Human plasma apolipoprotein E (apoE) is a low density lipoprotein (LDL) receptor ligand. It targets cholesterol-rich lipoproteins to LDL receptors on both hepatic and peripheral cells. The region of apoE responsible for its binding to the LDL receptor has been localized to amino acids 140–160. An apoE 141–155 monomeric peptide and a dimeric 141–155 tandem peptide were synthesized and tested for their inhibition of 125I-LDL degradation by human fibroblasts and human monocyte-like cells, THP-1. The monomer had no activity at 250 μM, but the dimer inhibited 125I-LDL degradation by 50% at 5 μM. The inhibition was specific for the LDL receptor because the dimer did not inhibit the degradation of 125I-acetylated LDL by scavenger receptors expressed by phorbol ester-stimulated THP-1 cells. As reported for native apoE, amino acid substitutions of Lys-143 → Ala, Leu-144 → Pro, and Arg-150 → Ala decreased the inhibitory effectiveness of the dimer. Furthermore, a trimer of the 141–155 sequence had a 20-fold greater inhibitory activity than the dimer. Studies with a radioiodinated dimer indicated that some of the inhibitory activity could be a result of the interaction of the dimer with LDL. However, direct binding of the 125I-dimeric peptide to THP-1 cells was observed as well. This binding was time-dependent, linear with increasing cell number, Ca2+- but not Mg2+-dependent, saturable, inhibited by lipoproteins, and increased by preculture of the cells in lipoprotein-depleted medium. Therefore, a synthetically prepared dimeric repeat of amino acid residues 141–155 of apoE binds the LDL receptor.

ApoE1 is a limiting factor for remnant receptor-mediated uptake of circulating cholesterol-rich lipoproteins. Two separate studies with hyperlipidemic rabbits demonstrated that a bolus intravenous administration of milligram amounts of apoE promoted a 20–40% decrease in total plasma cholesterol within 6 h (1, 2). The importance of the remnant receptor is highlighted by the fact that LDL receptor-mediated uptake was not a factor in either instance. Given the effect of a single injection of exogenous apoE on total cholesterol, it is possible that a sustained increase in plasma apoE could result in a consistently lower plasma cholesterol. Repeated intravenous administration of intact apoE would require large amounts of purified protein. A synthetic peptide that mimicked the receptor binding activity of native apoE could be used to examine the long term effects of repeated administration of apoE on plasma cholesterol. Furthermore, a synthetic peptide mimic with receptor binding activity could be used to determine the structural requirements of a receptor-competent ligand.

The structural features of apoE that specify its binding to hepatic remnant receptors are not known. However, the domain of apoE responsible for binding to the LDL receptor has been identified (3). This domain is a 20-amino acid region comprised of residues 140–160. Furthermore, there is evidence that this region is involved in remnant receptor binding. For example β-VLDL containing an apoE isoform, E2, which has <2% of wild type (E3) binding to the LDL receptor, is cleared by the remnant receptor at a reduced rate as well (4). This potential overlap of receptor specificity supports the feasibility of developing an apoE peptide that is both an LDL receptor and a remnant receptor ligand.

To date no fragments or synthetic peptides of apoE have been shown to have LDL receptor binding activity (5). Our assessment of the known characteristics of binding suggested that a key structural feature of the interaction is the multimeric nature of both the ligand and the ligand binding domains of the receptor. Up to four apoE molecules assembled on a phospholipid surface can bind a single LDL receptor, and when more than one of the apoE molecules bind, the apparent affinity is increased 25–50-fold (6). Therefore, the receptor competent form of apoE may be multimeric. If true, a peptide mimic also should contain multiple copies of the binding domain. To test this hypothesis peptides were synthesized that contained either a monomeric representation of the receptor binding domain, residues 141–155 (LRKLRRKRLRDADDL), or a dimeric linear tandem repeat of this sequence (i.e. 141–155-141–155). Following purification these peptides were tested for their ability to inhibit LDL binding and degradation by THP-1 cells and fibroblasts.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis**—All peptides were synthesized by the solid-phase method of Merrifield on an Applied Biosystems model 430A automated peptide synthesizer using hydroxybenzotriazole hydrate/dicyclohexylcarbodiimide activation. The peptides were purified by HPLC chromatography on a Water Autos 500 preparative HPLC column as described (7). The purity as obtained from analytical HPLC chromatograms and the amino acid composition of all peptides used in this study are listed in Table I. To prepare them for addition to the cells, all peptides were dissolved in PBS and dialyzed overnight.

