

HDL antielastase activity prevents smooth muscle cell anoikis, a potential new antiatherogenic property

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ABSTRACT Various studies using proteomic approaches have shown that HDL can carry many proteins other than its constitutive apolipoprotein A-I (apoA-I). Using mass spectrometry and Western blotting, we showed the presence of α_1 -antitrypsin (AAT) (SERPINA1, serpin peptidase inhibitor, clade A, an elastase inhibitor) in HDL, isolated either by ultracentrifugation or by selected-affinity immunosorption using an anti-apoA-I column. Furthermore, we report that HDL possesses potent antielastase activity. We further showed that only HDL but not LDL is able to bind AAT. HDL-associated AAT was able to inhibit extracellular matrix degradation, cell detachment, and apoptosis induced by elastase in human vascular smooth muscle cells (VSMCs) and in mammary artery cultured *ex vivo*. Degradation of fibronectin by elastase used as a marker of pericellular proteolysis was prevented by addition of HDL. Elastase present in aortic abdominal aneurysm (AAA) thrombus samples was also able to induce apoptosis of VSMCs in culture. This phenomenon was prevented by addition of HDL but not of LDL. Finally, we report that the proportion of AAT in HDL isolated from patients with an AAA is decreased relative to that from matched control subjects, suggesting a reduced capacity of HDL to inhibit elastase in these patients. In conclusion, our data provide evidence of a new potential antiatherogenic property of HDL attributable to AAT and its antielastase activity.—Ortiz-Muñoz, G., Houard, X., Martín-Ventura, J.-L., Ishida, B. Y., Loyau, S., Rossignol, P., Moreno, J.-A., Kane, J. P., Chalkley, R. J., Burlingame, A. L., Michel, J.-B., Meilhac, O. High-density lipoprotein antielastase activity prevents smooth muscle cell anoikis, a potential new antiatherogenic property. *FASEB J.* 23, 000–000 (2009). www.fasebj.org

inversely correlated with atherothrombotic risk in observational studies (1). Beneficial effects of HDL are principally attributed to reverse transport of cholesterol, even though other antiatherogenic properties are well documented, such as antioxidant, anti-inflammatory, or antithrombotic effects (2). Several studies using proteomic approaches on HDL from healthy subjects have identified α_1 -antitrypsin in HDL₂ and HDL₃ fractions (3, 4). This serine protease inhibitor is the natural circulating inhibitor of neutrophil elastase. We and others have reported that this protease is present in atherothrombotic lesions (5, 6) and circulating leukocyte elastase- α_1 -antitrypsin (AAT) complexes were correlated with carotid stenosis and a risk of myocardial infarction and stroke (7, 8). In addition, we have shown that neutrophil elastase present in the intraluminal thrombus of abdominal aortic aneurysms (AAAs) plays a pivotal role in the disappearance of arterial wall smooth muscle cells and subsequent absence of healing (9–11). Among the proteases reported to be present in the pathological arterial wall and able to induce apoptosis subsequent to extracellular matrix (ECM) degradation, elastase is one of the most potent (12). Polymorphonuclear neutrophils (PMNs) represent the main class of circulating leukocytes that are activated when they are trapped during the formation of the thrombus either in AAAs or in intraplaque hemorrhages, recently described as a driving force of atherosclerotic plaque evolution toward rupture (6, 13). PMN degranulation leads to the release of elastase in the extracellular compartment, which is able to degrade many proteins of the ECM such as elastin, fibronectin, thrombospondin, and vitronectin (14–16). Proteolysis

PLASMA LEVELS OF HDL CHOLESTEROL and its major protein apolipoprotein A-I (apoA-I) are consistently

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of the ECM destabilizes the arterial wall both directly by reducing its mechanical resistance and indirectly by inducing vascular cell apoptosis subsequent to rupture of cell-ECM contacts that normally convey survival signals (9). In the present study, we showed that AAT (SERPINA1, serpin peptidase inhibitor, clade A), the naturally occurring elastase inhibitor, is associated with HDL, and we tested the hypothesis that HDL could thereby inhibit elastase activity and its deleterious effects *in situ*, such as ECM degradation and smooth muscle cell anoikis. In addition, we assessed the levels of HDL and AAT-HDL in the plasma of patients with or without AAAs. Therefore, we describe here a new function of HDL, an antielastase activity possibly favoring arterial wall stabilization, that could account for its antiatherogenic potential.

MATERIALS AND METHODS

Reactives and cell culture

Human neutrophil elastase and AAT were from Calbiochem (San Diego, CA, USA). Human aortic vascular smooth muscle cells (VSMCs) (PromoCell GmbH, Heidelberg, Germany) were cultured in medium (PromoCell SM2) containing 10% FCS.

Isolation of lipoproteins

Lipoproteins were isolated from plasma of healthy volunteers sampled on EDTA by two different methods: ultracentrifugation and selected affinity immunosorption. Isolation of HDL by immunosorption was performed as described previously (17). In brief, an anti-apoA-I column was prepared by cross-linking rabbit polyclonal antibodies directed against apoA-I to Sepharose beads. A mock column without antibodies and an IgG column were prepared under the same conditions using nonrelevant IgG (Innovative Research, Novi, MI, USA). Plasma from healthy subjects (nonsmokers >50 yr old, with informed consent) was incubated overnight at 4°C with apoA-I and mock or IgG-Sepharose beads (1 ml of EDTA-plasma for 12.5 ml of beads) under gentle shaking. The columns were then rinsed three times with 5 volumes of saline (0.9% NaCl, 1 mM EDTA, and 0.025% Na₂S₂O₃) containing additional NaCl to reach a 0.5 M concentration. After a final wash with saline, the column was eluted with a solution containing 0.2 M acetic acid and 0.15 M NaCl, pH 3, and immediately buffered with Tris base to pH 7.9. HDL was then extensively rinsed with saline-EDTA and concentrated using a centrifugal concentrating device (cutoff 5 kDa; Vivascience, Stonehouse, UK). Alternatively, plasma density was adjusted to $d = 1.063$ with KBr and overlaid with KBr saline solution ($d = 1.063$). Ultracentrifugation was performed at 100,000 *g* for 20 h at 10°C. The upper lipoprotein fraction containing LDL was adjusted to a density of 1.25 g/ml with KBr and then overlaid with saline ($d = 1.006$) before ultracentrifugation at 100,000 *g* for 20 h at 10°C. After this step, the LDL fraction (orange layer) was recovered as a single band, and the KBr was eliminated by 3 washing steps using a centrifugal filter device. The density of the bottom fraction resulting from the first ultracentrifugation and containing HDL was adjusted to 1.25 g/ml with KBr and overlaid with KBr saline solution ($d = 1.21$). The second ultracentrifugation and subsequent washing steps were similar to those for LDL, except that HDL

fractions represent the top layer of the tube. When indicated, HDL₂ and HDL₃ were collected separately. All fractions were desalted either by dialysis against saline or by centrifugation and 3 washes with saline.

