Atherosclerosis 222 (2012) 360–366

Atherosclerosis

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

Inhibition of hepatic scavenger receptor-class B type I by RNA interference decreases atherosclerosis in rabbits

Egon Demetz[a,1], Ivan Tancevski[a,1], Kristina Duwensee[a], Ursula Stanzl[a], Eva Huber[a], Christiane Heim[a], Florian Handle[a], Markus Theurl[a], Andrea Schroll[a], Anne Tailleux[b], Hermann Dietrich[c], Josef R. Patsch[a], Philipp Eller[d], Andreas Ritsch[a,*]

[a] *Department of Internal Medicine I, Innsbruck Medical University, Innsbruck, Austria*
[b] *UMR Inserm 1011, Institut Pasteur de Lille, Université Lille 2, Lille, France*
[c] *Central Laboratory Animal Facilities, Innsbruck Medical University, Innsbruck, Austria*
[d] *Division of Angiology, Department of Internal Medicine, Medical University of Graz, Graz, Austria*

ARTICLE INFO

Article history:
Received 20 October 2010
Received in revised form 31 January 2012
Accepted 13 March 2012
Available online 23 March 2012

Keywords:
Gene therapy
Hypercholesterolemia
Cholesteryl ester transfer protein
HDL receptor
Rabbits

ABSTRACT

Objective: Scavenger receptor-class B type I (SR-BI), the receptor for HDL-cholesterol, plays a key role in HDL metabolism, whole body cholesterol homeostasis, and reverse cholesterol transport. We investigated the in vivo impact of hepatic SR-BI inhibition on lipoprotein metabolism and the development of atherosclerosis employing RNA interference.
Methods: Small hairpin RNA plasmid specific for rabbit SR-BI was complexed with galactosylated poly-L-lysine, allowing an organ-selective, receptor-mediated gene transfer. Rabbits were fed a cholesterol-rich diet, and were injected with plasmid-complexes once a week.
Results: After 2 weeks of treatment hepatic SR-BI mRNA levels were reduced by 80% accompanied by reduced SR-BI protein levels and a modulation of the lipoprotein profile. Rabbits treated with SR-BI-specific plasmid-complexes displayed higher cholesteryl ester transfer from HDL to apoB-containing lipoproteins, lower HDL-cholesterol, and higher VLDL-cholesterol levels, when compared to controls. In a long-term study, this gene therapeutic intervention led to a similar modulation of the lipoprotein profile, to lower total cholesterol levels, and most importantly to a 50% reduction of the relative atherosclerotic lesion area.
Conclusion: Our results are another indication that the role of SR-BI in lipoprotein metabolism and atherogenesis in rabbits – a CETP-expressing animal model displaying a manlike lipoprotein profile may be different from the one found in rodents.

1. Introduction

The first high-density lipoprotein (HDL) receptor to be discovered was the scavenger receptor-class B type I (SR-BI) [1]. The delivery of HDL cholesterol to cells via SR-BI is fundamentally distinct from the well-characterized endocytotic pathway mediated by the low-density lipoprotein (LDL) receptor. SR-BI mediates the high-affinity binding of HDL particles and the selective uptake of HDL derived lipids into cells [2]. The murine SR-BI cDNA has been identified by expression cloning [3], and several SR-BI homologues of mammalian species have been characterized. The human homologue of SR-BI was initially identified as CLA-1 and mapped to chromosome 12 [4,5]. In rodents, selective lipid uptake represents the major pathway for delivering cholesteryl esters to the liver and to steroidogenic tissues. A series of in vitro and in vivo studies clearly demonstrated SR-BI to function as a physiologically and pathophysiologically relevant receptor for HDL metabolism [6]. In apolipoprotein E-deficient mice, loss of SR-BI led to the early onset of occlusive atherosclerotic coronary artery disease, spontaneous myocardial infarction, severe cardiac dysfunction, and premature death [7–9]. On the other hand, gene transfer and hepatic overexpression of SR-BI reduced atherosclerosis in the cholesterol-fed LDL receptor-deficient mouse [10]. However, data from these studies are not fully applicable to the situation in humans for the following reasons: first, the lipoprotein profile of rodents is different from that of humans, with HDL being the predominant lipoprotein particle, which is probably due to the lack of cholesteryl ester transfer protein (CETP) in the plasma of rodents. Secondly, mice and rats are not prone to develop atherosclerosis. The lipoprotein profile of rabbits is more similar to humans than to rodents, as CETP mass and activity are present in rabbit plasma. Additionally, rabbits are a

* Corresponding author. Tel.: +43 512 504 23252; fax: +43 512 504 25608.
E-mail address: andreas.ritsch@i-med.ac.at (A. Ritsch).
[1] These authors contributed equally to the manuscript.

0021-9150/
doi:10.1016/j.atherosclerosis.2012.03.012

well-established model for atherosclerosis. Recently, we succeeded in cloning rabbit SR-BI cDNA and characterized its tissue expression [11]. Rabbit SR-BI cDNA shared a high overall homology with humans - higher than any of the so far identified homologues of other species. The high homology not only confirmed the isolation of rabbit SR-BI, but also promised a similar function when compared to the human homologue [11]. We therefore decided to extend our research to the rabbit system. Overexpression of SR-BI in rabbits using an adenoviral vector led to decreased levels of apoA-I and increased levels of LDL cholesterol, a lipoprotein pattern which is believed to enhance the development of atherosclerosis [12]. In this work we extended these studies in the rabbit system by investigating the impact of hepatic inhibition of SR-BI using the RNA interference technique [13–15].

2. Methods

2.1. Vector construction

Using computational analysis we designed 3 small hairpin RNA sequences targeting the rabbit SR-BI (GenBank AY283277) at the positions 214–232, 717–735, and 1559–1577, respectively (Suppl. Table 1). Corresponding DNA sequences were cloned into a pENTR plasmid (Invitrogen, Carlsbad, CA, USA) under control of the H1 polymerase II promoter. All plasmids were prepared using endotoxin-free Giga-preps (Macherey-Nagel, Dueren, Germany) and only plasmids with a ratio of absorbance at 260 nm/280 nm higher than 1.8 were used for transfection.

