Synthetic Amphipathic Helical Peptides That Mimic Apolipoprotein A-I in Clearing Cellular Cholesterol

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Abstract

Clearance of excess cholesterol from cells by HDL is facilitated by the interaction of HDL apolipoproteins with cell-surface binding sites or receptors, a process that may be important in preventing atherosclerosis. In this study, synthetic peptides containing 18-mer amphipathic helices of the class found in HDL apolipoproteins (class A) were tested for their abilities to remove cholesterol and phospholipid from cultured sterol-laden fibroblasts and macrophages and to interact with cell-surface HDL binding sites. Lipid-free peptides containing two identical tandem repeats of class A amphipathic helices promoted cholesterol and phospholipid efflux from cells and depleted cellular cholesterol accessible for esterification by acyl CoA/cholesterol acyltransferase, similar to what was observed for purified apolipoprotein A-I. Peptide-mediated removal of plasma membrane cholesterol and depletion of acyl CoA/cholesterol acyltransferase–accessible cholesterol appeared to occur by separate mechanisms, as the latter process was less dependent on extracellular phospholipid. The dimeric amphipathic helical peptides also competed for high-affinity HDL binding sites on cholesterol-loaded fibroblasts and displayed saturable high-affinity binding to the cell surface. In contrast, peptides with a single helix had little or no ability to remove cellular cholesterol and phospholipid, or to interact with HDL binding sites, suggesting that cooperativity between two or more helical repeats is required for these activities. Thus, synthetic peptides comprising dimers of a structural motif common to exchangeable apolipoproteins can mimic apolipoprotein A-I in both binding to putative cell-surface receptors and clearing cholesterol from cells. (*J. Clin. Invest.* 1994. 94:1698–1705.) Key words: synthetic peptides • amphipathic helices • high-density lipoprotein • cellular cholesterol efflux • HDL receptors

Introduction

The role of HDL in promoting the removal of excess cholesterol from cultured cells has been well established; however, mechanisms by which this occurs remain poorly understood. HDL appears to remove cholesterol from cells by both passive and active processes. Cholesterol that is synthesized by cells or ingested by receptor-mediated endocytosis of lipoproteins can be transported rapidly to the plasma membrane where it passively desorbs into the extracellular fluid and reabsorbs to phospholipid-containing particles (1, 2). When cells are overloaded with sterol, however, cholesterol accumulates within an intracellular pool that is accessible to the enzyme acyl CoA/cholesterol acyltransferase (ACAT),[1] thus allowing cells to store excess cholesterol as ester-rich lipid droplets. Under this condition, excretion of excess ACAT-accessible cholesterol can be facilitated by an active process (3, 4) that is stimulated by the interaction of HDL apolipoproteins with cell-surface binding sites or receptors (5).

A common secondary structural motif described for the plasma lipoprotein–associated apolipoproteins is the amphipathic helix (reviewed in 6 and 7). Based on the properties of amphipathic helices found in the exchangeable apolipoproteins, a synthetic peptide, designated 18A, was designed and synthesized (8–10). This class A amphipathic helical peptide has been shown to form stable complexes with phospholipids similar to those formed by apolipoprotein A-I, the major protein constituent of HDL (8–10). A dimer of 18A linked with a proline residue (37pA) has greater lipid affinity than 18A, presumably due to cooperativity provided by two covalently linked lipid-binding peptides. In contrast, an analogue of 18A (18R), with the position of charged residues reversed along the polar face of the helix, can only marginally interact with lipid (8–10). Synthetic amphipathic helices can mimic several of the biological effects of apo A-I, and the potencies of these effects are related to the lipid binding affinities of the synthetic peptides (11–15). The present study examines whether these synthetic amphipathic peptides could also mimic the ability of lipid-free apo A-I and HDL to promote efflux of excess cholesterol from cultured cells and to interact with HDL binding sites on the cell surface.

Methods

Lipoprotein and apolipoprotein isolation. LDL and HDL_3 (hereafter referred to as HDL) were prepared by sequential ultracentrifugation in the density intervals 1.019 to 1.063 and 1.125 to 1.21 g/ml, respectively. LDL was acetylated by the method of Goldstein et al. (16) and labeled

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1. *Abbreviation used in this paper:* ACAT, acyl CoA/cholesterol acyltransferase.

with [^{3}H]cholesteryl linoleate as described (17). HDL was iodinated by the iodine monochloride method described by Bilheimer (18). apo A-I was purified from HDL as previously described (4). Purified apo A-I exhibited a single band when examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis with Coomassie blue staining.

Phospholipid vesicles. Vesicles were prepared by the cholate dialysis method (19). For cell culture experiments, the vesicles were added to the medium immediately prior to adding the medium to cells.

Peptides. Peptides were synthesized and purified as described previously (9). The primary sequences of the peptides used in this study are shown in Table I.

