Cathepsin D, a Lysosomal Protease, Regulates ABCA1-mediated Lipid Efflux*§

Bassam Haidar†‡1, Robert S. Kiss‡1, Lea Sarov-Blat‡1, Roch Brunet§, Christopher Harder†, Ruth McPherson†, and Yves L. Marcel‡1,2

From the Lipoprotein and Atherosclerosis Research Group, University of Ottawa Heart Institute, and Departments of Biochemistry, Microbiology, and Immunology, and Pathology and Laboratory Medicine, and Medicine (Cardiology), University of Ottawa, Ottawa, Ontario, K1Y 4W7, Canada and GlaxoSmithKline, King of Prussia, Pennsylvania 19406

To identify genes involved in the regulation of plasma high density lipoprotein (HDL) cholesterol (HDL-C) levels, patients with low HDL-C and age- and sex-matched controls (normal HDL-C) were extensively characterized. Comparative transcriptome analysis was carried out in cholesterol-loaded monocyte-derived macrophages from low HDL subjects segregated into groups with or without cholesterol efflux defects or ABCA1 mutations. Clusters of differentially regulated genes were evident in the low HDL groups as compared with controls. Of particular note, expression of cathepsin D (CTSD), a lysosomal proteinase, was reduced by ~50% in monocyte-derived macrophages of low HDL-C subjects, most significantly those with cholesterol efflux defects but without mutations in ABCA1 (p < 0.01). These results were verified by reverse transcription-PCR and replicated in a second cohort. We show here that blocking the activity or expression of CTSD, by pepstatin or CTSD small interfering RNA, respectively, reduced ABCA1 expression and protein abundance in both macrophages and CHO cells and apolipoprotein A-I-mediated lipid efflux by more than 70%. Conversely, expression of CTSD increased both ABCA1 mRNA expression and cellular ABCA1 protein. Consistent with its role in the proteolytic processing of prosaposin, inactivation of CTSD function resulted in the accumulation of glycosphingolipid and free cholesterol in late endosomes/lysosomes, a phenotype similar to NPC1 deficiency. Inhibition of CTSD also caused retention of ABCA1 in lysosomal compartments, reducing its trafficking to the plasma membrane. These studies demonstrate a novel and potentially important role for CTSD in intracellular cholesterol trafficking and ABCA1-mediated efflux. Therefore, decreased CTSD expression may contribute to low plasma HDL-C levels.

High density lipoprotein cholesterol (HDL-C) deficiency is a major risk factor for cardiovascular diseases and has a complex metabolic and genetic etiology. Overproduction and/or impaired clearance of triglyceride-rich lipoproteins results in triglyceride enrichment of HDL and accelerated apoA-I catabolism and may account for low HDL-C in patients with other features of the metabolic syndrome. Low HDL can also result from mutations in a number of genes regulating HDL production or catabolism, including apolipoprotein A-I (1–4), lecithin:cholesterol acyltransferase (5–7), phospholipid transfer protein (8–10), and ATP-binding cassette protein A1 (11–19).

Defective cellular phospholipid and cholesterol efflux is also a common contributory phenotype in familial low HDL syndromes, and in several of these kindreds, affected subjects are heterozygous carriers of ABCA1 mutations (17, 20, 21). These genetic studies demonstrate the key role of ABCA1 in cholesterol efflux to apoA-I, but as we have reported previously (22), sequence variants in ABCA1 account for a small proportion of hypoalphalipoproteinemia. In further studies in a cohort of low HDL and control subjects previously characterized (Ref. 22 and Sarov-Blat et al.‡4 and Kiss et al.,5 submitted manuscripts), we have undertaken to identify other genes and gene products that contribute to lipid efflux and low HDL. High density oligonucleotide microarrays were used to analyze gene expression in cholesterol-loaded monocyte-derived macrophages (MDM) prepared from these subjects. Importantly, we demonstrate here that expression of cathepsin D (CTSD), a lysosomal proteinase (23), is reduced by 50% in the MDM of low HDL-C subjects, significantly in those with cholesterol efflux defects without mutations in ABCA1, compared with control subjects.

CTSD is involved in intracellular catabolism in lysosomal compartments but also functions in hormone and antigen processing (24–26). CTSD has been implicated in mitogenic activity in certain cancers (27, 28), which may be related to the secretion of...
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FIGURE 1. Cluster of the cathepsin family genes whose transcripts were decreased in low HDL groups (group A, low HDL, low efflux, no ABCA1 mutation; group B, low HDL, low efflux, ABCA1 mutation; group C, low HDL, normal efflux compared with control group D). Monocyte-derived macrophages isolated from patients with low HDL and age- and sex-matched controls were loaded with Ac-LDL, and global gene expression profiles were analyzed on duplicate GeneChip DNA-microarrays. A, hierarchical cluster dendrogram from TIGR MeV program for both genes and subjects. The cathepsin gene family was expressed similarly within low HDL groups and between control subjects. The red color represents up-regulated genes, whereas the green indicates down-regulated genes. The CTSD gene was the most differentially expressed member of the cathepsin gene family and was significantly down-regulated in groups A and B compared with control (p < 0.01 and p < 0.016, respectively). B, plot of the CTSD probe set relative expression in microarray for individual subjects and average CTSD mRNA expression value for each group.

