellular liberation of biologically active dexaP.

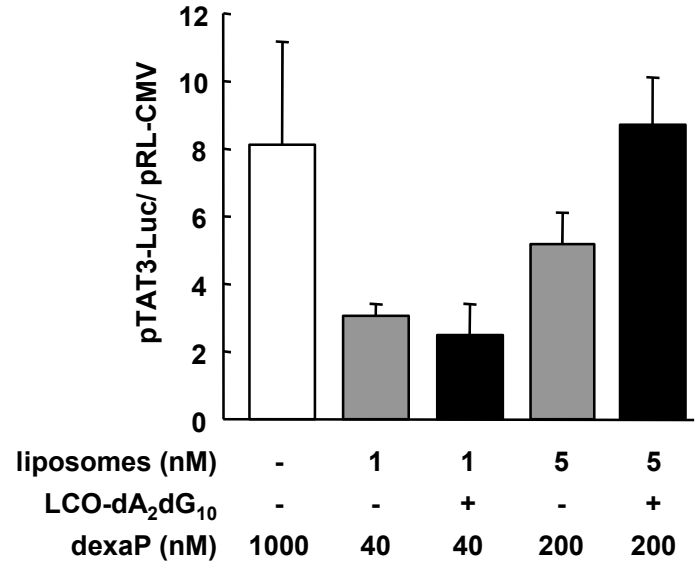

Figure 7. LCO-dA_2dG_{10} increases intracellular delivery of dexaP into RAW 264.7 cells. *RAW264.7 cells were cotransfected with a reporter gene construct encoding growth hormone responsive element-driven firefly luciferase (pTAT3-Luc), an expression vector containing the growth hormone receptor (pHR), and a construct encoding cytomegalovirus-driven renilla luciferase (pRL-CMV) as an internal control for transfection efficiency. After 16 h, cells were incubated (3 h) with dexaP or the indicated amounts of dexaP-containing liposomes in the absence or presence of LCO-dA_2dG_{10} (500 molecules per particle). After 16 h, cells were lysed, luciferase activity was determined, and corrected for the background activity in absence of dexaP. Values are means ± S.D. of three experiments.*

Discussion

Macrophages play an important role both in innate immunity, tumorigenesis, and the initiation and progression of atherosclerosis, prompting several research groups to develop macrophage-specific drug

carriers using scavenger receptors as a target. For example, maleylated bovine serum albumin ([38-40]) and oxLDL ([41]) have been used for the macrophage-targeted delivery of photosensitizers. Scavenger receptor-dependent uptake of photosensitizers could indeed be demonstrated both by macrophages *in vitro* ([39-41]) and into areas of intimal hyperplasia *in vivo* ([38]). However, the limited loading capacity of maleylated BSA and the potentially adverse properties of oxLDL impede their therapeutic applicability, which may be overcome by using synthetic particulate drug carriers.

In the present study, therefore, we evaluated the possibility to generate a macrophage-targeted liposomal carrier with both a high drug loading capacity and a high affinity for SRA. In addition, the versatile properties of liposomes allow for the incorporation of both hydrophilic, amphipathic and hydrophobic compounds into their aqueous core or phospholipid bilayer, thereby obviating the need for drug modification. We took advantage of the high SRA affinity of oligodeoxyriboguanines that have previously been shown to be very effective inhibitors of the binding and degradation of acLDL by SRA *in vitro*, by virtue of their high affinity for the highly cationic extracellular collagenous receptor domain ([32]). Since it appeared that dG_{10} is the minimal oligodeoxyriboguanine motif to display a high affinity for SRA ([32]), we have equipped this residue with the hydrophobic moiety LCO to enable anchoring onto the liposomal surface. We observed that the LCO-dA_2dG_{10} ligand dose-dependently enhanced the tissue distribution of liposomes *in vivo*, as related to the distribution of SRA in the different districts of the body. The involvement of SRA was underscored by the observation that the increased liposomal clearance rate could be completely blocked by polyI. Interestingly, the extent of SRA-dependent LCO-dA_2dG_{10}-induced liposome clearance by the liver (*i.e.* 63% of the injected dose) was much higher than we previously observed for free ^{32}P-labeled $dG_{10}dT_6$ (*i.e.* 32%) ([5]). Apparently, liposomal incorporation of dG_{10} further increases the intrinsic affinity of dG_{10} for SRA, which may relate to 1) the multivalent exposure on liposomes, 2) a more efficient formation of base-quartet-stabilized quadruplex structures or 3) oligonucleotide aggregation on the liposomal surface, which have all been shown to increase the affinity of oligodeoxyriboguanines for SRA ([32;42]). Thus, our data do not only confirm the high affinity of dG_{10} for SRA as observed by others ([32;43-45]), but also demonstrate for the first time the applicability of dG_{10} as an efficient targeting device for SRA *in vivo*.

Moreover, the high efficiency of dG_{10}-induced liposome targeting to SRA *in vivo* indicates that the LCO-dA_2dG_{10} ligand is retained on the liposomes upon intravenous administration, and does not readily redistribute to endogenous serum (lipo)proteins. Indeed, *in vitro* transfer studies in the presence of excess amounts of HDL showed only a minor

and slow redistribution of the LCO anchor from the liposomes to HDL (data not shown). This illustrates the broad applicability of this LCO group to anchor specific ligands for various receptors on macrophages and other cell types. In fact, we have previously reported that derivatization of triantennary galactosides with LCO affords a ligand for the highly efficient targeting of liposomes to the asialoglycoprotein receptor on hepatocytes *in vivo* ([33;34]).

It is intriguing to speculate about the mechanism underlying the high affinity of liposomal dG_{10} for SRA. Since SRA binds a broad array of (poly)anionic ligands such as LPS ([4]), polyI ([3]) and modified LDL ([7;8]), it may be argued that the ligands bind to the cationic extracellular collagenous domain of SRA simply through electrostatic interaction. However, the fact that polyA, polyC, and polyU do not bind to SRA suggests that the spatial distribution of the positively and negatively charged side chains in the collagenous domain, as well as the overall chemical structure, could play a critical role in the recognition of polyanions. Our surprising observation that liposomes bearing overall (oxPC, PC/PS) or clustered (LCO-EE) negative surface charges show an unaltered or marginally increased clearance, despite the fact that these modifications result in an at least equally high negative surface charge as compared to LCO-dA_2dG_{10} incorporation, demonstrates the high specificity of dG_{10} for SRA targeting *in vivo* and confirms that negative charges *per se* do not confer affinity for SRA.

In addition to SRA, CD36 has also been implicated in the binding and internalization of anionic ligands, such as acLDL and oxLDL ([19;20]), but also PS-containing liposomes ([23,24,46]). However, CD36 is probably not involved in the hepatic clearance of LCO-dA_2dG_{10}-exposing liposomes, as CD36 is not expressed by the liver ([21]). In addition, CD36 does not seem to contribute to the extrahepatic clearance of these liposomes (*e.g.* by the spleen) since the serum clearance was almost completely blocked by polyI, whereas CD36 is insensitive to polyI ([20]). Interestingly, inclusion of 50% PS into the liposomes did not increase their uptake by the liver substantially, indicating that PC/PS liposomes do not or only hardly interact with SRA. These data are in agreement with an earlier *in vitro* study showing that transfection of Chinese hamster ovary cells with SRA type I or II did not affect the uptake of PS-containing liposomes, whereas that of AcLDL was greatly enhanced ([48]). In contrast, PC/PS liposomes were selectively taken up by the spleen, which is in line with previous observations ([49]). Collectively, these data indicate that the expression pattern of SRA and CD36 differ markedly: whereas SRA is preferentially expressed by the liver, CD36 is mainly expressed by the spleen, thus offering the appealing opportunity to preferentially target specific subsets of the reticuloendothelial system.

In conclusion, we have demonstrated in the present study the feasibility to use a short oligodeoxyguanosine ligand (*i.e.* dG_{10}) for the efficient targeting of particulate systems via SRA to macrophages, thereby improving the intracellular delivery of encapsulated dexaP in a bioactive form. Our studies add to the recent *in vitro* observations that tethering of dG_{10} to antisense oligonucleotides ([43]) or dG_6 to CpG oligodeoxynucleotides ([44]) improves their SRA-mediated cellular delivery and efficacy, and that conjugation with dG_{10} markedly improves the macrophage-activating properties of muramyl dipeptide, resulting in an increased cytotoxic activity against tumor cells ([45]). Furthermore, we have now demonstrated for the first time that dG_{10} can be used for efficient SRA targeting *in vivo*. Since the versatility of LCO-dA_2dG_{10}-exposing liposomes allows for the encapsulation of a broad array of therapeutic agents, including photosensitizers, anti-inflammatory agents, and LXR agonists, the application of these liposomes for macrophage targeting *in vivo*, either systemically (intravenous administration) or locally (incubation within atherosclerotic areas), is warranted.

Acknowledgements

This work was supported by the Netherlands Heart Foundation (grants no 1999B149 and 2003T201), the Netherlands Organization for Scientific Research (grant no. 016-019-026 and VIDI 917.36.351) and the Leiden University Medical Center (Gisela Thier Fellowship to P.C.N.R.). J.W.J. is an established investigator of the Netherlands Heart Foundation (2001D032). The authors declare no conflict of interest.

Abbreviations

AcLDL, acetylated LDL; CO, cholesteryl oleate; dA_2dG_{10}, deoxy(adenosine)$_2$ deoxy(guanosine)$_{10}$; dexaP, dexamethasone phosphate; DMEM, Dulbecco's modified Eagle medium; DMF, N,N-dimethylformamide; EE, glutamyl glutamic acid; EYPC, egg yolk phosphatidylcholine; LCO, 3α(oleoyloxy)-5ß-cholanic acid (lithocholic oleate); oxPC, oxidized phosphatidylcholine; PBS, phosphate-buffered saline; polyI, polyinosinic acid; PS, phosphatidyl serine; SRA, scavenger receptor class A.

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Chapter 3

Specific and efficient targeting of adenovirus vectors to macrophages: application of a fusion protein between an adenovirus binding fragment and avidin, linked to a biotinylated oligonucleotide

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J Gene Med. 2006 Jun;8(6):668-78

Abstract

Background The application of serotype 5 adenoviruses (Ad5) in macrophages is hampered by the absence of the endogenous Coxsackie Adenovirus Receptor (CAR).

Methods To overcome this limitation, we first generated a linker protein consisting of the virus-binding domain of CAR and the C-terminus of avidin. Second, to target macrophages, this linker protein was equipped with the biotinylated (bio) oligonucleotide dA_6G_{10}, which was previously shown to display a high affinity for the scavenger receptor A (SR-A).

Results As compared to non-targeted virus, the linker protein equipped with biodA_6G_{10} showed a 500-fold increased reporter gene expression in mouse macrophage RAW264.7 cells. A linker protein equipped with a bio-dA_{16} control oligonucleotide was inactive. Moreover, the bio-dA_6G_{10} equipped linker showed a 390-fold increased luciferase expression in the macrophage cell line J774 and a, respectively, 276- and 150-fold increased reporter gene expression in primary peritoneal and bone marrow derived macrophages. Using bone marrow derived macrophages from SR-A knockout mice, it was shown that the dA_6G_{10}-mediated uptake is predominantly SR-A mediated.

Conclusions Thus, we have developed a novel tool to link biotinylated ligands to a virus-binding fragment of CAR and have exploited this linker protein to extend the applicability of Ad5 to infect transformed and primary macrophages.

Keywords: Targeting, Macrophages, Adenovirus, Gene Therapy, Atherosclerosis

Introduction

Adenovirus (Ad) mediated gene transfer is widely used as a powerful method to introduce novel genes in cells *in vitro* and *in vivo* (reviewed by ([1]). The vast majority of adenoviruses generated and used to date involve serotype 5 (Ad5). These Ad5 bind specifically to the Coxsackie Adenovirus receptor (CAR) ([2;3]). This interaction is mediated via the C-terminal knob domain of the Ad5 fiber protein and represents the first step in the uptake of Ad5. Subsequent interactions between Arg-Gly-Asp (RGD) motifs in the penton base of the viral particle and integrins αvβ3 and αvβ5 present on the host cell mediate internalization via receptor mediated endocytosis ([4-6]). After escape from the endosome, the virion is shuttled to the nuclear envelope after which its genome is translocated into the nucleus.

The tropism of Ad5 is determined by the tissue specific expression pattern of the CAR. This receptor is highly, but not exclusively, expressed on parenchymal cells in the liver, thus explaining the high level of infection of the liver after systemic application of Ad5 ([7]). However, many cell types, including tumors, endothelial cells ([8]) as well as macrophages ([9]) express CAR at very low levels, or not at all and are therefore resistant to adenoviral infection. Specific agents that target adenoviruses to cells of interest could, in theory, overcome this limitation. Ideally, retargeting induces affinity for a novel cell specific receptor and at the same time ablates the intrinsic affinity for the endogenous CAR. Two types of strategies have been used thus far to target adenoviruses to specific cells: genetic-based and conjugate-based targeting.

In genetic targeting, the viral capsid proteins responsible for binding to target cells are modified in such a way that affinity is induced for other cells. This modification can be achieved via pseudotyping ([10-14]) or via cloning ligands into the HI-loop ([15-19]) or the C-terminal domain of the fiber knob ([20-28]). For the latter two approaches, insertion of ligands must not interfere with fiber trimerisation during virus assembly ([20]). Thus, the challenge in the genetic approach is to avoid impairment of virus assembly and production by the inserted ligand. Moreover, each novel tropism requires that the virus is redesigned.

Conjugate-based targeting approaches involve the use of bifunctional linker proteins, which on the one hand bind to the adenovirus and on the other hand bind to the target cell. To confer affinity for the virus, either a Fab fragment of a virus binding (neutralizing) antibody ([29]), reviewed by ([30]) or the virusbinding domain of CAR ([31]) can be used (reviewed in ([32]). Introduction of a ligand for a cell specific receptor in the targeting protein induces a new tropism for the target cell. Generally, targeting proteins have been produced as a single protein, in which case

for each novel receptor a new targeting protein is designed, or the different components are covalently fused after production of the separate components. An extensive overview of conjugate based targeting strategies has been given by Krasnykh ([32]).

An alternative approach within the conjugate based targeting strategies, consists of the use of non-covalent cross-linking systems to target virus particles. This approach results in a flexible system to equip Ad with a variety of ligands. For example, Rogers used an anti-Ad Fab fragment coupled to phenylboronic acid (PBA) and attached FGF2 - dihydroxybenzohydroxamic acid (DHBHA) and thus exploited the binding of PBA to DHBHA to redirect Ad to FGF-receptor positive cells ([33]). Smith and Parrott applied the avidin-biotin interaction to retarget adenovirus vectors ([27;34]). Smith biotinylated adenovirus particles chemically and targeted them to biotinylated cells via an avidin bridge. When equipping the virus with biotinylated monoclonal antibodies anti-CD117, anti-CD34 or anti–CD44 via the avidin bridge, hematopoietic cells expressing the respective receptors could be infected, ([34]). Parrott et al. introduced a biotin acceptor peptide in the C-terminus of the fiber of Ad5 ([27]). After metabolic biotinylation of the virus particle, it could be equipped with a variety of ligands via an avidin bridge. Using this system, it was shown that Ad5 could be equipped with biotinylated monoclonal antibodies, biotinylated manose and biotinylated -oligonucleotides and targeted to primary dendritic cells.([27]). Introduction of the biotin acceptor peptide into capsid protein IX and the subsequent attachment of transferrin (via avidin) allowed for the targeting to CD71 positive cells ([35]). Here, we have further expanded upon the application of the avidin-biotin link to generate a conjugation based targeting system that can be applied without direct virus modification.

Thus, a bifunctional linker protein to target adenoviruses to novel receptors was developed. This linker protein consists of the virus-binding domain of the CAR genetically fused to avidin, which is used as a scaffold to dock novel ligands equipped with a biotin moiety. The genetic fusion between CAR and avidin ensures a single protein without the need for chemical modification. Moreover, many receptor-specific ligands are available commercially in biotinylated or easily biotinylatable form. We show the utility of the CAR-Avidin linker protein in combination with a biotinylated oligonucleotide dA_6G_{10}, that specifically binds to the scavenger receptor class A (SR-A), to redirect Ad5 to a variety of transformed and primary macrophages which are notoriously difficult to infect with Ad.

Materials and Methods

Cells Cos-1, Hela, J774 and RAW264.7 cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS (Invitrogen), 100 units/ ml Penicillin (Invitrogen), 100 µg/ml Streptomycin (Invitrogen) and glutamax (Invitrogen). Peritoneal macrophages from C57bl6 female mice were isolated 4 days after 1 ml of 3% Brewers Thioglycollate (Difco, Amsterdam, The Netherlands) was injected intraperitonealy. Peritoneum was flushed with 10 ml ice-cold culture medium (RPMI-1640 (Invitrogen) complemented with 6.5% FCS, 100 units/ ml Penicillin, 100 µg/ml Streptomycin and glutamax. After gentle but thorough washing cells were seeded in 12 well plates (Greiner), $5*10^5$ cells per well. Bone marrow cells were isolated by flushing femurs from C57bl6 or SR-A knockout mice with ice cold PBS. The femurs of the SR-A knockout mice were a generous gift from dr. Menno de Winther (Maastricht, The Netherlands). After washing with PBS, cells were cultured on bacterial plastic in RPMI-1640 complemented with 10% fetal calf serum, 100 units/ ml Penicillin, 100 µg/ml Streptomycin, glutamax and 15% L929 conditioned medium as a source for macrophage colony stimulating factor for 8 days. One day prior to experiments cells were detached from plastic with 4 mg/ml lidocain/ 10 mM EDTA in PBS and seeded into 12 well plates, $1.5*10^5$ cells per well. All cells were cultured in a humidified atmosphere of 5% CO_2

Viruses The generation of recombinant adenoviral vectors carrying the β-galactosidase gene (Ad.LacZ) under the control of the cytomegalovirus promoter has been described previously and were kindly provided by dr. Thomas Willnow and dr. Joachim Herz (Dallas, USA). The recombinant adenoviral vector expressing firefly luciferase under the control of the cytomegalovirus promoter (Ad.Luc) was kindly provided by prof. Rob Hoeben (Leiden, The Netherlands). Recombinant adenoviruses carrying the green fluorescent protein under control of the cytomegalovirus promoter (Ad.GFP) were constructed using the Ad-Easy-1 system as previously described ([36]). The recombinant adenovirus vectors were propagated on PerC6 cells ([37]) and titrated on the Ad5 E1-transformed human embryonic kidney cell line 911 as described previously ([38]). Briefly, for large-scale production of recombinant adenovirus lots, 1750 cm^2 of nearly confluent PerC6 monolayers were infected with adenovirus at a multiplicity of infection (MOI; in plaque forming units per cell) of 5-10. After 48 to 60 hours, the nearly completely detached cells were harvested and collected in 1 ml PBS/1% horse serum (Invitrogen). Virus was released from the producer cells by three rounds of freeze thawing and subsequent centrifugation. The lysate was subjected to two rounds of CsCl gradient purification. The CsCl was removed from the isolated virus bands by

extensive dialysis against storage buffer (25 mM Tris, 137 mM NaCl, 5 mMKCl, 0.73 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$; pH 7.45), at 4°C. Virus stocks were stored at -80°C. Routinely virus titers ranged from 1-5*10^{10} plaque forming units per ml (pfu /ml).

Design and cloning of the linker proteins. The extracellular domain of the Coxsackie Adenovirus Receptor (CAR) was obtained by PCR using pCAR as template using oligo's 5'-GCG GCC GCG GGT ACC CAC GGC ACG GCA G-3' and 5'-CTA GCT AGC AGC TTT ATT TGA AGG AGG GAC-3'. pCAR, an expression vector containing the entire Coxsackie adenovirus receptor cDNA, was kindly provided by prof. Rob Hoeben (Leiden, The Netherlands). The generated PCR fragment was cloned into pCRII Topo (Invitrogen) after which it was subcloned into pSG8, in front of the VSV and His6 tag already present, using restriction enzymes NotI and NheI, thus creating pSG8CAR. The pSG8 vector was a generous gift from prof. Henk Stunnenberg (Nijmegen, the Netherlands) The avidin fragment was obtained by RT-PCR on total RNA from chicken fibroblasts with random hexamer oligonucleotides and subsequent PCR using primers 5'-CGC GGA TCC GCC AGA AAG TGC TCG CTG -3' and 5'- CCA TCG ATG GTC ACT CCT TCT GTG TGC G -3'. The PCR fragment was cloned into cloning vector pBluescript (Stratagene, La Jolla, CA, USA) and subcloned in frame into pSG8CAR using restriction enzymes BamHI and ClaI. The CAR-Stop construct was obtained by site directed mutagenesis of the BamHI site in pSG8CAR from GGATCC to GTATCC resulting in a stop codon immediately behind the tags using the site directed mutagenesis kit from Promega (Madison, WI, USA) according to the protocol supplied by the manufacturer. All constructs were sequence verified.

