Cholesteryl Ester Loading of Mouse Peritoneal Macrophages Is Associated With Changes in the Expression or Modification of Specific Cellular Proteins, Including Increase in an α-Enolase Isoform

Lori A. Bottalico, Nancy C. Kendrick, Angelica Keller, Yueqing Li, and Ira Tabas

This report explores the hypothesis that massive cholesteryl ester (CE) accumulation in macrophages, such as that occurring in atheroma foam cells, results in changes in the expression or modification of specific cellular proteins. Two-dimensional (2-D) gel electrophoretic patterns of metabolically labeled cellular proteins from mouse peritoneal macrophages that were loaded with CE (through incubation with acetylated low-density lipoprotein [acetyl-LDL] for 4 days) were compared with those of control macrophages. Densitometric analysis of 2-D gel autoradiograms from the cell lysates revealed statistically significant changes in seven cellular proteins (five decreases and two increases). The changes in protein expression (foam cell versus control) ranged from a 458±164% (p<0.001) increase to a 35±34% (p<0.001) decrease (n=U). Incubation of macrophages with β-very low-density lipoprotein, which also increased the CE content of macrophages (albeit to a lesser extent than acetyl-LDL), resulted in changes in five of the seven proteins. In contrast, incubation of cells with LDL, fucoidan, or latex beads, none of which caused CE accumulation, did not lead to significant changes in four of these five proteins. One of these four proteins, which increased fourfold to fivefold in foam cells (Af=49,000; isoelectric point of 6.8), was purified by preparative 2-D gel electrophoresis. Internal amino acid sequence of cyanogen bromide fragments of this protein as well as Western blot analysis identified this protein as an isoform of α-enolase. The increased expression of this α-enolase isoform, which was seen as early as day 2 of acetyl-LDL incubation of the macrophages, was diminished by including an inhibitor of cholesterol esterification during the acetyl-LDL incubation period. In conclusion, macrophage foam cell formation is associated with distinct changes in protein expression, including a marked increase in an isoform of α-enolase, suggesting a specific biological adaptation to CE loading. (Arteriosclerosis and Thrombosis 1993;13:264-275)

KEY WORDS • macrophages • foam cells • α-enolase • two-dimensional gel electrophoresis • protein expression

The cholesteryl ester (CE)-loaded macrophage or "foam cell" is a prominent cell type in atherosclerotic lesions. Macrophage foam cells originate from blood-borne monocytes that have migrated into the endothelium, where they subsequently differentiate and become CE loaded. CE loading of macrophages is initiated by the endocytosis of certain "atherogenic" lipoproteins; the lipoprotein-derived CE is hydrolyzed inside the cell to free cholesterol, which together with endogenous cellular cholesterol, is reesterified to CE by the microsomal enzyme acyl-coenzyme A:cholesterol acyltransferase (ACAT). Much of the emphasis of research on foam cell formation has been on the identification of lipoproteins and their receptors that are thought to be involved in macrophage CE loading. These studies, using tissue culture macrophages, have revealed that modified forms of LDL (e.g., acetyl-low-density lipoprotein [acetyl-LDL]) can cause extensive foam cell formation in culture through uptake by the macrophage scavenger receptor. Furthermore, there is evidence to suggest that the scavenger receptor ligand, oxidized LDL, may have an important role in foam cell formation in vivo. Although under most circumstances native LDL does not lead to macrophage foam cell formation in culture, another lipoprotein ligand for the LDL receptor, β-very low-density lipoprotein (β-VLDL), does result in substantial CE loading of macrophages.
In contrast to the vast amount of information available on the identification of lipoproteins and receptors that may be involved in foam cell formation, very little is known about the functions of the macrophage foam cell or whether CE loading changes the physiology of the macrophage. This report begins to approach these important issues by determining whether CE loading of macrophages changes the patterns of protein expression. In particular, metabolically labeled cellular proteins of control and CE-loaded mouse peritoneal macrophages were separated by two-dimensional (2-D) gel electrophoresis, which has the power to resolve 1,000–2,000 individual proteins. This report describes specific changes in protein expression or modification that are associated with CE loading of macrophages. In addition, an isoform of α-enolase that is increased in foam cell macrophages is characterized.

**Methods**

**Materials**

Tissue culture media and reagents were obtained from GibCo Laboratories (Grand Island, N.Y.). Fetal calf serum (FCS) (lot No. 2151908) was obtained from Hyclone Laboratories (Logan, Utah). Lipoprotein-deficient serum (LPDS) was prepared from FCS by preparative ultracentrifugation (d=1.21 g/mL). Corning (24-mm-diameter) and Falcon (16-mm- and 35-mm-diameter) tissue culture dishes were purchased from Fisher Scientific Co. (Springfield, N.J.). 35S-labeled methionine/cysteine, [32P]orthophosphate, [1-14C]palmitate, [9,10-3H]myristate, and [9,10-3H]-palmitate were purchased from New England Nuclear (Boston, Mass.). Sodium dodecyl sulfate (SDS) and all organic solvents were purchased from Fisher. Osmotic lysis buffer (10 mM tris(hydroxymethyl)aminomethane [Tris, pH 7.4] and 0.3% SDS) and SDS boiling buffer (60 mM Tris [pH 6.8], 5% SDS, 5% β-mercaptoethanol, and 10% glycerol) were purchased from Kendrick Laboratories (Madison, Wis.). PPACK (N-phenylalanyl-propyl-arginyll choloromethyl ketone) was purchased from Calbiochem Biochemicals (San Diego, Calif.). All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.).

The rabbit anti-mouse α-enolase antiserum was prepared by injecting rabbits with purified mouse brain αα enolase as previously described; the antiserum recognized αα enolase but not γγ enolase. The cDNA probe specific for αα-enolase mRNA, corresponding to nucleotides 1,400–1,669 in the 3’ untranslated region, was generously provided by Dr. Monique Lazar, College de France, Paris; this sequence is available from EMBL/Gen Bank Data library under accession number X52379. Compound 58-035 (3-[decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenyl-ethyl]propanamide) was generously provided by Dr. John Heider of Sandoz, Inc. (East Hanover, N.J.). Stock solutions (5 mg/mL) were prepared in dimethyl sulfoxide.

