Cell Surface Proteoglycans Modulate Net Synthesis and Secretion of Macrophage Apolipoprotein E*

(Received for publication, August 1, 1995, and in revised form, March 13, 1996)

Madhuri Lucas and Theodore Mazzone†

From the Departments of Medicine and Biochemistry, Rush Medical College, Chicago, Illinois 60612

Using a macrophage cell line that constitutively expresses a human apolipoprotein E (apoE) cDNA, we have investigated the post-translational metabolism of endogenously produced apoE. Inhibition of lysosomal or cysteine proteases led to significant inhibition of apoE degradation but did not increase apoE secretion, indicating that cellular degradation is not limiting for apoE secretion in macrophages. Treatment of macrophages with inhibitors of proteoglycan synthesis (4-methylumbelliferyl-β-D-xyloside) or sulfation (sodium chlorate) enhanced the release of apoE from cells and significantly attenuated the increase in secretion produced by incubation with phosphatidylcholine vesicles (PV). These observations suggested that a significant fraction of the apoE retained by cells (and released by incubation with PV) was associated with proteoglycans. Treatment of cells with exogenous heparinase led to a greater than 4-fold increase in apoE secretion and similarly attenuated the response to PV, suggesting that apoE was trapped in an extracellular proteoglycan matrix. This conclusion was confirmed in studies showing that PV could enhance the release of apoE from cells during an incubation at 4 °C, but this enhanced release was abolished in proteoglycan-depleted cells. Incubation with lactoferrin at 4 or 37 °C produced a similar decrement in cellular apoE, again indicating the existence of a cell surface pool of apoE. Pulse-chase studies showed that the apoE trapped in the proteoglycan matrix was susceptible to rapid cellular degradation such that net synthesis of apoE (secreted plus cell-associated) was increased significantly in proteoglycan-depleted cells compared with control cells as early as 45 min during a chase period.

Previous work has shown that a substantial portion of apolipoprotein E (apoE) which is synthesized by macrophages is not secreted but is rapidly degraded (1). We have shown that the addition of HDL$_3$ or phosphatidylcholine vesicles (PV) can protect a portion of this apoE from degradation and promote its secretion (1). Cellular sterol balance, which modulates apoE synthesis and degradation of endogenously produced apoE, has been postulated to be important for cellular degradation of endogenously produced apoE based on studies using inhibitor molecules for these enzyme classes (2, 3).

The observation that a substantial portion of newly synthesized apoE is never secreted from macrophages raises the question of its potential function. ApoE has been postulated to modulate signaling of second messenger pathways in steroidogenic cells (4). Alternatively, intracellular apoE could be involved in the subcellular transport of lipid. Insight into potential functions could be obtained by identifying cellular compartments that retain the apoE that is not secreted. In hepatocytes, intracellular apoE has been localized to peroxisomes, cytosol, as well as elements of the endocytic and secretory apparatus (5, 6). These cells, however, have a unique role in assembling complex lipoprotein particles. Leblond and Marcel (7) have identified a plasma membrane pool of apoE in hepatocytes which appears to be important for selective uptake of cholesterol ester from HDL. Studies in mouse peritoneal macrophages have identified apoE in an endosomal/lysosomal compartment (3). Other studies, using human monocyte-derived macrophages, have identified focal accumulations of apoE associated with the plasma membrane within spaces formed by deep invaginations of this membrane (8).

Studies by Mahley and colleagues (9–11) have demonstrated that exogenously added apoE is avidly bound by the extracellular matrix of hepatocytes, especially by proteoglycans in the extracellular matrix. It has been postulated that these proteoglycans may serve as a high capacity reservoir for presenting apoE to specific cellular receptors (9–11). Studies by Stauderman et al. (12) have also documented an important role for extracellular proteoglycans in modulating hepatocyte apoE metabolism. In view of the above considerations and our previous observations regarding the effect of PV or HDL$_3$ on macrophage apoE secretion and degradation, we investigated a potential role for extracellular matrix in modulating the secretion and degradation of endogenously produced apoE in macrophages. The results of our investigations indicate that newly synthesized macrophage apoE is sequestered in a pericellular proteoglycan matrix and that the addition of PV stimulates release of this sequestered pool into the medium. Experimental interventions that inhibit the formation, or produce hydrolysis, of this proteoglycan matrix interfere with the sequestration of apoE and enhance its release into the medium, thereby attenuating the enhanced release of apoE in response to PV. Further, our studies demonstrate that apoE sequestered in the pericellular proteoglycan matrix is susceptible to rapid cellular degradation.