Production and purification of the linker protein. Cos-1 cells were transfected with pSG8CAR-Avidin or pSG8CAR-Stop using Fugene6 (Roche, Basel, Switzerland). Eight hours after transfection, cells were placed on serum-free and biotin-free culture medium for 32 hours after which the culture supernatant, containing the linker proteins, was harvested. Linker proteins were purified from the supernatant by immobilized metal affinity chromatography using Talon metal affinity resin (Clontech, Palo Alto, USA). Equilibrated culture supernatant (300 mM NaCl, pH = 7.0 and 20% glycerol) was incubated for 20 minutes at room temperature with Talon. After extensive rinsing with buffer (50 mM $NaPO_4$, 300 mM NaCl, 20% glycerol, pH = 7.0) the resin was pre-eluted with 4 volumes pre elution buffer (50 mM $NaPO_4$, 300 mM NaCl, 2,5 mM imidazole, 20% glycerol) after which it was eluted with 10 volumes elution buffer (50 mM $NaPO_4$, 300 mM NaCl, 150 mM imidazole, 20% glycerol). Samples of all purification steps were tested for the presence of the linker proteins via SDS-PAGE and western blotting analysis using Hybond ECL

nitro cellulose membranes (Amersham Biosciences, Buckinghamshire, UK) and antibodies P5D4 (α-VSV) and sheep-α-mouse-HRP for the CAR-Stop protein or α-Avidin (Abcam, Cambridge, UK) and goat-α-rabbit-HRP for the CAR-Avidin protein according to standard protocols ([39]). Analysis showed that the linker protein was only present in elution fractions 3 to 5 that were subsequently extensively dialyzed against PBS and used in all experiments. Western analysis of the CAR-Stop protein revealed a minor contamination of approximately 25 kD. Quantification of the band intensities was performed by analyzing the digital images with Tina® version 2.09 software (Raytest, Staubenhardt, Germany). The contaminating protein accounted for approximately 5 % of the total amount of protein.

Analysis of the linker protein. Elution samples that tested positive on western blotting analysis were submitted to a biotin binding assay. 10 µl samples of the indicated elution fractions or an avidin calibration range of 0.3 - 10 pM avidin were incubated with 0.2 µl 3H-Biotin (Du Pont NEN Research Products, Boston, MA, USA). After 1 hour the total reaction mixture was applied on a Sephadex G-50 column to separate the CAR-Avidin bound biotin from the free biotin. All elution fractions were counted for 3H-Biotin radioactivity using 5 ml of Hionic fluor scintillation cocktail (Packard Instrument Co., Perkin Elmer, Boston, MA, USA) in a Packard 1500 TriCarb liquid scintillation analyzer. The summed radioactivity in peak fractions 3 to 5 correlated with the amount of avidin present in the sample (R^2= 0.997). As having the highest concentrations elution fraction 3 was used for experiments and stored at –80°C. A yield in the order of 900-1000 µg was typical.

Affinity of the CAR-Avidin linker protein for the bio-dA_6G_{10} ligand was calculated from the occupied fraction at different linker protein to ligand ratio's using Graphpad Prism software (San Diego, CA, USA) and a one-site binding model.

Infection assays. Twenty-four hours before infection cells were seeded into 12 wells plates (Greiner, Sigma Aldrich, St Louis, MO, USA), RAW264.7, J774 and bone marrow derived macrophages at $2.0*10^5$ cells per well, Hela at $5.0*10^5$ cells per well. CAR-Avidin and bio-dA_{16} or bio-dA_6G_{10} were incubated at the indicated molar ratios for 1 hour in PBS at room temperature, after which Ad.Luc, Ad.GFP or Ad.LacZ were added and incubated for 1 hour. Cells were infected for 1 hour at 37°C with the virus-targeting protein complex after which the virus containing solution was replaced with normal culture medium. Experiments were terminated 36 hours after infection. Ad.luc infected cells were lysed in reporter lysis buffer (Promega) and luciferase activity in the lysate was determined using the luciferase reporter assay system (Promega) according to the

protocol supplied by the manufacturer. Luciferase activity was corrected for protein concentration determined by BCA assay (Pierce, Rockford, USA) using BSA as standard. Ad.LacZ infected cells were washed with PBS and fixed in 5.4% formaldehyde, 0.8% gluteraldehyde in PBS. LacZ expression was visualized by adding staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.2 mM $MgCl_2$, 0.1% 5-bromo-4-chloro-3-indolyl- β-D-galactoside (X-Gal) in PBS) to the wells until sufficient color intensity had been reached. Ad.GFP infected cells were rinsed once with PBS and subsequently fixed with 4% formaldehyde (Merck, Darmstadt, Germany) in PBS.

In vivo infection assays For the *in vivo* experiments, 10-12-wk-old female C57Bl/6 mice of weight 22-24 g from Broekman Instituut BV (Someren, The Netherlands) were used and fed *ad libitum* with regular chow diet. On day 0, mice were injected with either untargeted or targeted $1*10^9$ pfu Ad.LacZ. Generation of the targeted Ad.LacZ was performed as described above for the in vitro experiments. Five days after injection, the experiment was terminated and livers were removed and snap frozen in liquid nitrogen. 10µm sections were stained for LacZ activity. Sections were O/N incubated with staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.2 mM $MgCl_2$, 0.1% 5-bromo-4-chloro-3-indolyl- β-D-galactoside (X-Gal) in PBS).

Statistics Experiments were performed in triplicate and presented as mean ± standard deviation (s.d.). P-values were calculated by a two-tailed unpaired student's T-test. Data were considered to be significantly different when $p< 0.05$.

Results

Design and generation of the linker proteins To modulate the tropism of Ad5 to alternative cell surface receptors, a bifunctional linker protein was designed containing the extracellular domain of the CAR, a VSV tag, a His6 tag, and a biotin-binding avidin fragment (figure 1A). The extracellular domain of the CAR enables binding to the fiber knob ([2;40]), and is preceded by the original signal peptide to ensure effective secretion of the linker protein into tissue culture supernatant after transfection. The tags were introduced to facilitate visualization (VSV) and purification (His6) of the protein. Truncated chicken avidin (nucleotides 116 – 499) ([41]) was used as a scaffold to bind biotinylated ligands for receptors of interest. A negative control linker protein (CAR-Stop) was constructed by introducing a stop codon directly behind the His6 tag (figure 1A).

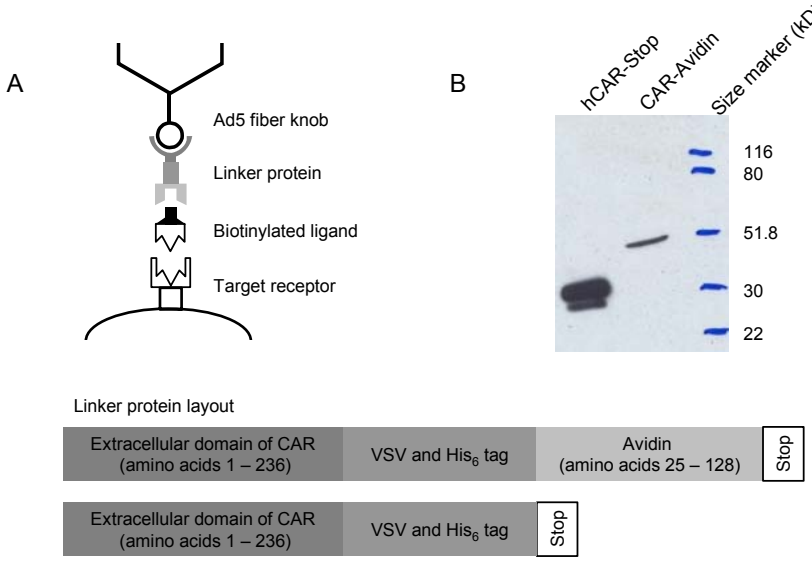

Figure 1. Linker protein design and characterization. (A) *The CAR-Avidin linker protein was constructed by cloning the extracellular domain of the CAR (first 236 aa, ■) into a pSG8 expression vector which contained the VSV and His tag (■). Avidin amino acids 25 to128 (■) were cloned immediately behind the tags. Ligands for specific receptors are attached to the avidin moiety of the linker protein via biotin (■). The CAR-Stop control protein was constructed by introducing a stop codon immediately behind the tags.* ***(B)*** *CAR-Avidin and CAR-Stop were produced by transient transfection in COS-1 cells. Presence of the proteins was shown by subjecting culture supernatant to SDS-PAGE (12%) and Western analysis using antibodies P5D4, directed against the VSV tag, and sheep-anti-mouse-HRP.*

Expression constructs encoding the linker proteins CAR-Avidin and CAR-Stop were transfected into Cos-1 cells and the supernatant was subjected to SDS-PAGE and Western blotting analysis. Figure 1B shows that the predominant products are 50 and 30 kD proteins, which is very close to the calculated mass of CAR-Avidin and CAR-Stop (45 kD and 28 kD respectively). Western analysis also indicated a minor protein contamination of approximately 25 kD in the CAR-Stop sample (approximately 5% of the total amount of protein within the sample). Large-scale protein productions were performed by transient transfection in Cos-1 cells under biotin-free and serum-free conditions and subsequent

purification of the linker proteins from the culture supernatant using His6 mediated immobilized metal affinity chromatography (IMAC).

Quantification and binding characteristics of the linker proteins The concentration of CAR-Avidin in the purified preparations was determined via a ^{3}H-biotin binding assay. The assay was calibrated using avidin. Bound ^{3}H-biotin was separated from unbound ^{3}H-biotin using gel filtration analysis (insert Fig. 2A). The radioactivity recovered in fractions 3-5 (bound ^{3}H-biotin) correlated excellently with the amount of avidin in the sample over a concentration range from 1.6 to 16.6 µg/ml (Fig 2).

The ability of the linker protein to bind both biotin and adenovirus was investigated by surface plasmon resonance spectrometry using a Biacore 2000 apparatus and a biotin coated sensor chip. Control injections of streptavidin led to rapid and avid association to the chip and confirmed biotin binding to the chip (data not shown). Injection of the CAR-Stop protein (figure 2B left panel, dark arrow) did not result in an increase in response units indicating that this protein did not bind to biotin present on the chip. Repeated injections of CAR-Avidin on the other hand clearly showed an increase in response units reflecting saturable binding of this linker protein to biotin on the chip (figure 2B left panel, light arrows). To determine the ability of CAR-Avidin to bind adenoviruses, CAR-Avidin was conjugated to a biotin coated sensor chip. Figure 2B (right panel) shows that Ad5 binds to CAR-Avidin coated chips but not to a streptavidin coated control chip. The affinity of the virus for CAR-Avidin appeared to be in the low nanomolar range, which is comparable to the reported affinity of the virus for the endogenous CAR (Kd: 3 nM, ([42]).

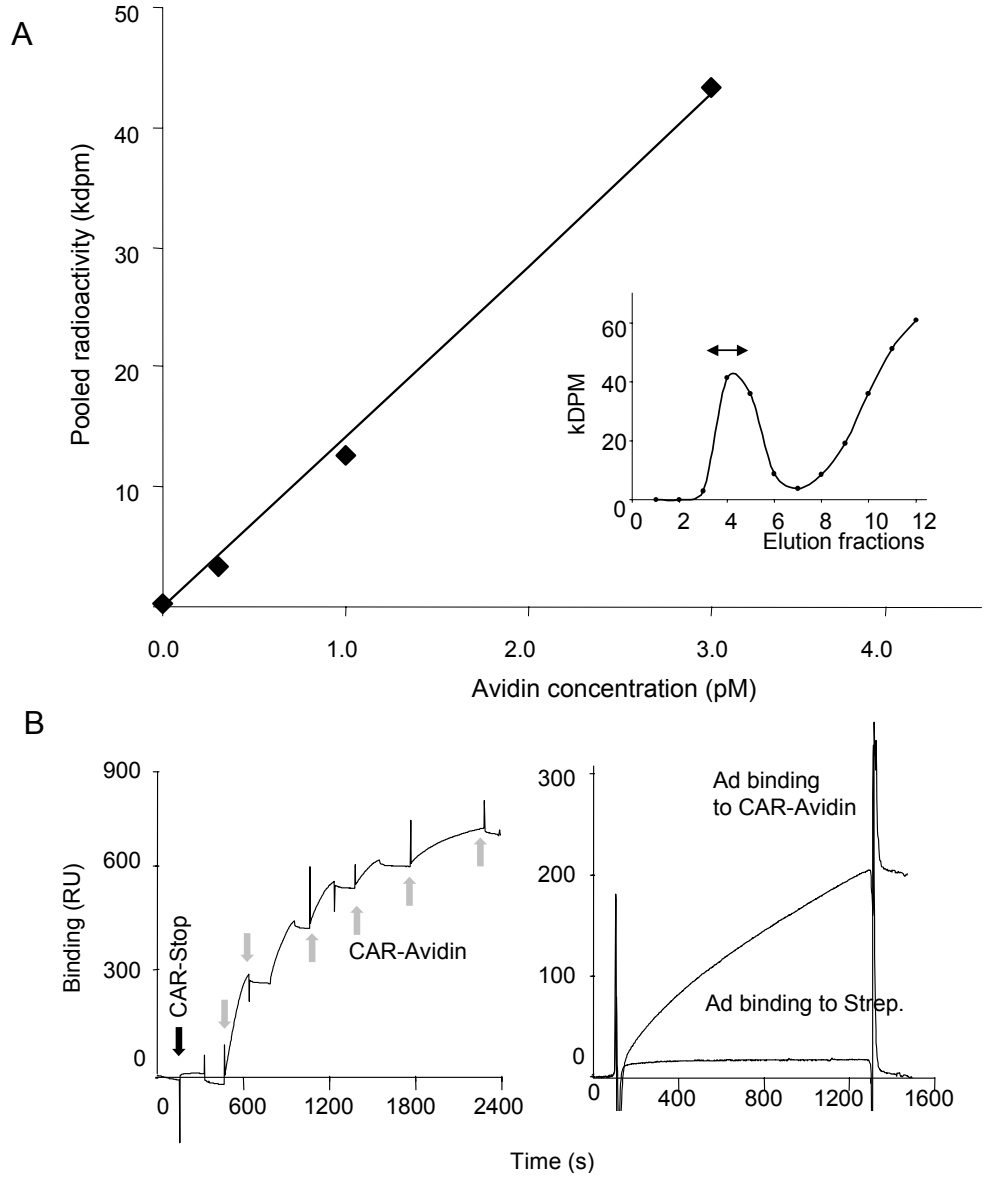

Figure 2. Quantification of the linker protein. *(**A**) CAR-Avidin and an avidin concentration range were incubated with 3H-Biotin. Total mixture was applied to a sephadex G50 column to separate avidin bound (small graph elution fractions 3-5) from the free 3H-Biotin (small graph elution fraction 8-12). The avidin associated radioactivity correlated to the avidin concentration (R^2= 0.997). (**B left**) Biotin was immobilized on the sensor*

*surface of a Biacore chip after which CAR-Stop (once, black arrow) or CAR-Avidin (5 times, gray arrows) were injected at a flow rate of 35 µl / min. The protein content of the purified CAR-Stop stock was assessed by western blot analysis and using CAR-Avidin as internal reference; equal amounts of CAR-Stop and CAR-Avidin protein were used (**B right**) Streptavidin or CAR-Avidin was bound to the sensor chip through conjugation to immobilized biotin. Ad5 was injected at a flow rate of 2 µl / min for 27 minutes.*

Generation of an adenovirus targeting construct The applicability of the linker protein to enhance the infection of macrophages was investigated using the mouse monocyte derived macrophage cell line RAW264.7. This cell line is refractory to adenovirus infection and only at very high, and cytotoxic, titers infection of less than 1% percent of cells can be observed (data not shown ([43]). On macrophages, the SR-A is an obvious candidate for targeting, since it is abundantly expressed and mediates the rapid internalization of bound substrates including modified lipoproteins, lipopolysaccharide, specific oligonucleotides and Fucoidan ([44]). A synthetic oligonucleotide ligand for SR-A was generated: dA_6G_{10} consisting of 6 deoxyadenosines serving as a spacer, and 10 deoxyguanosines, which confer specificity for the SR-A ([44]). It was biotinylated to allow docking on the CAR-Avidin linker protein. A synthetic oligonucleotide, bio-dA_{16}, served as a negative control since the adenosine stretch does not bind to SR-A ([44]) while having similar chemical and anionic properties.

Optimization of the infection protocol was performed in two steps. The optimal molar ratio between CAR-Avidin and ligand was determined. Hereto, CAR-Avidin at a fixed concentration of $5.0*10^{-8}$ M was incubated with varying amounts of bio-dA_6G_{10} and the residual biotin binding capacity was assessed in the ^{3}H-Biotin binding assay. Figure 3A shows that at a molar ratio of CAR-Avidin: bio-dA_6G_{10} higher than 1.0 the complex did not display any residual ^{3}H-Biotin binding capacity, indicating that at this ratio all biotin binding sites within the linker protein are occupied. From the saturation binding plot (the relative degree of occupation plotted against the concentration of bio-dA_6G_{10}, Fig. 3A, insert) we were able to calculate the apparent Kd for bio-dA_6G_{10} binding to CAR-Avidin ($2.3*10^{-9}$ M; R^2= 0.91). Thus, although the affinity of CAR-Avidin for bio-dA_6G_{10} is decreased as compared to the affinity of native avidin for biotin ($K_d = 10^{-15}$ M), the resulting interaction between the biotinylated ligand and CAR-Avidin is similar in avidity as compared to that of CAR to the adenovirus ($K_d = 3*10^{-9}$ M) and thus not likely to limit the adapter function *in vitro* or *in vivo*.

The binding equilibrium is sensitive to molar ratio's and the absolute concentration of the individual binding partners and for an effective association concentrations that exceed the dissociation constant are imperative. In our analyses, we therefore preferred to keep the CAR-Avidin/ligand concentration constant. Thus, an increasing amount of CAR-Avidin-dA_6G_{10} was added to a fixed amount of Ad.Luc and the level of gene transfer was determined. Luciferase activity was found to increase in a concentration dependent manner (Fig. 3B). At a concentration of 50 nM CAR-Avidin-dA_6G_{10} the luciferase activity reached 50% of the maximum activity, so in following experiments a concentration of 50 nM was used.