**Cell Culture**—THP-1 cells were acquired from American Type Culture Collection and cultured in RPMI-1640 medium supplemented with 10% FBS. Before assay the THP-1 cells were cultured overnight in serum-free RPMI-1640 medium supplemented with 1% Nutridoma-Hu to up-regulate LDL receptors. To induce scavenger receptor expression, THP-1 cells (5 × 10⁶/ml-16-mm well) were incubated in complete medium with PMA (10⁻⁷ M) for 4 days (8). Human fibroblasts were grown in Dulbecco’s minimal Eagle’s medium supplemented with 10% FBS. Fibroblasts were set up at 5 × 10⁴ cells/ml/
16-mm culture well and cultured for 4-6 days before transfer to 10% lipoprotein-depleted serum for overnight up-regulation of LDL receptors. Cellular protein was measured with the Bio-Rad protein assay reagent using bovine serum albumin as a standard.

**Lipoprotein Binding and Degradation**—LDL was isolated from human plasma by ultracentrifugation (8). The lipoproteins were radiolabeled using the iodine monochloride method (9) to a specific activity of 200-600 cpm/ng, and >99% of radioactivity was precipitated by 10% trichloroacetic acid. Cellular binding and degradation were assayed as described (9).

The effect of the peptides on the degradation of 125I-LDL by unstimulated THP-1 cells was examined (Fig. 1, top). Degradation was measured by incubating the cells with 5 µg of 125I-LDL at 37°C for 4 h in the absence or presence of the peptides. Neither a control apoA-I peptide, apoA-I 74-105 (Table I), nor the apoE 141-155 monomer inhibited 125I-LDL degradation. In contrast, the dimer inhibited 125I-LDL degradation by 50% at 5 µM. A 200-fold molar excess of the dimer compared with LDL was required to achieve 50% inhibition of degradation. Additional studies indicated that this inhibition was not a result of direct cellular toxicity, because cells in which the inhibitors had been washed away recovered their capacity to degrade 125I-LDL.

To demonstrate that the dimer was a specific inhibitor of LDL receptor-mediated events we tested three additional peptides. Two were synthesized as tandem repeats of other lysine- and arginine-rich sequences, e.g. (LQNRRGLD), and (LQRILAVERYLKDDQQL). Neither peptide at 100 µM had any effect on THP-1 cell 125I-LDL degradation suggesting that the activity of the apoE dimer was dependent on more than tandem repeats of lysine- and arginine-rich sequences. A final control peptide was tested and found to be inactive. This peptide was a single copy of the extended receptor binding region of apoE, residues 129-162. This peptide was tested to verify that the activity of the dimer was not just a reflection of stabilization of the α-helix-forming tendency of this region of apoE by the addition of flanking α-helix-forming residues (3).

To verify that the inhibition of LDL degradation was specific for the LDL receptor, we tested the effect of the dimer on scavenger receptor processing of 125I-LDL (Fig. 1, bottom). Neither native LDL nor the dimer inhibited the degradation of 125I-aLDL by PMA-stimulated THP-1 cells. At 12.5 µM the dimer had no effect on 125I-aLDL degradation, yet this concentration caused an 80% inhibition of 125I-LDL degradation (Fig. 1, top). Therefore, the dimer did not inhibit LDL degradation by nonspecific interference with receptor-mediated endocytosis.

LDL receptors on macrophages and fibroblasts differ in their apparent molecular weight and turnover (13). Therefore, we repeated the degradation assays using normal human fibroblasts to be certain that these differences did not alter the activity of the dimer. Fibroblast 125I-LDL degradation was inhibited 50% by LDL and dimer at 0.15 µM and 17 µM, respectively. Furthermore, fibroblast 125I-LDL binding at 4°C was inhibited by LDL and dimer at 0.01 µM and 14 µM, respectively. The inhibition was specific to the dimer because a control AI peptide, the 141-155 monomer, and the 129-162 monomer had no activity at up to 250 µM.

To confirm that the dimer could mimic apoE and bind the LDL receptor, we prepared three additional apoE peptides that contained single-amino acid substitutions at both positions in the tandemly repeated sequence. These peptides included substitution of the basic amino acids, Lys-143→Ala and Arg-150→Ala, and substitution of Leu-144 with an α-helix disrupting amino acid, Pro. As illustrated in Fig. 2, each of these substitutions reduced the ability of the dimer to inhibit the binding of 125I-LDL to fibroblasts. The Lys-143→Ala substitution had the smallest effect, whereas the Leu-144→Pro substitution had the greatest impact. The Leu-144→Pro-substituted dimer had no activity at 60 µM, an attenuation that was similar to that observed with a full-size apoE-Leu-144→Pro variant (14).