**Cells**

Monolayer cultures of resident mouse peritoneal macrophages were prepared from female ICR mice (22–25 g) as described previously. For metabolic labeling studies, 1×10⁶ cells were plated per 24-mm polyllysine-coated dish in Dulbecco's modified Eagle's medium (DMEM)/10% (vol/vol) FCS containing 100 units/mL penicillin, 100 μg/mL streptomycin, and 200 μM glutamine. For enolase enzyme assays and Northern blot analysis experiments, 2–3×10⁶ cells were plated per 35-mm polyllysine-coated dish. After 1 hour, the cells were washed twice with phosphate-buffered saline (PBS) and incubated for 4 days in a 37°C CO2 tissue culture incubator in DMEM/10% (vol/vol) LPDS in the absence or presence of lipoproteins. Fresh media were added on the second and fourth days.

**Lipoproteins**

Lipoproteins were isolated by preparative ultracentrifugation: LDL (density, 1.020–1.063 g/mL) from fresh human plasma and β-VLDL (density <1.006 g/mL) from the plasma of cholesterol-fed male New Zealand White rabbits (4–8 weeks on a diet of Purina rabbit chow supplemented with 0.2% cholesterol and 10% soybean oil [wt/wt]). Acety-LDL was prepared by acetylation of LDL with acetic anhydride as described previously. Acetyl-LDL preparations were tested for endotoxin using the Limulus lysate assay (Scientific Associates Inc., St. Louis, Mo.). There was <0.006 ng/mL endotoxin detected in 44 μg acety-LDL. All lipoprotein preparations were stored under argon at 4°C and used within 4 weeks.

**[35S]Methionine Pulse Labeling of Proteins**

On the morning of the fifth day of macrophage incubation, the media were removed and the cells washed with PBS. The cells were then incubated for 3 hours with DMEM (minus unlabeled methionine) containing 0.5 mL 300 μCi/mL 35S-labeled methionine/cysteine. In certain experiments, the incubation time was 6 hours, and the media contained 0.5% LPDS to keep the cells healthy for this extended period of time. No differences were observed between the 3- and 6-hour incubation conditions with respect to the synthetic patterns of the proteins of interest.

**Steady-State [35S]Methionine Labeling of Proteins**

One hour after plating, the macrophages were washed, then incubated for 4 days with DMEM/10% LPDS containing 20 μCi/mL. [35S]methionine/cysteine±25 μg/mL acetyl-LDL. Cells were fed with fresh media containing the 35S label±acetyl-LDL on days 2 and 4. For the time-course experiment, cells were prepared for 2-D gel electrophoresis on the indicated days after plating.

**[14C]Fatty Acid/[3H]Fatty Acid Pulse Labeling of Proteins**

On the fifth day of macrophage incubation, the media were removed and the cells washed with PBS. The cells were then incubated for 3 hours with DMEM/5% LPDS (minus methionine) containing 20 μCi/mL of [1-14C]-palmitic acid or 20 μCi/mL of [1-3H]myristic acid. In a second set of experiments, the foam cells were incubated for 3 hours with DMEM/5% LPDS containing 50 μCi/mL each [9,10-3H]-palmitate and [9,10-3H]-myristate.

**3P Labeling of Cells**

On the fourth night of macrophage incubation with acetyl-LDL, the media were removed and the cells...
washed with PBS. The cells were then incubated for 14 hours in DMEM that was 95% phosphate-free and contained 10% LPDS, 25 μg/mL acetyl-LDL, and 250 μCi/mL $[^{32}P]$orthophosphate. Alternatively, on the morning of the fifth day of macrophage incubation, the media were removed and the cells washed with PBS. The cells were then incubated for 3 hours with 95% phosphate-free DMEM containing 10% LPDS and 300 μCi/mL $[^{32}P]$orthophosphate.

2-D Gel Electrophoresis Sample Preparation

For the 2-D gels to be run at Kendrick Laboratories (see below), the labeled cell monolayers were washed three times with PBS on ice and solubilized in 100 μL preboiled (100°C) osmotic lysis buffer. An equal volume of SDS boiling buffer was then added. Alternatively, for the gels to be run at Cold Spring Harbor Laboratories (see below), cells were solubilized in preboiled 48 mM Tris-Cl, pH 8.0, 0.3% SDS, and 140 mM β-mercaptoethanol. DNase/RNase was also added to all cell lysates. The cell lysates were scraped with a rubber policeman, brought up and down in a 26-gauge needle, boiled, and stored at −70°C until 2-D gel electrophoresis was performed.

For preparative 2-D gel electrophoresis, $[^{32}P]$orthophosphate labeling experiments, and fatty acid labeling experiments, the cell lysates were lipid extracted. Briefly, cell lysates were solubilized in osmotic lysis buffer plus DNase/RNase and then lipid extracted in a 20-fold volume excess of 3:2 ethanol/ethyl ether at 4°C for 1 hour. The samples were then centrifuged at 1,500 rpm for 30 minutes to pellet the lipid-nonextractable material. The organic supernate was removed by suction, and the pellets were completely dried under nitrogen. The samples were then resuspended in a suitable volume of 1:1 SDS boiling buffer/double-distilled water (vol/vol) and boiled.

2-D Gel Electrophoresis and Autoradiography

2-D gel electrophoresis was performed at either Cold Spring Harbor Laboratories (Cold Spring Harbor, N.Y.) or Kendrick Laboratories (Madison, Wis.). The gels run at Cold Spring Harbor were run according to established procedures, with pH 4–8 or broad-range ampholytes in the first dimension and 10–12.5% acrylamide in the second dimension. The gels run at Kendrick Laboratories were run according to the method of O'Farrell as follows. Isoelectric focusing (IEF) was carried out in glass tubes of 2.0-mm inner diameter, using 2.0% Resolutes (pH 4–8; BDH from Hoefer Scientific Instruments, San Francisco, Calif.) for 9,600 volt-hours. One microgram of an IEF internal standard, vitamin D-dependent calcium binding protein, molecular weight 27,000, isoelectric point (pI) 5.2, was added to the samples. The final tube-gel pH gradient was measured by a surface pH electrode. After equilibration for 10 minutes in SDS sample buffer (10% glycerol, 50 mM dithiothreitol, 2.3% SDS, and 0.0625 M Tris [pH 6.8]), the tube gels were sealed to the top of a stacking gel that was on top of a 10% or 7% slab gel (0.75 mm thick), and SDS slab gel electrophoresis was carried out for about 4 hours at 12.5 mA per gel. Molecular weight standards were added to the agarose that sealed the tube gel to the slab gel. The slab gels were fixed and stained in a solution of 10% acetic acid, 50% ethanol, and 0.1% Coomassie brilliant blue R-250 overnight and then dried. Autoradiography was carried out using Kodak X-OMAT AR film, and the autoradiograms were analyzed by densitometry using a Molecular Dynamics computing densitometer model 300A with IMAGE-QUANT software. Molecular weight and pI values for protein spots of interest were determined by computer analysis of the autoradiograms using a set of protein standards at Cold Spring Harbor Laboratories.

