Macrophage Foam Cell Lipoprotein(a)/Apoprotein(a) Receptor
Cell-Surface Localization, Dependence of Induction on New Protein Synthesis, and Ligand Specificity

George A. Keesler, Yueqing Li, Paul J. Skiba, Gunther M. Fless, Ira Tabas

Abstract  Understanding the interaction of the atherogenic lipoprotein, lipoprotein(a) [Lp(a)], with macrophages may provide important insights into the physiology and pathophysiology of this lipoprotein. We have recently shown that cholesterol loading of macrophages, such as occurs in atheroma foam cells, leads to marked upregulation of a novel receptor activity for native Lp(a) and its plasminogen-like protein component, apoprotein(a) [apo(a)]. We show here that the Lp(a)/apo(a) receptor activity on cholesterol-loaded macrophages is trypsin sensitive, indicating that a cell-surface protein is involved and that the upregulation by cholesterol loading requires new protein synthesis. Ligand studies revealed that the foam cell receptor activity recognizes Lp(a) containing both small and large isoforms of apo(a) as well as rhesus monkey Lp(a), which contains an inactive kringle-4 sequence (K4y), lysine-binding domain. Elastase degradation products of plasminogen did not compete for 125I-labeled recombinant apo(a) [125I-r-apo(a)] internalization and degradation by foam cells, indicating that the K4y sequence, as well as the K5 and "protease" domains of apo(a), are not sufficient for receptor interaction. Consistent with these data, the degradation of 125I-r-apo(a) was completely blocked by an anti-Lp(a) polyclonal antibody that does not cross-react with plasminogen. Furthermore, the multiple sialic residues of apo(a) are also not involved in receptor interaction, since desialylated r-apo(a) interacted with foam cells as well as native r-apo(a). In contrast, reduced and denatured r-apo(a) was degraded by foam cells only slightly better than by control cells [28% increased degradation by foam cells versus 45% for native r-apo(a)], suggesting that the upregulated receptor activity recognizes certain secondary and tertiary structural features of apo(a). In summary, foam cells mediate the internalization and degradation of Lp(a) and apo(a) by a cell-surface receptor activity whose induction by cholesterol loading requires new protein synthesis. Remarkably, this receptor activity recognizes features of apo(a) that are distinct from those of plasminogen despite an 80% homology between these two proteins. (Arterioscler Thromb. 1994;14:1337-1345.)

Key Words  • atherosclerosis • lipoproteins • kringles • cholesterol

Lipoprotein(a) [Lp(a)] is a lipoprotein consisting of a low-density lipoprotein (LDL) particle covalently attached to a plasminogen-like glycoprotein, apoprotein(a) [apo(a)]. Elevated plasma levels of Lp(a) occur in ≈20% of the adult Caucasian population, and there appears to be an association between elevated Lp(a) levels and the occurrence of coronary atherosclerosis.1,2 Furthermore, cholesterol-fed apo(a) transgenic mice develop significantly more atherosclerosis than nontransgenic controls.3 Despite several important hypotheses and in vitro observations regarding possible roles of Lp(a) in atherosclerosis,4-7 neither the mechanism of Lp(a) atherogenicity nor the normal physiological roles of the lipoprotein are definitively known. Nonetheless, the interaction of Lp(a) with macrophages is thought to be important, since cholesteryl ester-filled macrophages (foam cells) are a prominent feature of atherosclerotic lesions.8-10 In fact, apo(a) has been found to colocalize with foam cells in these lesions.11

Previous studies have shown that cultured macrophages internalize and degrade native Lp(a) and apo(a) poorly.12-15 Recent work from our laboratory, however, revealed an Lp(a) receptor activity, different from known lipoprotein receptors, that can be induced in macrophages by cholesterol loading.16 The interaction of Lp(a) with cholesterol-loaded macrophages is solely dependent on the apo(a) moiety of Lp(a), since lipid-free recombinant apo(a) [rapo(a)] but not apo(a)-free Lp(a−) is recognized by the foam cell receptor activity.16

In the present study, we investigated whether a cell-surface protein is involved in the foam cell Lp(a)/apo(a) receptor activity and whether upregulation induced by cholesterol loading requires new protein synthesis. Furthermore, we explored several key properties of ligands that are needed for recognition by the foam cell receptor activity. Our data show that the Lp(a)/apo(a) receptor activity on cholesterol-loaded macrophages is trypsin sensitive, indicating that a cell-surface protein is involved and that the upregulation by cholesterol loading requires new protein synthesis. The ligand studies revealed that the foam cell receptor can recognize Lp(a) containing both small and large isoforms of apo(a). Moreover, whereas certain specific domains of apo(a) (eg, "protease" domain, kringle 5 [K5], K4y, and arginine-glycine-aspartic acid [RGD] site) and sialic acid residues appear not to be involved in this interaction, secondary and tertiary structural features of apo(a) are necessary for interaction with the upregu-
lated receptor activity on foam cells. Remarkably, these structural features of apo(a) that are recognized by the foam cell receptor activity are distinct from those of plasminogen even though apo(a) and plasminogen are 80% homologous.

