ATP-Binding Cassette Transporter A1 Expression and Apolipoprotein A-I Binding Are Impaired in Intima-Type Arterial Smooth Muscle Cells

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Background—Accumulation of excess cholesterol by intimal arterial smooth muscle cells (SMCs) contributes to the formation of foam cells in atherosclerotic lesions. The purpose of this study was to examine the expression and activity of ATP-binding cassette transporter A1 (ABCA1) in model intimal and medial arterial SMCs, in human atherosclerotic coronary artery intimal and medial layers, and in human intimal and medial SMCs.

Methods and Results—Model intimal arterial SMCs showed increased cholesteryl ester accumulation, absence of apolipoprotein A-I–mediated lipid efflux, markedly diminished ABCA1 expression, and poor apoA-I binding compared with medial-layer SMCs. Total ABCA1 mRNA and SMC-specific ABCA1 protein levels were diminished in the intimal layer compared with the medial layer of atherosclerotic human coronary arteries. Increased expression of ABCA1 by liver X receptor agonist treatment or gene transfection failed to correct apolipoprotein A-I binding, lipid efflux, or high-density lipoprotein particle formation by intima-type SMCs. In addition to impaired ABCA1 expression, intima-type SMCs appear to lack a critical binding factor or factors required for the apolipoprotein A-I–ABCA1 interaction, cholesteryl efflux, and high-density lipoprotein particle formation.

Conclusion—ABCA1 expression is reduced in cultured model intimal and human atherosclerotic lesion SMCs, suggesting that reduced ABCA1 activity contributes to smooth muscle foam cell formation in the intima. (Circulation. 2009;119:3223-3231.)

Key Words: apolipoproteins ■ atherosclerosis ■ cholesterol ■ lipoproteins ■ muscle, smooth

Smooth muscle cells (SMCs) constitute a, or the, major cell type in most stages of atherosclerosis.1 Relatively little is known, however, about cholesterol homeostasis in arterial SMCs compared with macrophages. Like macrophages, intima-type SMCs express scavenger receptors and develop into smooth muscle foam cells containing excess cholesteryl esters.2–6 Removal of cholesterol from SMCs, like other cells, is dependent on the binding of apolipoprotein A-I (apoA-I) to the cell surface and the transfer of cellular phospholipids and cholesterol to apoA-I through the actions of the ATP-binding cassette transporter A1 (ABCA1).7,8 Previous studies have reported marked variability in apoA-I binding and apoA-I–mediated lipid efflux from arterial SMCs obtained from different species,9–10 suggesting that the apoA-I–SMC interaction may differ depending on SMC phenotype. Recent studies have reported both increased and decreased expression of ABCA1 in atherosclerotic arteries,11–13 but the relative expression of ABCA1 in intimal versus medial layers has not been described. Although ABCA1 is expressed in SMCs originating from the medial layer of normal arteries,7 its expression in intimal SMCs has not been reported.

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In addition to excess cholesterol accumulation, intimal SMCs are characterized by a dedifferentiated state, an increased rate of proliferation, a loss of contractility, an increased synthesis of extracellular matrix components, and a reduced expression of SMC markers, including smooth muscle α-actin and smooth muscle myosin heavy chain.14,15 The marked differences in gene expression patterns have led some investigators to propose that SMCs populating the intima are of different clonal origins than those in the medial layer.14,16,17 Intimal SMCs of human origin are not available for use in continuous culture studies. In the present studies, we used clones of rat

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SMCs that exhibit features of human intimal SMCs, including accumulation of excess cholesteryl esters and reduction of smooth muscle α-actin and myosin heavy chain, and exhibit an epithelioid or cuboidal morphology as a model for cholesterol mobilization from intimal SMCs. We compared these results with the expression of ABCA1 in the intima and media and in intimal and medial SMCs of atherosclerotic human coronary arteries. Our results indicate a markedly reduced expression of ABCA1 and reduced binding and lipid mobilization by apoA-I from the model intimal SMCs and a reduction of ABCA1 expression in human atherosclerotic intima and intimal SMCs. Overexpression of ABCA1, however, failed to correct apoA-I binding to, or cholesterol efflux from, intima-phenotype SMCs.

Methods

SMC Cultures

Clonal SMC lines obtained from the medial layer of thoracic aortas of 12-day-old Wistar-Kyoto (WKY) rats (WKY12–22, epithelioid morphology) and 3-month-old rats (WKY3M–22, spindle morphology) were a generous gift from Dr Joan Lemire (University of Washington, Seattle). Cells were grown in DMEM/FBS and used between the 2nd and 10th passage after reestablishing the culture. Cells maintained a constant growth rate, morphology, ABCA1 expression, and apoA-I responses during these passages. Epithelioid SMCs showed a somewhat lower rate of cell proliferation at confluence (Figure I of the online-only Data Supplement). In some experiments, cells were loaded with excess nonlipoprotein cholesterol to generate SMC foam cells before incubation with apoA-I.10

Lipid Analyses

ApoA-I was obtained from the delipidated whole-protein fraction of human plasma high-density lipoprotein (HDL). Radiolabeled lipid efflux studies were performed as described in the Methods section of the online-only Data Supplement. Cholesterol mass was determined by gas chromatography.20 Cell surface cholesterol was determined with cholesterol oxidase as previously described.21 The rate of degradation of [14C]cholesteryl esters was assessed in the presence of an acyl CoA:cholesterol acyltransferase inhibitor as previously described.22 Two-dimensional gel electrophoresis of HDL particles was performed as described in the online Methods section.20

ABCA1 Analysis in SMCs

Determination of ABCA1 mRNA levels by quantitative real-time polymerase chain reaction and assessment of total, crude cell membrane, cell surface, and phosphorylated ABCA1 protein were as described in the Methods section in the online-only Data Supplement.