Two-dimensional (2-D) nondenaturing electrophoresis

Lipoproteins containing apoA-I (LpA-I) were purified from normolipidemic human plasma by anti-apoA-I immunosorption column chromatography. Residual serum albumin and immunoglobulins were removed by passage over anti-human serum albumin and protein A-Sepharose columns. The LpA-I fraction was analyzed by 2-D agarose × polyacrylamide gel (PAG) nondenaturing electrophoresis. LpA-I was electrophoresed in 0.8% agarose (w/v) (catalog no. 162-0126; Bio-Rad Laboratories, Hercules, CA, USA) prepared in 0.062 M Tris, 0.027 M *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine, and 0.005 M calcium lactate (pH 8.3) at a 15 V/cm field strength. The agarose strip was electrophoresed in a second-dimension linear gradient of PAG [0–30% total concentration of acrylamide + bisacrylamide (T)] to equilibrium (3000 V-h) at 5°C. A mixture of proteins was used to calibrate (Stoke's diameter) the second-dimension PAG and included ovalbumin (6.0 nm), BSA (7.1 nm), lactate dehydrogenase (8.1 nm), catalase (10.4 nm), ferritin (12.2 nm), thyroglobulin (17.0 nm), and LDL ($d = 1.030$ – 1.050 g/ml, 25 nm).

Mass spectrometry analysis of HDL species separated by 2-D nondenaturing gels

Proteins resolved by 2-D PAG were prepared for mass spectrometry by in-gel digestion with trypsin (catalog no. V5111; Promega Corp., Madison, WI, USA) (18). Peptides were separated by reverse-phase chromatography using an UltiMate HPLC system (Dionex, Sunnyvale, CA, USA). A C18 PepMap100 column (75 μm inside diameter × 15 cm) was used with a gradient from 2 to 30% acetonitrile/0.1% formic acid over 38 min, followed by an increase to 50% acetonitrile/0.1% formic acid over a further 2 min. Eluting peptides were introduced into an LTQ Orbitrap (Thermo Electron Corp., Waltham, MA, USA) in which data-dependent acquisition was used to fragment the 6 most abundant components observed in each survey scan, using dynamic exclusion of previously fragmented components. Raw data were converted to peaklists using Mascot Distiller 2.1.1.0 (<http://www.matrixscience.com/distiller>) and then analyzed using Protein Prospector 5.0 (<http://prospector2.ucsf.edu>) against the human entries of a database that consisted of the UniProt (<http://www.uniprot.org>) database downloaded on December 4, 2007, with a sequence shuffled/randomized decoy version concatenated onto the end of the database, giving a total of 152,244 entries searched. The concatenated database allowed for estimation of a peptide false-positive rate (19). Search parameters required tryptic cleavage specificity with up to one missed cleavage, precursor mass accuracy of within 20 ppm, and fragment mass accuracy of within 0.6 Da. Cysteine carbamidomethylation was searched for as a constant modification, and methionine oxidation, pyroglutamate formation from peptide N-terminal glutamines, and protein N-terminal acetylation were allowed for as variable modifications. Acceptance criteria were a minimum peptide score of 15, a minimum protein score of 22, and a maximum expectation value of 0.1. For all spots analyzed, a total of 1998 peptides above these thresholds were reported, which included three matches to the decoy part of the database. Hence, the peptide false-positive rate of identification for the dataset is approximately 0.3% [(3×2)/1998].

Western blots on 2-D nondenaturing gels

For immunoblots, calibrator proteins were biotinylated (catalog no. 170-6529; Bio-Rad Laboratories). Resolved proteins were electrophoretically transferred (55 V, 18 h, 10°C) onto nitrocellulose membranes (0.2 µm, catalog no. 162-0210; Bio-Rad Laboratories). Nonspecific binding was blocked with casein (25 mg/ml and 0.02 M Tris, pH 8.5). Membranes were probed with antibodies to apoA-I (custom produced goat polyclonal) and to AAT (mouse monoclonal, catalog no. 178260; Calbiochem), and bound antibodies were disclosed using biotinylated second antibodies, avidin-biotin-horseradish peroxidase conjugates (catalog no. 1852410; Pierce Chemical Co., Rockford, IL, USA), and 3,3'-diaminobenzidine (0.05%, w/v)/nickel chloride (2.5 mM)/H₂O₂ (0.05%, v/v) in 0.10 M imidazole (pH 7.0).

Western blot analysis after SDS-PAGE

HDL and LDL (5 µg) from 4 and 3 different preparations, respectively, were resolved by SDS-12% PAGE. After electrophoresis, proteins were transferred onto nitrocellulose membranes, blocked with 5% milk powder in Tris-buffer saline, pH 7.4/0.1% Tween 20, and then probed with either rabbit polyclonal anti- α_2 -antiplasmin (α_2 AP) (dilution 1:1000; Calbiochem) or rabbit polyclonal anti-AAT (dilution 1:1000; DakoCytomation A/S, Copenhagen, Denmark) and peroxidase-conjugated secondary antibody (dilution 1:2500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Purified AAT (200 ng; Calbiochem) and α_2 AP (100 ng; Calbiochem) were used as controls.

For detection of fibronectin fragments in cell culture supernatant, 5 µl of conditioned medium was analyzed by SDS-8%PAGE. The transblotted membranes were then probed with a rabbit polyclonal anti-human fibronectin (dilution 1:1000; Sigma-Aldrich Corp., St. Louis, MO, USA).

In all cases, an appropriate peroxidase-conjugated secondary antibody was used (dilution 1:2500; Jackson ImmunoResearch Laboratories) followed by ECL detection. Densitometry analysis was performed using a calibrated scanner (GS800; Bio-Rad).

Determination of elastase and plasmin activities

Human neutrophil elastase (10 nM; Calbiochem) was incubated with 1.5 mM of an elastase chromogenic substrate, MeO-Suc-Ala-Ala-Pro-Val-pNa (Calbiochem) in PBS (100 µl final volume). Plasmin (10 nM; American Diagnostica, Stamford, CT, USA) was incubated with 0.75 mM of the selective plasmin chromogenic substrate, CBS0065 (Diagnostica Stago, Asnières, France) in 50 mM phosphate buffer (pH 7.4) and 80 mM NaCl (100 µl final volume). AAT (40 nM), D-valyl-L-phenylalanyl-L-lysine chloromethylketone (10 µM; Calbiochem), a selective irreversible inhibitor of plasmin, HDL (50 µg or 1–4.5 µg for dose-response experiments), and LDL (50 µg) were preincubated with elastase or plasmin for 15 min at room temperature before the addition of the substrate. Human plasma (1:1000 dilution) or HDL from patients (1:4 dilution) was incubated in the presence of 10 nM elastase in the same conditions. Substrate hydrolysis was monitored for 2 h at 37°C by spectrophotometry at 405 and 490 nm. HDL antielastase activity was normalized to HDL-apoA-I quantity.

Cell detachment assay and apoptosis

Human VSMCs were grown to confluence in 12-well plates and serum-deprived for 24 h before stimulation. VSMCs were then incubated for 16 h with 10 nM elastase (Calbiochem) or

culture medium conditioned with intraluminal thrombus of human AAAs (1:5 dilution) with or without HDL and LDL (100 µg/ml). At the end of the experiment, cell supernatants were aspirated, centrifuged for 5 min at 3000 g, and analyzed for fibronectin proteolytic fragments by Western blot. Remaining viable adherent cells were washed with PBS and assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test as described previously (11). Alternatively, apoptosis was determined by the quantification of histone-associated DNA fragments using a photometric enzyme immunoassay (Cell Death Detection ELISA^{PLUS}; Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's instructions (20). Performing the trypan blue exclusion test on trypsinized cells confirmed that 95% of remaining adherent cells did not exhibit membrane permeabilization (21).

