methods

A novel enzyme immunoassay specific for ABCA1 protein quantification in human tissues and cells

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Abstract ATP-binding cassette transporter A1 (ABCA1) mediates the transport of cholesterol and phospholipids from cells to lipid-poor HDL and maintains cellular lipid homeostasis. Impaired ABCA1 function plays a role in lipid disorders, cardiovascular disease, atherosclerosis, and metabolic disorders. Despite the clinical importance of ABCA1, no method is available for quantifying ABCA1 protein. We developed a sensitive indirect competitive ELISA for measuring ABCA1 protein in human tissues using a commercial ABCA1 peptide and a polyclonal anti-ABCA1 antibody. The ELISA has a detection limit of 8 ng/well (0.08 mg/l) with a working range of 9–1000 ng/well (0.09–10 mg/l). Intra- and interassay coefficient of variations (CVs) were 6.4% and 9.6%, respectively. Good linearity (r = 0.97–0.99) was recorded in serial dilutions of human arterial and placental crude membrane preparations, and fibroblast lysates. The ELISA measurements for ABCA1 quantification in reference arterial tissues corresponded well with immunoblot analysis. The assay performance and clinical utility was evaluated with arterial tissues obtained from 15 controls and 44 patients with atherosclerotic plaques. ABCA1 protein concentrations in tissue lysates were significantly lower in patients (n = 24) as compared with controls (n = 5; 9.37 ± 0.82 vs. 17.03 ± 4.25 μg/g tissue; $P < 0.01$). The novel ELISA enables the quantification of ABCA1 protein in human tissues and confirms previous semiquantitative immunoblot results.—Paul, V., H. H. D. Meyer, K. Leidl, S. Soumian, and C. Albrecht. **A novel enzyme immunoassay specific for ABCA1 protein quantification in human tissues and cells. *J. Lipid Res.* 2008.** 49: **2259–2267.**

Supplementary key words ATP binding cassette transporter A1 • ELISA • carotid plaques

Maintenance of cellular cholesterol and phospholipid homeostasis is critical for human physiology and, if disturbed, leads to a variety of pathological conditions, including cardiovascular disease and atherosclerosis (1, 2). The ATP-binding cassette transporter A1 (ABCA1) is an integral membrane protein thought to be critical in maintaining cellular cholesterol and phospholipids homeostasis via promoting the efflux of intracellular phospholipids and cholesterol from peripheral tissues to extracellular lipid-free apolipoprotein A-I (apoA-I) and other apolipoproteins through reverse cholesterol transport (3–5).

The physiological importance of ABCA1 is demonstrated by mutation of *ABCA1* gene in patients with familial hypoalphalipoproteinemia and Tangier disease, a rare genetic disorder characterized by severe reductions in plasma HDL levels, accumulation of lipids in peripheral tissues and macrophages, and an increased risk of cardiovascular disease (6–8). Mice with a targeted knockout of ABCA1 in macrophages showed enhanced atherosclerosis (9–12). Conversely, overexpression of ABCA1 in mice increased plasma HDL levels and strikingly protects against atherosclerosis (13–15). Beside the established role of ABCA1 in lipid disorders, an impact of ABCA1 on insulin secretion and glucose homeostasis of pancreatic β cells has been reported (16). Moreover, it has been suggested that an impaired ABCA1 pathway could contribute to the increased atherogenesis associated with common inflammatory and metabolic disorders, such as diabetes or the metabolic syndrome (4).

ABCA1 mRNA is expressed abundantly in leukocytes, macrophages, liver, brain, lungs, placenta, and various other tissues (17, 18). The transcription of the ABCA1

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Abbreviations: ABCA1, ATP-binding cassette transporter A1; apoA-I, apolipoprotein A-I; BCA, bicinchoninic acid assay; BLU, Boehringer light units; CEA, carotid endarterectomy; CV, coefficient of variation; hF, human fibroblasts; HRP, horseradish peroxidase; LXR, liver X receptor alpha; PBST, phosphate buffered saline-tween 20; RXR, retinoid X receptor; TMB, tetramethylbenzidine.

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gene is regulated by cellular cholesterol levels via the liver X receptors (LXR) α and β and retinoid X receptor (RXR) (19, 20). Posttranscriptional processes affecting ABCA1 expression involve the modulation of ABCA1 protein stability. Hence, the ABCA1 protein degradation rate is controlled by phosphorylation of a PEST sequence in ABCA1 that directs calpain proteolysis (21, 22). Unsaturated fatty acids destabilize the ABCA1 protein through activation of protein kinase C δ pathway (23).

Attention has focused on ABCA1 because of its potential role in lipid metabolism, atherosclerosis, and metabolic disorders and the fact that therapeutic interventions could be targeted at regulatory pathways controlling ABCA1 expression by modulating pharmacological targets like nuclear LXR/RXR.

Quantification of ABCA1 is critical in studies related to its expression and regulation by metabolic factors, nutritional status, and new antiatherogenic drug candidates. As the ABCA1 protein levels in mouse and human tissues showed discordance with ABCA1 mRNA abundance (24, 25), it is important to establish a reproducible, quantitative assay for ABCA1 protein determination. Thus it was our aim to develop a sensitive quantitative ABCA1 ELISA, based on indirect competitive assay format for the reliable and rapid detection and quantification of ABCA1 protein in human arterial tissues. We validated this assay to extend its application to human placental tissue and cultured fibroblast cell lysates. The assay was also applied for ABCA1 protein quantification in human arterial tissues collected from patients with atherosclerotic plaques and respective controls.

