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Shedding of syndecan-1 from human hepatocytes alters VLDL clearance

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Abstract

We recently showed that the heparan sulfate proteoglycan syndecan-1 mediates hepatic clearance of triglyceride-rich lipoproteins in mice based on systemic deletion of syndecan-1 and hepatocyte-specific inactivation of sulfotransferases involved in heparan sulfate biosynthesis (MacArthur et al. (2007) *J. Clin. Invest.* 117:153–164; Stanford et al. (2009) *J. Clin. Invest.* 119:3236–3245; Stanford et al. (2010) *J. Biol. Chem.* 285:286–294). In this report we show that syndecan-1 expressed on primary human hepatocytes and Hep3B human hepatoma cells can mediate binding and uptake of VLDL. Syndecan-1 also undergoes spontaneous shedding from primary human and murine hepatocytes and Hep3B cells. In human cells, phorbol myristic acid (PMA) induces syndecan-1 shedding, resulting in accumulation of syndecan-1 ectodomains in the medium. Shedding occurs through a protein kinase C-dependent activation of A Disintegrin and Metalloproteinase-17. PMA-stimulation significantly decreases DiD-VLDL binding to cells, and shed syndecan-1 ectodomains bind to VLDL. Although mouse hepatocytes appear resistant to induced-shedding in vitro, injection of lipopolysaccharide into mice results in loss of hepatic syndecan-1, accumulation of ectodomains in the plasma, impaired VLDL catabolism, and hypertriglyceridemia.

Conclusion—These findings suggest that syndecan-1 mediates hepatic VLDL turnover in humans as well as in mice and that shedding might contribute to hypertriglyceridemia in patients with sepsis.

Keywords

Heparan sulfate; proteoglycans; triglycerides; lipoproteins; ADAM17

INTRODUCTORY STATEMENT

Hypertriglyceridemia is a common disorder that results from the accumulation of remnant triglyceride-rich lipoproteins (TRLs) in the circulation. TRLs include chylomicrons derived from dietary lipids, very low density lipoproteins (VLDL) from the liver, and the remnant particles that remain after the action of lipoprotein lipase in the vasculature. These remnant lipoproteins are cleared from the circulation via endocytic receptors in the liver, including

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the low density lipoprotein receptor (LDLR) (1, 2), the LDLR-related proteins (LRPs) (3, 4), lipolysis stimulated receptor (5) and one or more heparan sulfate proteoglycans (6, 7). The syndecans are type I transmembrane proteoglycans bearing up to three heparan sulfate chains and, in some family members, two chondroitin/dermatan sulfate chains (8). Genetic studies in which heparan sulfate assembly was selectively altered in mouse hepatocytes by Cre-loxP targeting of two sulfotransferases demonstrated the importance of the heparan sulfate chains in hepatic TRL clearance (9, 10). Furthermore, direct genetic evidence has been provided showing that syndecan-1 is the primary heparan sulfate proteoglycan mediating hepatic clearance of TRLs in mice (11). Its role in lipoprotein clearance in human hepatocytes is less clear, with some data suggesting that its primary role may be in binding of the remnants to the cell surface with subsequent transfer to other receptors (12, 13).

In many cultured cells, syndecan-1 is constitutively shed from the cell surface into the growth medium (14). Inducers such as growth factors, bacterial virulence factors, ceramide, and the protein kinase C agonist phorbol myristic acid (PMA) accelerate shedding by activation of one or more secreted or membrane-associated metalloproteinases including MMP-7, MMP-9, MMP-14, and ADAM-17 (14). Although the exact biological significance of syndecan-1 shedding is unclear in most cells, one important role appears to be in the regulation of chemokine activity during bacterial infection (15–17). The role of syndecan-1 shedding in lipoprotein metabolism has not been studied.

In this report, we examined the activity of syndecan-1 in TRL clearance in Hep3B human hepatoma cells and in primary human hepatocytes. We show that syndecan-1 is expressed by both human hepatoma cells and primary human hepatocytes and demonstrate its action as a TRL receptor. Syndecan-1 undergoes spontaneous proteolytic shedding from both human and mouse hepatocytes mediated by ADAM-17. In mice, LPS administration induces syndecan-1 shedding from the liver and reduces hepatic VLDL catabolism. These findings suggest that shedding of syndecan-1 may play a role in plasma lipid homeostasis.