Materials

Tissue culture media and reagents were obtained from GIBCO Laboratories, and fetal calf serum was purchased from Hyclone Laboratories. Lipoprotein-deficient serum (LPDS) was prepared from fetal calf serum by preparative ultracentrifugation (d, 1.21 g/mL). Clostridium perfringens neuraminidase, type X-A, attached to agarose beads was obtained from Sigma Chemical Co. All other reagents and chemicals were from Sigma.

Cells

All cells were maintained in a 37°C tissue culture incubator with a 5% CO2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) with penicillin (100 U/mL), streptomycin (100 µg/mL), and freshly added glucose (2 mmol/L). Resident mouse peritoneal macrophages from female ICR mice (20 to 25 g) were plated in DMEM/10% (vol/vol) fetal calf serum. After 1 hour for adherence, the cells were washed twice with phosphate-buffered saline (PBS) and then, unless otherwise indicated, preincubated for 2 to 4 days in DMEM/10% LPDS alone or containing 25 µg acetyl-LDL/mL. Fresh medium was added to the cells each day. In certain experiments, the acetyl-LDL was removed before the 125I-r-apo(a) degradation assay and substituted with lipoprotein-free cholesterol. This procedure was done because we were initially concerned that some of the 125I-r-apo(a) interaction with foam cells might be due to binding of the ligand to residual acetyl-LDL on the cell surface (cf Reference 19). Subsequent experiments indicated, however, that this type of interaction accounted for <10% of 125I-r-apo(a) degradation by foam cells (P.J.S. and I.T., unpublished data).

Lipoproteins and Apoprotein(a)

LP(a) with a total protein mass of 918 kD, obtained from the plasma of two subjects with a fast apo(a) polymorphism, was purified by lysine-Sepharse affinity chromatography and cesium chloride gradient centrifugation as described in detail by Snyder et al. LP(a) containing a slow ("S") isoform of apo(a), with a total protein mass of 1246 kD, was isolated from the plasma of a subject with two isoforms of apo(a) by the procedure of Fless and Snyder, which is slightly modified from the method referenced above. The lipoproteins isolated by ultracentrifugation at d=1.21 g/mL were dialyzed against 33 mmol/L phosphate, 1 mmol/L benzamidine, 0.01% NaN3, and 0.01% Na2EDTA, pH 7.4, and applied to a lysine-Sepharose column (20x2.5 cm) that was equilibrated in the same buffer. The LP(a) was eluted from the column with a 200-mL gradient of 0 to 100 mmol/L trans-4-(aminomethyl)cyclohexanecarboxylic acid. Consecutive 40-mL fractions were collected, made to 7.5% (wt/vol) with solid CsCl, and centrifuged for 20 hours at 400 000 rpm at 20°C in a Beckman 50.2 Ti rotor and then analyzed for apolipoprotein mass by gel filtration on a column (93x2.5 cm) of Sephadex G-75. The material that eluted with 200 mmol/L L-aminocaproic acid contained the Kl-3 (EDP I, M, 38 000 D), which did not bind to Sephadex G-75. The protein mass of LP(a) was determined before and after reduction with 5% 2-mercaptoethanol. Digestion of LP(a) with porcine elastase was performed according to the method of Sottrup-Jensen et al as described by Milanes and Ploeg. Briefly, 155 mg Glu-plasminogen in 21 mL of 0.3 mol/L NH4HCO3, pH 8.3, containing 7000 U of aprotinin (Trasylol) was incubated with 0.6 mg of porcine elastase and stirred gently for 5 hours at room temperature. The reaction was stopped with 2 mmol/L phenylmethylsulfonyl fluoride by stirring for 30 minutes. Subsequently, the mixture was made to 0.55 with solid NH4HCO3, and stirred overnight at 4°C before it was passed over a column (20x2.5 cm) of lysine-Sepharose equilibrated with 0.1 mol/L NH4HCO3, pH 8.3. The K5-protease domain (EDP III, M, 38 000 D), which did not bind to the column, was further purified by chromatography on Sephadex G-75. The material that eluted with 200 mmol/L L-aminocaproic acid contained the K1-3 (EDP I, M, 38 000 D) and K4 (EDP II, M, 11 000 D) domains of Glu-plasminogen. These were separated by gel filtration on a column (93×2.5 cm) of Sephadex G-75 equilibrated with 0.1 mol/L NH4HCO3. The purity of the three domains was judged to be >95% on the basis of SDS-PAGE using 8% to 25% Phast gels.

