Apolipoprotein AI Efficiently Binds to and Mediates Cholesterol and Phospholipid Efflux from Human but Not Rat Aortic Smooth Muscle Cells†

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Received July 27, 1999; Revised Manuscript Received September 24, 1999

ABSTRACT: Aortic smooth muscle cells (SMC) from several animal species have been reported to resist depletion of cellular cholesterol by the major apolipoprotein of HDL, apoAI. Resistance of SMC to this protective action of apoAI, if present in humans, could contribute to the overaccumulation of arterial wall cholesterol seen in atherosclerosis. We investigated the ability of human aortic medial SMC to bind and be depleted of cholesterol and phospholipids by apoAI. In contrast to rat aortic SMC, but similar to human fibroblasts, human SMC were readily depleted of cholesterol by apoAI, measured by a marked depletion of intracellular cholesterol available for esterification, and an increase in cholesterol efflux to the medium. Human SMC were also actively depleted of the phospholipids phosphatidylcholine and sphingomyelin by apoAI. In contrast, rat SMC released only a small fraction of these cellular phospholipids to apoAI-containing medium. ^{125}I-labeled apoAI bound with high affinity and specificity to human SMC, but failed to bind to rat SMC. Similar levels of expression of class B, type I scavenger receptor (SR-BI) and caveolin in human and rat SMC suggested these proteins do not account for the differences in apoAI binding or lipid efflux seen in these cells. An enhancer of apolipoprotein-mediated cholesterol efflux, tyrosyl radical-oxidized HDL, markedly amplified the depletion of cholesterol available for esterification in human SMC compared to HDL, but had no enhanced effect in rat SMC. These results show that human SMC bind and are readily depleted of cellular lipids by apoAI, and suggest that apoAI-mediated cholesterol efflux from arterial SMC may contribute significantly to the circulating pool of HDL cholesterol in vivo. The marked difference in apoAI binding to human and rat arterial SMC provides an excellent model to study the nature of the apoAI−cell binding interaction.

High-density lipoproteins (HDL)[1] are believed to protect against atherosclerosis in part by stimulating the removal of excess cholesterol from cells including macrophages and smooth muscle cells (SMC) in the artery wall (*1, 2*). HDL may promote this removal by acting as a passive acceptor of cholesterol moving down a concentration gradient from the cell surface to the HDL surface (*3*). HDL proteins, in particular apolipoprotein AI (apoAI), also appear to promote cholesterol removal from peripheral cells actively, through direct interactions with proteins or lipid domains on the cell surface (*4*−*7*). Amphipathic α-helical apolipoproteins such as apoAI, apparently working through these receptors, actively promote the depletion of the regulatory pool of cholesterol available for esterification by acyl-CoA:cholesterol acyltransferase (ACAT-accessible cholesterol) (*8*−*10*). In addition, lipid-free apolipoproteins can deplete plasma membrane cholesterol and phospholipids by a process referred to as "membrane microsolubilization" (*7, 11*).

The importance of lipid-free or lipid-poor apolipoproteins in HDL-mediated cellular lipid efflux is strongly supported by observations made in Tangier disease. Individuals with this disorder synthesize apoAI normally, but their nascent HDL are cleared rapidly from plasma and they have extremely low levels of HDL and apoAI [reviewed in (*12*)]. Cultured fibroblasts from patients with Tangier disease are not depleted of ACAT-accessible cholesterol by apoAI, and fail to release both cholesterol and phospholipids to medium containing apoAI (*10, 13*). Tangier fibroblasts do release cholesterol passively to HDL and phospholipid vesicles (*10, 13, 14*). The recent discovery that the ATP-binding cassette transporter protein 1 (*ABC1*) is mutated in both Tangier disease (*15*−*17*) and familial hypoalphalipoproteinemia (FHA) (*15*) suggests a likely role of this protein in the delivery of cellular lipids to apoAI. The previous finding of normal (*13*) or only slightly diminished (*10*) binding of apoAI to Tangier disease and FHA fibroblasts (*18*) suggests that *ABC1* may not be the initial binding protein for apoAI on the cell surface. It is possible that apoAI might still bind to lipid efflux-defective *ABC1*. The extremely low levels of HDL in these patients, however, suggest that the apoAI−cell interaction mediating lipid efflux is one, or possibly *the* most, important determinant of circulating HDL levels.

† This work was supported by the Heart and Stroke Foundation of Alberta and N.W.T. G.A.F. is a Scholar of the Medical Research Council of Canada, and Clinical Investigator of the Alberta Heritage Foundation for Medical Research.

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[1] Abbreviations: HDL, high-density lipoprotein(s); apo, apolipoprotein; ACAT, acyl-CoA:cholesterol acyltransferase; SMC, smooth muscle cells; LDL, low-density lipoprotein(s); DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; BSA, essentially fatty acid free bovine serum albumin; SR-BI/SR-BII, class B, type I or type II scavenger receptors.

