Investigation of plasma membrane-associated apolipoprotein E in primary macrophages

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Abstract. Our previous studies identified the lysosome as the compartment for degradation of newly synthesized apoE in primary macrophages. Lysosomal degradation of newly synthesized apoE is extensive and rapid (>50% in 60 min). In the present study we tested the hypothesis that the macrophage cell surface is part of the itinerary of apoE in its path to the lysosomes. We therefore examined the existence and size of the apoE pool associated with the macrophage cell surface. Such a pool may not only provide a mechanism of targeting apoE for lysosomal degradation, by endocytosis, but also have important implications for the metabolism of lipoproteins by macrophages. Treatment of macrophages with heparin (10 μg/ml and 5 mg/ml) and heparinase I (1 U/ml), which releases substantial amounts of apoE from HepG2 cells, results in no additional release of apoE from macrophages. Treatment of macrophages with xyloside (1 mM) or GRGDTP (500 μg/ml) does not decrease the extent of cell-associated apoE. Both immunogold labeling, followed by electron microscopy, and immunofluorescent labeling and light microscopy further confirm the lack of significant amounts of cell surface-associated apoE in macrophages. In contrast, immunolabeled apoE is readily observed in permeabilized cells. Taken together, these data indicate the absence of significant apoE–glycosaminoglycan interaction at the macrophage cell surface. The lack of such an interaction is likely due to a paucity of heparan sulfate proteoglycans on the macrophage cell surface, when compared to hepatocytes. Along with our previous observations (Deng, J., V. Rudick, and L. Dory, 1995. *J. Lipid Res.* **36:** 2129–2140), these results suggest direct targeting of a portion of newly synthesized apoE from *trans*-Golgi network to lysosomes for degradation, without involving the plasma membrane and endocytosis.—**Deng, J., V. Rudick, M. Rudick, and L. Dory.** Investigation of plasma membrane-associated apolipoprotein E in primary macrophages. *J. Lipid Res.* 1997. **38:** 217–227.

Supplementary key words HepG2 cells • xyloside • heparan sulfate proteoglycans • glycosaminoglycans • *trans*-Golgi network • lysosomes

Apolipoprotein E (apoE) is a component of cholesteryl ester-rich lipoproteins, such as VLDL, chylomicron remnants, and HDL (1,2), and it serves as a ligand for the removal of these particles from the circulation by the LDL receptor- and chylomicron remnant receptor-mediated pathways (3–5). Recently, attention has been focused on the association of apoE with the outer surface of the plasma membrane and its potential function in lipoprotein metabolism in several types of cells, including hepatocytes (6), HepG2 cells (7), and adrenocortical parenchymal cells (8). The apoE associated with the cell surface may play an important role in the binding of lipoproteins to the liver or adrenocortical plasma membrane and in the subsequent sequestration of cholesterol from these particles. The source of apoE associated with the cellular surface has yet to be identified; it may be derived from local synthesis or taken up from the general circulation.

The presence of cell surface-associated apoE in HepG2 cells was demonstrated biochemically (7). The membrane-associated apoE on these cells can be released by low concentrations of heparin, heparinase, and by xyloside treatment (7), suggesting that it is associated with the heparan sulfate moiety of cell surface-associated HSPG. There are several functional studies that provide circumstantial evidence for the existence and physiological significance of the membrane-associated apoE. For example, the uptake of HDL-derived cholesteryl ester by HepG2 cells is significantly inhib-

Abbreviations: apo, apolipoprotein; BSA, bovine serum albumin; CTAB, cetyltrimethylammonium bromide; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; EMEM, Eagle's minimum essential medium; ER, endoplasmic reticulum; EM, electron microscopy; FBS, fetal bovine serum; FITC, fluorescein-5-isothiocyanate; GAGs, glycosaminoglycans; GRGDTP, glycine-arginine-glycine-asparagine-threonine-proline; HDL, high density lipoprotein; HepG2, human hepatoma cell line; HSPG, heparan sulfate proteoglycans; IgG, immunoglobulin G; Mac-2, macrophage cell surface antigen 2; M-6-P, mannose-6-phosphate; PG, proteoglycans; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TGN, *trans*-Golgi network; xyloside, 4-methylumbelliferyl β-D-xyloside.

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ited when cells were treated with a monoclonal antibody against the heparin-binding domain of apoE (9). In addition, it has been shown that the binding of lipoprotein particles to HSPG on the cell surface is essential for the subsequent internalization of the β-VLDL remnant particles by HepG2 cells. The binding and internalization of the remnant particles were significantly enhanced by the addition of exogenous apoE (10–12). It thus appears that the specific interaction of lipoprotein-associated apoE with the lipoprotein receptor is insufficient for the efficient uptake of the remnant particles. Based on studies such as these, it has been proposed that the incoming VLDL and chylomicron remnants and possibly HDL particles enter the space of Disse and associate with the cell surface HSPG through apoE binding, thus becoming anchored to the membrane in the receptor vicinity (6, 9–12). Hence current biochemical and EM evidence, obtained from HepG2 cells (7, 9), liver slices (6), and adrenal tissue slices (8), identifies a distinct pool of apoE on the cell surface.

Previous studies in this laboratory established that in primary macrophages over 50% of the cellular apoE pool is degraded within a 60-min chase period intracellularly, prior to secretion, in the lysosomes (13, 14). We postulated two potential sorting routes for directing newly synthesized apoE to the lysosomal/endosomal compartment for degradation (13). One involves direct sorting of apoE from TGN to this compartment. The other involves secretion of apoE, followed by retention of a fraction of the secreted apoE on the cell surface, potentially by interacting with HSPG. The cell surface-associated apoE may be endocytosed and delivered to the lysosomes for degradation. In order to accommodate the extensive and rapid intracellular degradation of apoE, the latter mechanism requires that a substantial portion of cell-associated apoE be localized on the cell membrane. The present studies reveal that the small amount of apoE, if any, associated with the macrophage cell surface is insufficient to support the extent of degradation observed earlier (14). We conclude that the vast majority of the apoE destined for lysosomal degradation in primary macrophages is targeted there directly by a yet to be determined mechanism. This represents a novel finding for routing of a constitutively secreted protein.