Modifications of r-Apo(a)

Desialylation of 125I-r-apo(a) was performed as follows: 130 µg of 125I-r-apo(a) in 100 mmol/L sodium acetate (pH 6.5) was incubated at 37°C with 0.05 U of C. perfringens neuraminidase (attached to agarose beads) for 18 hours with constant shaking. At the end of the incubation period, the agarose-attached neuraminidase was removed by low-speed centrifugation. SDS-PAGE (3% to 12.5% gradient) indicated that this treatment did not result in proteolytic degradation of the 125I-r-apo(a). The actual amount of sialic acid released after neuraminidase treatment was 85% to 95% as determined by the method of Warren. For one experiment (see "Discussion"), a 0.75-mg aliquot of neuraminidase-treated 125I-r-apo(a) was incubated with 30 mL of endo-N-acetylgalactosaminidase (Streptococcus pneumoniae) and 20 U of peptide-N-glycosidase F (Flavobacterium meningosepticum) for 18 hours at 24°C, both enzymes were purchased from Oxford GlycoSystems, Inc. 125I-r-apo(a) was denatured, reduced, and
carboxymethylated according to Li et al. Briefly, \(^{125}\text{I}-r\text{-apo(a)}\) (150 \(\mu\text{g}\)) in 0.5 mol/L Tris-HCl, pH 8.0, was denatured by the addition of 6 mol/L guanidine-HCl and 50 mmol/L dithiothreitol. After incubation for 1 hour at 25°C under a nitrogen atmosphere, iodoacetic acid was added to a final concentration of 150 mmol/L, and the reactants were removed by dialysis. Authenticity of \(^{125}\text{I}-r\text{-apo(a)}\) reduction and denaturation was confirmed by SDS-PAGE.

**Antibodies**

Polyclonal IgG specific to the apo(a) moiety of human Lp(a) but nonreactive to plasminogen was prepared from goat anti-human Lp(a) serum that was passed sequentially over LDL, plasminogen, and Lp(a)-Sepharose 4B columns. The antibodies bound to the Lp(a)-Sepharose column were eluted with 0.1 mol/L glycine, pH 2.5, and were titrated immediately back to pH 7.4 with 1 mol/L tris(hydroxymethyl)aminomethane (Tris). The specificity of the antibody preparation was verified by Western blot and enzyme-linked immunosorbent assay. Rat anti-murine Fc-\(\gamma_2\) receptor antibody, 2.4 G2 (isotype 2B), was obtained from Pharmingen, and purified monoclonal murine IgG2a was from Chemicon International, Inc.

**\(^{125}\text{I}-\text{Lp(a)/Apo(a)}\) Degradation Assay**

Total cellular degradation of \(^{125}\text{I}-\text{labeled lipoprotein sample}\) was assayed as described by Goldstein et al. Cell monolayers were washed twice with PBS and once with DMEM/0.5% fatty acid-free bovine serum albumin (BSA) and then incubated with DMEM/0.2% BSA containing the indicated concentrations of \(^{125}\text{I}-\text{labeled Lp(a)}\) or \(r\text{-apo(a)}\). After a 3- to 5-hour incubation at 37°C, the amount of \(^{125}\text{I}-\text{labeled trichloroacetic acid-soluble material formed by the cells and excreted into the medium was assayed}\).

**Statistics**

Unless indicated otherwise, results are given as mean±SEM (n=3). Absent error bars signify SEM values smaller than the graphics symbols.

**Results**

**Trypsin and Cycloheximide Sensitivity of the Inducible Foam Cell Apo(a) Receptor Activity**

To determine whether a cell-surface protein was necessary for the foam cell Lp(a)/apo(a) receptor activity, receptor activity was assayed in unloaded (control) macrophages, foam cells, and trypsin-treated foam cells. The foam cell receptor activity described here recognizes the apo(a) moiety of Lp(a), so ligand degradation was assayed by use of \(^{125}\text{I}-\text{labeled r- apo(a)}\). The trypsin-treated foam cells appeared similar to the untreated foam cells by phase microscopy and excluded trypan blue. The data in Fig 1 show that foam cells degraded about fourfold more \(^{125}\text{I}-r\text{-apo(a)}\) than control macrophages, consistent with our previous findings.

Fig 1. Bar graph showing effect of trypsin treatment of foam cells on apolipoprotein(a) [apo(a)] receptor activity. Mouse peritoneal macrophages were incubated for 3 days in Dulbecco's modified Eagle's medium (DMEM)/10% lipoprotein-deficient serum alone (Control M0) or containing 25 \(\mu\text{g}\) acetyl-low-density lipoprotein/mL (Foam Cell). One set of foam cells was washed twice with phosphate-buffered saline (PBS) and incubated for 15 minutes at 37°C in DMEM/0.2% bovine serum albumin (BSA) containing 250 \(\mu\text{g}\) trypsin/mL; these cells were washed twice with DMEM/0.2% BSA and incubated an additional 15 minutes at 37°C in the same buffer containing 500 \(\mu\text{g}\) soybean trypsin inhibitor/mL (Trypsin-treated Foam Cell). All cells were incubated with 500 \(\mu\text{g}\) peroxidase-conjugated IgG for 1 hour at 37°C, washed twice with PBS, and incubated for 3 hours at 37°C in DMEM/0.2% BSA containing 25 \(\mu\text{g}\) soybean trypsin inhibitor/mL and 25 nmol/L \(^{125}\text{I}-\text{recombinant (r)-apo(a)}\) and assayed for total \(^{125}\text{I}-r\text{-apo(a)}\) degradation.

