Interaction with proteoglycans enhances the sterol efflux produced by endogenous expression of macrophage apoE

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Abstract **Endogenous expression of apolipoprotein (apo)E in macrophages facilitates cholesterol efflux in the presence and absence of extracellular sterol acceptors. A proteoglycan-associated pool of apoE has also been described. The relationship between a proteoglycan-associated pool of apoE and enhanced cholesterol efflux was investigated in these studies. Inhibition of proteoglycan expression reduced cholesterol efflux from apoE-expressing cells (J774E$^+$) in the presence and absence of HDL, but did not do so from non-expressing cells (J774E$^-$). The effect of proteoglycan depletion on sterol efflux from J774E$^+$ cells was confirmed by measuring differences in cell sterol mass, secreted sterol mass, and sterol efflux rates. Furthermore, apoE-containing particles secreted from proteoglycan-depleted J774E$^+$ cells were denser than those secreted from J774E$^+$ cells with intact proteoglycan expression. Also, in J774E$^+$ cells with intact proteoglycans, apoE particles isolated from the cell surface proteoglycan layer were denser than secreted particles. The apoE-lipid particles isolated from the cell surface proteoglycan layer had a lower lipid-to-apoE and cholesterol-to-apoE ratio compared with secreted particles. In distinction, proteoglycan depletion of J774E$^-$ cells did not reduce sterol efflux produced by the exogenous addition of apoE. These observations indicate that one mechanism by which endogenous expression of apoE facilitates effective cholesterol efflux from macrophages is related to its retention at the cell surface in a proteoglycan-associated pool. Further, our data suggest that apoE arrives at the cell surface in a relatively lipid-poor state, and that a proximate source of lipid available to the proteoglycan-bound apoE at the cell surface resides in the plasma membrane.**—Lin, C-Y., Z. H. Huang, and T. Mazzone. **Interaction with proteoglycans enhances the sterol efflux produced by endogenous expression of macrophage apoE.** ***J. Lipid Res.*** **2001.** 42: **1125–1133.**

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Macrophages play an important role in protecting the vascular wall from injury (1). In the presence of hyperlipemia, macrophages are among the first cells to accumulate in the vessel wall, and they express plasma membrane receptors that allow them to clear the extracellular space of denatured or oxidized lipoproteins. Macrophages are also the major source of secreted proteins that modulate vessel wall lipid/lipoprotein homeostasis including lipoprotein lipase and apolipoprotein (apo)E (2–4). The vessel wall expression of each of these proteins by macrophages produces effects on atherosclerosis that are distinct from those that can be attributed to systemic expression, as demonstrated in engineered mouse models (5–18). Macrophage-derived apoE, in particular, has been shown to have important effects on the susceptibility of the vessel wall to hyperlipemic injury, and to significantly modulate development of atherosclerotic lesions after such injury. The modulation of cholesterol efflux by endogenously produced apoE in the macrophage is one mechanism by which macrophages could influence vessel wall response to hyperlipemic injury. We and others demonstrated that endogenous expression of apoE by macrophages enhances efflux of cholesterol (19–23). This enhanced efflux can be observed in the absence of extracellular acceptors, but is magnified in the presence of such acceptors (e.g., HDL or phospholipid vesicles). In addition, the experimental characteristics of cholesterol efflux produced by endogenous expression of apoE can be distinguished from efflux produced by the exogenous addition of lipid-free apoE (23). This supports the notion that endogenous expression and exogenous addition of apoE may work by distinct mechanisms to produce cholesterol efflux from macrophage cells.

In previously reported studies, we demonstrated the usefulness of the transfected J774 model to study the effect of apoE expression on cholesterol flux in macrophages (21, 23, 24). We showed that cholesterol efflux produced

Abbreviations: ABC, ATP-binding cassette; ALDL, acetylated LDL; apo, apolipoprotein; HDL_3, apoE-free HDL_3; FCS, fetal calf serum; J774E$^+$, apoE-expressing J774 macrophages; J774E$^-$, nonexpressing macrophages; β-DX, 4-methyl-umbelliferyl-β-D-xyloside; SR-BI, scavenger receptor BI.