10.1021/bi991742b CCC: $18.00
Published on Web 11/11/1999

The importance of apoAI-mediated cellular lipid efflux in these studies using fibroblasts suggests the interaction of apoAI with other cell types in the artery wall would have significant impact on the atherosclerotic process, and the formation of HDL particles. Medial smooth muscle cells comprise the major cell type in the normal artery wall, and undergo migration to the intima, proliferation, and accumulation of lipids as the atherosclerotic lesion develops (*19*). Previous reports have indicated that aortic smooth muscle cells derived from a number of animal species (cow, rat, monkey, rabbit, and pig) are resistant to HDL- (*20*) and free apoAI-mediated cellular lipid efflux (*21*−*23*). This has led to a perception in the literature that smooth muscle cells in humans may also be resistant to apoAI-mediated lipid efflux (*7, 23*−*26*), and that this might explain the accumulation of excess cholesterol in these cells. Most studies of apoAI effects on animal aortic SMC have not correlated apoAI binding with lipid efflux, and no studies have correlated binding of human apoAI with lipid efflux from human aortic SMC.

In this study we have compared the ability of human and rat arterial SMC to bind and be depleted of cellular lipids by lipid-free human apoAI. Potential markers that might explain differences in the response of these cell types to apoAI have been explored, and the ability of tyrosyl radical-oxidized HDL, an enhancer of apolipoprotein-dependent cholesterol efflux (*27*−*29*), to deplete cholesterol from these cells was determined. Our results show that human medial aortic SMC bind and are readily depleted of cholesterol and phospholipids by apoAI.

EXPERIMENTAL PROCEDURES

Materials. Cholesterol, L-tyrosine, hydrogen peroxide (30%, ACS grade), diethylenetriaminepentaacetic acid (free acid form), and essentially fatty acid free bovine serum albumin (BSA) were from Sigma. [1-^{14}C]Oleate (55 mCi/mmol), [*methyl*-^{3}H]choline chloride (81 Ci/mmol), [1,2-^{3}H]-cholesterol (51 Ci/mmol), and ^{125}I (1968 Ci/mmol) were purchased from Amersham Pharmacia Biotech. Tissue culture medium was purchased from Bio-Whittaker, and fetal bovine serum was from Hyclone.

Lipoproteins and Apolipoproteins. HDL_3 ($d = 1.125-1.21$ g/mL, hereafter referred to as HDL) and LDL ($d = 1.019-1.063$ g/mL) were isolated by density gradient ultracentrifugation from pooled plasma of healthy male volunteers (*30*). HDL was subjected to heparin−agarose affinity chromatography to remove apoE- and apoB-containing particles (*31*). Tyrosyl radical-oxidized HDL was generated as described (*27*). The whole protein fraction of HDL was obtained by extraction of HDL with ether/acetone (1:3, v/v) and purified apoAI obtained using DEAE cellulose chromatography as described (*32*). Lipid-free rat apoAI was a gift from Dr. Norman Wong. ApoAI was iodinated using the iodine monochloride method (*33*). Protein content of HDL and free apoAI was determined by the method of Bradford (*34*) using BSA as standard.

Cell Culture. Human skin fibroblasts were obtained by skin biopsy from a normal subject. Human SMC line 1, from the arterial medial layer of adult human thoracic aorta, was a gift from Dr. John Oram, University of Washington, Seattle. Human SMC line 2 was from the American Tissue Culture Co. (ATCC, Rockville, MD) and was obtained from the abdominal aorta of an 11-month-old child. Rat arterial SMC line 1 was from the thoracic aorta of a 2-week-old Sprague-Dawley rat (*11*, a gift from Dr. Shinji Yokoyama, Nagoya City University), and line 2 was from ATCC and obtained from the thoracic aorta of a rat embryo. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum as described (*35*). Cells for experiments were plated at 15 000 cells/16 mm well or 50 000 cells/35 mm well and grown at 37 °C and 5% CO_2 to confluence (about 6 days for most cell lines). To cholesterol-load the cells, confluent cultures were washed twice with PBS containing 2 mg/mL BSA (PBS−BSA) and incubated for 24 h in DMEM containing 2 mg/mL BSA and 30 μg/mL cholesterol (added from a 10 mg/mL solution in ethanol). In some experiments, cholesterol enrichment was performed using cells grown from 60% to full confluence in DMEM/10% lipoprotein-deficient serum, rinsed twice with PBS−BSA, and then incubated with DMEM containing 1 mg/mL BSA and 100 μg/mL LDL for 24 h. To allow equilibration of added cholesterol, cell layers were rinsed twice with PBS−BSA and incubated for a further 24 h in DMEM containing 1 mg/mL BSA (DMEM−BSA).

Labeling of Cellular Cholesterol and Phospholipids. In the indicated experiments, rapidly growing cells were labeled during the last 40% of growth to confluence by addition of 0.2 μCi/mL [^{3}H]cholesterol as described (*28*). Cell layers were then rinsed twice with PBS−BSA prior to cholesterol-loading and equilibration as indicated above. Cellular phospholipids were labeled in cholesterol-loaded cells by addition of 1.0 μCi/mL [^{3}H]choline chloride to the DMEM−BSA medium during the 24 h equilibration incubations (*9*). Cells were washed 5 times with PBS−BSA before efflux incubations.