MATERIALS AND METHODS

Reagents

Heparin, chondroitin sulfate A, B (dermatan), C, heparan sulfate, heparinase I, 4-methylumbelliferyl β-D-xyloside (xyloside), glycine-arginine-glycine-asparagine-threonine-proline (GRGDTP), and purified rat and goat IgGs were obtained from Sigma Chemical Company. Rat anti-mouse macrophage cell surface antigen (anti-Mac-2) IgG was obtained from Boehringer Mannheim Biochemicals. EXPRE^{35}S^{35}S protein labeling mixture (specific activity of [^{35}S]methionine > 1,100 Ci/mmol) was purchased from NEN DuPont. Dulbecco's modified Eagle's medium (DMEM), methionine-free DMEM, Dulbecco's phosphate-buffered saline (DPBS), William's E medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco Life Technologies. Eagle's minimum essential medium (EMEM), methionine-free EMEM, heat-inactivated fetal bovine serum, amphotericin B, L-glutamine, collagen, collagenase, and trypsin in 1 × EDTA were also obtained from Sigma Chemical Company. Zysorbin was purchased from Zymed Laboratories, Inc. Immobilized rProtein A was obtained from RepliGen. Protein assay reagents for modified Lowry were obtained from Bio-Rad Life Science Research Products. Rabbit anti-goat IgG conjugated with colloid gold particles (Auro-Probe EM) was purchased from Amersham Life Sciences. Rhodamine-conjugated goat anti-rat IgG was purchased from Biodesign International. FITC-conjugated rabbit anti-goat IgG was purchased from Organon Teknika Corporation. All other reagents, such as hydrogen peroxide and salts, were purchased from Sigma Chemical Company.

Cell culture

Macrophage culture. Mouse peritoneal macrophages were obtained from male Swiss-Webster mice (14–18 g; Harlan) 4 days after an intraperitoneal injection of 1.5 ml of sterile 4% thioglycolate broth, essentially as previously described (15). On the second day of culture, cells were washed twice with DMEM and incubated in regular DMEM containing 25-hydroxycholesterol (0.8 μg/ml) for 24 h. This treatment results in a 3-fold transcriptional induction of apoE expression, although cellular sterol content or distribution remain unaffected (14). The cells were then washed and incubated in regular DMEM for an additional 24 h before the experiments.

HepG2 cell culture. HepG2 cells were maintained in T75 parent flasks in EMEM containing 10% heat-inactivated FBS, 4 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (2.5 μg/ml). When HepG2 cells were 85–90% confluent, they were trypsinized by treating the monolayer with 0.05% trypsin in 1 × EDTA, as described (7, 9). Briefly, 10 ml of trypsin solution was added to each T75 parent flask and the cells were incubated at 37°C for 8–10 min. Cells were then resuspended in EMEM with the same supplements and seeded (~100,000 cells/2 ml medium) in 6-well culture dishes. Culture medium was changed every 24 h. Experiments were carried out when

HepG2 cells reached 85–90% confluence. The cells were cultured in serum-free EMEM for 24 h before experiments.

Pulse-chase studies

Prior to labeling, cells were briefly incubated (10 min) in methionine-free DMEM. For short-term metabolic labeling, cells were pulsed for 60 min with [^{35}S]methionine (40 μCi/ml) in the presence or absence of indicated agents in methionine-free DMEM. After the pulse period, the cells were washed and then chased for up to 60 min in DMEM containing the appropriate agents. For long-term metabolic labeling, cells were pulsed with [^{35}S]methionine (50 μCi/ml) in the presence or absence of indicated agents in regular DMEM. At each time point of the chase period the cell-associated and medium [^{35}S]apoE was quantitatively immunoprecipitated using goat anti-rat apoE antibody, as previously described (14). The cell-associated or medium apoE activity at each time point is expressed as a percentage of the cell-associated activity at the beginning of the chase period (0 time chase). Our previous work established that the extent of [^{35}S]apoE degradation in the medium is negligible (14). Parallel studies on the fate of total cellular protein were also performed. Incorporation of [^{35}S]methionine into cellular and medium proteins was determined by trichloroacetic acid precipitation of an aliquot of cell lysate or medium, respectively, on Whatman #5 filter paper discs.

Immunofluorescence microscopy

Macrophages, grown on sterilized glass coverslips, were washed twice and incubated in DMEM for 1 h before fixation. All steps were performed at room temperature. After a brief rinse in DPBS, cells were fixed with freshly prepared 4% formaldehyde for 10 min, then washed twice with DPBS before either being incubated in DPBS or permeabilized with 0.2% Triton-X 100 for 10 min. Coverslips were then rinsed four times with DPBS over a 5-min period, followed by a 10-min incubation in 3% BSA in DPBS. All primary and secondary antibodies were diluted in DPBS containing 3% BSA so that the concentration of IgG in all primary antisera was equal. Intracellular and surface-associated apoE were labeled by incubating permeabilized and non-permeabilized cells, respectively, with purified goat anti-rat apoE IgG for 60 min. Nonspecific binding was determined similarly, except that non-immune goat IgG substituted for anti-apoE. Coverslips were then washed over 5 min in three changes of either 1% Triton-X 100 in DPBS, for permeabilized cells, or DPBS, for non-permeabilized macrophages, before being exposed to secondary antibody. ApoE was fluorescently labeled with rabbit anti-goat IgG conjugated to FITC, for 60 min. As a positive control, another series of coverslips containing fixed non-permeabilized cells was incubated with rat anti-mouse Mac-2, an antibody specific for thioglycolate-elicited macrophage cell surface antigen. The secondary antibody used with these coverslips was goat anti-rat IgG conjugated to rhodamine. All coverslips were washed in DPBS three times and then mounted in Mowiol containing DABCO (1,4-diazobicyclo-[2,2,2]-octane; Pierce Chemical Co.). Samples were observed with a Nikon Microphot FXA microscope and photographed using Kodak TMZ p3200 black and white negative film.