The non-viral vector was prepared using galactosylated poly-L-lysine, as previously described [16]. Briefly, 2 mg of poly-L-lysine hydrobromide (Sigma–Aldrich, St. Louis, MO, USA) were dissolved in 1 mL 10 mM sodium-phosphate (pH 7.2), and subsequently mixed with 85 μg of α-D-galactopyranosyl phenylisothiocyanate (Sigma St. Louis, MO, USA) in 1 mL acetone/dimethyl sulfoxide (Sigma St. Louis, MO, USA). The tube was shielded from light by aluminum foil, the solution was gently mixed over night at room temperature and dialyzed six times against 3 L of 5 mM NaCl using a 10 kDa cut off Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL, USA).

Three hundred μg of plasmid were dissolved in 700 mM NaCl, and vortexed at medium speed (1400 rpm). 95 μg of the galactosylated poly-L-lysine (in 150 μL of 700 mM NaCl) were added drop-wise and mixed by gently vortexing. Slow addition of the polycation resulted in the formation of a turbid solution which was subsequently dissolved by stepwise addition of nine 3 μL aliquots of 5 M NaCl. The shape of the galactosylated polylysine complexes was monitored by circular dichroism and electron microscopy (Philips EM 400, Fei Company Electron Optics, Eindhoven, Netherlands). Resulting particles had a diameter of 60–100 nm.

2.2. In vitro studies

The human hepatocellular carcinoma cell line (HuH-7, ECACC, HPA, UK) was cultivated in Dulbecco's Modified Eagle medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum, 1% glutamine and 1% penicillin/streptomycin (Biochrom AG, Berlin, Germany). To test our small hairpin RNA constructs, HuH-7 cells were stably transfected with the previously cloned pCR2.1 plasmid (Invitrogen, Carlsbad, CA, USA) coding for rabbit SR-BI by the use of a fish-transposon [17]. For in vitro studies, cells were transferred into 6-well plates. At a confluence of approximately 75%, they were incubated with 0, 4, 8, and 10 μg galactosylated poly-L-lysine–DNA complexes per 6-well diluted in 2 mL medium, respectively. The amount of rabbit SR-BI cDNA in transfected cells was measured by quantitative PCR.

2.3. In vivo studies

Male New Zealand White (NZW) rabbits (Charles River Laboratories, Kisslegg, Germany) were individually housed in 80 cm × 60 cm × 60 cm rabbit cages (Scanbur, Denmark) with ad libitum access to water in temperature- and humidity-controlled rooms with a 12 h light/dark cycle at the Central Laboratory Animal Facilities in Innsbruck under protocols approved by the Austrian Animal Care and Use Committee. At study termination, they were sacrificed by a threefold overdose of Ketasol/Xylasol (Graeub, Bern, Switzerland). Research was conducted in conformity with the Public Health Service Policy on Human Care and Use of Laboratory Animals.

We first performed a short-term study of 14 days. Animals were acclimated to vivarium conditions one week prior to experimentation, matched for body weight, and divided into 2 groups. Three male NZW rabbits on chow diet were intravenously injected with 30 μg/kg galactosylated poly-L-lysine pENTR214 complexes specific for rabbit SR-BI at days 1, 3, 7, and 13 of the study, while three control animals received 30 μg/kg of scrambled control vector. The animals were fasted for 5 h before collecting blood samples, which were taken twice a week. After 14 days, animals were fasted for 5 h, sacrificed, and tissue samples were snap-frozen.

In order to study the impact of our gene-therapeutical intervention on the development of atherosclerosis in rabbits on a cholesterol-rich diet, two independent long-term experiments were performed. After a lead-in phase of intravenous injections at days 1, 3, and 7, ten rabbits were weekly injected with galactosylated poly-L-lysine pENTR214 (30 μg/kg body weight), and 9 animals received a scrambled control once a week (30 μg/kg body weight). One week after the first Gal-PLL treatment all 19 male NZW rabbits were fed a Western type diet (Ssniff, Soest, Germany) supplemented with 4.9% coco-fat and 2% cholesterol. Food consumption was restricted to 100 g/day/animal. At 8 weeks of treatment, animals were fasted for 5 h, and killed by a threefold overdose of Ketasol/Xylasol (Graeub, Bern, Switzerland). Blood samples were immediately taken by cardiac puncture, plasma was isolated by centrifugation at 2500 rpm, and tissue samples were snap-frozen. The aortas were explanted, fixed with 4% formaldehyde solution (SAV, Flintsbach, Germany), and stained with Sudan IV (C. Roth, Karlsruhe, Germany). Lesion areas were quantified using Image-Pro Plus Software Version 5.1.2 (MediaCybernetics, MD) [18,19].

2.4. RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA from SR-BI expressing HuH-7 cells and liver specimens was prepared using High Pure Isolation Kit (Roche, Mannheim, Germany) and reversely transcribed with Omniscript-RT Kit (Qiagen, Hilden, Germany). Primers and TaqMan probes for SR-BI, ATP-binding cassette A1, low-density lipoprotein receptor, and cholesterol 7α-hydroxylase were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). Modulation of the gene expression was investigated by quantitative real-time Taqman or SybrGreen PCR (Eurogentec, San Diego, CA, USA) using the CFX96 PCR System (BioRad, Hercules, CA, USA) according to the user's manual. Average cycle (Ct) was calculated by CFX96 Manager. House keeping gene α-tubulin 1b was used as reference gene (Suppl. Table 1).