Cholesterol and Phospholipid Efflux. After the desired labeling protocol, cells were incubated in DMEM−BSA and the indicated concentration of apoAI or HDL. At the end of the incubation period, cell layers were rinsed twice with ice-cold PBS−BSA and twice with PBS. Cells were stored at −20 °C until extraction for lipid and protein content. Efflux media were collected and centrifuged (10 000 rpm for 10 min) to remove cell debris. The medium was then either counted directly (for cells labeled with [^{3}H]cholesterol) or extracted by the method of Folch et al. (for determination of medium phospholipids) (*36*). Cellular lipids were extracted, separated by thin-layer chromatography, and assayed for radioactivity as previously described (*28*). Cell proteins were determined by the method of Lowry et al. using BSA as standard (*37*).

Cholesterol Esterification Assay. To assess the ability of free apoAI or HDL to deplete cellular free cholesterol available for esterification, cholesterol-loaded cells were incubated for 16 h in DMEM−BSA and the indicated additions, washed once with PBS, and incubated for 1 h at 37 °C with DMEM containing 9 μM [^{14}C]oleate bound to 3 μM BSA (*8*). Cells were chilled on ice, rinsed twice with ice-cold PBS−BSA and twice with PBS, and stored at −20 °C until extraction. Cell lipids were extracted and separated by thin-layer chromatography, and the cholesterol ester spot was obtained for determination of radioactivity as described (*29*).

Cellular Binding of ApoAI. Total and specific (reversible) binding of apoAI to cells was determined as described (*38*). Cholesterol-loaded cells in 35 mm wells were incubated for

2 h at 0 °C in DMEM/BSA containing 25 mM HEPES and the indicated concentration of ^{125}I-apoAI. Cells were then washed 3 times with ice-cold PBS/BSA, and further incubated with DMEM/BSA (to assess total binding) or DMEM/BSA containing 50 μg/mL nonradiolabeled apoAI (to assess nonspecific binding) for 3 h at 0 °C. Cells were then rinsed 5 times with ice-cold PBS/BSA and twice with ice-cold PBS. Cell layers were dissolved in 0.1 N NaOH, and aliquots were taken for quantitation of radioactivity and protein.

Gel Electrophoresis and Immunoblotting. Cell membranes from human fibroblasts, human and rat SMC, primary rat sympathetic neurons (a gift from Dr. Jean Vance), rat liver, and Jurkat and BW5147 lymphocytes (a gift from Dr. Hanne Ostergaard) were obtained by sonication of isolated cells in 1 mL of extraction buffer (50 mM Tris-HCl, 500 ng/mL aprotinin, 1 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, 2 mM ethylene glycol tetraacetic acid, pH 7.4) for 12 s. The whole cell extract was centrifuged at 700 rpm at 4 °C for 10 min, and the supernatant was spun at 12 000 rpm at 4 °C for 20 min to obtain the membrane pellet. Isolated membranes were separated by SDS−PAGE using a 7% polyacrylamide gel under nonreducing conditions (*39*), and transferred to nitrocellulose for immunoblotting with anti-scavenger receptor class B type I or type II polyclonal antibody (Novus Biologicals) or anti-caveolin polyclonal antibody (Transduction Laboratories) as described (*40*). Bands were visualized using enzyme-linked chemiluminescence (Amersham).

Statistics. Results are expressed as the mean ± standard deviation. Differences between groups were calculated using ANOVA. Pairwise comparison was then conducted with a post-hoc analysis using Tukey's test.

RESULTS

Cholesterol Efflux to ApoAI from Human Aortic SMC. The efficient depletion of cholesteryl esters from cholesterol-loaded cells by HDL has been shown to depend on a competent apolipoprotein−cell interaction (*8, 10*). To determine the ability of apoAI to productively interact with human aortic SMC, we first assessed the ability of lipid-free apoAI to deplete the cholesterol substrate pool available for esterification by ACAT in two strains of these cells, compared to human skin fibroblasts and two strains of rat aortic SMC. Consistent with previous findings using a single strain of rat SMC (*41*), a 16 h incubation with apoAI depleted only a small percentage of cellular cholesterol available for esterification during a 1 h chase incubation with [^{14}C]oleate in the two rat SMC lines tested (Figure 1). In sharp contrast, both strains of human aortic SMC were depleted of 80% of this ACAT-accessible cholesterol pool by apoAI in the same interval, an extent similar to that seen with a human skin fibroblast cell line. Similar results were seen using cells enriched with cholesterol by incubation with LDL (data not shown). These results suggested the absence of any impairment in apoAI-mediated depletion of cholesterol available for esterification in the human arterial SMC lines. Lipid-free rat apoAI also failed to deplete the rat SMC of ACAT-accessible cholesterol (data not shown), suggesting that resistance of the rat cells was not due to structural differences between human and rat apoAI. Due to the consistency of results between strains of the two cell lines, subsequent experiments were done using one strain each of the human and rat SMC lines.