Transmission electron microscopy and immunocytochemistry

Macrophages grown on Lux plates (Nunc, Inc., Naperville, IL) were fixed in 3% glutaraldehyde in 0.1 M PIPES buffer, pH 7.3 for 1 h, followed by three 10-min washes in PIPES buffer alone. Cells were then post-fixed for 1 h in 1% osmium tetraoxide, rinsed with three 5-min washes of distilled water, and then stained for 1 h en bloc using a 0.5% aqueous uranyl acetate solution. After distilled water washes, the material was dehydrated in a series of graded ethanol solutions and propylene oxide before infiltration with Epon. The macrophages were then placed in fresh plastic before being polymerized at 65°C for 24 h. All procedures were performed at room temperature except for the polymerization.

Polymerized samples were sectioned on a Sorvall MT2-B Ultramicrotome (Research and Manufacturing, Tucson, AR) with a diamond knife. Thick sections (0.5–1.0 μm) were stained with 1% toluidine blue in a 1% sodium borate solution. Thin sections (60–100 nm) were cut, collected on uncoated 150 M nickel grids, and processed for immunocytochemistry. For this, grids containing the sections were washed with PBS; the aldehydes were quenched with 0.02 M glycine, blocked with 5% BSA in PBS, and then exposed to various dilutions of either goat anti-apoE IgG or goat non-immune IgG. After incubation for 1 h at room temperature, the grids were washed and then exposed to an anti-goat IgG 10 nm gold probe for 1 h. All sections were then washed, fixed in 1% glutaraldehyde, rinsed, and then stained with a 50% ethanolic saturated uranyl acetate solution for 5 min, followed by Sato's lead stain (16) for 5 min. Controls were performed by omitting either primary or secondary IgGs and substituting non-immune or unrelated antibodies in their place. Sections were then observed with a 910 Zeiss transmission electron microscope (Zeiss Instruments, Thornwood, NY) and pictures were taken using Kodak SO-163 negative film.

Analytical procedures

Protein determinations were carried out according to Lowry et al. (17) and SDS-PAGE by the method of

Laemmli (18). ApoE immunoprecipitates were resolved in reduced, SDS-containing 10% polyacrylamide gels. The immunoprecipitate-derived apoE bands, resolved by SDS-PAGE and identified by the use of molecular weight standards and purified apoE, were cut out, dissolved overnight in 30% hydrogen peroxide, and quantified by scintillation spectroscopy. The cell lysate and medium [^{35}S]protein precipitates on filter paper discs were washed extensively in 10% trichloroacetic acid (2 × 12 h), followed by two washes in 100% ethanol and a final wash in anhydrous ether. The dry discs were placed in scintillation vials and counted after the addition of scintillation fluid.

RESULTS

There are at least three potential binding sites for apoE on the cell surface. First, apoE may bind to heparan sulfate PGs using its own heparin-binding domain. This is the most likely binding site, as a heparin or heparinase-releasable pool of apoE has been reported in HepG2 cells (7). By adding exogenous heparin, the non-covalent interaction allows the displacement of apoE from the heparan sulfate moiety of the HSPG (19, 20). The association of apoE with GAGs other than heparan sulfate is less likely. Xyloside, by replacing the protein core (21, 22), reduces the availability of GAGs on the macrophage cell surface; treatment of macrophages with this compound should thus reduce the amount of cell-associated apoE, regardless of the type of GAG it is associated with. Third, apoE may bind to the protein core of proteoglycans via protein–protein interactions. This is the target of GRGDTP treatment. Some proteoglycans are anchored to the cell surface by interacting with the transmembrane integrin receptors, which recognize the arginine-glycine-asparagine (RGD) sequence, residing in the protein core of proteoglycans (23). These proteoglycans can thus be displaced by adding exogenous GRGDTP, a synthetic polypeptide more potent than the tripeptide RGD (24–26). The extent of cell surface-associated apoE should thus also be reduced in GRGDTP-treated cells.

Release of apolipoprotein E from the macrophage and HepG2 cell surface by heparin and heparinase treatment

In order to investigate the potential interaction of apoE with HSPG, macrophages were labeled with [^{35}S]methionine for 60 min and chased for 60 min in the presence of heparinase (1 U/ml) or heparin (10 μg/ml and 5 mg/ml). We found previously that labeling for 60 min is sufficient for the labeling of the complete cellular apoE pool (14). The effects of these treatments on the extent of cell-associated and medium apoE at the end of the chase period are shown in **Fig. 1A.** Neither heparinase nor heparin (at either concen-