EXPERIMENTAL PROCEDURES

Mice and animal husbandry

Wildtype C57BL/6 and *Ldlr*^{-/-} mice on a C57BL/6 background were purchased from The Jackson Laboratory. All animals were housed and bred in vivaria approved by the Association for Assessment and Accreditation of Laboratory Animal Care located in the School of Medicine, UCSD, following standards and procedures approved by the UCSD Institutional Animal Care and Use Committee. Mice were maintained on a 12-hour-light/12-hour-dark cycle, and fed ad libitum with water and standard rodent chow (Harlan Teklad).

Cell culture

Hep3B hepatocarcinoma cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, sodium pyruvate, penicillin and streptomycin. Normal human hepatocytes were obtained through the Liver Tissue Cell Distribution System (Pittsburgh, PA) funded by NIH Contract N01-DK-7-0004/HHSN267200700004C. Primary mouse hepatocytes were isolated and cultured as described previously (11) and used within 2 days of isolation.

Syndecan-1 shedding

To measure spontaneous syndecan-1 shedding, Hep3B cells were seeded in 12-well plates (3×10^5 cells) and incubated in serum-free medium. To induce shedding, cells were stimulated with 0.25 μ M phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich, Saint Louis, MO) for

1 hr at 37°C. The effect of various metalloproteinase inhibitors and signaling pathway inhibitors was surveyed by pretreating cells for 1 hr with 10 μ M of Marimastat (Tocris bio, Ellisville, MO), GI254023 or GW280264 (provided by Dr. Andreas Ludwig, RWTH Aachen University), Bisindolylmaleimide I (BIM I), U0126 and SB 203580 (EMD, San Diego, CA) or vehicle (dimethylsulfoxide, Sigma-Aldrich). In all shedding assays, conditioned media were collected and centrifuged to remove cell debris before further analysis.

Dot blotting

Conditioned media or diluted plasma samples were applied to cationic polyvinylidene difluoride-based membranes (Hybond-N+, Amersham Biosciences, Piscataway, NJ) under mild vacuum in a bio-dot apparatus (Bio-rad, Hercules, CA). The membrane was blocked in 5% non-fat milk buffer and incubated with human syndecan-1 mAb B-A38 (AbD Serotec, Raleigh, NC) or mouse syndecan-1 mAb 281-2 (BD Biosciences, San Diego, CA) and a secondary HRP-conjugated anti-mouse IgG (BD Bioscience, Franklin Lakes, NJ) or anti-rat IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were visualized by chemiluminescent substrate (Pierce, Rockford, IL) and analyzed with an Alpha Innotech Imager system (Cell Biosciences, Santa Clara, CA). All values were scaled relative to that obtained for the indicated control in each figure and expressed as a percentage.

Western blotting

Hepatocytes were incubated with a mixture of 2 mU/ml of heparin lyase I and II and 5 mU/ml of heparin lyase III in serum-free medium at 37°C for 15 min. Cells were subsequently lysed in RIPA buffer supplemented with 1X protease inhibitor cocktail (Sigma-Aldrich). Each sample (10 μ g of protein) was resolved on a 4–12% Bis-Tris NuPage gel (Invitrogen) and transferred to polyvinylidene difluoride membrane (PVDF; Bio-rad, Hercules, CA). The membrane was blocked with Super-Block buffer (Pierce). Blots were incubated with B-A38 [1:500] or anti-ADAM17 polyclonal antibody ab2051 [1:500] (Abcam, Cambridge, MA) and secondary antibodies (HRP-conjugated anti-mouse IgG (BD Biosciences). Reactive bands were visualized by chemiluminescence.

RNA interference

siRNAs specifically targeting human ADAM-17 (5'-AGAGAGUACAACUACAAA-3', 5'-GCAGUAAACAAUCAUCU-3', and 5'-GGAGAUUUGUUAUGAUA-3) and human syndecan-1 (5'-AGAUAUCACCUUGUCACA-3', 5'-CCAGUAGACCUUGUUAUCU-3' and 5'-GGAGACAGCAUCAGGGUU-3') were obtained from Integrated DNA Technologies (Coralville, IA). A scrambled siRNA was used as a control. All siRNAs were transfected into cells by using Transfectin (Integrated DNA Technologies, San Diego, CA).