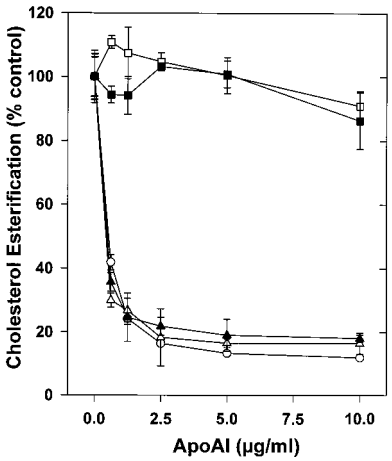

FIGURE 1: ApoAI-mediated depletion of cellular cholesterol available for esterification in human and rat arterial smooth muscle cells. Cells loaded with nonlipoprotein cholesterol were incubated with DMEM/BSA plus the indicated concentration of apoAI for 16 h. Cells were then washed and incubated for 1 h with [^{14}C]oleate, and cellular cholesteryl [^{14}C]oleate formed was measured as described under Experimental Procedures. (○) Human skin fibroblasts; (△) human arterial SMC line 1 (HASMC1); (▲) human arterial SMC line 2 (HASMC2); (□) rat arterial SMC line 1 (RASMC1); (■) rat arterial SMC line 2 (RASMC2). Results are the mean ± SD of quadruplicate determinations, expressed as percentage of picomoles of [^{14}C]oleate incorporated into cholesteryl ester per milligram of cell protein per hour in cells treated with DMEM/BSA alone (control), and are representative of three separate experiments. Error bars not shown are within the symbol dimensions. Results for both human SMC and fibroblast cell lines were significantly greater than both rat SMC lines at all concentrations of apoAI tested ($p < 0.001$).

Cholesterol efflux to apoAI was also determined using human and rat arterial SMC labeled with [^{3}H]cholesterol during growth. Cells were then incubated with lipid-free apoAI to assess cholesterol efflux to the medium. Over a 24 h incubation, the human SMC line released approximately 18% of total (cellular plus medium) [^{3}H]cholesterol to medium containing 10 μg/mL apoAI, as compared to 12% for human skin fibroblasts (Figure 2). The rat SMC released only 3.25% of cellular [^{3}H]cholesterol to medium containing this concentration of apoAI. The percent efflux to control medium containing 1 mg/mL BSA alone was 1.5% from these cells, similar to that seen for the other cell types. Similar levels of cholesterol efflux were obtained using cells in which the plasma membrane pool of cholesterol was specifically labeled with a brief (2 h) incubation with [^{3}H]cholesterol. In the cells labeled with [^{3}H]cholesterol during growth, apoAI depleted cholesterol from both the cellular esterified and free cholesterol pools in human smooth muscle cells and fibroblasts, whereas the small amount of cholesterol efflux to apoAI from rat smooth muscle cells was mainly from the cellular free cholesterol pool (data not shown). These results confirmed the ability of apoAI to readily deplete cellular cholesterol from human arterial SMC.

Phospholipid Efflux to ApoAI from Human and Rat Aortic SMC. The ability of apoAI to accept cellular cholesterol requires a competent acceptor surface, in the form of apoAI complexed with at least a small amount of phospholipid (*2, 4, 42*). The striking difference in the capacity of apoAI to

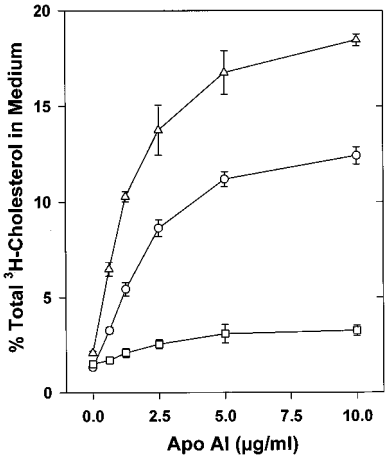

FIGURE 2: Cholesterol efflux to apoAI-containing medium from human and rat arterial smooth muscle cells. Cells were grown to confluence in medium containing [^{3}H]cholesterol, cholesterol-loaded, and incubated with DMEM/BSA containing the indicated concentration of apoAI. Following a 24 h incubation, the efflux medium was collected and centrifuged at 10 000 rpm for 10 min, and radiolabeled cholesterol in the supernatant was determined by scintillation counting. Results are expressed as the percentage of total medium and cellular [^{3}H]cholesterol released into the medium. Values are the mean ± SD of four determinations, representative of three experiments. (○) Human skin fibroblasts; (△) HASMC1; (□) RASMC1. Results for human SMC and fibroblasts are significantly greater than those for rat SMC at all concentrations of apoAI ($p < 0.001$).

remove cholesterol from human and rat SMC suggested that phospholipid efflux to apoAI was competent from the human cells but impaired from the rat cells. To test the ability of apoAI to remove cellular phospholipids from human and rat SMC lines, cholesterol-loaded cells labeled with [^{3}H]choline were incubated with apoAI for 24 h, and the percent of labeled phosphatidylcholine and sphingomyelin effluxed to the medium was determined. As shown in Figure 3, human SMC released a high percentage of both phospholipids to medium containing free apoAI during this interval. Phosphatidyl[^{3}H]choline efflux to apoAI from human fibroblasts was similar to that seen from the human SMC; [^{3}H]sphingomyelin release was lower in the human fibroblasts than SMC but still significant (15%). In sharp contrast, 10 μg/mL apoAI released only 2.9% more phosphatidyl[^{3}H]choline than control medium containing BSA alone from rat SMC; release of [^{3}H]sphingomyelin by apoAI from these cells was absent.