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in this model is very similar to that observed in human monocyte macrophages, in terms of amount of cholesterol and phospholipid secreted, the density of the apoE-containing lipid particle, and the response of cholesterol and phospholipid efflux to enriching cells with excess cholesterol. The availability of this transfected model offers a unique opportunity to evaluate the mechanism of endogenous apoE-induced sterol efflux from macrophages by comparing cells that express physiologic amounts of apoE with nontransfected parental cells that do not express apoE.

We also previously demonstrated the presence of a significant pool of apoE sequestered by proteoglycans on the macrophage cell surface in both the transfected J774 line and in human monocyte-derived macrophages (25). Using the transfected J774 macrophage model, it was recently demonstrated that apoE expression increases macrophage proteoglycan production and increases the length of glycosaminoglycan chains in proteoglycans (26). Conversely, inhibiting proteoglycan production by macrophages increases apoE secretion from cells and decreases its endogenous degradation (25). These observations suggest an important interaction between endogenously synthesized apoE and proteoglycans in macrophages. This interaction could have important implications for the binding of growth factors, or in cell-cell and cell-matrix associations in the vessel wall. In addition, as we discussed previously (23), observations in the literature suggest a potential role for cell surface apoE in producing cholesterol efflux from macrophages. For example, a cell surface pool of apoE, formed during movement of endogenously synthesized apoE across the pericellular space, could be optimally positioned to facilitate desorption of lipids from the plasma membrane or to enhance apoE interaction with ATP-binding cassette (ABC) transporters. In the current series of studies, we evaluate the importance of proteoglycan-apoE interactions, predominantly at the cell surface, for facilitating cholesterol efflux from macrophage cells.

MATERIALS AND METHODS

Materials

[^{3}H]cholesterol, [^{35}S]methionine, and the Enhanced Chemiluminescent kit were purchased from Amersham (Arlington Heights, IL). Aprotinin, leupeptin, PMSF, benzamidine, 4-methylumbelliferyl-β-D-xyloside (β-DX), heparinase, cholesterol, and peroxide-conjugated rabbit-anti-goat IgG were obtained from Sigma (St. Louis, MO). Coprostanol was obtained from Steraloids (Newport, RI). The goat anti-human apoE antiserum was obtained from International Immunology Co. (Murrieta, CA). All tissue culture reagents including DMEM, fetal calf serum (FCS), and neomycin were purchased from Gibco (Grand Island, NY), and all organic solvents including isopropanol, methanol, hexane, and chloroform were from Fisher Scientific Co. (Pittsburgh, PA). Acetylated LDL (ALDL) and apoE-free HDL_3 (HDL_3) were prepared as previously described (21, 23). HDL_3 was radiolabeled as previously described (24). Purified apoE from human VLDL was purchased from Calbiochem (San Diego, CA).

Cells

ApoE-expressing (J774E$^+$) and nonexpressing (J774E$^-$) macrophages have been previously characterized in detail (21, 23, 25–28), and were maintained in DMEM containing 10% FCS and neomycin (0.5 mg/ml) at 37°C in an atmosphere of 5% CO_2. One week prior to experiments, the cells were switched to neomycin-free growth medium. Where indicated, cells were enriched with cholesterol by incubating with ALDL at 80 μg/ml in DMEM containing 5% FCS. For studies in which cell surface proteoglycans were to be depleted, β-DX was added into the growth medium at final concentration of 1 mM 72 h prior to the start of the experiments; 2 h before the start of experiments, the cells were washed three times with DMEM containing 0.1% BSA, and incubated in the same medium containing 5 U/ml heparinase at 37°C for 2 h. We previously showed that treatment with β-DX reduces the synthesis of proteoglycans by up to 80%, and that treatment of J774E$^+$ cells with β-DX and heparinase significantly reduces cell surface apoE (25). Because apoE synthesis does not change after proteoglycan depletion, this reduction in cell surface trapping leads to increased apoE release from proteoglycan-depleted J774E$^+$ cells (25).