Preparative 2-D Gel Electrophoresis, Cyanogen Bromide Cleavage, Blotting, and Protein Microsequencing

To obtain enough material for protein sequencing, 31 2-D gels (approximately 300 μg total cell protein run per gel) were run at Kendrick Laboratories and stained with Coomassie blue as described above. The spot of interest was cut from each gel with a scalpel. The 31 spots were combined in a 1.2-ml Eppendorf tube and immediately dried under nitrogen to avoid oxidation of methionine residues. Cyanogen bromide cleavage was then carried out according to a modification developed at Kendrick Labs of previously described procedures, with Cyanogen bromide (75 μL of 50 mg/ml) in 70% formic acid was added to the tube containing the cutout spots, and cleavage was allowed to proceed for 15 hours in the dark at room temperature. The cleavage mixture was dried under nitrogen, then rehydrated and dried three times to remove the formic acid and cyanogen bromide. After the fourth dry-down, 75 μL SDS sample buffer was added and the acrylamide allowed to rehydrate for 2 hours. After rehydration, the acrylamide pieces were polymerized in the well of a 10% acrylamide stacking gel, overlaying a 1.5-mm-thick 16.5% acrylamide peptide gel poured the previous day. The peptide slab gel was run as described at 10 mA per gel overnight before transblotting onto polyvinylidene difluoride (PVDF) (Immobilon, Millipore Corp.). The transblotting was carried out using a Tris/glycine buffer. The PVDF blot was stained in 50% methanol and 0.1% Coomassie blue for 5 minutes, destained in 50% methanol for 2 minutes, and rinsed three times (5 minutes per rinse) in deionized water.

Protein bands were excised and subjected to N-terminal microsequencing using an Applied Biosystems sequencer (model 470A) equipped with an on-line phenylthiohydantoin analyzer (model 120A).

Fatty Acid Analysis of α-Enolase in Foam Cells

Delipidated cell lysates from $^{14}C$-fatty acid-labeled cells or $^3H$-fatty acid labeled cells were subjected to preparative 2-D gel electrophoresis as described above. Gel pieces containing spot A and spot B α-enolase (see text) were excised from preparative dried gels and allowed to rehydrate in double-distilled water for 15 minutes at 55°C for 15 minutes. The gel pieces were homogenized in 0.1% SDS/0.05 M NH4HCO3/5% β-mercaptoethanol, heated at 100°C for 5 minutes, then incubated overnight with shaking, in 0.2 mg/mL trypsin. The gel extract was then filtered through a 0.2-μm filter to obtain the trypsinized fragments, which were extracted with petroleum ether to remove any noncovalently associated label. The remaining aqueous solution was lyophilized, resuspended in 1 ml 6 M HCl containing 500 μg each of myristic and palmitic acid.
standards, and hydrolyzed at 110°C for 24 hours in a vacuum-sealed tube to break all lipids and peptides down to their simplest structural components. The hydrolysate was then extracted with petroleum ether three times. The ether extracts were dried under nitrogen and subjected to thin-layer chromatography using a petroleum ether/ethyl ether/acetic acid (80:20:1 vol/vol/vol) solvent system. The entire sample lane was scraped in sections and counted. The Rs value of the labeled hydrolysate sample was compared with those of the fatty acid standards run in adjacent lanes.

**Alkaline Phosphatase Treatment of Cell Lysates**

On the fifth day of macrophage incubation, the media were removed and the cells washed with PBS. The cells were then pulsed for 3 hours with [35S]methionine as described above. The cells were placed on ice, washed with PBS, and scraped into 10 mM Tris, pH 8.2 (containing DNase/RNase), with a rubber policeman. The following protease inhibitors were added at the indicated final concentrations: 12.5 μM pepstatin, 2 mM leupeptin, 2 mM phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 62.5 μg/mL antipain, and 2 μg/mL a-2 macroglobulin. The cell lysates were then sonicated for 10–15 seconds at 4°C. Sample volumes were adjusted to a final volume of 200 μL, with a final concentration of 50 mM Tris [pH 8], 1 mM ZnCl2, and 1 mM MgCl2, and were incubated with 10 units of alkaline phosphatase (Sigma, P2276) for 1.5 hours at 37°C. Samples not receiving alkaline phosphatase received instead the following phosphatase inhibitors in a volume corresponding to 50% of total binding values. The binding values measured in the presence of 6 mM e-amino-n-caproic acid were subtracted from the total binding values. The percent of total binding that was competed by e-amino-n-caproic acid was the same for control macrophages and foam cell macrophages: 85% for the 37°C experiments and 60% for the 4°C experiments.

**Whole-Cell [125I]-Plasminogen Binding**

[125I]-plasminogen was prepared by a modified chloramine T method.28 Cells were plated onto regular (i.e., not polylucite-coated) tissue culture wells for the whole-cell binding studies. Control macrophages and CE-loaded foam cells were washed with PBS on the morning of the fifth day in culture. For 4°C binding studies, cells were precooled in DMEM (minus lysine)/0.2% bovine serum albumin (BSA) at 4°C for 30 minutes. Cells were then incubated with 0.56 μM [125I]-plasminogen in DMEM (minus lysine)/0.2% BSA containing 10−3 M of the protease inhibitor PPACK for 1 hour. 6 mM e-amino-n-caproic acid was added to some wells to determine lysine site-specific binding.29 For 37°C binding studies, cells were incubated in DMEM (minus lysine)/0.2% BSA containing 10−3 M PPACK, 0.56 μM [125I]-plasminogen, and ±6 mM e-amino-n-caproic acid for 1 hour. After the binding experiments, cells were washed sequentially in PBS containing calcium and magnesium, then PBS/Ca²+/Mg²+ containing 0.2% BSA, and finally in PBS. The cell lysates were then solubilized in 0.2N NaOH and counted. The binding values measured in the presence of 6 mM e-amino-n-caproic acid were subtracted from the total binding values. The percent of total binding that was competed by e-amino-n-caproic acid was the same for control macrophages and foam cell macrophages: 85% for the 37°C experiments and 60% for the 4°C experiments.

