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BASIC STUDIES

Adenovirus-mediated hepatic syndecan-1 overexpression induces hepatocyte proliferation and hyperlipidaemia in mice

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Keywords
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Abbreviation:
Apo, apolipoprotein; HDL, high-density lipoprotein; HSPG, heparan sulfate proteoglycan; LDL, low-density lipoprotein; SDC-1, syndecan-1; SDC-1/ΔC, syndecan-1 mutant without C-terminal cytoplasmic tail; VLDL, very low-density lipoprotein

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Abstract

Background: Heparan sulfate proteoglycans (HSPGs) have been involved in the regulation of cell growth, apoptosis and lipid metabolism *in vitro*; however, their functional role *in vivo* remains unknown. *Aim:* Here, we describe hepatic tissue and lipid metabolism changes after liver overexpression of syndecan-1 (SDC-1), the main hepatic HSPG, in mice induced by adenoviral gene transfer. *Results:* SDC-1 overexpression was associated with marked hepatocyte proliferation, cell-isolated apoptosis and increased plasma alanine aminotransferase (ALT) levels. Additionally, SDC-1 liver overexpression significantly raised plasma cholesterol and triglyceride concentrations due to an increase in all lipoprotein particles, including the appearance of large and apolipoprotein (apo) E-enriched high-density lipoprotein (HDL) particles. Hepatic very low-density lipoprotein (VLDL) production was not affected by SDC-1 overexpression, suggesting a delayed plasma clearance of apo B lipoproteins as the underlying hyperlipidaemic mechanism. These pleotropic effects were qualitatively equivalent, even though less intense, in mice overexpressing a cytoplasmic C-terminal domain-deleted SDC-1. *Conclusions:* This is the first report *in vivo* of the biological effects induced by a specific HSPG in the liver, with potential implications in both regenerative biology and molecular lipidology.

Heparan sulfate proteoglycans (HSPGs) are involved in several key biological functions ranging from mechanical tissue support to cellular adhesion, growth, and differentiation (1). Thus, HSPG expression and function may be critical in development, regeneration and carcinogenesis *in vivo* (2, 3).

Although the liver contains a broad range of matrix and cell-associated proteoglycans (4), HSPGs are the most abundantly expressed in this tissue. Liver heparan sulfate (HS) chains are characterized by a very high sulfate content (5), determining enhanced *in vitro* affinity for a wide variety of ligands, including apolipoproteins (apo) (6) or viral (7, 8) and protozoan (9) pathogens as well as several growth factors (10, 11). These binding properties suggest the potential involvement of HSPGs, particularly those found on the hepatocellular surface, in various pathophysiological processes such as liver cancer (12), viral hepatitis (7, 8, 13), cirrhosis (14, 15) and hepatic regeneration (16). However, there is no evidence directly supporting any functional consequence of liver HSPGs *in vivo*.

Syndecans are the predominant form of HSPGs found in the liver. They are type-I transmembrane proteins substituted with HS chains in their extracellular domain, which enable them to bind extracellular

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matrix proteins (fibrilar collagen, fibronectin, tensacin, trombospondin), growth factors [fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), transforming growth factor-β1 and -β2, platelet-derived growth factor (PD6F), vascular endothelial growth factor (VEGF)], proteases (catepsin G, neutrophil elastase) and apo E and apo B, leading to ligand internalization as well as cell signaling (1, 2). It has been proposed that syndecans are actually coreceptors, favoring the interaction between ligands and their high-affinity cell surface receptors. Thus, syndecans can influence the concentration, stability, and spatial conformation of ligands and also facilitate ligand and/or receptor oligomerization (2). Additionally, the C-terminal cytoplasmic domain of syndecans binds intracellular proteins, such as PDZ proteins and protein kinase Cα (1), leading to receptor clustering, interaction with the actin cytoskeleton (17) and intracellular signaling (18).

Syndecan-1 (SDC-1), the prototype member of the transmembrane HSPG family (1), is the main proteoglycan expressed by hepatocytes both *in vitro* (19) and *in vivo* (20). Remarkably, SDC-1 is exclusively found on the sinusoidal (basolateral) hepatocellular surface, where it is directly and extensively exposed to many extracellular ligands that cross the fenestrated endothelium and the extracellular matrix of the space of Disse. Indeed, the extracellular SDC-1 core protein and its associated HS chains are integral components of the extracellular matrix of the liver.

SDC-1 has been systematically linked to cell growth regulation, participating in processes such as skin regeneration, inflammation and cancer (1, 21). Interestingly, tumor SDC-1 expression has been associated with histologic markers of cell growth, metastasis rate and clinical outcomes (22). However, the specific effect of SDC-1 on cell growth is not yet clear, mainly because it can either promote (23) or inhibit (24) cellular proliferation depending on the experimental conditions. Remarkably, there is no direct information on the role of SDC-1 in the regulation of hepatocyte proliferation *in vivo*.

On the other hand, HSPGs have been postulated as mediators of hepatic lipoprotein metabolism *in vivo* (25). In a classic experiment, Ji et al. (26) showed that direct infusion of HS-degrading enzymes into the portal circulation of mice dramatically reduced plasma clearance and hepatic uptake apo E-enriched lipoproteins, strongly suggesting a functional role for HSPGs in plasma lipoprotein metabolism. Unfortunately, this and other similar approaches used *in vitro* have not addressed the specific molecular identity of HSPGs that may account for their effects in lipid metabolism. As a sinusoidal transmembrane HSPG facing the plasma compartment, SDC-1 is a very attractive candidate to be involved in lipoprotein physiology.

The primary aim of this study was to evaluate the effects of SDC-1 overexpression in the liver after adenoviral gene transfer in mice, mainly focusing on hepatocyte proliferation and death. Additionally, we evaluated the impact of liver SDC-1 on lipid metabolism, including analyses of plasma total lipid levels, plasma lipoprotein cholesterol distribution and the hepatobiliary lipid phenotype.