VLDL binding and uptake

Human VLDL ($\delta < 1.006$ g/ml) was isolated from plasma by buoyant density ultracentrifugation as described (11) and quantified by BCA protein assay (Pierce). To label the particles, 1–2 mg of VLDL were combined with 100 μ L of 3 mg/mL 1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD; Invitrogen) in DMSO and then re-isolated by ultracentrifugation. After incubation with VLDL, cells were rinsed with PBS and lysed by adding 0.1 M NaOH plus 0.1% SDS for 40 min at room temperature (18). Fluorescence intensity was measured with appropriate excitation and emission filters in a plate reader (TECAN GENios Pro, Switzerland) and normalized to total cell protein. In some experiments, VLDL binding and uptake was measured by flow cytometry.

VLDL Plasma Clearance

Mice were fasted for 6 h and injected via the tail vein with 20 μ g of human VLDL protein. Serial samples were taken by tail vein bleeds and the amount of human VLDL remaining in the plasma was determined by sandwich enzyme-linked immunosorbent assay as previously described and plotted relative to VLDL detected at 2 min (19).

Ectodomain binding

To radiolabel glycosaminoglycans, hepatoma cells were incubated for 48 h in growth medium supplemented with 10% dialyzed fetal bovine serum containing 100 μ Ci/ml Na³⁵S]O₄ (PerkinElmer Life Science, Waltham, MA). Shed [³⁵S]syndecan-1 ectodomains were isolated from conditioned medium after incubation of cells with PMA. After 5 hours, proteoglycans were purified from the conditioned medium by anion exchange chromatography as described (20). Approximately 125 ng of syndecan-1 ectodomains (determined by Sdc-1 ELISA) were diluted in 200 μ L of a solution of δ = 1.019 g/ml iodixanol (OptiPrep Density Gradient Medium, Sigma-Aldrich) at the bottom of an ultracentrifuge tube in the presence or absence of 50 μ g VLDL and 50 μ g heparin. The tubes were then carefully filled with a solution of iodixanol (δ = 1.019 g/mL) and centrifuged at 45,000 rpm for 3 hrs at 18°C in a Beckman 50.4 Ti rotor. Fractions were removed sequentially from the top of the tubes and assayed for radioactivity by liquid scintillation counting.

Syndecan-1 shedding in vivo

C57Bl/6 mice, 8–10 weeks old, were injected intraperitoneally with 4.5 mg/kg *E. coli* lipopolysaccharide. Eighteen hours later, food was removed from the cages. After 6 hrs of fasting, the animals were sacrificed and their livers and blood were harvested. Syndecan-1 in plasma samples was measured by dot blotting. Syndecan-1 in liver sections was visualized using mAb 281-2 as described previously (11). Plasma triglyceride was measured using Triglyceride-SL Reagent (Genzyme Diagnostics, Framingham, MA) and cholesterol via Roche Cobas C111 analyzer (Roche Diagnostics, Indianapolis, IN).

Statistical analysis

All data were analyzed by unpaired one-tailed t-tests. $P < 0.05$ was considered statistically significant.

RESULTS

Human hepatocytes bind VLDL in a heparan sulfate-dependent manner

Binding of DiD-labeled VLDL (DiD-VLDL) at 4°C to Hep3B human hepatoma cells occurred in a saturable manner both with respect to time and concentration (Figure 1A and 1C, respectively). Treatment of the cells with a mixture of heparin lyases reduced binding by 80–90%, indicating that the majority of binding sites were attributable to heparan sulfate proteoglycans on the cell surface. Analysis of the binding curve in Figure 1C by a conventional single-site binding equation yielded a $B_{\max}$ value of 69 ± 12 μ g VLDL/mg cell protein and a K_D of 8.8 ± 3.4 μ g/ml ($R^2=0.9980$). Assuming that approximately 1.8 percent of the mass of VLDL particles is protein and an average molecular mass for VLDL of $\sim 2 \times 10^7$ Da (21), we calculated that Hep3B cells express $\sim 7 \times 10^5$ binding sites/cell attributable to heparan sulfate, similar to previous studies of mouse hepatocytes (11). Incubation of cells at 37°C increased the association of DiD-VLDL (Figure 1B and 1D) because of internalization of the particles, as reported previously (22). Inclusion of heparin (100 μ g/ml) blocked binding and uptake 5-fold as measured by flow cytometry (data not shown).