Cell Surface Binding of ApoA-I

Cells were incubated for 2 hours at 4°C in DMEM/BSA containing 25 mmol/L HEPES and 10 μg/mL IgG–apoA-I ±200 μg/mL unlabeled apoA-I. Cells were washed 5 times with cold PBS/BSA and twice with cold PBS before solubilization in 0.2N NaOH for quantification of radioactivity and protein. Cross-linking of apoA-I to ABCA1 was performed as described in the online-only Methods section.

ABCA1 Analysis in Atherosclerotic Human Coronary Arteries

Coronary artery sections from 14 patients with the primary diagnosis of coronary heart disease were obtained from the Cardiovascular Registry at St Paul’s Hospital, University of British Columbia (Table I of the online-only Data Supplement). Oil Red O staining, in situ hybridization of ABCA1 mRNA, immunohistochemistry for smooth muscle α-actin and ABCA1, and image analysis were performed as described in the online-only Data Supplement.

SMC Transfection

Cells were transiently transfected for 24 hours with control empty vector (pcDNA3.1) or full-length murine ABCA1 cDNA cloned in a pcDNA3.1 vector,23 a generous gift from Dr Nan Wang (Columbia University, New York, NY) with Lipofectamine 2000 (Invitrogen, Carlsbad, Calif).

Statistical Analysis

Results for cell culture data are expressed as mean±SD and for in situ hybridization and immunofluorescence data as mean±SEM. Significant differences between experimental groups were determined with the Student t test, with a value of P<0.05 considered significant.
Results

Cell Models

We previously determined that binding of apoA-I and apoA-I–mediated cholesterol and phospholipid efflux was intact in human but not rat SMC lines,10 but we did not correlate these results with features of medial or intimal SMCs in either species. In the absence of human intimal SMCs suitable for use in repeated cell culture studies, we studied clonal SMCs obtained from the aorta of 12-day-old WKY rats with features consistent with human intimal (plaque) SMCs14 and SMCs from bovine and pig arterial intima,24,25 including increased proliferation and proteoglycan production and reduced smooth muscle α-actin and smooth muscle myosin heavy chain expression, that demonstrate an epithelioid morphology in culture.18 We compared these with SMCs obtained from the medial layer of 3-month-old WKY rats exhibiting a spindle morphology as our medial SMC model (Figure 1A).18

Impaired ApoA-I–Mediated Phospholipid and Cholesterol Efflux From Epithelioid SMCs

Removal of cell phospholipids by apoA-I and other HDL apolipoproteins is required for initial HDL formation and to allow the developing HDL to solubilize cholesterol also
removed from cells. Spindle-morphology SMCs readily released both phosphatidylcholine and sphingomyelin, whereas epithelioid SMCs released little or none of these phospholipids to apoA-I (Figure 1B). Spindle SMCs similarly showed release of cholesterol mass to apoA-I, whereas epithelioid SMCs showed very little release of cholesterol to albumin alone or to apoA-I (Figure 1Ci). ApoA-I actively depleted cell cholesteryl ester mass from spindle SMCs but induced no reduction of the larger pool of cholesteryl ester mass seen in WKY rat epithelioid SMCs after loading with nonlipoprotein cholesterol (Figure 1Cii). Efflux of radiolabeled cholesterol to the medium and a decrease in radiolabeled cholesteryl esters in cells by apoA-I showed patterns similar to cholesterol mass changes in spindle and epithelioid SMCs (Figure II of the online-only Data Supplement). Additional clonal spindle and epithelioid SMCs obtained from Sprague-Dawley adult and pup rats, as well as spindle-morphology human arterial SMCs and the epithelioid human SMC clone HITA2,17 showed similar presence or absence of efflux of cell cholesterol to apoA-I, respectively (Figure III in the online-only Data Supplement). Neither the Sprague-Dawley rat or human HITA2 epithelioid SMCs, however, accumulated cholesteryl esters the way atherosclerotic and neointimal SMCs do4,5 (Figure III in the online-only Data Supplement). For this reason, the WKY rat epithelioid and spindle SMC lines were chosen for subsequent studies as our models for intimal and medial SMCs, respectively.

Decreased ABCA1 Expression and ApoA-I Binding in Epithelioid SMCs

ApoA-I-mediated phospholipid and cholesterol efflux is critically dependent on the membrane transporter ABCA1, the expression of which is normally increased with increasing cell cholesterol content.8 Consistent with our lipid efflux results, we found low basal and cholesterol-stimulated levels of ABCA1 mRNA and protein in epithelioid-morphology WKY rat SMCs (Figure 2A and 2B). Spindle SMCs showed high basal and further increased ABCA1 expression on cholesterol loading. Similar results were found for human and Sprague-Dawley rat SMCs, with spindle SMCs from both

Figure 4. ABCA1 and smooth muscle α-actin immunoreactivity in the intima (I) and media (M) of atherosclerotic human coronary arteries. Representative dark-field photomicrographs of smooth muscle α-actin (A; green) and ABCA1 (B; red) detected immunohistochemically. C, Color segmentation analysis showing colocalization of ABCA1 and smooth muscle α-actin (pink). The green line represents the internal elastic lamina. Scale bar in A = 50 μm. D, Hematoxylin and eosin–stained adjacent section highlighting the region shown in A through C. Scale bar in D = 200 μm. E, ABCA1 immunoreactivity as a percentage of total area is significantly higher in media (53%) compared with intima (31%), with intimal area measured as all area above the internal elastic lamina. F, Colocalization of ABCA1 and smooth muscle α-actin as a percentage of total smooth muscle α-actin-staining area indicates a significantly higher degree of ABCA1 immunoreactivity in SMCs in media (43%) vs intima (28%). E and F, Mean ± SEM of averages from the 14 cases, with at least 2 fields of view used to calculate an average for each section.
species showing increased ABCA1 protein with cholesterol loading and epithelioid cells showing an absence of increase in ABCA1 protein with cholesterol loading (Figure IV of the online-only Data Supplement).