EXPERIMENTAL PROCEDURES

Materials—The following reagents were used: [1,2-3H]cholesterol and [methyl-3H]choline chloride (PerkinElmer Life Sciences); minimal essential medium, RPMI, and antibiotic-antimycotic (Invitrogen); rabbit polyclonal anti-human apoA-I antibody (Calbiochem); rabbit polyclonal ABCA1, HSP60, and monoclonal anti-mouse CTSD (Novus Biologicals, Littleton, CO); streptavidin-horseradish peroxidase conjugate and protein G-Sepharose (Amersham Biosciences); pepstatin A, ceramide, and other reagents (Sigma); cathepsin D siRNA (Ambion, Austin, TX); TransIT-TKO transfection reagent (Mirus, Madison, WI); and SYBR Green kit (Roche Applied Science). GFP-ABCA1 human vector was kindly provided by Mason Freeman. C57Bl/6 and NPC1(−/−) mice were purchased from Jackson laboratory. The rabbit polyclonal anti-prosaposin antibody was kindly provided by Dr. Carlos Morales.

Selection of Low HDL-C Subjects and Controls—Patients referred to the Lipid Clinic of the University of Ottawa Heart Institute were selected based on the following criteria: Caucasian, HDL-C <5th percentile for age and sex; triglyceride <95th percentile (most <75th percentile); and LDL cholesterol <75th percentile, measured while not on treatment with lipid-modifying medications. Exclusion criteria included clinical conditions or medications causative of low HDL-C, including all forms of diabetes. There was no other selection bias, since most subjects were recruited in chronologic order of referral. The control group was selected among healthy and normolipemic volunteers of the same ethnic background recruited from the Ottawa region. The study was approved by the University of Ottawa Heart Institute Human Research Ethics Committee, and written informed consent was obtained from all participants.

RNA Isolation in Human Subjects—Macrophages were homogenized with Qiashredder, and total mRNA was isolated using the RNeasy mRNA isolation kit (according to the manufacturer’s instructions).

GeneChip Affymetrix Microarray—Gene expression profiles were analyzed by Affymetrix GeneChips, which cover 22,215 annotated genes. RNA quality and quantity was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and RiboGreen RNA quantitation reagent (Molecular Probes, Inc., Eugene, OR), respectively, in addition to measuring OD at 260 and 280 nm and 260/280 ratios. All samples possessed 18 and 28 S rRNA bands with no sign of RNA degradation. Affymetrix human U133A GeneChips (Affymetrix Inc., Santa Clara, CA) were used. Briefly, double-stranded cDNA was synthesized from each total RNA sample via oligo-T7-mediated reverse transcription. Biotin-labeled cRNA was synthesized using an in vitro transcription reaction with the Enzo BioArray high yield RNA transcript labeling kit (Enzo Diagnostics, Inc., Farmingdale, NY). Subsequently, the purified, biotin-labeled cRNA was fragmented into 50–150-nucleotide fragments. Fragmented, biotin-labeled cRNA (15 µg), together with a known spike (according to Affymetrix recommendations) was hybridized to a U133A GeneChip for 16–18 h at 45 °C. An Affymetrix GeneChip fluids station 450 was used to perform standard washing and streptavidin/phycocerythrin staining. The chips were scanned using an Affymetrix GeneChip scanner 3000.

TaqMan Quantitative Real Time PCR—Total RNA (1 µg) was converted to cDNA using a high capacity cDNA archive kit (Applied Biosystems), and the equivalent of 10 ng/well was arrayed into high density 384-well plates using a Biomek FX robot (Beckman Coulter), allowing expression profile generation for one gene at a time. Quantitative RT-PCR was carried out using a 7900HT Sequence Detector System (Applied Biosystems) in a 10-µl reaction volume. TaqMan Universal PCR
Master Mix 2X (Applied Biosystems) and universal PCR conditions recommended by the vendor were followed. SYBR Green Real Time PCR—mRNA was isolated from mammalian cells using Trizol reagent (Invitrogen, Burlington, Canada). CTSD mRNA, ABCA1, and GAPDH mRNA were quantified using the Stratagene SYBR Green kit on a Lightcycler real time PCR machine according to the manufacturer’s recommendations.

Cell Culture—Human blood monocytes were differentiated into macrophagic cells according to Cohen et al. (22). The monocytes were differentiated into macrophages over the course of 12 days in growth media, and the resulting cells were used in the efflux assay or for mRNA isolation. All macrophages were grown in RPMI medium supplemented with 10% pooled human serum, whereas J774 and CHO cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS). All cells were grown at 37 °C in 5% CO2 and incubated with penicillin/streptomycin. Bone marrow-derived macrophages were flushed from mouse femurs. Monocytes were differentiated into macrophages over the course of 12 days in growth media, and the resulting cells were used in the efflux assay or for mRNA isolation. Bone marrow-derived macrophages were flushed from mouse femurs. Macrophages were generated by incubating fetal liver cells (2×10^6 cells/ml) with DMEM, 10% FBS complemented with 15% L929 conditioned medium for 7 days. Three days prior to the isolation of peritoneal macrophages, mice were injected with 2 ml of 3% thioglycolate intraperitoneally. The animals were then euthanized with CO2, the peritoneal cavity was washed by an 8-ml injection of saline, and the cells were washed three times by centrifugation at 1000 g for 10 min with DMEM. The cells are then plated in 6-well plates containing DMEM, 10% FBS overnight.