Cell culture. Human skin fibroblasts were cultured and maintained as previously described (4, 5, 18). Usually 70,000 cells/2 ml medium in 35 mm dishes or 15,000 cells/1 ml medium in 16 mm wells were seeded and grown to confluence before use in experiments. Cells were loaded with nonlipoprotein cholesterol by incubation for 48 h with DME containing 2 mg/ml fatty acid-free BSA (Sigma Chemical Co., St. Louis, MO) and 30 μg/ml cholesterol. Cells were then incubated overnight (16–18 h) in DME containing 1 mg/ml BSA and 0.2 μCi/ml [^{3}H]cholesterol (40–60 Ci/mmol, Amersham Corp., Arlington Heights, IL) to label plasma membrane cholesterol. For experiments containing [^{3}H]choline-labeled phospholipids (see below), cells were labeled with 0.1 μCi/ml [^{14}C]cholesterol (50–60 Ci/mmol, Amersham Corp.). Choline-containing phospholipids were labeled by including 1 μCi/ml [^{3}H]choline chloride (75–85 Ci/mmol, Amersham Corp.) during the overnight incubation of fibroblasts. After incubation with radiolabel, cell layers were rinsed five times with PBS containing 0.1% BSA (PBS/BSA) before the addition of experimental media.

Resident mouse peritoneal macrophage cultures were established from unstimulated Swiss Webster mice by the method of Edelson and Cohn (20). Peritoneal cells were harvested from 20–25 mice in PBS, pooled at 4°C, collected by centrifugation (400 *g*, 10 min, 4°C), and washed once in DME. Cells were plated in DME containing 20% (vol/vol) FBS at 1×10^6 cells/0.5 ml/16 mm well. After incubation for 2 h at 37°C, nonadherent cells were removed by washing three times with DME. Adherent cells were maintained in culture with DME containing 20% total bovine serum for 5 d before use in experiments. Macrophages were cholesterol loaded by incubation with 50 μg/ml of [^{3}H]cholesteryl linoleate-labeled acetylated-LDL for 24 h. Cells were then washed two times with serum-free DME and incubated overnight with serum-free DME to allow equilibration of cholesterol pools.

Cholesterol and phospholipid efflux from cells. After the appropriate labeling protocol, cells were incubated at 37°C with DME containing 1 mg/ml BSA and the indicated additions. At the indicated times, the efflux media were collected and cell layers were rinsed three times with PBS. Media and cells were stored frozen at −20°C until extraction for lipid and protein content. Efflux media were extracted by the method of Folch et al. (21) and, in some experiments (labeled with cholesterol), counted directly after a brief centrifugation to remove cell debris. Cholesterol-labeled cell layers were extracted with hexane/isopropanol (3:2, vol/vol) as described (5, 17). Cells containing labeled phospholipids were extracted with 1 ml of isopropanol for 1 h, and then with hexane/isopropanol as above.

Sterol species were separated by thin layer chromatography on silica gel G plates developed in hexane/diethyl ether/methanol/acetic acid (120:30:10:1.5, vol:vol:vol:vol). Choline-containing phospholipids were separated by thin layer chromatography on silica gel H plates developed in chloroform/methanol/water (65:35:4, vol:vol:vol). Lipid spots were identified by staining with I_2 vapor and comigration with standards. After allowing I_2 stain to dissipate, appropriate spots were taken for determination of sterol mass and/or scintillation counting. Labeled cholesterol efflux and labeled phospholipid efflux were calculated as the percent of label appearing in the media, relative to the total (media + cell) radioactivity for each lipid.

Cell cholesterol esterification. To assess the relative activity of ACAT following incubation with test medium, cells were washed once with PBS and incubated for 1 h at 37°C with DME containing 9 μM ^{14}C-oleate (50–60 mCi/mmol, Amersham Corp.) bound to 3 μM BSA (5). Cells were chilled on ice and washed twice with PBS/BSA and twice with PBS. Cells were stored frozen at −20°C until extraction for lipid and protein as described above. Cell lipids were separated by thin layer chromatography as described above to determine cholesteryl ester radioactivity. Incorporation of radiolabel into cholesterol esters represents cholesterol esterification by ACAT, and results were quantitated as picomoles of [^{14}C]oleate incorporated into cholesteryl esters per milligram of cell protein.

Cell-surface binding of HDL and peptides. The specificity of high-affinity HDL binding sites on fibroblasts was tested using a sequential competitive binding assay (22). Cholesterol-loaded fibroblasts were washed twice with PBS/BSA and chilled on ice during a third wash. Cells were then incubated at 0°C (on ice) with Hepes-buffered DME containing 1 mg/ml BSA and the indicated concentration of competitor. After 3 h, the cells were rapidly washed three times with ice-cold PBS/BSA and incubated for 1 h at 0°C with Hepes-buffered DME containing 1 mg/ml BSA and 5 μg/ml of ^{125}I-HDL. Specific activity of ^{125}I-HDL ranged from 200 to 300 cpm/ng HDL protein with less than 2% of label associated with lipids. Cells were washed five times with ice-cold PBS/BSA and twice with PBS. Cell layers were dissolved in 0.1 M NaOH, and aliquots were taken to quantitate radioactivity and protein.

To measure direct binding of peptides 18A and 37pA to the cell surface, tyrosyl residues were labeled with ^{125}I by the iodine monochloride method (18, 23), and radiolabeled peptides were extensively dialyzed. The specific activities of the peptides ranged from 800 to 2,200 cpm/pmol. Fibroblasts were incubated with 10 nM to 2 μM radiolabeled peptide for 3 h at 0°C as described above for binding of ^{125}I-HDL. Iodination of the peptides had no effect on biological activity based on results showing that iodination of 37pA did not change its ability to inhibit cellular cholesterol esterification and that unlabeled 37pA blocked cell-surface binding of iodinated 37pA (data not shown).