A direct binding interaction between apoA-I and ABCA1 and/or a cellular association of apoA-I with lipid domains formed by ABCA1 are necessary for apoA-I–mediated lipid efflux. Spindle-morphology rat SMCs showed a much higher level of apoA-I binding than epithelioid SMCs before cholesterol loading and an approximate doubling of apoA-I binding after loading with nonlipoprotein cholesterol (Figure 2C). Despite no significant increase in ABCA1 expression in epithelioid SMCs after cholesterol loading, there was a small but significant increase in the low level of apoA-I binding to these cells after cholesterol loading (Figure 2C).

**Decreased ABCA1 Expression in Human Atherosclerotic Lesion Intima and Intimal SMCs**

To test the relevance of our results using cultured epithelioid and spindle arterial SMCs, we examined the relative expression of ABCA1 mRNA in the intimal and medial layers of coronary artery sections from 14 patients with various degrees of coronary artery disease resulting from native, as opposed to postangioplasty or posttransplantation, atherosclerosis (clinical details in Table I of the online-only Data Supplement). As previously demonstrated for transplant but not native human coronary atherosclerosis, staining with Oil Red O and costaining with smooth muscle α-actin showed a marked accumulation of lipid in intimal but minimal lipid accumulation in medial SMCs (Figure 3A and 3B). In situ hybridization revealed a striking decrease in ABCA1 mRNA levels in the intimal compared with medial layers of atherosclerotic human coronary arteries (Figure 3D). ABCA1 mRNA was present in an average of only 26% of total intimal area compared with 49% of total medial area from the 14 specimens (Figure 3E).

To test whether human intimal SMCs specifically exhibit diminished ABCA1 expression, we determined the relative level of ABCA1 protein in smooth muscle α-actin–positive cells in medial and intimal layers of coronary artery sections from the same 14 patients. Costaining of smooth muscle α-actin and ABCA1 revealed a relative paucity of ABCA1 protein in SMCs in the proximal intima (Figure 4C, top three fourths of the layer above the internal elastic lamina) relative to the deep intimal and medial layers. Image analysis of total ABCA1 immunoreactivity and colocalization of ABCA1 and smooth muscle α-actin per total smooth muscle α-actin–staining area in intimal or medial layers from 14 patients revealed significantly lower total ABCA1 and SMC-specific ABCA1 in atherosclerotic intima compared with medial arterial layers (Figure 4E and 4F, respectively). These findings are consistent with our results of diminished ABCA1 expression in model intimal rat and human epithelioid SMCs.

**Expression of ABCA1 Can Be Increased in Epithelioid SMCs**

Reduced ABCA1 expression and apoA-I binding provide potential explanations for the increased cholesterol accumulation seen in atherosclerotic intimal SMCs. To determine whether intimal phenotype SMCs might be amenable to treatments to increase lipid efflux and HDL formation, we treated cultured epithelioid SMCs with known agonists of ABCA1 expression. Liver X receptor (LXR) agonists 22-hydroxycholesterol and TO901317, retinoid X receptor agonist 9-cis-retinoic acid, and a combination of 22-hydroxycholesterol and retinoic acid increased ABCA1 protein levels in epithelioid SMCs to levels equal to or higher than those seen in unstimulated spindle SMCs (Figure 5). Unlike macrophages and human fibroblasts, 8-bromo-cAMP showed no ability to increase ABCA1 protein levels in epithelioid or spindle SMCs.

**Increased Expression of ABCA1 Fails to Correct ApoA-I–Mediated Cholesterol Efflux or ApoA-I Binding**

We next determined whether increased ABCA1 expression corrects binding and lipid efflux to apoA-I by epithelioid SMCs. LXR agonist TO901317 treatment increased total and cell surface ABCA1 in both spindle and epithelioid SMCs (Figure 6A). Consistent with this increase, spindle SMCs treated with the LXR agonist showed a 2-fold increase in cholesterol efflux to apoA-I (Figure 6B). Epithelioid cells, in contrast, showed no increase in cholesterol efflux to apoA-I despite increased ABCA1 expression, nor did they exhibit any correction of phosphatidylcholine efflux to apoA-I (Figure V of the online-only Data Supplement). The ability of apoA-I to be cross linked directly to ABCA1 increased after LXR agonist treatment of spindle SMCs but was low and increased minimally after agonist treatment of epithelioid SMCs (Figure VI in the online-only Data Supplement). The effects on lipid efflux were mirrored by changes in HDL particle formation as assessed by 2-dimensional gel electrophoresis. Spindle SMCs showed an increase in formation of all subspecies of α-HDL after LXR agonist treatment, whereas epithelioid SMCs showed pre-β but little or no α-HDL formation either before or after treatment with the agonist (Figure 7).

Efflux of cholesterol to HDL from epithelioid SMCs also was reduced, by ~40%; however, this rose to the same level...
as in spindle SMCs in the presence of an acyl CoA:cholesterol acyltransferase inhibitor (Figure VII in the online-only Data Supplement). This suggests that cholesterol can be mobilized to a pool available for HDL-dependent, but not apoA-I–dependent, efflux in epithelioid SMCs.