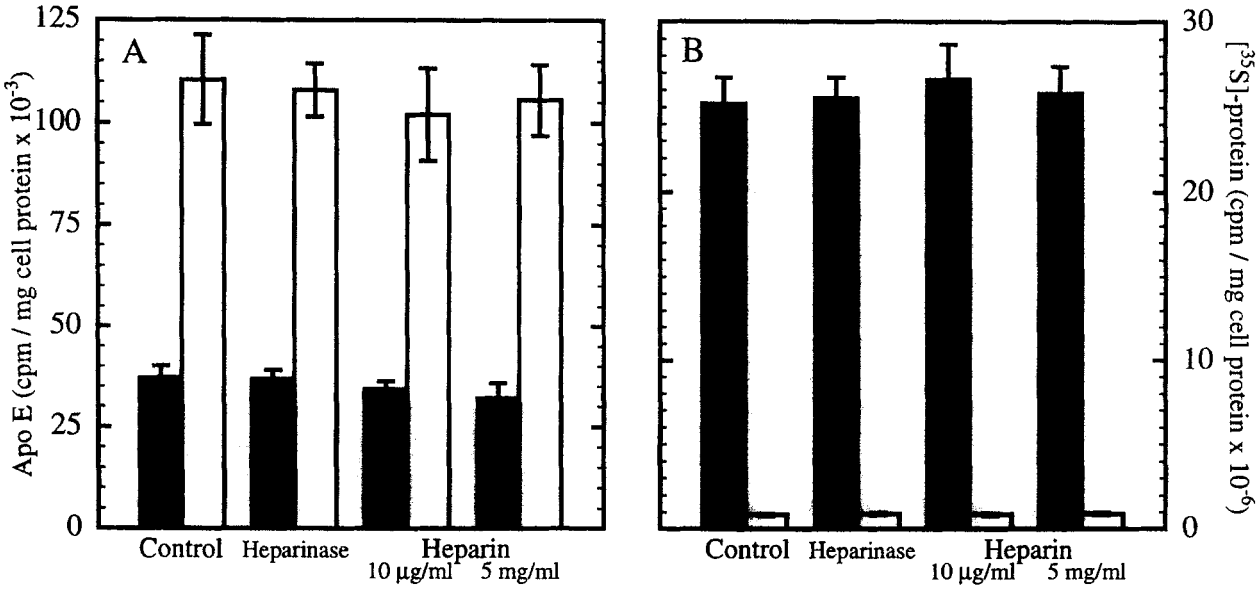

Fig. 1. Effect of heparin on apoE (A) and trichloroacetic acid-precipitable protein (B) distribution in macrophages. Macrophages were pulsed with 40 μCi/ml [^{35}S]methionine for 60 min. Heparin (10 μg/ml and 5 mg/ml) was present only during the 60-min chase. Cell-associated (closed bars) and medium (open bars) apoE at the end of the chase period were immunoprecipitated and quantified as described in Materials and Methods. The initial amount of cell-associated apoE was identical for each group. Each bar represents an average ± SEM of 6 wells. None of the experimental values is significantly different from the control values.

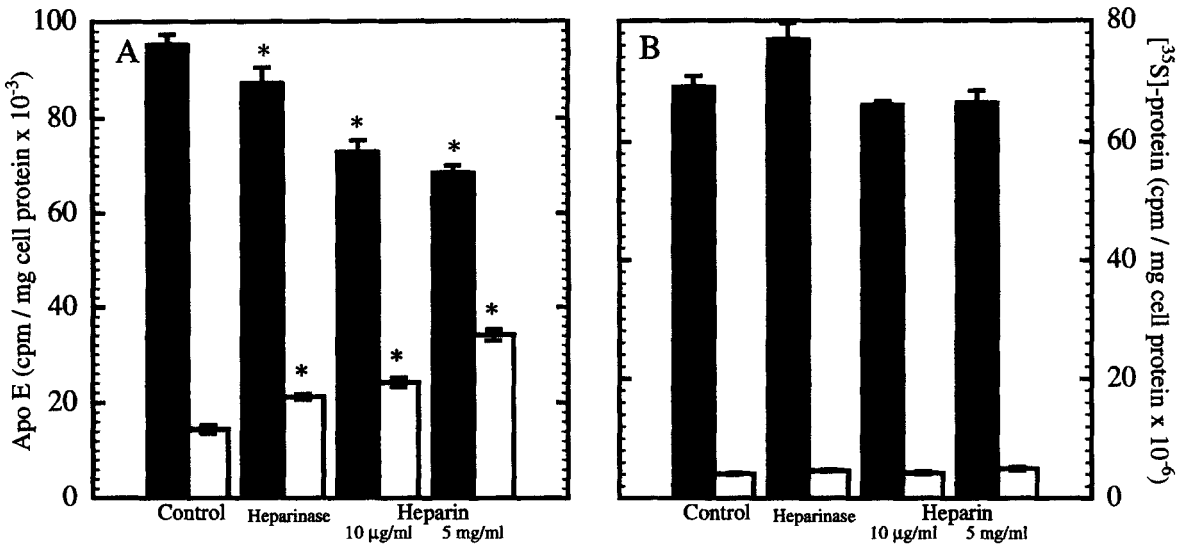

Fig. 2. Effect of heparin and heparinase I on apoE (A) and trichloroacetic acid-precipitable protein (B) distribution in HepG2 cells. HepG2 cells were cultured as described in Materials and Methods. Experimental design and procedures were identical to those described in the legend for Fig. 1. Heparinase I was used at 1 U/ml. Cell-associated (closed bars) and medium (open bars) apoE and trichloroacetic acid-precipitable proteins at the end of the 60-min chase period are shown. Each point represents an average ± SEM of 6 wells; *, significantly different from control values ($P < 0.05$).

tration) have any effect on the release of additional apoE from macrophages into the medium. Treatment of macrophages with heparin also has no effect on the distribution of total [^{35}S]protein (Fig. 1B).