Human hepatocytes express multiple heparan sulfate proteoglycans, including the membrane proteoglycans syndecan-1, -2, and -4 and glypicans-1 and -4, and the extracellular matrix proteoglycans perlecan, collagen 18, and agrin (23, 24) (Supplemental Figure S1). To examine the contribution of syndecan-1 to VLDL binding and uptake, we silenced its expression on Hep3B cells using specific siRNAs (Supplemental Fig. S2). DiD-VLDL binding at 4°C (Figure 1E) and uptake at 37°C (Figure 1F) was reduced compared to cells treated with a scrambled siRNA. Binding and uptake were diminished to a greater extent by heparan lyase digestion (Fig. 1E, F), suggesting that either the extent of syndecan-1 silencing was incomplete or that other heparan sulfate proteoglycans can mediate binding and uptake (6). Silencing of syndecan-4 did not result in reduction of binding, but other proteoglycans have not been examined (data not shown). The residual heparin lyase-insensitive component of binding/uptake presumably reflects other receptors, most likely LDL receptors and one or more members of the family of LDL receptor-related proteins (3, 4).

Hepatocytes shed syndecan-1

Syndecan-1 undergoes proteolytic shedding in many cells (14), resulting in the appearance in the medium of the extracellular domains of the protein containing the ligand binding heparan sulfate chains. To investigate whether human hepatocytes spontaneously shed syndecan-1, we collected conditioned growth media from Hep3B cells (Figure 2A) and primary human hepatocytes (Figure 2B) and measured syndecan-1 ectodomains by dot blotting. Syndecan-1 ectodomains accumulated progressively in the conditioned media over time in both types of cells. The broad-spectrum metalloproteinase inhibitor Marimastat blocked spontaneous shedding of syndecan-1 (Figure 2A and 2B), consistent with the idea that shedding depends on limited proteolysis.

Syndecan-1 shedding can be induced in cells by phorbol myristic acid (PMA) (14). In both Hep3B cells and primary human hepatocytes, PMA induced syndecan-1 shedding by ~10-fold (Figures 2C and 2D). Induction of shedding was dependent on both time of incubation and the concentration of PMA added to the medium (Supplemental Fig. S3). Syndecan-1 on the cell surface was diminished in a corresponding manner, consistent with shedding (Figures 2E and 2F). PMA is known to stimulate endocytosis, which could account for some reduction in cell surface expression of syndecan-1 as well (25). The protein kinase C inhibitor BIM I profoundly inhibited syndecan-1 ectodomain accumulation in conditioned media and prevented loss of syndecan-1 from the cell surface (Figure 3A and Supplemental Fig. S4A). In contrast, the MEK inhibitor U0216 and the p38 MAPK inhibitor SB203580 failed to inhibit PMA-induced shedding of syndecan-1.

Marimastat blocked syndecan-1 shedding induced by PMA and spontaneous shedding (Figure 2). To determine the relevant metalloproteinase(s), we tested two different enzyme inhibitors on Hep3B cells. GW280264, which inhibits both ADAM-17 and ADAM-10, profoundly diminished the accumulation of syndecan-1 ectodomain in conditioned media (Figure 3B) and prevented the PMA-induced loss of syndecan-1 from the cell surface (Supplemental Figure S4B). In contrast, the inhibitor GI254023, which preferentially blocks ADAM-10 but not ADAM-17, had no effect on PMA-induced shedding of syndecan-1. Silencing of ADAM-17 expression in Hep3B cells by siRNA reduced ADAM-17 expression ~6-fold and prevented PMA-induced accumulation of syndecan-1 ectodomains in the conditioned medium (Figure 3C) and prevented loss of syndecan-1 from the cell surface as compared to controls in which cells were treated with scrambled siRNA (Supplemental Figure 4C). Similar results were obtained with primary human hepatocytes (Figure 4). These findings indicated that ADAM-17 is the primary metalloproteinase responsible for PMA-induced syndecan-1 shedding in human hepatocytes.

Syndecan-1 shedding reduces VLDL binding and uptake

In view of the role for syndecan-1 in TRL binding and uptake (Figures 1 and 2, and (11)), we predicted that PMA-induced syndecan-1 shedding would affect VLDL binding and uptake. In Hep3B cells, PMA reduced VLDL binding and uptake by ~50% compared to untreated cells (Figure 5A and 5B). Treatment with heparin lyases reduced VLDL binding and uptake to a greater extent, most likely because of incomplete removal of proteoglycan receptors from the cell surface by PMA-induced shedding. Similar results were obtained in primary human hepatocytes (Figure 5C and 5D). Shed ectodomains did not inhibit binding when purified and added to fresh cells at their original concentration. PMA can also induce shedding of LRP-1, another TRL receptor, which might account for some decrease in binding (3, 26). However, the major remnant receptor appears to bear heparan sulfate based on the large inhibitory effect of heparin lyase-treatment (Fig. 1).