Transfection of SMCs with full-length ABCA1 cDNA to specifically upregulate ABCA1 expression similarly resulted in increased total and cell surface ABCA1 in spindle and epithelioid cells (Figure 8A). Protein kinase A–dependent phosphorylation of ABCA1, a determinant of constitutive

**Figure 6.** Cell surface localization of ABCA1 and cholesterol efflux from arterial SMCs treated with the LXR agonist TO901317. A. Confluent SMCs treated in the absence or presence of cholesterol and/or 10 μmol/L TO-901317 were biotinylated, and total and cell surface ABCA1 levels were determined by Western blotting as described in Methods. Protein disulfide isomerase (PDI) levels were determined as loading control. Results are representative of 2 experiments with similar results. B. Confluent SMCs labeled with [3H]cholesterol during growth were loaded with cholesterol, equilibrated, and incubated with the indicated concentration of apoA-I for 24 hours. Equilibration of cholesterol-loaded cells and apoA-I treatment were performed in the absence or presence of 10 μmol/L TO-901317. At the end of the incubation period, media were collected, and unesterified cholesterol (UC) in the medium and in cell cholesterol ester (CE) and UC were analyzed for [3H]cholesterol. Results are expressed as percentage of total cell plus medium [3H]sterol in the medium (a), cell CE (b), and cell UC (c) after subtraction of efflux to medium containing 1 mg/mL BSA alone. Values are the mean±SD of quadruplicate determinations and are representative of 2 experiments with similar results.

**Figure 7.** HDL particles formed by spindle and epithelioid arterial SMCs. Confluent SMCs were loaded with cholesterol, equilibrated for 24 hours, and incubated with 10 μg/mL apoA-I for 24 hours in the absence or presence of 10 μmol/L TO-901317 (LXR). Cell media were collected and analyzed for apoA-I–containing HDL particle species by 2-dimensional gel electrophoresis. Results are representative of 2 separate experiments with similar results.
ABCA1 activity,30 was also increased in transfected cells of both phenotypes. Despite appropriate cellular localization and phosphorylation, however, increased ABCA1 again failed to increase cholesterol efflux to apoA-I from epithelioid cells compared with the large increase seen in spindle SMCs (Figure 8B). In contrast to previous reports indicating increased apoA-I binding after transfection of ABCA1 in macrophages and HEK293 cells26,31 and despite increased cholesterol efflux in spindle SMCs, increased ABCA1 expression after transfection resulted in no increase in apoA-I binding to either spindle or epithelioid SMCs (Figure 8C). It is possible that despite the increase in total, cell surface, and phosphorylated ABCA1, ABCA1 remains inactive in epithelioid SMCs. Transfection with ABCA1 resulted in an increase in cholesterol oxidase–sensitive cholesterol in both spindle and epithelioid SMCs, suggesting that ABCA1 increased the mobilization of cholesterol to the outer leaflet of the plasma membrane in both cell types (Figure VIII A of the online-only Data Supplement). Hydrolysis of cholesteryl esters was intact in epithelioid SMCs, ruling out impairment of neutral cholesteryl ester hydrolase as a reason for impaired cholesterol efflux by these cells (Figure VIII B in the online-only Data Supplement).
form HDL particles with increased lipid content or ɑ-mobility on 2-dimensional gels. The ability of cells to generate ɑ- but not pre-β-mobility HDL has previously been shown to require ABCA1 activity. Consistent with low ABCA1 expression in cultured intima-type SMCs, immunohistochemistry of human atherosclerotic coronary arteries demonstrated reduced ABCA1 expression specifically in intimal SMCs. To correct for the reduced cell density and known reduction of smooth muscle ɑ-actin expression in intimal SMCs (Figure 4A), we normalized the colocalization of smooth muscle ɑ-actin and ABCA1 to total smooth muscle ɑ-actin-staining area in the intima and media. This analysis reveals a significant reduction of total intimal compared with medial SMC ABCA1 expression, despite the finding that deep intimal SMCs show relatively high ABCA1 expression compared with more proximal intimal SMC. Previous studies have reported both increased levels of ABCA1 mRNA and lower ABCA1 protein levels in whole atherosclerotic arterial extracts but did not determine ABCA1 mRNA or protein in specific arterial layers. Our results are the first demonstration of a reduction of intimal-compared with medial-layer ABCA1 mRNA and protein, specifically intimal SMC ABCA1 expression. In addition to reduced ABCA1 mRNA in intimal compared with medial layers, we cannot rule out the possibility that reduced ABCA1 stability also contributes to the lower ABCA1 protein in the intima and intimal-layer SMCs. Rong and colleagues have previously reported conversion of SMCs taken from the medial layer of adult mouse aorta to a macrophage-like state on cholesterol loading and ABCA1 expression by these cells. Although different subtypes of SMCs likely exist in the intima, our results indicate that impaired ABCA1 expression would explain the excess cholesterol accumulation in at least a subset of intimal SMCs.

The reduced expression of ABCA1 in rat epithelioid SMCs, also found in cultured human epithelioid SMCs (Figures III and IV in the online-only Data Supplement), was increased to levels seen in unstimulated spindle SMCs by agonists of nuclear receptors LXR and retinoid X receptor. Despite appropriate localization of the increased ABCA1 to the cell surface, this treatment failed to increase lipid efflux to apoA-I or HDL particle formation and increased the ability of apoA-I to be cross linked to ABCA1 only slightly. Similarly, transfection of full-length ABCA1, resulting in increased total, cell surface, and phosphorylated ABCA1, failed to increase both binding and cholesterol efflux to apoA-I by epithelioid SMCs. This result was seen despite evidence of activity of the transfected ABCA1 in increasing mobilization of cholesterol to a cholesterol oxidase–sensitive pool in both cell types. Therefore, reduced ABCA1 expression in model intimal SMCs does not appear to explain the reduced apoA-I binding to these cells. Correction of ABCA1 expression, localization, and apparent activity did not alter the low levels of apoA-I binding to these cells. These findings suggest that in addition to impaired ABCA1 expression, epithelioid SMCs lack a factor or factors, possibly a cell surface or cytoskeletal component, required for apoA-I to bind to cells and receive lipids made available for HDL formation by ABCA1. The striking absence of apoA-I binding in epithelioid SMCs compared with spindle SMCs despite normalization of ABCA1 expression provides an excellent model to study the nature of this additional binding factor or factors.

