LDL receptor binds newly synthesized apoE in macrophages: a precursor pool for apoE secretion

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Abstract **There appear to be multiple post-translational sites for regulation of macrophage apolipoprotein (apo)E secretion, including the presence of a distinct cell surface pool of apoE. Cell surface proteoglycans have been shown to be involved in forming this pool. The current studies were designed to investigate the role of an additional cell surface site, i.e., the low density lipoprotein (LDL) receptor. Antiserum to the LDL receptor displaced apoE from the macrophage cell surface and into the medium during a 4°C incubation from apoE-expressing J774 cells, from proteoglycan-depleted apoE-expressing J774 cells, and from human monocyte-derived macrophages. Similar results were obtained when purified monoclonal antibody to the LDL receptor was added to human monocyte-derived macrophages. J774 cells transfected to express an LDL receptor binding-defective mutant of apoE did not show a similar response to addition of LDL receptor antibody. Studies were conducted in which cells were pulse labeled for 30 min, followed by various periods of chase at 4°C or 37°C in the presence or absence of LDL receptor antibody. The results of these studies indicated that nascent macrophage-derived apoE binds to the LDL receptor, and that this apoE served as a precursor pool for apoE released into the medium. These studies establish a role for the LDL receptor in forming the cell surface pool of apoE and, along with data regarding the importance of proteoglycans, indicate that cell surface binding sites for nascent macrophage-derived apoE are heterogeneous. The heterogeneity of such sites could have implication for the size and turnover of this cell surface pool.**—Zhao, Y., and T. Mazzone. **LDL receptor binds newly synthesized apoE in macrophages: a precursor pool for apoE secretion.** ***J. Lipid Res.*** **1999.** 40: **1029–1035.**

Supplementary key words atherogenesis • atherosclerosis • vessel wall biology

Macrophage-derived apoE appears to have an important atheroprotective role when expressed in the vessel wall (1–6); and much of the vessel wall apoE is found in association with macrophages (7). There appear to be important post-transcriptional and post-translational loci for regulation of apoE secretion by macrophages (8–13). A significant portion of apoE synthesized by macrophages is never secreted and undergoes rapid cellular degradation (9), and the distribution of apoE between secretory and degradation pathways is subject to regulation by the intracellular sterol milieu (12). Whether apoE has an intracellular function, or exists in a discrete intracellular pool separate from the secretory pathway, is not yet completely clear. However, there does appear to be a distinct cell surface pool of macrophage apoE which has been identified in human monocyte-derived macrophages and in J774 cells transfected to express human apoE (10, 14, 15). Involvement of cell surface proteoglycans in the formation of the macrophage cell surface pool of apoE has been clearly established (10, 14, 15). However, additional cell surface binding sites present on macrophages that can bind apoE must also be considered. One of these is the LDL receptor, which is expressed in human monocyte-derived macrophages and in several macrophage cell lines including the J774 macrophage line (16, 17). The ability of apoE derived from lipoproteins to bind to the LDL receptor has been clearly demonstrated (18). However, binding activity is conformation-dependent and, specifically, depends on association with lipid, the size of the lipid particle, and the number of apoE molecules per particle (18). To evaluate a potential role for the macrophage LDL receptor in the cell surface sequestration of endogenously synthesized macrophage apoE, we utilized the J774 macrophage model transfected to express an amount of human apoE similar to that produced by human monocyte-derived macrophages. Our studies clearly show a role for the macrophage LDL receptor in establishing the cell surface pool of apoE, and further indicate that the apoE bound to the cell surface LDL receptor can serve as a precursor for secreted apoE.

METHODS

Materials

The polyclonal rabbit antisera to the LDL receptor was prepared as previously described (19, 20). When added to [^{35}S]methionine-

Abbreviations: FCS, fetal calf serum; LDL, low density lipoprotein: apo, apolipoprotein; BSA, bovine serum albumin; βDX, 4-methylumbelliferyl-β-d-xyloside.

