Modulation of estrogen signaling in hepatic and vascular tissue

Krom, Y.D.

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

Yvonne D. Krom[1$], J.C. Emile Gras[1$], Suzanne A Zadelaar [4], Ilze Bot [5], Rune R. Frants[1], Louis M. Havekes[2,3,4], Theo J. van Berkel[5], Erik A.L. Biessen[5] and Ko Willems van Dijk[1,3*]

Departments of Human Genetics[1], General Internal Medicine[2], Cardiology[3], Leiden University Medical Center, Leiden, The Netherlands

[4]TNO-Quality of Life, Gaubius Laboratory, Leiden, The Netherlands

[5]Division of Biofarmaceutics, Leiden Amsterdam Center for Drug Research, Leiden, The Netherlands

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]; (reviewed in [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.

Results

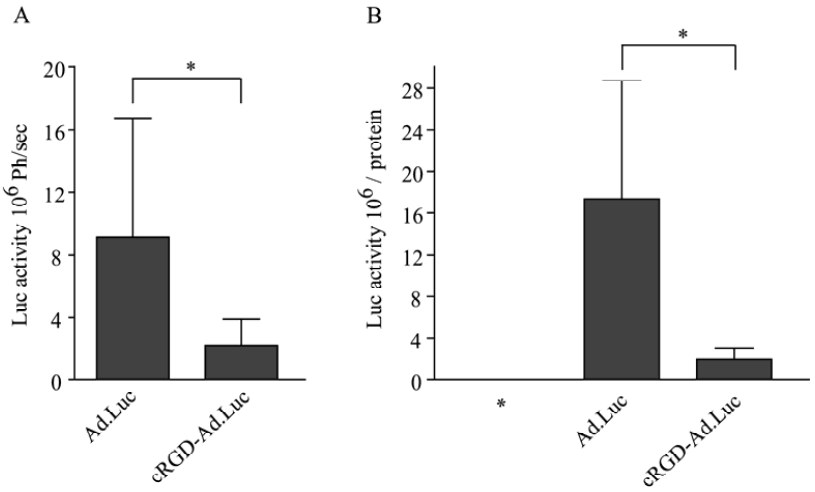

Figure 1. Effect of cRGD mediated targeting of adenovirus on luciferase expression in the liver. **(A)** Female C57Bl/6 mice received $2x10^9$ pfu Ad.Luc (i.p.). After 96 hours luciferin (150 mg/kg) was administered by i.p. injection and bioluminescent signals were recorded under full anesthesia for 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.

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). 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).

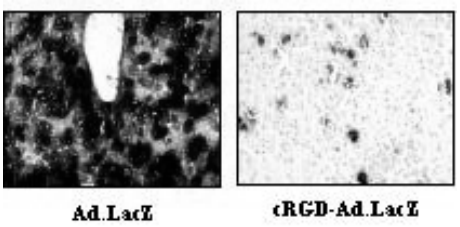

Figure 2. Effect of cRGD mediated targeting of adenovirus on ß-galactosidase expression in the liver. Female C57Bl/6 mice received cRGD-Ad.LacZ or untargeted Ad.LacZ ($1x10^9$ pfu; i.v. injection). Five days after infection mice were sacrificed, livers were excised and cryosections were made and stained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

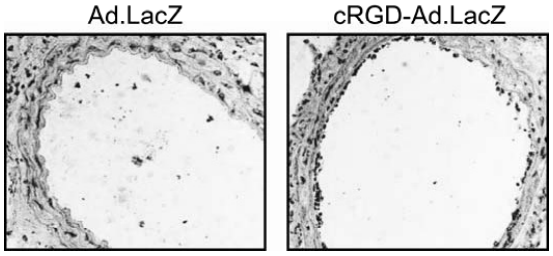

Figure 3. Effect of cRGD mediated targeting of adenovirus on LacZ expression in the vessel wall. The right common carotid artery of ApoE-/- mice was denuded by 3 rotational passes of a 0.36 mm guide wire. Subsequently, Ad (1.5×10^9 pfu) was instilled into the denuded common carotid artery segment via 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. As expected and