MATERIALS AND METHODS

Materials

A synthetic ABCA1 peptide (ab14148) containing the amino acid sequence AETSDGTLPAR (corresponding to residues 1259 to 1268 of the human *ABCA1* gene) and a polyclonal antihuman ABCA1 rabbit antibody (ab7360) raised against a synthetic peptide (immunogen) consisting of the amino acid sequence AETSDGTLPAP (derived from amino acid residues 1259 to 1269 of *ABCA1*) were purchased from Abcam, UK. A biotinylated secondary antirabbit IgG goat antibody (BA1000) was obtained from Vectors laboratories. Horseradish peroxidase (HRP)-conjugated antirabbit-IgG (sc2030) was from Santa Cruz, Germany. Triton X-100, n-Dodecyl-β-D-maltoside (DDM), benzonuclease, 3,3,5,5′-tetramethylbenzidine (TMB) were from Sigma, Germany. Complete protease inhibitor and streptavidin-HRP conjugate were from Roche, Germany. Lubrol 17A17 and BSA were obtained from Serva, Germany. All other chemicals were purchased from Merck, Germany unless stated otherwise.

Subjects: human tissues and cells

The origin and characteristics of patient and controls samples have been previously described by Albrecht et al. (25). Briefly, carotid plaques were collected from 20 patients (crude membrane preparations)/24 patients (tissue lysates) with internal carotid stenosis of >70% undergoing carotid endarterecotomy (CEA). Fifteen inferior normal mesenteric arteries dissected from colectomy specimens of subjects undergoing elective surgery served as controls. These were phenotypically free of symptomatic atherosclerotic disease as evidenced by history and physical examination. The CEA specimens consisted of the atheromatous plaques, together with adjacent intima and medial layers. The inferior mesenteric artery specimens were full-thickness and included the adventitial layer. All specimens were collected under the ethical approval from the Riverside Research Committee and informed consent from the subjects.

Human placental tissues were obtained after the normal vaginal delivery and immediately stored at −80°C. The tissue was kindly provided by Dr. Williamson (Institute of Reproductive and Developmental Biology, Imperial College London, UK).

Human fibroblasts (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium supplemented with L-glutamine (Gibco, Invitrogen, Germany), nonessential amino acids, and 10% fetal calf serum in a humidified 5% CO_2 atmosphere at 37°C. They were loaded with 15 μg/ml free cholesterol at confluence to increase ABCA1 expression and harvested after 24 h.

Preparation of crude membrane protein extracts

Arterial and placental tissues were pulverized in liquid nitrogen and stored at −80°C. Total crude membrane protein extractions were performed as described previously (25) with minor modifications. Briefly, 100 mg of pulverized tissue was homogenized in 1 ml ice-cold lysis buffer (50 mM mannitol, 2 mM EDTA, 50 mM Tris-HCl, pH 7.6) containing complete protease inhibitor in a Fastprep homogenizer followed by centrifugation (1,000 *g* for 10 min at 4°C). The supernatant was loaded onto a cushion of second ice-cold lysis buffer (300 mM mannitol, 2 mM EDTA, 50 mM Tris-HCl, pH 7.6) containing complete protease inhibitor. Total crude membrane protein fraction was pelleted by ultracentrifugation at 100,000 *g* for 1 h at 4°C in a Beckmann ultracentrifuge. The resulting pellet was resuspended and solubilized in 50 μl or 100 μl ice-cold phosphate buffered saline-tween 20 (PBST) extraction buffer (PBST pH 7.4 supplemented with complete protease inhibitor and 50 U of benzonuclease) containing either 1% w/v Triton X-100 or other detergents (DDM or Lubrol), and incubated for 30 min on ice. Protein concentrations were determined by bicinchoninic acid assay (26), with BSA as standard. Crude membrane protein extracts were stored overnight at −80°C and processed the next day.

Preparation of tissue lysates

Total tissue lysates from 100 mg pulverized arterial or placental tissues were prepared by washing with 500 μl ice-cold PBS containing complete protease inhibitor (30 s vortex step followed by centrifugation at 10,000 *g* for 5 min at 4°C; supernatant discarded). The washed tissue pellet was incubated with 400 μl ice-cold hypotonic PBS extraction buffer (8 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4, 2.7 mM KCl, 10 mM NaCl, pH 7.4 supplemented with complete protease inhibitor) containing either 1% w/v Triton X-100 or other detergents (DDM or Lubrol) for 60 min. This was followed by five homogenization cycles (20 s each and 10 min resting time on ice after each homogenization step) in a Fastprep homogenizer. The tissue homogenates were centrifuged at 15,000 *g* for 15 min at 4°C and the supernatants containing clear tissue lysate were collected. The remaining tissue pellets were re-extracted with 100 μl extraction buffer by repeating the above mentioned homogenization (once) and centrifugation steps. The clear supernatants were pooled, mixed, and stored at −80°C until used in ELISA.

Preparation of total cell lysates

Total cell lysates were prepared by incubating the cholesterol-loaded fibroblast cell pellet with 250 μl ice-cold lysis buffer

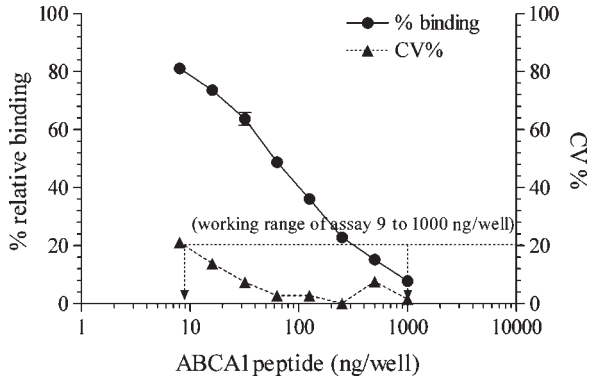

Fig. 1. Calibration curve for ABCA1 quantification in human tissues. A typical calibration curve prepared from serial dilutions (8–1,000 ng/well) of ABCA1 peptide in assay buffer. Coefficient of variation CV % represents the precision profile for replicates of the ABCA1 calibrators used in the calibration curve. The working range (9–1,000 ng/well) of the calibration curve was derived on the basis of <20 CV% between the replicates of ABCA1 calibrators.