Materials and methods

Recombinant adenoviruses

Rat SDC-1 cDNA was amplified by polymerase chain reaction (PCR) using the full sequence of rat SDC-1 cDNA, a kind gift from Dr. D.J. Carey (Weiss Center, Danville, PA, USA). All primers were designed on cDNA sequences available through GeneBank™ databases. SDC-1 primers were 5′-TATGGAAGCTTGTCC GGGCAGCATGACACGTG-3′ (forward) and 5′-TTA CCGATATCCTATTTCCCCATCAGGCGTAG-3′ (reverse). SDC-1/ΔC primers were 5′-TATGGAAGCTTG TCCGGGCAGCATGACACGTG -3′ (forward) and 5′-TAAC TGATATCTCACCGGTATAGCATGAAAGCCA C-3′ (reverse). Recombinant adenoviruses containing either rat SDC-1 full cDNA (Ad.SDC-1) or a mutant cDNA lacking the C-terminal cytoplasmic tail of rat SDC-1 (Ad.SDC-1/ΔC), both under control of the cytomegalovirus promoter, were generated by homologous recombination in bacterial cells using the AdEasy system generously provided by Dr. Bert Vogelstein (John Hopkins University, Baltimore, MD, USA) (27). An empty recombinant adenovirus (Ad.ΔE1), kindly donated by Dr. Karen Kozarsky (SmithKline Beecham Pharmaceuticals, King of Prussia, PA, USA), was used as the control adenovirus. Large-scale production of recombinant adenoviruses was performed in HEK293 cells by standard methods.

Animals

C57BL/6 male mice (8–12-week old) were maintained in a temperature- and humidity-controlled room with reverse light cycling and fed with a normal low-cholesterol chow diet (ProLab RMH 3000; PMI Feeds, St. Louis, MO, USA). For *in vivo* studies, mice ($n = 11$ and 13 for Ad.SDC-1 with 3 and 7 days post infection, respectively; $n = 7$ and 6 for SDC-1/ΔC with 3 and 7 days post infection, respectively) were anaesthetized by ether inhalation, a tail blood sample (100 μl) was taken for preinfection lipid and alanine

aminotransferase (ALT) determinations and 1×10^{11} viral particles of Ad.SDC-1, Ad.SDC-1/ΔC or Ad.ΔE1 (in 0.1 ml of saline buffer) were directly injected into the femoral vein exposed previously. Additional control animals were injected with 0.1 ml saline buffer. Three or 7 days after adenoviral infection, mice were fasted for 8 h, anaesthetized with an intraperitoneal injection of pentobarbital (45 mg/kg), the abdomen was opened and a gallbladder fistula was performed to collect hepatic bile for 30 min. Then, mice were euthanized by exsanguination (0.5–1 ml of blood) before the livers were removed. Three independent series of animals were infected and their samples were collected for 3-day as well as 7-day infections. Bile was maintained at $-20\,^{\circ}C$, while liver samples were flash frozen in liquid nitrogen and stored at $-70\,^{\circ}C$ for subsequent analyses. All chemical blood measurements and biochemical and morphological tissue analyses were performed separately for each animal. Samples were not pooled. Experimental procedures were approved by the Animal Research Advisory Committee of our institution.

Cell culture and adenoviral infection

HEK293 and HEPA 1–6 cells (ATCC, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO2/95% air. For infection, 60 mm culture plates (Orange Scientific, Braine-l'Alleud, Belgium) were seeded at 70% cell confluence. After 20 h of culture, cells were washed with PBS and exposed to 1.3×10^{9} viral particles of Ad.SDC-1, Ad.SDC-1/ΔC or Ad.ΔE1 suspended in 0.6 ml of 2% FBS/DMEM for 2 h. After this period, cells were supplemented with 4.5 ml of growth medium and maintained in culture for 24 h before Western blot analysis or biotin labeling.

Infected HEPA 1–6 cells were washed with cold PBS and labeled with a 0.83 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) solution for 1 h at 4 °C. Subsequently, cells were washed with 0.1 M glycine solution to stop labeling and lysed *in situ* with 10 mM Tris HCl pH 7.5, 1 mM $MgCl_2$, 0.5% NP40. Postnuclear lysates were fractioned with ImmunoPure Immobilized Streptavidin Gel (Pierce) following the manufacturer's recommendations and analyzed by SDC-1 Western blotting.

Northern blot analysis

An SDC-1 cDNA probe (939 bp) was prepared by PCR using the full sequence of rat SDC-1 cDNA cloned in pGEM-T vector as a template (Promega, Madison, WI, USA). An 18S rRNA probe was prepared by standard reverse transcriptase-PCR. While the SDC-1 cDNA probe was labeled with ^{32}P-α-dCTP by PCR, the 18S RNA probe was labeled with ^{32}P-α-dCTP by the random primer method (Promega). Northern blotting was performed as described previously (28–30). Signal quantification was performed by phosphor imaging with the GS-525 Molecular Image System (Bio-Rad, Hercules, CA, USA). SDC-1 mRNA expression levels were normalized to the signal generated from hybridization of the 18S RNA probe on the same filters.

Western blot analysis

Crude postnuclear lysates or total liver membranes were prepared from cells or tissues as described previously (28). 40 μg of protein/sample was size-fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted onto nitrocellulose with a rabbit polyclonal antibody against the extracellular domain of human SDC-1 (clone H-174, Santa Cruz Biotechnology, Santa Cruz, CA, USA). A rabbit polyclonal anti-ε-COP antibody was used as the protein loading control. Immunoblots were visualized by the enhanced chemiluminiscence procedure (Perkin Elmer Life Sciences, Boston, MA, USA) and quantified by the GS-525 Molecular Image System (Bio-Rad).

Genomic DNA electrophoresis

Nuclear DNA from liver samples was isolated and purified with DNAzol (Invitrogen, Carlsbad, CA, USA). DNA integrity was assessed by 1% agarose electrophoresis and ethidium bromide staining.

Caspase-3 activity determination

Colorimetric determination of caspase-3 activity was assessed with the CaspACE Assay System (Promega).

Hematoxilin/eosin staining and proliferating cell nuclear antigen immunohistochemistry

Liver sections were stained with haematoxylin/eosin and immunostained with anti-PCNA (Dako, Glostrup, Denmark).

Lipid and lipoprotein analysis

Plasma total cholesterol was determined by an enzymatic assay (29). Plasma lipoproteins were separated by Superose 6 fast-performance liquid chromatography (FPLC) (29), and total cholesterol content in lipoprotein fractions was enzymatically measured. Ten

microliter of FPLC fractions were separated by 10% SDS-PAGE and immunoblotted with polyclonal antibodies against apo E or apo A-I (Biodesign International, Saco, ME, USA). Immunoblots were visualized by the enhanced chemiluminiscence procedure (PerkinElmer).

Hepatic total, unesterified and esterified cholesterol, triglyceride, and phospholipid contents and biliary cholesterol concentrations were determined as described elsewhere (30). Bile acid contents in bile were measured with the 3α-hydroxysteroid dehydrogenase method (30). Biliary lipid outputs were calculated from biliary lipid concentrations and measured hepatic bile flows.