Detection of apoptosis *in situ*

Human mammary arteries were obtained from patients undergoing cardiac surgery at Bichat Hospital (Paris, France). These tissues are considered as surgical waste in accordance with French ethics laws (L.1211-3 to L.1211-7, L.1235-2, and L.1245-2) and the INSERM Ethics Committee. Equal segments of mammary arteries (5-mm rings), obtained by removal of the adventitia, were incubated with or without 10 nM elastase in the presence or absence of HDL or LDL (0.2 mg/ml each) for 24 h in serum-free RPMI 1640 medium at 37°C (5% CO₂). After incubation of mammary end-arteries with elastase, the tissue was fixed in 3.7% paraformaldehyde and embedded in paraffin. Immunohistochemical analysis was performed on 5-µm-thick sections using a monoclonal antibody to single-stranded DNA (Apostain; Alexis, Lüufeligen, Switzerland) as a marker of apoptosis *in situ* (22).

Preparation of conditioned medium from AAAs

AAA samples were obtained from patients undergoing surgery, who were enrolled in the REflet Sanguin de l'évolutivité des Anévrysmes de l'Aorte abdominale (RESAA) protocol (23). All patients gave their informed written consent, and the protocol was approved by a French ethics committee [Cochin Hospital Comité de Protection des Personnes se Prêtant à la Recherche Biomédicale (CCPPRB)]. AAA intraluminal thrombi sampled during surgery were incubated with 1 M acetate buffer, pH 4.5 (2 ml/g of wet tissue), for 2 h at room temperature. Extracts containing elastase were then dialyzed against PBS for culture assays as described previously (11).

HDL labeling with carbocyanines

HDL was incubated overnight at 37°C under gentle shaking with 10 µl/ml DiI₁₈ carbocyanines (Molecular Probes Inc., Eugene, OR, USA) and then separated by ultracentrifugation as described above. VSMCs were incubated with 100 µg/ml labeled HDL for 8 h. After 3 washes with PBS, cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (0.5 µg/ml for 10 min) and visualized under an epifluorescence microscope.

Immunocytofluorescence

For confocal microscopy, human VSMCs were plated onto Labtek slides and incubated with 50 µg/ml HDL for 4 h. The slides were then washed with PBS, fixed with 4% paraformaldehyde, blocked in 4% PBS-BSA, and incubated with goat

anti-AAT antibody and anti-apoA-I (Calbiochem) at a 1:50 dilution. Slides were then incubated with appropriate fluorescein 5-isothiocyanate- or tetramethyl rhodamine isothiocyanate-labeled secondary Ab (Sigma-Aldrich) at a 1:200 dilution for 1 h.

Aneurysm Metalloproteinases and Hypertension Study (AMETHYST) cohort

AMETHYST is an ongoing study promoted by INSERM that involves a cohort of patients with asymptomatic AAAs (with aortic diameter >5 cm) scheduled for endovascular repair within 1 mo. These patients were age and sex matched with healthy volunteers. All study participants gave informed consent. The study was approved by an ethics committee (CCPPRB, approval nos. 1930 and 1931).

Exclusion criteria for patients were cancer, infection, and any immune-mediated disease. Peripheral blood was drawn in standardized conditions (fasting subjects at rest for 10 min, between 8 and 10 AM), with minimal stasis, into prechilled EDTA tubes. No later than 30 min after collection, two centrifugations were performed to separate the plasma from the blood cells (2500 rpm, 15 min, 12°C and 2500 rpm, 15 min, 4°C). Plasma samples were stored at -80°C until used.

Determination of ApoA-I concentration

The apoA-I concentration was determined using an ELISA test from Mabtech AB (Nacka Strand, Sweden) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed with GraphPad InStat (GraphPad Software Inc., San Diego, CA, USA). For the comparison between patients with AAAs and age- and sex-matched control subjects, further adjustment for smoking habits was performed during the statistical analysis, without altering nontobacco-adjusted comparison results. All experiments were performed at least 3 times. Results are expressed as means \pm SD and were analyzed by ANOVA. Differences were considered significant when $P < 0.05$.

RESULTS

HDL but not LDL contains AAT

We used differential flotation properties of lipoproteins to isolate HDL by a 2-step ultracentrifugation tech-

nique on KBr, similar to that used by Karlsson *et al.* (3) to identify AAT in HDL by a proteomic approach. Here we show by Western blot that HDL isolated from plasma of four different subjects contains AAT whereas LDL isolated in the same conditions is devoid of this major plasma protein (Fig. 1A). A Western blot against α_2 AP performed on the same samples was not able to show the presence of this other abundant plasma protein either in LDL or in HDL. Despite its high concentration in plasma, AAT is unlikely to be a contaminant coisolated with HDL. Binding experiments were performed on ELISA plates coated with either apoA-I or AAT. ApoA-I is able to bind to AAT but in a nonsaturable fashion, suggesting a low-affinity or nonspecific binding (not shown). In a second step, we tested the ability of HDL to trap more AAT. For this purpose we incubated either HDL or LDL with purified AAT (1 mg:1 mg) for 16 h at 37°C under gentle agitation and then reisolated both lipoproteins by ultracentrifugation to get rid of free/unbound AAT. We show by Western blot that HDL but not LDL is able to bind and incorporate additional AAT (Fig. 1B). Our results suggest that, in contrast to LDL, HDL possesses an affinity for AAT. Finally, we show that AAT is more abundant in HDL₃ than in HDL₂ fractions (Fig. 1C).

AAT is present in α_1 fractions of HDL

In parallel, we isolated HDL by selected-affinity immunosorption using an anti-apoA-I column. Elution allowed recovery of the LpA-I. LpA-I species were separated by nondenaturing two-dimension electrophoresis gels, allowing each particle containing apoA-I to migrate according to its charge and size. The gels were either transblotted for Western blot analysis or stained by Coomassie blue for subsequent proteomic analysis (Fig. 2A). Spectra containing a good series of γ and β ions allowed identification of peptides from AAT and apoA-I (Fig. 2B). Mass spectrometry analysis performed on the different spots detected by colloidal Coomassie blue staining identified AAT in α_1 particles ranging in diameter from 7 to 10 nm Stokes radii; as expected, apoA-I was also identified in these particles. Twenty-