FIGURE 2. The modulation of CTSD activity affects apoA-I-mediated lipid efflux. Macrophage J774 cells were loaded with either LDL or AcLDL (100 μg/ml), labeled with [3H]cholesterol or [3H]choline, for 24 h. The next day, cells were washed with PBS and treated with pepstatin (0–10 μg/ml) or ceramide (0–20 μM) for 24 h in DMEM containing 0.2% BSA. The cholesterol and phospholipid radioactivity associated with apoA-I were measured in the medium, with or without pepstatin or ceramide, by counting immunoprecipitated lipids using a polyclonal anti-human apoA-I antiserum from sheep and protein G-Sepharose. Treatment of AcLDL-loaded J774 macrophages with ceramide increases the apoA-I-mediated cholesterol (A) and phospholipid (B) efflux in a dose-dependent manner. The inhibition of CTSD by pepstatin A in LDL-loaded J774 macrophages decreases the cholesterol (C) and the phospholipid (D) efflux in a dose-dependent manner. Analysis of cellular lipids by TLC shows that pepstatin treatment (10 μg) of J774 macrophages prevents cellular cholesteryl-ester mobilization (E) and slightly alters total free cholesterol (F). The data points represent the average ± S.D. of triplicate assays.
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60 \text{ kDa} & \quad \text{CTR} \\
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C

Cholesterol efflux in CHO cells

FIGURE 3. CTSD activity inhibition by pepstatin decreases ABCA1 mass and efflux. J774 macrophages (A) were treated with 0, 5, and 10 μg/ml pepstatin A, respectively, for 36 h in DMEM. CHO cells were treated with 0 and 10 μg/ml (B) or with 0, 5, and 10 μg/ml (C) pepstatin A in RPMI medium containing 0.2% BSA. Western blots were performed on the cell lysates using a rabbit anti-ABCA1 polyclonal antibody, and HSP60 was used for as a control for loading (A and B). The results are representative of three independent experiments. CHO cells labeled with [3H]cholesterol and treated with pepstatin in B were assayed for cholesterol efflux (C).

Lipid Analysis—Free cholesterol and cholesteryl esters were extracted and separated by TLC on Sil-G TLC plates (EMD Chemicals, Darmstadt, Germany) using the solvent system hexane/diethyl ether/acetic acid (105:45:1.5) (35). Lipid bands were visualized with iodine vapors, isolated, and analyzed by β-scintillation counting.

siRNA Transfection and Expression Vectors—Cathepsin D siRNA obtained from Ambion (Austin, TX) was transfected into macrophage J774 cells using the TransIT-TKO transfection reagent (Mirus, Madison, WI). Briefly, 100 nM siRNA and 4 μl of the transfection reagent were both mixed with 100 μl of serum-free DMEM for 5 min. The solutions were then incubated at room temperature for 20 min. This mixture was applied to the DMEM, 10% FBS medium in each well of a 12-well plate (for cholesterol efflux) or 6-well plate (for RT-PCR and Western blotting) and incubated for 36 h. J774 or CHO cells were transiently transfected with human cathepsin D vector using Lipofectamine 2000 according to the manufacturer’s recommendations.

Cell Surface Biotinylation and Western Blotting—Biotinylation was performed as described earlier (36) with slight modification. J774 or CHO cells were treated with or without pepstatin for 36 h, and surface proteins were biotinylated with 500 μg/ml sulfo-NHS-LC-biotin (Pierce) for 30 min at 4 °C on a platform rotator. Cells were then washed with ice-cold quench buffer (1 mM Tris-HCl (pH 7.5)) and once with ice-cold phosphate-buffered saline. The cells were swollen on ice for 10 min and then homogenized with 20 strokes in a tight fitting Dounce homogenizer. After centrifugation at 2000 × g, at 4 °C, for 10 min to remove unbroken cells and nuclei, the supernatant was recentrifuged at 100,000 × g, at 4 °C, for 60 min. The resulting supernatant was discarded, and the final membrane pellet was resuspended in 250 μl of immunoprecipitation buffer; 150 μg of protein was added to 30 μl of streptavidin-Sepharose (Amersham Biosciences) and incubated overnight on a platform mixer at 4 °C. The gel was pelleted and washed five times with immunoprecipitation buffer. The Western blotting technique was performed as described before (36). Briefly, cells were washed twice with ice-cold PBS and scraped into 0.5 ml of radioimmunoprecipitation buffer and homogenized with 20 strokes in a tight fitting Dounce homogenizer. After centrifugation at 2000 × g, at 4 °C, for 10 min to remove unbroken cells and nuclei, the protein concentration was determined by a Lowry assay. The same amounts of proteins were electrophoresed on a SDS-polyacrylamide gel and transferred to nitrocellulose at 125 V for 4 h. The nitrocellulose membranes were then blocked with 5% milk in Tris-base-buffered solution and incubated overnight with CTSD, HSP60, ABCA1, or prosaposin antibodies. Horseradish peroxidase-linked secondary antibodies (Amersham Biosciences) were used, and bands were detected by chemiluminescence (Supersignal substrate; Pierce).

Fluorescence Microscopy—Cells were seeded on 35-mm dishes with attached glass coverslips (Fisher) and transfected with ABCA1-GFP. The coverslips were then mounted in a temperature-controlled chamber (37 °C) in regular growth medium supplemented with 20 mM HEPES (pH 7.4) and visualized with an Olympus ×100 oil immersion objective, numerical aperture 1.4, on an Olympus IX80 laser-scanning confocal microscope operated by FV1000 software version 4.0 (TILL Photonics, Heidelberg, Germany). The protocol for recording images documenting filipin staining consisted of exciting the fluorophore with a Polychrome IV monochromometer at 380 nm with 1000-ms exposure time, utilizing a Fura filter cube (Chroma). Images were merged as RGB files using the TILLvisION software. The images were exported from the microscope software and processed with Adobe Pho-
Cells were stimulated with or without 0.5 mM of 8-bromo-cAMP for 24 h in the presence or absence of pepstatin.