Very low-density lipoprotein (VLDL) production analysis

Three days after infection, mice were fasted for 8 h and injected with 400 mg/kg solution of Triton WR1339 (Sigma-Aldrich Corp, St. Louis, MO, USA) into the femoral vein. Two hours later, the animals were euthanized, plasma was removed and VLDL was isolated by ultracentrifugation and cholesterol and triglycerides were assessed as described (29).

Statistical analysis

The results are presented as mean ± standard error. The statistical difference between means of the different experimental groups was analyzed by the Student *t* test. A difference was considered to be statistically significant when $P < 0.05$. Statistical analysis and graphs were performed with GRAPHPAD PRISM 4.0 software (GraphPad Software Inc., San Diego, CA, USA).

Results

Recombinant adenovirus-mediated hepatic overexpression of SDC-1 *in vivo*

In order to explore the functional role of SDC-1 in the liver, we infected 8–12-week-old male C57BL/6 mice with recombinant adenoviruses for wild type and mutant tailless forms of SDC-1 (Ad.SDC-1 and Ad.SDC-1/ΔC respectively). Systemic inoculation of both Ad.SDC-1 and Ad.SDC-1/ΔC resulted in significant overexpression of the corresponding transcripts (Fig. 1A) and core proteins (Fig. 1B). As reported previously, endogenous levels of SDC-1 were very low in livers from control-infected mice (31) (Fig. 1A and B). Overexpressed SDC-1 and SDC-1/ΔC core proteins were processed and glycanated, as evidenced by the high-molecular-weight smear that HSPGs exhibit in Western blots. The molecular weight for SDC-1 core

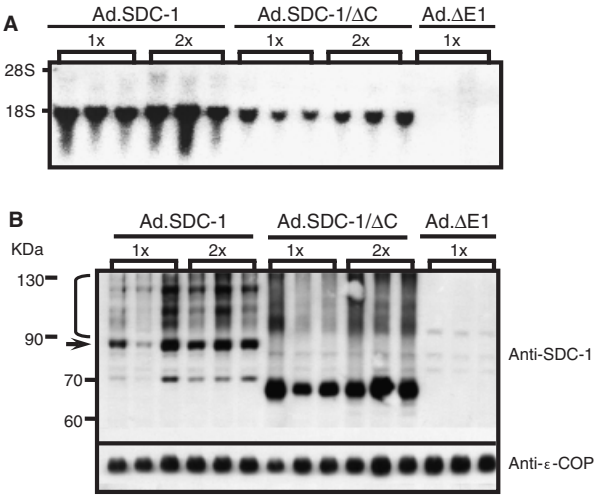

Fig. 1. Recombinant adenovirus-mediated overexpression of wild-type or tailless syndecan-1 (SDC-1) in the murine liver. C57BL/6 mice received a single ($1 \times = 10^{11}$ adenoviral particles) or double ($2 \times$) dose of recombinant adenovirus for wild-type or C-terminal tailless mutant SDC-1 (Ad.SDC-1 and Ad.SDC-1/ΔC, respectively). Control animals were infected with 10^{11} particles of a adenovirus without transgene (Ad.ΔE1). Three days after the infection, mice were euthanized to study liver expression of SDC-1 or syndecan-1 mutant without C-terminal cytoplasmic tail (SDC-1/ΔC) by Northern (A) and Western (B) blot. In panel B protein loading was controlled by ε-COP expression analysis. The bracket indicates high-molecular-weight glycanated syndecan 1 species. The arrow shows the SDC-1 core protein. Blots show representative results of three independent animals for each experimental group.

protein was 88 kDa, whereas that for the C-terminal tailless mutant was 68 KDa, which is concordant with formerly published data (17). Interestingly, wild-type SDC-1 appears to be more efficiently glycanated than SDC-1/ΔC as indicated by the greater high molecular/core protein ratios.

Because the anti-SDC-1 antibodies were not suitable to immunostain this proteoglycan in liver sections and cultured cells, the subcellular distribution of transgenic proteoglycans in hepatocytes was assessed in cultured Hepa 1–6 cells infected with Ad.SDC-1 and Ad.SDC-1/ΔC. After infection, cell surface proteins were biotinylated and subsequently precipitated with streptavidin, separated by SDS-PAGE and immunoblotted with anti-SDC-1. Wild-type and truncated forms of this proteoglycan were significantly overexpressed in whole lysates of cultured Hepa 1–6 cells. SDC-1 was predominantly recovered in the streptavidin pellet, whereas poorly glycanated SDC-1/ΔC was enriched in the non precipitable fraction (Fig. 2A and B respectively). Then, mature transgenic wild-type SDC-1 was mainly localized on the hepatocellular surface, whereas the C-terminal deleted

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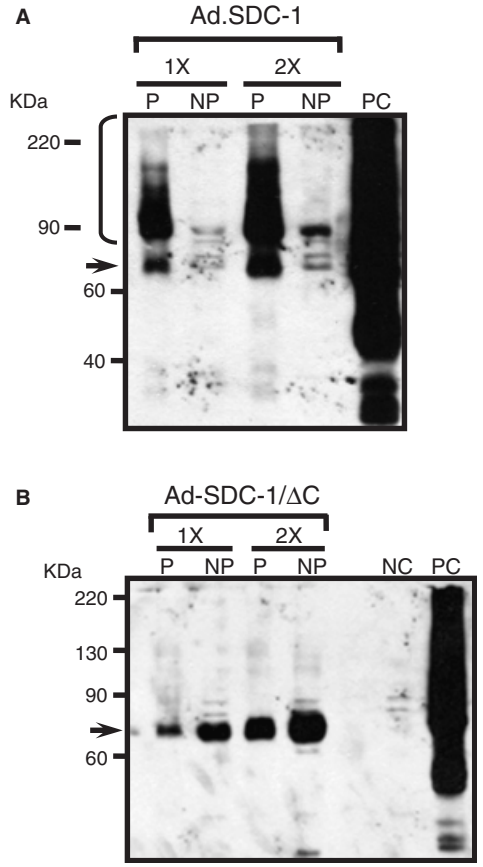

Fig. 2. Subcellular destination of syndecan-1 (SDC-1) and syndecan-1 mutant without C-terminal cytoplasmic tail (SDC-1/ΔC) in recombinant adenovirus-infected Hepa 1–6 cells. Cultured hepatoma Hepa 1–6 cells were infected with a single ($1\times = 6.5 \times 10^7$ particles/cm^2) or double ($2\times$) dose of Ad.SDC-1 (A) or Ad.SDC-1/ΔC (B). Fourty-eight hours post infection, cell surface was biotinylated, streptavidin precipitated and analyzed by Western blot for SDC-1 expression (see Materials and Methods). P, streptavidin pelleted; NP, non streptavidin precipitated; PC,positive control with whole lysates of Ad.SDC-1-infected Hepa 1–6 cells; NC, negative control with whole lysates of Ad.ΔE1-infected Hepa 1–6 cells. The bracket indicates high-molecular-weight glycanated syndecan-1 species. The arrow shows the SDC-1 and SDC-1/ΔC core protein.

mutant seemed to be mainly intracellular and poorly glycanated.