Binding of ApoAI to Human and Rat SMC. The marked differences in apoAI-mediated efflux of cholesterol and phospholipids from human and rat arterial SMC suggested that the initial apoAI−cell interaction is competent in the human but impaired in the rat cells. To assess the ability of these cells to bind apoAI, cholesterol-loaded cells were incubated with iodinated apoAI, followed by chase incubation with DMEM/BSA to measure total binding, or excess unlabeled apoAI to displace reversibly (specifically) bound ^{125}I-apoAI. Total and specific binding of apoAI revealed high-affinity, partially saturable binding for both human SMC and fibroblasts (Figure 4). Total and specific binding of apoAI to rat SMC was almost completely absent. Incubations with higher concentrations of ^{125}I-apoAI did not increase the apoAI bound per milligram of cell protein to the rat SMC. Binding studies with the other human and rat SMC lines showed similar results (data not shown). Our results show that the apoAI interaction with cell surface binding sites is intact in human arterial SMC, but negligible or absent in these rat SMC. These findings suggest that binding of apoAI to SMC is necessary for effective efflux of cellular lipids to apoAI.

Expression of SR-BI and Caveolin by Human and Rat SMC. Recent reports suggest that, in addition to mediating the specific uptake of HDL cholesteryl esters (*43*), class B, type I scavenger receptors (SR-BI) may play a role in cholesterol efflux from cells to HDL (*44*). Caveolin is a cellular protein believed to deliver intracellular cholesterol to cholesterol-rich domains of the plasma membrane (caveolae) (*45*, *46*), where it can be offloaded to HDL (*47*). To investigate whether differences in apoAI-mediated lipid efflux from human and rat SMC might be due to differences in the levels of expression of these proteins, we examined isolated cell membranes of these cells for the presence of SR-BI and caveolin. We did not find detectable levels of SR-BI in human or rat SMC, nor in normal human skin fibroblasts (Figure 5A). SR-BII was also not detected in these cell lines (data not shown). SR-BI is not detectable in cultured rat sympathetic neurons (negative control) (E. Posse de Chaves and J. Vance, personal communication), whereas murine liver expresses high levels of SR-B1 (*43*). An antibody detecting caveolin isoforms 1−3 revealed the presence of caveolin in both human and rat SMC, and in human fibroblasts as previously reported (*47*) (Figure 5B). Jurkat and BW5147 lymphocytes, known to lack caveolin and caveolae (*48*, *49*), were negative controls. These results suggest that the differences in apoAI-mediated lipid efflux between human and rat SMC are not explained by variable expression of SR-BI, SR-BII, or caveolin.

Depletion of SMC of ACAT-Accessible Cholesterol by Tyrosylated HDL. Previous results from our laboratory showed that HDL oxidized by peroxidase-generated tyrosyl radical (tyrosylated HDL) markedly increases cellular cholesterol efflux by enhancing the delivery of ACAT-accessible cholesterol to efflux-available sites in the plasma membrane (*28*). This effect is due to modification of HDL apolipoproteins, specifically formation of apoAI−apoAII heterodimers (*29*), and is not associated with an increased ability of tyrosylated HDL to passively accept plasma membrane cholesterol or phospholipids (*28*). The ability of human SMC to efficiently release lipids to apoAI suggested this effect would be enhanced by tyrosylated HDL. Figure 6A shows that human SMC, as previously shown for human fibroblasts and mouse macrophages (*27*), had a marked decrease in the pool of cholesterol available for esterification following incubation with tyrosylated HDL, compared to cells incubated with HDL. Consistent with results obtained with free apoAI in rat SMC (Figure 1), tyrosylated HDL and control HDL had a similar and diminished capacity to deplete these cells of cholesterol available for esterification (Figure 6B). The level of depletion of ACAT-accessible cholesterol in rat SMC was similar to that seen by passive (non-apolipoprotein-dependent) desorption in normal fibroblasts incubated with trypsin-treated HDL (*8*), or Tangier disease fibroblasts incubated with intact HDL (*10*). These results further support the presence of a competent apolipoprotein−cell interaction in human but not rat arterial SMC.