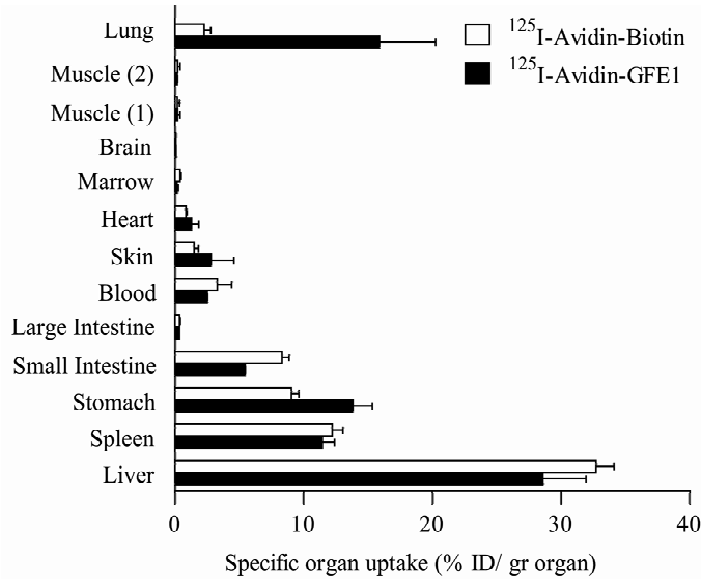

Figure 4. Biodistribution of ^{125}I-Avidin-GFE1 after intravenous injection into mice. ^{125}I-avidin-biotin or ^{125}I-avidin-GFE1 (molar ratio of 1: 1; 156749 dpm, in 100 µl PBS) was injected intravenously into female C57Bl/6 mice. Tissue distribution was determined 1h after injection. Tissue accumulation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

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.

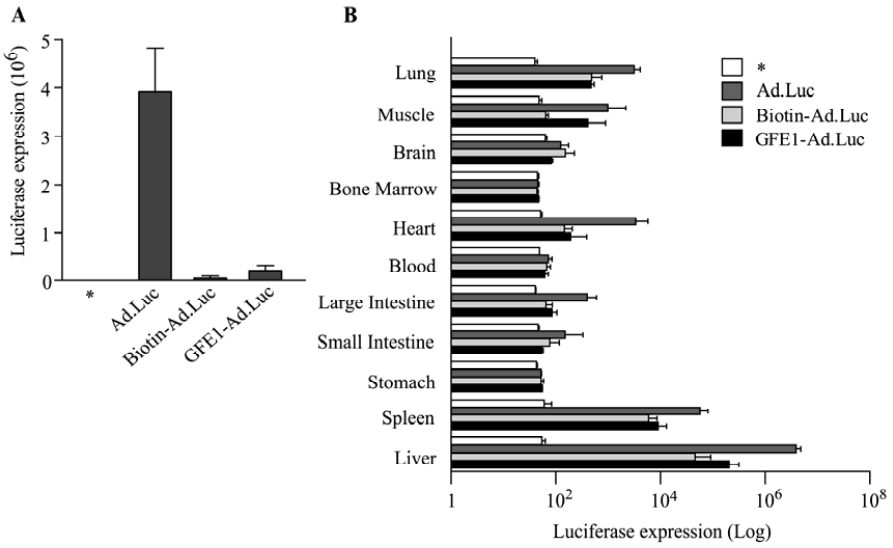

Figure 5. Effect of GFE1 mediated targeting of adenovirus on biodistribution luciferase expression. **(A)** untargeted Ad.Luc, biotin-Ad.Luc or GFE1-Ad.Luc were systemically administered to female C57Bl/6. Liver lysates were prepared 120 hours after infection by homogenisation and subsequent freeze-thawing. Supernatants were used for determining luciferase activity. **(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.