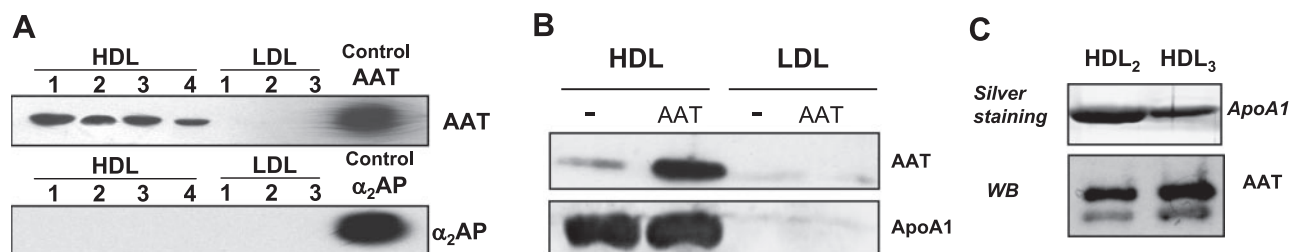


Figure 1. HDL isolated by ultracentrifugation contains AAT. *A*) HDL and LDL from human plasma were isolated by 2-step ultracentrifugation on KBr gradient density. Four different batches of lipoproteins (5 μ g) were immunoblotted with AAT (200 ng) and α_2 AP (100 ng) antibodies. HDL contained AAT but no α_2 AP. *B*) HDL and LDL (100 μ g) were incubated overnight (16 h) with 100 μ g of AAT in a total volume of 200 μ l at 37°C under gentle shaking. Both lipoproteins were then isolated by ultracentrifugation, and 1 μ g of each was loaded for Western blot analysis against AAT and apoA-I. Results are representative of 3 independent experiments. *C*) HDL₂ and HDL₃ fractions isolated by ultracentrifugation were either silver-stained (top panel) or submitted to Western blot (WB) analysis using an anti-AAT antibody (bottom panel). Gels shown are representative of 4 independent experiments.

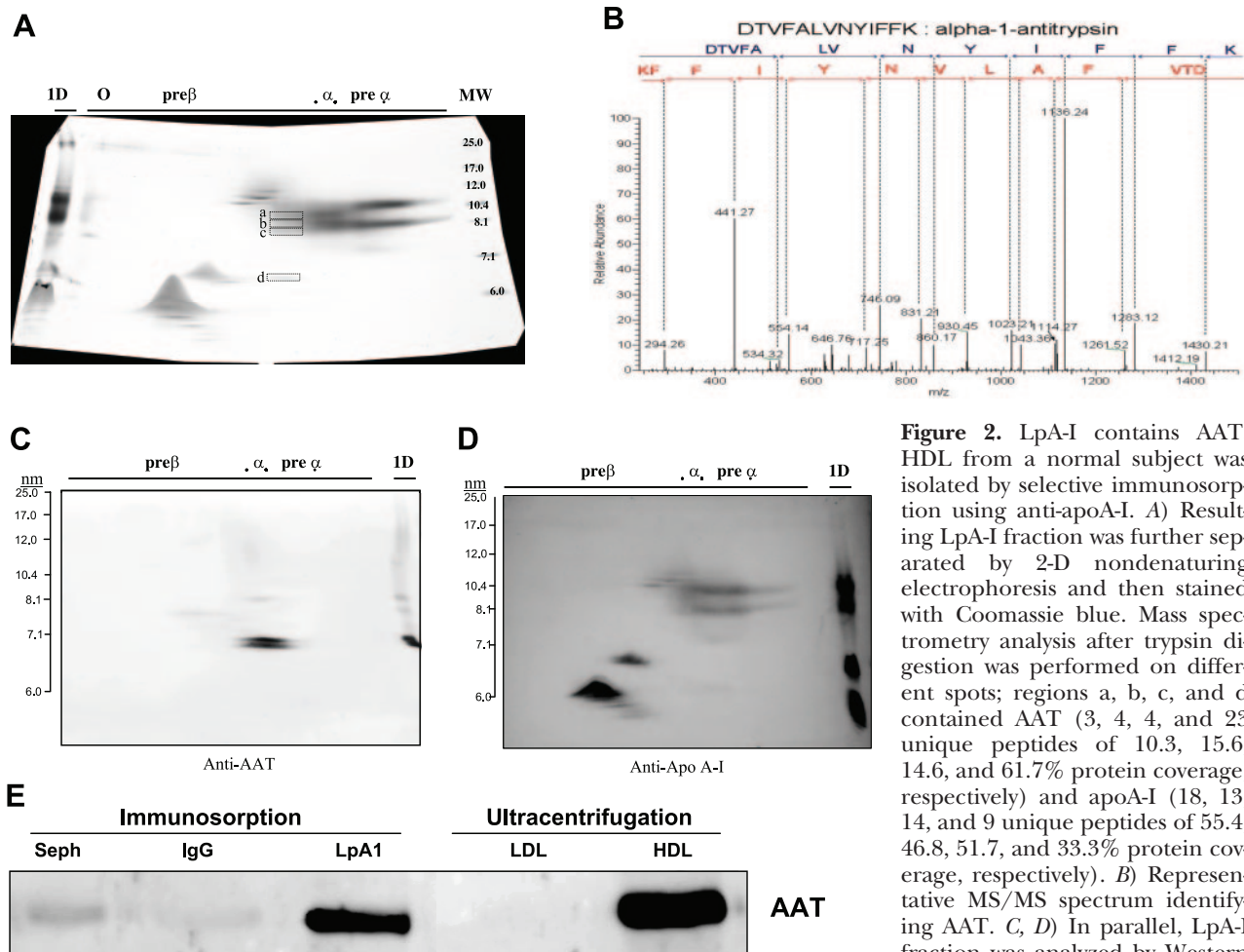


Figure 2. LpA-I contains AAT. HDL from a normal subject was isolated by selective immunosorption using anti-apoA-I. *A*) Resulting LpA-I fraction was further separated by 2-D nondenaturing electrophoresis and then stained with Coomassie blue. Mass spectrometry analysis after trypsin digestion was performed on different spots; regions a, b, c, and d contained AAT (3, 4, 4, and 23 unique peptides of 10.3, 15.6, 14.6, and 61.7% protein coverage, respectively) and apoA-I (18, 13, 14, and 9 unique peptides of 55.4, 46.8, 51.7, and 33.3% protein coverage, respectively). *B*) Representative MS/MS spectrum identifying AAT. *C, D*) In parallel, LpA-I fraction was analyzed by Western

blot using anti-AAT (*C*) and anti-apoA-I antibodies (*D*). *E*) SDS-PAGE followed by Western blot confirmed the presence of AAT in HDL, isolated both by immunosorption and ultracentrifugation. A mock column without anti-apoA-I antibody (Seph) and a nonrelevant IgG column were used to assess nonspecific binding of HDL to the Sepharose beads and immunoglobulins.

three unique AAT peptides were identified in the smallest 7-nm-diameter particle, whereas 3 to 4 peptides were identified in the other larger 8- to 10-nm particles, suggesting that the predominant LpA-I AAT mass is localized in the 7-nm LpA-I particle. ApoA-I composition in these particles varied, with fewer unique peptides being identified in the 7-nm LpA-I particles than in the larger 8- to 10-nm particles (8 *vs.* 13–18, respectively). The 7-nm LpA-I particle composition included lesser amounts of paraoxonase, apoA-IV, apoD, apoC-III, factor V, and α_1 -acid glycoprotein 1. Western blot against AAT showed that the 7- to 10-nm particles of α_1 electrophoretic mobility contained AAT that colocalized with anti-apoA-I staining, confirming the results obtained by mass spectrometry (Fig. 2*C, D*). The weak immunoreactivity against apoA-I on the blot is probably due to the fact that the antibodies directed against apoA-I do not react well on blots after 2-D electrophoresis under nondenaturing conditions. AAT was also detected to a lesser extent in the large α fraction. Regular Western blot after SDS-PAGE separation confirmed the presence of AAT only in LpA-I isolated by the anti-apoA-I column relative to a mock

column without antibody. HDL isolated by ultracentrifugation from the same plasma was also positive for AAT (Fig. 2*E*).