EXPERIMENTAL PROCEDURES

Materials—4-Methylumbelliferyl-β-D-xyloside (mDX), sodium chlorate, ALLN, monensin, chloroquine, ammonium chloride, and lactoferrin were obtained from Sigma. All other materials were from sources.

---

* This work was supported by National Institutes of Health Grant HL 39653. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Rush Medical College, 1653 West Congress Parkway, Chicago, IL 60612. Tel.: 312-942-6163; Fax: 312-563-2096.

1 The abbreviations used are: apoE, apolipoprotein E; HDL$_3$, high density lipoprotein 3; PV, phosphatidylcholine vesicles; mDX, 4-methylumbelliferyl-β-D-xylanoside; ALLN, N-acetyl-leucyl-leucyl-methioninal; dpm, disintegrations/min; LR, low density lipoprotein receptor-related protein.
Proteoglycans Modulate Macrophage ApoE Turnover

The human apoE-producing J 774 cell line used for these studies constitutively expresses a human apoE cDNA and has been characterized in detail previously (1). We have reported previously that this cell line, when incubated under standard growth conditions, produces 900 ng of apoE/mg of cell protein/24 h, an amount similar to that produced by mature cholesterol-loaded human monocyte-derived macrophages in culture. Our previous observation that addition of PV or HDL could preserve a substantial amount of newly synthesized apoE from degradation and enhance its secretion (1) led us to ask which of these effects was primary; i.e., was apoE preserved from degradation because its secretion was enhanced, or was secretion limited by competing cellular degradative pathways? The data in Tables I–III address this question. We used two different classes of protease inhibitors that have been shown to prevent the degradation of newly synthesized apoE: chloroquine and ammonium chloride as inhibitors of lysosomal proteases, and ALLN as a cysteine protease inhibitor. As shown in Tables I and II, these agents prevent degradation of newly synthesized apoE in macrophages that constitutively express a human apoE cDNA, leading to its accumulation within the cell. However, this intracellular stabilization and subsequent accumulation do not lead to a substantial increase in the secretion of apoE, especially when compared with the effect of PV on apoE secretion. Therefore, intracellular degradation is not rate-limiting for apoE secretion. This result could be explained if, after completion of its synthesis, apoE is committed to separate cellular pools destined for secretion or degradation which are not in equilibrium. We assessed this by evaluating the effect of monensin (1 μM) or PV (1 mg/ml) 24 h at 37°C. Where indicated, monensin (1 μM) or PV (1 mg/ml) were added during the chase period only. Values shown are the mean ± S.D. from triplicate cultures.

### Table I

| Effect of cysteine protease inhibition by ALLN on apoE degradation and secretion |
|------------------|------------------|------------------|------------------|
|                  | Medium apoE | Cell apoE |
| Control          | 2,470 ± 84   | 2,913 ± 133 |
| PV               | 6,197 ± 77   | 3,247 ± 509 |
| ALLN             | 3,144 ± 585  | 4,443 ± 609  |

### Table II

| Effect of lysosomal protease inhibitors on apoE degradation and secretion |
|------------------|------------------|------------------|
|                  | Medium apoE | Cell apoE |
| Control          | 1,131 ± 56   | 846 ± 74   |
| PV               | 6,319 ± 140  | 837 ± 49  |
| CH + NH₄Cl       | 715 ± 145    | 2,113 ± 11 |

### Table III

| Newly synthesized apoE is present in a post-Golgi pool available for rapid secretion |
|------------------|------------------|------------------|
|                  | Medium apoE | Change with PV |
| Control          | 672 ± 88     | 1                |
| PV               | 1,957 ± 257  | 2.9              |
| Monensin         | 416 ± 39     | 1                |
| Monensin + PV    | 1,072 ± 95   | 2.6              |
Inhibition of proteoglycan synthesis enhances apoE secretion.

Cells were seeded and grown as described under “Experimental Procedures.” Cells were incubated in growth medium alone or with 1 mM βDX for 72 h prior to the start of a 45-min labeling period. Where indicated, 1 mg/ml PV was added during the 45-min chase period. Values shown are the mean ± S.D. from triplicate cultures.