Conclusions
We have demonstrated that spindle-morphology, media-phenotype SMCs exhibit high levels of ABCA1 expression, apoA-I binding, and efflux of cellular lipids to form HDL particles, whereas intima-phenotype SMCs lack these features. We have also shown that correction of ABCA1 expression fails to correct apoA-I binding or HDL production by intima-type cells. Human atherosclerotic intimal SMCs similarly exhibit impaired ABCA1 expression, suggesting a novel explanation for the development of intimal smooth muscle foam cells in vivo.

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Disclosures
None.

References

Disclosures
None.
CLINICAL PERSPECTIVE

Arterial smooth muscle cells (SMCs) are a major cellular component of atherosclerotic lesions and, like macrophages, accumulate excess cholesterol. This study provides evidence that expression of the major mediator of cholesterol removal from cells and the rate-limiting protein in high-density lipoprotein particle formation, ATP-binding cassette transporter A1 (ABCA1), is reduced in intima-type cultured SMCs, in human coronary atherosclerotic intima, and specifically in atherosclerotic intimomedial SMCs. Remarkably, overexpression of ABCA1 in cultured intima-type SMCs failed to correct the binding of the main protein of high-density lipoprotein, apolipoprotein A-I, to the cells or cholesterol efflux from these cells. These results provide a previously unknown explanation for the accumulation of excess cholesterol in intimal smooth muscle foam cells and suggest that differences in gene and protein expression by medial and intimal SMCs might identify factors required for new high-density lipoprotein particle formation. Identification of these factors would provide novel targets for raising high-density lipoprotein clinically for the prevention of atherosclerosis.
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Data Supplement

Expanded Methods

Cell Culture

Human aortic smooth muscle cells (HASMC) grown from the abdominal aorta of an 11-month-old child (CRL-1999) were obtained from American Type Culture Collection and exhibited a spindle morphology. HITA2 cells are a clonal cell line isolated from human internal thoracic artery obtained at the time of coronary artery bypass surgery that exhibit an epithelioid morphology. Clonal SMC lines obtained from the medial layer of thoracic aortas of 12-day- (SD12d, epithelioid morphology) and 3-month-old (SD3M-23, spindle morphology) Sprague-Dawley rats were a gift of Dr. Joan Lemire, University of Washington. All cell lines were used between the second and tenth passages after reestablishing cultures, expressed smooth muscle α-actin, and maintained a constant growth rate and cell morphology during the studies. Cell proliferation was determined using incorporation of $^3$H-Thymidine when cells were confluent, and normalized to DNA content.

Phospholipid Efflux Assay

Cells were seeded on 35-mm wells and grown to confluence in DMEM containing 10% FBS. Cells were then cholesterol loaded and incubated with 4 μCi/ml $[^3]$H-choline
chloride (NEN Life Science Products, Boston, MA) in DMEM/BSA during the 24 h equilibration period to radiolabel choline-derived phospholipids. After rinsing 5 times with PBS/BSA, cells were incubated for 24 h in DMEM/BSA containing 0-10 µg/ml apoA-I. At the end of the incubation, media were collected and centrifuged (3,000 rpm for 10 min) to remove cell debris, and cell layers were rinsed twice with iced PBS/BSA and twice with iced PBS. Medium and cellular lipids were extracted, separated by thin layer chromatography, and assayed for $[^{3}\text{H}]$choline-containing phospholipids as described.$^4$ Cell proteins were determined using BSA as standard.

**Cholesterol Efflux Assay**

Cells were seeded on 16-mm wells and radiolabeled during the last 40% of growth to confluence in DMEM containing 10% FBS and 0.3 µCi/ml $[^{3}\text{H}]$cholesterol (NEN Life Science Products, Boston, MA) to radiolabel cell cholesterol pools. Confluent cells were then cholesterol loaded with unlabeled non-lipoprotein cholesterol and equilibrated prior to incubation with DMEM containing 1 mg/ml fatty acid-free bovine serum albumin (DMEM/BSA) containing 0-10 µg/ml apoA-I for 24 h.$^5$ At the end of the incubation, media were collected and centrifuged (3,000 rpm for 10 min) to remove cell debris, and radioactivity in the medium was measured by liquid scintillation counting. Cell layers were rinsed twice with iced phosphate buffered saline containing 1 mg/ml bovine serum albumin (PBS/BSA) and twice with iced PBS, and stored at −20 °C until lipid extraction. Cellular lipids were extracted and separated by thin layer chromatography, and assayed for $[^{3}\text{H}]$cholesterol and $[^{3}\text{H}]$cholesteryl ester as described.$^4$ Cell proteins were determined using BSA as standard.$^6$ Efflux measurements done in the presence of the
ACAT inhibitor Sandoz 58-035 (Sigma) to inhibit cholesterol esterification were done using 2μg/mL 58-035 during equilibration of cells following cholesterol loading and apoA-I or HDL2-mediated efflux periods.

**Cholesterol Mass Determination**

Cells were seeded on 60-mm dishes and grown to confluence in DMEM containing 10% FBS. Following cholesterol loading and equilibration cells were rinsed twice with PBS/BSA and incubated for 24 h in DMEM/BSA with or without 10 µg/ml apoA-I. At the end of the incubation, media and cells were collected for analyses. Phospholipids from media or cell homogenate were digested by phospholipase C, and total lipids were extracted in the presence of tridecanoin as the internal standard. Samples were derivatized with Sylon BFT (Supelco, Bellefonte, PA) and analyzed by gas chromatography [Agilent Technologies, 6890 Series equipped with a Zebron capillary column (ZB-5, 15 m x 0.32 mm x 0.25 µm) and connected to a flame ionization detector, Palo Alto, CA]. The oven temperature was raised from 170 to 290 °C at 20 °C/min, and then to 340°C at 10 °C/min where the temperature was kept for 24 min. Helium was used as a carrier gas. The gas chromatography was operated in the constant flow mode with the flow rate of 4.5 ml He/min. The injector was operated in the split mode and was kept at 325 °C, and the detector was kept at 350 °C.