The dimer could interfere with LDL binding to the LDL receptor through an association with the receptor binding domain of apoB on LDL or through binding to the cellular LDL receptor. To distinguish between these two possibilities, we prepared a radiiodinated form of the dimer. This peptide was synthesized with an additional amino-terminal tyrosine residue and was iodinated with 125I. Full activity of a mock-
LDL Receptor Active ApoE Peptide

Specific amino acid substitutions in the dimer altered its ability to inhibit 125I-LDL binding to fibroblasts. The peptides: dimer (●), Lys-143 → Ala (△), Leu-144 → Pro (■), and Arg-150 → Leu (★), were incubated for 1 h at 4 °C with fibroblasts in the presence of 5 μg/ml of 125I-LDL. Buffer control cultures bound 1.95 ± 0.12 μg of 125I-LDL/mg of cell protein. Each point is the average of three replicates per treatment ± standard deviation.

The trimer peptide was a more potent inhibitor of fibroblast LDL degradation than the dimer peptide. The peptides: dimer (●), trimer (△), monomer (■), A174-105 (▲), and LDL (▼), were incubated with fibroblasts that had been plated at 10^4/0.2 ml/well of a 96-well culture plate. LDL degradation was measured after 5 h at 37 °C with 2 μg/ml of 125I-LDL. Buffer control cultures degraded 2.26 ± 0.16 μg of 125I-LDL/mg of cell protein. Each point is the average of five replicates per treatment ± standard deviation.

Iodinated peptide was documented by observing comparable levels of inhibition of both LDL binding and LDL degradation. To assess the association of the 125I-dimer with lipoproteins, 259 ng of the radiolabeled peptide were mixed with 200 μg of VLDL, LDL, or HDL. After 1 h at 37 °C the apoA-I-containing lipoproteins were immunoprecipitated with optimal amounts of a polyvalent apoA-I-specific antiserum and the apoB-containing lipoproteins precipitated with phosphotungstic acid and MgCl₂. Whereas less than 0.7% of the added 125I-dimer was associated with HDL, under the conditions studied 5 and 39% of the dimer were found associated with VLDL and LDL, respectively. Calculated as the number of peptide molecules bound per lipoprotein particle, VLDL, LDL, and HDL each contained 1.8, 0.25, and 0.0043 dimers per particle, respectively. Therefore, because the 10 μM concentration of dimer required to observe inhibition of 125I-LDL binding could result in lipoprotein association, the possibility exists that the dimer inhibited the cellular binding and degradation of 125I-LDL by binding to the lipoprotein.

The direct binding of the 125I-dimer to dividing THP-1 cells was measured. It was linear with cell number, saturable, and reached an apparent steady state within 40 min at 4 °C. 125I-dimer binding to THP-1 cells was specifically inhibited by apoB- and apoE-containing lipoproteins with VLDL > LDL >> HDL. A 2-fold increase in bound peptide was detected.
when the Ca\textsuperscript{2+} concentration was increased from 0.1 to 2.0 mM, but Mg\textsuperscript{2+} at 2.0 mM had no effect. Finally, if THP-1 cells were cultured in lipoprotein-free serum for 96 h before \textsuperscript{125}I-LDL and \textsuperscript{125}I-dimer binding was assayed, a 2-3 fold increase was observed with both ligands, indicating up-regulation of both the LDL receptor and dimer binding sites. When the binding of \textsuperscript{125}I-dimer to THP-1 cells cultured in LDL-free medium was studied in the presence of 2.0 mM Ca\textsuperscript{2+} and in the absence and presence of 500 \mu g/ml of VLDL, specific saturable binding was observed with a \textit{K}_d of 7.3 x 10\textsuperscript{-8} M and 2 x 10\textsuperscript{-6} dimers bound/cell.

The binding of apoE-containing lipoproteins to the LDL receptor has a number of distinguishing characteristics. Binding is saturable, Ca\textsuperscript{2+}-dependent, up-regulated by culture of the cells in LDL-deficient serum, inhibitable by both apoB- and apoE-containing lipoproteins, and altered by specific single-amino acid substitutions in the primary sequence of apoE (15). Because each of these distinguishing properties could be observed with the dimer, binding of the peptide to LDL receptors on both fibroblasts and THP-1 cells is highly likely.

There is abundant evidence to suggest that lipoprotein metabolism is influenced by the number of apoE molecules per particle. First, reducing the number of apoE molecules per phospholipid complex results in decreased receptor binding (16). Second, hepatic VLDL uptake is enhanced further to test this concept with peptides a linear trimer of the 141-155 sequence was synthesized and its activity compared to the LDL receptor, the improved binding of particles with multiple copies of apoE may be due to the close association of two or more copies of apoE on the particle surface such that multiple interaction sites are formed between lipoprotein and receptor.

Our data suggest that the dimer binds the LDL receptor; however, there is reason to speculate that the LDL receptor is not the only fibroblast or THP-1 binding site. LRP is expressed by fibroblasts (19) and monocyte/macrophages (20). Because LRP contains reiterated sequences that are homologous to the ligand binding domains of the LDL receptor, it is possible that the dimer also binds LRP. Studies are under way with LDL receptor-deficient fibroblasts to determine if the dimer binds LRP.

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