Lactoferrin has been shown to bind to cell proteoglycans and the LRP in hepatocytes (11). We therefore evaluated its effect on macrophage apoE secretion. As shown in Table V, lactoferrin at 100 μg/ml increased apoE secretion into the medium by 2.5-fold. However, reduction of cell proteoglycans by prior incubation in βDX did not appear to attenuate the effect of lactoferrin on apoE release. In fact, lactoferrin displaced an additional 3,192 dpm of apoE from βDX-treated cells compared with 906 dpm in control cells. This observation not only indicates the existence of an alternate binding site shared by endogenously produced apoE and lactoferrin, but also suggests a synergistic interaction between alternate site(s) and proteoglycans. As would be expected, lactoferrin caused a reduction in cell-associated apoE. The lowest cell-associated apoE was observed in cells grown in βDX and chased in the presence of lactoferrin. The next series of experiments were designed to more precisely define an extracellular versus intracellular location for the newly synthesized apoE that is released from cells by lactoferrin or by depletion of proteoglycans.

For the experiments shown in Table VI, cells were chased at 37 or 4°C, with or without lactoferrin. As shown, lactoferrin is equally effective at 4 or 37°C in reducing cell-associated apoE. Because cellular secretion is arrested at 4°C, this result indicates that lactoferrin is displacing apoE from an extracellular pool. We next assessed the accessibility of the proteoglycans responsible for sequestering apoE to exogenously added heparinase. As shown in Fig. 2, treatment with exogenous heparinase during a 60-min pulse period led to a 4.8-fold increase of apoE secretion during a subsequent 45-min chase in which heparinase was not present. Further, treatment with heparinase during the pulse period significantly blunted the effect of PV added only during the subsequent chase period. Therefore,
predigestion of extracellular proteoglycans during the pulse period led to enhanced apoE secretion during the subsequent chase period and attenuated the ability of PV to enhance apoE secretion further.

We next considered whether the observed effects of βDX or sodium chlorate could be ascribed to effects on an extracellular proteoglycan pool, or if it was necessary to consider a role for these agents in altering the intracellular processing of apoE. Cells were preincubated with no addition, or with βDX or sodium chlorate to inhibit the synthesis and/or sulfation of proteoglycans respectively (Fig. 3). During the pulse period, cells were treated with exogenous heparinase for 45 min. The results in Fig. 3 are expressed as fold change with heparinase treatment compared with the same preincubation condition without heparinase treatment. Cells not preincubated in βDX or sodium chlorate released substantially more apoE during the chase period after treatment with exogenous heparinase during the preceding 45 min. However, cells preincubated in βDX or sodium chlorate showed no increase in apoE release after the addition of PV because production of this pool has been inhibited.

Next we directly assessed the effect of proteoglycans on macrophage apoE turnover in more detail. In cells depleted of proteoglycans (Fig. 5) by a 3-day incubation in 1 mM βDX the amount of apoE secreted into the medium is higher at every point beyond 20 min compared with control cells. Further, the accumulation of apoE in the medium in βDX-treated cells appears to be linear for 120 min, whereas in control cells the amount of apoE in the medium appears to plateau and changes very little between 20 and 120 min. ApoE associated with the cell is also similar in the two groups at 20 min, but in proteoglycan-depleted cells it falls rapidly over the next 25 min to a level that is approached more slowly by control cells over the next 100 min. At 120 min, control and proteoglycan-depleted cells have similar levels of cell-associated apoE, but secreted apoE is 2.5-fold higher in the proteoglycan-depleted cells. These data indicate that newly synthesized apoE trapped in the extracellular proteoglycan matrix is susceptible to rapid cellular degradation.

**FIG. 2.** Pretreatment with heparinase enhances subsequent release of apoE from macrophages. Cells were seeded and grown as described under “Experimental Procedures.” Cultures were pulse labeled for 60 min. Where indicated, freshly made heparinase (Hep), 3 units/ml, was added only during the pulse labeling period. Cultures were then chased for 45 min with or without 1 mg/ml PV as indicated. Values shown are the mean ± S.D. from triplicate cultures.

**FIG. 3.** Pretreatment with heparinase does not enhance subsequent release of apoE from proteoglycan-depleted cells. Cells were seeded and grown as described in the legend to Fig. 2. Cultures were preincubated for 72 h in growth medium alone or with 1 mM βDX or 30 mM sodium chlorate (NaCl). Where indicated, freshly made heparinase (Hep), 3 units/ml, was added only during a 60-min labeling period. Cells were then washed and chased for 45 min with no additions. Autoradiographs were quantitated by laser densitometry and results expressed as fold change with heparinase compared with the same preincubation condition without heparinase treatment. Values shown are the mean ± S.D. from triplicate cultures.
The data in this manuscript indicate that a substantial portion of the apoE that is newly synthesized by macrophages is sequestered in an extracellular proteoglycan pool. The extracellular location of this pool is confirmed by its accessibility to exogenously added heparinase (Figs. 2 and 3) and by the results of experiments utilizing chase incubations at 4 °C (Table VI and Fig. 4). The rapidity with which this pool turns over is indicated by the data in Fig. 5; after 45 min of chase time there is already a significant difference in the net accumulation of apoE.
apoE between proteoglycan-depleted and control cells. Sequestration of apoE within this extracellular proteoglycan matrix, therefore, renders it susceptible to rapid cellular degradation.