To determine whether the upregulation of the apo(a) receptor activity that occurs with macrophage cholesterol loading requires new protein synthesis, \(^{125}\text{I}-r\text{-apo(a)}\) degradation was compared in unloaded (control) macrophages, cholesterol-loaded macrophages (foam cells), and macrophages that were loaded with cholesterol in the presence of the protein synthesis inhibitor cycloheximide (Fig 2A). Note that cycloheximide does not block the ability of acetyl-LDL to load macrophages with cholesterol.

The data in Fig 1A show that incubation of cycloheximide during the cholesterol loading period almost totally blocked the upregulation of receptor activity. In contrast to these data, experiments using \(^{125}\text{I}-\text{acetyl-LDL}\) as the ligand (Fig 2B) showed that cholesterol loading did not lead to upregulation of the macrophage scavenger receptor (cf Reference 16), and foam cell scavenger receptor activity was not blocked by cycloheximide. Thus, the upregulation of apo(a) receptor activity on macrophages that occurs with cholesterol loading requires new protein synthesis.

The data in Fig 2A demonstrate another new point about the induction of the foam cell apo(a) receptor activity. Our previous studies used macrophages that had been subjected to relatively prolonged incubation periods (ie, 2 to 4 days) with lipoproteins or cholesterol. In the experiment in Fig 2A, however, macrophages were incubated with acetyl-LDL for only 4.5 hours. The data show that even after a relatively short period of cholesterol loading, apo(a) receptor activity was upregulated more than fourfold.
Degradation of Different Forms of Lp(a) by Foam Cells

Lp(a) is found to have a high degree of size heterogeneity, which is dependent on the number of repeating K42 units in the apo(a) moiety of Lp(a). Our previous work examined Lp(a) with only the smaller, more abundant isoforms of apo(a) (ie, fast ["F"] and "B"). To determine whether the foam cell receptor activity recognized Lp(a) with a larger isoform of apo(a), the degradation by foam cells of Lp(a) containing the small, fast form of apo(a) was compared with the degradation of Lp(a) containing a large, slow form. The data in Fig 3 show that Lp(a) with the larger, slow apo(a) was degraded by foam cells even somewhat better than Lp(a) with the smaller, fast apo(a). Thus, the foam cell Lp(a) receptor activity recognizes Lp(a) containing both small and large isoforms of apo(a).

Lp(a) is also found in rhesus monkeys, although the apo(a) moiety of this Lp(a) differs in several respects from human apo(a), including an inactive lysine-binding domain at K437 and a modification of the RGD peptide sequence at K435 of human apo(a). The data in Fig 3 show that foam cells degraded rhesus monkey Lp(a) to the same degree as human (fast isoform) Lp(a). Thus, neither an active K437 lysine-binding domain nor an intact K435 RGD sequence appears to be necessary for interaction with the foam cell Lp(a) receptor activity.

Ligand Competition and Antibody Studies

Since apo(a) shares 80% homology with plasminogen, we sought to determine whether the foam cell Lp(a)/apo(a) receptor activity recognized certain structural features of individual plasminogen domains. Although holoplasminogen does not appear to interact with the receptor activity, it was possible that certain apo(a)-like regions of plasminogen, separate from the conformational constraints of the whole molecule, could be recognized by the foam cell receptor. Thus, individual peptide segments of plasminogen, generated by elastase digestion, were tested for their ability to competitively inhibit 125I-r-apo(a) degradation by foam cells. These elastase degradation products (EDPs) in-
clude three sequences: EDP I, containing kringle 1, 2, and 3 [which are not found in apo(a)]; EDP II, containing kringle 4 (specifically K4$_3$); and EDP III, containing both kringle 5 and the protease domain.\textsuperscript{26} Fragments EDP II and EDP III inhibited the binding of 125I-plasminogen to U937 cells.\textsuperscript{24} The data in Fig 4A show, as before, that cholesterol loading of macrophages caused upregulation of an apo(a) receptor activity, and degradation of 125I-r-apo(a) by the cholesterol-loaded foam cells was markedly inhibited by excess unlabeled r-apo(a). In contrast, the same molar excess of each of the three elastase degradation products of plasminogen did not inhibit degradation of 125I-r-apo(a) by the foam cells. 125I-r-apo(a) degradation by foam cells was inhibited, however, by a polyclonal antibody that recognizes the apo(a) moiety of human Lp(a) but that does not cross-react with plasminogen (Fig 4B). Thus, although apo(a) shares extensive homology with plasminogen, the foam cell apo(a) receptor does not recognize sequences in apo(a) that are common to plasminogen.