Cholesterol efflux kinetics

Cells were plated as previously described and labeled with [^{3}H]cholesterol (2 μCi/ml) for 48 h in DMEM plus 10% FCS (23). [^{3}H]cholesterol was added in ethanol vehicle and the final ethanol concentration in the medium was <0.4%. Prior to the start of efflux incubation, the cells were rinsed three times with DMEM plus 0.1% BSA, incubated in DMEM plus 0.5% BSA for 2 h, and then rinsed again in DMEM alone. The efflux time course was initiated by the addition of efflux medium (DMEM + 0.1% BSA) to the culture, and aliquots of the medium were sampled at the time point indicated. Medium samples were centrifuged at 2,500 rpm × 15 min to pellet detached cells. ^{3}H radioactivity in the supernatant was quantitated by liquid scintillation counting. Efflux is expressed as a percentage, calculated as [^{3}H]cholesterol radioactivity released divided by that measured in cells at the beginning of the efflux incubation. Cell monolayers were washed three times with PBS, dissolved in 0.1 N NaOH, and assayed for protein and cholesterol content.

[^{35}S]apoE and ^{3}H lipid labeling, isolation, and analysis of macrophage-derived apoE particles

ApoE-expressing macrophages were grown in DMEM containing 10% FCS until 80% confluence, and switched to DMEM containing 5% lipoprotein-deficient FCS for 8 h to up-regulate de novo sterol synthesis. The cells were labeled with [^{35}S]methionine (100 μCi/ml) and [^{3}H]sodium acetate (40 μCi/ml) for 18 h in methionine-free DMEM supplemented with 10 μM cold methionine and 0.1% BSA. After three washes with PBS plus 0.1% BSA at 37°C, the cells were incubated for an additional 1 h in the methionine-free DMEM containing the same amount of [^{35}S]methionine and [^{3}H]sodium acetate. This medium (i.e., secreted fraction) was collected into prechilled tubes containing a protease-inhibitor cocktail (aprotinin, PMSF, leupeptin, and benzamidine). Cell monolayers were subsequently treated with heparinase (5 U/ml) in DMEM plus 0.1% BSA for 30 min at 37°C. This fraction (cell surface) was collected as noted above. Both fractions were centrifuged at 2,500 rpm × 15 min to pellet detached cells. ApoE-lipid particles in each fraction were then recovered by immobilization on protein G agarose beads coated with apoE antiserum in detergent-free buffer. The pelleted beads were extensively washed with PBS, and resuspended in 0.2 ml PBS; the pellets were then split into two equal volumes. From one set of samples, ^{3}H-labeled lipid components were extracted using hexane–isopropanol (3:2, v/v), separated by TLC

(hexane–acetone–acetic acid, 70:30:2, v/v/v), and radioactivity in cholesterol was quantified by liquid scintillation counting. From the other set of samples, immunoprecipitated apoE was released from the beads by boiling in a dissociation buffer containing 5% SDS, 0.12 M Tris-HCl (pH 6.8), and β-mecaptoethanol (40 μl/ml) for 15 min, and subjected to SDS-PAGE. ApoE-associated ^{35}S radioactivity in apoE was detected and quantified with a Molecular Dynamics Storm Phosphorimager using ImageQuant software. This immunoisolation procedure allows for highly specific and quantitative recovery of biosynthetically labeled apoE (27, 29).