The control subjects were divided into four age- and sex-matched groups based on the presence or absence of low HDL-C, cholesterol efflux defect, activity and generates less active forms of CTSD (38) (supplemental Fig. 1). These observations led to the hypothesis that reduced expression of CTSD may play a role in plasma HDL levels. 4

For TaqMan analysis, copy numbers of a given mRNA detected in each sample were calculated after normalization to the average log of the expression of three housekeeping genes (cyclophilin, glyceraldehyde-3-phosphate dehydrogenase, and B-actin). A one-way analysis of variance test, followed by Dunnett’s test for multiple comparisons, was run separately for each gene to determine significant differences between groups.

RESULTS

Cathepsin D Gene Expression Is Decreased in Monocyte-derived Macrophages of Low HDL Subjects—Subjects were divided into four age- and sex-matched groups based on the presence or absence of low HDL-C, cholesterol efflux defect, and functional mutations in ABCA1 as follows: group A, low HDL, low efflux, no ABCA1 mutation; group B, low HDL, low efflux, ABCA1 mutation; group C, low HDL, normal efflux; group D, normal HDL, normal efflux. The control subjects (group D) had normal plasma levels of triglycerides and LDL cholesterol, similar to low HDL subjects and, by definition, higher HDL-C and hence higher total cholesterol concentrations. The three low HDL groups demonstrated very similar lipid values, and all groups were closely matched for age, sex, and body mass index (data not shown). 4

MDM from low HDL and control subjects were loaded with AcLDL; mRNA was isolated from the cells of each subject and analyzed using duplicate Affymetrix U133A oligonucleotide arrays (see “Experimental Procedures”). Comparative gene expression analysis revealed the differential regulation of several pathways reported elsewhere. 4 In particular, a cluster of cathepsin genes, which are major aspartic proteases of endolysosomes, was found to be down-regulated in MDM of low HDL-C groups compared with control (Fig. 1A; red represents higher relative expression, and green represents lower relative expression for each gene comparing all of the subjects for that gene). CTSD was significantly decreased in efflux-defective subjects compared with control (p < 0.01) (Fig. 1B). These differences were confirmed by real time PCR (group A, p < 0.01; group B, p < 0.016), and both microarray results and real time PCR values were also recapitulated in a second cohort for group A (RT-PCR series 2, p < 0.05) but not for group B, for which no additional subject was available. The decrease in CTSD observed in the low HDL group with normal efflux (group C) was not significant in either microarray series 1 (Fig. 1, A and B) or series 2 or in RT-PCR assays. Similarly, ABCA1 expression was not significantly changed in groups A and C (data not shown). These observations led to the hypothesis that reduced expression of CTSD may play a role in plasma HDL levels.

CTSD Activity Modulates ABCA1-mediated Lipid Efflux—CTSD, an aspartic protease involved in the proteolytic degradation of proteins in endosome-lysosome compartments, binds ceramide with high affinity (37). The interaction of ceramide with CTSD results in autocatalytic proteolysis of the 52-kDa prepro-CTSD to form the enzymatically active 48/32-kDa isoforms of CTSD (37). Pepstatin is also a potent inhibitor of CTSD, which binds CTSD and inhibits its autacatalytic activity and generates less active forms of CTSD (38) (supplemental Fig. 1). To investigate the effect of CTSD modulators on lipid efflux, J774 macrophages were radioactively labeled by incubation with LDL (100 μg/ml) equilibrated with [3H]cholesterol or with medium containing [3H]choline for 24 h.

FIGURE 4. Pepstatin decreases the ABCA1-mediated lipid efflux in different macrophage cell lines. J774 cells were stimulated with or without 0.5 mM of 8-bromo-cAMP for 24 h in the presence or absence of pepstatin. Cholesterol and the phospholipid efflux assays were performed as described for Fig. 2. Pepstatin decreases the ABCA1-dependent cholesterol (A) and phospholipid (B) efflux in J774. Pepstatin also reduces cholesterol efflux in BMDM (C) and in LDL- or Ac-LDL-loaded peritoneal macrophages (D). The data points represent the average ± S.D. of triplicate assays.
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**FIGURE 5. Knockdown of CTSD expression decreases ABCA1 expression and apoA-I-mediated lipid efflux.** J774 cells were treated with 50 or 100 nM CTSD siRNA or scrambled dsRNA for 36 h, and apoA-I-mediated lipid efflux was assayed as described for Fig. 2. The addition of CTSD-specific siRNA reduces cholesterol efflux (A) and phospholipid efflux (B) in dose-dependent manners. At 100 nM CTSD siRNA, cholesterol efflux is reduced by ~80%, and phospholipid efflux is reduced by ~50%. Note the unchanged cholesterol or phospholipid efflux with the addition of scrambled dsRNA. Each value is the mean ± S.D. of triplicates. Western blot experiment of ABCA1, CTSD, and HSP60 shows that siRNA CTSD reduces ABCA1 protein in a dose-dependent manner (C) as well as CTSD compared with control. HSP60 was used as a control for loading. In addition, siRNA completely blocked CTSD transcription (D). Results are representative of two similar experiments.