**Effect of Apo(a) Desialylation and Reduction/Denaturation**

Since apo(a) is a richly sialylated glycoprotein,\textsuperscript{24,33} it was necessary to determine whether the foam cell receptor activity was a lectin that recognized the sialic acid residues of apo(a). Therefore, an aliquot of 125I-r-apo(a) was treated with neuraminidase such that 85% to 95% of the sialic acid residues were removed. The data in Fig 5 show that neuraminidase-treated 125I-r-apo(a), like untreated 125I-r-apo(a), was degraded quite well by foam cells but not by unloaded macrophages. In fact, total degradation of neuraminidase-treated 125I-r-apo(a) by foam cells was \( \approx \)1.5-fold greater than that of untreated 125I-r-apo(a). When the “specific” component of this value was determined by competition with excess unlabeled native r-apo(a), however, the resulting value was very similar to that of untreated 125I-r-apo(a). Thus, although a portion of neuraminidase-treated 125I-r-apo(a) may bind to other sites on foam cells in addition to the upregulated apo(a) receptor activity, the data indicate that the sialic residues of apo(a) are not necessary for interaction with the upregulated foam cell apo(a) receptor activity. In an additional experiment to test the possible role of other sugar moieties known to be present on apo(a),\textsuperscript{32,34} we followed the strategy of Hajjar and Reynolds.\textsuperscript{35} These investigators demonstrated that a 250-fold molar excess of d-fucose or d-galactose inhibited the binding of 125I-labeled tissue plasminogen activator to HepG2 cells. The data in the Table show that under conditions in which a 50-fold molar excess of unlabeled r-apo(a) inhibited 125I-r-apo(a) degradation by foam cells by 81%, none of five monosaccharides (d-fucose, d-galactose, d-mannose, d-galactosamine, or d-glucosamine) in 250-fold molar excess demonstrated any significant degree of inhibition of 125I-r-apo(a) degradation by the foam cells.

Last, we sought to determine whether the interaction of the foam cell receptor activity with apo(a), which has multiple disulfide bonds,\textsuperscript{24,33} was dependent on its secondary and tertiary structure. Therefore, an aliquot of 125I-r-apo(a) was reduced, denatured, and carboxymeth-
Many questions concerning the possible functional roles and mechanisms of Lp(a) in atherogenesis, inflammation, and other areas currently remain unanswered. Since macrophages are prominent in both atherosclerotic and inflammatory lesions and since Lp(a) is colocalized with macrophage foam cells in atheromatous lesions, understanding the interaction of Lp(a) with macrophage foam cells is crucial. The data in Fig 6 demonstrate two points: first, whereas the degradation of native 125I-r-apo(a) was increased by approximately 450% in foam cells, the degradation of reduced and denatured 125I-r-apo(a) was increased by only 28% in foam cells; second, the total amount of reduced and denatured 125I-r-apo(a) degraded by the foam cells was approximately half that of native 125I-r-apo(a). Thus, the foam cell receptor activity appears to recognize certain features in the secondary and/or tertiary structure of apo(a). The approximately 50% degradation observed with reduced and denatured 125I-r-apo(a) may represent either residual recognition by the foam cell apo(a) receptor or recognition by another receptor activity, since, unlike native 125I-r-apo(a), interaction with unlabeled macrophages was almost as great as with foam cells.

### Discussion

The possible functional roles and mechanisms of Lp(a) in atherogenesis, inflammation, and other areas currently remain unanswered. Since macrophages are prominent in both atherosclerotic and inflammatory lesions and since Lp(a) is colocalized with macrophage foam cells in atheromatous lesions, understanding the interaction of Lp(a) with macrophage foam cells is crucial.

#### Table: Effect of Monosaccharides on the Degradation of 125I-r-Apo(a) by Foam Cells

<table>
<thead>
<tr>
<th>Macrophage</th>
<th>Unlabeled Competitor</th>
<th>125I-r-Apo(a) Degradation, pmol·mg Cell DNA⁻¹·h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>None</td>
<td>34.0</td>
</tr>
<tr>
<td>Foam cell</td>
<td>r-Apo(a)</td>
<td>31.2</td>
</tr>
<tr>
<td>Foam cell</td>
<td>D-Fucose</td>
<td>164.7</td>
</tr>
<tr>
<td>Foam cell</td>
<td>D-Glucosamine</td>
<td>170.1</td>
</tr>
<tr>
<td>Foam cell</td>
<td>D-Manneose</td>
<td>164.5</td>
</tr>
<tr>
<td>Foam cell</td>
<td>D-Galactosamine</td>
<td>195.5</td>
</tr>
<tr>
<td>Foam cell</td>
<td>D-Glucosamine</td>
<td>158.0</td>
</tr>
</tbody>
</table>