Ye and colleagues have studied the effect of protease inhibitors on apoE degradation in hepatocytes and Chinese hamster ovary cells (2). Both ALLN and lysosomal inhibitors increased apoE accumulation within cells. In our studies, we show a similar effect of ALLN and lysosomal inhibitors on macrophage apoE accumulation, and we demonstrate that such cellular accumulation is not reflected in increased apoE secretion. Degradation of apoE by cysteine proteases or lysosomal proteases therefore is not rate-limiting for apoE secretion.

Others have previously studied the role of proteoglycans on hepatocyte secretion of apoE (12). However, cellular apoE degradation and turnover data were not reported. In these studies, heparan sulfate or heparin increased apoE secretion into the medium. Preincubation of cells in βDX attenuated the effect of heparin, but βDX alone did not increase the amount of apoE secreted into the medium. On the basis of this observation, it was suggested that apoE and proteoglycans are transported to the plasma membrane as a complex (12). This result is different from what we have observed in macrophages in which preincubation with βDX or sodium chlorate or treatment with heparinase directly increase the amount of apoE which is secreted into the medium. In macrophages, therefore, our data suggest that newly synthesized apoE becomes trapped in a preexisting pool of pericellular proteoglycans.

Mahley and co-workers (9–11) have reported extensively on the importance of extracellular proteoglycans for modulating cellular metabolism of exogenously added apoE, or lipoprotein ligands containing apoE, by hepatocytes. They have shown that cell surface proteoglycans act as an important intermediary serving to present apoE to specific cell surface receptors, for example the LRP. In one series of studies, it was demonstrated that lactoferrin inhibited remnant lipoprotein (and apoE) binding to hepatocytes and Chinese hamster ovary cells by interacting with both proteoglycans and the LRP (11). These observations are of specific interest for the interpretation of the data shown in Table V. Lactoferrin alone increased apoE released into the medium and decreased cell-associated apoE. This is consistent with its interaction with proteoglycans, or the LRP, leading to displacement of apoE from these sites. However, depletion of cellular proteoglycans does not attenuate the effect of lactoferrin on the release on apoE. Further, the displacement of apoE from proteoglycan-depleted cells appears to be enhanced compared with control cells (based on the apoE dpm displaced). This suggests that displacement from the non-proteoglycan site may in fact be facilitated in the absence of proteoglycans. This is consistent with the hypothesis put forth by Mahley and co-workers (9–11) that proteoglycans and cell surface receptors interact synergistically to bind and degrade apoE.

The data in Fig. 5 indicate that the pool of extracellular proteoglycans that sequester apoE are involved in facilitating its rapid cellular degradation. This would suggest that these proteoglycans are intimately associated with, or perhaps even anchored in, the plasma membrane. The rapidity of apoE turnover in this pool may also indicate the involvement of one of the cell surface endocytic receptors for apoE (e.g. LRP, low density lipoprotein receptor, very low density lipoprotein receptor) acting in concert with cell surface proteoglycans. Receptors exhibit rapid lateral mobility in the plasma membrane (for review, see Ref. 20). Because of this, apoE bound to abundant proteoglycan binding sites will have frequent encounters with higher affinity endocytic receptors that can rapidly internalize and degrade apoE.