HDL-associated AAT displays antielastase activity *in vitro*

In a second step, we tested the potential of HDL-associated AAT to inhibit elastase activity. *In vitro*, leukocyte elastase (30 nM) was incubated with HDL isolated from healthy subjects, and its activity was assessed using a chromogenic substrate, MeO-Suc-Ala-Ala-Pro-Val-pNa. Elastase was dose dependently inhibited by HDL, and almost total inhibition of elastase activity was reached, ranging from 5 to 20 $\mu\text{g}/\text{ml}$ HDL, depending on the batch of HDL (isolated from different healthy subjects). In contrast, 50 $\mu\text{g}/\text{ml}$ of LDL from 3 different healthy subjects did not exhibit any antielastase activity (Fig. 3*A*). Similar results were obtained using conditioned media from luminal layers of human AAA intraluminal thrombi or supernatants of activated neutrophils as sources of leukocyte elastase

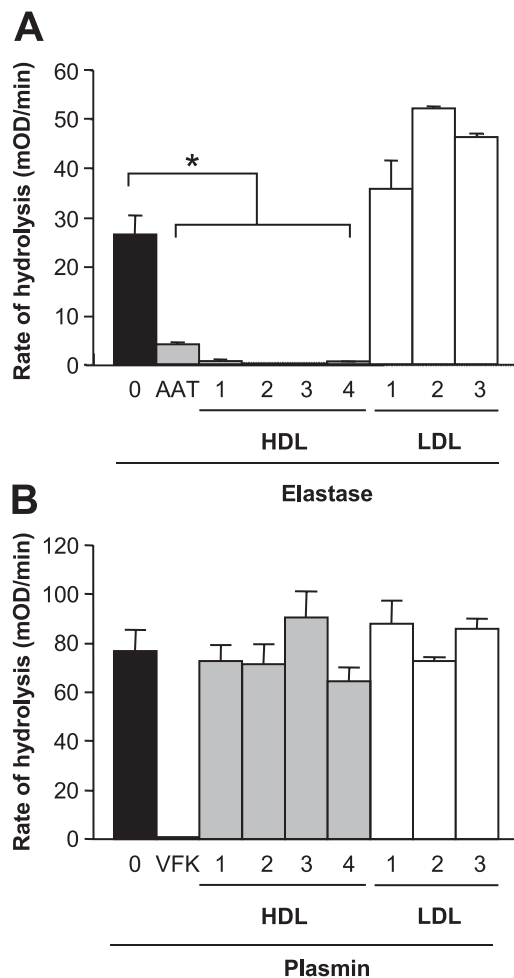


Figure 3. HDL, but not LDL, exhibits antielastase activity. *A*) Leukocyte elastase (10 nM) was incubated with a chromogenic substrate (MeO-Suc-Ala-Ala-Pro-Val-pNa) in the presence or absence of 50 $\mu\text{g}/\text{mL}$ HDL (4 batches) or LDL (3 batches). AAT (10 nM) was used as a positive control of elastase inhibition. Data for rate of hydrolysis [mean optical density (mOD)/min] are means \pm SD of 4 experiments performed in triplicate. $*P < 0.01$ vs. elastase alone. *B*) Plasmin activity is not affected by incubation with HDL or LDL (50 $\mu\text{g}/\text{mL}$). Val-Phe-Lys peptide (VFK) was used as plasmin inhibitor.

(data not shown). Both HDL and LDL were unable to inhibit plasmin activity (Fig. 3B).

HDL prevents elastase-induced VSMC anoikis

As described previously, purified leukocyte elastase (9) or that present in the thrombus of AAAs (11) is able to induce detachment and subsequent death of human VSMCs by degradation of the ECM (anoikis). We used this model to test the potential of HDL to prevent detachment induced by leukocyte elastase. In Fig. 4A–C, E, we show that VSMC viability was decreased by incubation with 10 nM elastase and that this effect was prevented in the presence of HDL in a dose-dependent manner (24 ± 9 vs. $98 \pm 2\%$ for coinubation with 100 $\mu\text{g}/\text{mL}$ HDL; $P < 0.005$). However, LDL had no effect

on VSMC detachment induced by elastase. In contrast to LDL, HDL was able to almost totally prevent fibronectin degradation induced by elastase, suggesting a direct inhibition of pericellular matrix proteolysis (Fig. 4D). Next, we assessed the protective effect of HDL on cells incubated with medium conditioned by the luminal layer of AAA intraluminal thrombus. We reported previously that leukocyte elastase was present chiefly in the luminal layer, relative to the intermediate and abluminal layers of the AAA thrombus and that it was able to induce detachment of VSMCs (11). Here we show that HDL could thwart this phenomenon and inhibit apoptosis induced by incubation with conditioned medium from the luminal layer of AAA thrombus (Fig. 4F) ($P < 0.05$). Finally, *ex vivo* incubation of mammary end-arteries with elastase led to VSMC apoptosis, detectable within the tissue, and this effect was inhibited by coinubation with HDL, but not with LDL. This is shown by the absence of nuclei positive for Apostain when mammary arteries were incubated with elastase in the presence of HDL (Fig. 4G).

Because HDL is known to exhibit antiapoptotic effects that are not reported to be mediated by AAT, including intracellular effects, we tested whether preincubation of the VSMCs with HDL (pulse-chase) was sufficient to prevent apoptosis induced by elastase. The cells were incubated for either 4 or 16 h with 100 $\mu\text{g}/\text{mL}$ HDL, carefully rinsed 3 times with PBS, and then incubated with 10 mM elastase (Fig. 5A). We show that a remnant antiapoptotic effect could be observed when cells were pretreated for 16 h with HDL ($41 \pm 8.2\%$ of inhibition). We checked that HDL was internalized by the cells using red carbocyanine-labeled HDL (Fig. 5B) and showed by confocal microscopy that apoA-I colocalized with AAT within the cells (Fig. 5C).