2.5. Western blotting

Western blotting was performed as previously described [12]. Briefly, 10 μg of membrane protein extracts were analyzed using 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and

blotted on nitrocellulose transfer membrane (Whatman, Dassel, Germany). For detection of SR-BI, we used a rabbit anti-rabbit antibody developed in our laboratory at a final concentration of 0.8 μg/mL [11]. The secondary antibody, a polyclonal horse radish peroxidase conjugated goat anti-rabbit antibody (Dako, Glostrup, Denmark), was diluted to a final concentration of 0.1 μg/mL. The chemoluminescent reaction was performed using Super Signal West Dura Reagent (Pierce, Rockford, IL, USA), and blots were visualized by Fluor-S-Imager using Quantity One V4.1 software (BioRad, Hercules, CA, USA).

2.6. Lipid parameters

Total cholesterol and triglycerides were measured in whole plasma of each animal employing Roche commercial kits (Roche, Mannheim, Germany). Free cholesterol was determined using Cholesterol/Cholesteryl Ester Quantitation Kit II according to the users manual (BioVision, CA). Additionally, pooled plasma of each group was subjected to a fast protein liquid chromatography (FPLC) fractionation analysis with two tandem Superose 6 columns (GE Healthcare, Uppsala, Sweden) as described previously [12]. To enable FPLC analysis of hyperlipidemic samples chylomicrons had to be removed by centrifugation (90 min, 3000 × *g*, 4 °C) and an aliquot adjusted to the initial plasma volume was applied to the Superose 6 columns. Additionally, lipoprotein fractions from hyperlipidemic plasma samples were isolated by stepwise ultracentrifugation. Accordingly, two 3.5 ml aliquots of each plasma sample were filled to polycarbonate thick-wall ultracentrifugation tubes (Beckman) and submitted to ultracentrifugation (30 min, 50.000 rpm, 15 °C, Rotor Beckman 50.4 Ti). 0.5 ml of the supernatant were collected and combined to yield 1 ml chylomicron/VLDL solution. The density of the remaining plasma solution was adjusted with NaBr to 1.063 g/ml. Two aliquots of 3.5 ml of this solution were again submitted to ultracentrifugation (16 h, 50.000 rpm, 15 °C, Rotor Beckman 50.4 Ti). 0.5 ml of the supernatant were collected and combined to yield 1 ml LDL solution. To prepare HDL the density of the lower fractions (total volume of 3 ml) was adjusted to 1.21 using NaBr. This solution was equally distributed to two thick-walled tubes and submitted to ultracentrifugation (same conditions as above). 0.5 ml of the supernatant were collected and combined to yield 1 ml HDL solution. Lipoprotein fractions were dialyzed three times against 1 l of PBS using Mini Dialysis Kit 8 kDa (GE Healthcare) and stored at 4 °C. For determination of particle size aliquots of the HDL solution adjusted to the initial plasma volume were analyzed by FPLC. Apolipoprotein measurements were performed by an immunonephelometric assay [20,21]. Plasma activity of cholesterol ester transfer protein was measured with a commercial CETP Activity Assay Kit (BioVision, San Francisco, CA, USA) according to the manufacturer's manual and quantified using a fluorescent reader (Tecan Infinite M200, Maennedorf, Switzerland). Cholesteryl ester transfer was performed as described [12,22]. Serum alanine-aminotransferase, serum aspartate-aminotransferase, γ-glutamyl transferase, alkaline phosphatase and C-reactive protein were measured in a Roche MODULAR Hitachi P800/Elecsys E170 apparatus (Roche, Mannheim, Germany).

2.7. Statistics

Values are presented as mean ± SD. Results were analyzed with unpaired Student's *t*-tests whenever the data were normally distributed. Shapiro–Wilk W testing was performed to check the normality of the data. In the event that a group failed to pass the normality test, the Wilcoxon–Mann–Whitney-*U* test was performed. A difference was considered statistically significant when $P < 0.05$. Statistical analyses were performed using SPSS version 15.0 (Chicago, IL, USA).

3. Results

In vitro and in vivo inhibition of SR-BI was accomplished by RNA interference. As vectors for our experiments we chose non-viral galactosylated poly-L-lysine DNA complexes, as they constitute particles of discrete size and shape suitable for receptor-mediated endocytosis. These particles are selectively targeted to hepatocytes via the asialoglycoprotein receptor, thus allowing an organ-selective, receptor-mediated gene transfer. The complexes were constructed as previously described by Perales et al. [16], and their structure was analyzed by circular dichroism and by electron microscopy (Suppl. Fig. 1).

3.1. In vitro studies

We tested 3 galactosylated poly-L-lysine DNA complexes in vitro in the human hepatocyte cell line HuH-7 stably transfected with rabbit SR-BI (HuH-7/rSR-BI). At a confluence of 75%, HuH-7/rSR-BI cells were exposed to increasing amounts of pENTR214-, pENTR717-, and pENTR1559-complexes, respectively. While small hairpin RNA against the SR-BI nucleotides 717–735 and 1559–1577 did not show any significant effect on gene expression, respectively, hepatocytes transfected with plasmid pENTR214 showed a substantial decrease of the rabbit SR-BI protein expression after an incubation period of two days (Suppl. Fig. 2). Therefore, all further experiments were performed using the pENTR214 plasmid targeting SR-BI nucleotide positions 214–232.

3.2. In vivo studies

After these preliminary in vitro studies, we investigated the short-term effects of this gene-therapeutical intervention in vivo. As animal model we chose rabbits, as they display a human-like lipoprotein profile, express CETP, and develop atherosclerosis upon a cholesterol-rich diet. After 14 days of treatment, we found a marked downregulation of hepatic SR-BI mRNA by 78.0 ± 0.9% ($p = 0.005$) (Fig. 1A), and a corresponding decrease of hepatic SR-BI protein content (Fig. 2). In other organs including spleen, lung and adrenals SR-BI expression was not inhibited upon siRNA treatment, with an even increased SR-BI expression in spleen (Suppl. Fig. 3A). The modulation of hepatic SR-BI expression was associated with a shift in the lipoprotein profile. Rabbits treated with SR-BI-specific small hairpin RNA had significantly lower HDL-cholesterol, and significantly higher VLDL-cholesterol levels when compared to scrambled siRNA treated controls (Fig. 1B). There was no significant change in the activity of CETP per se or in the total plasma triglyceride levels after two weeks of treatment (Table 1). Hepatic expression of the LDL receptor, ATP-binding cassette transporter A1, and cholesterol 7α-hydroxylase was not altered in the two trial arms (data not shown). However, in siRNA treated animals we found a tendency for increased initial CE transfer and a significant 37% increase in total cholesteryl ester transfer (Fig. 1C).