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labeled lysates of J774 cells, or in Western blots of unlabeled cell lysates, this antibody recognizes a unique protein that migrates at the molecular weight of the LDL receptor. Purified monoclonal antibody (IgGC7) to the LDL receptor (21) and purified monoclonal antibody to human cardiac myoglobin (used as an antibody control) were purchased from American Research Products (Belmont, MA). Ascites fluid containing a monoclonal antibody to the LDL receptor binding site of apoE (1D7) was purchased from the University of Ottawa Heart Institute (22). The antibody was purified on protein G Sepharose by standard methods. [^{35}S] methionine was purchased from Amersham Co. (Arlington Heights, IL). The goat-derived anti-human apoE antisera was obtained from International Immunology Co. (Murrieta, CA). All tissue culture reagents were obtained from Gibco (Grand Island, NY). All other materials were from previously described sources (8–12, 14).

J774 macrophages and human monocyte-derived macrophages

Mouse J774 macrophage cells stably transfected to express a human apoE3 cDNA have been previously described in detail (9). Under standard growth conditions, these cells secrete approximately 900 ng of apoE/mg cell protein over 24 h, which is an amount similar to that produced by mature human monocyte-derived macrophages in culture (15). These cells were maintained in selection medium containing DMEM with 10% FCS, and 0.5 mg/ml neomycin at 37°C in 5% CO_2. One week prior to initiation of experiments, the selection medium was replaced by 10% FCS in DMEM. Freshly isolated human monocytes were purified by elutriation, and were >94% pure as previously described (23). After differentiation to macrophages for 4 days in 10% pooled human AB serum and 20% FCS, the cells were utilized for experiments. J774 cells transfected to express an LDL receptor binding-defective mutant apoE were generated using transfection protocols previously described (9). The mutant apoE was produced by polymerase chain reaction-based mutagenesis using standard molecular biology techniques (24). In this mutant apoE, arginine 145 is changed to cysteine. The decreased binding to the LDL receptor for this form of mutant apoE has been previously characterized (18, 25). For some experiments, cells were depleted of proteoglycans by previously described techniques (10).

Immunoprecipitation of apoE from medium and cell lysates

ApoE was quantitatively immunoprecipitated from macrophage cell lysates and macrophage cell culture medium by methods previously described in detail (9). In each experiment, immunoprecipitations were started using equal numbers of trichloroacetic acid-precipitable counts. Therefore, changes in apoE synthesis and secretion are already corrected for changes in total protein synthesis and secretion. For experiments, macrophages were seeded in 6-well plates at 2 million cells per well until approximately 95% confluent before starting the labeling incubations. After immunoprecipitation, the apoE–antibody complex was separated on SDS polyacrylamide gels, and the radioactive signal present in apoE was detected and quantified using a Molecular Dynamics Phosphorimager with ImageQuant Software. The results are expressed in cpm scanning units. Statistical comparisons for significance were performed using the non-paired two-tailed Student's *t*-test; a value of $P < 0.05$ was considered significant.

RESULTS

We have previously identified a cell surface pool of apoE bound to cell surface proteoglycans (10). ApoE can also, when in the proper conformation, bind to the LDL receptor (18). This receptor is expressed in macrophages, including human monocyte-derived macrophages and various macrophage cell lines. We, therefore, evaluated the role of the macrophage cell surface LDL receptor in the binding of endogenously synthesized apoE. **Figure 1** shows the results of an experiment in which apoE-expressing J774 cells were labeled for 5 h with radioactive methionine at 37°C, and then incubated for 2 h at 4°C in the presence of increasing concentrations of nonimmune serum, or a polyclonal antibody to the LDL receptor. After the 2-h incubation at 4°C, the media were harvested for quantitative immunoprecipitation of apoE. As shown, inclusion of the LDL receptor antisera produced a concentration-dependent increase in apoE released into the medium. The nonimmune serum was without substantial effect compared to the BSA control. We used the LDL receptor antiserum and nonimmune serum at 20 μl/ml in future experiments.

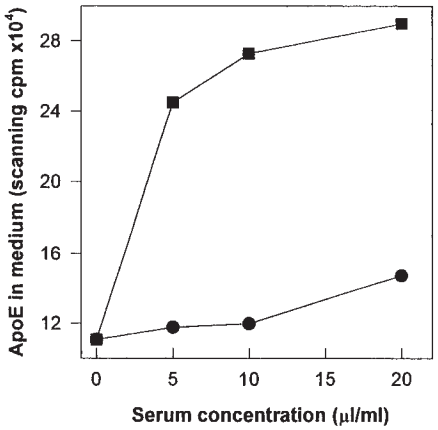

Fig. 1. Effect of LDL receptor antiserum on apoE released from the cell surface of apoE expressing J774 macrophages. ApoE-expressing J774 cells were seeded in 6-well plates, and grown as described in Methods. The cells were labeled by incubation with 100 μCi/ml of [^{35}S] methionine in medium containing 5 μm cold methionine for 5 h. After washing the cell monolayer, the cells were incubated for an additional 2 h at 4°C with DMEM containing 0.1% BSA, with LDL receptor antisera (squares) or nonimmune serum (circles) at the indicated concentrations (5–20 μl/ml). At the end of the 2-h incubation period, cell media were harvested for quantitation of apoE. Values shown are mean of duplicate samples.