As a positive control for the lack of effect of heparin or heparinase I on apoE release by macrophages, experiments of identical design were carried out with HepG2 cells. As shown in **Fig. 2A,** treatment of HepG2 cells with heparinase I results in a 11% decrease in cell-associated apoE and a nearly 50% increase in the medium apoE recovery. The effect of heparin treatment is even more obvious. At low concentrations heparin reduces cell-associated apoE content by over 25% and increases medium apoE by 60%. At high concentrations heparin reduces cell-associated apoE content by over 30% and increases medium apoE by 130%, a profound effect. Note that the recovery of total apoE (cell-associated plus medium) relative to the control ranges from 86 to 96%, suggesting that cell surface-associated apoE in HepG2 cells is not destined for degradation. These data confirm previous observations of the presence of a pool of apoE that can be rapidly displaced by exogenous heparin in HepG2 cells (7). As in the case of macrophages, there are no significant changes in the distribution of total [^{35}S]proteins in the heparin-treated HepG2 cells (Fig. 2B). It should be noted that a 60-min labeling period does not completely label the cellular pool of apoE in HepG2 cells (180 min are required, data not shown); the profound effects observed in these studies underestimate the extent of apoE association with the surface of HepG2 cells.

Effect of xyloside and GRGDTP on apolipoprotein E distribution

The experiments described above indicate that apoE does not associate with the heparan sulfate moiety of HSPG on the macrophage cell surface. We therefore decided to investigate the potential role of GAGs other than heparan sulfate. We took advantage of the mechanism of action of xyloside, an inhibitor of proteoglycan assembly (22). In order to assure a significant turnover of membrane proteoglycans, macrophages were labeled, in the presence of xyloside (1 mM), for 24 h. The extent of cell-associated [^{35}S]apoE at the end of the 24-h labeling period was compared in control cells, untreated cells, and in xyloside-treated cells. In contrast to its effect on apoE association with the HepG2 cell membrane (7), xyloside treatment has no effect on the extent of cell-associated apoE in macrophages (shown in **Fig. 3**). Identical results were obtained using macrophages exposed to xyloside for up to 72 h (data not shown). Xyloside treatment has no effect on the extent of accumulation of [^{35}S]apoE in the medium.

In order to examine potential protein–protein interactions of apoE with the core protein components of membrane proteoglycans, macrophages were also labeled with [^{35}S]methionine, for 24 h, in the presence (500 µg/ml) or absence of GRGDTP, a hexapeptide ca-

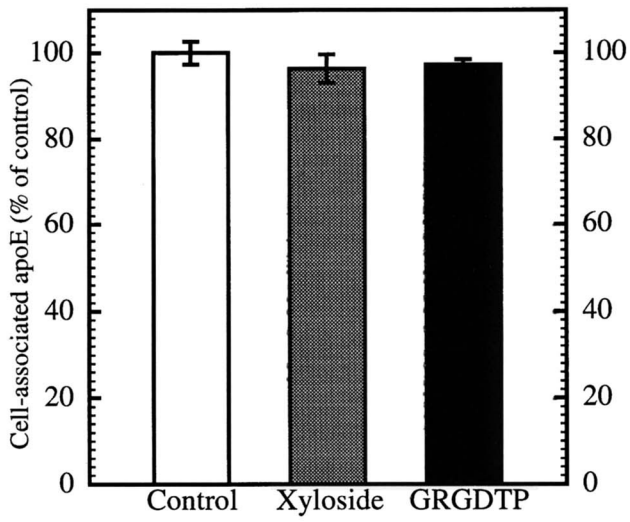

Fig. 3. Effect of xyloside and GRGDTP on cell-associated apoE. Macrophages were pulsed with 50 μCi [^{35}S]methionine/ml regular DMEM for 24 h in the presence or absence of xyloside (1 mM, stippled bar) or GRGDTP (500 μg/ml, closed bars). Cell-associated apoE at the end of the labeling period was quantified by immunoprecipitation. Each bar represents an average ± SEM of 6 wells. None of the experimental values is significantly different from the control value.

pable of displacing anchored proteoglycans, including fibronectin, vitronectin and collagen I (25, 26). The presence of GRGDTP, as shown in Fig. 3, has no effect on the extent of cell-associated [^{35}S]apoE in macrophages. Consistent with this finding, GRGDTP has no effect on the extent of apoE accumulation in the medium (data not shown).

Light microscopy of immunofluorescent macrophages

The extent of apoE association with the macrophage cell surface was also examined using immunocytochemistry. Macrophages grown on coverslips were fixed, either permeabilized or not, to compare intracellular versus surface labeling, respectively, and then incubated with specific or non-immune IgGs, as described in Materials and Methods. As shown in **Fig. 4A,** non-permeabilized macrophage cell surfaces were intensely labeled with rhodamine, after incubation with anti-Mac-2 IgG, an antibody that recognizes thioglycolate-elicited macrophage cell surface antigen. In comparison, little rhodamine staining was observed with cells in which non-immune IgG substituted for anti-Mac-2 (Fig. 4B). Thus, under the conditions of the experiment, if a surface antigen is present and free to react, it can be visualized at the plasma membrane.

The extent of surface labeling (FITC) when anti-apoE was used (Fig. 4C) is only barely discernibly higher than when non-immune IgG prepared in the same species (goat in this case) is used in its place (Fig. 4D), suggesting that there is little, if any, apoE free to interact with specific antibody at the surface of non-permeabilized macrophages. In contrast, intracellular labeling of apoE is evident in permeabilized cells (Fig. 4E). In these macrophages, fluorescence is primarily localized to perinuclear regions and is therefore probably associated with compartments of the secretory pathway. Negligible staining is seen in the permeabilized control cells (Fig. 4F). It should be noted that immunofluorescence studies of this nature are difficult in macrophages as these cells possess the Fc receptor. Varying amounts of nonspecific IgG-cell membrane interactions may be observed, depending on the source of the IgG used. In any case, these experiments provide, at best, an overestimate of surface-bound antigens. Based on a qualitative comparison of the amount of immunofluorescent apoE in permeabilized cells versus non-permeabilized cells, the amount of surface-bound apoE appears to be well below 5%.