**Northern Blot Analysis**

Resident mouse peritoneal macrophages were incubated in the absence or presence of 25 μg/mL acetylated-LDL as described above. On the fifth day of culture, total RNA was isolated from the cells according to the procedure of Chomczynski and Sacchi30 and subjected to Northern blot analysis as described previously.31 RNA was normalized by loading equal masses of total RNA as determined by absorbance at 260 nm. A 270-base cDNA corresponding to the 3'-untranslated region of mouse α-enolase mRNA was radio-labeled with 32P using a random priming method32 and hybridized to the membrane at 42°C in the presence of 40% formamide. The membrane was washed with 2x saline-sodium citrate containing 0.1% SDS at 50°C and exposed to an x-ray film. The relative intensities of the bands were determined by densitometric scanning of the autoradiograms using a Molecular Dynamics computing densitometer (model 300A) with image-quanta software.

**[125I]-Plasminogen Ligand Blotting**

[35S]methionine steady-state–labeled cell lysates were prepared for 2-D gel electrophoresis, and the gels were run as described above. The slab gels were transferred to transfer buffer (12.5 mM Tris [pH 8.8], 86 mM glycine, and 10% methanol) and transblotted onto PVDF paper overnight at 4°C at 200 mA and 100 V per two gels. The entire sample lane was exposed to an x-ray film. The autoradiograms containing DNAse/RNase), with a rubber policeman. The following protease inhibitors were added at the indicated final concentrations: 12.5 μM pepstatin, 2 mM leupeptin, 2 mM phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 62.5 μg/mL antipain, and 2 μg/mL α-2 macroglobulin. The cell lysates were then sonicated for 10–15 seconds at 4°C. Sample volumes were adjusted to a final volume of 200 μL, with a final concentration of 50 mM Tris [pH 8], 1 mM ZnCl2, and 1 mM MgCl2, and were incubated with 10 units of alkaline phosphatase (Sigma, P2276) for 1.5 hours at 37°C. Samples not receiving alkaline phosphatase received instead the following phosphatase inhibitors in a volume corresponding to 50% of total binding values. The binding values measured in the presence of 6 mM e-amino-n-caproic acid were subtracted from the total binding values. The percent of total binding that was competed by e-amino-n-caproic acid was the same for control macrophages and foam cell macrophages: 85% for the 37°C experiments and 60% for the 4°C experiments.

**Western Blot Analysis**

[35S]methionine-labeled cell lysates were prepared for 2-D gel electrophoresis, and the gels were run as described above. The slab gels were transferred to transfer buffer (12.5 mM Tris [pH 8.8], 86 mM glycine, and 10% methanol) and transblotted onto PVDF paper overnight at 4°C at 200 mA and 100 V per two gels. The PVDF membrane was then air dried, wrapped in plastic, and exposed to film at −70°C to obtain the [35S]methionine-labeled protein pattern. Western blot analysis was carried out using rabbit anti-mouse α-enolase antisem (1:2,000 dilution) according to the methods of the Amer sham Co., Arlington Heights, Ill.). The antibody-antigen complex was visualized by color reaction of a horseradish peroxidase–coupled secondary (anti-rabbit) antibody with N'-N'-diaminobenzidine.

**Enolase Enzyme Assays**

Cytosolic enolase activity was measured spectrophotometrically at 240 nm as the conversion of sodium 2-phospho-d-glycerate to phosphoenolpyruvate as described previously.26 The reaction was linear for 5 minutes for both control and experimental samples; the time chosen for determination of enolase activity was 4 minutes.
incubation in PBS/0.2% BSA, the blots were incubated in 7.5–10 μM 125I-plasminogen in PBS/0.2% BSA for 3–4 hours at room temperature. The blots were then washed extensively (1–2 hours with four to five buffer changes) in PBS/0.2% BSA with 0.5 M NaCl. The blots were then covered with plastic wrap and two sheets of 3-mm filter paper so that only 125I-derived radioactivity would be exposed to film at -70°C.

**Cellular Lipid and Protein Assays**

Cell monolayers were extracted twice with 1 mL hexane/isopropanol (3:2 vol/vol) for 1 hour at room temperature. The extracts were dried under nitrogen and analyzed by gas-liquid chromatography as described previously. 33 β-Sitosterol was included in the extracts as an internal standard. Cell extracts were assayed for protein by the method of Lowry et al. 34

**Results**

**Changes in Protein Synthesis Pattern Observed With Acetyl-LDL Incubation of Mouse Peritoneal Macrophages**

Resident mouse peritoneal macrophages were incubated for 4 days in the absence or presence of 25 μg/mL acetyl-LDL. On the fifth day, the cells were metabolically labeled with 35S-methionine/cysteine for 3 or 6 hours, and the cell lysates were then subjected to 2-D gel electrophoresis. In the absence and presence of acetyl-LDL, the cells contained 9 and 554 μg CE per milligram of cell protein, respectively. The macrophages incubated with acetyl-LDL had multiple cytoplasmic inclusions visible by phase-contrast microscopy. Thus, by both biochemical and morphological criteria, the macrophages incubated with acetyl-LDL were foam cells.

Figure 1 shows representative autoradiograms of 35S-labeled proteins from cell lysates of control macrophages (panel A) and acetyl-LDL–incubated cells (panel B). Equal trichloroacetic acid–precipitable counts were loaded onto 2-D gels for the control and foam cell samples. To correct for any pipetting errors, however, the raw densitometry data on each gel were normalized to at least four protein spots that demonstrated no change between foam cell and control samples in each experiment. The intensities of spots analyzed by densitometry were within the linear range of the densitometer. Densitometric analysis of individual pairs of autoradiograms revealed 10–20 protein spots that were increased or decreased in foam cells versus control macrophages. However, careful analysis of pairs of autoradiograms from nine to 12 separate experiments revealed reproducible changes in the intensity of eight protein spots (five decreases and three increases) in the foam cells. One of these eight spots that increased in foam cells had the M₈ and pI of apoprotein E (marked by the solid arrowhead in Figure 1B). Since the induction of apoprotein E in cholesterol-loaded macrophages has already been established, 31,32 an analysis of the other seven spots (labeled 1C–7C in Figure 1) was carried out.

The M₈'s, pI's, and average fold changes of these seven spots are listed in Table 1. The data reveal that...
the proteins with altered expression had $M_r$'s ranging from 21,450 to 80,840 and $p_i$'s from 5.22 to 6.80. The changes in protein expression (foam cell versus control) ranged from a 458±164% ($p<0.001$) increase to a 35±34% ($p<0.001$) decrease.