We next confirmed these results using human monocyte-derived macrophages. In these cells (**Fig. 2**) the addition of the LDL receptor antisera at 4°C significantly reduced the amount of apoE in the cell lysate (by 30%), and significantly increased that released into the medium (by 50%). In **Fig. 3**, this experiment was repeated using a purified monoclonal antibody previously shown to recognize the ligand binding domain of the LDL receptor (21). Inclusion of the monoclonal antibody to the LDL receptor led to a significant increase in apoE released into the medium (by 57%), and a significant reduction in cell monolayer apoE (by 18%). The addition of the control antibody (to human cardiac myoglobin) had no effect on apoE in the cell monolayer or medium compared to BSA alone. The results in Figs. 1–3 show that an antibody to

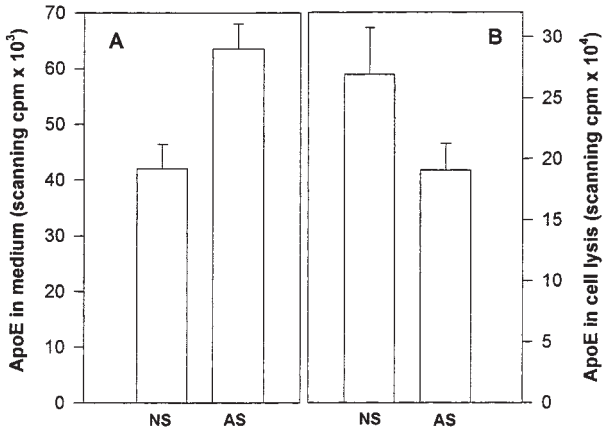

Fig. 2. Effect of LDL receptor antiserum on apoE released from the cell surface of human monocyte-derived macrophages. Human monocytes were seeded in 6-well plates and differentiated in the presence of 10% pooled human serum and 20% fetal bovine serum for 4 days. After washing, the cells were labeled by incubation at 37°C for 5 h with 200 μCi/ml of [^{35}S] methionine in medium containing 2.5 μm cold methionine. After washing the cell monolayer, the cells were incubated at 4°C for 2 h in medium containing 0.1% BSA, and either 20 μl/ml of LDL receptor antisera (AS) or nonimmune serum (NS). The media (panel A) and cells (panel B) were then harvested for quantitation of apoE. Values shown are mean ± SD from triplicate samples. The difference between AS vs. NS in the cell monolayer is significant at $P < 0.05$; and in medium at $P < 0.01$.