Hepatomegaly and increased hepatocyte proliferation and apoptosis in mice with overexpression of syndecan-1

Macroscopic assessment of SDC-1 or SDC-1/ΔC overexpressing livers revealed a significant increase in average liver mass and liver/body weight ratio, which were directly proportional to overexpression levels and postinfection time (Fig. 3A). Histopathologically, there was a significant increase in the presence of hepatocyte mitotic rate in Ad.SDC-1-infected mice (Fig. 3B). To further evaluate hepatocyte proliferation, PCNA nuclear expression, a reliable marker for cells that actively synthesize DNA, was assessed. Consistent with histopathology, SDC-1 overexpressing livers showed enhanced nuclear PCNA staining (Fig. 3C). Noticeably, hepatomegaly and hepatocyte proliferative responses were significantly less pronounced in SDC-1/ΔC overexpressing mice than those overexpressing wild-type SDC-1.

The well-known cytopathic effects of adenoviral vectors led us to measure plasma ALT levels, a standard marker of hepatocellular damage. Overexpressing SDC-1 or SDC-1/ΔC mice significantly increased plasma ALT levels, which were directly proportional to the transgene overexpression levels and postinfection time (Fig. 4). This hypertransaminasaemic response was stronger in SDC-1 than SDC-1/ΔC overexpressing mice. Importantly, mice infected with control adenovirus without a transgene (Ad.ΔE1) also exhibited plasma ALT elevations relative to animals injected with PBS only (Fig. 4). This hypertransaminasaemia was, nonetheless, negligible compared with that elicited by SDC-1 or SDC-1/ΔC. This latter finding, together with the scarcity of histological evidence of hepatitis or hepatocellular necrosis (Fig. 5A), suggests a primary effect of SDC-1 and SDC-1/ΔC overexpression on liver cell proliferation per se beyond the cytopathic effect induced by adenoviral infection.

Histological analysis also revealed increased number of isolated apoptotic cells (apoptotic bodies) in SDC-1 overexpressing livers compared with control-infected mice (Fig. 5A). To further confirm this morphological finding using biochemical approaches, we assessed DNA fragmentation by agarose gel electrophoresis and measured caspase-3 activation in liver samples. Figure 5B shows no evidence of apoptotic internucleosomal DNA fragmentation (DNA ladder) or unspecific DNA degradation, discarding massive apoptosis or necrosis in both SDC-1 and SDC-1/ΔC overexpressing mice as it was suggested by histopathology. In contrast, the high-sensitivity caspase assay revealed a twofold significant increase in caspase-3 activity (Fig. 5C) in liver samples obtained from mice with SDC-1 overexpression, which was consistent with the presence of isolated apoptotic cells.

Plasma, liver and biliary lipid metabolism in mice with hepatic SDC-1 overexpression

Independent lines of evidence have suggested that HSPGs bind lipoproteins through interactions