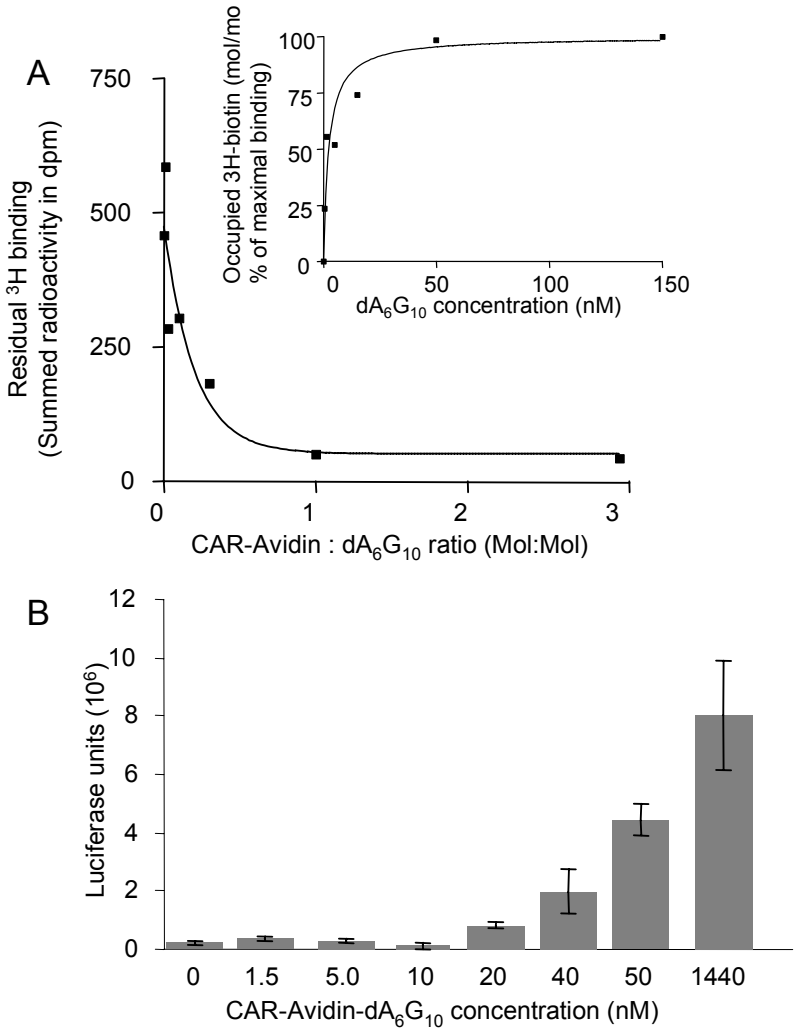

Figure 3. Optimization of the linker protein to ligand ratios and targeting construct to virus ratios. *(**A**) Optimal CAR-Avidin to dA_6G_{10} ratios was determined by adding CAR-Avidin (concentration $5.0*10^{-8}$) to dA_6G_{10} in a molar ratio varying from 0.01 to 3. Subsequently the residual and the occupied ^{3}H-Biotin binding was determined (R^2= 0.85). (**B**) The optimal CAR-Avidin-dA_6G_{10} concentration was determined by adding CARAvidin-dA_6G_{10} (at a concentration ranging from $7.5*10^{-8}$ to $1.5*10^{-6}$ M) to Ad.Luc. after which RAW264.7 cells were infected (MOI of 100) with the virus suspension. Cells were lysed and luciferase activity was determined*

and corrected for protein concentration. Results are mean ± s.d. of triplicate experiments.

Characterization of adenovirus targeting specificity The specificity of the CAR-Avidin-dA6G10 conjugate was investigated by comparison with CAR-Avidin-dA16. PolyA has been reported to lack affinity for the SR-A (44). Thus bio-dA16 or bio-dA6G10 equipped CAR-Avidin was incubated with Ad.Luc and its effect on adenoviral gene transfer was assessed as described. Infection of RAW264.7 cells at a multiplicity of infection (MOI) of 100 plaque forming units per cell (pfu) with CAR-Avidin-dA16 targeted Ad.Luc resulted in a luciferase activity that was comparable to non-targeted virus, whereas CAR-Avidin-dA6G10 targeted virus showed a 45 fold increase in luciferase activity (P= 0.0001) establishing that the cellular infection with Ad.Luc via dA6G10 is specific and most likely involves the SR-A (Fig. 4A).

Retargeting implies acquisition of novel specificity and concomitant loss of the intrinsic specificity. To investigate whether the linker protein ablated the affinity for cells expressing the CAR, Ad.Luc was incubated with the CAR-Stop protein after which the complex was used to infect Hela cells (MOI 230). Hela cells are known to express CAR and are therefore readily infected even at low adenovirus titers ([2]). Figure 4B shows a 75% decrease in luciferase expression after addition of the CAR-Stop protein to Ad.Luc (P = 0.0003) indicating loss of affinity for the endogenous CAR.

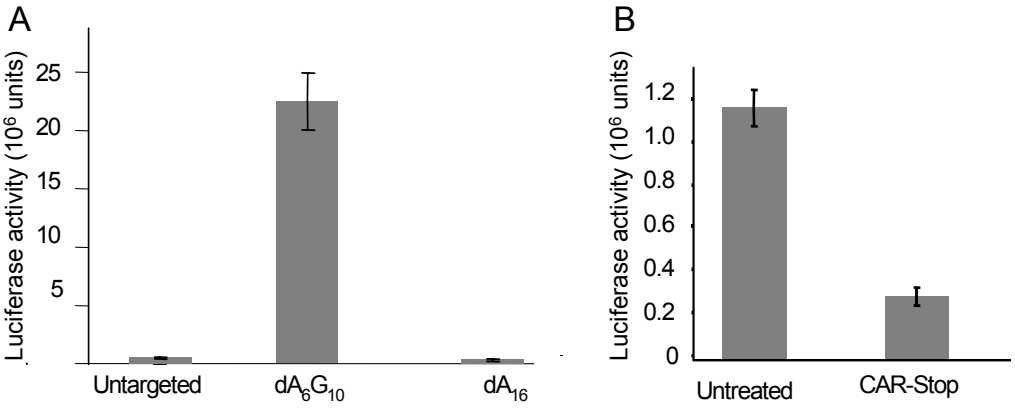

Figure 4. Effect of CAR-Avidin-dA$_6$G$_{10}$ targeting on gene activity in different cell lines *(**A**) CAR-Avidin-dA$_6$G$_{10}$ or CAR-Avidin-dA$_{16}$ (1:1 M:M) was added to Ad.Luc after which RAW264.7 cells were infected (MOI 100). Cells were lysed and luciferase activity was determined and*

*corrected for protein concentration. Results are mean ± s.d. of triplicate experiments. (**B**) Hela cells were infected with Ad.Luc (MOI of 277) pre incubated in the presence (right bar) or absence (right bar) of the CAR-Stop protein. For the pre incubation the same protein concentration had been used as is used for the CAR-Avidin. Cells were lysed and luciferase activity was determined and corrected for protein concentration. Results are mean ± s.d. of triplicate experiments.*

To investigate whether gene transfer to primary cells could be enhanced using CAR-Avidin-dA_6G_{10}, an adenovirus expressing GFP (Ad.GFP) was targeted to RAW264.7 cells and peritoneal macrophages. Scoring the GFP positive cells revealed a 21-fold increase in GFP positive RAW264.7 cells (P = 0.02) and 237-fold increase in GFP positive peritoneal macrophages (p = 0.04) after targeting with CAR-Avidin-dA_6G_{10} as compared to non-targeted Ad.GFP (Fig 5A). To investigate whether the CAR-Avidin-dA_6G_{10} construct could also facilitate the adenoviral infection of other transformed or primary macrophages, RAW264.7 cells, J774 cells and bone marrow derived macrophages were infected as previously described at increasing MOI (50, 100, 200 pfu per cell). CAR-Avidin-dA_6G_{10} increased the infection efficiency by 536-fold in RAW264.7 cells ($p < 0.04$), up by 392-fold in J774 cells ($p < 0.013$) and by 101-fold in bone marrow derived macrophages ($p < 0.013$) (Fig. 5B; please note the logarithmic scale of this figure).

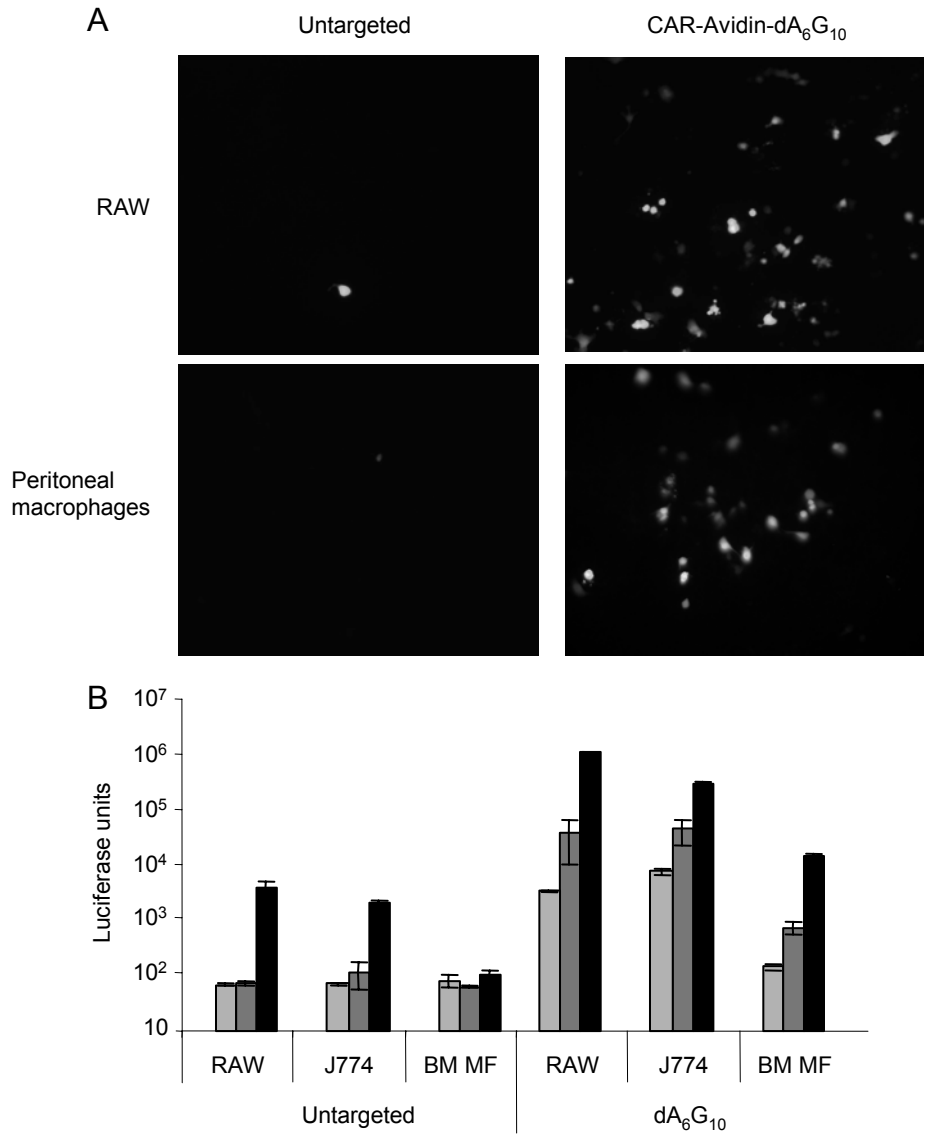

Figure 5. Effect of CAR-Avidin-dA$_6$G$_{10}$ targeted infection of different cell types with Ad.Luc *(**A**) CAR-Avidin was added to dA$_6$G$_{10}$ at a molar ratio of 1:1. Ad.GFP was incubated with CAR-Avidin-dA$_6$G$_{10}$ at a concentration of 5.0*10^{-8} M after which RAW264.7, and peritoneal macrophages were infected (MOI 100). 40 hours after infection wells were checked for GFP positive cells. (**B**) CAR-Avidin was added to dA$_6$G$_{10}$ at a molar ratio of 1:1. Ad.Luc was incubated with CARAvidin-dA$_6$G$_{10}$ at a*

*concentration of 5.0*10^{-8} M after which RAW264.7, J774 and bone marrow derived macrophages (BM MF) were infected with different MOI's (50, 100, 200). Cells were lysed and luciferase activity was determined and corrected for protein concentration. Results are mean ± s.d. of triplicate experiments. Please note that the scale of this figure is logarithmic in contrast to scales of the other figures in this paper.*

To further investigate the specificity for the SR-A, the dA_6G_{10} equipped linker protein was used to target Ad.Luc to bone marrow derived macrophages isolated from wt or SR-A-/- mice. Figure 6 shows a clear increase in infection efficiency in macrophages isolated from the wt mice whereas this increase is much more modest in the macrophages isolated from SR-A-/- mice when compared to untargeted Ad.Luc, indicating that the increased infection efficiency via CAR-Avidin-A_6G_{10} is predominantly SR-A mediated. The infection efficiency of targeted Ad.Luc was found to be significantly higher than that of untargeted virus at MOI > 100 pfu per cell ($p < 0.02$).

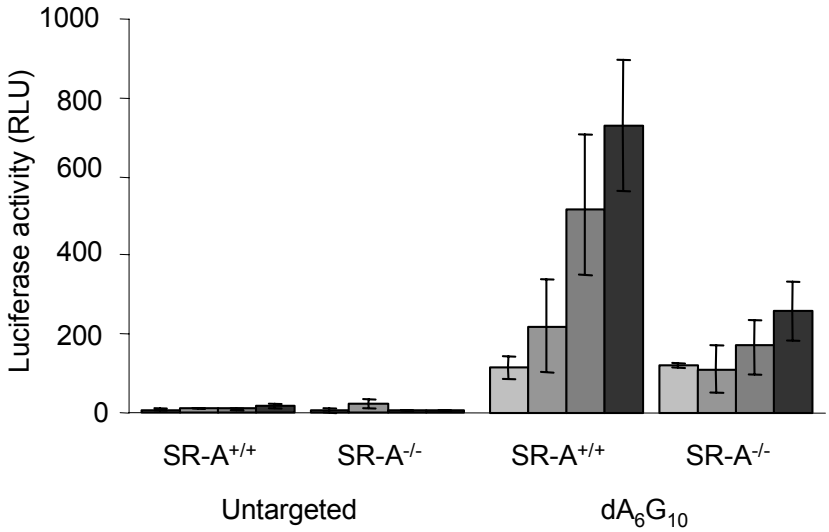

Figure 6. Effect of CAR-Avidin-dA$_6$G$_{10}$ targeting on Ad.Luc infection in macrophages isolated from SR-A+/+ versus SR-A-/- mice *CAR-Avidin was added to dA$_6$G$_{10}$ at a molar ratio of 1:1. Ad.Luc was incubated with CAR-AvidindA$_6$G$_{10}$ at a concentration of 5.0*10^{-8} M after which bone marrow derived macrophages isolated from either wt or SR-A-/- were infected with different MOI's (50, 100, 200, 400 pfu/cell). Cells were lysed and luciferase activity was determined and corrected for*

*protein concentration. Results are mean ± s.d. of triplicate experiments. * indicates p<0.02*

To investigate whether the targeting would change the biodistribution of Ad, wild type mice received either untargeted or CAR-Avidin-dA_6G_{10} targeted Ad.LacZ. Five days after injection, clear redistribution of β-galactosidase staining within the liver was observed. After infection with untargeted virus, all parenchymal liver cells were found to stain blue (fig 7, left panel). Targeting led to a reduced infection of the parenchymal cells, whereas that of non-parenchymal cells was increased (fig 7, right panel). Based on morphology and localization within the tissue, these non-parenchymal cells most likely are Kuppfer cells (fig 7, right panel, dark arrow). These data suggest that the virus-targeting complex is stable in vivo and suggest that it might be possible to redirect Ad5 from parenchymal liver cells to Kuppfer cells.

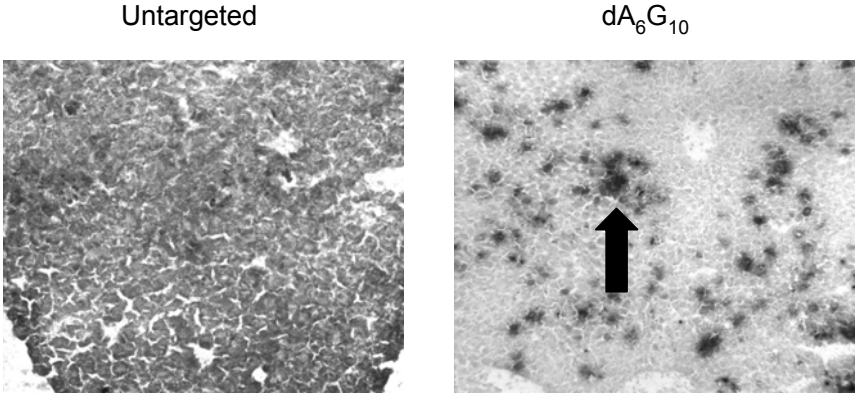

Figure 7 Effect of CAR-Avidin-dA_6G_{10} targeting of Ad.LacZ on biodistribution in the liver in vivo. *Wt female mice received a dose of $1*10^9$ pfu of untargeted or targeted Ad.LacZ. CAR-Avidin was added to dA_6G_{10} at a molar ratio of 1:1. Ad.LacZ was incubated with CAR-Avidin-dA_6G_{10} at a concentration of $5.0*10^{-8}$ M. Five days after infection mice were sacrificed and livers were isolated and stained for LacZ activity. The experiment was performed in duplo, representative sections are shown at a 100 times magnification.*

Discussion

In this paper we present the generation and characterization of a CAR-Avidin linker protein to expand the applicability of adenoviruses to cells that normally are refractory to infection. We show that equipping the

linker with an SR-A ligand (oligonucleotide bio-dA_6G_{10}) results in a targeting construct which increases the Ad5 mediated reporter gene transfer to both primary and transformed macrophages by up to 500-fold. Thus, in contrast to untargeted adenovirus, the CAR-dA_6G_{10} construct results in significant levels of adenovirus mediated gene transfer to macrophages.

Although the dA_6G_{10} ligand is highly specific for macrophages *in vitro* and *in vivo* ([44]), our analysis of the receptor specificity using macrophages derived from SR-A knockout mice suggests that the effect is not completely SR-A specific, since the SR-A -/- macrophages are also infected by the dA_6G_{10} equipped Ad, albeit at a significantly lower level (fig 6). This may be explained by the presence of alternative members of the (presumably) scavenger receptor family also capable of taking up polyG oligonucleotides ([45]). In the constitutive absence of the SR-A, these receptors may even be upregulated as part of a compensatory pathway. On the other hand, these alternative receptors may contribute to the uptake of the dA_6G_{10}-equiped Ad even in the presence of the SR-A. Evidence for a constitutive background activity comes from the observation that at low MOI both SR-A-/- and SR-A+/+ macrophages are infected by dA_6G_{10}-equiped Ad at a low level.

Macrophages play, via the secretion of proinflammatory cytokines, a role in a wide variety of biological and pathogenic processes including angiogenesis, inflammation, HIV infection, chronic granulomatous disease, atherosclerosis, lysosomal storage disorders, diabetes and lupus erythematosus. Because of this central role, much effort has been put into finding approaches to modulate gene expression in macrophages. One of the approaches to intervene in macrophage biology involves the use of gene therapy (reviewed by ([43]). Several strategies have been pursued to facilitate the use of adenovirus vectors for the modulation of macrophage gene expression, including the insertion of a poly-lysine stretch in the fiber knob ([20]). Although successful, this strategy is not easily applicable to the majority of existing and available adenoviral vectors.

The CAR-Avidin linker protein equipped with a biotinylated ligand is physically separated from the virus. This renders the targeting construct applicable to all Ad5 based vectors and in addition precludes problems associated with the generation and propagation of some of the genetically modified viruses ([19;46]). The majority of cell lines used for generating and propagating recombinant adenoviruses (911, 293 and PerC6) are infected via interaction of the virus with the CAR receptor present on the packaging cell line. Because the virus itself remains unaltered, virus assembly or propagation in the commonly used adenovirus production cell lines will not be affected.

Binding of native biotin to avidin results in the formation of avidin-biotin tetramers ([47]). Addition of sugar moieties, or other compounds, to avidin or biotin is known to affect the binding between avidin and biotin in a negative manner ([48]) and the femtomolar affinity between avidin and biotin is generally solely reached with unmodified components. Our biotin binding experiments revealed a K_d of 2.3 nM for interaction of CAR-Avidin with biotin-dA_6G_{10}. In analogy, the dissociation constant of a biotinylated 17-mer peptide was found to be as low as 0.15 nM (data not shown). Thus, the affinity of avidin for biotin indeed appears to be affected by attached structures. However, while the affinity may be lower than that of native avidin and biotin, it is significantly higher than that of monomeric avidin and biotin ($K_d = 10^{-7}$ M) and, in fact, is comparable to the affinity of CAR for Ad5 (3 nM). We thus conclude that the high stability of the CAR-avidin and bio-ligand interaction will not limit application of the targeting construct. The nM affinity of CAR-Avidin for bio-dA_6G_{10} is suggestive for the formation of multimers, although this remains to be demonstrated directly.

The avidin docking scaffold will allow the use of a whole range of biotinylated ligands including glycosides ([49-51]), peptides ([20;52-55]), entire proteins ([29;56-58]) or oligonucleotides ([44;59;60]) to confer affinity for the cell of interest. The affinity of the avidin biotin dyad, even after attachment of bulky structures, warrants sufficiently tight association between the adenovirus-binding moiety and the novel homing device and obviates complex coupling reactions and purification steps (reviewed by ([32]). The avidin-biotin binding takes place at room temperature in any physiological buffer and unbound biotinylated ligands, if present, can be removed via dialysis.