To determine which of these changes was correlated with CE loading, the cells were incubated for 4 days with 25 $\mu$g/mL of another atherogenic lipoprotein, $\beta$-VLDL. $\beta$-VLDL incubation results in macrophage CE accumulation (289 $\mu$g CE per milligram of cell protein), albeit to a lesser extent than acetyl-LDL incubation (above). As shown by the data in Table 2, most of the proteins that were increased or decreased in response to acetyl-LDL incubation were increased or decreased, respectively, in macrophages incubated with $\beta$-VLDL. Although the magnitude of the changes was less than that observed with acetyl-LDL treatment in some cases, it appears that the changes in at least five of the proteins (1C, 2C, 3C, 5C, and 7C) were correlated with CE accumulation (i.e., regardless of the lipoprotein ligand and endocytic pathway used).

A series of control incubations with LDL, fucoidan, or latex beads was then carried out to determine whether the changes in proteins 1C, 2C, 3C, 5C, and 7C (above) were correlated with CE loading. To control for lipoprotein addition, cells were incubated for 4 days with 25 $\mu$g/mL native LDL, which does not result in CE accumulation (10 $\mu$g CE per milligram of cell protein). To control for a scavenger receptor-ligand interaction (i.e., versus CE loading per se), cells were treated for 4 days with 25 $\mu$g/mL fucoidan, a nonlipoprotein ligand for the scavenger (i.e., acetyl-LDL) receptor, which results in no CE accumulation by the cell. Last, latex beads are phagocytosed by macrophages and remain as cytoplasmic inclusions, resulting in an increase in macrophage size (as observed with phase-contrast microscopy) similar to that observed when macrophages are CE loaded with acetyl-LDL. Therefore, cells were incubated with 0.6-$\mu$m latex beads to determine whether these morphological changes alone, in the absence of CE accumulation, could result in any of the changes in protein expression. Analysis of autoradiograms from these control experiments, compared with those from macrophages incubated with medium alone, revealed that four of the five protein spots that changed with both acetyl-LDL and $\beta$-VLDL incubation (above) did not significantly change with either LDL, fucoidan, or latex bead incubation. Only spot 5C, which decreased 35% and 38% in macrophages incubated with acetyl-LDL and $\beta$-VLDL, respectively, decreased 72% ($n=2$ with identical values for the duplicates) in fucoidan-incubated cells. Spot 5C was unaffected by LDL or latex bead incubation.

In summary, 2-D gel electrophoresis of metabolically labeled cellular proteins from acetyl-LDL-incubated macrophages demonstrated reproducible and significant changes in several protein spots. Five of these proteins (1C, 2C, 3C, 5C, and 7C) were similarly altered.

### Table 1. Newly Synthesized Cellular Proteins That Change in Response to 4-Day Acetyl-LDL Incubation of Mouse Peritoneal Macrophages

<table>
<thead>
<tr>
<th>Spot</th>
<th>$p_i$</th>
<th>$M_r$ (D)</th>
<th>n</th>
<th>Relative densitometric value of foam cell spots (% of control macrophage spots)</th>
</tr>
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<tbody>
<tr>
<td>1C</td>
<td>6.75</td>
<td>80,840</td>
<td>9</td>
<td>53±17 ($p&lt;0.001$)</td>
</tr>
<tr>
<td>2C</td>
<td>6.76</td>
<td>50,818</td>
<td>11</td>
<td>77±26 ($p&lt;0.002$)</td>
</tr>
<tr>
<td>3C</td>
<td>6.80</td>
<td>49,001</td>
<td>11</td>
<td>458±164 ($p&lt;0.001$)</td>
</tr>
<tr>
<td>4C</td>
<td>5.87</td>
<td>43,316</td>
<td>12</td>
<td>361±180 ($p&lt;0.001$)</td>
</tr>
<tr>
<td>5C</td>
<td>5.22</td>
<td>43,114</td>
<td>11</td>
<td>35±34 ($p&lt;0.001$)</td>
</tr>
<tr>
<td>6C</td>
<td>6.56</td>
<td>29,931</td>
<td>12</td>
<td>66±28 ($p&lt;0.002$)</td>
</tr>
<tr>
<td>7C</td>
<td>5.69</td>
<td>21,460</td>
<td>11</td>
<td>46±22 ($p&lt;0.001$)</td>
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$p_i$, isoelectric point. Macrophages were incubated for 4 days in the absence (control) or presence of 25 $\mu$g/mL acetyl-low density lipoprotein (acetyl-LDL), then labeled for 3–6 hours with $[^{35}]$S)methionine/ cysteine. The cell lysates were subjected to two-dimensional gel electrophoresis, and the autoradiograms of the gels were analyzed by laser densitometry. Equal counts per minute were loaded for the control and foam cell gels. To correct for any pipetting errors, however, the raw densitometry data on each gel were normalized to at least four protein spots that demonstrated no change between foam cell and control samples in each experiment. Twelve separate experiments comparing control and acetyl-LDL-loaded cells were performed. For some of the proteins, analysis was performed on fewer than 12 experiments either because the protein of interest was inadvertently run off the gels or because the protein spots on the autoradiograms were too faint to analyze. Otherwise, all of the spots analyzed were within the linear range of the densitometer. The data are presented as the mean±SD of the amount of newly synthesized protein in foam cells expressed as a percentage of the amount in control macrophages. A two-tailed $t$ test was performed to assess the statistical significance of the data.

$n$, Number of separate autoradiograms analyzed.

*In one experiment included in the statistical analysis, an increase in protein 2C in foam cells vs. control macrophages (146%) was observed. If this experiment had not been included in the statistical analysis ($n=10$), the mean±SD would have been 70±13 ($p<0.001$).

*In two experiments included in the statistical analysis, protein 5C did not change in response to acetyl-LDL treatment. If these experiments had not been included in the statistical analysis ($n=10$), the mean±SD would have been 21±8 ($p<0.001$).