Other methods. Protein concentrations were estimated by the method of Lowry et al. (24) using BSA as the standard. Statistical differences were calculated by Student's *t* test. Kinetic analyses of peptide binding and lipid efflux were performed using the SAAM30 computer program.

Results

The effects of HDL, apo A-I, and synthetic amphipathic helical peptides on cholesterol clearance from cultured human skin fibroblasts were determined by two independent assays. First, as an index of cholesterol efflux from the plasma membrane, the appearance of radiolabeled cholesterol in the medium was measured after cholesterol-loaded cells were labeled overnight with trace quantities of [^{3}H]cholesterol. Second, depletion of cellular pools accessible for esterification by ACAT was estimated by the decrease in incorporation of [^{14}C]oleate into cholesteryl esters. Results show that HDL and lipid-free apo A-I can promote [^{3}H]cholesterol efflux and decrease cholesterol esterification (Fig. 1, *A* and *B*). Both apo A-I and HDL had maximal effects on cholesterol esterification at ~ 400 nM, although HDL had a greater capacity to promote [^{3}H]cholesterol efflux, presumably because of exchange between HDL cholesterol and plasma membrane tracer (1, 2).

The synthetic peptides 18A and the charge-reversed analogue 18R (see Table I) had no significant effect on cholesterol efflux or esterification, even at molar concentrations 20-fold higher than that for HDL (Fig. 1 *C*). To increase the α-helical potential and the lipid-binding properties of 18A, the NH_2- and COOH-terminal groups were protected by acetyl and NH_2 groups, respectively (15). This modification of peptide 18A did not affect cholesterol efflux from cells (Fig. 1 *E*). The increased cholesterol esterification caused by Ac-18A-NH_2 appeared to reflect enhanced uptake of [^{14}C]oleate as indicated by a parallel increase in incorporation of label into triglycerides (data not

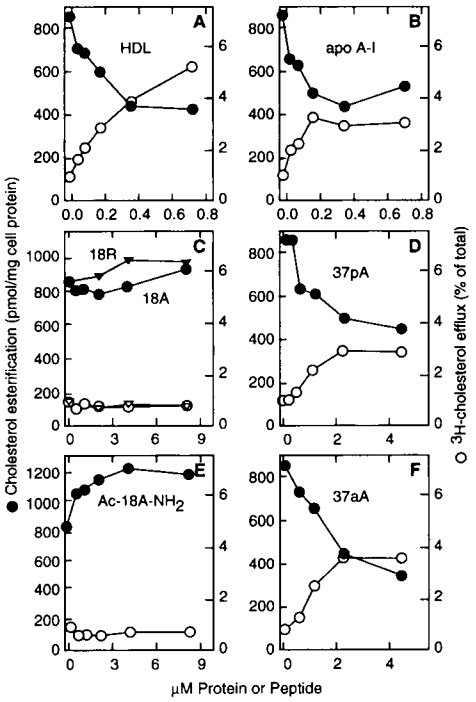

Figure 1. Effect of HDL, apo A-I, and synthetic amphipathic helical peptides on cellular cholesterol efflux and esterification. Cholesterol-loaded human skin fibroblasts, labeled with [^{3}H]cholesterol, were incubated with serum-free DME containing 1 mg/ml BSA and the indicated concentrations of HDL or peptide. After 6 h, the medium was removed and cells were further incubated for 1 h with medium containing ^{14}C-oleate to measure cholesterol esterification by ACAT. Cell and medium lipids were extracted and separated by thin-layer chromatography, and cholesterol and cholesteryl ester radioactivity was quantitated. Cholesterol efflux (*open symbols*) was calculated as the percent of total (cell and medium) [^{3}H]cholesterol appearing in the medium. Total [^{3}H]-cholesterol counts were 18,605±1,211 cpm/dish. Cholesterol esterification (*closed symbols*) was calculated as picomoles of ^{14}C-oleate incorporated into cholesteryl esters normalized for cell protein. The results are the means of duplicate dishes representative of two to five experiments. The coefficients of variation for cholesterol efflux and cholesterol esterification assays were 5.9% and 6.9%, respectively.

shown). None of the other peptides tested had any effect on incorporation of oleate into triglycerides compared to controls. Thus, even though Ac-18A-NH$_2$ appeared to perturb the lipid environment of the plasma membrane, it was no more active than 18A or 18R in promoting cholesterol efflux.

In contrast to the monomers, dimers of 18A linked with proline (37pA) or alanine (37aA) were able to inhibit cholesterol esterification and promote [^{3}H]cholesterol efflux (Fig. 1, *D* and *F*). The maximum extent of depletion of ACAT substrate for these peptides was comparable to that for HDL and apo A-I. Time course studies at saturating concentrations of HDL, apo A-I, and 37pA demonstrated equivalent rates for the decrease in cholesterol esterification over 16 h of incubation, while 18A remained without effect (data not shown).