125I-r-Apo(a) indicates recombinant apolipoprotein(a), Mouse peritoneal macrophages were incubated for 2 days in Dulbecco's modified Eagle's medium (DMEM)/10% lipoprotein-deficient serum alone (Control Mφ) or in the presence of 25 μg acetyl-low-density lipoprotein/mL (Foam Cell), and the cells were washed twice with phosphate-buffered saline. The control macrophages and one set of foam cells were incubated for 3 hours at 37°C with DMEM/0.2% bovine serum albumin containing 10 nmol/L 125I-r-apo(a). The rest of the foam cells were incubated with 10 nmol/L 125I-r-apo(a) plus a 50-molar excess of unlabeled r-apo(a) or a 250-molar excess of the indicated monosaccharides. Total degradation of 125I-r-apo(a) was then assayed. The values shown are duplicates, which varied by an average of <10%.

Macrophages may provide important insight into the pathophysiology and physiology of Lp(a). Our recent report of an Lp(a)/apo(a) receptor activity on macrophages that is upregulated by cholesterol loading provided the first convincing evidence that macrophages are able to internalize and lysosomally degrade native Lp(a).
Lp(a) and r-apo(a) in a regulated fashion. We now show that the receptor activity is sensitive to cell-surface proteolysis, that the cholesterol-induced upregulation is dependent on new protein synthesis, that foam cells can interact with different-size isoforms of Lp(a) as well as rhesus monkey Lp(a), and that the receptor activity recognizes certain structural features of apo(a) that are distinct from those of plasminogen even though the two proteins share 80% homology.

The most likely interpretation of the cell-surface proteolysis data is that the internalization and degradation of r-apo(a) by foam cells are mediated by a cell-surface receptor protein. Along these lines, we have prepared detergent-solubilized membrane proteins from foam cells, separated these proteins by native PAGE, and probed blots of these native gels with $^{125}$I-r-apo(a). These ligand blots have revealed a single band that binds $^{125}$I-r-apo(a) with many of the characteristics demonstrated for $^{125}$I-r-apo(a) interaction with intact foam cells, including calcium dependence, tryptophan sensitivity, and displacement by excess unlabeled r-apo(a) (G.A. Keesler, Y. Li, D.L. Eaton, and I. Tabas, unpublished data). Protein purification and inhibitory antibody generation will be needed, however, before we can determine whether this band includes a protein that is, in fact, responsible for the foam cell Lp(a)/apo(a) receptor activity. Nonetheless, our present data have revealed two important characteristics of the foam cell receptor activity in addition to its sensitivity to cell-surface proteolysis: induction can occur after a relatively short period of cholesterol loading (ie, 4.5 hours versus 2 to 4 days in our previous study), and cholesterol-mediated induction requires new protein synthesis (Fig 2). The requirement for new protein synthesis indicates that cholesterol or an intracellular cholesterol metabolite (eg, an oxysterol $^{37}$), rather than simply causing a preexisting cell-surface protein to become “exposed,” leads to the induction of either the Lp(a)/apo(a) receptor molecule itself or a protein that is critical for receptor function.

The ligand studies in this report have also revealed some important properties of the foam cell Lp(a)/apo(a) receptor activity. First, whereas our previous report used Lp(a) containing only small isoforms of apo(a) (ie, “F” and “B”), the data in Fig 3 reveal that foam cells can also internalize and degrade Lp(a) containing a very large (“S”) isoform of apo(a). In fact, the data show that the Lp(a) with the large isoform was internalized and degraded to an even greater extent (=1.6-fold, $P<.002$) by foam cells than Lp(a) with the small isoform. The reason for this difference is not known. If the concentration of $^{125}$I-Lp(a) used in this experiment (25 nmol/L) was below receptor saturation, one interpretation of these data could be that the Lp(a) with the large isoform had a greater affinity for the receptor than Lp(a) with the small isoform [eg, by virtue of a greater number of receptor-binding sites per Lp(a) particle resulting from the extra copies of K4 on the large isoform of apo(a)] (cf Reference 38 and below). Alternatively, if 25 nmol/L Lp(a) was above receptor saturation, this might mean that additional receptor sites were available for the large isoform of apo(a) [eg, by recognition of the large isoform apo(a) by a subpopulation of cell-surface apo(a) receptors that do not readily interact with the small isoform]. Since the fold increase in $^{125}$I-Lp(a) degradation by foam cells versus control macrophages in this experiment was even greater for Lp(a) with the large isoform (approximately eightfold) than for Lp(a) with the small isoform (approximately fourfold) (data not shown), these putative additional receptor sites would also be subject to cholesterol-induced upregulation. We attempted 4°C binding studies to distinguish between these and other possibilities; however, despite the high degree of specificity of the receptor activity as demonstrated by the 37°C $^{125}$I-r-apo(a) degradation data (eg, Fig 4), the degree of nonspecific binding of r-apo(a) to foam cells at 4°C was too high for accurate analysis (G.A. Keesler, Y. Li, D.L. Eaton, and I. Tabas, unpublished data). Nonetheless, in view of the tremendous size heterogeneity of Lp(a),$^{1,2}$ we now know from the data in Fig 3 that the interaction of foam cells with Lp(a) is not limited to Lp(a) containing small isoforms of apo(a).