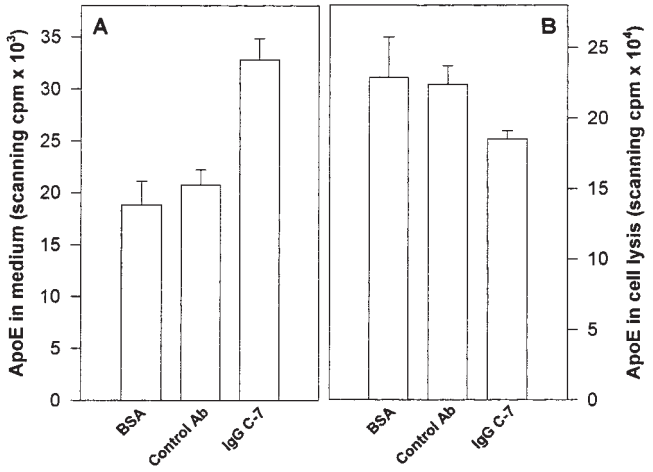

Fig. 3. Effect of monoclonal antibody to the LDL receptor on apoE released from the cell surface of human monocyte-derived macrophages. Human monocytes were seeded, differentiated, and labeled as described to the legend in Fig. 2. After washing the cell monolayer, the cells were incubated at 4°C for 2 h in medium containing 0.1% BSA with or without 12.5 μg/ml of purified monoclonal antibody to the LDL receptor (IgGC7) or 12.5 μg/ml of purified monoclonal antibody to human cardiac myoglobin (control). The cells and medium were then harvested for quantitation of apoE as described in Methods. Values shown are the mean ± SD from quadruplicate samples. Panel A shows results of apoE from the medium. Panel B shows results of apoE from the cell monolayer. The difference between IgGC7 vs. the IgG control in the medium and in the cells are each significant at $P < 0.01$.

the LDL receptor (monoclonal or polyclonal antisera) can displace apoE from the macrophage cell surface into the medium during a 4°C incubation. Additional experiments were performed to corroborate this conclusion. First, incubation of apoE-expressing J774 cells with acetylated LDL, in order to down-regulate LDL receptor protein expression, eliminated the ability of the polyclonal antisera to displace apoE from the cell surface during 4°C incubations (not shown). We also used a monoclonal antibody directed to an epitope of the apoE particle (amino acids 143–150) previously identified as important for binding to the LDL receptor protein (22). Incubation with this antibody also significantly released apoE from the cell monolayer during 4°C incubations. Finally, we evaluated the apoE–LDL receptor interaction using cells which express a mutant apoE, previously shown to be defective in binding to the LDL receptor, i.e., a mutant in which arginine 145 is changed to cysteine. Addition of LDL receptor antiserum to J774 macrophage cells expressing this LDL receptor binding-defective apoE mutant produced no change in the cell or medium content of apoE (**Fig. 4**). In addition to confirming the conclusion from the above experiments, these results also provided evidence that the interaction between apoE and the LDL receptor, which is disrupted by the LDL receptor antisera, is a direct one.

The issue of direct versus indirect interaction between apoE and the LDL receptor was next evaluated by examining a potential role of cell surface proteoglycans in participating in the LDL receptor/apoE interaction. This was of interest because of the important role played by cell surface proteoglycans in enhancing the interaction of apoE-containing lipoproteins with another cell surface receptor; the LDL receptor-related protein (26). For the experiment shown in **Fig. 5**, we compared the results of the addition of LDL receptor antisera, or nonimmune serum, to cells in which cell surface proteoglycans were intact (labeled +PG), or to cells in which cell surface proteoglycans had been reduced by preincubation in βDX and treatment with heparinase (labeled −PG). The addition of LDL receptor antisera to proteoglycan-replete cells produced a 77% increase in the medium content of apoE at 4°C. After depletion of proteoglycans, the addition of the LDL receptor antisera produced a 68% increase in medium content of apoE. Thus, the increase of apoE released from the cell surface by the antisera is similar whether cells are proteoglycan-replete or depleted. This result indicated that binding of the nascent apoE particle to the LDL receptor is not influenced by the presence of cell surface proteoglycans.

The above results, indicating that LDL receptors on the surface of macrophages can bind endogenously synthesized apoE, raised a question regarding the fate of apoE bound to the cell surface at this site. In addition, because of the long labeling period (5 h) used in the above experiments, it is unclear how rapidly newly synthesized apoE is bound to cell surface LDL receptors. In order to address these issues, we performed a series of pulse/chase experiments, and the results of representative experiments are shown in Figs. 6 and 7. For the experiment shown in **Fig.**

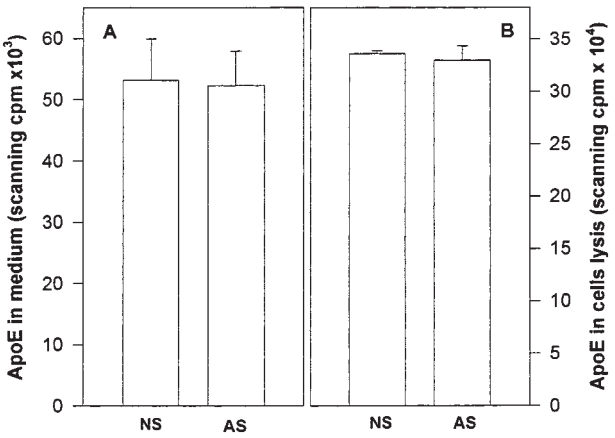

Fig. 4. Effect of LDL receptor antisera on the release of mutant apoE from the macrophage cell surface. J774 macrophages transfected with an LDL receptor binding-defective mutant form of apoE were grown as described in the legend to Fig. 1. Cells were labeled for 5 h with 100 μCi/ml of [^{35}S] methionine in medium containing 5 μm cold methionine. After washing, cells were incubated for an additional 2 h at 4°C with 20 μl/ml of LDL receptor antisera (AS) or nonimmune serum (NS). At the end of that time, media and cells were harvested for quantitation of apoE. Values shown are mean ± SD from triplicate samples. There are no significant differences between experimental conditions.