Density distribution of apoE-lipid and HDL_3 particles

To assess the effect of proteoglycan depletion on the density of the secreted apoE-lipid particles, apoE-expressing macrophages were plated in T-75-cm^2 flasks at 3 to 4 million cells per flask in DMEM plus 10% FCS until 60–70% confluence. Control apoE-expressing cells are incubated for an additional 72 h in growth medium prior to the start of the apoE particle collection. For proteoglycan-depleted apoE-expressing cells, β-DX at 1 mM was included in this 72-h incubation, and cells were treated with heparinase (5 U/ml) for 2 h at 37°C prior to start of the apoE particle collection. Cells were washed extensively, and 0.1% BSA in DMEM was added to start the apoE particle collection. After 24 h, the medium was harvested into prechilled tubes containing pepstatin (5 μg/ml), leupeptin (5 μg/ml), benzamidine (10 μg/ml), aprotinin (0.1 mg/ml), and PMSF (0.5 mM). The samples were centrifuged at 2,500 rpm × 15 min (4°C) to pellet floating cells. The supernatant was recovered and subjected to KBr density-gradient ultra-centrifugation as previously described (23). Fractions were exhaustively dialyzed against saline/EDTA (4°C) to remove KBr, and lyophilized using a speed vacuum. The lyophilized fractions were resuspended in an equal volume of PBS (0.2 ml); an aliquot was taken for SDS-PAGE and Western blotting in which a goat-anti-human apoE antiserum was used as the primary antibody, and horseradish peroxidase-conjugated rabbit-anti-goat IgG as the secondary antibody. ApoE bands were visualized using an enhanced chemiluminescent kit from Amersham. Quantitation was performed using a UMAX scanner and Zero Dscan software (Scanalytics Inc., Fairfax, VA).

In experiments designed to evaluate the density of proteoglycan-associated cell surface apoE, the cells were plated and grown as described above without β-DX. The surface proteoglycan-associated apoE was harvested from the cell surface by heparinase treatment (5 U/ml, 37°C for 30 min). ApoE-lipid particles released from the cell surface were collected for density-gradient ultracentrifugation, SDS-PAGE, and Western-blotting analysis as described above. For HDL_3 density distribution, iodinated HDL_3 was incubated with cells for 24 h as described in the figure legends. Cell culture medium was recovered and the distribution of radioactivity along a KBr density gradient was determined.

Other procedures

Cholesterol mass in extracts of cells or media was measured by gas-liquid chromatography using coprostanol as the internal standard (23). Protein mass was measured using the method of Lowry using BSA as standard. Statistical significance was evaluated by ANOVA using SPSS software (SPSS Inc., Chicago, IL).

RESULTS

Depletion of cellular proteoglycans reduces sterol efflux from $J774E^+$ cells to HDL_3

In the first series of experiments, we incubated radiolabeled HDL_3 with $J774E^+$ and $J774E^-$ cells. Both cell types