The data in Fig 3 also show that rhesus monkey Lp(a) is internalized and degraded by foam cells to a similar degree as human Lp(a). This finding indicates that certain features on human apo(a) that are altered or absent on rhesus monkey apo(a) are not necessary for recognition by the foam cell receptor. These features include a cysteine-to-histidine substitution in the catalytic triad of the protease region of simian apo(a), absence of the K5-like domain, absence of a key tryptophan residue involved in a lysine-binding site, and an absence of the large isoform of apo(a) (cf Reference 38 and below). The alterations in the lysine-binding domains of simian apo(a) are particularly important: these changes, especially those in K437, are thought to be responsible for the decreased ability of monkey Lp(a) versus human Lp(a) to bind to lysine-Sepharose and U937 cells.$^{21}$ Thus, the fact that rhesus monkey Lp(a) interacts well with foam cells, together with our previous finding that the lysine analogue, e-aminocaproic acid, does not inhibit the interaction of human Lp(a) with foam cells,$^{16}$ indicates that interaction of apo(a) with the foam cell receptor does not involve the lysine-binding domains of apo(a).

Our ligand studies have also explored in detail the issue of whether the foam cell receptor activity recognizes a site on apo(a) that is in common with plasminogen, which shares 80% homology with apo(a).$^{38}$ Our previous publication mentioned that plasminogen did not compete for $^{125}$I-Lp(a) binding to foam cells.$^{16}$ This experiment, however, used a 4°C binding assay, which we now know includes nonspecific interactions (G.A. Keesler, Y. Li, D.L. Eaton, and I. Tabas, unpublished data). Furthermore, as mentioned in the “Results,” the conformational constraints of the intact plasminogen molecule may have masked its ability to competitively inhibit $^{125}$I-r-apo(a) uptake by foam cells even if there were a plasminogen-like region in apo(a) that was important in receptor binding. Thus, for these reasons together with the remarkably high degree of homology between apo(a) and plasminogen,$^{38}$ we thought that this issue needed to be investigated further. In the present report, we present several pieces of evidence that, when viewed together, strongly indicate that the receptor recognition site or sites on apo(a) include features of the molecule that are different from those shared with plasminogen. First, simian Lp(a), which lacks both the K5 domain of plasminogen and important structural features of the K4 domain of plasminogen (see above),
interacts well with foam cells (Fig 3). Second, individual elastase degradation products of plasminogen, which inhibit the binding of \(^{125}\text{I}-\text{plasminogen}\) to U937 cells, did not inhibit the degradation of \(^{125}\text{I}-\text{r-apo(a)}\) by foam cells (Fig 4A). Third, a polyclonal anti-Lp(a) antibody that recognizes apo(a) but not plasminogen blocked the degradation of \(^{125}\text{I}-\text{r-apo(a)}\) by foam cells (Fig 4B). Thus, the foam cell receptor probably recognizes some site or sites in apo(a) that are not homologous with plasminogen despite the extensive homology between the two proteins.