HDL from patients with AAAs carries less AAT than that from healthy subjects

As in other forms of atherothrombosis, it has been reported that levels of HDL were lower in patients with AAAs than in normal subjects (24, 25) and that leukocyte elastase is involved in pathophysiology of AAAs (11). Here, we report that patients with AAAs have significantly lower HDL than healthy control subjects (1.11 ± 0.23 mM, $n = 13$, vs. 1.35 ± 0.3 mM, $n = 23$; $P = 0.017$). Plasma apoA-I levels were also $68 \pm 2.6\%$ lower in patients with AAAs than in control subjects (Fig. 6A) ($P < 0.0001$). We tested the hypothesis that HDL from patients with AAAs could carry less AAT than that from control subjects. For this purpose, we isolated HDL from each individual (patients with AAAs or matched control subjects) and assessed the presence of AAT by Western blot, which was normalized to apoA-I content quantified by ELISA ($29 \pm 0.59\%$ reduction vs. that for matched control subjects) ($P < 0.0001$). Figure 6B shows that patients with aneurysms of diameter > 5 cm had significantly less AAT associated with their HDL than the control group. Accordingly, the elastase inhibitory potential associated with HDL was

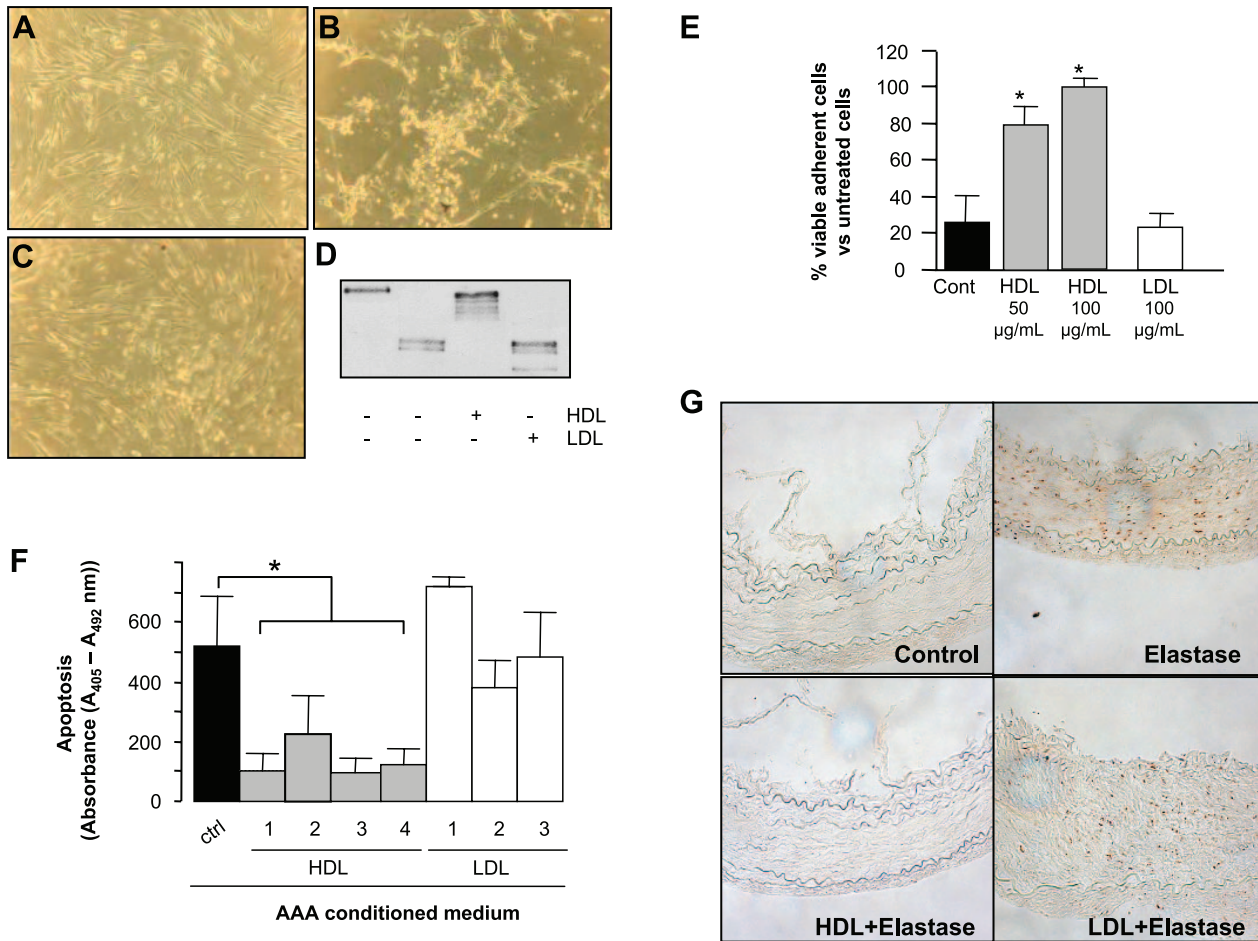


Figure 4. HDL inhibits anoikis induced by leukocyte elastase. *A–C*) Photomicrographs of human VSMCs in culture. *A*) Untreated control cells. *B*) VSMCs treated with elastase (10 nM) alone for 16 h. *C*) VSMCs treated with elastase in the presence of 50 $\mu\text{g/mL}$ HDL. *D*) Incubation with HDL prevents fibronectin degradation by leukocyte elastase. Representative Western blot detecting fibronectin in the cell culture supernatant. *E*) Viable adherent VSMCs were quantified by using the MTT test after treatment with elastase (10 nM). Results are expressed as percentages of untreated control cells. Columns represent means \pm SD of 4 separate experiments performed in triplicate. $*P \leq 0.005$ vs. elastase. *F*) Quantification of apoptosis in cells incubated with medium conditioned by the luminal layer of AAA intraluminal thrombus for 24 h in the presence or absence of 100 $\mu\text{g/mL}$ HDL or LDL from 4 and 3 different subjects, respectively. Results are expressed as percentages of untreated cells [control (ctrl) absorbance: 0.082 ± 0.028 nm]. Columns represent means \pm SD of 2 separate experiments performed in duplicate with medium conditioned by AAA intraluminal thrombus from 5 patients. $*P < 0.05$ vs. treatment without HDL. *G*) Detection of apoptotic nuclei by Apostain (brown indicates positive nuclei) after incubation of human mammary end-arteries with 10 nM elastase \pm 50 $\mu\text{g/mL}$ HDL or LDL for 24 h.

lower in patients than in control subjects (Fig. 6C), whereas global plasma antielastase activity was similar in the two groups (Fig. 6D), stressing the importance of the compartmentalization tissue *vs.* plasma.

DISCUSSION

The beneficial role of HDL in atherosclerosis is well documented, especially in relation to its function as a reverse transporter of cholesterol. Increasing evidence suggests that HDL may have other antiatherogenic properties such as prevention and correction of endothelial dysfunction and anti-inflammatory and antioxidant effects (26). Recent proteomic studies have focused on lipoproteins with the aim of identifying proteins associated with either HDL or LDL (3, 27). At

least two studies have shown that AAT was present in HDL isolated either by ultracentrifugation or by affinity chromatography (3, 4). In the present study, we sought to validate proteomic data using antigenic and enzymatic methods and then evaluated potential antielastase activity of HDL associated with the presence of AAT. Preparative and analytical ultracentrifugation remains the predominant method to isolate HDL. This method is based on flotation properties of lipoproteins during the ultracentrifugation process in a gradient of KBr of appropriate densities. It has been shown that during this process, proteins can dissociate from HDL and that native particles could be disrupted or discarded (28, 29). In addition, contamination by plasma proteins may occur during the collection of HDL fractions. For this purpose, in addition to ultracentrifugation, we also used selected affinity immunosorption