(10 mM Tris HCl pH7.4, containing 10 mM NaCl, 1.5 mM $MgCl_2$, 1% (w/v) Triton X-100 and complete protease inhibitor or hypotonic PBS extraction buffer) on ice for 60 min with vortexing every 10 min. Supernatants were collected after centrifugation at 15,000 *g* at 4°C for 10 min and stored at −80°C until used in ELISA.

ELISA development

The ELISA was based on an immobilized antigen that competes with the analyte for specific capture antibody. The captured antibody was further quantified with a secondary biotinylated detection antibody by using streptavidin-HRP and TMB substrate reaction. The ELISA was established in three steps: *a*) the different concentrations of coating ABCA1 peptide and ABCA1 specific capture antibody were tested in a checkerboard design to select the optimal combination of coating ABCA1 peptide and specific capture antibody, *b*) the optimal dilutions of the detection (secondary biotinylated) antibody was established, and *c*) the characteristics of the assay were studied.

Enzyme immunoassay procedure

To quantify ABCA1 protein, microtiter plates (Nunc Maxisorp™–Immuno Plates, Denmark) were coated with 100 μl 0.06 mg/l ABCA1 peptide in 50 mM carbonate/bicarbonate buffer pH 9.6 overnight at 4°C. The plates were then washed twice with 300 μl/well of PBST (8 mM $Na_2HPO_4.2H_2O$, 1.5 mM K_2HPO_4, 2.7 mM KCl, 137 mM NaCl, 0.1% Tween 20, pH 7.4) in a 96-well plate washer (SLT Lab Instruments, Germany). Plates were blocked with 300 μl/well of 10 g/l BSA in PBST for 1 h at room temperature. After two washings, the calibrators (ABCA1 peptide 8–1000 ng/100 μl PBST and 40 μl extraction buffer/well), the unknown samples (crude membrane protein extract/cell or tissue lysate 40 μl and 100 μl PBST/well), and known control samples (ABCA1 peptide spiked crude membrane protein extract; 100 μl and 40 μl extraction buffer/well) were incubated in respective wells along with 100 μl anti-ABCA1 rabbit antibody (2 μg/l diluted in PBST) for 5 h at room temperature while shaking on an ELISA plate shaker. Afterwards four washings were performed. All further incubations were carried out at room temperature. After incubation with 100 μl/well of goat antirabbit IgG biotinylated antibody (10 μg/l diluted in PBST) for 1 h, again four washings were performed. The plates were incubated with 100 μl/well of streptavidin-HRP conjugate (1:15000 diluted in PBST) for 20 min and again washed four times. Thereafter, the plates were incubated with 150 μl/well of TMB substrate solution for 40 min in dark. The enzymatic reaction was stopped by the addition of 50 μl/well of 2 M H_2SO_4, and absorbance was measured at 450 nm in an ELISA plate reader (Sunrise™, Tecan, Austria). The ABCA1 peptide calibration curve was generated by the use of the online four-parameter Marquardt analysis program (Megallan V6.1 software, Tecan, Austria). ABCA1 protein concentrations of unknown samples were calculated from the calibration curve. All data were calculated as ABCA1 peptide/well and finally expressed as ABCA1 protein ng/μg membrane protein and μg/g of tissue.

Validation of ELISA

The synthetic ABCA1 peptide was used as calibrator in the ABCA1 ELISA to generate a dose-response curve. Calibrators of concentrations ranging from 0.08 to 10 mg/l were prepared from a stock solution of 1g/l ABCA1 peptide; dilutions were performed with ice-cold PBST (assay buffer). Each calibrator concentration (100 μl) and 40 μl extraction buffer were used per well. The extraction buffer (40 μl) and assay buffer (100 μl) was considered as a zero calibrator. The detection limit was estimated as the minimum analyte concentration evoking a response significantly different from that of the zero calibrator (3 SD above the mean for a zero calibrator).

TABLE 1. Assay precision for ABCA1 ELISA in human arterial tissues

	Within run (Intraassay)[b]		Between runs (Interassays)[c]	
Sample[a]	Mean (SD) ABCA1 measured (ng/10 μg crude membrane protein)	CV (%)	Mean (SD) ABCA1 measured (ng/10 μg crude membrane protein)	CV (%)
A1	16.1 (2.2)	13.4	14.7 (2.1)	14.1
A1	161.4 (7.3)	4.5	160.5 (7.6)	4.7
A1	600.2 (26.0)	4.3	633.5 (60.6)	9.6
A2	16.2 (1.2)	7.7	15.3 (2.1)	13.6
A2	201.9 (5.1)	2.5	196.4 (15.5)	7.9
A2	672.2 (39.0)	5.8	677.5 (32.0)	4.7
A3	27.3 (3.3)	12.1	27.0 (3.3)	12.1
A3	209.2 (10.8)	5.2	213.5 (14.0)	6.6
A3	788.9 (16.5)	2.1	859.3 (109.3)	12.8
	Mean	6.4	Mean	9.6

[a] Crude membrane protein pools from three different human carotid artery plaques tissues (A1, A2, and A3) spiked with ABCA1 peptide at concentrations of approximately 20, 200, and 800 ng/10 μg crude membrane protein.
[b] Ten determinants (in duplicate) assayed in the same assay.
[c] Fifteen determinants (in duplicate) assayed in five assays performed on different days.