The model we propose for post-translational regulation of apoE by pericellular proteoglycans is presented in Fig. 6. In the macrophage, newly synthesized apoE may be directed to the cell surface (b) or may remain intracellular (a). However, additional investigation is required to confirm the existence of a pool of apoE that remains (and functions?) intracellular without ever reaching the cell surface (as indicated by the question mark). ApoE directed to the cell surface may be secreted into the medium (b3) or trapped at the cell surface (b1, b2). Our data indicate that a preexisting pool of pericellular proteoglycans is important for pericellular trapping of apoE (b1); however, there also appears to be evidence for alternate cell surface binding site(s) that function to sequester newly synthesized apoE at the cell surface (b2). The addition of PV or lactoferrin stimulates release of apoE from cell surface binding sites into the medium. ApoE that is sequestered at the cell surface is susceptible to rapid cellular degradation (c, d). Whether apoE bound to pericellular proteoglycans must first be transferred to endocytic cell surface receptors (e.g. the LRP) prior to cellular degradation (c) or can be internalized directly by plasma membrane-bound proteoglycans (d) requires additional investigation. Questions that also require further investigation include how apoE is sorted at the membrane between secreted and sequestered pools and whether PV and lactoferrin release apoE from b1 and b2 sites with equal efficacy. Further, the identity of the proteoglycan(s) that bind apoE and whether these are integral plasma membrane proteins will require additional study. This model, supported by the data from our studies, proposes that the pericellular proteoglycan matrix represents an important locus for post-translational control of macrophage apoE expression. Changes in extracellular matrix composition which accompany macrophage differentiation or activation (21–23) can thereby significantly modulate the net accumulation of apoE at any tissue site where macrophages are a significant source of apoE, for example in the atherosclerotic vessel wall (24). Further, binding of macrophage-derived apoE in the matrix could alter the biological activity of cytokines or...
growth factors by displacing them from matrix binding sites and altering their interaction with their respective cell surface receptors. In addition, apoE in pericellular matrix could influence macrophage interaction with other lipoprotein and non-lipoprotein ligands that can directly interact with apoE.

Acknowledgment—We thank Beverly Burge for typing the manuscript.

REFERENCES

1. Mazzone, T., Pustelnikas, L., and Reardon, C. A. (1992) J. Biol. Chem. 267, 1081–1087
2. Ye, S. Q., Reardon, C. A., and Getz, G. S. (1993) J. Biol. Chem. 268, 8497–8502
3. Deng, J., Rudick, V., and Dory, L. (1995) J. Lipid Res. 36, 2129–2140
4. Reyland, M. E., and Williams, D. L. (1991) J. Biol. Chem. 266, 21099–21104
5. Dahan, S., Ahluwalia, J. P., Wong, L., Posner, B. I., and Bergeron, J. J. M. (1994) J. Cell Biol. 127, 1859–1869
6. Hamilton, R. L., Wong, J. S., Guo, L. S. S., Krisans, S., and Havel, R. J. (1990) J. Lipid Res. 31, 1589–1603
7. Leblond, L., and Marot, Y. L. (1993) J. Biol. Chem. 268, 1670–1676
8. Kruth, H. S., Skarlatos, S. I., Lilly, K., Chang, J., and Ifrim, I. (1995) J. Cell Biol. 129, 133–145
9. Ji, Z. S., Bredt, W. J., Miranda, R. D., Hussain, M. M., Innerarity, T. L., and Mahley, R. W. (1993) J. Biol. Chem. 268, 10160–10167
10. Ji, Z. S., Fazio, S., Lee, Y. L., and Mahley, R. W. (1994) J. Biol. Chem. 269, 2764–2772
11. Ji, Z. S., and Mahley, R. W. (1994) Arterioscler. Thromb. 14, 2025–2032
12. Stauderman, M. L., Brown, T. L., Balasubramanian, A., and Harmony, J. A. K. (1993) J. Lipid Res. 34, 190–200
13. Mazzone, T., and Reardon, C. (1994) J. Lipid Res. 35, 1345–1353
14. Batzri, S., and Korn, E. D. (1973) Biochim. Biophys. Acta 298, 1015–1019
15. Carey, D. J., Rafferty, C. M., and Todd, M. S. (1987) J. Cell Biol. 105, 1013–1021
16. Shimada, K., and Ozawa, T. (1987) Arteriosclerosis 7, 627–636
17. Rapraeger, A. (1989) J. Cell Biol. 109, 2509–2518
18. Humphries, D. E., Sugumaran, G., and Silbert, J. E. (1989) Methods Enzymol. 179, 428–434
19. Rapraeger, A. C., Krufka, A., and Olwin, B. B. (1991) Science 252, 1705–1708
20. Schlesinger, J., Lax, L., and Lenmon, M. (1995) Cell 83, 357–360
21. Edwards, I. J., Xu, H., Obunike, J. C., Goldberg, I. J., and Wagner, W. D. (1995) Arterioscler. Thromb. Vasc. Biol. 15, 400–409
22. Yeaman, C., and Rapraeger, A. C. (1993) J. Cell. Physiol. 157, 413–425
23. Laskin, J. D., Dokidis, A., Gardner, C. R., and Laskin, D. L. (1991) Hepatology 14, 306–312
24. Rosenfeld, M. E., Butler, S., Ord, V. A., Lipton, B. A., Dyer, C. A., Curtiss, L. K., Palinski, W., and Witztum, J. L. (1993) Arterioscler. Thromb. 13, 1382–1389