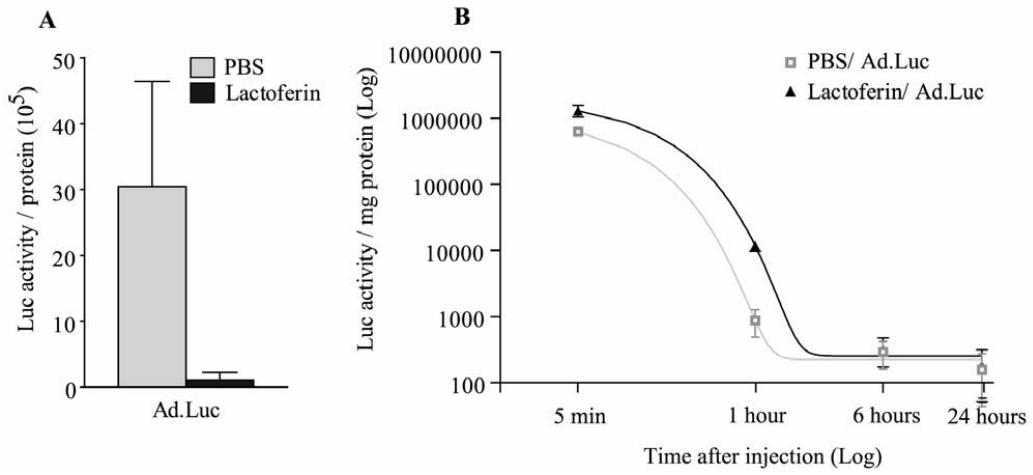

Figure 6. Effect of systemic lactoferrin treatment on Ad half-life after i.v. administration. (A) Mice received bovine lactoferin (i.v., 70mg/kg) (grey line, triangles) or PBS (black line, squares) 2 minutes before Ad.Luc ($1{,}5.10^9$ pfu) administration. Presence of circulating infectious particles 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.

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 x 10^{10} to 1 x 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.

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), Dutch Heart Foundation (NHS 2001-141, NHS 2003T201 and NHS 99-194) and the Center of Medical Systems Biology (CMSB) established by the Netherlands Genomics Initiative/Netherlands Organization for Scientific Research (NGI/NWO).

References

1. van Dijk KW, Kypreos KE, d'Oliveira C, Fallaux FJ: **Adenovirus-mediated gene transfer.** *Methods Mol Biol* 2003, **209:** 231-247.

2. Roelvink PW, Lizonova A, Lee JG, Li Y, Bergelson JM, Finberg RW, Brough DE, Kovesdi I, Wickham TJ: **The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F.** *J Virol* 1998, **72:** 7909-7915.

3. Roelvink PW, Mi LG, Einfeld DA, Kovesdi I, Wickham TJ: **Identification of a conserved receptor-binding site on the fiber proteins of CAR-recognizing adenoviridae.** *Science* 1999, **286:** 1568-1571.

4. Havenga MJ, Lemckert AA, Grimbergen JM, Vogels R, Huisman LG, Valerio D, Bout A, Quax PH: **Improved adenovirus vectors for infection of cardiovascular tissues.** *J Virol* 2001, **75:** 3335-3342.

5. Knaan-Shanzer S, van dV, I, Havenga MJ, Lemckert AA, De Vries AA, Valerio D: **Highly efficient targeted transduction of undifferentiated human hematopoietic cells by adenoviral vectors displaying fiber knobs of subgroup B.** *Hum Gene Ther* 2001, **12:** 1989-2005.

6. Rea D, Havenga MJ, van Den AM, Sutmuller RP, Lemckert A, Hoeben RC, Bout A, Melief CJ, Offringa R: **Highly efficient transduction of human monocyte-derived dendritic cells with subgroup B fiber-modified adenovirus vectors enhances transgene-encoded antigen presentation to cytotoxic T cells.** *J Immunol* 2001, **166:** 5236-5244.

7. Ophorst OJ, Kostense S, Goudsmit J, De Swart RL, Verhaagh S, Zakhartchouk A, Van Meijer M, Sprangers M, Van Amerongen G, Yuksel S, Osterhaus AD, Havenga MJ: **An adenoviral type 5 vector carrying a type 35 fiber as a vaccine vehicle: DC targeting, cross neutralization, and immunogenicity.** *Vaccine* 2004, **22:** 3035-3044.