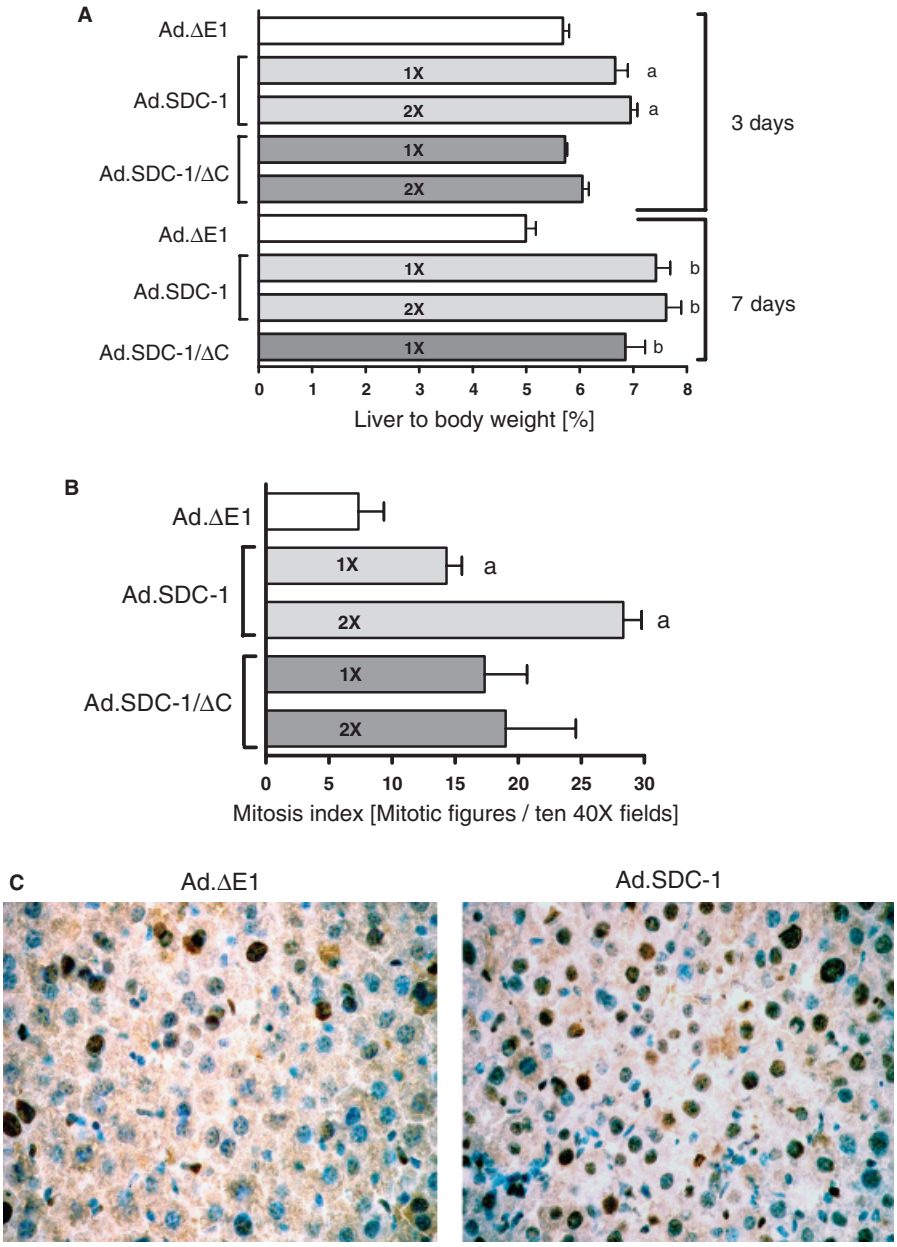

Fig. 3. Liver overexpression of syndecan-1 (SDC-1) induces hepatomegaly and hepatocyte proliferation. C57BL/6 mice were infected with a single (1×) or double (2×) intravenous dose of Ad.SDC-1 or Ad.SDC-1/ΔC. Control animals were infected with Ad.ΔE1. Three or seven days post infection, animals were euthanized and the livers were extracted for morphological analysis. Fresh organ weight was immediately assessed after surgery (A). Liver sections were haematoxylin–eosin stained and mitotic figures were directly counted at 3 days post infection (B). Alternatively, nuclear PCNA expression was evaluated by immunostaining at 3 days post infection (C). 'a' and 'b' indicate p value < 0.05 compared with Ad.ΔE1 mice after 3 or 7 days post infection respectively.

between specific negatively charged HS chain sequences and positively charged apolipoprotein ligands present on the lipoprotein surface (6). Because this interaction may have a physiological impact on hepatic lipoprotein metabolism, we evaluated plasma and biliary lipid phenotypes of mice with hepatic SDC-1 overexpression.

Mice overexpressing SDC-1 exhibited a significant increase in plasma total cholesterol and triglyceride levels (Fig. 6). This mixed hyperlipidaemic phenotype directly depended on both SDC-1 expression levels as well postinfection time. In fact, cholesterolemia increased by 41% at three days and 134% at seven days after infection in high SDC-1 overexpressors.

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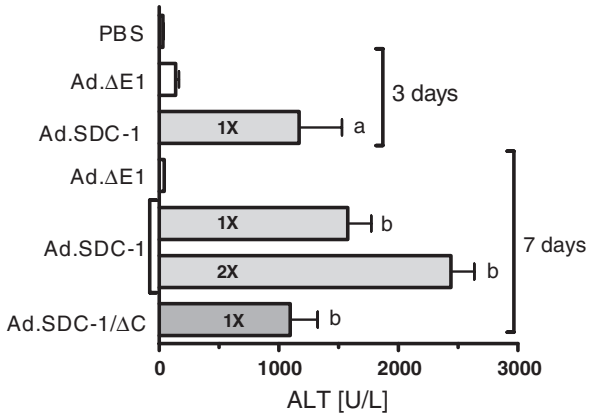

Fig. 4. Liver overexpression of syndecan-1 (SDC-1) and syndecan-1 mutant without C-terminal cytoplasmic tail (SDC-1/ΔC) increases plasma ALT in mice. C57BL/6 mice were infected with a single (1 ×) or double (2 ×) intravenous dose of Ad.SDC-1 or Ad.SDC-1/ΔC. Control animals were infected with Ad.ΔE1. Three or seven days post infection, animals were euthanized and plasma extracted for plasma ALT analysis. 'a' and 'b' indicate p value < 0.05 compared with Ad.ΔE1 mice after 3 or 7 days postinfection respectively.

Triglycerides increased by 65% at seven days after viral inoculation in mice with high levels of expression of the SCD-1 transgene (Fig. 6, inset). Plasma unesterified to total cholesterol ratio was normal in SDC-1 overexpressing mice (results now shown), excluding a significant cholestatic abnormality as the major cause of the lipid changes found in these animals.

To further explore the plasma lipid phenotype of SDC-1 overexpressing mice, we analyzed the relative lipoprotein cholesterol distribution by FPLC. As shown in Fig. 7A, SDC-1 overexpression increased plasma cholesterol content in all lipoprotein fractions, particularly IDL/low-density lipoprotein (LDL) and high-density lipoprotein (HDL). In addition, the elution peak of HDL was significantly expanded to the left, suggesting the presence of more heterogenous and larger HDL particles. Indeed, Western blotting detected apo A-I in these enlarged lipoproteins, establishing their identity as HDL (Fig. 7B). Additionally, apo E was increased in both HDL and LDL even though a more dramatic enrichment within the range of the LDL particle size was evident (Fig. 7B). As shown for histopathology, SDC-1/ΔC overexpression induced qualitatively similar, but quantitative less intense total plasma lipid and lipoprotein changes to those detected in wild-type SDC-1 overexpressing mice (Fig. 6 and 7A).

The significant increase in plasma IDL/LDL cholesterol and triglycerides caused by SDC-1 overexpression may be due either to increased hepatic VLDL production or delayed lipoprotein clearance. To distinguish between these two mechanisms, we measured hepatic VLDL production in Ad.SDC-1 and control-infected mice after Triton WR1339-mediated inhibition of lipoprotein lipase, the main remodeling enzyme involved in VLDL catabolism. Remarkably, no significant differences were found in VLDL cholesterol (Fig. 8A) or VLDL triglyceride (Fig. 8B) accumulation in plasma of SDC-1 overexpressing mice compared with controls. Then, the increased IDL/LDL phenotype caused by SDC-1 is likely due to delayed IDL lipoprotein remodeling and/or LDL clearance from plasma.

In addition, SDC-1 or SDC^2-1/ΔC overexpression did not affect liver total cholesterol content (including unesterified cholesterol to cholesteryl ester ratio), bile flow or biliary cholesterol and bile salt secretion (not shown).

Discussion

Here, we report a significant proliferative response associated with isolated apoptosis of hepatocytes after SDC-1 overexpression *in vivo*, which is consistent with the well-known growth factor-modulating activity reported for HSPGs *in vitro* (1, 2). Additionally, we found a hyperlipemic phenotype that was unexpected based on the current working hypotheses on the role of HSPGs in lipoprotein metabolism.