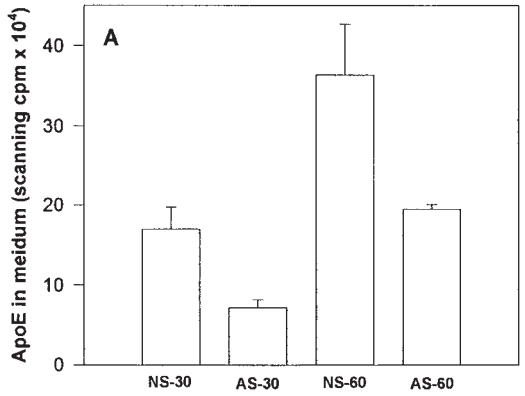

Fig. 6. Effect of preincubation in LDL receptor antiserum on the subsequent release of newly synthesized apoE from macrophages. ApoE-expressing J774 cells were seeded in 6-well plates as described in Fig. 1. Cells were labeled with medium containing 200 μCi/ml of [^{35}S] methionine at 37°C for 30 min. At the end of that time, cells were washed with PBS at 4°C and subsequently incubated for an additional 2 h at 4°C with DMEM containing 0.1% BSA with either 20 μl/ml of LDL receptor antisera (AS) or nonimmune serum (NS). At the end of 2 h, cells were washed and placed at 37°C in chase medium that contained 0.1% BSA and DMEM with 500 μm unlabeled methionine but no antiserum. Cells were then incubated at 37°C for an additional 30 or 60 min as indicated. At the end of the chase period, media were recovered for quantitation of apoE. Values shown are mean ± SD from triplicate samples. The differences between NS-30 vs. AS-30 are significant at $P < 0.01$ and that between NS-60 vs. AS-60 at $P < 0.01$.

6, cells were pulse-labeled for 30 min. and then rapidly cooled to 4°C for a 2-h incubation with LDL receptor antisera or nonimmune serum. At the end of that time, the cells were washed and placed in chase medium already at 37°C for an additional 30 min or 60 min. The chase medium contained only BSA and excess unlabeled methionine, but no antibody. During this 30-min chase, significantly less apoE was released from the cells that had been preincubated in the LDL receptor antisera, compared to cells that had been preincubated at 4°C with nonimmune serum. This result suggested that a portion of the apoE removed from the cell surface during the 4°C incubation was likely destined for secretion. Moreover, between 30 and 60 min, more apoE continued to be released from the cells preincubated with nonimmune serum than from cells preincubated at 4°C with the LDL receptor antisera. This is consistent with the explanation that nascent labeled apoE present inside the cell at the end of the 2 h incubation at 4°C was not released into the medium but was sequestered at LDL receptor binding sites made available by the release of apoE from these sites during the preincubation in LDL receptor antisera.