The CAR-avidin based targeting strategy to adapt the tropism of Ad vectors, that is presented in this study, differs in essential aspects from previously described approaches based on avidin and biotin. As compared to the approach of Smith et al., which uses chemically biotinylated Ad vectors and avidin partially occupied with biotinylated ligands ([34]), the application of a CAR-avidin adapter obviates the chemical modification step of the ad vector and at the same time ablates the intrinsic affinity for cellular CAR. In addition, with regards to the maximum number and specific sites of attachment, the CAR-Avidin approach is more well defined. On the other hand, the approach using biotinylated Ad vectors does not require the generation of the CAR-Avidin linker protein. An alternative targeting approach is based on a biotin acceptor peptide that has been inserted into the virus knob ([27]) or into protein IX ([35]) of the Ad vector and that is biotinylated metabolically during production of the virus. Similar to Smith et al., coupling of biotinylated ligands to the Ad vector is

achieved via an avidin bridge. This genetic approach results in well defined targeting complexes and obviates the need for linker proteins such as CAR-Avidin. On the other hand, genetic targeting is not readily applicable to already available Ad5 type vectors and thus requires regeneration of the Ad vectors. Thus, the specific requirements for the Ad targeting that are desired will determine which system is most appropriate.

The *in vivo* experiment using the dA_6G_{10} equipped linker protein with adenoviruses shows that the virus-linker protein complex remains stable after *in vivo* administration and, based on morphological characteristics, suggest a redistribution of the virus from the parenchymal liver cells to, most likely, Kuppfer cells (Fig 7). From these data, it cannot be concluded whether the redistribution of the Ad is caused by the specificity of dA_6G_{10} equipped Ad for Kuppfer cells, or whether the redistribution is caused by de-targeting of the endogenous CAR on the liver parenchymal cells. The preliminary observation that dA_{16} equipped Ad resulted in low to very low level infection of Kuppfer cell like cells (data not shown) indicates that part of the dA_6G_{10}-specific Kuppfer cell infection is caused by nonspecific uptake as a consequence of parenchymal cell-specific CAR de-targeting.

In conclusion, we have demonstrated the application of a CAR-Avidin linker protein in association with a biotinylated ligand to mediate adenovirus infection. Equipping the linker protein with an appropriate biotinylated ligand can considerably increase adenoviral gene transfer to refractory cell types. The CAR-avidin linker protein is, when equipped with a biotinylated dA_6G_{10} oligonucleotide, able to increase the infection efficiency of multiple primary and transformed macrophage cell lines. The versatility of the avidin moiety within the linker protein readily allows an expansion of the number and nature of the ligands that can be attached via a biotin moiety, thus enabling the application of the targeting protein for adenoviral infection of a wide variety of cell types.

Acknowledgements

This research is conducted in the framework of the “Leiden Center for Cardiovascular Research LUMC-TNO” and is financially supported by the Netherlands Heart Foundation (grant no. 1999B149). Rob Hoeben (Leiden University Medical Center, Leiden, The Netherlands) is kindly acknowledged for providing the Coxsackie adenovirus receptor cDNA and the recombinant adenovirus expressing luciferase. The pSG8 expression vector was a kind gift from Henk Stunnenberg (Nijmegen University, Nijmegen, the Netherlands). Menno de Winther (Department of Molecular Genetics, Maastricht University, Maastricht, The Netherlands) is kindly acknowledged for supplying femurs from SR-A knockout mice.

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Chapter 4

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

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Biochemical and Biophysical Research Communications 338 (2005) 847–854

Abstract
Vascular smooth muscle (VSMC) and endothelial cells (EC) are particularly resistant to infection by type 5 adenovirus (Ad) vectors. To overcome this limitation and target Ad vectors to ubiquitously expressed aVb3/5 integrins, we have generated a linker protein consisting of the extracellular domain of the coxsackie adenovirus receptor (CAR) connected via avidin to a biotinylated cyclic (c) RGD peptide. After optimization of CAR to cRGD and to Ad coupling, infection of mouse heart endothelial cells (H5V) could be augmented significantly, as demonstrated by 600-fold increased transgene expression levels. In EOMAs, a hemangioendothelioma-derived cell line, the fraction of infected cells was enhanced 4- to 6-fold. Furthermore, the fraction of infected primary mouse VSMC was increased from virtually 0% to 25%. Finally, in human umbilical vein endothelial cells, the number of GFP positive cells was enhanced from 2% to 75%. In conclusion, CAR-cRGD is a versatile and highly efficient construct to target Ad vectors to both transformed and primary VSMC and EC.

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

Introduction
Recombinant type 5 adenovirus (Ad) vectors are extensively used to modulate gene expression in a wide variety of cells and organs, both in vitro and in vivo. Part of this popularity can be ascribed to their relatively straightforward generation and amplification to high titers ([1]). Ad entry and infection of cells requires at least two distinct interactions. First, attachment of the virus particle occurs via interaction of its fiber knob with the Coxsackie adenovirus receptor (CAR) present on the cell surface ([2–5]). Second, the Arg-Gly-Asp (RGD) motifs present in the viral penton base will bind to aVb3 and aVb5 integrins on the target cell surface and trigger internalization via receptor-mediated endocytosis ([6–8]). In addition, recent data have shown the involvement of heparan sulfate glycosaminoglycans (HSGs) in adenoviral entry in vivo ([9]). Recombinant Ad vectors encoding numerous wild type and mutant genes, as well as short hairpin RNA molecules have been generated. However, the application of Ad vectors in CAR negative cell lines, such as vascular smooth muscle cells (VSMC) and endothelial cells (EC) ([10–12]) is hampered by low infection efficiencies at low multiplicity of infection (MOI) and Ad associated cytotoxicity at high MOI. To expand the applicability of Ad-mediated gene transfer, various strategies to modify Ad tropism have been undertaken. In the genetic

modification approach, peptide ligands have been incorporated into the HI-loop of the Ad fiber knob ([13-16]), added to the C-terminus of the fiber knob ([17]) or inserted into the hexon protein ([18]). However, it is not possible to predict which peptide or protein ligands will be tolerated and do not disturb fiber trimerization and/ or capsid function. In addition, for each specific targeting application, rederivatization of the original recombinant Ad vectors is obligatory. Alternatively, bifunctional targeting proteins have been generated consisting of an Ad-binding domain coupled to a peptide or protein that confers a novel specificity ([19]). This strategy enables the utilization of existing recombinant Ad vectors, but the generation of the bifunctional targeting protein may require chemical linkage and subsequent purification steps. In addition, Parrot and co-workers have introduced a novel approach to target viral vectors. They launched the concept of metabolically biotinylated vectors ([20;21]) and demonstrated the utility of the avidin–biotin based system for vector targeting. Recently we have combined the advantages of the latter two targeting strategies, by developing a bi-functional linker protein that exploited the avidin–biotin concept (Gras, personal communication). This linker protein consists of the extracellular domain of the CAR fused to chicken avidin, which functions as a universal docking site for biotinylated ligands. It was demonstrated that a biotinylated dA6G10 oligonucleotide coupled to the CAR-Avidin linker confers macrophage specificity (Gras, personal communication). In this study, the CAR-Avidin linker protein is coupled to a biotinylated cyclic RGD peptide (bio-cRGD) to increase infection efficiency of EC and VSMC. This cRGD peptide has a high affinity for αVβb3 and αVβb5 integrins ([22]), which are expressed ubiquitously on transformed cell lines and most primary cells. It is demonstrated that linking of Ad to the CAR-cRGD targeting construct resulted in a highly significant improvement of infection efficiencies of transformed and primary VSMC and EC at all MOI used.

Materials and methods

Cell culture. Chinese hamster ovary (CHO) cells, H5V (mouse endothelial cell line derived from heart) and EOMA (mouse hemangioma-derived micro vascular cell line), were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL). Ramos cells (Burkitt lymphoma cells) and K-562 cells (chronic myelogenous leukemia cells from blast crisis) were cultured in RPMI 1640 medium. All media were supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 lg/ml streptomycin, and glutamax (Invitrogen). Human umbilical vein endothelial cells (HUVECs) were a generous gift from E. Pieterman (TNO Prevention and Health, Leiden, The

Netherlands) and were isolated as previously described ([23;24]) and grown in medium 199 with 10% human serum. Mouse VSMC were isolated from aorta from male C57Bl6 mice as previously described ([25]) and cultured in DMEM with 10% newborn calf serum (NCS). All cells were maintained at 37 °C in a humidified atmosphere of 5% CO2.

Production of recombinant Ad vectors. Recombinant E1, E3-deleted Ad-vectors expressing b-galactosidase gene (Ad.LacZ) and firefly luciferase (Ad.Luc) under the control of the cytomegalovirus promoter (CMV) were kindly provided by, respectively, Dr. Willnow (Houston, USA) and Dr. Hoeben (LUMC, Leiden, The Netherlands). Recombinant adenovirus vector carrying the green fluorescent protein under control of CMV (Ad.GFP) was constructed using the Ad-Easy-1 system as previously described by ([26]). Additionally, the viruses were propagated in PERC6 cells as described ([27]). The purification process involved two rounds of CsCl ultracentrifugation and dialysis against dialysis buffer (25 mmol/L Tris, 137 mmol/L NaCl, 5 mmol/L KCl, 0.73 mmol/L NaH2PO4, 0.9 mmol/L CaCl2, and 0.5 mmol/L MgCl2, pH 7.45) followed by dialysis against the same buffer supplemented with sucrose (50 g/L). Plaque titration was performed on 911 cells according to standard techniques ([28]). Aliquots of 50 ll virus were stored at -80 °C. Generally, virus titers of the stocks varied from $1 * 10^{10}$ to $1 * 10^{11}$ pfu/ml.

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

Production and purification CAR-Avidin linker protein. For production Cos-1 cells were transfected with pSG8CAR-Avidin using Fugene6 (Roche, Basel, Switzerland). Forty hours after transfection (serum-free, biotin-free culture medium), the supernatant, containing the linker proteins, was harvested. Linker proteins were purified from the supernatant by immobilized metal affinity chromatography using Talon metal affinity resin (Clontech, Palo Alto, USA). Equilibrated culture supernatant (300 mM

NaCl, pH 7.0, and 20% glycerol) was incubated with Talon, 20 min at room temperature. After extensive rinsing (50 mM NaPO4, 300 mM NaCl, and 20% glycerol, pH 7.0), resin was pre-eluted (4 volumes; 50 mM NaPO4, 300 mM NaCl, 2,5 mM imidazole, and 20% glycerol) prior to its elution (10 volumes; 50 mM NaPO4, 300 mM NaCl, 150 mM imidazole, and 20% glycerol). Presence of linker protein in the purified samples was detected by SDS–PAGE and Western blotting analysis using Hybond ECL nitro cellulose membranes (Amersham Biosciences, Buckinghamshire, UK) and antibodies P5D4 (a-VSV) or a-Avidin (Abcam, Cambridge, UK). Elution fractions 3–5 contained the linker protein and were dialyzed against PBS.

Quantification of CAR-Avidin linker protein. The linker protein was quantified by a biotin binding assay. Ten microliters of the linker protein elution fraction or an avidin calibration range of 0.3–10 pM avidin was incubated with 0.2 µl [^{3}H]Biotin (Du Pont NEN Research Products, Boston, MA, USA) for 1 h. The total reaction mixture was applied on a Sephadex G-50 column to separate CAR-Avidin bound biotin from the free biotin. The elution fractions were counted for [3H]biotin radioactivity using 5 ml of Hionic fluor scintillation cocktail (Packard Instrument, Perkin Elmer, Boston, MA, USA) in a Packard 1500 TriCarb liquid scintillation analyzer. The summed radioactivity in peak fractions 3–5 correlated with the amount of avidin present in the sample (R2 = 0.997). Elution fraction 3, which had the highest concentrations, was used for experiments and stored at -80 °C. A yield of the order of 900–1000 µg was typical.

Biotin binding assay. CAR-Avidin (5 ll of 30 nM) was incubated for 1 h at RT with bio-cRGD (cdFK(e-C6-biotin)RGD), from Asynth Service BV (Roosendaal, Netherlands), at molar ratios ranging from 1:0.001 to 1:3, after which 2 µl of [^{3}H]biotin (NEN) was added and the mixture was incubated again for 1 h. To separate the CAR-Avidin-(^{3}H- or cRGD-) biotin bound fractions from free [^{3}H]biotin, the mixture was applied on a Sephadex G-50 column. [^{3}H]Biotin radioactivity in the elution fractions was measured after addition of 5 ml Hionic Fluor scintillation cocktail (Packard Instrument) in a Packard 1500 tricarb liquid scintillation analyzer. The summed radioactivity in peak fractions 4–6 corresponded to the [^{3}H]biotin binding capacity of the CAR-Avidin. This value is plotted for each sample containing different molar ratios of CAR-Avidin to biocRGD (1:0.001 to 1:3).

Infection assay. Twenty-four hours before infection, cells were seeded into 12 well plates (Greiner). The CHO, H5V, and HUVEC at 4 * 10^4, VSMC at 6 * 10^4, Ramos and K-562 at 1 * 10^5, and EOMA at 1.2 * 10^5 cells per well. At the day of infection, three wells were tryp to calculate the number of cells. After that, CAR-Avidin was incubated for 1 h at RT with

bio-cRGD in a total volume of 50 µl PBS. Then, the CAR-cRGD targeting construct was added and incubated for 1 h with different amounts of Ad.Luc, Ad.GFP or Ad.LacZ. Subsequently 300 ll of cRGD-Ad diluted in PBS/2% horse serum was added to the cells. After 1 h at 37 °C, the media were changed and infection efficiency was determined 40 h after infection. Ramos and K-562 (suspension) cells were washed by centrifugation for 5 min at 1000 rpm in between the incubation steps. Ad.luc infected cells were lysed in 300 µl reporter lysis buffer (Promega). Luc activity (Promega) and protein content (BCA assay, Pierce) were measured according to the protocol supplied by the manufacturer. The Ad.LacZ infected cells were washed with PBS and fixed for 5 min at 4 °C in 5.4% formaldehyde, 0.8% glutaraldehyde in PBS after which staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.2 mM MgCl2, and 0.1% 5-bromo-4-chloro-3-indolyl-b-D-galactoside (X-Gal) in PBS) was added. After 4 h and LacZ positive cells were visual and scored microscopically. Ad.GFP infected cells were trypsinized gently, homogenized in PBS supplemented with 2% fetal calf serum, and kept on ice until further analysis by flow cytometry (Becton–Dickinson). GFP fluorescence was detected at 530/30 nm FACscan (FL1 channel) following excitation with an argon ion laser source at 488 nm. The forward-scatter/side-scatter plot was gated to exclude cellular debris from the analysis. The number of events/FL1 (which reflects the fluorescence intensity) is plotted against the total number of cells, and the percentage of GFP-positive cells was determined. For each sample, 10,000 events were collected.

Statistical analysis. Results are presented as means ± SD values of three samples. The significance of differences between the experimental groups was calculated using a two-tailed Student□s t test. The level of statistical significance of the difference was set at $P < 0.05$.

Results

Generation and optimization of the adenovirus targeting construct To target Ad to αVβ3/5 integrins, the bi-functional linker protein CAR-Avidin was equipped with the targeting peptide bio-0?>cRGD to yield CAR-cRGD. TcRGD, resulting in a complete occupation of all available biotin-binding sites, was determined by a [^{3}H]biotin binding assay. Figure 1A shows the amount of [^{3}H]biotin that is still able to bind to CAR-Avidin at a molar ratio of CAR-Avidin to bio-RGD ranging from 1:0.001 to 1:3. At a molar ratio of more than 1:0.3, no residual [^{3}H]biotin-binding capacity could be detected, indicating that at this ratio all biotinbinding sites of CAR-Avidin were occupied. In all the following experiments, a slight

excess of CAR-Avidin to bio-cRGD was used (ratio 1:1) to generate the CAR-cRGD targeting construct. To determine the optimal ratio of Ad to CAR-cRGD targeting construct, a fixed amount of Ad.LacZ was incubated with various concentrations of CAR-cRGD and added to αVβ3/5-positive mouse heart endothelial cells (H5V) ([29]). Two days after infection, the cells were fixed and stained for LacZ activity. The number of LacZ positive cells was enhanced in a CAR-cRGD concentration dependent manner (Fig. 1B) ranging from 1 to 270 positive cells per microscope field. The increase in infection efficiency leveled off at a concentration of 1.2 µM CAR-cRGD. Therefore in the subsequent experiments, a concentration of 1.2 µM CAR-cRGD was used.

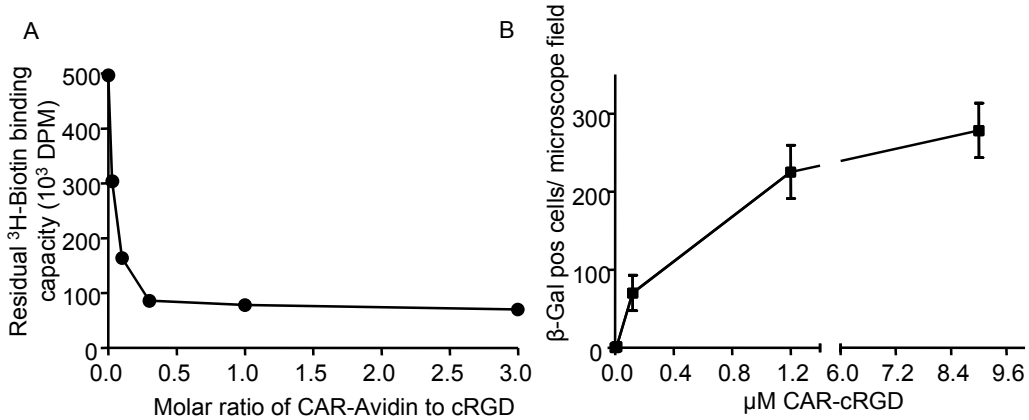

Figure 1. Optimization of Ad: CAR-Avidin/cRGD ratio. *(**A**) CAR-Avidin (30 nM) preincubated with different molar ratios of biotin-cRGD was incubated with an excess of [^{3}H]biotin. CAR-biotin (-^{3}H or -cRGD) radioactivity was counted. (**B**) Ad.LacZ was preincubated with either BSA (Ad) or with different amounts of the targeting construct, CAR-cRGD (cRGD-Ad), and subsequently added at a titer of MOI 1000 to mouse endothelial cells for 1 h. Forty hours post-infection, cells were fixed and stained for LacZ activity for 4 h. Multiple microscope fields were counted for positive cells. Values represent means ± SD of three samples.*

cRGD mediated adenoviral gene transfer in CAR deficient and αVβ3/5 positive cells The ability of the CAR-cRGD targeting construct to achieve CAR-independent gene transfer was determined in the CAR-negative, but αVβ3/5 positive, cell line CHO ([30]). Near confluent CHO cells were infected with untargeted Ad versus targeted Ad-vectors expressing luciferase (cRGD-Ad.Luc) (MOI 100–2500). Forty hours after infection, cell lysates

were evaluated for luciferase expression (Fig. 2A). As expected, Ad.Luc was incapable of infecting CHO cells even at an MOI of up to 500. In contrast, already at MOI 100, the cRGD-Ad.Luc vector resulted in a 3 to 4 log-fold enhancement of luciferase expression, demonstrating that the cRGD-equipped Ad.Luc achieves gene transfer through a CAR-independent cell entry pathway. The specificity of targeting Ad to αVβ3/5 integrins was investigated by comparing gene transfer of Ad versus cRGD-equipped Ad in human leukemia cell lines, Ramos and K-562. In both cell lines moderate levels of CAR are present, however only K-562 cells express αVβ3 and αVβ5 integrins ([31]). Ad.GFP plus or minus cRGD was applied to both cell lines at an MOI ranging from 100 to 2500. After 24 h, the cells were monitored for GFP expression by FACS analysis (Figs. 2B and C). In both the αVβ3/5 integrin negative as well as positive cell line, gene transfer mediated by unmodified Ad.GFP was low but dose-dependently increased. In Ramos cells, which do not express αVβ3/5 integrins, cRGD-mediated gene transfer did not increase infection efficiency at MOI 500, as compared to unmodified Ad. Moreover, at MOI 2500 cRGD-mediated gene transfer remained low, resulting in a significantly lower percentage of GFP positive cells as compared to unmodified Ad.GFP. In contrast, in the αVβ3/5 integrin positive K-562 cell line, cRGD-mediated gene transfer resulted in an approximately 10-fold increase in the number of GFP positive cells as compared to those infected with untargeted Ad.GFP at all MOIs used. Thus, cRGD-Ad markedly enhanced gene transfer only in the αVβ3/5 integrin positive K-562 cell line, suggesting that the αVβ3/5 integrins are involved in the uptake of cRGD equipped Ad vectors.