With regard to the proliferative phenotype, even though we cannot rule out a regenerative reactive response in our experiments, the remarkable histological disparity between hepatocyte proliferation and cell death strongly suggests a primary effect of hepatic SDC-1 overexpression on cell growth regulation. SDC-1 binds FGF2 and HGF through its HS moieties and presents these trophic factors to specific cell surface receptors, promoting cell proliferation *in vitro* (23, 32). Additional evidence also supports a primary effect of SDC-1 in cell growth regulation *in vivo*. Alexander et al. (33) found that SDC-1 is a critical determinant of mammary epithelium carcinogenesis in Wnt-1 overexpressing mice. Moreover, increased bone marrow SDC-1 expression positively correlates with neo-angiogenic response and bone marrow HGF and FGF2 levels in multiple myeloma (34), which are negatively associated with survival in human subjects with this disease (35). Even more relevant to our work, it has been reported that SDC-1 gene expression is positively regulated after partial hepatectomy in rats (36), a response that is likely secondary to increases in growth factor and interleukin levels within the liver that enhance SDC-1 transcription (37), strongly

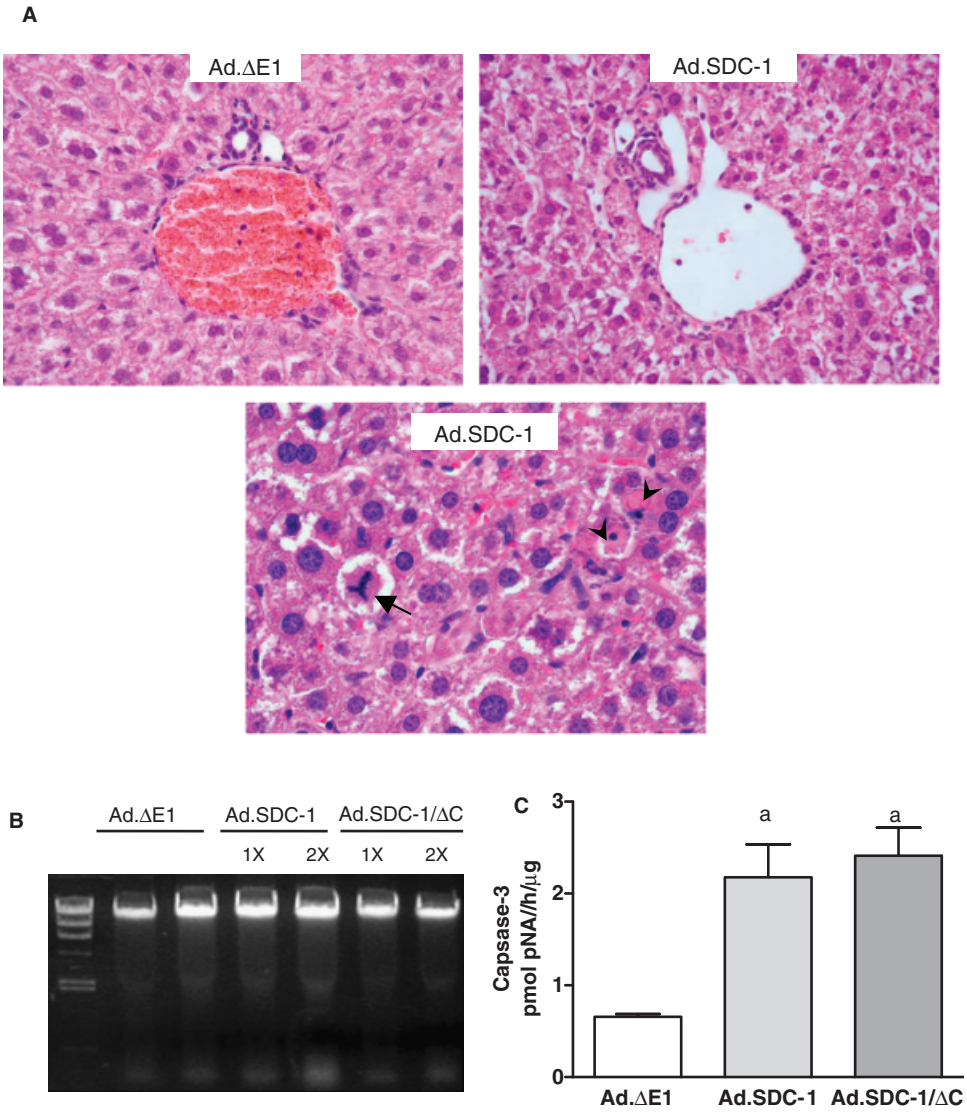

Fig. 5. Liver overexpression of syndecan-1 (SDC-1) and syndecan-1 mutant without C-terminal cytoplasmic tail (SDC-1/ΔC) increases apoptosis in mice. C57BL/6 mice were infected with a single (1 ×) or double (2 ×) intravenous dose of Ad.SDC-1 or Ad.SDC-1/ΔC. Control animals were infected with Ad.ΔE1. Three days postinfection, animals were euthanized and the livers were extracted for further analysis. Haematoxylin–eosin-stained liver sections were directly analyzed for apoptotic bodies. The arrowheads show apoptotic bodies, and the whole arrow indicates an atypical mitosis (A). DNA aliquots from frozen liver samples were electrophoresed in agarose gels and stained with ethidium bromide for visualization (B). Liver caspase-3 activity was colorimetrically assessed (C). 'a' indicates p value < 0.05 compared with Ad.ΔE1 mice.

supporting a role for SDC-1 in hepatocyte proliferation and liver regeneration.

In contrast, there is less clear information about any specific role(s) that HSPGs may play in apoptosis. This is mainly because conflicting evidence suggests both pro-apoptotic and anti-apoptotic functions. It has been shown that SDC-2 overexpression is associated with increased apoptosis rate in cultured osteosarcoma cells (38) and that glypican-3 gene mutation causes the congenital overgrowth Simpson–Golabi–Bemhel syndrome, suggesting diminished apoptosis during development (39). However, glycome and transcriptome analyses in endothelial cells exposed to pro-apoptotic stimuli show that HS degradation may be a key step underlying programmed cell death (40), suggesting that HSPGs, under normal conditions, promote cell survival, protecting from apoptosis. Indeed, a positive correlation has been reported