In order to study the effect of hepatic SR-BI inhibition on the development of atherosclerosis, we put the rabbits on a Western type diet supplemented with 2% cholesterol for 8 weeks. Two independent long-term experiments were performed. The animals were injected either with galactosylated poly-L-lysine-pENTR214 complexes (30 μg/kg body weight; $n = 10$), or its scrambled control construct ($n = 9$). To check for potential toxic effects of repeated treatment with poly-L complexes, we measured serum levels of aspartate-aminotransferase (AST), alanine-aminotransferase (ALT), γ-glutamyl transferase, alkaline phosphatase and C-reactive protein. The observed moderately increased serum levels of AST

Table 1
Biochemical characteristics of rabbits after 2 weeks of RNA interference treatment.

	Controls ($n=3$)	siRNA ($n=3$)	p-value
Plasma cholesterol (mg/dL)	80.0 ± 10.0	58.9 ± 12.7	0.087
Plasma triglycerides (mg/dL)	132.0 ± 112.6	130.6 ± 64	0.99
ApoA-I (mg/dL)	102.0 ± 10.4	93.0 ± 5.5	0.23
ApoB (mg/dL)	76.0 ± 5.5	93.0 ± 39.1	0.49
CETP-activity (pmol/μL/h)	17.5 ± 4.5	17.7 ± 0.9	0.86
Lipoprotein lipase (nmol/ml/mmin)	90 ± 5	111 ± 14	0.073

Data presented in % are normalized to the respective controls. siRNA = small interfering RNA.

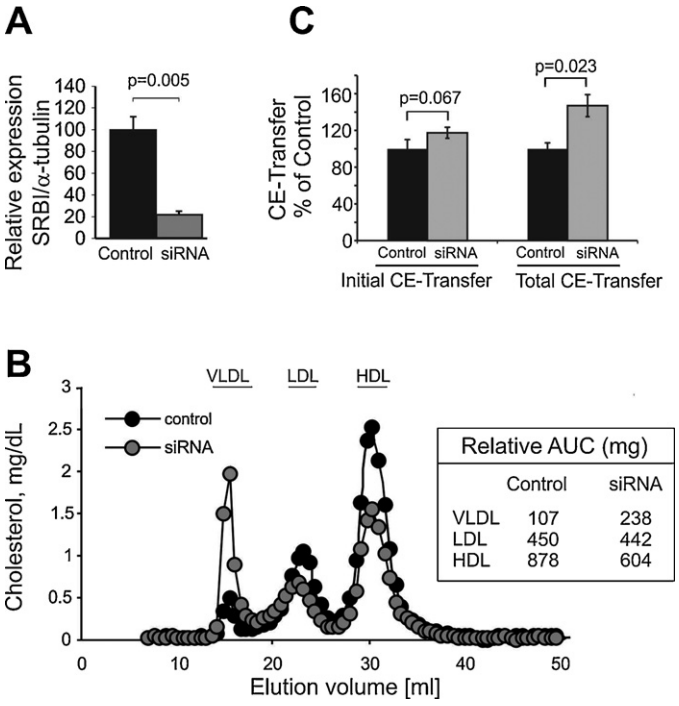

Fig. 1. Down-regulation of hepatic SR-BI expression on RNA level (A) was associated with a shift in the lipoprotein profile (B) and increased total CE-transfer (C) after two weeks of treatment with SR-BI specific galactosylated poly-L-lysine pENTR214 complexes (grey circles), when compared to the scrambled controls (black circles). Plasma was pooled from three animals for chromatography analysis. siRNA = small interfering RNA.

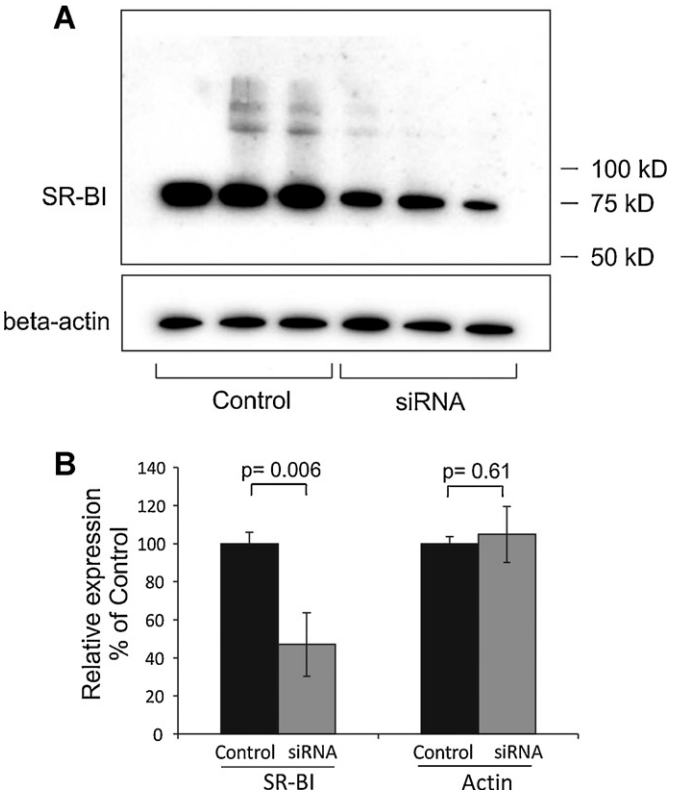

Fig. 2. Down-regulation of hepatic SR-BI expression on protein level after two weeks of treatment with SR-BI specific galactosylated poly-L-lysine pENTR214 complexes (siRNA), when compared to the scrambled controls (Control). A representative blot (A) and quantification (B) of 3 independent experiments are shown. Positions of molecular weight marker bands are indicated.