8. Schoggins JW, Gall JG, Falck-Pedersen E: **Subgroup B and F fiber chimeras eliminate normal adenovirus type 5 vector transduction in vitro and in vivo.** *J Virol* 2003, **77:** 1039-1048.

9. Dmitriev I, Krasnykh V, Miller CR, Wang M, Kashentseva E, Mikheeva G, Belousova N, Curiel DT: **An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism.** *J Virol* 1998, **72:** 9706-9713.

10. Krasnykh V, Dmitriev I, Mikheeva G, Miller CR, Belousova N, Curiel DT: **Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob.** *J Virol* 1998, **72:** 1844-1852.

11. Belousova N, Krendelchtchikova V, Curiel DT, Krasnykh V: **Modulation of adenovirus vector tropism via incorporation of polypeptide ligands into the fiber protein.** *J Virol* 2002, **76:** 8621-8631.

12. Wu H, Seki T, Dmitriev I, Uil T, Kashentseva E, Han T, Curiel DT: **Double modification of adenovirus fiber with RGD and polylysine motifs improves coxsackievirus-adenovirus receptor-independent gene transfer efficiency.** *Hum Gene Ther* 2002, **13:** 1647-1653.

13. Nicklin SA, Von Seggern DJ, Work LM, Pek DC, Dominiczak AF, Nemerow GR, Baker AH: **Ablating adenovirus type 5 fiber-CAR binding and HI loop insertion of the SIGYPLP peptide generate an endothelial cell-selective adenovirus.** *Mol Ther* 2001, **4:** 534-542.

14. Wickham TJ, Tzeng E, Shears LL, Roelvink PW, Li Y, Lee GM, Brough DE, Lizonova A, Kovesdi I: **Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins.** *J Virol* 1997, **71:** 8221-8229.

15. Hidaka C, Milano E, Leopold PL, Bergelson JM, Hackett NR, Finberg RW, Wickham TJ, Kovesdi I, Roelvink P, Crystal RG: **CAR-dependent and CAR-independent pathways of adenovirus vector-mediated gene transfer and expression in human fibroblasts.** *J Clin Invest* 1999, **103:** 579-587.

16. van Deutekom JC, Cao B, Pruchnic R, Wickham TJ, Kovesdi I, Huard J: **Extended tropism of an adenoviral vector does not circumvent the maturation-dependent transducibility of mouse skeletal muscle.** *J Gene Med* 1999, **1:** 393-399.

17. Bouri K, Feero WG, Myerburg MM, Wickham TJ, Kovesdi I, Hoffman EP, Clemens PR: **Polylysine modification of adenoviral fiber protein enhances muscle cell transduction.** *Hum Gene Ther* 1999, **10:** 1633-1640.

18. Gonzalez R, Vereecque R, Wickham TJ, Facon T, Hetuin D, Kovesdi I, Bauters F, Fenaux P, Quesnel B: **Transduction of bone marrow cells by the AdZ.F(pK7) modified adenovirus demonstrates preferential gene transfer in myeloma cells.** *Hum Gene Ther* 1999, **10:** 2709-2717.

19. Gonzalez R, Vereecque R, Wickham TJ, Vanrumbeke M, Kovesdi I, Bauters F, Fenaux P, Quesnel B: **Increased gene transfer in acute myeloid leukemic cells by an adenovirus vector containing a modified fiber protein.** *Gene Ther* 1999, **6:** 314-320.

20. Yoshida Y, Sadata A, Zhang W, Saito K, Shinoura N, Hamada H: **Generation of fiber-mutant recombinant adenoviruses for gene therapy of malignant glioma.** *Hum Gene Ther* 1998, **9:** 2503-2515.

21. Parrott MB, Adams KE, Mercier GT, Mok H, Campos SK, Barry MA: **Metabolically biotinylated adenovirus for cell targeting, ligand screening, and vector purification.** *Mol Ther* 2003, **8:** 688-700.

22. Perlman H, Liu H, Georganas C, Woods JM, Amin MA, Koch AE, Wickham T, Kovesdi I, Mano T, Walsh K, Pope RM: **Modifications in adenoviral coat fiber proteins and transcriptional regulatory sequences enhance transgene expression.** *J Rheumatol* 2002, **29:** 1593-1600.