**Quantification of ABCA1 mRNA**

To determine ABCA1 mRNA levels, two micrograms of RNA were reverse-transcribed with Superscript cDNA synthesis reagents (Invitrogen, Burlington, ON). Quantitative
real-time PCR was performed using Platinum SYBR Green qPCR Supermix (Invitrogen) and a Rotor-Gene 3000 instrument (Corbett Research, Kirkland, PQ). Cyclophilin was used as a PCR amplification control. The primers used are as follows: ABCA1, 5'-AGT ACC CCA GCC TGG AAC TT (forward), 5'-TGG GTT TCC TTC CAT ACA GCG (reverse); cyclophilin, 5'-TCC AAA GAC AGC AGA AAA CTT TCG (forward), 5'-TCT TCT TGC TGG TCT TGC CAT TCC (reverse).

**Western Blot Analysis of ABCA1**

To determine total ABCA1 protein levels, cells were lysed in RIPA buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, and 4 mM EDTA] containing complete protease inhibitors (Roche Molecular Biochemicals, Laval, QC). The total protein extracts were subjected to Western blotting with anti-ABCA1 antibody (1:1000, Novus Biologicals, Littleton, CO). The blots were reprobed with anti-protein disulfide isomerase antibody (1:2000, StressGen Biotechnologies, Victoria, BC) to verify equal protein loading. To determine ABCA1 protein levels in crude membrane fraction, cells were homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing complete protease inhibitors and 1 mM EDTA. The nuclear fraction was removed by centrifugation for 2 min at 5000 rpm, and the supernatant was subsequently centrifuged for 30 min at 14,000 rpm. The pellet was then resuspended in the homogenizing buffer with 0.45 M urea, 0.1 % Triton X-100, 0.2 % SDS and 0.05 % dithiothreitol prior to performing Western blotting with anti-ABCA1 antibody. To determine cell surface ABCA1, cell surface proteins were biotinylated using a cell surface protein biotinylation and purification kit (Pierce, Rockford, IL). Biotinylated proteins were isolated according to the manufacturer’s protocol and subjected to Western
blotting with anti-ABCA1 antibody. The blots were reprobed with anti-heat shock protein 90 antibody (1:1000, Sigma, Oakville, ON) to verify no cytosolic protein in the purified samples. To determine the status of ABCA1 phosphorylation, the total protein extracts were subjected to Western blotting with anti-phospho-(Ser/Thr) PKA substrate antibody (1:1000, Cell Signaling Technology, Beverly, MA).7

**Oil Red O and SM-α-actin staining**

OCT embedded frozen sections were cut, fixed with 10% formalin for 5 minutes at room temperature, and stained histochemically with Oil Red O alone and counterstained with hematoxylin and eosin, or double-stained, immunohistochemically using a serial section with smooth muscle-α actin antibody, and then histochemically with Oil Red O. Briefly, slides were blocked with Dako universal block for 30 minutes, and then incubated with smooth muscle-α actin antibody overnight. HRP conjugated goat anti-mouse IgG secondary antibody was applied to the sections followed by DAB incubation for 10 minutes. Sections were then processed for Oil Red O staining and counterstaining with hematoxylin and eosin.

**In situ hybridization**

Human ABCA1 cDNA was a kind gift from Dr. M. Hayden (Centre for Molecular Medicine and Therapeutics, University of British Columbia, Vancouver, British Columbia, Canada) and was cloned into a pBlueScript-KS vector. Probes recognizing the cDNA sequence of human ABCA1 (N-terminal nt 1006-2898) were prepared by *in vitro* transcription.8 Tissue sections from human artery and mouse brain were used as positive tissue controls. Omission of the probe served as the negative control for the *in situ*
hybridization protocol. Paraffin-embedded tissue sections were dewaxed, rehydrated and then incubated with proteinase K, dehydrated, and hybridized with the appropriate probes. Hybridization was allowed to proceed at 55° C overnight, followed by stringent washing in 50% formamide/2x SSC and several changes of 2x SSC and 2x SSC. After hybridization, alkaline phosphatase-conjugated anti-digoxigenin antibody was applied to the tissue sections for 30 minutes and staining visualized with the color substrate Vector Red (Vector Laboratories).

**Immunohistochemistry**

Formalin-fixed tissues were paraffin-embedded, sectioned and mounted on glass slides. Sections were dewaxed and rehydrated, then immunohistochemical staining for smooth muscle-\(\alpha\) actin and ABCA1 was performed using Shandon disposable immunostaining coverplates (Thermo Shandon, Inc., Pittsburgh, PA). Briefly, slides were blocked with 10% normal donkey serum for one hour, then co-incubated with monoclonal mouse anti-human smooth muscle (SM) \(\alpha\)-actin (Sigma) and rabbit polyclonal anti-ABCA1 antibody (Novus Biologicals) overnight. AlexaFluor® 488-conjugated donkey anti-mouse IgG and AlexaFluor® 594-conjugated donkey anti-rabbit IgG were used to detect smooth muscle-\(\alpha\) actin and ABCA1, respectively by dual immunofluorescent staining. Nuclei were stained using Hoechst 33342, then slides were mounted using SloFade® mounting reagent (Molecular Probes). Isotype-matched IgG and omission of the primary antibody were used as negative controls.