TABLE 2. Analytical recovery of ABCA1 peptide calibrator added to crude membrane protein extracts (10 μg) of three different arterial tissues

	ABCA1 (ng/10 μg crude membrane protein)		
Arterial tissue	Amount added	Amount measured[a]	Recovery (%)
A4	800	600.2 ± 26.02	75.0
A4	200	161.4 ± 7.27	80.7
A4	20	17.43 ± 3.07	87.2
A5	800	638.9 ± 73.93	79.9
A5	200	201.9 ± 5.1	101.0
A5	20	15.99 ± 2.87	80.0
A6	800	788.9 ± 16.5	98.6
A6	200	209.2 ± 10.5	104.6
A6	20	21.85 ± 2.65	109.3
		Mean	90.7

[a] Mean ± SD of 10 replicates in duplicates.

Precision

The intra-assay and interassay precision of the ABCA1 protein ELISA was determined by using an ABCA1 peptide calibrator and three different arterial tissue membrane preparations (A1, A2, and A3) spiked with ABCA1 peptide. The intraassay (within-run) precision [coefficient of variations; (CVs)] was determined by analyzing ABCA1 peptide-spiked arterial tissue membrane protein pools at three different concentrations (~20, 200, and 800 ng ABCA1 peptide/10 μg membrane protein) in 10 replicates within the same assay. The interassay (between-run) precision was determined by testing three aliquots of each ABCA1 peptide pool on different days in a total of five separate assays. The mean absorbances from duplicate wells were used to calculate 10 (within-run) and 15 (between-run) separate values for each ABCA1 peptide pool.

Analytical recovery

To measure the analytical recovery, various amounts of ABCA1 peptide (~20, 200, and 800 ng/10 μg crude membrane protein) were added to total crude membrane extracts of three different arterial tissues (A4, A5, and A6) containing undetectable amounts of ABCA1 protein. ABCA1 concentrations were then measured and the percentage recovery rates were calculated.

Linearity

Total crude membrane extracts from two human arterial tissues (A7 and A8; initial ABCA1 concentration 700.2 ng/5 μg crude membrane protein and 238.0 ng/20 μg crude membrane protein, respectively), one human placental tissue (hP; initial ABCA1 concentration 319.2 ng/20 μg crude membrane protein) and one cholesterol-loaded human fibroblasts cell lysate (initial ABCA1 concentration 302.0 ng/20 μl cell lysate) were serially diluted 1:1 in PBST extraction buffer containing 1% Triton X-100, and the concentrations of ABCA1 protein determined using the indirect competitive ELISA described above. The experimental values obtained were plotted against expected values considering the highest undiluted concentration as 1.

Extraction efficiency

Three different detergents (Triton X-100, DDM, and Lubrol) at 1% (w/v) concentration levels in PBS hypotonic extraction buffer were tested for the extraction efficiency of ABCA1 protein from human placental tissues (n = 2) by using direct tissue extraction (tissue lysate) and crude membrane preparation protocols. The effect of the employed detergents on the sensitivity of the calibration curve and the linearity of dilution of tissue lysates with hypotonic PBS extraction buffer in ELISA was also studied.

Immunoblotting

Western blot analysis for ABCA1 protein was performed as described previously with minor modifications (25). Briefly, 50 μg crude membrane protein fractions were resolved by 7% SDS-PAGE and immunodetected by using a polyclonal anti-ABCA1 antibody and a secondary antibody HRP-conjugated anti-rabbit-IgG. Blots were developed using SuperSignal® West Pico chemiluminescent substrate (Pierce, Perbio, Germany) and visualized on Lumi-film (chemiluminescent detection film, Roche, Germany). For semiquantitative measurements, the developed films were scanned and analyzed with a Lumi-Imager™ (Boehringer, Germany) using Lumi Analyst 3.0 software (Boehringer, Germany).

Competitive immunoinhibition for binding site recognition on the ABCA1 antibody

To demonstrate the same epitope recognition site for ABCA1 protein in the analyte and ABCA1 peptide calibrator on the polyclonal ABCA1 antibody, 15 μl cell lysates of cholesterol loaded human fibroblasts (hFI and hFII containing 66 and 33 ng ABCA1 protein as measured by ELISA) were resolved on 7% SDS-PAGE. Immunoblots were probed with the primary ABCA1 antibody solution containing various concentrations of ABCA1 peptide (0; 35; 70 ng). Peptide was incubated with the antibody for 2 h at 6° to 8°C prior to probing with the blots.

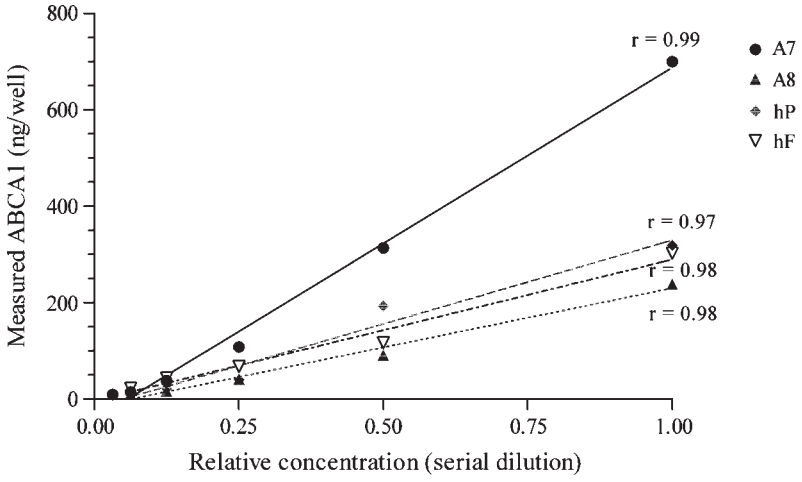

Fig. 2. Analytical linearity of ABCA1 protein concentrations in human tissue extracts (membrane preparations and cell lysate). A7 and A8 are the crude membrane preparations from inferior mesenteric arteries. hP and hF represent human placental tissue crude membrane preparations and human fibroblast cell lysate, respectively. Crude membrane extracts and cell lysate were serially diluted with assay buffer and respective ABCA1 concentrations were measured using ELISA.