Transmission electron microscopy of immunogold-labeled apoE in macrophages

Using transmission electron microscopy of thin frozen sections of macrophages, we previously demonstrated the presence of immunogold-labeled apoE in various subcellular compartments, including small vesicles and the endosomal/lysosomal compartment (13). However, the cellular morphology in cryopreserved material is not as good as in glutaraldehyde-fixed, plastic-embedded specimens. As our antibody against apoE was still able to recognize the antigen under the latter conditions with a minimal amount of background labeling due to gold sticking nonspecifically to the plastic, we elected to use plastic sections, in which the morphology was excellent, to further localize intracellular and any surface-associated apoE there might be at the ultrastructural level. The scarcity or absence of significant amounts of apoE on the macrophage cell surface seen in these Epon-embedded cells after immunostaining **(Fig. 5)** confirms what we observed previously in the frozen sections (13) and what we saw at the light microscopic level (Fig. 4). As can be seen in Fig. 5, immunogold-labeled apoE is clearly visible in the interior of the cell in the large membrane-bounded compartments (Fig. 5A and B) that we reported seeing in frozen sections (13), but little or no gold-labeled apoE is evident associated with the outer surface of the cell membrane (Fig. 5A and C).

DISCUSSION

There are several mechanisms by which apoE may be anchored to the cell surface. ApoE may bind to the cell