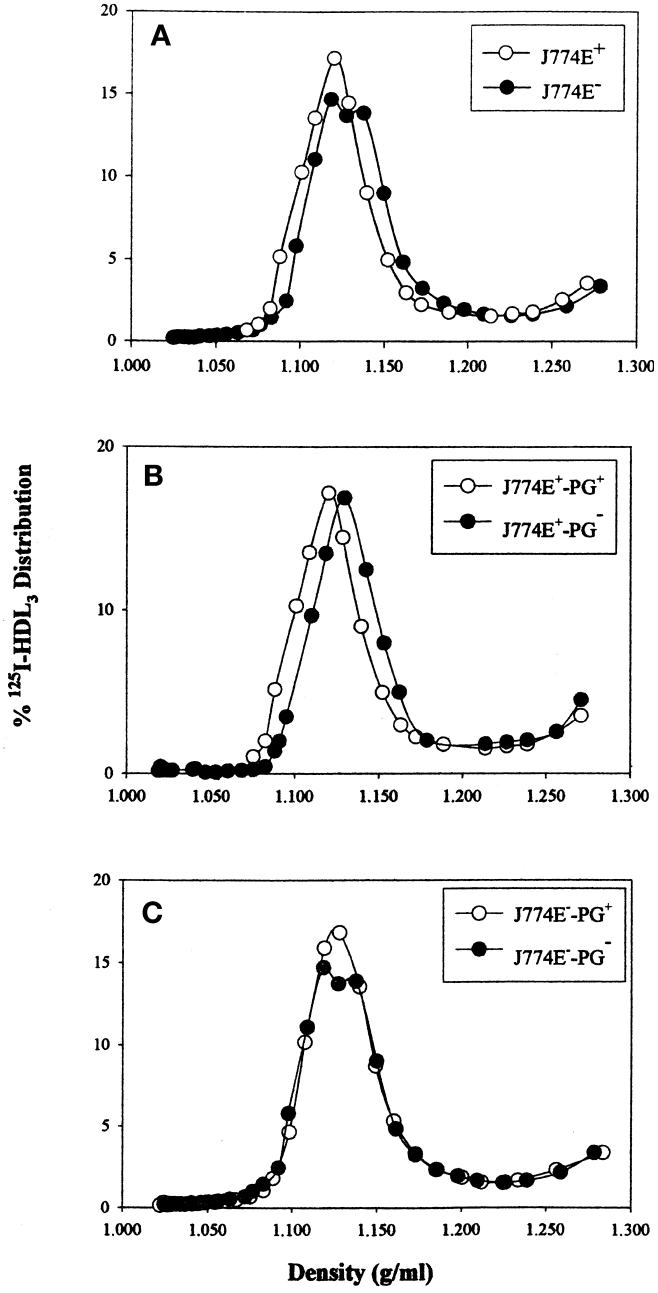

Fig. 1. Density distribution of HDL_3 after incubation with control or proteoglycan-depleted $J774E^+$ and $J774E^-$ cells. $J774E^+$ and $J774E^-$ cells were grown in 5% FCS plus 80 μg/ml ALDL, alone or with 1 mM β-DX. After 48 h, medium was aspirated and cells treated with β-DX were incubated in 5 U/ml of heparinase for 2 h at 37°C. All cells were extensively washed, and 0.1% BSA with 40 μg ^{125}I HDL_3 and 1 μg/ml S58035 was added to all cultures. In addition, cultures previously treated with β-DX and heparinase received 1 mM β-DX. After 24 h, the medium was collected, cells were removed by centrifugation, and the density distribution of radioactivity in HDL_3 was measured through a KBr gradient as described in Materials and Methods. A: The density distribution of ^{125}I HDL after incubation with $J774E^+$ or $J774E^-$ cells. B: The density distribution of ^{125}I HDL_3 after incubation with $J774E^+$ cells or proteoglycan-depleted $J774E^+$ cells. C: The density distribution of ^{125}I HDL_3 after incubation with $J774E^-$ or proteoglycan-depleted $J774E^-$ cells. The density gradients shown are from a representative analysis from a total of three experiments performed. PG^+, cells with intact proteoglycans; PG^-, cells with depleted proteoglycans.

TABLE 1. Proteoglycan depletion reduces HDL_3-mediated cholesterol efflux only from J774E$^+$ macrophages

	J774E$^+$		J774E$^-$	
Total Cellular Cholesterol	Control	Proteoglycan-Depleted	Control	Proteoglycan-Depleted
	μg/mg cell protein			
Before incubation with HDL_3	63.96 ± 3.35[a]	69.47 ± 4.45	69.91 ± 3.87	62.28 ± 1.18
After incubation with HDL_3	51.14 ± 4.18[a]	69.41 ± 2.35	65.37 ± 4.04	62.77 ± 1.39