**Image analysis**
Slides were viewed using a Nikon Eclipse TE300 inverted microscope, illuminated by a Nikon Super High Pressure Mercury Lamp, filtered using DAPI, FITC, or rhodamine filter sets, and captured using a Spot digital camera (Diagnostic Instruments). The intensity of the staining of ABCA1 protein and mRNA in intima and media of each section was assessed quantitatively in darkfield micrographs using *ImagePro Plus®* image analysis software. Briefly, areas of interest (AOI) were selected, separating the intima and media of vessels using the internal and external elastic laminae, as identified from adjacent sections stained with Movat’s pentachrome. For immunohistochemistry, an image segmentation file identifying ABCA1 positivity and ABCA1 and smooth muscle-α actin co-localization were created, with a minimum threshold cutoff of 75/255 using 8-bit images. The ABCA1 positive area or ABCA1 co-localized with smooth muscle-α actin was determined by the imaging software as a percentage of the total area of the AOI. Values were tabulated in Microsoft Excel and the average values for the intima and media were obtained and represented as average ABCA1 positive area as a percentage of total area (both mRNA and protein expression) or as average co-localized area (ABCA1 + smooth muscle-α actin) as a percentage of total area (protein expression).

**Cross-linking of ABCA1 and apoA-I**

Chemical cross-linking of ABCA1 and apoA-I was performed following the method of Denis et al. with slight modifications. Confluent SMC were loaded with non-lipoprotein cholesterol and then equilibrated for 24 h in DMEM/2 mg/ml BSA prior to incubation with 10 μg/ml apoA-I for 2 h at 37°C. Cells were treated in the absence or presence of 10 μM LXR agonist TO901317 during the equilibration and apoA-I incubations.
then placed on ice, washed, incubated with dithiobis(succinimidylpropionate) (DSP) for 1 h as described. Cell homogenates were incubated for 1 h in the presence or absence of β-mercaptoethanol (5% v/v) prior to separation by 4-20% gradient gel SDS-PAGE and Western blot analysis as described above or with anti-apo-A-I antibody (Calbiochem, Mississauga, ON).

Two-dimensional gel electrophoresis of HDL particles

Cells were incubated for 24 h with 10 µg/ml apoA-I in the presence or absence of 10 µM TO-901317. At the end of the incubation, the media were collected and centrifuged at 2000 rpm for 10 min at 4ºC to remove cell debris and the supernatant was concentrated 10-fold using Amicon Ultra-4 centrifugal filter, MWCO 10,000 (Millipore, Cambridge, ON). HDL particles in equivalent volumes of the concentrated media were analyzed by two-dimensional non-denaturing gel electrophoresis and Western blot with anti-apoA-I antibody as previously described, but substituting the secondary [125I]-labeled donkey anti-rabbit antibody for anti-rabbit IgG-peroxidase antibody (Sigma-Aldrich, Oakville, ON), and detecting chemiluminescence using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL).

References


**Data Supplement Figure I.**

[^3]H]Thymidine incorporation into spindle and epithelioid SMC. Confluent dishes of spindle and epithelioid cells were incubated with[^3]H]thymidine to determine the rate of cell proliferation. Epithelioid cells had a lower rate of proliferation after reaching confluence (p < 0.02). Mean ± SD of triplicate determinations; representative of two experiments.
**Data Supplement Figure II.** Impaired apoA-I-mediated cholesterol efflux from Wistar-Kyoto epithelioid SMC. SMC from 12-day-old (epithelioid) or 3-month-old (spindle) Wistar Kyoto rat aorta were labeled with $[^3]$Hcholesterol during growth, loaded with unlabeled cholesterol for 24 h, equilibrated for 24 h, and incubated with DMEM containing 1 mg/ml fatty acid-free bovine serum albumin (DMEM/BSA) and the indicated concentration of apoA-I for 24 h. At the end of the incubation, the medium was removed and cholesteryl ester (CE) in cells and unesterified cholesterol (UC) in cells and media were analyzed for $[^3]$Hcholesterol. Results were expressed as percent of total cell plus medium $[^3]$Hsterol in the medium (A), cell CE (B), and cell UC (C) following subtraction of efflux to medium containing 1 mg/ml BSA alone. Results represent averages ± SD of three separate experiments performed in quadruplicate. Note the increased radiolabel in the Cell CE pool of the epithelioid SMC.

**Data Supplement Figure III.** Impaired apoA-I-mediated cholesterol efflux from human and Sprague-Dawley rat epithelioid arterial SMC. Human arterial smooth muscle cells (HASMC, spindle morphology), human internal thoracic artery SMC (HITA2, epithelioid morphology), and arterial SMC from 3-month-old (SD3M-23, spindle morphology) and 12-day-old (SD12d, epithelioid morphology) Sprague-Dawley rats were labeled with $[^3]$Hcholesterol, cholesterol loaded, incubated with apoA-I, and medium and cellular lipid analyses performed as in Data Supplement Figure I legend. Results represent mean ± SD of quadruplicate determinations and are representative of two separate experiments with similar results. Note the absence of accumulation of radiolabeled CE in the HITA2 human epithelioid SMC.
**Data Supplement Figure IV.** ABCA1 protein levels in spindle and epithelioid human and Sprague-Dawley rat arterial SMC. Human HASMC (spindle) and HITA2 (epithelioid), and Sprague-Dawley rat SD3M-23 (spindle) and SD12d (epithelioid) cells were grown to confluence in DMEM/10% FBS, then incubated with or without 30 µg/ml cholesterol for 24 h and equilibrated in DMEM/BSA for 24 h prior to determination of ABCA1 protein levels. Western blotting of 50 µg of cellular membrane protein was performed using a rabbit polyclonal antibody to ABCA1. Results are representative of two experiments with similar results.

**Data Supplement Table I.** Clinical characteristics of patients with native atherosclerosis used to perform coronary artery intimal and medial ABCA1 and smooth muscle-α actin determinations.