treated with ceramide, an activator of CTSD (37), or pepstatin A, a specific high affinity inhibitor of CTSD (38), for 24 h in DMEM containing 0.2% BSA, prior to the efflux assay. Ceramide increased the apoA-I-mediated efflux of cholesterol (Fig. 2A) and phospholipid (Fig. 2B) in a dose-dependent manner by more than 80%. In contrast, inhibition of CTSD by pepstatin A resulted in a dose-dependent decrease in the efflux of both cholesterol (Fig. 2C) and phospholipid (Fig. 2D). Treatment with pepstatin A also impaired cholesteryl ester mobilization (Fig. 2E) and slightly decreased cellular free cholesterol (Fig. 2F). These experiments clearly show that the modulation of CTSD activity affects apoA-I-mediated efflux of cholesterol and phospholipid.

We next investigated whether the impaired apoA-I-mediated efflux by pepstatin A was associated with decreased ABCA1 protein. These studies demonstrated that treatment with pepstatin A (10 μg/ml) significantly reduced ABCA1 protein in J774 macrophages (Fig. 3A) and to an even greater extent in CHO cells (Fig. 3B), where it was also accompanied by decreased cholesterol efflux (Fig. 3C). Concurrently, cell surface ABCA1 was also reduced upon treatment with pepstatin A (data not shown). These results are consistent with the hypothesis that suppression of apoA-I-mediated lipid efflux by pepstatin A is the result of decreased cellular ABCA1 protein. J774 macrophages were treated with or without a stimulator of cholesterol efflux, cAMP, for 24 h in the presence or absence of pepstatin A, and cholesterol and phospholipid effuxes were determined. In both conditions, pepstatin A significantly decreased apoA-I-dependent efflux of cholesterol (Fig. 4A) and phospholipid (Fig. 4B) in J774 cells. Pepstatin A similarly reduced cholesterol efflux from bone marrow-derived-macrophages (Fig. 4C) and in LDL or AcLDL treated peritoneal macrophages (Fig. 4D). These results indicate that CTSD promotes lipid efflux through the ABCA1-dependent pathway in primary macrophages and in immortalized J774 cells. Determination of cholesterol efflux and ABCA1 protein level in J774 cells at three doses of pepstatin demonstrated a highly significant correlation of the two parameters (data not shown).

**Modulation of the Expression Level of CTSD Regulates ApoA-I-Mediated Lipid Efflux**—For a more direct assay of the effect of CTSD functional deficiency on apoA-I-mediated lipid efflux, J774 cells were treated with siRNA CTSD or dsRNA CTSD for 36 h. The addition of CTSD-specific siRNA significantly reduced cholesterol efflux in a dose-dependent manner (Fig. 5A) and phospholipid efflux (Fig. 5B). At 100 nM, CTSD siRNA decreased cholesterol efflux by ~80% and phospholipid efflux by about 50%. In contrast, the scrambled dsRNA had no effect on these parameters.

Western blot analysis of CTSD and ABCA1 after siRNA treatment demonstrated decreased expression of CTSD with siRNA but not dsRNA (Fig. 5D) and parallel changes in ABCA1 protein mass (Fig. 5C). These results further support the notion that CTSD regulates ABCA1 levels and cholesterol efflux.

To explore the potential role of CTSD overexpression on lipid efflux and ABCA1 protein, CHO or J774 cells were transfected with a human full-length CTSD plasmid for 24 h, lipid efflux was assayed, and ABCA1 protein level was determined. Consistent with the siRNA results, transfection of human CTSD in CHO cells increased apoA-I-mediated lipid efflux (Fig. 6, A and B) and the cellular level of ABCA1 protein (Fig. 6C). Having shown that CTSD increases both ABCA1 protein and cholesterol efflux, we next determined whether CTSD exerted its effect directly on ABCA1 gene transcription. Macrophages were treated with either pepstatin A or by overexpression of human CTSD for 24 h, and ABCA1 mRNA levels were measured by RT-PCR. As expected, ABCA1 expression was significantly increased (>50%) by CTSD overexpression and was significantly decreased (<50%) by pepstatin treatment (Fig. 6D). Overall, these findings demonstrate that CTSD induces cholesterol efflux at least in part by regulating ABCA1 transcription.
CTSD increased the level of both pro-SAP and saposins, and these human CTSD. Interestingly, we also found that overexpression of into proteolytic products of Western blotting. As shown in Fig. 7, prosaposin was hydrolyzed expression of CTSD, pro-SAP, and the control protein HSP60 by transiently overexpressed CTSD in CHO cells and quantified the CTSD regulates the proteolysis of pro-SAP in our model, we tran- sient saposins is dependent on CTSD (39). To examine whether the addition of exogenous ceramide would also enhance cholesterol efflux in NPC1-deficient cells, we isolated bone marrow-derived macrophages from control and NPC1-defi- cient mice and measured apoA-I-mediated cholesterol efflux. The values increases were proportional to the level of CTSD (Fig. 7, top). These data verify that CTSD functions in the proteolytic processing of prosaposin. Interestingly, prosaposin mRNA expression was found to be decreased in the microarrays for series 1 and 2 for low HDL subjects compared with control subjects (p < 0.001 and p < 0.03, respectively).