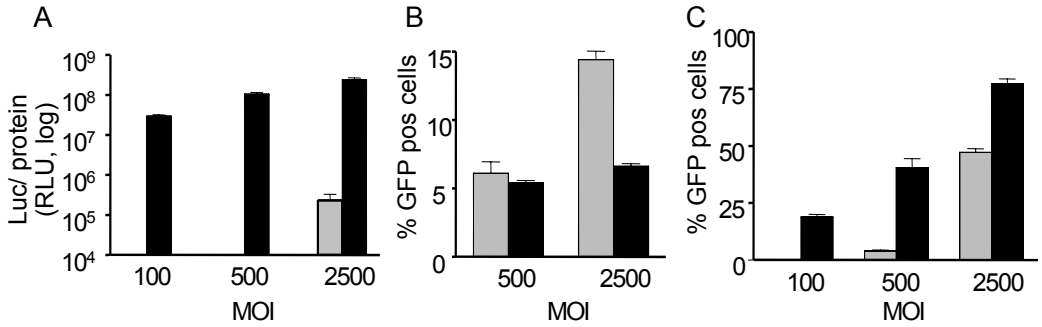

Figure 2. Analysis of aVb3-/aVb5-integrin and CAR-dependent gene transfer of cRGD equipped Ad. *For all subfigures ▒ Ad.Luc, ■ cRGD-Ad.Luc (**A**) CAR-negative CHO cells were exposed for 1 h to Ad.luc, preincubated with BSA (Ad) or 1200 nM CARcRGD (1:1 M) (cRGD-Ad) at different MOI. Forty hours after infection luciferase expression was measured and corrected for protein levels. Values represent means ± SD of three samples. (**B**) Ramos and (**C**) K-562 cells were infected with an increasing titer of unmodified Ad (Ad.GFP) or cRGD equipped Ad-vector (cRGD-Ad.GFP). CAR-Avidin was prebound to cyclic RGD at a 1:1 molar ratio and 1200 nM of the complex was incubated with Ad. Twenty-four hours after infection FACs analysis was performed. Values represent means ± SD of three samples.*

Quantification of targeting efficiency in transformed vascular cell lines The optimized targeting conditions were used to determine the efficiency of Ad-mediated gene delivery to murine vascular cell lines. Mouse heart endothelial (H5V) cells ([32]) were incubated with increasing concentrations of Ad.Luc or cRGD-Ad.Luc. Luciferase expression levels showed a titer-dependent increase. As compared to gene delivery with untargeted Ad.Luc, cRGD-Ad.Luc showed a 59-fold increased luciferase expression at MOI 250 and a 650-fold increased luciferase expression at MOI 2500 (Fig. 3A). To verify these results, the experiment was reproduced in a second mouse cell line, the hemangioma-derived micro vascular endothelial cell line (EOMA) ([33;34]). The EOMA cells were infected with either Ad.GFP or cRGD-equipped Ad.GFP virus. FACS analysis showed that EOMA cells were refractory to infection with untargeted Ad.GFP, as judged by 8% GFP positive cells at the relatively high MOI of 1000. However, infection efficiency increased 4-to 6-fold after equipping Ad.GFP with cRGD (Fig. 3B).

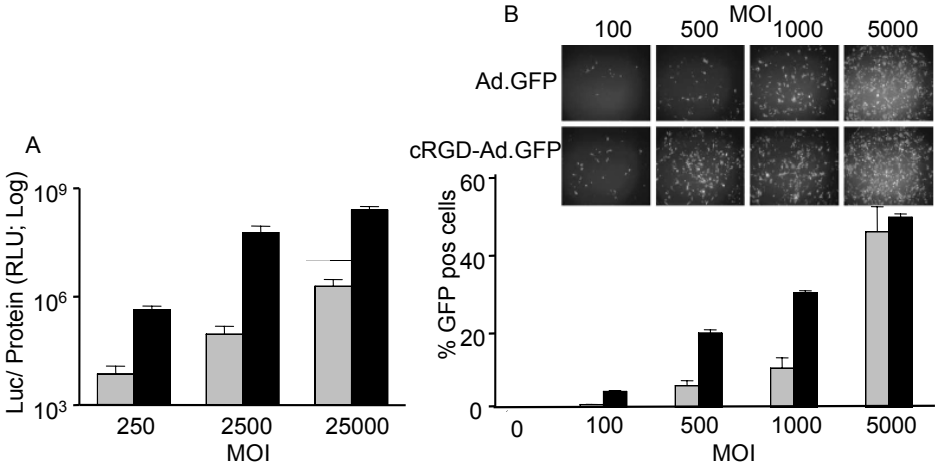

Figure 3. Ad mediated gene transfer of cRGD equipped vectors to mouse EC. *For all subfigures ▒ Ad.Luc, ■ cRGD-Ad.Luc (**A**) Ad.Luc was pre-incubated with either BSA (Ad.Luc) or 1200 nM of CARcRGD (1:1) (cRGD-Ad.Luc) and the complex was exposed to H5V cells at different MOI. Luciferase expression was measured 40 h post-infection. Results were normalized for protein concentration. (**B**) EOMA cells were exposed to different titers of Ad.GFP or cRGD equipped Ad.GFP. cRGD was bound to CAR-Avidin at a 1:1 molar ratio and 1200 nM of the CAR-cRGD conjugate was incubated with Ad.GFP. Values represent means ± SD of three samples.*

Quantification of targeting efficiency in primary mouse VSMs and human EC Next, cRGD-mediated Ad targeting to primary vascular cells was examined. Primary mouse VSMC isolated from aorta and HUVECs were infected with either Ad.GFP or Ad.GFP equipped with the CAR-cRGD construct. Two days after infection, FACS analysis was performed to determine GFP expression levels. Primary VSMC were highly resistant to infection, as only 0.02% of the cells were infected with untargeted Ad.GFP (MOI 500) and 13% at high MOI (2500). In contrast, cRGD-Ad.GFP mediated gene transfer resulted in a titer-dependent increase in GFP positive cells up to 46.5% at MOI 2500 (Fig. 4A). At MOI 500, this amounted to a 25-fold increased infection efficiency of primary VSMC using cRGD equipped Advectors. Improvement of gene transfer was also tested in HUVECs. Gene transfer using untargeted Ad.GFP resulted in a very low percentage of GFP positive cells (2%), while cRGD targeting of Ad led to a 36-fold increase (MOI 100) of infected HUVECs (Fig. 4B).

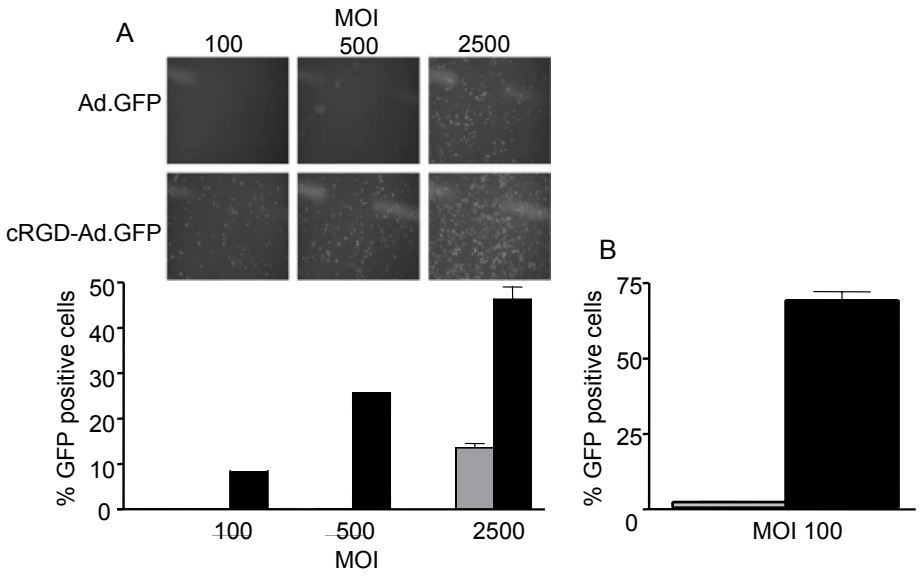

Figure 4. Ad mediated gene transfer of cRGD equipped vectors to primary cells. *(**A**) For all subfigures ▒ Ad.Luc, ■ cRGD-Ad.Luc. Mouse vascular smooth muscle cells and (**B**) HUVECs were exposed for 1 h to different MOI of Ad.GFP or to cRGD-Ad.GFP. CAR-Avidin was bound to cyclic RGD at a 1:1 molar ratio and 1200 nM of this complex was incubated with Ad.GFP. Forty hours after infection FACs analysis was performed. Values represent means ± SD of three samples.*

Discussion

In the present study, we show that coupling of recombinant Ad vectors to a CAR-cRGD linker protein results in a significantly improved infection efficiency of both transformed and primary VSMC and EC. Conjugation of CAR-cRGD to Ad reporter vectors markedly enhanced gene transfer to the established endothelial cell lines H5V and EOMA (up to 59-fold), as well as to primary HUVEC (36-fold) and VSMC (25-fold). This was associated with a considerable increase in the percentage of infected cells. Thus, the CAR-cRGD targeting construct expands the utility of Ad vectors to CAR-negative cell types that do express αVβ3- and αVβ5 integrins. The biotin–avidin based coupling of a ligand to the CAR adaptor molecule is straightforward and highly efficient due to the femtomolar affinity of biotin for avidin ([35;36]). As compared to chemical modifications, this obviates the use of complex reaction mixtures and purification steps and enables simple quantification of CAR adaptor concentrations and

optimal CAR-Avidin/biotin-cRGD ratios by a [^{3}H]biotin binding assay. Moreover, the CARAvidin adaptor may be coupled to a wide variety of biotinylatable ligands and has recently been successfully applied to target Ad vectors to macrophages using a biotinylated oligonucleotide (Gras, personal communication). Application of cRGD-Ad vectors expressing GFP increased the number of infected vascular cells rather than that it boosted gene expression in a limited cell population, as compared with untargeted Ad (Figs. 3B, 4A and B). Thus, CAR-cRGD mediated targeting of recombinant Ad vectors allows the use of considerably reduced MOIs to obtain near-quantitative gene transfer, thereby decreasing the vector related toxicity. This is particularly important for those cells that are sensitive to Ad-mediated toxicity. For example, only 2% of HUVECs were infected with unmodified Ad.GFP at MOI 100, whereas the same titer of cRGD equipped Ad.GFP resulted in 75% infected cells (Fig. 4B). To obtain a similar level of infection with unmodified Ad.GFP, MOI's > 1000 would be required, which coincides with cytotoxicity (data not shown). In addition to a lower virus dose, the near quantitative infection of HUVECs enables the application of Ad vectors encoding inserts that require quantitative infection, such as short hairpin (sh)RNA constructs to knock down gene function. αVβ3- and αVβ5 integrins are known to be up-regulated on proliferating EC and subsequently have been exploited as targets to develop anticancer drugs. For this purpose, linear- and cyclic RGD peptides have been developed and used as a targeting moiety to selectively deliver drugs to angiogenic blood vessels ([37;38]). Pfaff et al. ([22]) have shown that a cyclic RGD peptide displayed a higher affinity for αVβ3- and αVβ5 integrins than the linear RGD peptide. We have confirmed this observation, showing a 4-fold increased LacZ activity utilizing cRGD-Ad vectors compared to linear RGD equipped Ad vectors (data not shown). The mechanism of cRGD-Ad mediated gene transfer was further characterized by infection of cell lines that differ in their expression levels of aVb3/5 integrins and CAR. CHO and K-562 cells, which express αVβ3/5 integrins but not CAR, were efficiently transduced by cRGD-Ad vectors. On the other hand, Ramos cells, which do not express aVb3/5 integrins, were almost completely resistant to infection by cRGD targeted Ad vectors. These results demonstrated that the entry route of our CAR-cRGD targeted Ad vectors is CAR independent and most likely mediated via aVb3/5 integrins. Conversely, in all these three cell lines very low infection efficiencies were obtained for untargeted Ad vectors. At the high MOI of 2.500, only the integrin expressing cell lines, CHO and K-562, were infectable. Apparently, at this very high MOI, the local concentration of Ad particles was high enough to bind via their RGD motifs present in the viral penton base to the αVβ3/5 integrins and trigger internalization. In

several cell types and tissues which represent important targets for gene therapy, like the vascular system, the expression level of the endogenous adenovirus receptor CAR is low ([39;12;40]). On the other hand, αVβ3/5 integrins are abundantly expressed on activated and proliferating EC and VSMC, which are present during angiogenesis, neovascularization, and inflammation ([41–43]). In vitro the majority of proliferating cells express αVβ3/5 integrins. Therefore, the bifunctional linker protein carrying specificity for Ad vectors on the one hand and for αVβ3/5 integrins on the other hand greatly expands the applicability of conventional Ad vectors. In addition to providing Ad vectors with a novel tropism, the CAR.cRGD construct likely prevents binding of the Ad vectors to CAR (data not shown) and thus ablates the intrinsic specificity. This may be useful in vivo, in applications where CAR mediated uptake is undesired.

In summary, we have demonstrated the feasibility of the CAR.cRGD construct to target Ad-vectors with high efficiency to transformed endothelial cell lines as well as to primary endothelial and smooth muscle cells. It is conceivable that additional Ad resistant and aVb3/5 integrin expressing cell lines and tissues may become amenable to Ad infection via this strategy.

Acknowledgments

This work was performed in the framework of the Leiden Center for Cardiovascular Research LUMC-TNO and supported by grants from the Dutch Organization for Scientific Research (NWO 902-26-220), Netherlands Heart Foundation (NHS 2001-141, NHS 2003T201 and NHS 1999B194) and the Center of Medical Systems Biology (CMSB) established by the Netherlands Genomics Initiative/ Netherlands Organization for Scientific Research (NGI/NWO).

Abbreviations

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

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Chapter 5

Targeting adenovirus vectors reduces liver tropism but does not enhance specific organ uptake.

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Abstract

Systemic administration of adenovirus (Ad) vectors results in gene delivery to the liver. To modify Ad vector tropism, we have generated a linker protein consisting of the virus binding domain of the coxsacky adenovirus receptor (CAR) genetically fused to avidin. In association with a biotinylated ligand, this CAR-Avidin linker protein can successfully retarget Ad vectors *in vitro*. Here, we set out to apply this targeting strategy *in vivo*. Two biotinylated peptide ligands were used to, respectively, target integrin $\alpha_V\beta_{3/5}$ expressing cells and lung endothelium. Systemic administration of both types of targeted Ad vectors resulted in an up to 85-fold reduced hepatic transgene expression. However, neither of the targeted Ad vectors resulted in increased transgene expression in the intended target tissue. Moreover, a substantial portion of the targeted Ad could not be recovered from any of the organs, indicative of efficient Ad neutralization. Indeed we observed that the maximum half-life of Ad in the circulation after systemic lactoferin treatment, which completely blocks hepatic Ad uptake was 8' as compared to 6' for untargeted Ad suggesting the presence of efficient extrahepatic elimination pathways. Apparently, rapid neutralization of targeted Ad in the circulation efficiently prevents uptake by target organs other than the liver.

Introduction

Adenovirus (Ad) mediated gene transfer is widely used as a powerful method to modulate gene expression *in vitro* and *in vivo* (reviewed by ([1])) The vast majority of Ad used to date involves serotype 5 (Ad5). Application of Ad5 vectors is dependent on the expression of the cognate receptor, the Coxsackie Adenovirus Receptor (CAR) by the target cell ([2;3]). Infection of CAR deficient cells, such as many tumours, endothelial and hematopoietic cells, with Ad vectors is very ineffective and can be achieved only at high multiplicities of infection. Thus efficient infection of these cells requires modulation of Ad tropism.

In vitro, several targeting approaches have been proven successful. In one of the strategies the capsid protein is genetic modified by inserting peptide ligands ([4-22]). Another approach for targeting Ad vectors is based on conjugates. Here, the vector is equipped with a bifunctional adapter molecule able to bind the virus on the one hand and a marker protein on the target cell on the other hand ([23]); (reviewed by ([24;25-28;29])). The adapter can either associate with the native virus or with chemically or genetically modified capsid proteins. This targeting approach is more versatile than the genetic modification based strategy, as it results in a flexible targeting system able to confer/ accommodate infection of a variety of cell types via the addition of different ligands.

Recently, we described the generation and *in vitro* characterization of linker protein CAR-Avidin for the targeting of Ad5 to alternative cell types. This linker protein consists of the virus-binding moiety of the endogenous receptor CAR, genetically fused to the biotin-binding moiety of avidin. Equipping CAR-Avidin with the oligodeoxy nucleotide ligand dA_6dG_{10} or the cyclic peptide ligand GRGDSP (cRGD) resulted in efficient targeting *in vitro* of both transformed and primary macrophages ([30]) and to both transformed and primary vascular smooth muscle and endothelial cells ([31]), respectively. The aim of the current study was to determine whether *in vivo* targeting of Ad vectors to extrahepatic tissue such as carotid artery or alveolar cells, can be effected via the CAR-Avidin linker protein.

Methods

Cells Cos-1, H5V and EOMA cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS (Invitrogen), 100 units/ ml Penicillin (Invitrogen), 100 µg/ml Streptomycin (Invitrogen) and glutamax (Invitrogen). Mouse VSMC were isolated from aorta from male C57Bl6 mice as previously described [46] and cultured in DMEM with 10% newborn calf serum (NCS). One day prior to transfection Cos-1 cells were detached from plastic with 1% Trypsine/ 10 mM EDTA in PBS and seeded to 50% confluency. Cells were cultured in a humidified atmosphere of 5% CO_2

Production of recombinant Ad vectors. Recombinant E1, E3-deleted Ad-vectors expressing beta-galactosidase gene (Ad.LacZ) and firefly luciferase (Ad.Luc) under the control of the cytomegalovirus promoter (CMV) were kindly provided by respectively Dr. Willnow (Houston, USA) and Dr. Hoeben (LUMC, Leiden, The Netherlands). Additionally, the Ad vectors were propagated in PERC6 cells as described [47]. The purification process involved two rounds of CsCl ultra centrifugation and dialysis against dialysis buffer (25 mmol/l Tris, 137 mmol/l NaCl, 5 mmol/l KCl, 0.73 mmol/l NaH_2PO_4, 0.9 mmol/l $CaCl_2$, and 0.5 mmol/l $MgCl_2$, pH 7.45) followed by dialysis against the same buffer supplemented with sucrose (50 g/l). Plaque titration was performed on 911 cells according to standard techniques ([48]). Aliquots of 50 µl virus were stored at -80°C. Generally, virus titers of the stocks varied from 1×10^{10} to 1×10^{11} plaque forming units per ml (pfu/ml).

Production, purification and characterization of the linker protein The CAR-Avidin linker protein was produced, purified and characterized as previously described [30]. In short: CAR-Avidin was produced by transient transfection of Cos-1 cells with pSG8CAR-Avidin using Fugene6 (Roche,

Basel, Switzerland) under serum free conditions. Thirty two hours after transfection the linker protein was harvested and purified from the supernatant by immobilized metal affinity chromatography using Talon metal affinity resin (Clontech, Palo Alto, USA). Culture supernatant was equilibrated (addition of 5M NaCl to an end concentration of 300 mM NaCl, pH was adjusted to 7.00 using 50% HCl in PBS, 100% glycerol was added to an end concentration of and 20% glycerol) and incubated for 20 minutes at room temperature with Talon. After extensive rinsing with buffer (50 mM $NaPO_4$, 300 mM NaCl, 20% glycerol, pH = 7.0), washing with 4 volumes pre-elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 2,5 mM imidazole, 20% glycerol) the resin was eluted with 10 volumes elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 150 mM imidazole, 20% glycerol). SDS-PAGE and western blotting analysis of all purification steps and elution fractions showed that the linker protein was only present in elution fractions 3 to 5 that were subsequently extensively dialyzed against PBS and used in all experiments. The concentration of the active component was determined in the biotin binding assay as previously described using avidin (Sigma Aldrich, St Louis, MO, USA) as standard.