Concentrations of ABCA1 protein in human arterial tissues from patients and controls

Crude membrane protein extracts. ABCA1 protein concentrations in crude membrane extracts obtained from carotid atherosclerotic plaques (n = 20) and control arterial tissues (n = 10) was measured by ABCA1 ELISA (calibration curve and linearity, see Fig. 1 and Fig. 2) using extraction buffer (PBST pH 7.4 supplemented with complete protease inhibitor, 50 U of benzonuclease) containing 1% w/v Triton X-100.

Tissue lysates

In a separate approach and a different set of patient samples, ABCA1 protein concentrations were also measured in direct tissue lysates of carotid atherosclerotic plaques (n = 24) and control arterial tissues (n = 5). The tissue lysates were prepared by direct extraction using PBS hypotonic extraction buffer and 1% DDM (calibration curve and linearity, see Fig. 3). The small number of controls as compared with atherosclerotic tissues used in this assay was due to technical reasons based on severe limitations regarding the availability and quantity of control tissues.

Statistical analysis

Statistical significance between patients and controls was determined by a two-tailed unpaired Student's *t*-test (GraphPad Prism Software, San Diego, CA). Results are reported as mean ± SEM. *P* values < 0.05 were considered significant.

RESULTS

ELISA development and validation

During the development of our ELISA assay, we found that the highest signal for ABCA1 protein was obtained

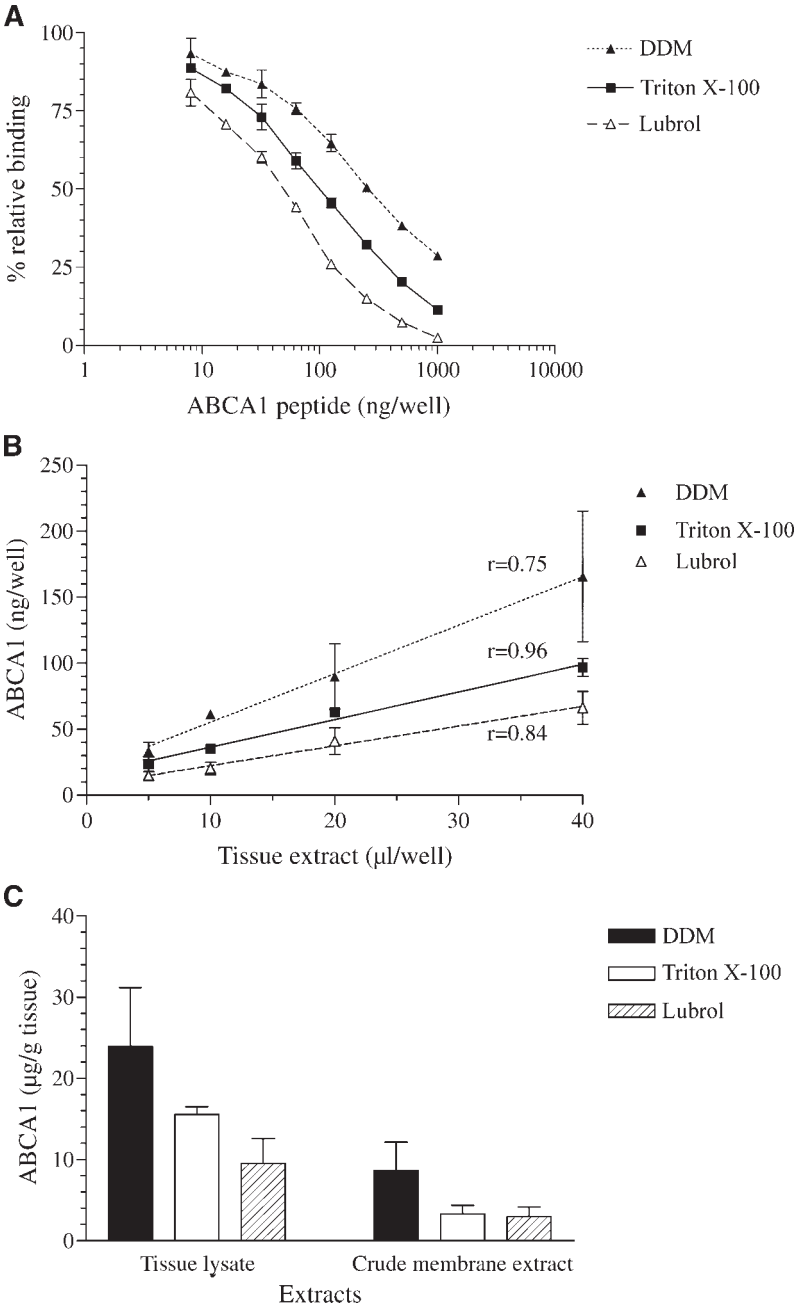

Fig. 3. Extraction and solubilization efficiency of ABCA1 protein from human placental tissue lysates and crude membrane preparations. Three different detergents (DDM, Triton X-100, and Lubrol) were included at 1% w/v in hypotonic PBS extraction buffer to evaluate the extraction/solubilization efficiency. Panel A represents the influence of the specific detergent on the sensitivity of calibration curve; panel B represents the dilution linearity of tissue lysates with detergent specific extraction buffer; panel C shows the amount of ABCA1 protein measured in placental tissue lysates and crude membrane extracts using different detergents. ABCA1 protein was measured in ELISA and values are expressed as means ± SEM of two placental tissues.

with the reagent concentrations and sample dilutions as described in Material and Methods (ELISA procedure).