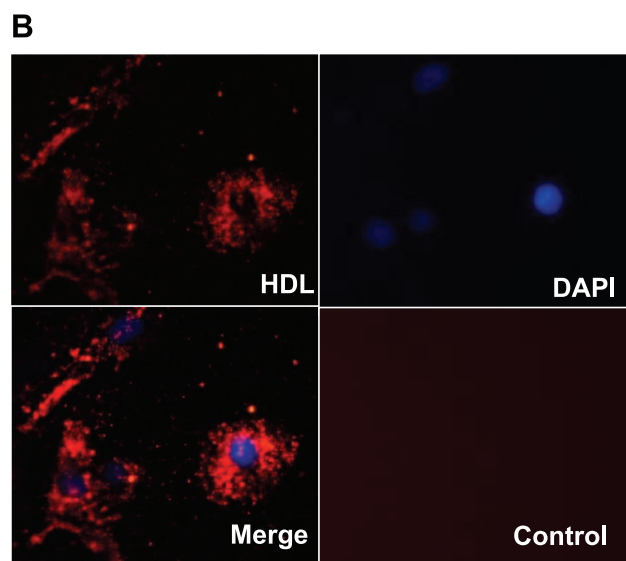
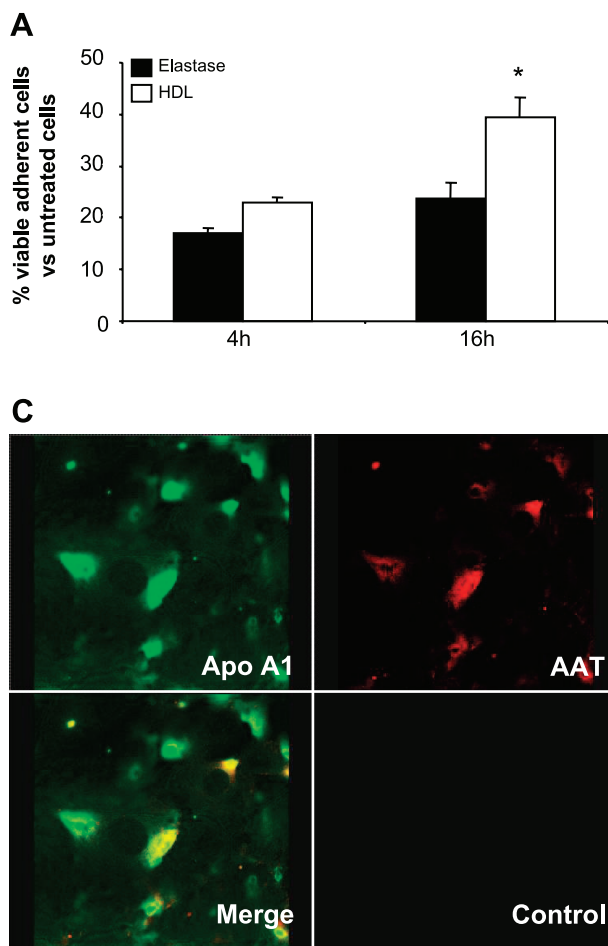


Figure 5. Intracellular HDL prevents elastase-induced apoptosis. *A*) MTT test: HDL (100 $\mu\text{g/ml}$) was preincubated for 4 and 16 h, and then the cells were rinsed before incubation with 10 nM elastase for 16 h. Values are means \pm SD. * $P < 0.005$ vs. elastase alone. *B*) HDL (100 $\mu\text{g/ml}$) labeled with DiIC₁₈ carbocyanines was incubated for 8 h with VSMCs, counterstained with DAPI, and observed under an epifluorescence microscope. *C*) Double immunostaining was performed for apoA-I and AAT after incubation with 50 $\mu\text{g/ml}$ HDL for 4 h. Observations were made by confocal microscopy (green, apoA-I; red, AAT; yellow, colocalization).

followed by 2-D nondenaturing electrophoresis to verify the presence of AAT associated with HDL. The use of an anti-apoA-I column allowed isolation of the LpA-I fraction that was used directly or after further separation of the different particles by nondenaturing 2-D-PAGE for detection of AAT by Western blot. AAT was confirmed to be present mostly in the HDL₃ fraction and in the LpA-I fraction with α_1 electrophoretic mobility. In previous reports, AAT was found in HDL₃ (4) (however, the authors did not analyze the HDL₂ fraction) and in both HDL₃ and HDL₂ fractions (3). Immunisorption using anti-apoA-I column allowed us to identify AAT in small α_1 HDL particles and, to a lesser extent, within pre- α particles (data not shown). This result is consistent with the fact that α_1 HDL is contained in the HDL₃ fraction. In addition, the levels of α_1 and pre- α particles were reported to be decreased in patients with coronary artery disease (30).

This decrease was shown by Western blot, but also *via* identification by tandem mass spectrometry (MS/MS) analysis. The full list of proteins identified by MS/MS in HDL is not provided here because this identification is not the aim of the present manuscript. However, AAT was identified in HDL from three healthy people by mass spectrometry with a high degree of confidence. For example, peptides spanning 54.3% of the sequence of AAT were identified in one patient, and the spectral assignment to the peptide DTVFALVNYIFFK shown in

Fig. 2D was matched with an expectation value of 1.0×10^{-5} ; *i.e.*, there is only a 1 in 10,000 chance that it is incorrect. With the immunisorption technique, followed by separation according to charge and size under nondenaturing conditions, any contaminant AAT would be discarded, suggesting that its association with HDL is not an artifact. In contrast to the results obtained by Vaisar *et al.* (4), we were unable to show the presence of α_2 AP in HDL either by Western blot (Fig. 1) or by mass spectrometry, which could be explained by the fact that in their study, this antiprotease was identified in HDL from patients with coronary artery disease.

ApoA-I being the major protein of HDL, we hypothesized that AAT may display an affinity for this apolipoprotein. However, we could not demonstrate any specific binding of AAT to apoA-I, suggesting that AAT may interact with other protein/lipidic partners within HDL that need to be identified. This result suggests that AAT is not likely to interfere with apoA-I binding to its receptor, which mediates HDL reverse transport cholesterol function. Finally, affinity of AAT for HDL was further demonstrated in the experiment of coinubation/reisolation. We were able to enrich HDL but not LDL with AAT by overnight incubation at 37°C followed by ultracentrifugation for isolation. This result shows that circulating HDLs may not be saturated with AAT, and their capacity to bind and transport AAT may be modulated under pathological conditions.