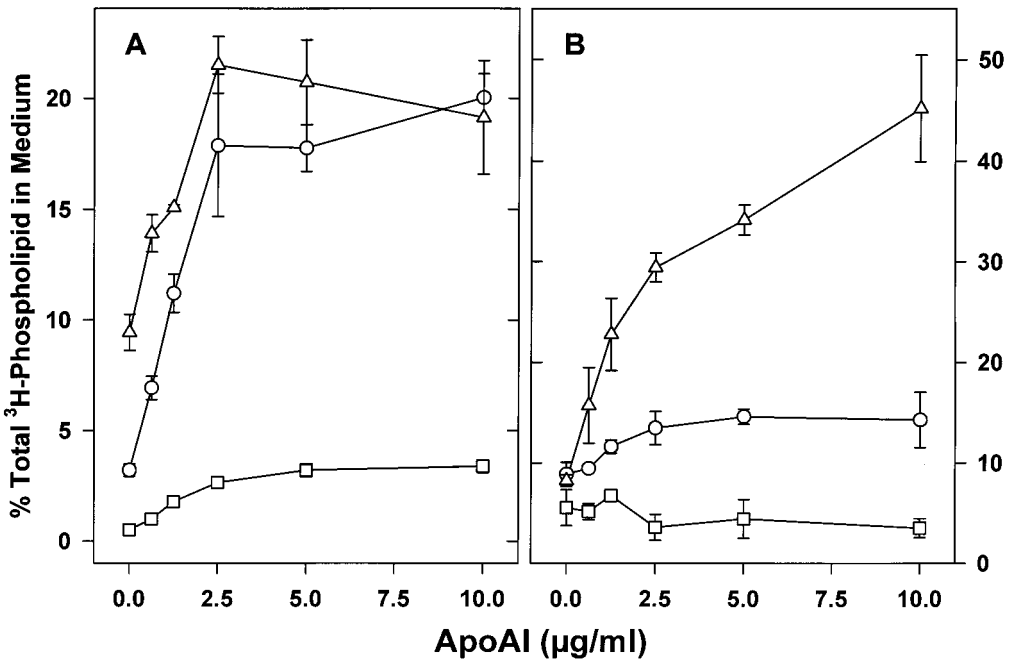

FIGURE 3: Phospholipid efflux to apoAI-containing medium from human and rat arterial smooth muscle cells. Cells were incubated with [^{3}H]choline during the 24 h equilibration period following cholesterol loading, washed extensively, and incubated for 24 h with DMEM/BSA containing the indicated concentration of apoAI. Medium and cellular phosphatidyl[^{3}H]choline (A) and [^{3}H]sphingomyelin (B) were measured as described under Experimental Procedures. Values are the mean ± SD of four determinations, expressed as the percentage of total cellular plus medium radiolabeled phospholipid appearing in the medium, and are representative of two experiments. (○) Human skin fibroblasts; (△) HASMC1; (□) RASMC1. Efflux of both phospholipids to apoAI was significantly lower from rat SMC than either of the human cell lines at all concentrations ($p < 0.001$).

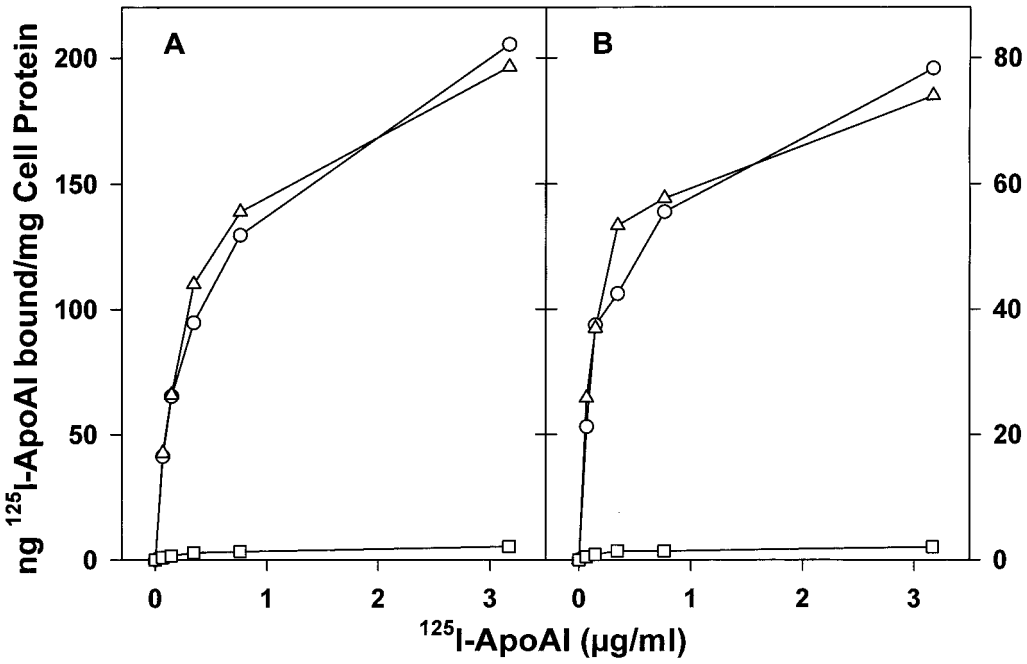

FIGURE 4: Binding of ^{125}I-apoAI to human and rat arterial smooth muscle cells. Cholesterol-loaded cells were incubated with DMEM/BSA plus the indicated concentration of radiolabeled apoAI for 2 h at 0 °C. After extensive washing, cells were incubated a further 3 h at 0 °C in DMEM/BSA alone (A) or with 50 μg/mL unlabeled apoAI (B) to displace reversibly bound ^{125}I-apoAI. Reversible (specific) binding (B) was estimated by subtracting nondisplaceable (nonspecific) binding from the total binding (A), using the data in panel A. Values are the mean of duplicate determinations, representative of three experiments. (○) Human skin fibroblasts; (△) HASMC1; (□) RASMC1.