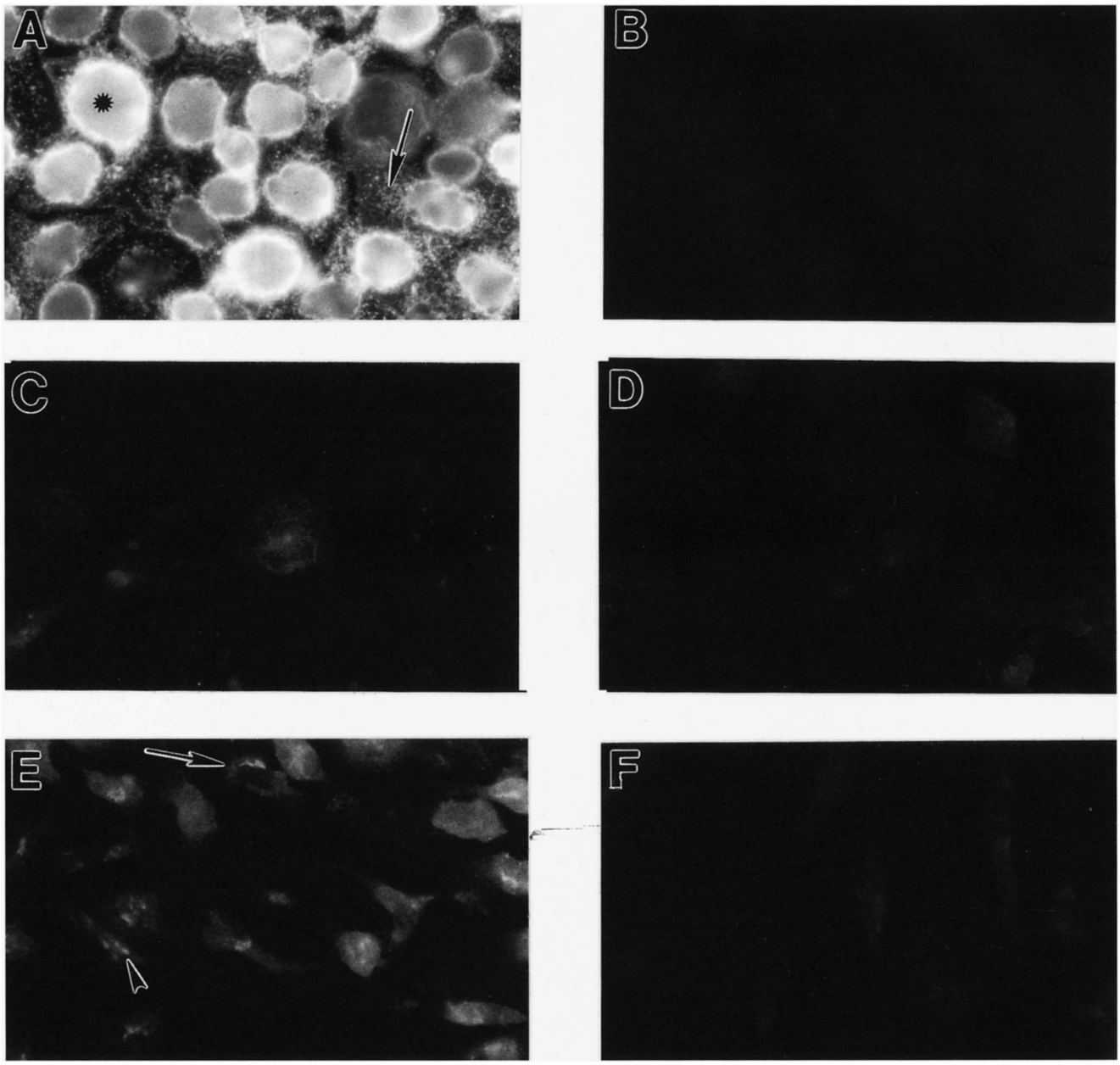

Fig. 4. Localization of immunofluorescent apoE in macrophages. Macrophages were grown, fixed, processed, and apoE localized with FITC while a macrophage-specific cell surface marker was labeled with rhodamine as described in Materials and Methods. All primary, secondary, and non-immune IgGs were diluted 1:100 in DPBS containing 3% BSA and equal exposure times were used for each experimental and control group. (A and B): Nonpermeabilized macrophages were treated with primary rat anti-mouse Mac-2 IgG (thioglycolate-elicited macrophage cell surface marker), followed by rhodamine-conjugated goat anti-rat IgG (A) or with non-immune purified rat IgG substituted for the primary antibody (B). The growth pattern of the macrophage is such that, although attached to the substratum, the cells are, for the most part, globular in shape with looped cytoplasmic extensions at the periphery. This morphology can be discerned in the staining pattern (A), where the globular portion of the cell (*) stains intensely and the extensions (arrow) exhibit ''stringy'' fluorescence. Control nonpermeabilized cells (B) lack surface staining. (C and D): Nonpermeabilized macrophages were treated with either purified primary goat anti-rat apoE IgG, followed by FITC-conjugated rabbit anti-goat IgG (C) or non-immune IgG substituted for the primary antibody (D). Lack of surface-associated fluorescence is evident in both experimental and control cells. (E and F): Macrophages treated as in C and D except that the cells have been permeabilized to allow intracellular staining. Fluorescence associated with apoE is evident in perinuclear (E; arrow) and large cytoplasmic (E; arrowhead) compartments. No comparable stained structures are evident in control cells (F).

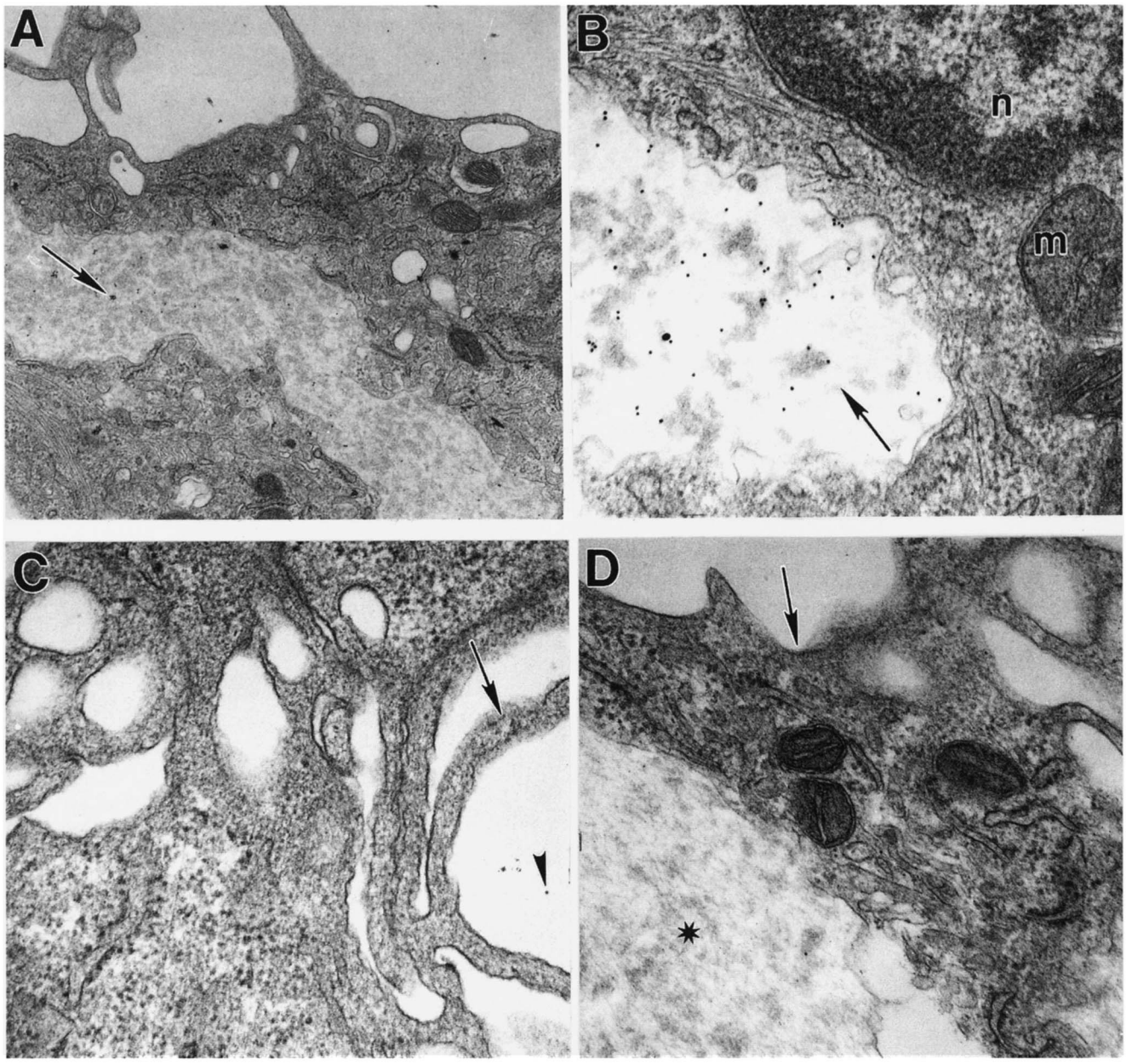

Fig. 5. Immunoelectron microscopy of the macrophage. Fixation, embedding, and protocol for immunostaining the cells is described in Materials and Methods. A 1:1000 dilution of polyclonal goat anti-apoE antibody, followed by incubation with a 1:35 dilution of 10 nm gold-conjugated anti-goat IgG was used except as noted. A: Low magnification (0.76 μm) view showing lack of specific apoE-gold complex at the cell surface, including along the cytoplasmic extensions. In contrast, significant amounts of apoE-associated gold may be seen in the large membrane-bounded compartment (arrow). B: Higher magnification view (302 nm) of a second membrane-bounded compartment containing apoE-gold (arrow); nucleus (n); mitochondrion (m). C: Higher magnification view (302 nm) of the cell surface and areas of looping cell extensions (arrow). The arrowhead points to a gold particle on the plastic. D: Higher magnification view (302 nm) of a macrophage in which non-immune goat IgG was substituted for the primary antibody. The cell surface is indicated by the arrow; the * identifies a membrane-bounded compartment similar to that shown in B. Note the relative absence of nonspecific gold label in this control.