**Data Supplement Figure V.** Absence of enhanced phospholipid efflux from epithelioid SMC following treatment with LXR agonist. SMC grown and labeled with [³H]choline as per Methods were incubated in the absence of 10 µM TO901317 during choline labeling and incubation with apoA-I for 24 h. Epithelioid SMC showed no increase in [³H]phosphatidylcholine efflux with LXR agonist treatment. Efflux from untreated spindle SMC was greater than untreated or treated epithelioid cells at apoA-I levels ≥ 2.5 µg/ml, p < 0.03. Efflux from treated spindle SMC was greater than untreated spindle cells with apoA-I ≥ 2.5 µg/ml, p < 0.03. Mean ± SD of triplicate determinations; representative of two experiments.
Data Supplement Figure VI. Cross-linking of ABCA1 and apoA-I in spindle and epithelioid SMC. Cholesterol-loaded SMC were incubated in the absence or presence of 10 μM TO901317 during equilibration and incubation with apoA-I, followed by cross-linking with the thiol-sensitive, bifunctional cross-linker DSP as described above. ApoA-I crosslinking to ABCA1 increased in spindle SMC, but did not increase or increased only minimally in epithelioid cells with increasing ABCA1 expression, as shown by the anti-apoA-I immunoblot performed in the absence or presence of β-mercaptoethanol (β-ME) to cleave the ABCA1-apoAI bond. Results are representative of three experiments with similar results.

Data Supplement Figure VII. Increased cholesterol efflux from epithelioid SMC treated with ACAT inhibitor Sandoz 58-035. Cells labeled with [3H]cholesterol and cholesterol loaded were incubated in the absence or presence of 2 μg/mL Sandoz 58-035 during the equilibration period and 24 h efflux period using 10 μg/ml apoA-I or 40 μg/ml HDL2. Cholesterol efflux to apoA-I rose following treatment of both cell types with ACAT inhibitor (*, **, p < 0.03 and p < 0.01 respectively), but remained very low from epithelioid SMC. Cholesterol efflux to HDL2 was lower in epithelioid than spindle SMC in the absence of ACAT inhibitor (p < 0.005), but rose to a similar level as spindle SMC following treatment with ACAT inhibitor. Mean ± SD of triplicate determinations; representative of two experiments.
Data Supplement Figure VIII. Cholesterol-oxidase sensitive cellular cholesterol and cholesteryl ester hydrolysis in spindle and epithelioid SMC. A, Cells were grown to ~60% confluence on 35-mm wells and radiolabeled for 24 h by addition of 0.3 µCi/ml $[^3]$Hcholesterol in DMEM containing 10% FBS. At the end of the radiolabeling, cells reached ~90% confluence and were transfected for 24 h with full-length murine ABCA1 cDNA cloned in a pcDNA3.1 vector or with empty vector (Mock) using Lipofectamine 2000. To measure cell-surface cholesterol redistribution, cells were washed once with PBS and then incubated with 1 unit/ml cholesterol oxidase (Calbiochem) in DMEM at 37 °C for 10 min. Cells were then washed twice with PBS; cellular lipids were extracted, and $[^3]$Hcholestenone was measured after isolation by TLC. Cholestanone as a fraction of cholestanone plus cell unesterified cholesterol, both normalized to cell protein, was 12.21 ± 1.03% and 14.21 ± 0.40% for spindle SMC, and 12.09 ± 1.04% and 16.13 ± 0.17% for epithelioid SMC, pre- and post-infection respectively. Results are representative of two experiments performed in triplicate with similar results. To measure ABCA1 expression levels, homogenates from cells grown and transfected under the same conditions but without $[^3]$Hcholesterol were assessed for ABCA1 protein levels by Western blotting as indicated above. B, Cells grown to confluence in DMEM containing 10% FBS were rinsed twice with PBS/BSA and incubated with DMEM containing 30 µg/ml cholesterol, 9 µM $[^14]$Coleate, and 5 mg/ml BSA for 24 h. Cells were then rinsed twice with PBS/BSA, equilibrated for 24 h in the same medium without 30 µg/ml cholesterol, rinsed three times with PBS/BSA, and incubated in DMEM/BSA containing 2 µg/ml of acyl CoA:cholesterol acyltransferase (ACAT) inhibitor Sandoz 58-035 (a generous gift from Sandoz Pharmaceuticlals) with or without 10 µg/ml apoA-I up
to 48 h. After the indicated intervals, media were removed and cell layers were rinsed twice with iced PBS/BSA and twice with iced PBS, and cellular lipids were extracted and analyzed for residual cholesteryl $[^{14}\text{C}]$oleate. Results are representative of two experiments with similar results.
Data Supplement Figure I

Spindle Epithelioid


Spindle Epithelioid

*
Data Supplement Figure II
A. Medium

B. Cell CE

C. Cell UC

Data Supplement Figure III

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Data Supplement Figure IV
Data Supplement Table I. Clinical characteristics of patients.

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Data Supplement Figure V

ApoA-I (μg/mL)

[3H]Phosphatidylcholine Efflux (%)

- Spindle
- Spindle + TO901317
- Epithelioid
- Epithelioid + TO901317
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Data Supplement Figure VII

The diagram shows the percentage of total [3H]sterol in different cell types and conditions. The x-axis represents different treatments: ApoA-I 10 μg/mL and HDL 40 μg/mL. The y-axis represents the percentage of total [3H]sterol. The treatment groups include Spindle, Spindle + 58035, Epithelioid, and Epithelioid + 58035.

Significance levels are indicated with asterisks: * for p < 0.05, ** for p < 0.01, and *** for p < 0.001.
Spindle Epithelioid
ABCA1 protein

Mock Mock ABCA1 ABCA1

$[^3]H$Cholestenone
(DPMX10^-3/mg cell protein)

0 20 40 60 80

Mock transfection
ABCA1 transfection

* * $^p<0.05$

ABCA1 protein

Epithelioid (- ApoA-I)
Epithelioid (+ ApoA-I)
Spindle (- ApoA-I)
Spindle (+ ApoA-I)

pmol $[^14]C$oleate/mg cell protein

0 500 1000 1500 2000 2500

0 10 20 30 40 50

Data Supplement Figure VIII

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