and ALT in both groups are in line with previous studies by us, where high cholesterol/high fat diet had the same effect in New Zealand White Rabbits [18]. However, all other parameters were not elevated upon treatment suggesting that that rabbits treated with PLL-Gal complexes did not develop severe cholestasis or systemic inflammation (Table 2). Additionally, we observed a steady increase in body weight in both groups of animals (Suppl. Fig. 4A). We monitored total cholesterol levels over time during the experiment (Fig. 3A). As observed in the short term experiment, there was again a shift in the lipoprotein profile from HDL-cholesterol to VLDL-cholesterol after two weeks of treatment (data not shown). Lower HDL-cholesterol levels were associated with decreased

Table 2
Biochemical characteristics of rabbits after 8 weeks of RNA interference treatment.

	Controls ($n=5$)	siRNA ($n=5$)	p-value
Plasma cholesterol (mg/dL)	4800 ± 2074	4689 ± 1687	0.93
Plasma triglycerides (mg/dL)	560 ± 625	212 ± 109	0.35
Free cholesterol (mg/dL)	2638 ± 1115	2287 ± 685	0.28
Alanine-aminotransferase (U/L)	68.8 ± 42.0	92.2 ± 70.8	0.54
Aspartate-aminotransferase (U/L)	57.2 ± 14.8	66.4 ± 26.1	0.56
γ-glutamyl transferase (U/L)	8.4 ± 2.0	11.4 ± 3.0	0.10
Alkaline phosphatase (U/L)	21.2 ± 15.0	16.2 ± 11.0	0.58
C-reactive protein (mg/dL)	N.D.	N.D.	
Initial cholesteryl ester transfer (%)	77.2 ± 9.6	80.2 ± 5.1	0.56
Total cholesteryl ester transfer (%)	72.5 ± 25.1	77.8 ± 18.7	0.72
CETP-activity (pmol/μL/h)	76.0 ± 18.5	63.7 ± 6.4	0.20

siRNA = small interfering RNA; N.D. = non detectable.

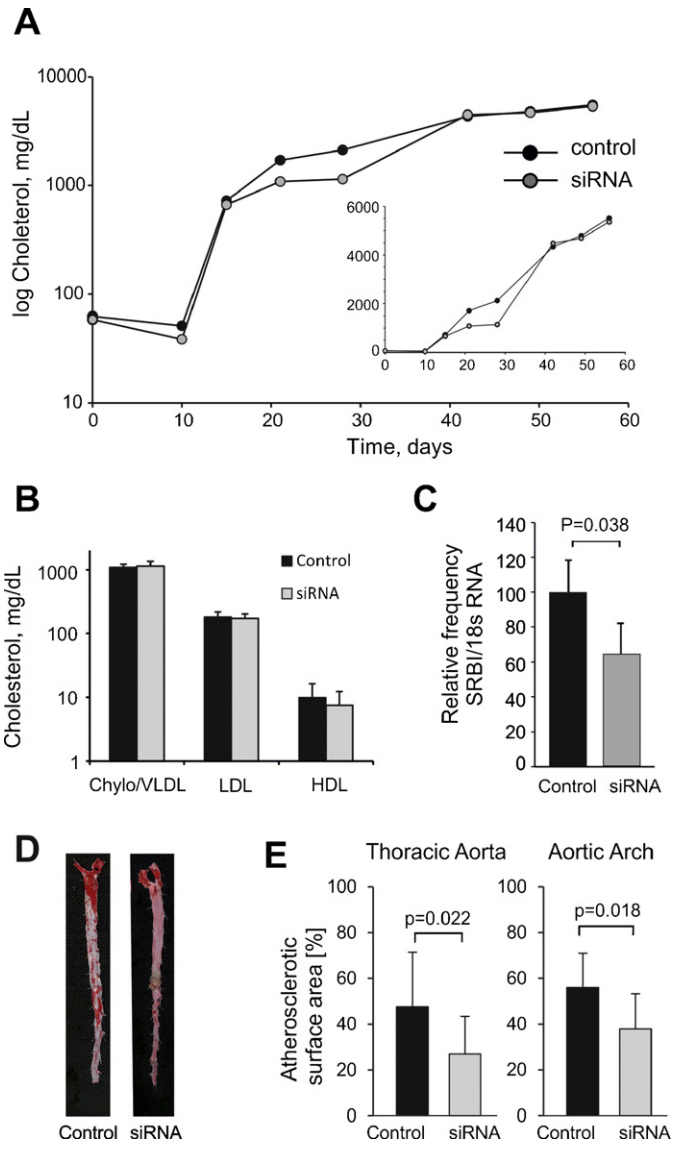

Fig. 3. Rabbits on cholesterol-rich diet were treated with SR-BI specific small hairpin RNA (grey circles) and respective scrambled controls (black circles) for 8 weeks. Total cholesterol levels were analyzed weekly (A). The insert shows the same curves in linear scale. Cholesterol measurements of lipoproteins fractions isolated from hyperlipidemic plasma samples by stepwise ultracentrifugation are shown in (B). Down-regulation of hepatic SR-BI expression on RNA level was shown by real-time PCR (C). Two representative aortas with Sudan IV stained lipid depositions in the intima (D), and the relative areas of atherosclerotic plaques in whole thoracic aortas and in corresponding aortic arches are presented (E). siRNA = small interfering RNA.

apoA-I levels (83% of control; $p = 0.057$) in the treatment group. However, HDL particle size was not altered upon siRNA treatment for two weeks (Suppl. Fig. 4B). These changes resulted in reduced total cholesterol levels. Animals treated with SR-BI-specific small hairpin RNA had total cholesterol levels of 38.4 ± 4.7 mg/dL, while controls displayed cholesterol levels of 51.2 ± 6.6 mg/dL ($p = 0.014$). This difference disappeared at the end of the experiment, when the total cholesterol levels rose to a plateau of nearly 5000 mg/dL in both trial arms (Fig. 3A; Table 2). No differences in levels of lipoprotein fractions isolated by stepwise ultracentrifugation were detected at this stage of the experiment (Fig. 3B). This was confirmed by FPLC analysis of chylomicron depleted plasma samples (Suppl Fig. 4C). The same was true for levels of free cholesterol as well as for initial and total CE transfer (Table 2). Biochemical characteristics of the animals at the end of the experiment are given in Table 2.