Macrophages were incubated for 72 h in DMEM with 10% FCS. For proteoglycan depletion, 1 mM β-DX was included. Forty-eight hours prior to the start of the efflux incubation, ALDL was added to the culture medium of all cells to a final concentration of 80 μg/ml. The cells were then washed and incubated in DMEM with 0.1% BSA for 2 h. Heparinase (5 U/ml) was included in cells preincubated with β-DX. Cells were subsequently incubated in DMEM with 0.1% BSA plus apoE-free HDL_3 (40 μg/ml). β-DX (1 mM) was included in proteoglycan-depleted cells. After 24 h, cellular lipids were extracted and quantified. Data shown are mean ± SD from triplicate samples.
[a] $P < 0.05$.

were cholesterol loaded by incubation with ALDL prior to the start of the incubation with radiolabeled HDL_3. Each cell type was also examined with and without proteoglycan depletion. As noted in Materials and Methods, proteoglycan depletion was accomplished by treatment with heparinase and β-DX. The latter compound substitutes for the core protein moiety of proteoglycans in the cell and significantly reduces their synthesis and appearance on the cell surface (25). After 24-h incubation with labeled HDL_3, the HDL_3 was recovered from cell culture medium and resolved on a KBr density gradient. As shown in **Fig. 1A**, after incubation with J774E$^+$ cells, the HDL_3 had a more buoyant density compared with HDL_3 incubated with J774E$^-$ cells. As shown in Fig. 1B, depletion of proteoglycans from the J774E$^+$ cells resulted in a shift of radiolabeled HDL_3 to denser fractions. As shown in Fig. 1C, similar proteoglycan depletion of J774E$^-$ cells did not shift HDL_3 to a denser fraction. These results (representative of a total of three separate experiments) are consistent with the interpretation that HDL_3 acquires more cellular lipid from J774E$^+$ cells than from J774E$^-$ cells (Fig. 1A), and that proteoglycan depletion reduces lipid efflux from J774E$^+$ cells but not from J774E$^-$ cells (Fig. 1B and C). Next, this issue was evaluated in another way. The change in cellular cholesterol mass in cholesterol-enriched macrophages was measured in control and proteoglycan-depleted J774E$^+$ and in control and proteoglycan-depleted J774E$^-$ cells during a 24-h incubation in HDL_3. The results of a representative experiment are shown in **Table 1**. In J774E$^+$ cells, there is a substantial decrease in total cellular cholesterol during the 24-h incubation period in HDL_3. However, this decrease is significantly attenuated in J774E$^+$ cells depleted of proteoglycans. There was no significant change in total cellular cholesterol in J774E$^-$ cells or in proteoglycan-depleted J774E$^-$ cells over the 24-h incubation period in HDL_3.

Depletion of cellular proteoglycans reduces sterol efflux from J774E$^+$ in the absence of extracellular sterol acceptors

We next evaluated the effect of proteoglycan depletion on cholesterol efflux in the absence of a specific extracellular sterol acceptor. In addition, these experiments were performed in cells with basal levels of cholesterol in order to determine whether cholesterol loading was required to observe differences. Because cell cholesterol would likely not change substantially due to homeostatic changes in cell cholesterol synthesis, for this experiment, we measured the mass of cholesterol secreted into the medium during a 24-h incubation. As shown in **Table 2**, the highest amount of cholesterol was secreted from J774E$^+$ cells with intact proteoglycans; this amount was significantly reduced by proteoglycan depletion. The level of cholesterol secreted from J774E$^-$ cells was lower than J774E$^+$ cells and was not affected by proteoglycan depletion. We next evaluated the effect of proteoglycan depletion on cholesterol efflux using a radiolabeled kinetic efflux assay. J774E$^+$ and J774E$^-$ cells with or without proteoglycan

TABLE 2. Proteoglycan depletion decreases spontaneous cholesterol efflux only from J774E$^+$ macrophages