†In one experiment included in the statistical analysis, a slight increase (133%) in protein 6C was observed in foam cells vs. macrophages. If this experiment had not been included in the statistical analysis ($n=11$), the mean±SD would have been 60±19 ($p<0.001$).
TABLE 2. Expression of Newly Synthesized Proteins 1C-7C in Mouse Peritoneal Macrophages Incubated for 4 Days With β-VLDL

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<thead>
<tr>
<th>Spot</th>
<th>n</th>
<th>Relative densitometric value of spots from β-VLDL–incubated cells (% of control macrophage value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1C</td>
<td>5</td>
<td>54±50* (NS)</td>
</tr>
<tr>
<td>2C</td>
<td>5</td>
<td>73±13 (p&lt;0.01)</td>
</tr>
<tr>
<td>3C</td>
<td>5</td>
<td>248±39 (p&lt;0.002)</td>
</tr>
<tr>
<td>4C</td>
<td>5</td>
<td>144±59 (NS)</td>
</tr>
<tr>
<td>5C</td>
<td>5</td>
<td>38±22 (p&lt;0.005)</td>
</tr>
<tr>
<td>6C</td>
<td>5</td>
<td>88±50* (NS)</td>
</tr>
<tr>
<td>7C</td>
<td>5</td>
<td>41±20 (p&lt;0.005)</td>
</tr>
</tbody>
</table>

Macrophages were incubated for 4 days in the absence (control) or presence of 25 μg/mL β-very low density lipoprotein (β-VLDL) and labeled on the fifth day for 3 hours with [35S]methionine/cysteine. The cell lysates were subjected to two-dimensional gel electrophoresis and autoradiography, and spots 1C–7C of the autoradiograms (see Table 1) were analyzed by laser densitometry exactly as described in Table 1. Five separate experiments comparing control and β-VLDL–loaded cells were performed. The data are reported as mean±SD of the amount of newly synthesized protein in β-VLDL–incubated cells expressed as a percentage of the amount in control macrophages. A two-tailed t test was performed to assess the statistical significance of the data (NS, not significant).

n, Number of separate autoradiograms analyzed.

*In one experiment included in the statistical analysis, an increase in protein 1C in β-VLDL–incubated cells vs. control macrophages (146%) was observed. If this experiment had not been included in the statistical analysis (n=4), the mean±SD would have been 33±15 (p<0.005).

†In one experiment included in the statistical analysis, β-VLDL–incubated cells demonstrated a 173% increase in spot 6C. If this experiment had not been included in the statistical analysis (n=4), the mean±SD would have been 67±17 (p<0.005).

in β-VLDL–incubated macrophages. Furthermore, expression of at least four of these proteins (1C, 2C, 3C, and 7C) was not significantly affected by incubation with either LDL, fucoidan, or latex bead treatment. These data suggest that the changes in the synthesis of proteins 1C, 2C, 3C, and 7C may be correlated with macrophage CE loading.

Identification of a Cellular Foam Cell Protein as α-Enolase

Protein 3C demonstrated the greatest synthetic change in acetyl-LDL foam cells (fourfold to fivefold increase). This protein also increased significantly with β-VLDL treatment (twofold to threefold) but not with LDL, fucoidan, or latex bead incubation. Thus, the increase in the expression of protein 3C is correlated with macrophage CE accumulation. For these reasons, protein 3C was chosen for identification.

To obtain enough material for amino acid sequencing, preparative 2-D gel electrophoresis was carried out. Because Cold Spring Harbor Laboratories was not equipped to run preparative 2-D gels, it was necessary to carry out this part of the project at Kendrick Laboratories. In the Kendrick gel system, a protein spot with M, and pI similar to protein 3C (originally observed on gels run at Cold Spring Harbor) was identified (Figure 2B, spot labeled A). The spot identified on the Kendrick gels demonstrated an increase with acetyl-LDL incubation (406±108%; p<0.002, n=7) and did not change with LDL treatment. Note that the protein patterns from the Cold Spring Harbor gels in Figure 1 differ slightly from those of the Kendrick gels in Figure 2. It is not uncommon for proteins to migrate differently when the 2-D gel running conditions are changed from one system to another.

Initial attempts to sequence this protein (from 2-D gels blotted onto PVDF membrane) revealed that the amino terminus was blocked, and thus it was necessary to obtain an internal peptide sequence through cyanogen bromide cleavage at methionine residues. Protein spots were pooled and digested in situ with cyanogen bromide, and the resulting fragments were then electrophoretically separated on a slab gel and blotted onto a PVDF membrane. The amino acid sequence was obtained from two of the peptides. From amino acid residue matching, it was revealed that this particular foam cell–associated protein was α-enolase (Figure 3A). To confirm this identification, protein blots of 2-D Kendrick gels of 35S-labeled cellular proteins from foam cell macrophages were subjected to autoradiography and Western blot analysis using an antisera specific for the α-isofrom of enolase.12,13 The Western blot data (Figure 3C) revealed the presence of a band of reactive material with the same M, as α-enolase but varying in pI. In this band, there were two prominent spots (labeled spot A and spot B) that reacted with the anti-α-enolase antisera. By aligning the Western blot with the autoradiogram, one of the antisera-reacting spots (spot A) corresponded to the foam cell–inducible protein described above (i.e., the spot labeled A in Figure 2). The other major antisera-reacting spot in Figure 3C (spot B) corresponded to a protein spot on the 2-D gel autoradiograms (labeled B in Figure 2) that did not increase with cholesterol loading of the macrophages and was approximately threefold more abundant than spot A, as determined by steady-state labeling experiments. To confirm the identity of spot B as α-enolase, internal amino acid sequence data from this protein were obtained as described above. Spot B gave a cyanogen bromide fragment pattern identical to that of spot A, and internal amino acid sequence data confirmed that spot B was in fact α-enolase (Figure 3B). Therefore, in macrophages, one can distinguish at least two forms of α-enolase, one of which increases during foam cell formation.

Further Characterization of Spot A α-Enolase

In all of the experiments described above, cells that were maintained in the absence or presence of lipoproteins were pulse labeled on the fifth day for 3–6 hours. Hence, the data reflected proteins being newly synthesized on the fifth day. To determine whether the total amount of spot A α-enolase was increased in foam cells versus control macrophages, control and foam cell macrophage types were steady-state labeled with [35S]methionine for 2, 4, and 5 days. The cellular proteins were analyzed by 2-D gel electrophoresis as described above. The percent increases of spot A (Figure 2) in foam cells versus control macrophages at 2, 4, and 5 days were 302%, 343%, and 392%, respectively. Thus, the steady-state level of spot A α-enolase was increased in CE-loaded macrophages, and this increase was seen as early as 2 days after acetyl-LDL incubation.
To more definitively determine the role of CE accumulation in spot A α-enolase induction, an experiment was conducted in which macrophages were incubated for 4 days with 25 μg/mL acetyl-LDL in the absence or presence of the ACAT inhibitor 58-035 (5 μg/mL). Cells incubated in the presence of acetyl-LDL plus the ACAT inhibitor contained significantly more free cholesterol and less CE than the cells incubated with acetyl-LDL alone. The macrophages incubated with acetyl-LDL plus 58-035 expressed 44% less of spot A α-enolase than the macrophages incubated with acetyl-LDL alone. Therefore, the increase observed in the synthesis of this protein appears to be correlated with CE loading per se rather than to the accumulation of free cholesterol or some other lipoprotein component.