SiRNA mediated inhibition of hepatic SR-BI on RNA level was still effective at that time of treatment (Fig. 3C). Most importantly, animals treated with SR-BI specific small hairpin RNA displayed a significant reduction in the relative atherosclerotic lesion area within both thoracic aortas and corresponding aortic arches, respectively (Fig. 3D and E). Individual results from two consecutive experiments are shown in Suppl. Fig. 5

4. Discussion

Hepatic inhibition of SR-BI expression did not increase HDL-cholesterol levels in our animal model of hypercholesterolemic rabbits, but reduced atherosclerosis. The association of low HDL-cholesterol levels with attenuated atherosclerosis is paradoxical in light of human epidemiological data.

After 14 days of treatment with SR-Bi specific hairpin RNA, we found a marked downregulation of hepatic SR-BI mRNA and a corresponding decrease of hepatic SR-BI protein content. SR-BI expression was not inhibited in lung and adrenals reflecting that the major part of PLL-Gal complexes indeed was taken up in the liver. Surprisingly, we found an increased SR-BI expression in spleen extracts, which may be due to increased SR-BI expression in residing macrophages. Macrophage SR-BI has been shown to bind HDL and other lipoproteins, such as native LDL, acetylated LDL, and oxidized LDL [23], enabling bi-directional flux of cholesterol. In murine RAW macrophages and in differentiated human macrophages, SR-BI was shown to be downregulated by incubation with oxidized LDL particles [24]. Since our shRNA vectors were designed to selectively target liver cells, and since in the spleen SR-BI is mainly expressed in monocytes/macrophages, we speculate that the observed upregulation of SR-BI in spleen might be due to decreased loading of macrophages with apoB-containing lipoproteins. Increased SR-BI in macrophages might have enhanced HDL-mediated cholesterol efflux, thereby inhibiting excessive cholesterol accumulation in the vasculature. In line with this hypothesis, Van Eck et al. have shown that the presence of SR-BI in bone marrow-derived cells in LDLr KO mice decreased lesion development after 9 and 12 weeks of Western-type diet feeding [25].

Our results are in contrast to the findings in the SR-BI KO mice that develop a markedly accelerated atherosclerosis [7–9]. This difference in species is probably due to the lack of CETP in mice. Several studies in transgenic mice were focused on the influence of CETP and SR-BI expression on reverse cholesterol transport and the development of atherosclerosis. Previously, Tanigawa et al. showed that CETP expression in SR-BI KO mice restores proper RCT mechanism [26]. In addition, Harder et al. observed a decrease in atherosclerotic lesion development in CETP transgenic SR-BI KO mice, suggesting a direct link between RCT and atherogenesis in these mice [27]. In contrast, Hildebrand et al. found no protective effect of CETP in SR-BI KO mice, despite normalization of the lipoprotein profile and enhanced flux of cholesterol from HDL to VLDL particles. The authors concluded that in contrast to the work by Harder et al. where mice expressed CETP in the liver only, ubiquitous expression of CETP including organs such as liver, spleen, small intestine, kidney, adipose tissue, and macrophages may have differentially affected atherosclerosis development [28]. In studies of Bouhassani et al, cross-breeding of hypomorphic and whole-body SR-BI knockout mice with CETP transgenic animals led to a reduction in atherosclerosis development. The same authors showed that in liver-selective SR-BI KO mice, CETP expression reduces plasma ^{3}H-cholesterol levels after injection of ^{3}H-cholesterol-labeled macrophages, indicating enhanced plasma clearance via CETP [29]. However, in contrast to the studies by Rader's

laboratory, macrophage-to-feces RCT was not improved. Different tissue distribution as well as level of transgene expression may at least partially be responsible for these differences.

Rabbits do express CETP, and thus might be able to bypass the inability of the liver to take up HDL-cholesterol via SR-BI, when this HDL receptor is knocked down. As in normolipidemic healthy humans [30], the CETP pathway seems to be critical for hepatic clearance of HDL-derived cholesteryl esters. Indeed, in our rabbits treated with SR-BI-specific small hairpin RNA we found an increased total transfer of cholesteryl esters. As total transfer of CE is mainly determined by the presence of triglyceride-rich acceptor lipoproteins, this is in good agreement with the found increased VLDL-cholesterol level and unchanged CETP-activity – the latter reflecting CETP concentration in plasma. One explanation might be that in our specific experimental design this alternative transport route of HDL-derived cholesterol via apoB-containing lipoproteins to the liver could be more efficient than the classic route via SR-BI resulting in a decrease of total plasma cholesterol levels and ultimately to the inhibition of atherosclerosis development. Further experiments including HDL-turnover studies and measurement of cholesterol output would be required to prove this hypothesis.