The synthetic peptides were compared to HDL and apo A-I for their ability to promote clearance of cholesterol from sterol-laden mouse peritoneal macrophages, a cultured cell model with features common to foam cells of atherosclerotic lesions (16). Cells were overloaded with cholesterol by incubation with acetylated-LDL containing [^{3}H]cholesteryl linoleate to radiolabel cellular pools of free and esterified cholesterol by the scavenger receptor pathway (16, 25). HDL and apo A-I were able to promote efflux of [^{3}H]cholesterol into the medium and decrease the amount of cholesterol available for esterification by ACAT (Fig. 2, *A* and *B*). Peptides 37pA and 37aA were also effective in promoting [^{3}H]cholesterol efflux and decreasing [^{14}C]oleate

Table I. Amino Acid Sequence of Synthetic Peptides

Peptide	Sequence
18A	− + − + − Asp-Trp-Leu-Lys-Ala-Phe-Tyr-Asp-Lys-Val-Ala-Glu- + + − Lys-Leu-Lys-Glu-Ala-Phe
18R	+ − + − + Lys-Trp-Leu-Asp-Ala-Phe-Tyr-Lys-Asp-Val-Ala-Lys- − − + Glu-Leu-Glu-Lys-Ala-Phe
37pA	18A-Pro-18A
37aA	18A-Ala-18A
Ac-18A-NH$_2$	Acetyl-18A-NH$_2$

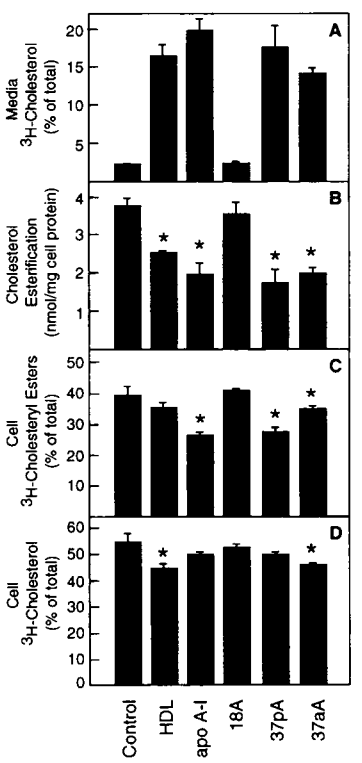

Figure 2. Effect of synthetic amphipathic peptides on cholesterol efflux from mouse peritoneal macrophages. 5-d-old macrophage cultures were incubated for 24 h with serum-free DME containing 0.5 mg/ml BSA and 50 μg/ml acetylated-LDL, which had been labeled with [^{3}H]cholesteryl linoleate. Cells were washed and incubated with medium for 18 h to allow equilibration of cholesterol pools. Cells were incubated with medium containing 0.5 mg/ml BSA alone (control) or with the following additions: 0.36 μM HDL, 0.36 μM apo A-I, 9.0 μM 18A, 4.5 μM 37pA, and 4.5 μM 37aA. After 6 h, the efflux medium was removed and cells were pulse-labeled with ^{14}C-oleate to determine cholesterol esterification by ACAT. Media and cell lipids were extracted and subjected to thin-layer chromatography to determine medium (*A*) and cellular (*D*) [^{3}H]-cholesterol, cellular [^{3}H]cholesteryl esters (*B*), and ^{14}C-oleate incorporated into cellular cholesteryl esters (*C*). Results are the mean±SD of three dishes. Total [^{3}H]cholesterol radioactivity was 5,770±157 cpm/dish for all dishes. Values marked with an asterisk are significantly different from controls by Student's *t* test; $P < 0.005$ in (*B*), $P < 0.05$ in (*C*) and (*D*). In (*A*), all values except those for 18A are significantly different from controls ($P < 0.0005$).

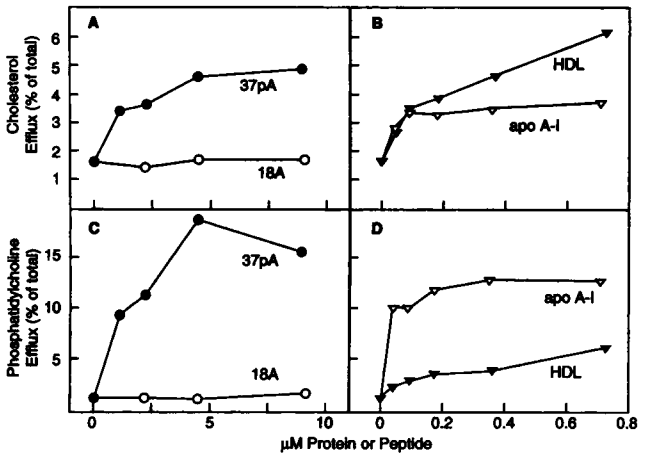

Figure 3. Comparison of the effects of HDL, apo A-I, and synthetic peptides on cholesterol and phosphatidylcholine efflux from cholesterol-loaded fibroblasts. Cholesterol-loaded fibroblasts were incubated for 18 h with serum-free medium containing 1 mg/ml BSA, 0.1 μCi/ml [^{14}C]-cholesterol, and 1.0 μCi/ml [^{3}H]choline chloride (to label cellular phosphatidylcholine). Labeled cells were then incubated with the indicated concentrations of HDL or peptides for 6 h. Cell and medium lipids were extracted and separated by thin-layer chromatography to quantitate [^{14}C]cholesterol (*A* and *B*) and [^{3}H]phosphatidylcholine (*C* and *D*) (as described in Methods). Efflux was calculated as the percent of total (cell and medium) label appearing in the medium. For [^{3}H]-phosphatidylcholine and [^{14}C]cholesterol, total counts were 4,049±121 and 13,337±368 cpm/dish, respectively (±SEM). Results are the mean of duplicate dishes. The coefficient of variation was 10.0% and 9.6% for phospholipid and cholesterol efflux assays, respectively.