surface-anchored heparan sulfate with its heparin binding domain (19, 20). The role of HSPG in anchoring apoE to the HepG2 cell surface was previously reported (7) and confirmed by the present studies. Incubation of HepG2 cells with even low concentrations of heparin leads to a significant increase in apoE release into the medium. In contrast, heparin or heparinase have no effect on apoE release into the medium by macrophages. The lack of xyloside or GRGDTP effect on the extent of cell-associated apoE in macrophages further leads us

to conclude that apoE–PG interactions essentially do not take place on the macrophage cell surface. Based on accepted biochemical approaches, we were unable to detect any surface-associated apoE in macrophages. Morphological evidence, including light microscopy of fluorescently labeled cells or gold labeling, followed by electron microscopy, support these findings. Immunofluorescent studies in macrophages are complicated by the presence of Fc receptors on the cell surface and the results overestimate the extent of antigen association. A qualitative comparison of the amount of fluorescence in non-permeabilized cells (intracellular apoE) versus permeabilized cells indicates that the surface might contain less than 5% of the total cellular pool of apoE.

The contrasting findings regarding the association of apoE with the hepatocyte and macrophage cell surface may well be due to significant differences in the extent and kinds of GAG synthesized by hepatocytes and by macrophages. It is well established that the major GAG synthesized and secreted by hepatocytes is heparan sulfate (27–29). In contrast, macrophages synthesize and secrete mostly chondroitin sulfate and dermatan sulfate PG, neither of which binds apoE significantly. In macrophages the proportion of heparan sulfate PG of the total PG synthesis varies from 10 to 25% (30–33). HSPG is thus a much less prominent PG in the macrophage repertoire when compared to that of the hepatocytes. Studies in our laboratory confirmed these observations and revealed that GAG synthesis in HepG2 cells is nearly 10-fold higher than in primary mouse macrophages when an equal number of cells is compared (M. Rudick and L. Dory unpublished observations). Taken together, these observations provide a convincing explanation for the lack of significant cell surface-associated apoE in macrophages.

The lack of a significant association of apoE with the macrophage cell surface is not unexpected. Unlike hepatocytes or adrenal cells, macrophages participate in lipoprotein metabolism essentially as scavengers of excess modified lipoproteins. Indeed, the level of expression of LDL or remnant receptors in macrophages is low or nonexistent under in vivo conditions (34, 35). The major route of lipoprotein internalization is the apoE-independent, scavenger receptor pathway (36). A major function of apoE produced in macrophages may well be to promote efflux of cholesterol and its delivery to hepatocytes and not to help trap additional cholesterol.

The lack of surface-associated apoE in primary macrophages is in direct contrast to the recently reported association of apoE with the pericellular matrix of macrophage-like cell lines, THP-1, or transfected (with human apoE) J774 cells (37). The apparent difference may well be explained by changes in the expression in cell surface GAGs, as reported for tumor cells (38). Furthermore, there appeared to be numerous differences in the regulation of apoE expression and unesterified cholesterol metabolism between primary macrophages and macrophage-like cell lines. Thus, for instance, J774 cells do not express endogenous apoE at all. The expression of apoE by THP-1 cells is induced by phorbol ester treatment (39), a treatment that profoundly depresses apoE secretion by primary macrophages (40). In a potentially related observation, neither J774 or THP-1 cells are capable of mobilizing unesterified cholesterol into an HDL-accessible compartment for efflux (41, 42). These observations suggest that studies on the effect of HDL on apoE expression and cholesterol efflux carried out in macrophage-like cell lines should be interpreted with caution.

Our findings have important implications for the processing of apoE by macrophages. We previously reported that over 50% of newly synthesized apoE in macrophages was degraded in lysosomes in a 60-min period (13, 14). We proposed two potential pathways apoE may take to the lysosomal compartment: *a*) newly synthesized apoE may be routed first to the cell membrane, where a portion is released into the medium, while the remainder is endocytosed into the lysosomes, or *b*) direct targeting from the *trans*-Golgi network to lysosomes. In light of the extensive and rapid apoE degradation, the former hypothesis requires that a substantial portion of cell-associated apoE be localized on the cell surface. Clearly, our findings are not consistent with this hypothesis; instead it appears that a portion of newly synthesized apoE is targeted to the lysosomal compartment directly by a novel mechanism. The mechanism of such targeting is not clear, but it is clearly pH-sensitive (13) and may involve an intracellular receptor for apoE. One potential candidate is the 59 kDa apoE-binding protein residing in the ER (43). Another potential candidate is the lysosomal form of prosaposin. Prosaposin is a glycoprotein able to interact with proteins and lipids destined for lysosomes, such as lysosomal sphingolipid hydrolases and glycolipids, especially glycosphingolipids (44, 45). Prosaposin has been proposed to function as a carrier protein to target certain lysosomal components to lysosomes (45, 46). It is possible that prosaposin may also play a role in targeting apoE to lysosomes, either by direct protein–protein interaction or by binding to the phospholipids coating these transport vesicles.

In summary, apoE is secreted by macrophages without a significant amount of apoE becoming associated with the cell surface. A substantial amount of newly synthesized apoE is degraded intracellularly, prior to secretion, in lysosomes. Our current results indicate that apoE destined for lysosomal degradation is targeted di-

rectly from the TGN, without reaching the cell surface.

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