Although the ADAM-17 cleavage site in syndecan-1 is unknown (27), it presumably lies in the membrane proximal region, thus liberating the ectodomain with covalently attached heparan sulfate chains. Based on previous genetic studies of mouse syndecan-1 (11), the heparan sulfate chains make up the TRL-binding domain of the syndecan-1 receptors. Thus, we predicted that shed syndecan-1 ectodomains from hepatocytes would retain the capacity to bind VLDL. To test this hypothesis, we incubated hepatoma cells with $^{35}\text{SO}_4$ to radiolabel the heparan sulfate chains on syndecan-1, triggered shedding with PMA and purified the ^{35}S -labeled ectodomains from the medium by anion exchange chromatography. ^{35}S -labeled ectodomains were then mixed with VLDL, placed at the bottom of a centrifuge tubes and overlaid with a solution of iodixanol ($\delta = 1.019 \text{ g/ml}$). Ultracentrifugation resulted in the appearance of ~65% of counts in the top four fractions, whereas in the absence of VLDL less than 5% of the counts were found in the top fractions (Figure 6). Dot blot analysis of the pooled top four fractions showed syndecan-1 ectodomains were present in samples containing VLDL (inset). Inclusion of 50 μg of heparin in the binding solution reduced the recovery of ^{35}S -ectodomains in the top fractions, consistent with the idea that heparin prevented the association of the particles with the heparan sulfate chains on the ectodomains. Interestingly, when the mixture of VLDL and ^{35}S -ectodomains was overlaid with a lower density solution ($\delta = 1.006 \text{ g/ml}$), flotation of ^{35}S -ectodomains did not occur (data not shown), consistent with the idea that the association of ectodomains with VLDL caused a shift in the buoyant density of the complex.

LPS causes syndecan-1 shedding in mice and increases plasma triglycerides

Based on these findings, we predicted that the steady state level of plasma triglycerides might depend on the extent of syndecan-1 shedding that occurs in the liver. To examine this possibility, we isolated hepatocytes from wildtype C57Bl/6 mice and examined syndecan-1 shedding. Syndecan-1 was constitutively shed from the cells based on the appearance of ectodomains in the conditioned media (Figure 7A). However, in contrast to human hepatocytes, shedding was insensitive to Marimastat (or PMA stimulation, data not shown). Other factors previously shown to induce shedding in other cells types such as insulin, LPS, and $\text{TNF-}\alpha$, also were without effect (28–30).

Hayashida et al. recently showed that in mice injection of LPS induces syndecan-1 shedding in liver (16). We confirmed this finding by dot blotting of plasma samples 24 hrs after injection of 4.5 mg LPS/kg into mice, compared to the level in mice injected with vehicle (Figure 7B). LPS injection also resulted in dramatic loss of syndecan-1 in the liver, as measured by immunohistochemistry (Figure 7C). Analysis of fasting plasma lipids showed that triglyceride levels increased 2-fold in mice injected with LPS (Figure 8A), whereas plasma total cholesterol did not change (Figure 8B). The accumulation of plasma lipids was greatly accentuated under these conditions by deletion of the LDL receptor, which resembles

the phenotype of compound mutants lacking hepatic heparan sulfate and LDL receptors (Supplemental Fig. S5 and (9)). When the animals were challenged with human VLDL, the injected particles cleared less extensively in the LPS-injected mice (Fig. 8C, AUC = 9200 ± 400 for LPS injected mice vs. 7000 ± 400 for the control), consistent with a loss of syndecan-1 clearance receptors in the liver.

Discussion

In this report, we have shown that the heparan sulfate proteoglycan syndecan-1 mediates clearance of VLDL in human hepatoma cells and primary human hepatocytes. Furthermore, we demonstrated that shed syndecan-1 ectodomains from human hepatoma cells bind VLDL particles in vitro (Figure 6), consistent with the idea that ectodomains contain the ligand binding site of the syndecan-1 receptor. Importantly, stimulation of shedding of syndecan-1 in mice was accompanied by an increase in fasting triglycerides (Figure 8). Proteoglycan receptors appear to dominate the receptor landscape on human hepatocytes as on murine hepatocytes, representing at least 90% of the binding sites (11).