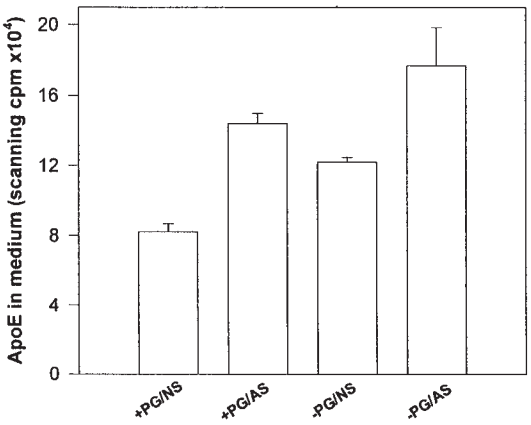

Fig. 5. Effect of LDL receptor antiserum on release of apoE in proteoglycan-depleted cells. ApoE-expressing J774 cells were seeded and grown as described in the legend to Fig. 1. Some cultures were depleted of proteoglycans by incubation for 48 h in 1 mm of βDX (designated as −PG). Cells were then labeled for 5 h with [^{35}S]methionine as described in the legend to Fig. 1. After the labeling period, cells that had been preincubated in βDX were incubated in freshly made heparinase (4 U/ml) for 1 h at 37°C. The monolayers were then washed extensively, and incubated at 4°C for 2 h in medium containing 0.1% BSA and 20 μl/ml of nonimmune serum (NS) or LDL receptor antiserum (AS). At the end of 2 h, medium was collected and apoE was quantitated. The values shown are the mean ± SD from triplicate samples. The difference between +PG/NS vs. +PG/AS is significant at $P < 0.01$. The difference between −PG/NS vs. −PG/AS is also significant at $P < 0.01$.

This issue was further evaluated by performing a similar experiment in which we also included LDL receptor antibody during the chase. The results of this experiment are shown in **Fig. 7**. Cells were pulse-labeled for 30 min and then incubated for 2 h with or without LDL receptor antisera at 4°C. At the end of that time, cells were placed in chase medium at 37°C with or without LDL receptor antisera for an additional 45-min chase period before harvesting the medium for quantitation of apoE. The effect of including the antisera only during the 4°C preincubation is shown by comparing the first two bars (NS/NS vs. AS/NS), and are completely consistent with the results shown

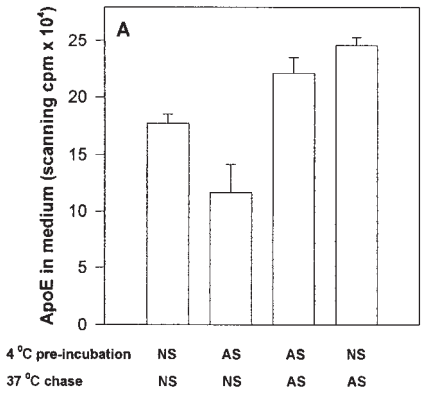

Fig. 7. Pulse/chase evaluation of the amount of newly synthesized apoE bound at the macrophage cell surface LDL receptor. ApoE-expressing J774 cells were grown and labeled as described in the legend to Fig. 6. After labeling, cells were incubated for 2 h at 4°C as described in the legend to Fig. 6 in the presence of 20 μl/ml of antisera (AS) or nonimmune serum (NS) as indicated. At the end of the 4°C incubation, cells were chased at 37°C in 01.% BSA with 500 μm cold methionine in the presence of 20 μl/ml LDL receptor antisera (AS) or non-immune serum (NS) for 45 min as indicated. At the end of this chase time, the media were harvested for quantitation of apoE. Values shown are mean ± SD from triplicate samples. The difference between NS/NS vs. AS/NS is significant at $P < 0.01$; that between AS/NS vs. AS/AS is significant at $P < 0.005$.

in Fig. 6. When LDL receptor antibody is included both in the 4°C preincubation and in the chase incubation at 37°C, the amount released during the 45-min chase is significantly greater than when antibody is present only during the 4°C preincubation (compare AS/NS vs. AS/AS). This indicates that the decreased apoE secretion observed in the AS/NS experimental condition was due to enhanced sequestration of nascent apoE by the LDL receptor during the chase. The last bar in this figure shows the result of including the antisera only during the chase incubation. The most apoE is released from cells treated in this manner because no apoE was lost during the 2-h preincubation at 4°C, and apoE binding to the LDL receptor was blocked during the 45-min chase.

DISCUSSION

A cell surface pool of apoE has been shown to be present in a variety of cell types, including hepatocytes, adrenal cells, and macrophages (10, 14, 15, 27–29). With respect to macrophages, the cell surface pool was first demonstrated in the J774 macrophage cell line that was transfected to express human apoE (10). Proteoglycans were identified as contributing to the formation of this pool, and it was further shown that cell surface apoE could be displaced by addition of phospholipid vesicles. A proteoglycan-associated cell surface pool of apoE has also been demonstrated in human monocyte-derived macrophages (14, 15). Interestingly, the study of human monocyte-derived macrophages that express the E2, E3, or E4 isoform of apoE produced results indicating that these naturally occurring mutations of apoE influence the size of the cell surface pool of apoE (15). In these studies, it was also demonstrated that the addition of suramin could displace apoE from the macrophage surface (15).