Ligands The lung specific GFE1 peptide (CGFECVRQCPERC;[32]) was synthesized as N-terminally biotinylated peptide by standard Fmoc based solid-phase chemistry and provided by J.W. Drijfhout (LUMC, Leiden, The Netherlands). The quality was checked by high resolution LC-MS mass spectroscopy. The bio-cRGD (cdFK(e-C6-biotin)RGD) was obtained from Asynth Service BV (Roosendaal, Netherlands).

Targeting conditions CAR-Avidin and peptide ligands were incubated at a 1: 1 molar ratio for 1 hour at room temperature, as was determined in previous experiments [30,31]). Complex formation of adenovirus and CAR-Avidin-ligand was facilitated by incubating CAR-Avidin-ligand at a concentration of 50 nM with the appropriate amount of virus for 1 hour at room temperature.

Bioluminescent reporter imaging 12 wks old female C57Bl/6JIco mice (Charles river, The Netherlands), fed standard chow diet (Hope Farms, Woerden, NL) *ad libitum,* were injected with Ad.Luc ($2x10^9$ pfu). Bioluminescent signals were determined 4 days after Ad injections using the Xenogen IVIS imaging system (IVIS 100). Approximately 5 minutes before imaging the living mice were injected luciferin, (150 mg/kg) intraperitoneally (ip). The mice were anaesthetized with isofluorane/oxygen and placed on the imaging stage. Total photon emission of each animal was acquired for 1 minute. Captured images were quantified using the Living Image software (Xenogen Corp, Almeda, CA) and the IGOR software (WaveMetrics Corp, Lake Oswego, OR). Bioluminiscence from the region of interest was expressed via a pseudo color scale (Red most intense and Blue least intense luminescence) and

data were presented as the cumulative photon counts collected within each region of interest. Because layers of tissue may limit photon emission from inner organs, 4 days after Ad injection the livers of mice were dissected to verify the results from the bioluminescent reporter imaging experiment by determination of the luciferase activity in liver lysates.

Luciferase enzymatic assay The liver extracts were prepared by homogenisation with the minibead beater in reporter lysis buffer (Promega), two cycles of freeze-thawing and 2 min. of centrifugation at maximum speed. Supernatants were used for determining protein-normalized luciferase activity by adding 100 µl luciferyl-CoA (Promega) to 20 µl of liver extract in a monolight luminometer (BD Biosciences). Protein content was measured in a 96-well microtiter plate using the BCA protein assay kit (Pierce). Absorbance at 562 nm was determined in a microplate reader.

Local gene transfer 9-10 weeks old ApoE-/- mice, fed regular chow diet *ad libitum*, were used for the local gene transfer studies. Local gene transfer was ensured using a procedure developed by Von der Thüsen ([49]) In short: with use of a midline neck incision, the left external carotid artery was looped proximally and tied off distally with 6-0 silk suture (Ethicon). Additional 6-0 silk ties were looped round the common and internal carotid arteries for temporary vascular control during the procedure. A transverse arteriotomy was made in the left external carotid artery, and a 0.36-mm flexible angioplasty guidewire was advanced by 1 cm via a transverse arteriotomy of the external carotid artery, and endothelial denudation of the common carotid artery was achieved by 3 rotational passes. In one experiment, the animals were inoculated i.v. with 1,5 $\times 10^9$ pfu of Ad.LacZ or cRGD-Ad.lacZ in 200 µl of phosphate buffered saline one and five hours after denudation. In a second experiment, immediately after angioplasty, 10 µl of adenoviral suspension (1.5×10^9 pfu/ml) was instilled into the right common carotid artery via the external carotid. The suspension was left *in situ* for 10 min and was subsequently drawn off before ligation of the external carotid and closure of the skin wound with silk sutures.

Tissue harvesting and histological analysis Five days after Ad incubations, carotid artery specimens were obtained and transverse 5 µm cryosections prepared after in situ perfusion fixation with formalin as described ([50]). Cryosections were routinely stained with hematoxylin (Sigma Diagnostics) and eosin (Merck Diagnostica, Darmstadt, Germany). ß-Galactosidase was demonstrated by incubation with staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.2 mM $MgCl_2$, 0.1% 5-bromo-4-chloro-3-indolyl- β-D-galactoside (X-Gal) in PBS) at 37°C

O/N. Sections were stained immuno histochemically with antibodies against α-SM-actin (clone 1A4; diluted 1:500; Sigma) and CD31(rat anti-mouse, BD pharmingen; diluted 1:200). To detect specific Ab binding goat anti-mouse IgG peroxidase conjugate (dilution 1:100; Nordic, Tilburg, the Netherlands) was used as secondary antibodies, with 3,3'-diamino-benzidine, nitro blue tetrazolium as enzyme substrates (all Sigma) and for CD31 the ABC-AP kit and Vector-Red Substrate was used (Vector laboratories).

Biodistribution of GFE1 Recombinant avidin (SIGMA, St. Louis, USA) was radioiodinated at pH 10.0 with carrier free ^{125}I according to a modification [51] of the ICI method [52]. Free ^{125}I was removed by Sephadex G-25 gel filtration. GFE1 was incubated with ^{125}I- Avidin at a molar ratio of 1: 1. For the *in vivo* bio-distribution experiments, 10-12-wk-old female C57Bl/6 mice of weight 22-24 g from Broekman Instituut BV (Someren, The Netherlands) were used and fed *ad libitum* with regular chow diet. Mice were anaesthetized by subcutaneous injection of ketamine (75 mg/kg, Eurovet), droperidol (1 mg/kg), fluanisone (0.75 mg/kg), and fentanyl (0.04 mg/kg) (all from Janssen-Cilag, Beerse Belgium). Mice were injected with indicated ligand via the tail vein. One hour after injection the experiment was terminated, organs were removed and the organ bound radioactivity determined.

Bio-distribution of targeted Ad For the *in vivo* virus bio-distribution experiments, 10-12-wk-old female C57Bl/6 mice of weight 22-24 g from Broekman Instituut BV (Someren, The Netherlands) were used and fed *ad libitum* with regular chow diet. On day 0, mice were injected with $1*10^9$ pfu of the appropriately targeted Ad.Luc or 100µl PBS in case of the uninfected control. Five days after injection, the experiment was terminated, the organs were removed and snap frozen in liquid nitrogen. Proteins were isolated after homogenisation of organ samples and subsequent solubilization in 1* reporter lysis buffer (Promega, Madison, WI, USA). Luciferase activity was determined according to the protocol supplied by the manufacturer.

Decay study using lactoferrin 16-17 weeks old C57Bl/6JIco mice (Charles river, The Netherlands) were injected intravenously either with dissolvent or bovine lactoferrine (Serva, Brunschwig Chemie), 70mg/kg at t = -2 min. At t = 0 min, both groups of mice received $1{,}5.10^9$ pfu/mice Ad.Luc intravenously. Blood samples were taken by tail bleeding at 5 min, 60 min, 6 hours and 24 hours. At day 5 liver, heart, spleen and lung were isolated. To assess the presence of circulating infectious particles, AT3 cells were incubated for 1 hour with the blood samples. After 24 hours, protein extracts were prepared by addition of reporter lyses buffer (Promega) to the AT3 cells and two cycles of freeze-thawing followed by 2 min. of centrifugation at maximum speed. Supernatants were used for

determining protein-normalized luciferase activity by adding 100 µl luciferyl-CoA (Promega) to 20 µl of AT3 extract in a monolight luminometer (BD Biosciences). Protein content was measured in a 96-well microtiter plate using the BCA protein assay kit (Pierce). Absorbance at 562 nm was determined in a microplate reader. $T_{½}$ were calculated from the luciferase activity at different timepoints using Graphpad Prism, software and a one-phase exponential decay model.

Statistics Experiments were performed in triplicate and presented as mean ± standard deviation (s.d.). P-values were calculated by a two-tailed unpaired student's T-test. Data were considered to be significantly different when $P< 0.05$, indicated with an asterix (*) in the figures.

Results

Systemic administration of cRGD equiped Ad into mice. To examine the capability of integrin targeted Ad vectors to mediate extrahepatic gene transfer and reduce the liver uptake normally seen after systemic application of Ad, either untargeted Ad expressing luciferase (Ad.Luc) or Ad.Luc equipped with CAR-Avidin-cRGD (cRGD-Ad.Luc) was injected intravenously into mice. Two and four days after injection, *in vivo* gene transfer was monitored in situ via a high resolution CCD camera. Luciferase expression accumulated in time and was solely observed in the liver and not in other organs of both the Ad.Luc as well as cRGD-Ad.Luc treated mice. Compared to Ad.Luc treated mice, luciferase expression was decreased 3-fold in mice that had received cRGD-Ad.Luc (Fig. 1A).

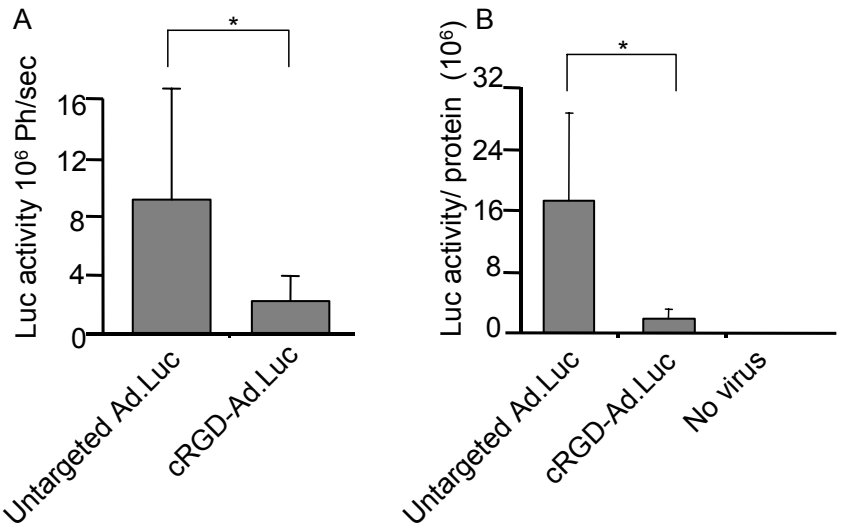

Figure 1. Effect of cRGD mediated targeting of adenovirus on luciferase expression in the liver. (A) *Female C57Bl/6 mice received 2x10^9 pfu Ad.Luc (i.p.). After 96 hours luciferin (150 mg/kg) was administered by i.p. injection and bioluminescent signals were recorded under full anesthesia for 1 minute. Data are presented as cumulated photon counts.* ***(B)*** *Liver lysates were prepared by homogenisation and subsequent freeze-thawing. Supernatants were used for determining luciferase activity using. Luciferase activity was corrected for protein concentration using BSA as standard. P<0.05 is indicated by an asterix.*

Because layers of tissue may limit photon emission from inner organs, luciferase activity was also measured in liver lysates. These data confirmed that the liver had indeed been infected and that transgene expression by liver was considerably reduced after cRGD targeting (11.5 fold; P<0.01) (Fig 1B).

To determine whether the decreased luc activity after targeting was caused by a reduction in the percentage of infected cells and not only by a reduction of virus particles entering a cell, untargeted Ad expressing β-galactosidase (Ad.lacZ) or targeted Ad.LacZ (cRGD-Ad.LacZ) was injected systemically. Four days after intravenous administration, β-galactosidase staining of the livers of mice that had received cRGD-Ad.LacZ revealed only 1% LacZ$^+$ cells. In comparison, approximately 80% of hepatic cells stained positive for β-galactosidase Fig. 2). In addition, with cRGD targeted Ad the cellular staining intensity seemed to be quenched as compared to untargeted Ad (Fig. 2).

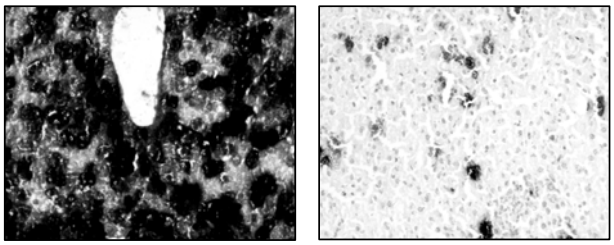

Figure 2. Effect of cRGD mediated targeting of adenovirus on ß-galactosidase expression in the liver. *Female C57Bl/6 mice received cRGD-Ad.LacZ or untargeted Ad.LacZ (1x10^9 pfu; i.v. injection). Five days after infection mice were sacrificed, livers were excised and cryosections were made and stained for ß-galactosidase or with hematoxylin/ eosin.*

Administration of cRGD equipped Ad to mice with carotid artery injury. To determine whether the endothelium constituted a barrier to infection, a carotid artery segment was injured by guide wiring prior to systemic virus administration. The guide wire injury will result in activation of flanking endothelial cells and medial vascular smooth muscle cells (VSMC) and subsequently in an increase in $\alpha_V\beta_{3/5}$ integrin expression. Systemic administration of cRGD-Ad.LacZ 1 or 5 hours after denudation of the internal carotid artery did not result in an increased amount of LacZ$^+$ cells in the vessel wall (data not shown). Because the anatomical position of the carotid artery could be incompatible with the dynamics of Ad infection, we also administrated Ad focally by instillation in an uninjured or a denuded carotid artery segment. Similar to above, cRGD equipped Ad vectors did not enhance transgene expression in intact endothelium nor in denudated vessels (Fig 3).

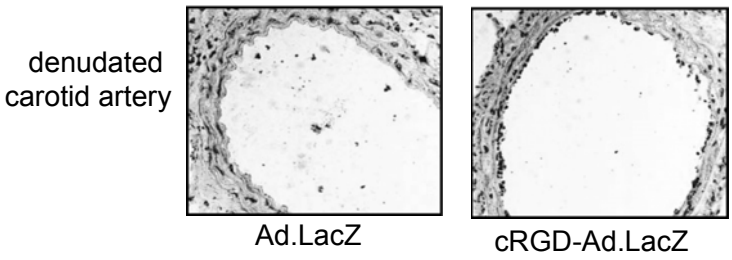

Figure 3. Effect of cRGD mediated targeting of adenovirus on LacZ expression in the vessel wall. *The right common carotid artery of ApoE-/- mice was denuded by 3 rotational passes of a 0.36 mm guide wire. Subsequently, Ad (1.5 × 10^9 pfu) was instilled into the denuded common carotid artery segment via the external carotid artery after prior ligation of the common carotid artery proximal and distal to the bifurcation point. The Ad was left in situ for 15' and removed. Five days after infection mice were sacrificed, tissues were isolated and cryosections of the common carotid arteries were stained for ß-galactosidase or with hematoxylin/ eosin*

Lung specific targeting of Ad vectors. To determine whether Ad retargeting would be successful with an alternative ligand for a more accessible organ, we have explored the potential of a lung specific peptide, GFE1 [32], in CAR-Avidin aided gene transfer in vivo. First, biodistribution to the lung of this peptide was confirmed by systemic administration of biotinylated GFE1 coupled to ^{125}I labelled avidin. Figure 4 shows the specific organ uptake of the avidin$^{125\text{-}I}$ bound peptide 1 hour after systemic injection. Biotin was included as a negative control.

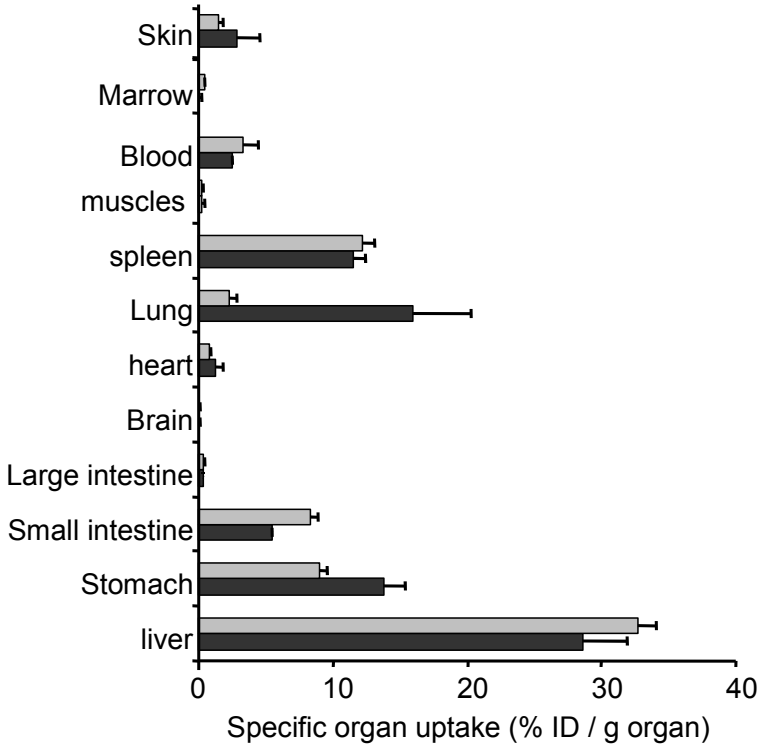

Figure 4. Biodistribution of ^{125}I-Avidin-GFE1 after intravenous injection into mice. *^{125}I-avidin-biotin (light grey), or ^{125}I-avidin-GFE1 (dark grey) (molar ratio of 1: 1; 156749 dpm, in 100 µl PBS) was injected intravenously into female C57Bl/6 mice. Tissue distribution was determined 1h after injection. Tissue accumulation is expressed as % of the injected dose per gram wet tissue and was corrected for radioactivity associated with tissue-entrapped plasma.*

As expected and already reported by Trepel et al., avidin bound GFE1 showed a much higher lung uptake than the biotin control (7-fold increase). Second, Ad.Luc was equipped with GFE1 (GFE1-Ad.Luc) for in vivo application. Biotin saturated CAR-Avidin (biotin-Ad.Luc) and untargeted Ad.Luc served as control. Five days after systemic injection, luciferase activity was determined in different organs. In comparison to Ad.Luc administration, the mice treated with biotin-Ad.Luc and GFE1-Ad.Luc displayed an 85- and 19- fold reduction in hepatic luciferase activity, respectively (Fig. 5A). Figure 5B shows the overall organ distribution of the luciferase expression after administration of GFE1-

Ad.Luc, biotin-Ad.Luc or Ad.Luc. In most tissues, including lung, luciferase expression was found to be lower in the GFE1-Ad.Luc than when in Ad.Luc treated mice. Thus, similar to the results obtained with the cRGD ligand, the GFE1 peptide did not enhance specific organ uptake.