DISCUSSION

In this study, we have shown that human arterial smooth muscle cells, in contrast to smooth muscle cells obtained from the aortas of numerous animal species, exhibit efficient cellular lipid efflux to human apoAI. Human SMC bind apoAI and release cellular cholesterol and phospholipids to an extent similar to that seen with normal human skin fibroblasts, whereas rat SMC released very low levels of cholesterol and phospholipids to both human and rat free apoAI.

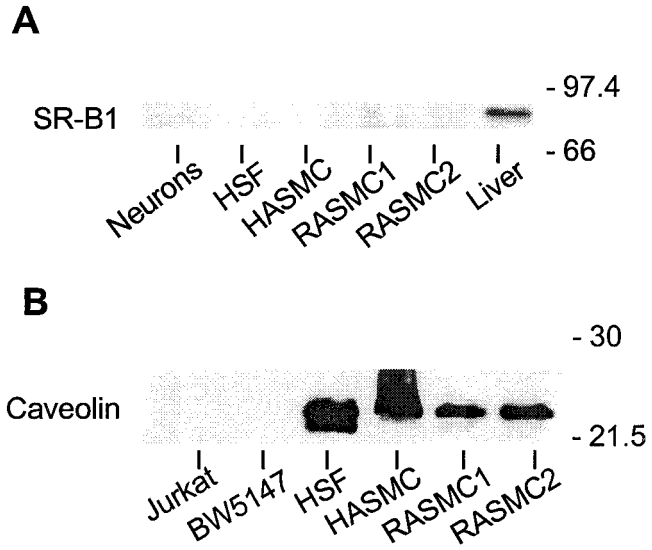

FIGURE 5: Expression of SR-BI and caveolin by human and rat aortic smooth muscle cells. Representative immunoblots for the indicated cell lines. 50 μg of isolated membranes from each cell type was resolved by SDS−PAGE before transfer to nitrocellulose and immunoblotting for SR-BI (A) or caveolin (B) as described under Experimental Procedures. Neurons, primary rat sympathetic neurons (negative control for SR-BI; see text); HSF, human skin fibroblasts; HASMC/RASMC, human and rat aortic smooth muscle cells, respectively; Liver, rat liver (positive control for SR-BI); Jurkat and BW5147, T-lymphocyte cell lines (negative controls for caveolin). Molecular mass markers (kDa) are indicated on the right.

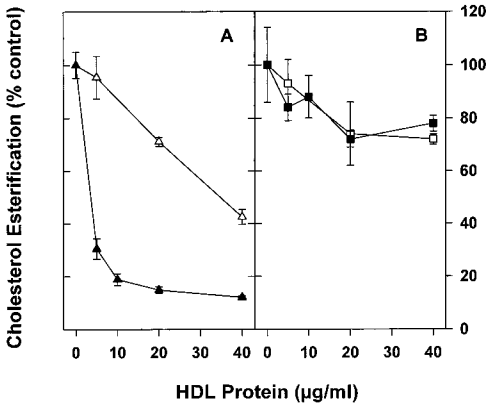

FIGURE 6: HDL- and tyrosylated HDL-mediated depletion of cellular cholesterol available for esterification in human and rat arterial smooth muscle cells. Cholesterol-loaded human (A) and rat (B) SMC were incubated with DMEM/BSA (control) plus the indicated concentration of HDL (open symbols) or tyrosyl radical-oxidized HDL (closed symbols) for 16 h, and then incubated with [^{14}C]oleate for 1 h as in Figure 1 to measure incorporation into cholesteryl [^{14}C]esters. Results are expressed as in Figure 1 and are the mean ± SD of four determinations, representative of two experiments. (△) HASMC1; (□) RASMC1. Results for tyrosylated HDL were significantly greater than HDL in the human SMC at all concentrations ($p < 0.001$).

The level of binding of apoAI to rat SMC was negligible, likely accounting for the inability of these cells to release significant amounts of cellular lipids to free apoAI.

Consistent with an intact apolipoprotein−cell interaction in human SMC, tyrosylated HDL markedly enhanced the depletion of ACAT-accessible cholesterol from these cells. Conversely, the level of depletion of ACAT-accessible cholesterol from rat SMC by both HDL and tyrosylated HDL was markedly diminished, and similar to levels seen in normal human fibroblasts incubated with trypsinized HDL particles (*8*), or in Tangier disease fibroblasts incubated with intact HDL particles (*10*). These results complement previous findings in rat SMC, that while nonspecific diffusional efflux of cellular lipids to HDL is intact, apolipoprotein-mediated cellular cholesterol efflux is absent (*21*, *41*).

Previous reports describing impaired cellular lipid efflux from arterial smooth muscle cells suggested different potential reasons for this defect. Savion and Kotev-Emeth concluded that impaired cholesterol efflux from bovine arterial SMC compared to bovine vascular endothelial cells was likely due to diminished levels of HDL binding to SMC (*20*). Yokoyama and colleagues concluded that diminished apoAI-mediated cholesterol efflux from rat SMC (*21*, *22*) was due to a defect in a protein kinase C-mediated signaling cascade necessary for translocation of ACAT-accessible cholesterol to the cell surface (*41*, *50*). The appearance of some cellular phospholipid in apoAI medium in their experiments was interpreted to indicate the presence of competent apoAI binding, but direct measurements of apoAI binding to rat SMC and percent of total cell phospholipid effluxed were not reported. Our results suggest that a defect in binding of apoAI to the rat SMC surface is the primary defect, with the lack of downstream activation of PKC to signal intracellular cholesterol efflux being a secondary defect. The low but measurable levels of phosphatidylcholine and cholesterol efflux to free apoAI in the absence of competent binding to rat SMC in our experiments may be due to receptor-independent microsolubilization of membrane lipids by apoAI (*7*), or possibly passive desorption of a small amount of cell surface phospholipid and cholesterol to apoAI. The level of phospholipid efflux in particular, however, was insufficient to generate effective acceptor particles or significant amounts of cholesterol efflux in the absence of a productive apoAI−cell surface binding interaction.