Until now, two functions have been attributed to α-enolase: cytosolic glycolytic enzyme activity and plasma membrane plasminogen binding activity. However, neither of these two functions was increased in foam cells versus control macrophages. In two separate experiments, control macrophage enolase activity values were 0.50 and 0.61 μmol/mg per minute, and foam cell enolase activity values were 0.48 and 0.68 μmol/mg per minute. With respect to 4°C plasminogen binding activity, control macrophages and foam cells bound 335±11 and 310±21 ng 125I-plasminogen per milligram

FIGURE 2. Autoradiograms of two-dimensional electrophoretic gels (Kendrick Laboratories) of 35S-labeled cellular proteins from control (panel A) and acetyl-low density lipoprotein (acetyl-LDL)-incubated (panel B) macrophages. Resident mouse peritoneal macrophages were incubated for 4 days in the absence (control) or presence of 25 μg/mL acetyl-LDL. The cells were then labeled for 3–6 hours with 35S-labeled methionine/cysteine, and the cellular proteins were subjected to two-dimensional gel electrophoresis (Kendrick Laboratories). Approximately 4 million cpm were loaded per gel. The autoradiogram exposure time was 1 day. Each autoradiogram, from left to right, runs from acidic to basic pH. The labels A and B refer to protein spots referred to in the text.
FIGURE 3. Amino acid sequences from cyanogen bromide peptide fragments of foam cell protein spots A (panel A) and B (panel B) and a Western blot (panel C) of a two-dimensional gel of foam cell proteins using rabbit anti-mouse α-enolase antiserum. Protein spot 3C (spot A, Figure 2) (panel A) and the protein spot denoted as spot B in Figure 2 and in Figure 3C (panel B) from acetyl-low density lipoprotein (acetyl-LDL)-induced foam cells were purified by preparative two-dimensional gel electrophoresis, and cyanogen bromide–generated fragments were subjected to amino acid microsequencing as described in “Methods.” The amino acid sequences of the peptide fragments are compared with the known sequence of mouse α-enolase obtained from the Protein Identification Resource of the National Biomedical Research Foundation. However, the sequence data shown were determined before comparison with the known α-enolase sequence. An x denotes an amino acid residue whose identity was not certain during the first reading of the sequencing data; in several cases (e.g., those x residues corresponding to D and G of α-enolase), a second reading was consistent with the known α-enolase residues. The arrows denote amino acid residues that specifically identify the peptides as originating from the α isoform of enolase (i.e., as opposed to β or γ). The positions of methionine residues (i.e., the sites of cyanogen bromide cleavage) in the known mouse α-enolase are noted for reference. Given the very small amount of material available for analysis, the amount of protein loaded into the sequencer was not assayed, so initial yields of each sample could not be determined. Representative repetitive yields were as follows: spot A, sequence 245=82% (Y8/Y13); spot B, sequence 245=92% (F7IF17); and spot B, sequence 98=96% (N5/N12). Panel C: [35S]methionine pulse-labeled cell lysates were prepared for two-dimensional gel electrophoresis and the gels run at Kendrick Labs as described above. The slab gels were transblotted onto polyvinylidene difluoride (PVDF) paper overnight as described in “Methods.” The PVDF membrane was then air dried, wrapped in plastic, and exposed to film at −70°C to obtain the autoradiogram for identification of the spots of interest. Western blot analysis was carried out according to the methods of the Amersham Western Blotting Detection Kit (Amersham Co., Arlington Heights, Ill.) using rabbit antiserum specific to α-enolase at a 1:2,000 dilution. The antibody–antigen complex was visualized by color reaction of a horseradish peroxidase–coupled secondary (anti-rabbit) antibody with N′N′-diaminobenzidine. Spots A and B (see Figure 2) were identified by aligning the antibody-reactive spots with the 35S-methionine–labeled protein spots A and B on the autoradiogram (see Figure 2B).

As described above (Figures 2 and 3), at least two forms of α-enolase can be distinguished in macrophages. Potential structural differences between the two forms were investigated to determine if the forms might result from differences in cotranslational/posttranslational modification. In fact, the idea that the increase in spot A α-enolase in foam cells might be due to an induction of a particular cotranslational/posttranslational modification of α-enolase, rather than an increase in de novo synthesis, was supported by the finding that

of cell protein, respectively (mean±SD, n=3). At 37°C, the control and foam cell macrophages bound 1,828±475 and 1,601±263 ng 125I-plasminogen per milligram, respectively. Thus, the functional significance of the increase in spot A α-enolase in CE-loaded macrophages is yet to be determined.
α-enolase mRNA, as assayed by Northern blot analysis using a cDNA probe specific for α-enolase mRNA (see “Methods” and Reference 14), was not increased in foam cells compared with control macrophages. Two possible cotranslational/posttranslational modifications that could potentially alter the pI of α-enolase without changing the MΣ include phosphorylation and fatty acylation. To determine the possible role of phosphorylation, foam cells were labeled for either 3 hours or overnight with [32P]orthophosphate and subjected to preparative 2-D gel electrophoresis. The Coomassie-stained gels were aligned with the corresponding autoradiograms to determine whether spots A and B incorporated the [32P] label. Neither α-enolase spot, however, incorporated the [32P]orthophosphate label. Consistent with these data, the migratory patterns of spots A and B were not altered on 2-D gels from foam cell lysates incubated with alkaline phosphatase compared with foam cell lysates incubated under identical conditions in the presence of phosphatase inhibitors (see “Methods”). In a second set of experiments, incubation of foam cells with [3H]palmitate and [3H]myristate resulted in incorporation of label into both spots A and B α-enolase. However, α-enolase spots A and B, derived from foam cell lysates that had been incubated with 3C-labeled fatty acids (or Ï²-labeled fatty acids), did not demonstrate intact fatty acid release once they were purified and subjected to acid hydrolysis. Thus, a modification other than phosphorylation or fatty acylation, such as acetylation or an amino acid substitution, may be responsible for the difference in the pI between spot A and spot B α-enolase.