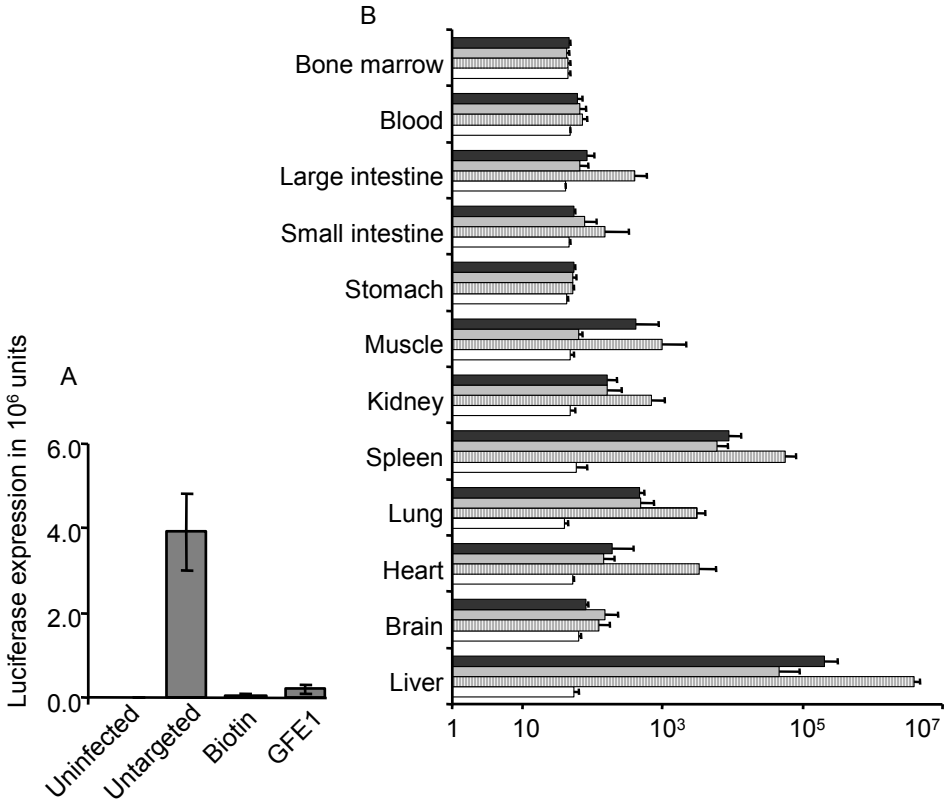

Figure 5. Effect of GFE1 mediated targeting of adenovirus on biodistribution luciferase expression. (A) *untargeted Ad.Luc (dashed bars), biotin-Ad.Luc (light grey) or GFE1-Ad.Luc (dark grey) were systemically administered to female C57Bl/6. Liver lysates were prepared 120 hours after infection by homogenisation and subsequent freeze-thawing. Supernatants were used for determining luciferase activity. Samples of untreated mice were included as controls (open bars).* ***(B)*** *The organ distribution profile of luciferase expression 120h after i.v. administration of GFE1-Ad.Luc, biotin-Ad.Luc, untargeted Ad.Luc or buffer (uninfected) to female C57Bl/6 mice is plotted. Mind the logarithmic X-axis.*

Determining Ad stability *in vivo*. As the previous experiments made clear, a substantial portion of Ad could not be recovered from any of the organs, therefore *in vivo* kinetics studies were performed. Untargeted Ad.Luc had a half-life of approximately 6 minutes *in vivo* (fig. 6A) and was efficiently cleared by the liver resulting in efficient infection. When animals were pre-treated systemically with lactoferrin, hepatic uptake of Ad virus particles was nearly completely blocked (fig. 6B). Under these conditions however, half-life of untargeted virus was only increased to 8 minutes (fig. 6A). This relatively minor increase in half-life indicates that neutralization of virus in the systemic circulation is extremely fast.

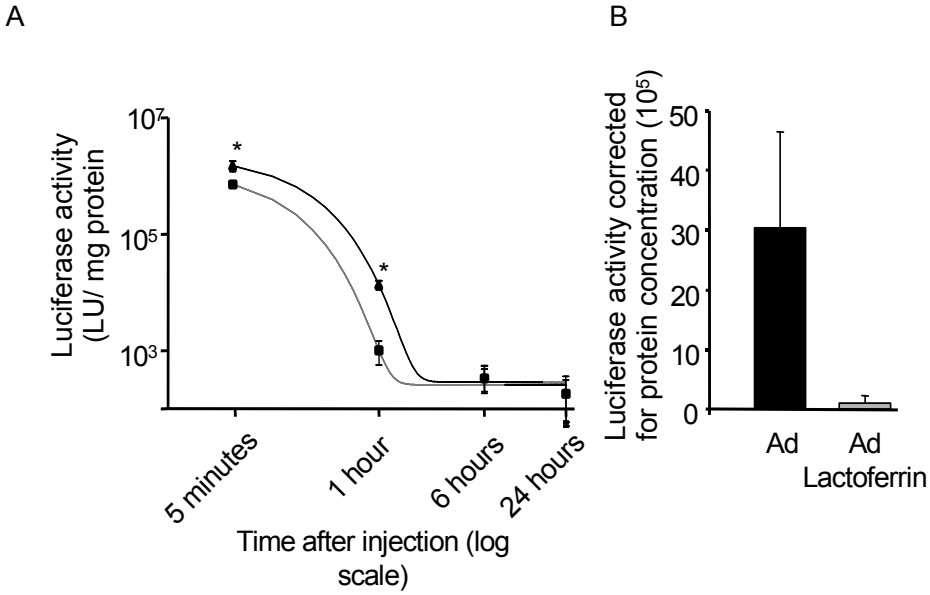

Figure 6. Effect of systemic lactoferrin treatment on Ad half-life after i.v. administration. (A) *Mice received bovine lactoferin (i.v., 70mg/kg) (black line, triangles) or PBS (grey line, squares) 2 minutes before Ad.Luc (1,5.10^9 pfu) administration. Presence of circulating infectious particles was determined by blood sampling and subsequent incubation of the samples on AT3 cells. Luciferase activity in AT3 cells is corrected for protein concentration and plotted against the time of blood sampling.* ***(B)*** *Luciferase activity in livers of mice determined 5 days after lactoferrin and Ad.Luc administration. Luciferase activity was determined as previously mentioned.*

Discussion

In this paper we report our efforts to target adenovirus vectors to alternative cell types *in vivo*. Ad linked to either the integrin binding peptide cRGD ([33;34]) or the lung specific peptide GFE1 ([32]) via CAR-Avidin was able to reduce liver uptake after systemic administration. While cRGD-Ad has already been shown to be effective in delivering genes to vascular cells *in vitro* ([14;35]) and GFE1 was demonstrated to be lung specific *in vivo*, neither cRGD-Ad nor GFE1-Ad was able to increase transgene expression by respective target tissue *in vivo*. In addition we demonstrate that the half-life of adenovirus in blood is rather short even after ablation of liver uptake by lactoferrin suggesting that other elimination pathways are functional in the clearance of adenovirus. This could contribute to the apparent failure of efficient target organ uptake *in vivo*.

Because of the clinical relevance, efficient *in vivo* targeting – in other words enhanced target organ uptake and quenching of the intrinsic tropism - of Ad vectors is highly desired. Though attempted extensively, successful targeting of Ad *in vivo* is limited to local ([36-38]) or intra-organ injections ([39]) of the virus. To our knowledge, increased target organ uptake of Ad after systemic injection has only been shown by Izumi and colleagues ([40]), who administered engineered Ad.luc containing CD40 on their fiber proteins in transgenic mice with lung vasculature specific CD40 expression. The detour Izumi took to accomplish retargeting demonstrates the difficulty in retargeting Ad in vivo.

In the current paper we succeeded in efficiently reducing liver uptake by applying the CAR-Avidin linker protein equipped with ligands for alternative receptors to Ad. Apparently, the CAR-Avidin linker protein is capabable of blocking the interaction of Ad fiber knob with its natural receptor, CAR. As both untargeted Ad and cRGD-Ad were from the same batch, we can exclude that batch-related factors are underlying the observed phenomena. Parallel in vitro studies confirmed that the cRGD Ad complexes were still functional, as they significantly enhanced gene transfer to vascular cells *in vitro*. Moreover, electron microscopy studies revealed that cRGD equipped Ad did not form large aggregates >100-150 nm, that are unable to penetrate the fenestrae in the liver (data not shown). Thus, a major requirement for successful retargeting of Ad vectors to specific cell and tissue targets has been achieved.

To redirect Ad vectors we have linked cRGD to Ad vectors via the CAR-Avidin linker protein. This peptide was shown to display a high affinity for $\alpha_V\beta_{3/5}$ integrins expressed on activated (angiogenic) endothelial cells and has been widely exploited in for targeting strategies of Ad ([33;34]) and other drug carriers i.e. liposomes ([41]). In the current study, cRGD equipped Ad vectors have been used to target mechanically injured carotid arteries. Unfortunately, cRGD-Ad failed to infect both quiescent

endothelial cells as well as activated endothelial cells flanking the site of injury after systemic administration *in vivo*. Furthermore, medial vascular smooth muscle cells at the site of injury were not infected as well. Since erythrocytes ubiquitously express integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ it is conceivable that the cRGD ligand is an inappropriate targeting molecule for systemic application, and this could explain our negative results. This is in line with findings of Haubner and colleagues ([42]), who have investigated several RGD based compounds. Interestingly, we were unable to detect any infection of vascular cells by cRGD-Ad even after local incubation in the absence of erythrocytes, suggesting that erythrocyte scavenging cannot be held accountable for the lack of vascular targeting by Ad in vivo. Several studies have demonstrated enhanced $\alpha_V\beta_{3/5}$ expression on endothelial and vascular smooth muscle cells after injury ([43-45]). However, $\alpha_V\beta_{3/5}$ mediated uptake is relatively slow and we cannot exclude the possibility that the time frame of upregulation of $\alpha_V\beta_{3/5}$ expression is incompatible with the currently applied infection protocol.

To avoid the possibility of erythrocyte mediated sequestration of cRGD-Ad or poor transendothelial permeation, a lung specific targeting moiety was used. The lung is a relatively large organ with high blood flow and thus easily accessible. Our targeting moiety was proven to be effective, since iodinated avidin-bioGFE1 was specifically taken up by lung. Nevertheless, the GFE1-Ad did not enhance transgene expression in the lung. It is possible that the in vivo stability of the ad vector and/or the local lung-specific blood flow conditions are incompatible with the attachment and uptake of the virus particles by the target cells.

Completely blocking liver uptake with lactoferin had no dramatic effect on the half-life of the untargeted virus (only a 33% increase). Due to the high blood flow through the liver (24% of the cardiac output) the slightly prolonged retention in the blood is probably not sufficient for the aimed target organs to take up the virus. Upon inhibition of liver uptake, Ad is rapidly neutralized by erythrocytes through binding of RGD motives to αvβ3/ αvβ5. This compromises virus stability after intravenous administration. So any retargeting approach will have to compete with systemic neutralization, and apparently the two ligands we have selected are not capable of doing this.

In conclusion CAR-Avidin has shown to efficiently detarget Ad from the liver upon equipping the Ad with novel ligands. This decrease in liver tropism however, was not accompanied by an increased transgene expression in novel target cells.

Acknowledgements

This work was performed in the framework of the Leiden Center for Cardiovascular Research LUMC-TNO and supported by grants from the Dutch Organization for Scientific Research (NWO 902-26-220), Netherlands Heart Foundation (NHS 2001-141, NHS 2003T201 and NHS 99B149) and the Center of Medical Systems Biology (CMSB) established by the Netherlands Genomics Initiative/ Netherlands Organization for Scientific Research (NGI/NWO).

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Chapter 6

General discussion and future perspectives

General discussion and future perspectives

In this thesis, the development of a targeting system for adenovirus (Ad) vectors is described. Adenovirus vectors (Ad) are powerful tools to modulate gene expression and are promising tools for (gene) therapeutic applications. Their applicability however, depends on the expression of the Coxsackie Adenovirus Receptor (CAR), by the target cell. Many cells interesting for either research or gene therapy do not, or only at low levels express CAR rendering them resistant to Ad mediated gene transfer.

In chapter 2, a poly-A containing oligonucleotide ligand is characterized using liposomes as a model to present the ligand to the cells. Interestingly, the SRA-specific LCO-dA_2dG_{10}-mediated liposome clearance by the liver appeared to be higher than was previously observed for uncomplexed $dG_{10}dT_6$ by Biessen and colleagues ([1]). This demonstrates that increasing the avidity by clustering ligands on the liposome surface has a positive effect on the recognition and uptake by scavenger receptors. One of the further advantages of liposomes as ligand testing system, is that they can be produced relatively easily and at low cost. Moreover, liposomes are slowly cleared from the circulation (facilitating the investigation of targeting efficiency *in vivo*), and they can be radiolabed with various means. Although ligand-equiped liposomes are relatively poor agents to functionally deliver gene expression constructs *in vivo*, recent research in fusogenic peptides indicates that using liposomes for the delivery of genetic material is feasible ([2-5]).

Targeting any drug to a specific cell type requires cell type-specific receptors and ligands. These receptors and ligands need to be characterized extensively to determine their tissue distribution pattern in different stages of development and pathology. This information is required to maximize the dose-response ratio and to minimize adverse effects of targeted drug therapy. Thus far, targeting approaches are hampered by the relative absence of both cell type-specific receptors and receptor-specific ligands for targeting receptors.

Before the advent of genomics technology, selecting cell-type specific receptors and ligands from a large set of candidates was a rather laborious task. By exploiting databases with cell type specific gene and protein expression patterns obtained by micro-array and proteomics technologies, it can be expected that candidate selection will be more straight forward in the foreseeable future. In addition to database searches, selection strategies have been developed for the identification of receptor or cell-type specific ligands. One such section strategy is the phage display technology. These phages display ligands in their capsid proteins and thus present peptides in a natural conformation. The

selection strategy is based on repeated rounds of binding of a pool of different phages and subsequent re-isolation of these bound phages.

Ideally, primary cells should be used for gathering information on cell type-specific receptors and ligands. Culturing cells *ex vivo* unambiguously induces changes in the protein expression profile of the cell, specifically cell surface receptors that are now in contact with an artificial medium (being either or both the tissue culture plastic and the growth medium). This is demonstrated by integrin expression by endothelial cells (see below). For targeting to be effective, especially variations between comparable cell types need to be mapped in detail, i.e. endothelial cells in different organs or vessel types, differences between normal and tumor cells. Since it is also likely that the method used for isolation of specific cell types or tissues affects the expression of specific proteins (i.e. enzymatic treatments), it is obvious that it remains a major challenge to identify suitable candidate receptors and ligands.

The CAR-Avidin linker protein described in chapter 3 is designed to be highly flexible and versatile. The avidin moiety in the linker protein can accommodate the attachment of a variety of ligands to the virus including poly-nucleotides (Chapter 3) and peptides (Chapter 4) via biotin. Since the CAR-Avidin linker protein, the biotinylated ligand and the virus are all generated as separate entities, the targeting system can be applied to all pre-existing Ad5 vectors without the need to redesign and modify any of the components.

One of the motivations to apply the avidin-biotin bridge in our targeting strategy was also the multimerization of biotin-bound ligands to avidin. Multimerization greatly improves the avidity of the complex. For example, Molenaar and colleagues observed that (avidin-mediated) multimers of the peptide ligand TM-11 displayed a much higher affinity for P-Selectin than monomers ([6]), Thoma and colleagues show an increased affinity of an E-Selectin inhibitor when presented as a multimers ([7]). Although the affinity of CAR-avidin for biotin indicates that multimerization has taken place (fig 3 chapter 3), we failed to supply biochemical evidence that multimerization had occurred. This is likely caused by the stickiness of the chicken avidin fragment that was applied in the linker protein. Since streptavidin has been reported to be less sticky, it is possible that incorporation of a streptavidin fragment in our construct further improves the applicability.

For research purposes, the CAR-Avidin strategy enables testing of a variety of vectors with different gene expression constructs and virus backbones. Likewise, any biotinylatable ligand can be implemented relatively easily. Peptide ligands that are developed in such a manner can

subsequently be utilized in a genetic targeting strategy, by for example, cloning into the HI loop. Once the best virus backbone, promoter, transgene and ligand combination are fully characterized and clinical application becomes feasible, a 1 or 2 component system may be favorable. Fewer components mean fewer variables to take into account resulting in a simpler, better-defined system. Direct incorporation of the novel ligand into the fiber and simultaneously prevent CAR mediated uptake, would result in a single component system. In the case of oligonucleotides or other chemical compounds serving as ligands, a tool to couple them to the virus is still required. Biotinylation of the ligand in combination with incorporation of a biotin binding peptide in the fiber, as was demonstrated by Parrot and colleagues ([8]), results in a 2-component system.

Combining physical targeting with CAR-Avidin and genetic targeting by cell type specific promoters can further improve the specificity of targeting Ad vectors. Any virus taken up by cells other than the intended target tissue, would remain silent since the non-target cells do not have the appropriate transcription machinery to ensure transgene expression. The efficacy of genetic targeting is demonstrated by Curiel and colleagues ([9-12]) and Haisma and colleagues ([10;13]).

The *in vivo* targeting experiments reported in chapter 5 demonstrate that expression in the liver was dramatically reduced upon equipping Ad with the linker protein in combination with various ligands. This linker protein was designed to block the CAR mediated uptake and apparently was able to do so. Alternative Ad vector uptake pathways have been described, such as sulfate glycosaminoglycan-mediated uptake ([14]). However, our results indicate that these pathways do not play a quantitative role in hepatic uptake of targeted Ad vectors.

An extensive effort was made to target Ad vectors towards the endothelium in the carotid artery by equipping the vector with a ligand for the integrins $\alpha_V\beta_{3/5}$ (chapter 5). However, gene delivery to endothelial cells in both normal and injured carotid arteries after systemic administration was never observed. Integrins have long been regarded as prime targets to mediate the uptake of (Ad) vectors (reviewed in ([15]) and ([16]) or other drugs ([17]) ([18]) for several reasons: (I) Integrins are abundantly expressed by a broad range of cell types, (II) Upon binding integrins are capable of internalizing particles, (III) They recognize short peptide motifs, making it relatively simple to target them. Most of the research in targeting Ad vectors focusses on *in vitro* targeting before applying the ligands *in vivo*. Many *in vitro* cell culture systems however, select towards adhering cells. Since integrins mediate cell survival ([19]), adherence and proliferation

([20;21]), it is likely that integrin expression is influenced by the *in vitro* tissue culture conditions. Published data on integrin expression *in vivo* may be somewhat contaminated by the information available from in vitro studies, and should therefore be handled with care. Besides altered integrin expression it is conceivable that the expression of other surface proteins is altered as well, emphasizing the need for using primary cells when selecting surface structures for the targeting of vectors. However, since we have not mapped the integrin expression of endothelial or vascular smooth muscle cells in our studies, we cannot fully address these issues.

Since erythrocytes ubiquitously express integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ it is conceivable that the RGD-equiped Ad vectors are sequestered by erythrocytes after systemic application and thus are rapidly inactivated. Akiyama has shown that to increase the effective Ad dose in the blood it is necessary to block both CAR mediated uptake as well as $\alpha_v\beta_{3/5}$ mediated infection (Akiyama, 14). However, this does not explain our negative results obtained after local incubation in the absence of erythrocytes. Interestingly, others have obtained similar negative results in their efforts to locally target integrins on vascular cells ([22]). Although enhanced $\alpha_V\beta_{3/5}$ expression on endothelial and vascular smooth muscle cells after injury has been demonstrated, we cannot exclude the possibility that the time frame of upregulation of $\alpha_V\beta_{3/5}$ expression is incompatible with the applied infection protocols.

An alternative explanation for the absence of target tissue uptake of targeted Ad could be the local flow conditions (too fast or turbulent), in combination with the high blood flow through the liver (24% of the cardiac output). Particles need time to bind the receptor and be internalized by the target cell, and although the retention in the blood is somewhat prolonged, unfavorable flow conditions may prohibit binding and uptake resulting in failure of the target cells to express the transgene. Unfavorable local flow conditions alone however, cannot account for the absence of increased Ad mediated transgene expression in the lung. The GFE1 ligand used for these experiments was isolated from a phage display library; Phages capable of binding endothelium in the lung were isolated and purified. The receptor-ligand combination is thus capable of binding spherical “particles”. Since phages are roughly similar in size to Ad GFE1, size alone cannot explain the failure of this targeting approach for targeting lung endothelium. Apparently other processes in the pathway that should result in transgene expression are inhibited in GFE1 mediated targeting of Ad.

The in vivo data in chapter 3 indicate that targeting Ad vectors to Kuppfer cells via dA_6G_{10} results in increased transgene expression. Tao

and colleagues have shown that under normal conditions, viral uptake by Kuppfer cells does not result in transgene expression ([23]). Furthermore, observations from Akiyama showed that the administration of double ablated vectors, unable to be taken up via CAR and $\alpha_V\beta_{3/5}$ integrins, and thus taken up via alternative routes, does not result in transgene expression ([24]). This suggests that transgene expression is dependent on the intra-cellular trafficking of the virus: while uptake generally leads to degradation of the virus, dA_6G_{10} mediated uptake via the scavenger receptor results in transgene expression.

The primary goal in targeting research is obviously the ability of the combination of vector and targeting system to enhance transgene expression in the target cell. This endpoint however, is the result of a sequence of events including *in vivo* vector transportation, vector binding, uptake, intra-cellular trafficking and expression of the transgene. Any problem in this chain of events can cause failure to reach this goal. All steps, from initial administration to transgene expression, should therefore be closely monitored. Recently, non-invasive *in vivo* imaging of fluorescently labeled markers using highly sensitive CCD cameras has become feasible ([25;26]). This technology enables more direct monitoring of the fate of targeted vectors after *in vivo* adminstration, making it possible to distinguish the different steps resulting in transgene expression. This should give in more insight into the critical steps in ad vector targeting.