What might be the receptor-recognition site or sites in apo(a)? One difference between apo(a) and plasminogen is the presence of \(N\)-linked oligosaccharide groups on the K4 domains of apo(a) but not on the K4 domain of plasminogen. In addition, the interkringle sequences of apo(a) have many more \(O\)-linked oligosaccharides than the interkringle sequences of plasminogen. To begin to address the possible importance of the sugar residues of apo(a) in interaction with the foam cell receptor, we tested the possibility that the exposed sialic acid residues of the oligosaccharides on r-apo(a) were necessary for the interaction of r-apo(a) with foam cells. The data in Fig 5, however, indicate that this is not the case, since \(^{125}\text{I}-\text{r-apo(a)}\) in which \(\approx 90\%\) of the sialic acid residues had been removed demonstrated no decrease in ability of the r-apo(a) to be internalized and degraded by foam cells. In addition, as shown in the Table, other sugar moieties known to be present on apo(a) did not compete for the interaction of \(^{125}\text{I}-\text{r-apo(a)}\) with foam cells (cf Reference 35). We also attempted to remove oligosaccharides from the ligand by treating desialylated \(^{125}\text{I}-\text{r-apo(a)}\) with a combination of endo-\(\alpha\)-N-acetylglactosaminidase (\(O\)-glycanase) and peptidase-N-glycosidase F. Although these enzymes work optimally on reduced protein substrates, we had to carry out the glycosidase reactions on native \(^{125}\text{I}-\text{r-apo(a)}\), since reduction of apo(a) alters its interaction with macrophages and foam cells (see Fig 6). As expected, therefore, SDS-PAGE analysis of the treated \(^{125}\text{I}-\text{r-apo(a)}\) revealed that oligosaccharide removal was not complete. Nonetheless, there was a greater decrease in the apparent \(M_r\) of the treated \(^{125}\text{I}-\text{r-apo(a)}\) compared with desialylated \(^{125}\text{I}-\text{r-apo(a)}\), indicating additional sugar removal. Under these conditions, glycosidase treatment did not decrease the interaction of apo(a) with foam cells: the upregulation of ligand degradation in foam cells compared with control macrophages was 4.8-fold for untreated \(^{125}\text{I}-\text{r-apo(a)}\) and 5.2-fold for glycosidase-treated \(^{125}\text{I}-\text{r-apo(a)}\) (data not shown). Thus, the sugar chains removed by this treatment, which probably include exposed oligosaccharides in regions of apo(a) lacking disulfide bonds (eg, the \(O\)-linked oligosaccharides in the interkringle sequences), are not necessary for the interaction of apo(a) with foam cells.

Also relevant to the carbohydrate issue are the data in Fig 6, which show that \(^{125}\text{I}-\text{r-apo(a)}\) degradation by foam cells was decreased by half when the ligand was reduced and denatured. In fact, the residual degradation observed may represent another receptor activity, since it is not substantially upregulated by cholesterol loading. Since reduction and denaturation of apo(a) should not alter the sugar moieties of apo(a) and might even increase the exposure of the oligosaccharide chains, it is unlikely that the receptor recognizes exposed sugar groups. Rather, since kringles are known to contain disulfide bonds that impart a particular tertiary structure to the peptide, the data may indicate that certain aspects of the kringle structures of apo(a) are important in receptor recognition.

In this regard, perhaps the most obvious difference between the foam cell ligand apo(a) and the nonligand plasminogen is the large number of K4 peptides in tandem in apo(a) versus a single K4 peptide in plasminogen. Thus, the foam cell receptor might recognize a motif containing two or more K4 segments linked together; this motif might include the kringle structures themselves or, possibly, one or more of the interkringle sequences, which also differ from the interkringle sequences of plasminogen. Consistent with these ideas is the finding that under conditions in which a 50-fold molar excess of unlabeled r-apo(a) caused 83\% inhibition of \(^{125}\text{I}-\text{r-apo(a)}\) degradation by foam cells, a 400-fold molar excess of a single-copy recombinant K4 peptide (cf Reference 28) caused only 21\% inhibition (G.A.K., Y.L, G.M.F., and I.T., unpublished data). Although the relatively weak competitive inhibition by the recombinant peptide might be explained by its expression in a noneukaryotic system (ie, not glycosylated), the peptide is recognized by a monoclonal antibody that can distinguish native from reduced K4, and the recombinant kringle possesses other features that suggest that its overall conformation is normal. Thus, two or more kringles in tandem may be necessary for optimal interaction with the foam cell receptor. Further testing of this idea, including the determination of whether specific K4 sequences or interkringle sequences are involved and how many K4 sequences in tandem are necessary for receptor interaction, must await the availability of a series of recombinant kringle constructs.

In summary, we have demonstrated several important characteristics of the foam cell Lp(a)/apo(a) receptor activity, including its susceptibility to cell-surface proteolysis, the dependence of its upregulation on new protein synthesis, and its recognition of structural features on apo(a) that are not shared with its homologue, plasminogen. Current work is directed toward purifying the receptor protein and determining the functional roles of the receptor activity. With respect to functional roles, it is possible that the receptor may allow macrophage foam cells to scavenge Lp(a) or apo(a) and thus prevent possible antifibrinolytic \(\text{or smooth muscle cell proliferative effects}\) of these molecules. The receptor activity may also be involved in some aspect of the inflammatory response, since interferon-gamma down-regulates the Lp(a)/apo(a) receptor (Reference 40 and P.J. Skiba, G.A. Keesler, and I. Tabas, manuscript submitted). Furthermore, since the receptor activity is seen in mouse macrophages (in addition to human macrophages) and since mice normally do not have Lp(a) or apo(a), the receptor undoubtedly has at least one other ligand. The identification of this putative ligand and the determination of its possible relationship to foam cells and atherosclerosis may give further insight into the physiology of the foam cell Lp(a)/apo(a) receptor activity.

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