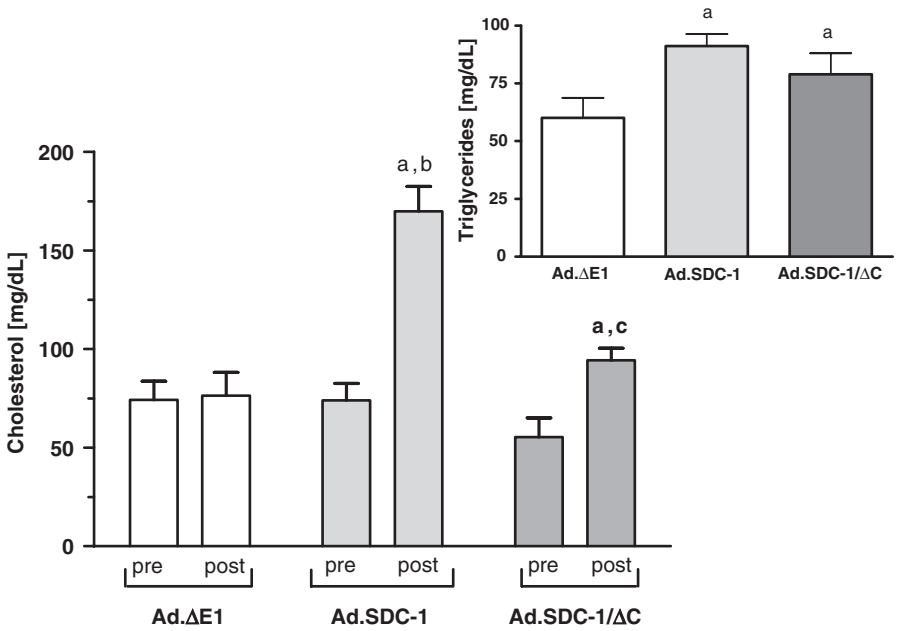

Fig. 6. Liver overexpression of syndecan-1 (SDC-1) and syndecan-1 mutant without C-terminal cytoplasmic tail (SDC-1/ΔC) increases plasma cholesterol and triglycerides. Before infection, tail vein blood samples from C57BL/6 mice were extracted and analyzed for preinfection (pre) plasma lipids. Seven days after infection with 10^{11} intravenous particles of Ad.SDC-1, Ad.SDC-1/ΔC or empty adenovirus Ad.ΔE1, animals were euthanized for plasma lipid analysis (post). 'a' indicates p value < 0.05 compared with Ad.ΔE1 mice, 'b' and 'c' denote $p < 0.05$ with regard to the same mice before infection (pre) with Ad.SDC-1 or Ad.SDC-1/ΔC respectively.

between heparanase gene expression and the amount of apoptotic cells in human liver carcinoma specimens (41). Moreover, cultured hepatoma cells transfected with hSulf1 sulfatase have shown reduced activity of proliferative signaling pathways and increased sensibility to apoptotic death after the use of cytotoxic drugs (42).

Our results support that hepatic SDC-1 expression exhibit direct pro-apoptotic activity in the murine liver *in vivo*. Additional evidence derived from other cellular types (23, 32, 33) is also consistent with the pro-apoptotic phenotype observed in SDC-1 overexpressing mice. It is possible that hepatic SDC-1 HS chains modulate the interactions between soluble ligands (i.e. growth factors) and hepatocellular receptors, resulting either in enhanced cell growth and/or apoptosis depending on the presence or absence of additional tissular growth factors or extracellular matrix proteins. In this regard, it has been described that, in contrast to cell surface-bound SDC-1, its soluble ectodomain released by shedding strongly inhibits heparin-mediated FGF-2 cell proliferation *in vitro* (43). Notably, degradation of sulfated HS regions of soluble SDC-1 by platelet heparanase fully restores FGF-2 mitogenicity (44). Thus, further detailed molecular studies are necessary to elucidate the molecular mechanisms that may explain the complex response induced by SDC-1 on hepatocyte proliferation and apoptosis regulation.

With regard to the lipid phenotype, the working hypothesis for HSPG function predicts that hepatic overexpression of SDC-1, a putative lipoprotein coreceptor, would reduce plasma cholesterol levels as a consequence of increased lipoprotein cholesterol uptake leading to potential changes in hepatic and/or biliary cholesterol content. Unexpectedly, lipid changes observed in SDC-1 overexpressing mice were exactly the opposite: plasma cholesterol and triglycerides increased and hepatic/biliary cholesterol and bile salts levels were not changed. Cholesterol increases in all lipoprotein fractions associated with larger HDL particle size and apo E enrichment of HDL and IDL/LDL suggest that the mechanism underlying the hyperlipemic phenotype induced by SDC-1 may primarily be an impairment in plasma lipoprotein remodeling and/or clearance, rather than increased hepatic lipoprotein synthesis. This hypothesis is supported by the unchanged rate of hepatic VLDL cholesterol and triglyceride secretion when SDC-1 overexpressing mice were compared with controls.

Considering the well-known high-capacity/low-affinity ability of HSPGs to bind lipoproteins, we propose that the lipid phenotype reported here may result from

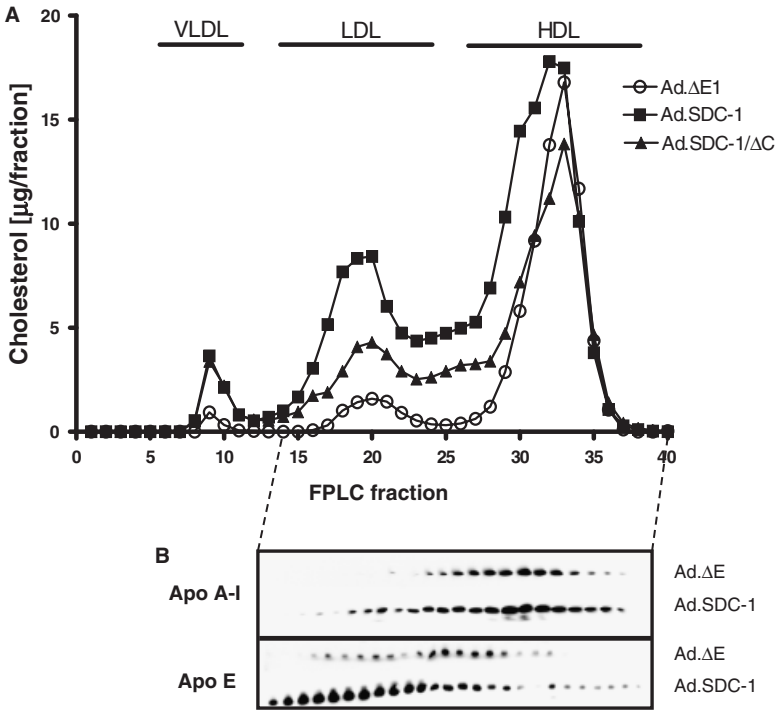

Fig. 7. Liver overexpression of syndecan-1 (SDC-1) and syndecan-1 mutant without C-terminal cytoplasmic tail (SDC-1/ΔC) changes plasma lipoprotein cholesterol and apolipoprotein (apo) profiles. C57BL/6 mice were infected with 10^{11} intravenous particles of Ad.SDC-1 or Ad.SDC-1/ΔC. Control animals were infected with Ad.ΔE1. Seven days post infection, animals were euthanized and plasma was fractioned by fast-performance liquid chromatography (FPLC). Cholesterol content (A) and Western blot analysis for apo A-I and apo E (B) were assessed in each FPLC fraction.