A somewhat disturbing picture arises from these data: Will retargeting *in vivo* ever be achievable? Successful attempts published thus far for ad targeting *in vivo* are extremely scarce. Our data seem to indicate that an important problems lies in the rapid inactivation of ad vectors after systemic administration. Detargeted Ad vectors (in the presence of lactoferrin) are cleared with a half life of 8 minutes, versus a half life of 6 minutes for hepatic clearance of control Ad vectors. Whether scavenging by erythrocytes or Kupfer cells in the liver is responsible for this phenomenon remains to be determined. Nevertheless, insight in the mechanism responsible for Ad neutralization will be required for Ad targeting to become effective.

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Chapter 7

Summary

Summary

In this thesis, the development of a targeting system for adenovirus (Ad) vectors is described. Ad vectors are powerful tools to modulate gene transcription both *in vitro* and *in vivo* in both quiescent and dividing cells. Their applicability however, depends on the expression of the Coxsackie Adenovirus Receptor (CAR), by the target cell. Parenchymal cells in the liver and a variety of other cell types express CAR. However many cells relevant to (gene) therapy, including endothelial cells, macrophages, vascular smooth muscle cells and many tumors do not, or only at low levels express CAR rendering them resistant to Ad mediated intervention. Efficient and specific intervention starts with the delivery of the vector to the appropriate cells only. For this targeting to be successful, affinity for the CAR must be ablated and at the same time the vector should be equipped with an appropriate ligand to induce affinity for the target cell.

In **chapter 2,** the development of a ligand for macrophages *in vitro* and *in vivo* is described. Macrophages play, via the secretion of pro-inflammatory cytokines, a central role in a wide variety of biological and pathogenic processes including innate immunity, tumorigenesis, and the initiation and progression of atherosclerosis, prompting several research groups to develop macrophage-specific drug carriers, using scavenger receptors as a target. Oligodeoxyriboguanines have a known affinity for the scavenger receptor class A (SR-A) abundantly expressed by macrophages *in vitro.* Pearson and colleagues found that dG_{10} is the minimal oligodeoxyriboguanine motif to display a high affinity for SRA {Pearson, 1993 124 /id}. This residue was coupled to a dA_2 spacer and the hydrophobic lithochololeate (LCO) anchor to ensure anchoring into the surface of the liposome. The LCO-dA_2dG_{10} ligand dose-dependently enhanced the tissue distribution of liposomes *in vivo* in a strictly SR-A - dependent manner, since the increased liposomal clearance rate by the liver could be completely blocked by polyI. These data demonstrate the applicability of dG_{10}-based ligands for targeting to SR-A.

Having a ligand for the delivery of spherical particles via SR-A to macrophages in hand, we set out in **chapter 3** to develop and characterize the linker protein CAR-Avidin to target adenovirus vectors. This linker protein is composed of the virus-binding domain of the CAR genetically fused to avidin. Equipping this linker protein with a suitable, biotinylated ligand results in a targeting protein capable of retargeting Ad vectors. The utilization of this linker protein is demonstrated by equipping it with the slightly modified dA_2G_{10} ligand, the spacer is increased from dA_2 to dA_6 to ensure clear separation of the SR-A binding moiety dG_{10} from the bulky avidin-biotin moiety in the linker protein. An up to 500-fold increased transgene expression in both primary and transformed macrophages known to be notoriously difficult to infect with adenovirus vectors, *in vitro*

was found. Preliminary *in vivo* experiments suggest a redistribution in the liver uptake from parenchymal cells to Kupffer cells, based on β-Galactosidase expression and cell morphology. Experiments with macrophages from SR-A knockout mice however, suggest that the dA_6G_{10} mediated uptake, though highly macrophage specific *in vitro* as well as *in vivo*, is not completely SR-A specific. It appeared that SR-$A^{-/-}$ macrophages are also infected by the dA_6G_{10} equipped Ad, though at a significantly lower level.

In **chapter 4** the versatility of the linker protein CAR-Avidin is demonstrated. Since most chemical substances can be biotinylated, a variety of compounds, i.e. peptides, oligonucleotides, proteins, antibodies and chemically synthesized compounds can be used to target Ad vectors. Here, we describe the utilization of the biotinylated circular peptide ligand RGD to facilitate infection via the $\alpha_V\beta_{3/5}$ integrins of several endothelial cell lines and vascular smooth muscle cells *in vitro*. Equipping reporter Ad vectors with cRGD via CAR-Avidin markedly enhanced gene transfer to the established endothelial cell lines H5V (up to 600fold increase in reporter gene expression) and EOMA (4 to 6-fold increase), as well as to primary HUVEC (from 2% to 75% of cells infected upon targeting) and VSMC (from 0% to 25% of cells infected).

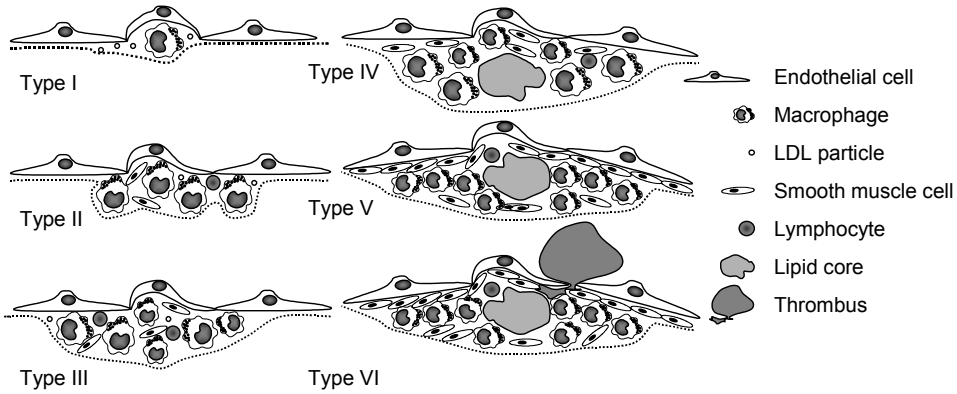
In **chapter 5** we report our efforts to retarget adenovirus vectors using the linker protein CAR-Avidin. Experiments with the reporter gene luciferase expressing adenovirus equipped with cRGD or biotin via CAR-Avidin- indicate an up to 85-fold reduction in liver infection after systemic administration. This observation is supported when using the GFE1 ligand, indicating that addition of novel ligands to the virus knob of adenovirus vectors reduces the liver uptake of the vectors *in vivo*. Though successful *in vitro,* the cRGD nor the GFE1 peptide ligand were able to increase Ad uptake by the target tissues *in vivo*. This inability to increase adenovirus vector mediated transgene expression by target tissues cannot be ascribed to inaccessibility of the ligand for the receptor or complex formation due to addition of the linker protein. Furthermore, blocking liver all uptake by lactoferin resulted in a minor increased virus half-life, suggesting the presence of efficient extrahepatic elimination pathways. Apparently, rapid neutralization of (targeted) Ad in the circulation efficiently prevents uptake by target organs other than the liver.

Taken together, our data show that targeting of Ad *in vitro* by the CAR-Avidin technology to primary endothelial cells, primary macrophages, primary vascular smooth muscle cells, endothelial cell lines, and macrophage cell lines appears to be successful. The CAR-Avidin linker protein proves to be a versatile tool to target Ad to previously refractory cells. When applied *in vivo* however, the CAR-Avidin technology leads to a

reduced transgene expression by the liver, but does not result in tissue-specific targeting of the virus.

Nederlandstalige samenvatting

Atherosclerose, ook wel aderverkalking genoemd is een ziekte die wordt gekenmerkt door de vorming van vetophopingen, zogenaamde plaques, in de middelgrote slagaders. Met name de vaten die het hart van bloed voorzien blijken erg gevoelig voor het ontstaan van plaques die kunnen ontstaan door vele factoren waaronder overgewicht, te hoge bloeddruk en verhoogd LDL-cholesterol (het “slechte cholesterol”). Door deze factoren stapelen vetten zich op op op specifieke plaatsen in da vaatwand waardoor de cellen die de vaten aan de binnenkant bekleden (endotheel cellen) chronisch geïrriteerd raken. Andere cellen in de vaatwand, macrofagen, hebben tot taak om stoffen die niet thuis horen in een bepaald orgaan of weefsel op te ruimen. Macrofagen zijn echter niet in staat de cholesterol af te breken en slaan het dus op, het worden dan zogenaamde “schuimcellen”. Deze schuimcellen zorgen, samen met de geïrriteerde endotheel cellen voor een ontstekingsreactie in de vaatwand. De cellen van het immuun systeem komen op deze ontsteking af en activeren de vaatwand nog meer waardoor een vicieuze cirkel ontstaat die uiteindelijk resulteert in vernauwing van het bloedvat (fig. 1).



Figuur 1 *Ontsteking reactie in de vaatwand die leidt tot vernauwing*

Een plaque is een zwakke plek die kan barsten waardoor het ontstekingsmateriaal in het vat terechtkomt. Wordt het vat helemaal geblokkeerd dan ontstaat een hartinfarct. Gebeurt dit in de hersenen dan spreekt men van een herseninfarct.
Omdat de endotheelcellen en de macrofagen zo’n cruciale rol spelen in het ontstaan én het in stand houden van atherosclerose zijn ze interessant voor (gen)therapeutische interventie.

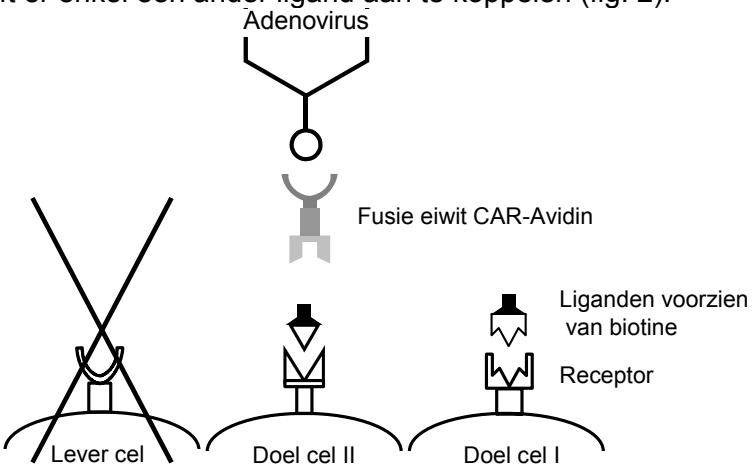
In dit proefschrift wordt een nieuwe technologie beschreven om adenovirussen selectief door bepaalde cellen opgenomen te krijgen (“targeten”). Adenovirussen zijn krachtige instrumenten om cellen te beïnvloeden. Door ze aan te passen en uit te rusten met het genetisch

materiaal (DNA) voor therapeutisch interessante eiwitten zijn ze in staat cellen nieuwe eiwitten te laten maken (gentherapie), of de productie van andere eiwitten te remmen. Ook kunnen ze na aanpassing bijvoorbeeld het groei gedrag van cellen beïnvloeden waardoor ze bijvoorbeeld ook gebruikt kunnen worden voor de behandeling van kanker. Het adenovirus is in staat om zowel delende als niet delende cellen te infecteren en te beïnvloeden. Om adenovirussen te kunnen gebruiken moeten de cellen die ze moeten gaan beïnvloeden een specifieke receptor op het oppervlak hebben, de Coxsackie Adenovirus Receptor (CAR). Een interactie tussen deze receptor en een eiwit aan de buitenkant van het virus (ligand) zorgt ervoor dat het virus aan de cel bindt om deze vervolgens binnen te dringen. Eenmaal in de cel zorgt het virus ervoor dat het DNA dat het virus meeneemt gebruikt wordt om nieuwe eiwitten te gaan maken die een positief effect hebben op de cel.
De eerder genoemde CAR komt vooral, maar niet uitsluitend op de lever voor. Indien het originele virus zou worden gebruikt wordt dus voornamelijk de lever bereikt. Als dit orgaan ook het bedoelde orgaan voor de therapie is, is dit geen probleem. Veel andere cellen echter, die interessant zijn voor (gen)therapie hebben deze CAR niet op hun oppervlak en zijn dus niet of zeer moeilijk te infecteren door het virus. Tot deze groep behoren onder andere de cellen die de binnenkant van de bloedvaten bekleden (endotheel cellen) en veel soorten tumorcellen. Als je dus een orgaan, anders dan de lever, wil bereiken zul je een manier moeten vinden om (I) de opname door de lever te blokkeren en (II) de opname door de CAR negatieve cel mogelijk te maken.

In **hoofdstuk 2** wordt beschreven hoe een ligand is gevonden en onderzocht dat gebruikt kan worden het adenovirus in staat te stellen macrofagen te infecteren. Macrofagen hebben een bepaalde receptor op hun oppervlak, de “Scavenger receptor” (SR-A). Deze receptor komt bijna uitsluitend voor op macrofagen en kan daarom goed gebruikt worden om opname van het virus door deze cellen te bevorderen. Pearson en collegae hebben ontdekt dat een bepaalde stof, oligodeoxyriboguanine (dG_{10}) erg sterk bindt aan deze receptor. Om te onderzoeken of deze interactie tussen de SR-A en dG_{10} geëxploiteerd kon worden voor de targeting van adenovirussen is een zijstap gemaakt naar model deeltjes met een vergelijkbare grootte, liposomen. Liposomen zijn kleine vetachtige deeltjes die gemakkelijk gemaakt kunnen worden en gebruikt kunnen worden om geneesmiddelen door het lichaam te vervoeren. Voor deze fase van het onderzoek is gebruik gemaakt van liposomen omdat ze relatief gemakkelijk, goedkoop en snel te maken zijn. Daarnaast zijn ze gemakkelijk te merken (labelen) waardoor goed te onderzoeken is in welke organen ze worden opgenomen. De liposomen die uitgerust waren met dG_{10} bleken via de SR-A opgenomen te worden door de lever van

muizen. Deze conclusie kon worden getrokken omdat voorbehandeling van de dieren met een stof waarvan bekent is dat hij bindt aan de SR-A de liposoom opname door de lever sterk reduceerde. Deze gegevens laten zien dat het dG_{10} een veelbelovend ligand is voor gestuurde opname van adenovirussen.

In **hoofdstuk 3** wordt de ontwikkeling van CAR-Avidin beschreven. CAR-Avidin is een fusie eiwit dat bestaat uit het virus bindende deel van de CAR receptor, en het eiwit avidine. Avidine is een eiwit dat een erg sterke binding kan aangaan met biotine. Biotine is een klein molecuul dat gemakkelijk langs chemische weg aan liganden gekoppeld kan worden. Op deze manier ontstaat een systeem dat werkt als dubbelzijdig plakband: de ene kant plakt aan het virus, de andere kant plakt aan een ligand waarvoor de receptor aanwezig is op de cel of het weefsel dat je wilt infecteren. Doordat het ligand niet direct aan het virus bindende deel vast is gemaakt, is een flexibel systeem ontstaan waarbij je voor een ander cel type niet het hele fusie eiwit opnieuw hoeft te ontwerpen en produceren, je hoeft er enkel een ander ligand aan te koppelen (fig. 2).



***Figuur 2** Het principe van het "dubbelzijdig plakband" CAR-Avidine*

Om adenovirussen macrofagen te kunnen laten infecteren is het dG_{10} ligand uit hoofdstuk 2 gebruikt. Er is een stukje aan vast gezet (dA_6) om ervoor te zorgen dat het ligand niet "klem" komt te zitten in het avidine en daardoor de avidine-biotine interactie of de dG_{10}-SR-A interactie negatief beïnvloedt. Een adenovirus is vervolgens uitgerust CAR-Avidin-dA_6G_{10} en getest op diverse typen macrofagen. Dit virus zorgt ervoor dat de geïnfecteerde cellen luciferine gaan maken, een eiwit dat makkelijk meetbaar is. CAR-Avidin-dA_6G_{10} bleek in staat om ervoor te zorgen dat adenovirussen zowel macrofaag lijnen als primaire macrofagen konden infecteren waardoor de luciferine activiteit tot 500-maal hoger was in

vergelijking tot virussen die niet waren uitgerust met CAR-Avidin-dA_6G_{10}. CAR-Avidin-dA_6G_{10} is ook in muizen getest waarbij het opnamepatroon van het adenovirus door de lever verschoven leek naar in de lever aanwezige macrofagen. Experimenten met macrofagen afkomstig van muizen die geen SR-A hebben laten echter zien dat, hoewel het dG10 ligand erg macrofaag specifiek is, het ook bindt aan andere receptoren dan SR-A, mogelijk andere typen scavenger receptoren.

In **hoofdstuk 4** wordt de flexibiliteit van CAR-Avidin beschreven aan de hand van experimenten in endotheelcellen. Omdat endotheelcellen de CAR niet op hun oppervlak hebben is het normaal gesproken praktisch onmogelijk voor adenovirussen ze te infecteren. Zelfs bij grote hoeveelheden virus wordt maar een fractie van de cellen geïnfecteerd. Door CAR-Avidin nu uit te rusten met een ander soort ligand, genaamd cRGD, blijkt het mogelijk voor adenovirussen endotheelcellen te infecteren. Dit ligand blijkt selectief te binden aan verschillende typen endotheelcellen, zowel van muizen als van mensen en zo de infectie sterk te verhogen (toename van 2% naar 75% van de aanwezige cellen). Op primaire gladde spiercellen (ander type cel in de wand van bloedvaten) bleek een toename de hoeveelheid geïnfecteerde cellen van praktisch 0 naar 25% wanneer het adenovirus werd uitgerust met CAR-Avidin-dA_6G_{10}.

In **hoofdstuk 5** worden de pogingen om adenovirussen in muizen te targeten naar specifieke cellen/ organen beschreven. Omdat de dA_6G_{10} en RGD liganden in staat bleken om de infectie van adenovirussen *in vitro* te bevorderen werd tevens onderzocht of deze liganden ook in staat waren om adenovirussen in intacte dieren naar andere organen te sturen. Zoals eerder aangegeven worden deze virussen normaal gesproken opgenomen door de lever. Door de adenovirussen echter uit te rusten met cRGD of GFE1 (een ligand waarvan bewezen is dat het in de longen wordt opgenomen) via CAR-Avidin bleek de leveropname met 91.3% verminderd. Deze afname in leveropname ging echter niet gepaard met een verhoogde opname in de logen of andere organen. Het vermoeden bestaat dat er een competitie tussen het gewenste doelorgaan (bijvoorbeeld de longen) en cellen in het bloed die het virus opruimen nu het virus niet meer snel en efficiënt door de lever wordt opgenomen. De doelorganen zijn blijkbaar dus niet in staat om deze strijd te winnen.

De hier beschreven technologie maakt het mogelijk om adenovirussen onder kweek omstandigheden voor een breder spectrum aan cel typen toe te passen dan tot nu toe het geval was. Om deze reden zal het bijdragen aan het onderzoek waarin men bepaalde cellen graag zou willen moduleren door middel van adenovirussen maar dat tot nu toe niet kon omdat de cellen niet te infecteren bleken met deze virussen.

Curriculum Vitae

Emile Gras werd op 6 december 1974 geboren in Utrecht. Na het behalen van het HAVO diploma in 1992 aan het Cals College in Nieuwegein en het VWO diploma in 1994 aan de Nassau Scholengemeenschap in Breda werd gestart met de studie Biomedische Wetenschappen aan de Universitiet Leiden. Tijdens deze studie werden 3 onderzoeksstages verricht. De eerste bij de faculteit Geneeskunde van de Universiteit Leiden (nu LUMC) onder leiding van dr. A.G. Jochemsen, de tweede bij de faculteit Wiskunde en Natuurwetenschappen onderleiding van dr. P.C.N. Rensen. De laatste stage werd verricht bij TNO – Quality of life onder leiding van dr. P.H.A. Quax waarna in 2000 het doctoraal examen werd behaald. Vervolgens is hij begonnen als assistent in opleiding bij de afdeling Humane genetica van het LUMC op het door dr. ir. Willems van Dijk en dr. ir. Biessen geleidde project: “Adenoviral gene delivery to atherosclerotic lesions to restore endothelial function”. Sinds 2005 is hij werkzaam als trial en datamanager voor de unit Trials & Statistiek bij het Erasmus MC – Daniel den Hoed waar hij zich bezig houdt met de organisatie, logistiek en uitvoering van klinische studies.