23. Douglas JT, Rogers BE, Rosenfeld ME, Michael SI, Feng M, Curiel DT: **Targeted gene delivery by tropism-modified adenoviral vectors.** *Nat Biotechnol* 1996, **14:** 1574-1578.

24. Curiel DT: **Strategies to adapt adenoviral vectors for targeted delivery.** *Ann N Y Acad Sci* 1999, **886:** 158-171.

25. Parrott MB, Adams KE, Mercier GT, Mok H, Campos SK, Barry MA: **Metabolically biotinylated adenovirus for cell targeting, ligand screening, and vector purification.** *Mol Ther* 2003, **8:** 688-700.

26. Pereboev AV, Nagle JM, Shakhmatov MA, Triozzi PL, Matthews QL, Kawakami Y, Curiel DT, Blackwell JL: **Enhanced gene transfer to mouse dendritic cells using adenoviral vectors coated with a novel adapter molecule.** *Mol Ther* 2004, **9:** 712-720.

27. Rogers BE, Douglas JT, Ahlem C, Buchsbaum DJ, Frincke J, Curiel DT: **Use of a novel cross-linking method to modify adenovirus tropism.** *Gene Ther* 1997, **4:** 1387-1392.

28. Smith JS, Keller JR, Lohrey NC, McCauslin CS, Ortiz M, Cowan K, Spence SE: **Redirected infection of directly biotinylated recombinant adenovirus vectors through cell surface receptors and antigens.** *Proc Natl Acad Sci U S A* 1999, **96:** 8855-8860.

29. Krasnykh V, J.T.Douglas: **Targeted adenoviral vectors I: transductional targeting.** In *Adenoviral vectors for gene therapy*. Edited by D.T.Curiel, J.T.Douglas. San Diego: Academic Press, Inc; 2002:205-245.

30. Emile Gras JC, Verkuijlen P, Frants RR, Havekes LM, van Berkel TJ, Biessen EA, van Dijk KW: **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.** *J Gene Med* 2006, **8:** 668-678.

31. Krom YD, Gras JC, Frants RR, Havekes LM, van Berkel TJ, Biessen EA, van Dijk KW: **Efficient targeting of adenoviral vectors to integrin positive vascular cells utilizing a CAR-cyclic RGD linker protein.** *Biochem Biophys Res Commun* 2005, **338:** 847-854.

32. Trepel M, Grifman M, Weitzman MD, Pasqualini R: **Molecular adaptors for vascular-targeted adenoviral gene delivery.** *Hum Gene Ther* 2000, **11:** 1971-1981.

33. Wickham TJ, Mathias P, Cheresh DA, Nemerow GR: **Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment.** *Cell* 1993, **73:** 309-319.

34. Wickham TJ, Carrion ME, Kovesdi I: **Targeting of adenovirus penton base to new receptors through replacement of its RGD motif with other receptor-specific peptide motifs.** *Gene Ther* 1995, **2:** 750-756.

35. Vigne E, Mahfouz I, Dedieu JF, Brie A, Perricaudet M, Yeh P: **RGD inclusion in the hexon monomer provides adenovirus type 5-based vectors with a fiber knob-independent pathway for infection.** *J Virol* 1999, **73:** 5156-5161.

36. Uchida H, Tanaka T, Sasaki K, Kato K, Dehari H, Ito Y, Kobune M, Miyagishi M, Taira K, Tahara H, Hamada H: **Adenovirus-mediated transfer of siRNA against survivin induced apoptosis and attenuated tumor cell growth in vitro and in vivo.** *Mol Ther* 2004, **10:** 162-171.

37. Rein DT, Breidenbach M, Wu H, Han T, Haviv YS, Wang M, Kirby TO, Kawakami Y, Dall P, Alvarez RD, Curiel DT: **Gene transfer to cervical cancer with fiber-modified adenoviruses.** *Int J Cancer* 2004, **111:** 698-704.

38. Witlox AM, van Beusechem VW, Molenaar B, Bras H, Schaap GR, Alemany R, Curiel DT, Pinedo HM, Wuisman PI, Gerritsen WR: **Conditionally replicative adenovirus with tropism expanded towards integrins inhibits osteosarcoma tumor growth in vitro and in vivo.** *Clin Cancer Res* 2004, **10:** 61-67.