**Inhibition of CTSD Causes the Intracellular Accumulation of Cholesterol and Sphingolipids**—Since saposins are sphingolipid-binding proteins involved in the degradation of glycosphingolipids, we hypothe- sized that dysregulation of the expression of pro-SAP or its co-activator CTSD could impair glycosphingolipid (GSL) metabolism and cholesterol traffic. Indeed, the cellular transport and storage of cholesterol is linked to GSL metabolism (40–43), and the accumulation of GSL in human macrophage foam cells and fibroblasts causes the trapping of choles- terol in endosome/lysosome compart- ments (41, 43). Thus, in CHO cells, we assessed the effect of pepstatin treatment on the cellular localization of GSL using BODIPY-lactosylceramide and free cholesterol accumulation using filipin staining. We demonstrated perinuclear fluo- rescence of BODIPY-lactosylceramide (BODIPY Lac-cer) in control cells (Fig. 8A), whereas, in cells treated with pepstatin A, there was predomi- nantly bright punctate endosome/lysosome fluorescence (Fig. 8B) similar to the cellular phenotype of Niemann-Pick type C disease (NPC) (44). Extensive studies have also shown the accumulation of GSL and cholesterol in late endosomes/lysosomes in NPC cells (45). To test whether the addition of exogenous ceramide would improve cholesterol efflux in NPC1-deficient cells, we isolated bone marrow-derived macrophages from control and NPC1-defi- cient mice and measured apoA-I-mediated cholesterol efflux. The addition of ceramide (20 μM) or cAMP increased cholesterol efflux in control cells (2-fold) but not in the NPC1-deficient cells (Fig. 8C). As expected, pepstatin also tended to cause enlarged punctate fluorescence of filipin-stained free cholesterol in endosome/lysos- some-like structures, whereas in control cells cholesterol distribu- tion was diffuse throughout the cell (data not shown). Taken together, these results demonstrate that inactivation of CTSD function results in the accumulation of GSL and free cholesterol in late endosomes/lysosomes.

**Inhibition of CTSD Causes the Mistargeting of ABCA1 Pro-tein to Late Endosomes/Lysosomes**—ABCA1 is normally localized at the cell surface and in the late endosomal com-
To test whether inhibition of CTSD by pepstatin affected ABCA1 localization, we followed the distribution of ABCA1 in CHO cells by confocal microscopy using GFP-ABCA1 and Lysotracker red as a lysosomal marker. In these experiments, cycloheximide treatment was omitted to avoid interference with the degradation of lysosomal enzymes. Under these conditions, we observed no co-localization of ABCA1 with Lysotracker red (Fig. 9A) in control cells, but in cells treated with pepstatin, ABCA1 was found to colocalize extensively with lysosomes, as indicated by the yellow punctate fluorescence (Fig. 9B). These results show that inhibition of CTSD activity causes trapping of ABCA1 in a lysosomal compartment, impairing its trafficking to the plasma membrane.

DISCUSSION

These studies were designed to identify and evaluate novel candidate genes relevant to HDL metabolism and the etiology of low HDL syndromes. By focusing on genes differentially expressed in a group of subjects with low HDL, we identified a cluster of cathepsin genes. Notably, mRNA expression of CTSD, the major aspartic protease of endolysosomes (25), was decreased in the low HDL groups compared with control subjects and significantly in groups with low efflux ($p < 0.01$) (Fig. 1). These results were confirmed in a second cohort that included a group with low HDL and efflux defect but no mutation in ABCA1, whereas the group with low HDL and normal efflux did not present with decreased CTSD. Although the decreased CTSD could not be verified in a second group with low HDL and efflux with ABCA1 mutations (for lack of subjects), the initial observation (Fig. 1B) suggests the possibility of an independent decrease in CTSD that compounds the ABCA1 defect. Alternatively, the decreased ABCA1 activity may decrease CTSD through a feedback loop.

Based on these observations, we hypothesized that CTSD activity might affect cellular cholesterol transport or efflux. CTSD binding of ceramide affects its autocatalytic activity (37), and in cells with acid sphingomyelinase deficiency, low endogenous ceramide (the product of the digestion of sphingomyelin) is associated with decreased CTSD activity (37). We showed that exogenous ceramide increases cholesterol and phospholipid efflux by increasing the autocatalytic activity of CTSD (supplemental Fig. 1). In agreement with our results, Witting et al. (47) have shown that the addition of ceramide increases cholesterol efflux and cell surface ABCA1. Alternatively, ceramide may also affect the cholesterol efflux through the feedback activation of acid sphingomyelinase (48).

CTSD has been shown to bind and stimulate the proteolytic processing of proSAP (39). The proSAP knock-out mice (49) and tissues from individuals with proSAP deficiency (50, 51) are characterized by GSL accumulation (40–43). Recently, our laboratory showed that the inhibition of prosaposin delivery to late endosomes by LRPI and its adaptor, GULP, causes the accumulation of GSL and free cholesterol in late endosomes, resulting in down-regulation of ABCA1 and decreased cholesterol efflux (52). Consistent with these observations, we demonstrate that the inhibition of CTSD is also accompanied by the accumula-
The identification of CTSD as an activator of pro-SAP has provided important insight into the mechanism by which CTSD modulates the metabolism of cellular cholesterol and GSL. Pro-SAP is the precursor of four protein cofactors, saposins A, B, C, and D, that are required for the enzymatic hydrolysis of sphingolipids by specific lysosomal hydrolases. Saposins have important functions in the degradation of ceramide (saposin D), glucocerebroside (saposin C), sulfatide (saposin B), and glycosylceramide (saposin C) (53). Recently, saposin B was also shown to bind and transfer phospholipids from one membrane to another (54), a process that can occur in lysosome/late endosome compartment and is specific for either phosphatidylcholine or phosphatidylinositol. Here, we show that overexpression of CTSD increases cellular protein levels of both pro-SAP and saposins, which control sphingolipid transport and the cholesterol efflux pathway.