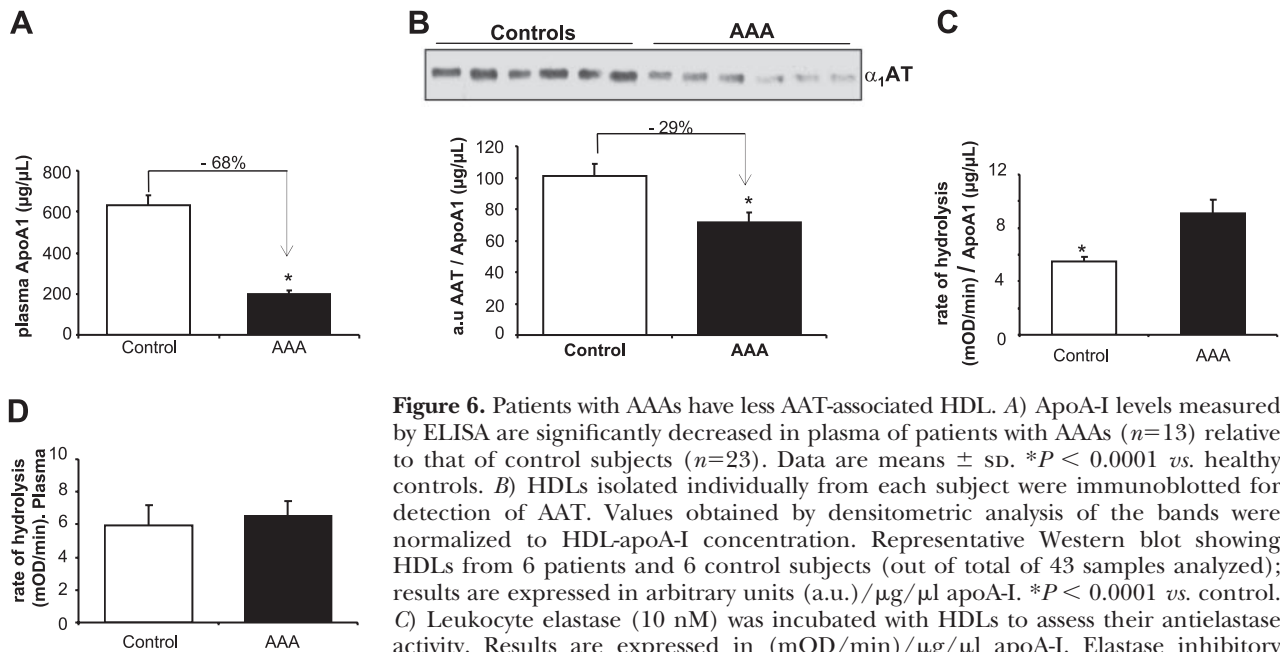


Figure 6. Patients with AAAs have less AAT-associated HDL. *A*) ApoA-I levels measured by ELISA are significantly decreased in plasma of patients with AAAs ($n=13$) relative to that of control subjects ($n=23$). Data are means \pm SD. $*P < 0.0001$ vs. healthy controls. *B*) HDLs isolated individually from each subject were immunoblotted for detection of AAT. Values obtained by densitometric analysis of the bands were normalized to HDL-apoA-I concentration. Representative Western blot showing HDLs from 6 patients and 6 control subjects (out of total of 43 samples analyzed); results are expressed in arbitrary units (a.u.)/ $\mu\text{g}/\mu\text{L}$ apoA-I. $*P < 0.0001$ vs. control. *C*) Leukocyte elastase (10 nM) was incubated with HDLs to assess their antielastase activity. Results are expressed in (mOD/min)/ $\mu\text{g}/\mu\text{L}$ apoA-I. Elastase inhibitory potential is significantly reduced in HDL from patients with AAAs. $*P < 0.0001$ vs. control group. *D*) Plasma antielastase activity was tested *in vitro* by incubating leukocyte elastase with diluted plasma (1:1000) from healthy control subjects and patients with AAAs. Results are expressed in mOD/min.

AAT is the major physiological circulating inhibitor of elastase. Leukocyte elastase has been reported recently to be a potential predictive marker of incident myocardial infarction and stroke in the Edinburgh artery study (8). Earlier, leukocyte elastase levels were shown to be correlated with the severity of peripheral artery disease (31). Furthermore, the elastase inhibitory capacity of sera has been shown to be significantly elevated in atherosclerotic patients (32). This finding could be interpreted as a systemic response to the increased levels of elastase in this pathological condition (7). We and others have suggested that the elastase content of the mural thrombus in AAAs could play a major role in the progression of the pathological condition by preventing thrombus colonization and cicatrization and by inducing smooth muscle cell death by detachment (11, 33). Here we show that HDL is able to inhibit elastase activity and its deleterious effects on human VSMCs *in vitro* and on *ex vivo* human mammary arteries. Purified elastase, or that present in medium conditioned by the luminal part of the aneurysmal thrombus, induced ECM degradation and apoptosis by detachment of VSMCs (anoikis). HDL-associated AAT was able to blunt these effects in a dose-dependent manner at concentrations that are physiologically relevant (50–100 $\mu\text{g}/\text{ml}$, whereas low HDL concentrations in a normal individual are approximately 350 $\mu\text{g}/\text{ml}$). That HDL inhibits elastase activity by a mechanism other than *via* AAT cannot be totally excluded. However, the antielastase potential of HDL and its antiapoptotic effect on VSMCs were correlated with the amount of AAT present in the different batches of HDL used (data not shown).

Interestingly, VSMCs preincubated with HDL were partially protected from elastase-induced cell death,

suggesting that HDL can interfere with intracellular events triggered by elastase. Confocal microscopy allowed us to show that AAT was internalized with HDL and colocalized with apoA-I. AAT could directly inhibit caspase 3 activity as described in a model of apoptosis induced by staurosporine in lung endothelial cells (34). Besides its direct extracellular antiprotease effects, AAT may thus exhibit intracellular antiapoptotic properties. It has been reported recently that AAT can be taken up by endothelial cells and protects them from apoptosis induced by cigarette smoke (35). The antiapoptotic potential of HDL due, for example, to paraoxonase activity cannot be excluded in a process in which oxidative stress is involved.

AAT has been reported to inhibit chymase (36), a protease potentially involved in atherosclerosis (37). Mast cells, which represent the major source of chymase, were recently shown to modulate AAA progression in a mouse model (38). HDL-associated AAT could thus be beneficial by inhibiting both leukocyte elastase and mast cell chymase in atherothrombosis.

The relation between the lipid profile and the presence of AAAs is not clear. A study by Hobbs *et al.* (39) suggested a correlation between LDL cholesterol levels and the presence of small AAAs, whereas levels of HDL were unchanged in patients with AAAs vs. matched healthy subjects. Conversely, earlier studies had shown an inverse correlation between HDL cholesterol and the presence of AAAs, reporting that patients with an AAA had lower HDL levels compared with healthy subjects (24, 25). In the present study, we confirmed that HDL levels and, more specifically, circulating apoA-I levels, are decreased in patients with AAAs relative to those in control subjects. Moreover, after normalization to apoA-I levels, we found that HDL

from patients with AAAs contained less associated AAT than that from healthy subjects. We hypothesized that HDL could be a physiological vector of antiproteases, including AAT, which could act locally within atherothrombotic lesions. Normal plasma concentrations of AAT range from 1 to 3 g/L, of which we estimated that <1% is carried by circulating HDL. These high concentrations of free AAT in plasma may explain why no difference was observed in patients with AAAs *vs.* control subjects in terms of global plasma antielastase activity. Despite the high levels of circulating AAT, in pathological conditions and within the vascular compartment, the balance may be in favor of elastase. In these conditions, HDL-associated antielastase activity could play a pivotal antiatherogenic role.

In conclusion, we report here a new antiprotease activity for HDL that is able to inhibit leukocyte elastase and its associated deleterious effects on vascular cells. We speculate that a decreased level of HDL and associated AAT in patients with AAAs may account for less effective protection against elastase in the vascular wall, favoring the progression of this disease. The potential antiatherogenic role of HDL-associated AAT merits more extensive studies in the context of atherothrombosis and particularly with reference to the stabilization of the plaque, as well as in AAAs. FJ

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