incorporation into cholesterol esters. Peptide 18A was again without effect. The appearance of [^{3}H]cholesterol in the medium could be accounted for by decreases in both cell free and esterified cholesterol (Fig. 2, *C* and *D*). apo A-I, 37pA, and 37aA caused a significant decrease in esterified cholesterol content. HDL was less effective at cholesteryl ester clearance, and efflux was due mostly to a loss of free cholesterol. It is noteworthy that the decrease in cell [^{3}H]cholesteryl esters by the active peptides and HDL strongly correlated (r = 0.794) with the decrease in ACAT-mediated cholesterol esterification. These data demonstrate that apo A-I, 37pA, and 37aA have similar abilities to promote clearance of excess cholesterol from macrophages.

Peptides 18A and 37pA were compared to HDL and apo A-I for their ability to promote cholesterol and phospholipid efflux from cholesterol-loaded fibroblasts (Fig. 3). Dose response curves showed that HDL, apo A-I, and 37pA promoted efflux of both radiolabeled cholesterol and phosphatidylcholine. At saturating concentrations, all three acceptors were able to promote cholesterol efflux to nearly the same extent. In contrast, HDL was much less effective at removing cellular phosphatidylcholine compared to the lipid-free peptides, presumably because its apolipoproteins were already associated with phospholipids. Peptide 18A did not promote efflux of either phosphatidylcholine or cholesterol at the concentrations tested.

Results shown in Fig. 3 suggest that removal of cholesterol from cells by apo A-I or 37pA may be secondary to formation of phospholipid-rich particles that act as cholesterol acceptors. To further test for this possibility, we compared the ability of 37pA to stimulate cellular cholesterol and phospholipid efflux in the presence or absence of an extracellular source of phospholipid. Addition of phosphatidylcholine vesicles alone to the medium promoted efflux of [^{3}H]cholesterol from the plasma membranes of fibroblasts (0 μM 37pA, Fig. 4 *A*). In contrast, the vesicles had no measurable effect on efflux of [^{3}H]-phosphatidylcholine during the incubations (Fig. 4 *B*) nor on cholesterol esterification by ACAT (Fig. 4 *C*) following the 6-h incubations. In the presence of phosphatidylcholine vesicles, 37pA had no additional ability to stimulate [^{3}H]cholesterol efflux (compared to vesicles alone) at concentrations up to 2 μM. This peptide, however, was still capable of stimulating cellular [^{3}H]phosphatidylcholine efflux and inhibiting cholesterol esterification when vesicles were included in the medium, although the half-maximal concentration was increased by two- to threefold. This concentration shift may have been caused by the interaction of 37pA with phospholipid vesicles. These results support the concept that efflux of plasma membrane cholesterol depends largely on the presence of extracellular phospholipid particles, whereas both efflux of plasma membrane phospholipids and removal of ACAT-accessible cholesterol occur by a separate mechanism, possibly involving the direct interaction of amphipathic helices with cell-surface binding sites. The different mechanisms for removal of [^{3}H]cholesterol and ACAT-accessible cholesterol suggest that these sterols reside in different cellular pools.

We have previously demonstrated that binding of HDL to putative cell-surface receptors is required to promote removal of excess cholesterol from the ACAT substrate pools, and that intact apolipoproteins are required for binding (5). To test the possibility that apo A-I and the synthetic peptides are operating by the same mechanism, we compared the ability of different peptides to interact with high-affinity HDL binding sites on the surface of cholesterol-loaded fibroblasts. For these studies, we

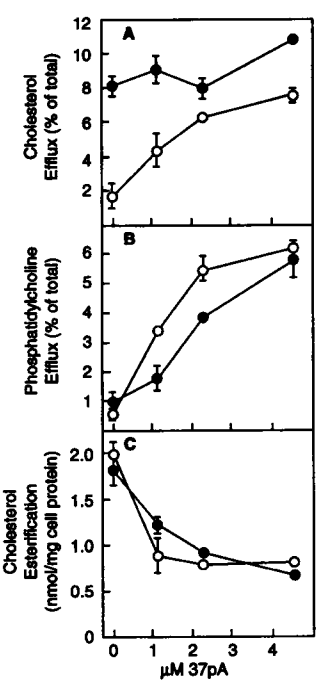

Figure 4. Effect of 37pA on cholesterol efflux (*A*), phosphatidylcholine efflux (*B*), and cholesterol esterification (*C*) in the presence of phospholipid vesicles. Parallel cultures of cholesterol-loaded human skin fibroblasts were labeled with either 0.2 μCi/ml [^{3}H]cholesterol or 1.0 μCi/ml [^{3}H]choline chloride for 18 h as described in Methods. The cells were then incubated for 6 h at 37°C with the indicated concentrations of 37pA in the presence (*closed symbols*) or absence (*open symbols*) of 100 μg/ml of phosphatidylcholine vesicles added to the medium immediately prior to the incubations. After incubation, one set of dishes was pulsed with ^{14}C-oleate to measure cholesterol esterification as described for Fig. 1. Efflux media and cell layers were analyzed for cholesterol, cholesteryl ester, and phosphatidylcholine radioactivity. For [^{3}H]phosphatidylcholine and [^{3}H]cholesterol, total counts were 13,848±1,469 and 14,114±135 cpm/dish, respectively (±SEM).