Sphingolipid storage diseases are lysosomal storage disorders. One of the most common, Gaucher’s disease, is caused by deficiency of glucocerebrosidase, a lysosomal acid β-glucosidase, which results in the accumulation of glucocerebrosidase in lysosomes. Notably, affected subjects have decreased plasma concentrations of HDL cholesterol (55, 56). Niemann-Pick, type A or B, is caused by altered function of acid sphingomyelinase, which metabolizes the sphingomyelin to ceramide. The saposins A, C, and D are responsible for the hydrolytic activation of this pathway (57, 58). Individuals with this disease also exhibit low HDL-cholesterol levels (59), as do patients with NPC disease (60). Furthermore, it has been reported that macrophages from acid sphingomyelinase knock-out mice accumulate sphingomyelin and exhibit defective cholesterol trafficking and efflux (61). Fibroblasts treated with lactosylceramide or with an inhibitor of glucocerebrosidase also accumulate glycosphingolipids, which results in inhibition of cholesterol efflux by the apoA-I/ABCA1 pathway (62). Thus, disruption of one of the pathways of both BODIPY-lactosylceramide and free cholesterol in structures resembling endosome/lysosome compartments reminiscent of the cellular phenotype of NPC disease (44).
of sphingolipid metabolism can cause GSL and free cholesterol accumulation and alter cellular lipid homeostasis and HDL concentrations (Fig. 10). In this study, the low HDL subjects with efflux defects have low CTSD expression, suggesting a correlation between CTSD expression and HDL levels. This low HDL phenotype could also be additive with decreased expression of prosaposin (50). ABCA1 expression levels are not significantly decreased in group A (data not shown), suggesting that decreased CTSD may also affect efflux by dysregulation of ABCA1 trafficking rather than expression.

The expression and regulation of cathepsins are complex. They can act as transcriptional activators (63), and there can be cross-talks between cathepsins, where for example cathepsin L is involved in CTSD processing and regulation (64). The CTSD gene is controlled by a mixed promoter that contains features of both housekeeping and regulated genes (65–68). It is regulated by estrogens (66, 69) and by retinoids (65, 70). Here, a decrease in CTSD results in decreased stasis and inflammation (71).