Detection limit and precision of ELISA

The detection limit of the assay was 8 ng/well (corresponding to 0.08 mg/l). The dynamic working range of the calibration curve (calculated from the <20% CVs for duplicate analyses of the calibrators) was 9 to 1,000 ng/well (corresponding to 0.09 to 10 mg/l). A typical calibration curve is shown in **Fig. 1** together with the CVs for duplicate analyses. For routine quantification of ABCA1 protein in human tissue samples, the results were expressed as concentration of ABCA1 peptide in ng/well and finally displayed as ABCA1 protein in ng/μg crude membrane protein and μg/g tissue using crude membrane preparations and tissue lysates. The within-run and between-runs assay imprecision (CV) were <15% (**Table 1**).

Analytical recovery and linearity

Recoveries of exogenously added ABCA1 peptide to three different arterial tissue membrane protein extracts ranged from 75% to 109.3% with a mean recovery of 90.7% (**Table 2**). Dilution curves of crude membrane protein extracts from two arterial tissues (A7 and A8), human placental tissue (hP), and total cell lysate from cholesterol-loaded human fibroblasts (hF) displayed good linearity (r = 0.97 to 0.99; **Fig. 2**).

Extraction efficiency

Among the three detergents, DDM was the most efficient detergent, followed by Triton X-100 and lubrol in extracting ABCA1 from placental tissues. DDM extracted 2.52-fold and Triton X-100 1.64-fold higher ABCA1 protein (mean ABCA1 protein concentration/g tissue) in placental tissues in comparison to lubrol (**Fig. 3C**). A similar trend was observed for the solubilization of ABCA1 from crude membrane preparations with the highest efficiency demonstrated with DDM, followed by Triton X-100 and lubrol. DDM solubilized 2.91-fold and Triton X-100 1.1-fold higher ABCA1 protein (mean ABCA1 protein concentration/g tissue) from crude membrane preparations in comparison to lubrol (Fig. 3C). The effects of the employed detergents on the sensitivity of the calibration curve and the dilution linearity of the tissue lysates in ELISA are depicted in Fig. 3A and 3B. Surprisingly, using human placental tissue approximately 50% less ABCA1 protein was detected in crude membranes as compared with direct placental tissue lysates.

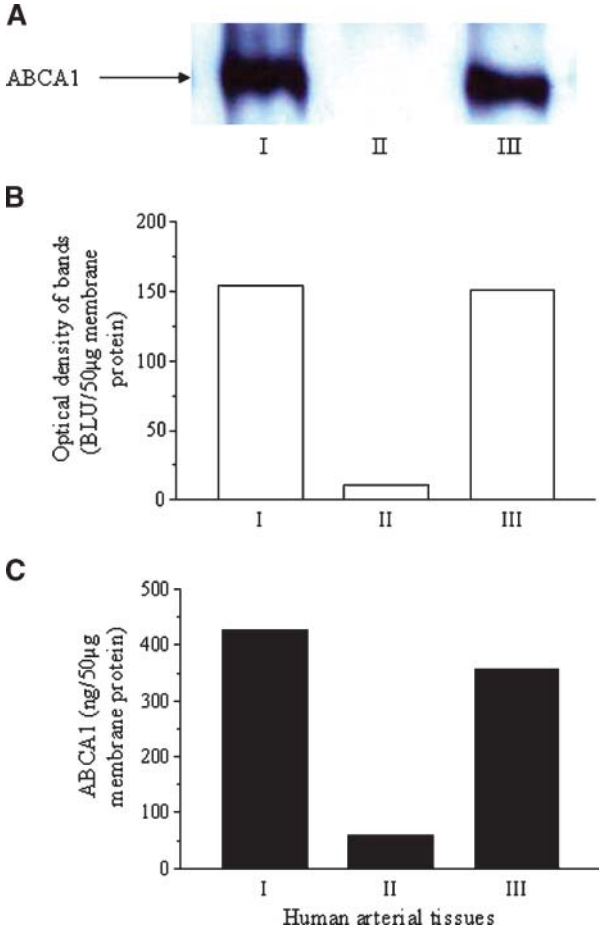

Fig. 4. ABCA1 protein expression in human arterial tissues. Sample I, II, and III represent three different specimens of human arterial tissues. ABCA1 was quantified in crude membrane preparations (50 μg/sample) using a commercial antibody against ABCA1. A: Representative immunoblot analysis of ABCA1 protein expression in human arteries by reducing SDS-PAGE. B: Semiquantitative analysis of ABCA1 protein based on optical density measurements of the bands obtained by immunoblotting in A using Lumi-Imager™ (Boehringer). Optical densities of the bands are expressed as arbitrary units (BLU; Boehringer light units) after background noise reduction. C: Quantitative measurements for ABCA1 protein expression (ng /50 μg membrane protein) using ELISA.