Earlier studies of TRL uptake in human hepatocytes focused on hepatoma cell lines such as HepG2 and Hep3B derived from well-differentiated human hepatocellular carcinomas (12, 31, 32) or more rarely on primary hepatocytes (33). The data demonstrated that the addition of apoE and lipoprotein lipase to particles enhanced their uptake, and that under these conditions uptake occurred in a manner dependent on heparan sulfate. Similar observations were made using non-hepatic cell lines, which also suggested the participation of various syndecans (34–39) and perlecan (34, 35, 37, 40). Zeng et al showed that syndecan-1 can mediate binding and uptake of chylomicron remnants by HepG2 liver cells in vitro based on antisense and antibody inhibition experiments (12). In this report, we have shown that syndecan-1 on primary human hepatocytes as well as Hep3B cells can bind and take up native TRL particles derived from fasted donors. Prior genetic studies in mice and silencing experiments in human hepatocytes suggest that other HSPGs probably do not fulfill this role, emphasizing that syndecan-1 may be the dominant proteoglycan clearance receptor in humans as well as in mice (11).

It has been estimated that as much as 4% of all cell-surface receptors undergo regulated proteolytic shedding (41). Syndecan-1 also undergoes proteolytic processing, resulting in shedding of ectodomains containing the attached glycosaminoglycan chains (42). The shedding process occurs in hepatocytes and appears to be mediated by ADAM-17 both under basal conditions (data not shown) and after PMA stimulation (Figs. 3 and 4). The inducibility of syndecan-1 shedding by inflammation, ischemia, glucose and insulin suggests that shed ectodomains might have functional significance (28, 43–46). Shed syndecan-1 ectodomains might bind plasma lipoproteins in the space of Disse and prevent their escape back into the plasma or facilitate their further processing prior to uptake. Because shedding of ectodomains increases plasma triglycerides (Figure 8), shedding would appear to serve an alternative role, e.g. increasing the circulatory half-life of TRLs for more complete utilization of triglycerides in peripheral tissues. Studies are underway using recombinant forms of syndecan-1 that fail to shed or which are shed constitutively in order to study this question.

Shedding of syndecan-1 in hepatocytes depends on ADAM17, which can be induced by treatment with PMA through a pathway dependent on protein kinase C. ADAM17, also known as tumor necrosis factor- α converting enzyme (TACE), processes membrane bound pro-TNF- α , releasing the soluble active form of TNF- α , as well as other factors involved in inflammation, including L-selectin (46–48), LRP1 (43), TNF- α receptors TNFR1 and TNFR2 (49), CD30 (50), and interleukin-6 receptor IL-6R (51). Park and colleagues have

demonstrated that the role of syndecan-1 ectodomains in lung inflammation is to modulate the availability of chemokines (52). It is also well known that LPS can bind to lipoproteins, which serves as a sink for this potent inflammatory mediator (53). Thus, one can imagine that activation of shedding and the ensuing increase in plasma TRLs might serve a protective role during infection. The observation that LPS induces shedding, elevates plasma TRLs, and reduces VLDL clearance is consistent with this hypothesis and suggests that syndecan-1 shedding might be responsible in part for the elevation of plasma triglycerides in bacterial sepsis (54, 55). Further studies are planned to determine whether syndecan-1 shedding contributes to the hyperlipidemia of sepsis in human patients and whether it might explain other idiopathic forms of hypertriglyceridemia, a common side effect of many drugs (56).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TRLs	triglyceride-rich lipoproteins
VLDL	very low density lipoproteins
LDLR	low density lipoprotein receptor
LRPs	LDLR-related proteins
PMA	phorbol-12-myristate-13-acetate
DiD	1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine perchlorate
BIM I	Bisindolylmaleimide I
LPS	lipopolysaccharide
ADAM	a disintegrin and metalloproteinase
HSPGs	heparan sulfate proteoglycans

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Figure 1. VLDL binding and uptake by human Hep3B hepatoma cells

Binding and uptake of DiD-VLDL was measured at 4°C (A, C) and 37°C (B, D), respectively, before (circles) or after (squares) treatment with heparin lyases. In A and B, cells were incubated with 100 µg/ml DiD-VLDL for indicated time. In C and D, the cells were incubated with indicated concentration of DiD-VLDL for 1 hour. (n = 3). In E and F, cells were treated with a scrambled siRNA (sc-siRNA, open bars), siRNA to syndecan-1 (siSDC-1, filled bars) or heparin lyase (hatched bars). Binding (E) and binding/uptake (F) were measured by incubating treated cells with 100 µg/ml DiD-VLDL for 1 hour. (n = 4). The values represent the average fluorescence intensity normalized to cell protein. Error bars indicate standard deviation.