In conclusion, these studies show for the first time that CTSD regulates intracellular transport of phospholipid and cholesterol and ABCA1-mediated lipid efflux. The addition of pepstatin A, an inhibitor of CTSD, impaired apoA-I-mediated efflux in CHO cells and in macrophage cell lines. Specific siRNA oligonucleotides had a similar effect, causing a marked reduction in CHO cells and in macrophage cell lines. Specific siRNA oligonucleotides had a similar effect, causing a marked reduction in CHO cells and in macrophage cell lines. Specific siRNA oligonucleotides had a similar effect, causing a marked reduction in CHO cells and in macrophage cell lines. Specific siRNA oligonucleotides had a similar effect, causing a marked reduction in CHO cells and in macrophage cell lines.
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Sohar, I., Lobel, P., and Johnson, G. S. (2006) Mol. Genet. Metab. 87, 341–348
33. O’Brien, J. S., and Kishimoto, Y. (1991) FASEB J. 5, 301–308
34. Kishimoto, Y., Hiraia, M., and O’Brien, J. S. (1992) J. Lipid Res. 33, 1255–1267
35. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. 37, 911–917
36. Haidar, B., Denis, M., Marcil, M., Krimbou, L., and Genest, J., Jr. (2004) J. Biol. Chem. 279, 9963–9969
37. Heinrich, M., Wickel, M., Schneider-Brachert, W., Sandberg, C., Gahr, J., Schwindner, R., Weber, T., Saftig, P., Peters, C., Brunner, J., Kronke, M., and Schütze, S. (1999) EMBO J. 18, 5252–5263
38. Conner, G. E. (1989) Biochem. J. 263, 601–604
39. Gopalakrishnan, M. M., Grosch, H. W., Locatelli-Hoops, S., Werth, N., Smolenova, E., Nettersheim, M., Sandhoff, K., and Hasilik, A. (2004) Biochem. J. 383, 507–515
40. Puri, V., Watanabe, R., Dominguez, M., Sun, X., Wheatley, C. L., Marks, D. L., and Pagano, R. E. (1999) Nat. Cell Biol. 1, 386–388
41. Garner, B., Mellor, H. R., Butters, T. D., Dwek, R. A., and Platt, F. M. (2002) Biochem. Biophys. Res. Commun. 290, 1361–1367
42. Silence, D. J., Puri, V., Butters, T. D., Dwek, R. A., Pagano, R. E., and Platt, F. M. (2002) J. Lipid Res. 43, 1837–1845
43. Puri, V., Jefferson, J. R., Singh, R. D., Wheatley, C. L., Marks, D. L., and Pagano, R. E. (2003) J. Biol. Chem. 278, 20961–20970
44. te Vruchte, D., Lloyd-Evans, E., Veldman, R. J., Neville, D. C., Dwek, R. A., Platt, F. M., van Blitterswijk, W. J., and Silence, D. J. (2004) J. Biol. Chem. 279, 26167–26175
45. Pagano, R. E. (2003) Philos. Trans. R. Soc. Lond. B Biol. Sci. 358, 885–891
46. Neufeld, E. B., Remaley, A. T., Demosky, S. J., Stonik, J. A., Cooney, A. M., Comly, M., Dwyer, N. K., Zhang, M., Blanchette-Mackie, J., Santamarina-Fojo, S., and Brewer, H. B., Jr. (2001) J. Biol. Chem. 276, 27584–27590
47. Witting, S. R., Maiorano, J. N., and Davidson, W. S. (2003) J. Biol. Chem. 278, 40121–40127
48. Deigner, H. P., Claus, R., Bonaterra, G. A., Gehrke, C., Bibak, N., Blass, M., Cantz, M., Metz, J., and Kinscherf, R. (2001) FASEB J. 15, 807–814
49. Fujita, N., Suzuki, K., Vanier, M. T., Popko, B., Maeda, N., Klein, A., Henseler, M., Sandhoff, K., Nakayasu, H., and Suzuki, K. (1996) Hum. Mol. Genet. 5, 711–725
50. Elleder, M., Jerabkova, M., Befekadu, A., Hrebicek, M., Berna, L., Ledvinaova, J., Hulkaova, H., Rosewich, H., Schymik, N., Paton, B. C., and Harzer, K. (2005) Neuropediatrics 36, 171–180
51. Hulkaova, H., Cervenkova, M., Ledvinaova, J., Tochackova, M., Hrebicek, M., Poupetova, H., Befekadu, A., Berna, L., Paton, B. C., Harzer, K., Boor, A., Smid, F., and Elleder, M. (2001) Hum. Mol. Genet. 10, 927–940
52. Kiss, R. S., Ma, Z., Nakada-Tsukui, K., Brugnera, E., Vassiliou, G., McBride, H. M., Ravichandran, K. S., and Marcel, Y. L. (2006) J. Biol. Chem. 281, 12081–12092
53. Sandhoff, K., and Kolter, T. (2003) Philos. Trans. R. Soc. Lond. B Biol. Sci. 358, 847–861
54. Ciaffoni, F., Tatti, M., Boe, A., Salvori, R., Fuharty, A., Sonnino, S., and Vaccaro, A. M. (2006) J. Lipid Res. 47, 1045–1053
55. Pocovi, M., Cenarro, A., Civeira, F., Torralba, M. A., Perez-Calvo, J. I., Mozas, P., Giraldo, P., Giralt, M., Myers, R. H., Cupples, L. A., and Ordonez, J. M. (1998) Lancet 351, 1919–1923
56. Ginsberg, H., Grabowski, G. A., Gibson, J. C., Fagerstrom, R., Goldblatt, J., Gilbert, H. S., and Desnick, R. J. (1984) Clin. Genet. 26, 109–116
57. Forst, W., and Sandhoff, K. (1992) Biochim. Biophys. Acta 1126, 1–16
58. Sandhoff, K., and Kolter, T. (1996) Trends Cell Biol. 6, 98–103
59. McGovern, M. M., Pohl-Worgall, T., Deckelbaum, R. J., Simpson, W., Mendelson, D., Desnick, R. J., Schuchman, E. H., and Wasserstein, M. P. (2004) J. Pediatr. 145, 77–81
60. Choi, H. Y., Karten, B., Chan, T., Vance, J. E., Greer, W. L., Heidenreich, R. A., Garver, W. S., and Francis, G. A. (2003) J. Biol. Chem., 61. Leventhal, A. R., Chen, W., Tall, A. R., and Tabas, I. (2001) J. Biol. Chem. 276, 44976–44983
62. Glaros, E. N., Kim, W. S., Quinn, C. M., Wong, J., Gelissen, I., Jessup, W., and Garner, B. (2005) J. Biol. Chem. 280, 24515–24523
63. Chapman, H. A. (2004) Dev. Cell 6, 610–611
64. Wille, A., Gerber, A., Heimburg, A., Reisenauer, A., Peters, C., Saftig, P., Reinheckel, T., Welte, T., and Buhling, E. (2004) Biochim. Biophys. Acta 385, 665–670
65. Atkins, K. B., and Troen, B. R. (1995) Cell Growth Differ. 6, 871–877
66. May, F. E., Smith, D. J., and Westley, B. R. (1993) Gene (Amst.) 134, 277–282
67. Cavaileas, V., Augereau, P., and Rochefort, H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 203–207
68. Redecker, B., Heckendorf, B., Grosch, H. W., Mersmann, G., and Hasilik, A. (1991) DNA Cell Biol. 10, 423–431
69. Augereau, P., Miralles, F., Cavailles, V., Gaudelet, C., Peters, C., Saftig, P., Reinheckel, T., Welte, T., and Buhling, E. (2004) Biochim. Biophys. Acta 385, 665–670
70. Augereau, P., Miralles, F., Cavailles, V., Gaudelet, C., Parker, M., and Rochefort, H. (1994) Trends Cell Biol. 6, 871–877
71. Sheik, M. S., Augereau, P., Chalbos, D., Garcia, M., and Rochefort, H. (1996) J. Steroid Biochem. Mol. Biol. 57, 283–291
72. Zelcer, N., and Tontonoz, P. (2006) J. Clin. Invest. 116, 607–614