However, our results are in line with the findings by Schwartz et al. who demonstrated that in humans the vast majority of HDL-cholesteryl esters that are secreted into bile are transported to the liver by apoB-containing lipoproteins [30]. Additionally, patients carrying a heterozygous loss of function SR-BI mutation showed increased HDL plasma levels and reduced capacity for efflux of cholesterol from macrophages [31]. These patients had no significant increase in atherosclerosis. This is not in line with data from experiments in SR-BI knock-out mice, where increased levels of HDL were accompanied by increased development of atherosclerosis, which itself can be explained by the absence of CETP in murine plasma. Accordingly, as mentioned above, cross-breeding of SR-BI knockout mice with CETP transgenic animals led to a reduction in atherosclerosis development. Another explanation might be a low statistical power of the human study given the small number of carriers and their relatively young age [27]. Additionally, the possible benefit of increased HDL levels in P297S carriers may be reversed by the concomitant decrease in cholesterol efflux capacity, as well as by decreased platelet and adrenal function in these patients. This might also represent an explanation for the different results found in our study in NZW rabbits, as we used a liver selective system of SR-BI inhibition, which most likely did not affect parameters of platelet and adrenal function. The liver-specific type of SR-BI inhibition may also be responsible for the fact that HDL-cholesterol levels were not increased in our long-term experiments.

After 2 weeks of treatment hepatic SR-BI mRNA levels were reduced by 80% accompanied by reduced SR-BI protein, lower HDL-cholesterol, and higher VLDL-cholesterol levels. Considering the time-course of total cholesterol levels in our long-term experiments, this situation seems to change in the presence of excessive plasma cholesterol levels. Indeed, between week 5 and 8 on cholesterol-rich diet, the rabbits reached a hypercholesterolemic plateau without any significant difference in total cholesterol or HDL-cholesterol levels despite the ongoing intravenous injection of the galactosylated poly-L-lysine pENTR214 complexes. Thus, our gene-therapeutical intervention cannot overcome overt hypercholesterolemia.

Our study does have some limitations, and thus caution is advised when trying to directly extrapolate the presented results to human biology. Firstly, due to the method used, SR-BI inhibition by siRNA was only partial and organ-specific in our animal model. Secondly, New Zealand White rabbits naturally express CETP, but develop atherosclerosis only when fed a Western type diet. Feeding rabbits with a cholesterol-rich diet is associated with hypercholesterolemia, and unnaturally elevated levels of VLDL and chylomicron-like lipoproteins [32]. In contrast, humans at high cardiovascular risk present with pathologically elevated LDL-cholesterol levels. As a consequence, Western type diet fed rabbits represent an atherosclerosis model of lipid-deposition. They develop lipid-rich, collagen- and fibrinogen-poor atherosclerotic plaques in the aortic arch and thoracic aorta, rather than heterogeneous plaques as do humans [32,33]. Thirdly, atherogenesis in humans is driven by chronic oxidative, hemodynamic, or biochemical stimuli (from smoking, hypertension, dyslipidemia) and inflammatory factors, leading to the formation of a fibrous cap over the developing atheromatous plaque, triggering atherothrombosis of the coronary artery and eventually myocardial infarction upon rupture of the cap [34]. It may readily be envisioned that the corresponding scenario in NZW rabbits is displaying several differences to the one observed in humans.

In summary, the present studies show that the in vivo injection of galactosylated poly-L-lysine-pENTR214 complexes is safe and effective to inhibit the hepatic expression of SR-BI. Furthermore, treatment of cholesterol fed rabbits with inhibitory complexes was associated with less atherosclerotic plaques in the aortas. In addition to our data on SR-BI expression in rabbits using an adenoviral vector [12], this work is again supporting the view that the role of SR-BI in lipoprotein metabolism and atherogenesis in rabbits is different from the one seen in rodents.

Conflict of interest

All authors declared that there is not conflict of interest.

Acknowledgments

We are very grateful to Prof. N. Romani for assistance with the electron microscopy.

This work was supported by the Hans & Blanca Moser Stiftung (No. 61–1994/95 to I.T.), by the Medizinische Forschungsfoerderung Innsbruck (MFI No. 4316 to I.T.), by the Jubiläumsfond der Oesterreichischen Nationalbank (OENB, No. 12156 to I.T. and A.R.) and by the Fonds zur Foerderung der wissenschaftlichen Forschung (FWF, P19999-B05 to A.R.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2012.03.012.

References

[1] Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, Krieger M. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science 1996;271:518–20.
[2] Krieger M. Scavenger receptor class B type I is a multiligand HDL receptor that influences diverse physiologic systems. The Journal of Clinical Investigation 2001;108:793–7.
[3] Acton SL, Scherer PE, Lodish HF, Krieger M. Expression cloning of SR-BI, a CD36-related class B scavenger receptor. The Journal of Biological Chemistry 1994;269:21003–9.
[4] Calvo D, Vega MA. Identification, primary structure, and distribution of CLA-1, a novel member of the CD36/LIMPII gene family. The Journal of Biological Chemistry 1993;268:18929–35.
[5] Cao G, Garcia CK, Wyne KL, Schultz RA, Parker KL, Hobbs HH. Structure and localization of the human gene encoding SR-BI/CLA-1. Evidence for transcriptional control by steroidogenic factor 1. The Journal of Biological Chemistry 1997;272:33068–76.
[6] Trigatti BL, Krieger M, Rigotti A. Influence of the HDL receptor SR-BI on lipoprotein metabolism and atherosclerosis. Arteriosclerosis, Thrombosis, and Vascular Biology 2003;23:1732–8.
[7] Braun A, Trigatti BL, Post MJ, et al. Loss of SR-BI expression leads to the early onset of occlusive atherosclerotic coronary artery disease, spontaneous myocardial infarctions, severe cardiac dysfunction, and premature death in apolipoprotein E-deficient mice. Circulation Research 2002;90:270–6.