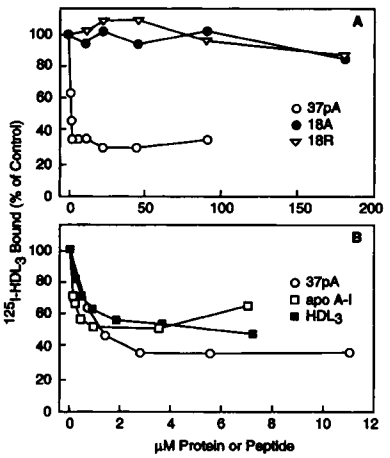

Figure 5. Interaction of synthetic amphipathic helical peptides with cell-surface HDL binding sites. Cholesterol-loaded fibroblasts were chilled on ice and incubated with ice-cold serum-free medium containing 1 mg/ml BSA and the indicated concentrations of 37pA, 18A, 18R, apo A-I, or HDL. After 3 h, dishes were rinsed three times with PBS/BSA, 1 ml of serum-free medium containing 5 μg/ml ^{125}I-labeled HDL was added to each dish, and incubations were continued for 1 h on ice. Cells were then washed five times with PBS/BSA and twice with PBS and dissolved in 0.1 M NaOH, and aliquots were taken for ^{125}I and protein determinations. Results are means of duplicate dishes (3.8% coefficient of variation) representing at least three separate experiments. In the absence of competitors, cells bound 33±3 ng ^{125}I-labeled HDL/mg cell protein (n = 6).

used a sequential competitive binding protocol to assay for the ability of different peptides to block high-affinity HDL binding. Cells were first exposed at 0°C to increasing concentrations of unlabeled competitor and, after removal of unbound competitor, were then incubated at 0°C with ^{125}I-HDL at a concentration near its high-affinity K_d. Because high-affinity HDL binding is essentially irreversible at 0°C (26, 27), molecules that bind to these sites with similar kinetics as HDL should remain bound during the second 0°C incubation and block high-affinity binding of ^{125}I-HDL. It was necessary to use a sequential competition assay rather than a more conventional simultaneous incubation to minimize the interaction of peptides and ^{125}I-HDL in solution, which may produce artifacts due to the displacement of ^{125}I-apolipoproteins from HDL particles (10, 23). A similar sequential competition assay has been used previously to identify receptor ligands in plasma that interact with other plasma components (22).

Results using the sequential competitive binding assay showed that neither 18A nor 18R could prevent the subsequent binding of ^{125}I-HDL (Fig. 5 *A*). In contrast, apo A-I and 37pA effectively blocked sites recognized by ^{125}I-HDL (Fig. 5, *A* and *B*). These sites were also blocked by 37aA (data not shown). The concentrations for half-maximal inhibition of ^{125}I-HDL binding by HDL, apo A-I, and 37pA were 3×10^{-8} M, 1×10^{-8} M, and 5×10^{-8} M, respectively. These results indicate that high-affinity HDL binding sites on cholesterol-loaded fibroblasts also interact with apo A-I, 37pA, and 37aA, but not with 18A and 18R.

We also examined direct binding of iodinated 37pA and 18A to cholesterol-loaded fibroblasts (Fig. 6). Both peptides bound extensively to cells, but with different saturation kinetics. Over a concentration range of 0.01 to 2.0 μM, 18A binding was nearly linear. At higher concentrations (not shown), some saturability was evident, but these high-capacity binding sites had a relatively low-affinity (apparent K_d of 10^{-5} M). In contrast, computer analysis of ^{125}I-37pA binding indicated that the concentration curve best fit a single saturable binding component with a K_d of $\sim 2 \times 10^{-7}$ M, plus a linear (nonspecific) component. Thus, 37pA appears to interact with saturable cell-surface binding sites that have a much higher affinity than those for 18A.

We characterized the specificity of the 37pA binding sites by competitive binding studies. To assess competition by HDL, we used the sequential competitive binding assay to avoid potential interactions between iodinated 37pA and lipoprotein particles. At a concentration of iodinated 37pA near its K_d for high-affinity binding (10^{-7} M), pretreatment of cholesterol-loaded fibroblasts with unlabeled 37pA and 37aA blocked 40% of the iodinated 37pA binding in a concentration-dependent and saturable manner (Fig. 7). Pretreatment with 18A had no effect on 37pA binding, even at concentrations of 18A that exhibited twofold greater total cellular binding than 37pA (Fig. 6). In contrast, pretreatment of cells with apo A-I or HDL blocked approximately 20% of the 37pA binding sites (half that blocked by 37pA). These results suggest that 37pA is interacting with multiple high-affinity binding sites on cholesterol-loaded fibroblasts, none of which interact with 18A with the same kinetic properties. A subset of these sites, however, appears to bind both HDL and apo A-I, consistent with results showing that 37pA interacts with the high-affinity HDL binding sites.