Levels of phospholipid efflux like those seen to rat SMC in the present study were also reported in probucol-treated macrophages, where probucol induced a similar defect in apoAI binding and apoAI-mediated depletion of the ACAT substrate pool in mouse peritoneal macrophages (*38*). The primary defect induced by probucol in that study was concluded to be a block in apoAI binding to the cell surface, consistent with our findings in rat SMC. Another study using probucol, however, found that this agent inhibited apoAI-mediated efflux prior to demonstrable changes in apoAI binding. These results suggest probucol may alter either the pool of lipids available for efflux to apoAI or the lipid environment necessary for proper function of the putative apoAI receptor (*26*).

The reason apoAI fails to deplete rat and other animal arterial SMC of cellular lipids but efficiently depletes human SMC lipids is unclear. One possibility is that the response to apoAI is dependent on the stage of cell differentiation (*51*). In our study, we obtained similar responses to apoAI using human SMC obtained from the thoracic aorta of an adult and abdominal aorta of an 11-month-old child. The rat SMC, obtained from the thoracic aortas of a 2-week-old rat and rat embryo, both failed to bind and release significant amounts of lipids to apoAI. The cells used in our studies were all derived from the arterial medial layer, and appeared homogeneous in culture. The possibility that rat SMC from

the same (or different) anatomic site but older animals might respond differently to apoAI could be tested. It is also possible that alternate SMC phenotypes found in human atherosclerotic lesions might be resistant to apoAI-mediated cholesterol efflux, and this will also need to be assessed. Interspecies differences in apoAI are a potential but unlikely explanation for the different responses we found between human and rat SMC. Human apoAI readily depletes rat and mouse macrophages of excess cholesterol (*50, 52*), suggesting that the absence of a response to apoAI by rat SMC was not due to the use of human apoAI in our studies. The rat SMC lines used in our studies also failed to mobilize ACAT-accessible cholesterol in response to rat apoAI.

Regardless of the stage of differentiation of the rat and human SMC used here, the difference in their responses to apoAI represents an excellent opportunity to study the initial apoAI−cell interaction. The nature of the plasma membrane receptor or lipid domain responsible for the differential response to apoAI in these cell lines is not yet known. We found a similar absence of expression of SR-BI and SR-BII in our human and rat cell lines, suggesting this protein could not explain the differences in apoAI-mediated lipid efflux seen. Both apoAI-responsive and nonresponsive SMC used in this study expressed the proposed cholesterol transport protein caveolin, suggesting defective delivery of intracellular cholesterol to apoAI-accessible plasma membrane sites also did not explain these differences. We did not assess our cells for morphologic caveolae; however, no cell line expressing caveolin has yet been found that lacks cell surface caveolae (E. Smart, personal communication). The presence of caveolin on the cytoplasmic surface of the plasma membrane and the lack of a transmembrane domain (*53*) suggest this protein is an unlikely candidate as a direct apoAI receptor. The presence of normal or only slightly diminished binding of apoAI to fibroblasts from patients with Tangier disease and familial hypoalphalipoproteinemia (*10, 13, 18*) suggests that ATP-binding cassette transporter 1 (*ABC1*) mutations in these conditions (*15*−*17*) do not explain the differences we observed in the initial binding and lipid efflux to apoAI by human and rat SMC. The ability of even lipid efflux-defective *ABC1* to retain an ability to act as an apoAI receptor, however, will need to be tested.

The current results suggest that apoAI-mediated lipid efflux from human medial SMC is competent, and can be enhanced by a modified form of HDL that increases cholesterol efflux due to modifications in its apolipoprotein structure. The potential for SMC in normal and atherosclerotic human arteries to be efficiently depleted of lipids by apoAI and HDL in the first stages of reverse cholesterol transport would therefore seem likely. The accumulation of excess cholesterol in arterial SMC and macrophages in human atheromata are believed to be formed by similar mechanisms, i.e., through uptake of oxidized or aggregated LDL particles by scavenger receptors or the α2-macroglobulin receptor/LDL receptor-related protein (alpha 2 MR/LRP) on the cell surface (*54*−*56*). The differences in apoAI binding in human and rat aortic SMC, and the critical importance of this binding to the efficient depletion of cellular lipids, suggest an important new model to study the apoAI receptor or binding domain in the plasma membrane responsible for this interaction.

ACKNOWLEDGMENT

We thank Doug Watters and Ryan Perry for excellent technical assistance, and Drs. Robert Ryan and Luis Agellon for critical review of the manuscript.

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BI991742B