competition of lipoprotein binding between overexpressed SDC-1 and specific high-affinity lipoprotein receptors normally found on the sinusoidal surface of hepatocytes. This competition would lead to diminished hepatic lipoprotein processing and uptake and their subsequent accumulation in the plasma. Alternatively, hepatic SDC-1 overexpression on the basolateral hepatocyte surface may have mimicked a reduction/lack of endothelial fenestrae by increasing electronegativity within the space of Disse, impairing the normal passage of lipoproteins from plasma through the sinusoidal endothelium toward the hepatocyte cell surface. Indeed, this potential mechanism may be analogous to the 'liver sieve hypothesis' that postulates that anatomic stretching of the hepatic sinusoidal endothelium fenestrae leads to reduced porosity of the liver sieve, impaired hepatic lipoprotein uptake and increasing plasma lipoproteins, mainly in the form of large-size particle fractions (45). Interestingly, liver sinusoidal defenestration after either synthetic surfactant intravenous infusion (46) or genetic downregulation of liver VEGF signaling (47) induced a strong hyperlipidaemic response and reduced liver uptake of fluorescent-labeled lipoproteins associated with perturbed hepatic lipid homeostasis.

Even though it is possible that hepatic tissue changes secondary to adenovirus-mediated SDC-1 overexpression reported here may have influenced plasma lipoprotein metabolism, precluding definitive mechanistic interpretations on the lipid phenotype, it has systematically been reported that acute hepatitis of both viral and alcoholic etiology (48–50) as well as chronic viral hepatitis and liver cirrhosis (51, 52) are usually associated with a reduction, rather than an increase, in plasma total and HDL cholesterol in humans. On the other hand, the influence of liver proliferation and/or regeneration on lipid metabolism is not yet well defined. In fact, while hepatic cholesterol biosynthesis (53) and LDL receptor mRNA levels (54) seem to increase during accelerated liver cell turnover, possibly secondary to a higher cholesterol requirement for membrane synthesis, plasma cholesterol changes show major discrepancies depending on the experimental models used. For example, whereas liver regeneration secondary to partial hepatectomy (55) and xenobiotic-induced hepatocyte preneoplastic cellular growth (56)

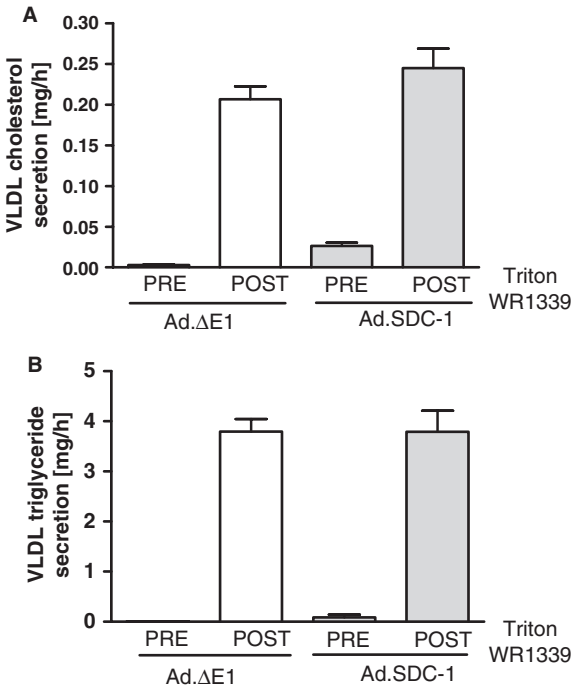

Fig. 8. Liver overexpression of syndecan-1 (SDC-1) and syndecan-1 mutant without C-terminal cytoplasmic tail (SDC-1/ΔC) does not change hepatic very low-density lipoprotein (VLDL) cholesterol and triglyceride secretion. C57BL/6 mice were infected with 10^{11} intravenous particles of Ad.SDC-1, Ad. SDC-1/ΔC or Ad.ΔE1. Three days post infection, peripheral lipoprotein lipase activity was inhibited by intravenous injection of Triton WR1339. Blood samples pre- and 2 h post infusion of Triton WR1339 were taken for plasma VLDL cholesterol (A) and triglyceride (B) measurements.

correlated with a hypocholesterolemic phenotype, intravenous infusion of human recombinant HGF increased hepatocyte proliferation and was associated with cholesterol, triglyceride, and phospholipid enrichment of plasma lipoproteins and an increase in hepatic cholesterol synthesis and VLDL secretion in both mice and cultured hepatocytes (57, 58). In this context, it is possible that the SDC-1 overexpression-dependent phenotypes observed in these mice may be explained by unifying hypothesis in which SDC-1 facilitated HGF activity acting as a coreceptor for this growth factor, which led to enhanced intrahepatic HGF/c-Met signaling as the initiating event for liver cell proliferation and hyperlipemia *in vivo*. This hypothesis awaits further testing.

With regard to the abnormal hepatic posttranslational processing of SDC-1 lacking its C-terminal intracellular domain, we do not have a definitive explanation. The functional role of the C-terminal domain of SDC-1 as a determinant of proper subcellular destination, glycanation and cell signaling of this HSPG is controversial (17, 59). Regardless of the underlying mechanism, experiments overexpressing this tailless mutant form of SDC-1 showed a qualitatively identical, even though of a lower magnitude, response in liver cell proliferation and plasma lipid phenotype. We hypothesize that this finding may be explained by the significant lower expression and less glycanated SDC-1/ΔC on the hepatocellular surface (Fig. 2A and B) as a consequence of defects in intrahepatic trafficking and/or posttranslational modification. However, we cannot rule out that the attenuated effects of SDC-1/ΔC on hepatocyte cell growth and lipid metabolism were owing to decreased intrinsic activity of this HSPG as a consequence of the lack of its C-terminal cytoplasmic domain itself or impaired interaction with intrahepatic adaptor proteins.

In summary, this study is the first report that directly evaluates the impact of liver overexpression of a specific hepatic HSPG such as SDC-1 on hepatocyte proliferation and plasma and biliary lipids levels. Even though our findings seem to be relevant for regenerative liver biology, further research using alternative molecular and pharmacological strategies to modulate SDC-1 expression in the liver will be needed to gain more insights into the actual importance and applications of SDC-1 function to liver physiology and pathology *in vivo*.

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