39. Oberholzer C, Tschoeke SK, Bahjat K, LaFace D, Hutchins B, Clare-Salzler MJ, Moldawer LL, Oberholzer A: **In vivo transduction of thymic dendritic cells with adenovirus and its potential use in acute inflammatory diseases.** *Scand J Immunol* 2005, **61:** 309-315.

40. Izumi M, Kawakami Y, Glasgow JN, Belousova N, Everts M, Kim-Park S, Yamamoto S, Wang M, Le LP, Reynolds PN, Curiel DT: **In vivo analysis of a genetically modified adenoviral vector targeted to human CD40 using a novel transient transgenic model.** *J Gene Med* 2005, **7:** 1517-1525.

41. Schiffelers RM, Molema G, ten Hagen TL, Janssen AP, Schraa AJ, Kok RJ, Koning GA, Storm G: **Ligand-targeted liposomes directed against pathological vasculature.** *J Liposome Res* 2002, **12:** 129-135.

42. Haubner RH, Wester HJ, Weber WA, Schwaiger M: **Radiotracer-based strategies to image angiogenesis.** *Q J Nucl Med* 2003, **47:** 189-199.

43. Dufourcq P, Couffinhal T, Alzieu P, Daret D, Moreau C, Duplaa C, Bonnet J: **Vitronectin is up-regulated after vascular injury and vitronectin blockade prevents neointima formation.** *Cardiovasc Res* 2002, **53:** 952-962.

44. Kappert K, Blaschke F, Meehan WP, Kawano H, Grill M, Fleck E, Hsueh WA, Law RE, Graf K: **Integrins alphavbeta3 and alphavbeta5 mediate VSMC migration and are elevated during neointima formation in the rat aorta.** *Basic Res Cardiol* 2001, **96:** 42-49.

45. Li JM, Fan LM, Shah A, Brooks G: **Targeting alphavbeta3 and alpha5beta1 for gene delivery to proliferating VSMCs: synergistic effect of TGF-beta1.** *Am J Physiol Heart Circ Physiol* 2003, **285:** H1123-H1131.

46. Michon IN, Hauer AD, der Thusen JH, Molenaar TJ, van Berkel TJ, Biessen EA, Kuiper J: **Targeting of peptides to restenotic vascular smooth muscle cells using phage display in vitro and in vivo.** *Biochim Biophys Acta* 2002, **1591:** 87-97.

47. Fallaux FJ, Bout A, van dV, I, van den Wollenberg DJ, Hehir KM, Keegan J, Auger C, Cramer SJ, van Ormondt H, van der Eb AJ, Valerio D, Hoeben RC: **New helper cells and**

matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. *Hum Gene Ther* 1998, **9:** 1909-1917.

48. Fallaux FJ, Kranenburg O, Cramer SJ, Houweling A, van Ormondt H, Hoeben RC, van der Eb AJ: **Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors.** *Hum Gene Ther* 1996, **7:** 215-222.

49. de Nooijer R, Verkleij CJ, der Thusen JH, Jukema JW, van der Wall EE, van Berkel TJ, Baker AH, Biessen EA: **Lesional overexpression of matrix metalloproteinase-9 promotes intraplaque hemorrhage in advanced lesions but not at earlier stages of atherogenesis.** *Arterioscler Thromb Vasc Biol* 2006, **26:** 340-346.

50. der Thusen JH, van Berkel TJ, Biessen EA: **Induction of rapid atherogenesis by perivascular carotid collar placement in apolipoprotein E-deficient and low-density lipoprotein receptor-deficient mice.** *Circulation* 2001, **103:** 1164-1170.

51. van Tol A, Van Gent T, 't Hooft FM, Vlaspolder F: **High density lipoprotein catabolism before and after partial hepatectomy.** *Atherosclerosis* 1978, **29:** 439-448.

52. McFarlane AS: **Efficient trace-labelling of proteins with iodine.** *Nature* 1958, **182:** 53.