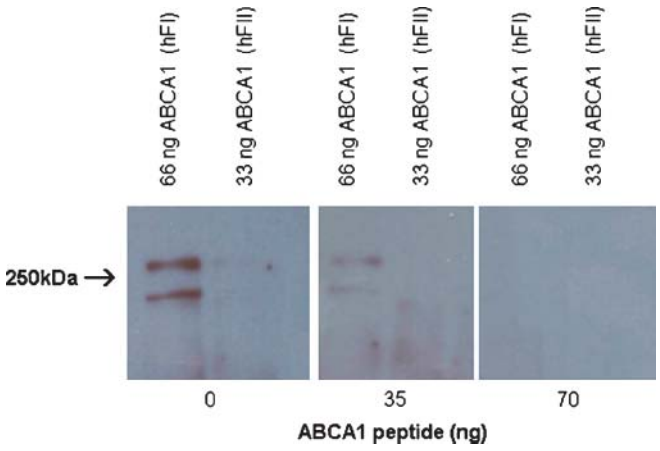

Fig. 5. Competitive immunoinhibition of antibody binding to ABCA1 protein in cholesterol loaded human fibroblasts by ABCA1 peptide (calibrator). Cell lysates (15 μl) from cholesterol loaded human fibroblasts containing 66 ng (hFI) and 33 ng (hFII) ABCA1 protein (as quantified by ELISA) were resolved on 7% SDS-PAGE. Immunoblots were probed with solutions of ABCA1 polyclonal antibody [diluted 1:2,000 in 3 ml phosphate buffered saline-tween 20 (PBST)] containing 0 ng, 35 ng, and 70 ng of ABCA1 peptide calibrator. Protein bands on blots were visualized by chemiluminescence.

Comparison of immunoblotting and ELISA

Of 15 human arterial tissue specimens previously analyzed by immunoblotting (25), we determined ABCA1 protein in three representative samples (I, II, III) both by Western blotting and ELISA technique using fresh crude membrane protein preparations (50 μg). These samples were previously demonstrated to have either nondetectable or on intermediate level expressed ABCA1 protein concentrations. The optical density of bands obtained by immunoblotting (**Fig. 4A**) was measured using a Lumi-Imager system (Fig. 4B). As shown in Fig. 4, we observed corresponding results for ABCA1 protein expression with the calibrated quantitative ELISA (Fig. 4C) and the semiquantitative immunoblot technique.

Competitive immunoinhibition for binding site recognition on the ABCA1 antibody

Addition of ABCA1 peptide (calibrator) in antibody solution inhibited the binding of ABCA1 antibody to ABCA1 protein in fibroblast lysates (hFI and hFII) on immunoblots with increasing peptide concentrations (**Fig. 5**). A significant competition was observed at a peptide concentration of 70 ng, which completely inhibited the antibody binding to the equivalent amount (66 ng; measured in ELISA) of ABCA1 protein in 15 μl electrophorized fibroblast lysate (hFI). This experiment confirms that the polyclonal ABCA1 antibody recognizes the same epitope in the reference 11-mer ABCA1 peptide and the holo ABCA1 protein (around 250 kDa) in the biological sample.

Concentrations of ABCA1 protein in human arterial tissues from patients and controls

Crude membrane preparations. ABCA1 protein concentrations in crude membrane extracts of carotid atherosclerotic plaques (n = 20) was significantly lower ($P < 0.05$) than in control arterial tissues (n = 10). The mean ABCA1 protein concentrations were 3.58 ± 1.42 vs. 35.58 ± 14.47 ng/μg of crude membrane protein ($P < 0.01$; **Fig. 6A**) and 4.16 ± 1.50 vs. 57.68 ± 31.53 μg/g of arterial tissue ($P < 0.05$; Fig. 6B) in patients and controls, respectively. In crude membrane protein preparations concentrations of ABCA1 protein varied from undetectable levels (<0.8 ng/μg) to levels as high as 133.13 ng/μg in controls and from undetectable levels to 23.01 ng/μg in patients.

Tissue lysates

In another separate approach and a different set of patient samples, ABCA1 protein concentrations were also measured in tissue lysates of carotid atherosclerotic plaques (n = 24) and control arterial tissues (n = 5). The mean ABCA1 protein concentration in tissue lysates obtained from direct extraction of the patients' carotid atherosclerotic plaques was significantly lower than in control arterial tissues (9.37 ± 0.82 vs. 17.03 ± 4.25 μg/g; $P < 0.01$; Fig. 6C) and confirmed previous results obtained in crude membrane preparations (see previous discussion and Fig. 6B).

DISCUSSION

The identification of ABCA1 as an essential integral membrane protein for the removal of intracellular cholesterol and phospholipids through HDL formation has generated great interest in understanding its functions in various tissues and cells associated with cellular lipid transport and metabolism. It has also been suggested that an impaired ABCA1 pathway could contribute to the increased atherogenesis associated with common inflammatory and metabolic disorders such as diabetes or the metabolic syndrome (4). Therefore, the identification of new pharmacological agents that regulate ABCA1 gene expression and protein synthesis will be a major challenge in treating disorders like atherosclerosis (27–30), neurodegenerative diseases (31), Type 2 diabetes (16), as well as placenta (32) and lung (33) malfunctions. These in-