Multiple sites need to be considered for maintaining the cell surface pool of apoE in macrophages. Proteoglycans are involved based on studies using inhibitors of proteoglycan synthesis and sulfation or treatment with enzymes that degrade extracellular proteoglycans (e.g., heparinase) (10, 14, 15). However, there are other cell surface sites expressed in macrophages that can bind apoE. One of these is the LDL receptor. Expression of this receptor has been clearly demonstrated in macrophage cell lines, including the J774 cell line, and in human monocyte-derived macrophages (16, 17). It is also of interest that the LDL receptor is highly expressed in non-macrophage cells that make abundant apoE and that express a significant apoE cell surface pool; i.e., hepatocytes and steroidogenic cells.

The binding of purified apoE to the LDL receptor has been studied in detail (18). This binding is conformation-dependent and, specifically, depends on association with lipid, the size of the apoE-lipid particle, and the number of apoE molecules per particle. The receptor binding site for apoE has been ascribed, predominantly, to amino acids 136–150 by mutational analysis and monoclonal antibody mapping. ApoE does not bind with high affinity to the LDL receptor in the lipid-free state. Our results indicate, that there is significant binding of macrophage-derived apoE to the LDL receptor within 30–60 min after it is synthesized. This suggests that newly synthesized apoE becomes associated with lipid and is in the proper conformation for LDL receptor binding either prior to, coincident with, or very soon after arriving at the plasma membrane. Further, after demonstrating significant binding of newly synthesized apoE to the LDL receptor, our initial prediction was that apoE thus bound would be rapidly returned to the cell for degradation. While our results do not exclude such a path for a portion of the nascent apoE bound to the LDL receptor, they clearly indicate that some of this apoE will be secreted.

The involvement of the LDL receptor in modulating the secretion of macrophage apoE may change during evolution of the atherosclerotic plaque lesion. Such an interaction may be more important during the early infiltration and differentiation of monocyte-macrophages in the vessel wall compared to terminally differentiated foam cells in which LDL receptor expression would be low. The latter circumstance could represent an adaptation to maximize apoE secretion by eliminating expression of one of its cell surface binding sites. In addition, the presence of other physiologic ligands for the LDL receptor (e.g., LDL or remnant particles) could modulate the role of the LDL receptor in sequestering newly synthesized apoE. Characteristics for these competing interactions at the LDL receptor of the macrophage would depend not only on the abundance of each ligand, but on the effective

concentration at the cell surface and relative affinities of each potential ligand.

We have interpreted the results of our pulse/chase studies as demonstrating the binding of nascent apoE to the macrophage LDL receptor. While our results clearly indicate that the binding of newly synthesized apoE occurs very soon after apoE is synthesized, they cannot rule out a very rapid release and re-uptake of apoE which may occur in the pericellular space. It is interesting to speculate based on the results in Figs. 6 and 7, however, that LDL receptor binding sites on the cell surface must reach a threshold of occupancy before apoE will be released from the macrophage cell surface into the medium. How newly synthesized apoE is released from the cell surface LDL receptor and into the medium prior to endocytosis will require further investigation. The concentration of apoE at the cell surface may reach a sufficient level such that the equilibrium shifts to favor release. Alternatively, the apoE particle bound at the cell surface may be remodeled, perhaps by changes in lipid content, and this results in a modified apoE conformation with less affinity for the LDL receptor. The newly synthesized nascent apoE particle would then have higher affinity and displace the more mature and remodeled apoE particle from the LDL receptor.

Cell surface apoE in the macrophage could represent a metabolically distinct pool of apoE. Furthermore, apoE bound to different cell surface sites could be metabolically heterogeneous. Differential regulation of specific apoE cell surface binding sites could, therefore, alter the size and/or turnover of the cell surface pool of apoE, even separate from any changes in macrophage apoE synthesis. Differences have already been shown to exist in the capacity of the various naturally occurring apoE isoforms to form a cell surface pool in the macrophage (15), and we have already demonstrated the importance of this cell surface pool for HDL_3 binding to the macrophage (14). Modulation of the size and turnover of the cell surface pool of apoE in macrophages could have important implications for the metabolism of other lipoproteins by macrophages, as well as the interaction of macrophages with other cells or with cell-derived matrix components.

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