Discussion

Results presented in this report demonstrate that the synthetic peptides 37pA and 37aA, composed of two 18-mer class A amphipathic helical repeats (6–11), promoted cholesterol efflux and inhibited cellular cholesterol esterification in both fibroblasts and macrophages. These effects were similar to those mediated by lipid-free apo A-I and HDL, except that HDL had a greater capacity to promote efflux of plasma membrane cholesterol, probably due to exchange of cholesterol between

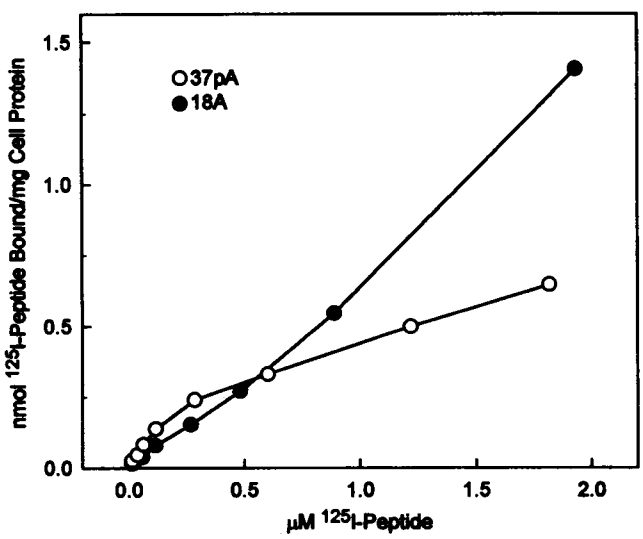

Figure 6. Cell-surface binding of iodinated 18A and 37pA to cholesterol-loaded fibroblasts. Cholesterol-loaded human skin fibroblasts were incubated with increasing concentration of iodinated peptide for 3 h at 4°C. Then, cells were washed, and radioactivity was determined as described in Fig. 5. The specific activities of ^{125}I-labeled 18A and ^{125}I-labeled 37pA were 810 and 2,100 cpm/pmol, respectively. Each value represents the mean of duplicate dishes (5.5% coefficient of variation).

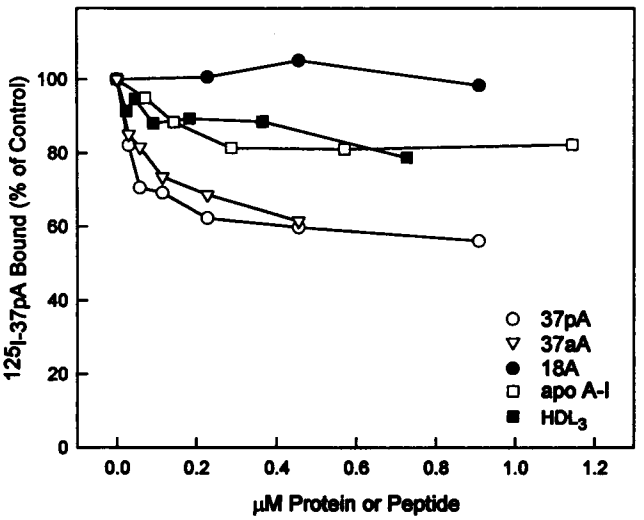

Figure 7. Interaction of HDL, apo A-I, and synthetic peptides with cell surface 37pA binding sites. Cholesterol-loaded fibroblasts were chilled on ice and incubated with ice-cold serum-free medium containing 1 mg/ml BSA and the indicated concentrations of 37pA, 37aA, 18A, apo A-I, or HDL. After 3 h, dishes were rinsed three times with PBS/BSA, 1 ml of serum-free medium containing 0.5 μg/ml (0.11 μM) ^{125}I-labeled 37pA was added to each dish, and incubations were continued for 1 h on ice. Cells were treated and radioactivity determined as described in Fig. 5. Total cell binding of ^{125}I-labeled 37pA (specific activity of 2,100 cpm/pmol) in the absence of competitor was 64 pmol/mg cell protein. Each value represents the mean of duplicate dishes (3.5% coefficient of variation).

the lipoprotein particle and cells. Additionally, 37pA and 37aA could promote efflux of cellular [^{3}H]phosphatidylcholine, similar to the effects of apo A-I, while HDL had a limited capacity for phospholipid efflux. In contrast to the peptide dimers, peptides containing only a single 18-mer amphipathic helix (18A and Ac-18A-NH_2) had little or no ability to stimulate cholesterol and phospholipid efflux and to deplete cells of substrate for cholesterol esterification by ACAT. Thus, although these monomers have been shown to interact with lipids and have biological activity (8–15), they were almost completely inactive in mimicking apo A-I or HDL in stimulating lipid transport from cells.

Exposure of fibroblasts to apo A-I or 37pA increased efflux from the plasma membrane of both cholesterol and phosphatidylcholine in parallel to depletion of ACAT-accessible cholesterol, implying that these three lipid transport processes are mediated by the same cellular interactions. Several other lines of evidence, however, suggest that these processes are occurring by independent mechanisms. First, removal of cholesterol from either the plasma membrane or ACAT substrate pools does not require simultaneous release of plasma membrane phospholipids, as evidenced by results showing that HDL stimulated efflux of both sources of cholesterol to the same extent as 37pA and apo A-I but had markedly less ability to promote efflux of cellular phospholipids. Second, efflux of radiolabeled cholesterol from the plasma membrane can be dissociated from both phospholipid efflux and depletion of ACAT-accessible cholesterol. For example, addition of phospholipid vesicles to the medium increased cholesterol efflux from the plasma membrane severalfold within 6 h, but there was no significant effect on phosphatidylcholine efflux or depletion of ACAT-accessible cholesterol. Conversely, addition of 37pA to medium containing phospholipid vesicles increased cellular phosphatidylcholine efflux severalfold and depleted over half of the ACAT-accessible cholesterol when there was no further increase in cholesterol efflux from the plasma membrane.