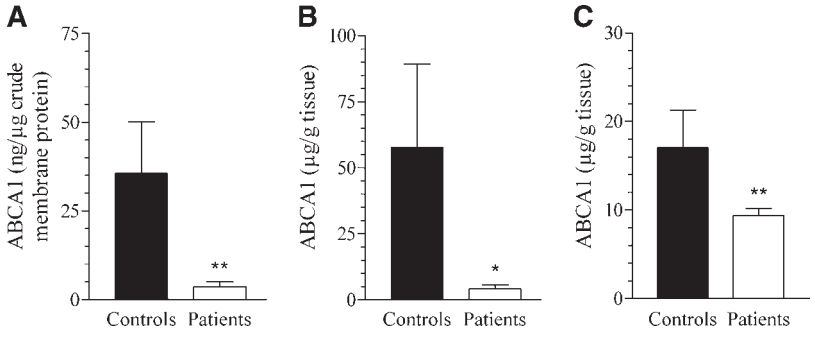

Fig. 6. ABCA1 protein levels in human arterial tissues of controls and patients with atherosclerotic plaques. ABCA1 protein levels are significantly higher in controls as compared with patients with carotid atherosclerotic plaques. Relative ABCA1 protein concentrations of controls (n = 10) and patients (n = 20) measured in crude membrane preparations are presented as ng/μg of crude membrane (A) and μg/g tissue (B) (here, ABCA1 concentrations per g of tissues were extrapolated from the amount of tissue used to one μg of crude membrane protein). Panel C shows the amounts of ABCA1 protein measured in direct tissue lysates of controls (n = 5) and patients (n = 24). Crude membrane proteins and tissue lysates from arteries were analyzed with ELISA using ABCA1 peptide standard (calibrator) and antihuman ABCA1 rabbit polyclonal antibody. Data are represented as mean values ± SEM. * $P < 0.05$ level of significance; ** $P < 0.01$.

vestigations require a specific quantitative detection of ABCA1 protein.

In a present study, we developed a novel sensitive ELISA for measuring ABCA1 protein concentrations in human arterial tissue extracts. The ABCA1 ELISA exhibited a high analytical and functional sensitivity, low imprecision, good linearity, and satisfactory analytical recovery. The analytical recovery reflects the accuracy and reproducibility of the ELISA values obtained from a spiked pool of crude membrane preparations. Furthermore, the linear quantification of ABCA1 protein from crude membrane and tissue extracts of human placenta and fibroblast lysates showed the possibility of extending the application of this ELISA to these and potentially other cell types and tissues.

As ABCA1 protein is mostly localized at the cell surface plasma membrane and intracellular vesicles (8, 34), the crude membrane preparation protocol was adopted to concentrate ABCA1 in the membrane extracts. Membrane protein enrichment could have an additional benefit in concentrating ABCA1 protein especially in carotid plaque tissues where ABCA1 protein expression was found to be low (25). Hence, in order to validate our present ABCA1 protein ELISA results in arterial tissues and compare them with the previous semiquantitative immunoblot analysis obtained in our laboratories (25), we initially used the crude membrane preparations to keep similar experimental conditions. However, we also evaluated the extraction and solubilisation efficiency of ABCA1 protein for the optimal choice of the detergent both in crude membrane extracts and direct tissue lysates of human placenta. The detergent selection was based on the reports of previous studies (35, 36). In our study, the DDM was the most efficient detergent, as judged by the amount of ABCA1 protein in placental tissue lysates and crude membrane protein extracts. Despite of the higher extraction and solubilization efficiency, DDM also affected the sensitivity of calibration curve. In comparison to DDM, Triton X-100 showed moderate extraction efficiency but resulted in a more sensitive calibration curve, hence, could be a suitable alternative for the more expensive DDM.

As our ABCA1 ELISA quantifications were based on the synthetic ABCA1 peptide calibrator, it was crucial to demonstrate that the antibody recognizes the same epitope in ABCA1 present in a biological sample and the reference peptide. Complete competitive inhibition of the binding site for ABCA1 protein (66 ng; around 250 kDa whole protein molecule) on the ABCA1 antibody by the addition of ABCA1 peptide (calibrator; 70 ng) in antibody solution was evidenced by immunoblotting. These experiments confirmed the same epitope (antigen recognition site) on the antibody for the ABCA1 peptide and ABCA1 protein in a biological sample. The ratio of amount of peptide required to completely inhibit the binding of antibody to the amount of ABCA1 protein on immunoblot was 1.06. This suggests that 1 mol of peptide (calibrator) is equivalent to approximately 1 mol of ABCA1 protein measured in the analyte.

Using this ELISA, the ABCA1 protein quantification in crude membrane preparations and direct arterial tissue extracts of CEA patients and controls revealed significantly lower levels of ABCA1 protein in plaques as compared with control arteries. Decreased ABCA1 protein levels in tissue of carotid plaques collected from the patients with advanced atherosclerosis confirms previous findings (25) for ABCA1 protein expression based on semiquantitative Western blot analysis. There is evidence that the extensive lipid accumulation in advanced atherosclerotic plaques despite of inducing *ABCA1* transcription is associated with impaired lipid efflux from foam cells most likely due to reduced levels of ABCA1 protein (reviewed in 37) and/or reduced activity (38, 39). In advanced atherosclerotic lesions, macrophages accumulate large amounts of free cholesterol (40), which leads to the degradation of ABCA1 protein in these macrophages (41). Moreover, long fatty acids present in the plaques (42) may promote macrophage ABCA1 protein degradation by phosphorylation of a PEST sequence in ABCA1 (23).

In conclusion, the newly established ABCA1 ELISA allows a reliable, specific detection and quantification of ABCA1 protein in human arterial tissues. Using this technique, we were able to confirm and extend previous semiquantitative Western blot data (25) on ABCA1 protein expression to quantitative levels in human carotid arteries. The good linearity of ABCA1 protein dilutions obtained with human placental tissue membrane proteins, direct tissue lysates, and fibroblast cell lysates extends the applicability of this ELISA to these and potentially other cell types and tissues.

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