Discussion

The goal of this project was to explore the hypothesis that massive CE loading of macrophages, an event that occurs prominently in atherosclerotic lesions,1–3 leads to a change in the pattern of protein expression or protein modification by the cells. 2-D gel electrophoresis of metabolically labeled cellular proteins demonstrated reproducible and significant changes in several protein spots with acetyl-LDL incubation of the macrophages. Five of these proteins were similarly altered (i.e., either increased or decreased) in β-VLDL–incubated macrophages. Furthermore, the expression of at least four of these proteins, one of which was identified as an isofrom of α-enolase (spot A; see above) that was increased in foam cells, was not affected by incubation with either LDL, fucoidan, or latex bead treatment. Thus, for at least four of the proteins altered in acetyl-LDL–treated cells, there is evidence to suggest a correlation between CE loading and a change in protein expression. In addition, macrophages incubated with acetyl-LDL in the presence of the ACAT inhibitor 58-035 contained less of spot A α-enolase than macrophages incubated with acetyl-LDL alone. Therefore, the increase observed in the synthesis of at least this protein appears to be due to CE loading per se rather than to the accumulation of some other lipoprotein component such as free cholesterol.

In previous work in other laboratories, acetyl-LDL incubation of mouse peritoneal macrophages has been shown to increase the synthesis and secretion of apoprotein E.46,37 We also found an increase in foam cells of a protein with the MΣ and pI of apoprotein E (Figure 1B, solid arrowhead). Further work by others in this area revealed that the accumulation of ACAT-derived CE by the macrophages did not appear to be the key regulatory event because ACAT inhibition did not diminish the response; rather, free cholesterol levels in the cell may be more important in this regard.38 Werb and colleagues,36 using one-dimensional SDS polyacrylamide gel electrophoresis, have reported that acetyl-LDL did not change the pattern of cellular proteins other than apoprotein E in mouse peritoneal macrophages but did increase the appearance of other secreted proteins, none of whose identities have been published. Interestingly, the secretion of these proteins was also induced by various non–cholesterol-containing macrophage ligands (i.e., latex beads). In another study, Hamilton et al39 examined the effect of lipoprotein incubation on the inflammatory response of mouse macrophages. These authors found that the induction of tumor necrosis factor-α mRNA in stimulated macrophages was suppressed by incubation with oxidized LDL but not with acetyl-LDL. Thus, the effect was independent of foam cell formation.

Mouse macrophages contain two proteins with the same MΣ but different pIs that are recognized by anti-α-enolase antiserum (Figure 3C): a protein that is increased in foam cells (spot A in Figure 2) and another, more abundant, protein that is not increased in foam cells (spot B in Figure 2). Interestingly, Zeitoun et al40 and Heydorn et al41 have published Western blots showing an apparently similar heterogeneity of α-enolase from cerebral tissue. In these cases, the relative intensity of the more acidic α-enolase (apparently equivalent or similar to spot A α-enolase reported here) was very low and thus was similar to the situation with control (i.e., non–foam cell) macrophages. In addition, the more acidic spot identified by the antibody in these studies was not characterized further. Herein, the two protein spots were identified by antisemur specific for α-enolase and were found to have the same cyanogen bromide peptide profile and the identical amino acid sequence in two peptide fragments, including several amidate residues that distinguish the α-isofrom from β- or γ-enolase (Figure 3C). Thus, this report establishes by peptide fragmentation and amino acid sequence data that, at least in macrophages, the more acidic spot (i.e., spot A) is, in fact, a form of α-enolase. Furthermore, this is the first report showing that the more acidic form, which is usually a very minor form of α-enolase (see above), can be substantially increased under a particular condition—i.e., CE loading. Interestingly, the Western blot also revealed the presence of other reactive material with the same MΣ as α-enolase but with a more acidic pI than spot A (Figure 3C). Whether this material contains other isoforms of α-enolase, as determined by amino acid sequencing, has not been determined.

What might be the structural basis for the difference between spot A and spot B α-enolase? Although it is possible that more extensive amino acid analysis would have revealed minor amino acid sequence differences between the two forms, the idea that the different pIs resulted from differences in cotranslational or posttranslational modification was investigated. Differences in glycosylation, including sialylation, are unlikely because the two α-enolases have the same apparent MΣ (Figure 2). Furthermore, we have not been able to
implicate differences in phosphorylation as a cause for the different forms of α-enolase because neither form incorporated \(^{32}P\)orthophosphate label and alkaline phosphatase treatment of foam cell lysates before 2-D gel electrophoresis did not alter the migratory pattern of either α-enolase protein spot. Last, we have demonstrated incorporation of \(^{14}C\) radioactivity from \(^{14}C\)-labeled palmitic and myristic acid into both α-enolase isoforms. However, we were not able to demonstrate intact fatty acid release from a hydrolysate of purified α-enolase. Thus, it is possible that the fatty acid label was converted to labeled acetate, which was then used to acetylate α-enolase; if so, different degrees of acetylation might, in theory, provide a basis for the observed α-enolase heterogeneity.

The increase in spot A α-enolase in foam cells could be due to either increased de novo synthesis or increased cotranslational/posttranslational modification of preexisting α-enolase. The latter possibility was suggested by the absence of an increase in either α-enolase functional activity (above) or α-enolase mRNA (above) in foam cells versus control macrophages. However, if spot A α-enolase was derived from preexisting α-enolase, it is surprising that a decrease in spot B α-enolase in CE-loaded macrophages was not seen. How might these points be resolved? It is possible, given the greater abundance of spot B versus spot A α-enolase, that a decrease in spot B proportionate to the increase in spot A in foam cells would have been difficult to detect. Alternatively, with respect to the functional activity data, spot A α-enolase may simply be inactive in these particular functions (see below). Regarding the Northern blot data, the lack of increase in mRNA may be related to changes in mRNA translatability. Further work is needed to resolve these issues.

As mentioned above, there was no increase in either α-enolase enzymatic activity or plasminogen binding activity in foam cells. One possibility for the plasminogen binding results could be that spot A α-enolase was not membrane bound. However, 2-D gels of membrane and cytosolic fractions of foam cell lysates revealed that both isoforms of α-enolase were present in both fractions. Alternatively, spot A α-enolase might be modified in a way that leads to less plasminogen binding (e.g., lysine modification; see Reference 29). This possibility was supported by ligand blot data (using foam cell lysates), which indicated that spot A α-enolase bound fivefold to sixfold less \(^{125}I\)-plasminogen per amount of steady-state protein than spot B α-enolase (authors’ unpublished data). The possibility that spot A α-enolase may have an as-yet-undiscovered function in foam cells is currently under investigation.

In conclusion, cultured foam cell macrophages have been shown to have changes in the expression or modification of a specific set of cellular proteins. Thus, when arterial wall macrophages accumulate CE during atherogenesis, the accumulation of the lipid may lead to an increase or decrease of specific proteins or isoforms of proteins. These alterations in foam cell proteins could, in theory, have important physiological consequences related to atherogenesis.

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