[8] Rigotti A, Trigatti BL, Penman M, Rayburn H, Herz J, Krieger M. A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. Proceedings of the National Academy of Sciences of the United States of America 1997;94:12610–5.
[9] Zhang W, Yancey PG, Su YR, et al. Inactivation of macrophage scavenger receptor class B type I promotes atherosclerotic lesion development in apolipoprotein E-deficient mice. Circulation 2003;108:2258–63.
[10] Kozarsky KF, Donahee MH, Glick JM, Krieger M, Rader DJ. Gene transfer and hepatic overexpression of the HDL receptor SR-BI reduces atherosclerosis in the cholesterol-fed LDL receptor-deficient mouse. Arteriosclerosis, Thrombosis, and Vascular Biology 2000;20:721–7.
[11] Ritsch A, Tancevski I, Schgoer W, et al. Molecular characterization of rabbit scavenger receptor class B types I and II: portal to central vein gradient of expression in the liver. The Journal of Lipid Research 2004;45:214–22.
[12] Tancevski I, Frank S, Massoner P, et al. Increased plasma levels of LDL cholesterol in rabbits after adenoviral overexpression of human scavenger receptor class B type I. The Journal of Molecular Medicine 2005;83:927–32.
[13] Rubinson DA, Dillon CP, Kwiatkowski AV, et al. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nature Genetics 2003;33:401–6.
[14] Stewart SA, Dykxhoorn DM, Palliser D, et al. Lentivirus-delivered stable gene silencing by RNAi in primary cells. RNA 2003;9:493–501.
[15] Sugano M, Makino N. Changes in plasma lipoprotein cholesterol levels by antisense oligodeoxynucleotides against cholesteryl ester transfer protein in cholesterol-fed rabbits. The Journal of Biological Chemistry 1996;271:19080–3. Issn: 10021-19258.
[16] Perales JC, Ferkol T, Beegen H, Ratnoff OD, Hanson RW. Gene transfer in vivo: sustained expression and regulation of genes introduced into the liver by receptor-targeted uptake. Proceedings of the National Academy of Sciences of the United States of America 1994;91:4086–90.
[17] Ivics Z, Hackett PB, Plasterk RH, Izsvak Z. Molecular reconstruction of Sleeping Beauty, a Tc1-like transposon from fish, and its transposition in human cells. Cell 1997;91:501–10.
[18] Tancevski I, Wehinger A, Demetz E, et al. The thyromimetic T-0681 protects from atherosclerosis. The Journal of Lipid Research 2009;50:938–44.
[19] Tangirala RK, Rubin EM, Palinski W. Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. The Journal of Lipid Research 1995;36:2320–8.
[20] Pruvot I, Fievet C, Durieux C, Vu Dac N, Fruchart JC. Electroimmuno- and immunonephelometric assays of apolipoprotein A-I by using a mixture of monoclonal antibodies. Clinical Chemistry 1988;34:2048–52.
[21] Tailleux A, Labbe J, Mallet C, Percevault F, Fruchart JC, Fievet C. Measurement of rabbit apolipoprotein B by use of electroimmunodiffusion and immunonephelometric assays. CompMED 2000;50:309–13.
[22] Kaser S, Sandhofer A, Holzl B, et al. Phospholipid and cholesteryl ester transfer are increased in lipoprotein lipase deficiency. Journal of Internal Medicine 2003;253:208–16.
[23] Sun B, Boyanovsky BB, Connelly MA, Shridas P, van der Westhuyzen DR, Webb NR. Distinct mechanisms for OxLDL uptake and cellular trafficking by class B scavenger receptors CD36 and SR-BI. The Journal of Lipid Research 2007;48:2560–70.
[24] Han J, Nicholson AC, Zhou X, Feng J, Gotto Jr AM, Hajjar DP. Oxidized low density lipoprotein decreases macrophage expression of scavenger receptor B-I. The Journal of Biological Chemistry 2001;276:16567–72.
[25] Van Eck M, Bos IS, Hildebrand RB, Van Rij BT, Van Berkel TJ. Dual role for scavenger receptor class B, type I on bone marrow-derived cells in atherosclerotic lesion development. The American Journal of Pathology 2004;165:785–94.
[26] Tanigawa H, Billheimer JT, Tohyama J, Zhang Y, Rothblat G, Rader DJ. Expression of cholesteryl ester transfer protein in mice promotes macrophage reverse cholesterol transport. Circulation 2007;116:1267–73.
[27] Harder C, Lau P, Meng A, Whitman SC, McPherson R. Cholesteryl ester transfer protein (CETP) expression protects against diet induced atherosclerosis in SR-BI deficient mice. Arteriosclerosis, Thrombosis, and Vascular Biology 2007;27:858–64.
[28] Hildebrand RB, Lammers B, Meurs I, et al. Restoration of high-density lipoprotein levels by cholesteryl ester transfer protein expression in scavenger receptor class B type I (SR-BI) knockout mice does not normalize pathologies associated with SR-BI deficiency. Arteriosclerosis, Thrombosis, and Vascular Biology 2010;30:1439–45.
[29] El Bouhassani M, Gilibert S, Moreau M, et al. Cholesteryl ester transfer protein expression partially attenuates the adverse effects of SR-BI receptor deficiency on cholesterol metabolism and atherosclerosis. The Journal of Biological Chemistry 2011;286:17227–38.
[30] Schwartz CC, VandenBroek JM, Cooper PS. Lipoprotein cholesteryl ester production, transfer, and output in vivo in humans. The Journal of Lipid Research 2004;45:1594–607.
[31] Vergeer M, Korporaal SJ, Franssen R, et al. Genetic variant of the scavenger receptor BI in humans. The New England Journal of Medicine 2011;364:136–45.
[32] Moghadasian MH, Frohlich JJ, McManus BM. Advances in experimental dyslipidemia and atherosclerosis. Laboratory Investigation 2001;81:1173–83.
[33] Riedmuller K, Metz S, Bonaterra GA, et al. Cholesterol diet and effect of long-term withdrawal on plaque development and composition in the thoracic aorta of New Zealand White rabbits. Atherosclerosis 2010;210:407–13.
[34] Nabel EG, Braunwald E. A tale of coronary artery disease and myocardial infarction. The New England Journal of Medicine 2012;366:54–63.