One explanation for these findings is that, of these three lipid transport processes, only phospholipid efflux and depletion of ACAT-accessible cholesterol involve interactions of amphipathic helices with cell-surface binding sites. Promotion of cholesterol efflux from the plasma membrane may be a secondary response to formation of membrane-derived phospholipid complexes generated by reversible cell-surface binding of peptides or lipid-free apolipoproteins. These newly formed particles may act as sterol acceptors and enhance passive diffusion of cholesterol from the cell surface (1, 2). This two-step lipid transport has been postulated for promotion of cholesterol efflux from cells by various lipid-free apolipoproteins (25, 28–31). It is likely, however, that removal of plasma membrane phospholipid is significant only for those apolipoproteins that are lipid-deficient. In contrast, the interaction of apolipoproteins with cells depletes ACAT-accessible cholesterol even when the apolipoproteins are associated with HDL phospholipid. This process may be initiated by the release of intracellular signals which activate a cholesterol excretory pathway (3, 4).

Competitive binding studies showed that apo A-I, 37pA, and 37aA blocked the interaction of HDL with its high-affinity binding sites on cholesterol-loaded fibroblasts, while 18A had no effect on HDL binding. These results strongly suggest that the interaction of HDL with its cell-surface binding site is mediated by two or more tandem repeats of class A amphipathic helices within HDL apolipoproteins. Thus, peptides containing two of these amphipathic helices can mimic apo A-I in both stimulating lipid transport from cells and in binding to high-affinity HDL binding sites. The higher efficacy of the dimeric peptides compared to the monomers cannot be explained by an extension of the polar and nonpolar faces along the helical axis, for linkage of the two 18-mer helices by a single amino acid causes a 35° rotation of these faces. Moreover, linkage by proline compared to alanine causes a "kink" between the axes, yet 37pA and 37aA were equally active. It is most likely that cooperativity between two or more amphipathic helices is required for optimum binding to high-affinity sites on the cell surface, as has been described previously for the interaction of HDL apolipoproteins with cells (28, 32). This property may confer specificity for lipid-poor subclasses of HDL particles which have more helices exposed for cellular interactions (7, 33, 34).

Studies with iodinated peptides revealed that both 18A and 37pA bind extensively to the surface of cholesterol-loaded fibroblasts, but only 37pA binds to saturable, high-affinity sites. High-affinity binding of 37pA was not blocked by 18A and was only partially blocked by HDL and apo A-I, suggesting that none of these 37pA binding sites interacts with 18A and only a subset interacts with HDL apolipoproteins. Therefore, although both apo A-I and 37pA are recognized by HDL binding sites, 37pA appears to have unique properties that promote its binding to additional cell-surface sites that do not bind 18A, apo A-I, or HDL.

Previous studies showed that modifying HDL so as to decrease its interaction with cellular high-affinity binding sites also reduces its ability to remove excess cellular cholesterol (4, 5, 35–37), suggesting that these two processes are closely

coupled. The findings that both high-affinity binding to cells and removal of cellular cholesterol require two or more tandem amphipathic helices further support this concept. The current study, however, does not provide direct evidence that HDL, apo A-I, and dimeric peptides are removing cellular lipids through their interactions with a common binding site. The interactions of amphipathic helices with the plasma membrane are complex and appear to involve multiple binding sites, and the relative contribution of each of these sites to lipid transport processes is unknown. In addition, comparisons of saturation curves revealed that the apparent half-maximal concentrations for inhibition of HDL binding were approximately fivefold lower than those for removing cholesterol. However, this may reflect some of the special conditions of these assays, particularly the different temperatures used (37°C vs. 0°C). The kinetics of high-affinity HDL binding is highly temperature sensitive, in that the dissociation rate is much faster at 37°C than at 0°C (26, 27). Moreover, the active conformation of lipid-free amphipathic peptides in solution may vary with temperature. Despite these differences between assays, the relative efficacy of the peptides was similar for each process. The half-maximal molar concentrations for stimulation of cholesterol efflux and inhibition of HDL binding were approximately fivefold higher for 37pA than for apo A-I. This may be related to the fact that apo A-I has a molecular mass six times that of 37pA and contains four times the number of amphipathic helices. These and other peptide analogues will be useful tools for testing the hypothesis that HDL apolipoproteins stimulate clearance of cellular cholesterol through the cooperative interaction of their amphipathic helices with cell-surface binding sites or receptors.

Numerous population studies have shown an inverse correlation between plasma HDL levels and risk for coronary heart disease, suggesting that HDL protects against atherosclerosis. This may be related to the ability of HDL apolipoproteins to stimulate clearance of cholesterol from peripheral cells, particularly those of the artery wall. The finding that synthetic peptides with structural features of HDL apolipoproteins can mimic the biological activity of HDL raises the possibility that specific agonists could be developed for the cellular cholesterol excretory pathway. These agonists may have therapeutic benefits for the prevention of coronary heart disease.

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