Figure 2. Shedding of syndecan-1 from human hepatocytes

Conditioned medium was collected from Hep3B human hepatoma cells (A) and primary human hepatocytes (B) incubated with (squares) or without (circles) the metalloproteinase inhibitor Marimastat (MM). At the indicated time points, shed syndecan-1 was measured by dot blotting. Hep3B cells (C,E) and primary human hepatocytes (D,F) were treated with 0.25 μ M PMA for 1 hour in the presence (shaded bars) or absence (filled bars) of Marimastat (MM). Conditioned media was collected and subjected to dot blotting for shed syndecan-1 (C,D) and cell lysates were prepared after treatment of the cells with heparin lyases in order to measure syndecan-1 core protein by western blotting (E,F). Each data point or bar represents the average $\pm$ standard deviation ($n = 3$).

Figure 3. ADAM-17 mediates PMA-induced shedding of syndecan-1

(A) Different signaling pathway inhibitors were tested for their ability to inhibit syndecan-1 shedding induced by PMA. (B) Hep3B cells were pretreated with different metalloproteinase inhibitors and then stimulated with 0.25 μ M PMA for 1 hour. Syndecan-1 shedding was measured by dot blotting of the medium. (C) ADAM-17 was silenced by siRNA and the extent of syndecan-1 shedding after PMA stimulation was measured. The extent of silencing of ADAM-17 was measured by western blotting. All values represent the average $\pm$ standard deviation (n=3).

Figure 4. PMA induces syndecan-1 shedding on primary human hepatocytes

Primary human hepatocytes cells were pretreated with different metalloproteinase inhibitors and then stimulated with 0.25 μ M PMA for 1 hour. Syndecan-1 shedding was measured by dot blotting of the medium (A) and by western blotting of syndecan-1 in the cell lysate (B). All values represent the average $\pm$ standard deviation (n=3).

Figure 5. PMA-induced shedding reduces VLDL binding and uptake

Hep3B cells (A,B) and primary human hepatocytes (C,D) were treated with 0.25 μ M PMA for 1 hour (filled bars) or heparin lyase for 30 minutes (hatched bars) and then incubated with DiD-VLDL (100 μ g/ml) for 1 hour at 4°C for binding (A,C) or at 37°C for binding and uptake (B,D). The fluorescence intensity was quantitated and normalized to cell protein. Controls cells were not treated with PMA (open bars). All values represent the average $\pm$ standard deviation ($n = 3$).

Figure 6. Shed syndecan-1 binds VLDL

³⁵S-labeled syndecan-1 ectodomains were combined with 50 μ g of human VLDL in the presence (squares) or absence (filled circles) of 50 μ g of heparin. A sample of ectodomains was incubated with buffer alone as a control (open circles). The samples were then adjusted to $d = 1.019$ g/ml with iodixanol and centrifuged. Fractions taken sequentially from the top of the tubes were assayed for radioactivity and syndecan-1 ectodomains (embedded dot blot figure).

Figure 7. LPS causes syndecan-1 shedding in the liver

Conditioned medium was collected from primary mouse hepatocytes incubated in the presence (squares) or absence (circles) of Marimastat (MM). (A) Shed syndecan-1 was measured by dot blotting ($n = 3$). (B) Mice were injected intraperitoneally with 4.5 mg LPS/kg or saline and plasma syndecan-1 was measured by dot blotting 24 hours later. All values represent the average $\pm$ standard deviation ($n = 5$) (C) The livers were harvested, fixed in paraformaldehyde, and immunostained for mouse syndecan-1 using mAb 281-2. Bar = 50 microns.

Figure 8. LPS increases plasma triglycerides in mice

Wildtype or *Ldlr*^{-/-} mice were injected intraperitoneally with 4.5 mg LPS/kg or PBS.

Eighteen hours later, the animals were fasted for six hours and plasma triglycerides (A) and total cholesterol (B) were determined. In (C), similarly treated animals were injected with 20 μ g of human VLDL and at the indicated times the amount of remaining human apoB was determined by ELISA. Each value represents the average $\pm$ standard deviation ($n = 3$ mice).