	J774E$^+$		J774E$^-$	
Total Cholesterol	Control	Proteoglycan-Depleted	Control	Proteoglycan-Depleted
	μg/mg cell protein			
Cell	24.25 ± 3.20	25.31 ± 1.45	27.13 ± 4.28	26.11 ± 2.27
Medium	1.47 ± 0.31[a]	0.71 ± 0.12[a]	0.74 ± 0.08	0.68 ± 0.20

Macrophages were depleted of proteoglycans using β-DX (1 mM for 72 h), and heparinase (5 U/ml for 2 h), as noted in the legend to Table 1. Cells were then washed extensively and incubated for an additional 24 h in DMEM with 0.1% BSA. β-DX (1 mM) was included in proteoglycan-depleted cells. After this 24 h incubation, the medium was collected, and total cholesterol in media and cells was measured as described in Materials and Methods. Data shown are mean ± SD from triplicate samples.
[a] $P < 0.01$.

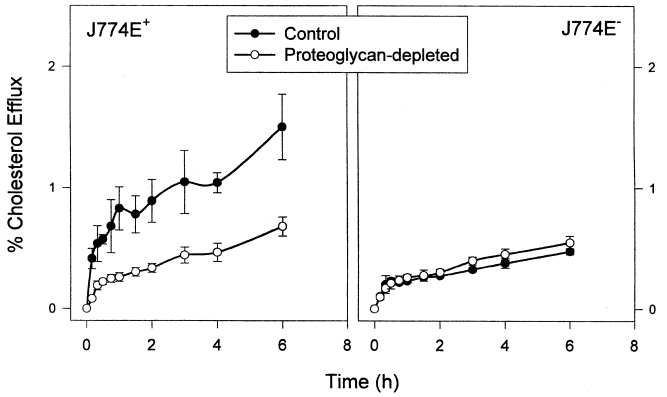

Fig. 2. Effect of proteoglycan depletion on cholesterol efflux kinetics in J774 macrophages. Cells with basal levels of cholesterol were grown and labeled as described in Materials and Methods. Seventy-two hours before the start of the efflux assay, β-DX (1 mM) was added to cells for proteoglycan depletion. Cells were radiolabeled with [^{3}H]cholesterol (2 μCi/ml) in DMEM plus 10% FCS for 48 h. Cells were then extensively washed and incubated in DMEM with 0.1% BSA for 2 h. Heparinase (5 U/ml) was included in cells previously incubated in β-DX. The cholesterol efflux assay was initiated by adding fresh DMEM with 0.1% BSA. β-DX (1 mM) was added to proteoglycan-depleted cells. The appearance of labeled cholesterol was measured in the medium at the time points indicated in the figure. Data shown are mean ± SD from triplicate samples. The specific activities for cellular cholesterol (dpm/ng) in the four groups are as follows: J774E$^+$ control cells, 35.0 ± 1.3; J774E$^+$ proteoglycan-depleted cells, 41.4 ± 3.5; J774E$^-$ control cells, 30.1 ± 2.1; J774E$^-$ proteoglycan-depleted cells, 32.4 ± 2.3.

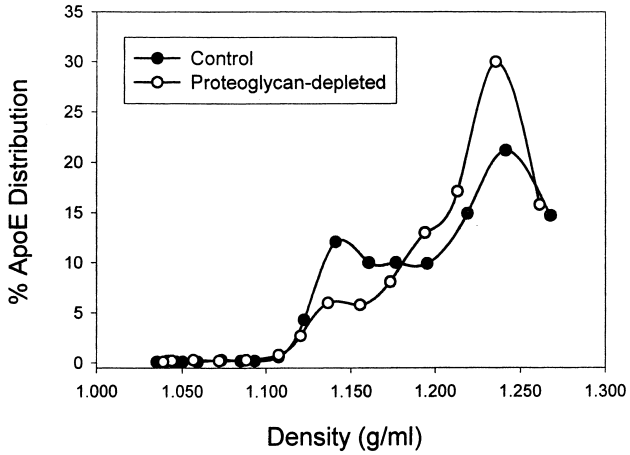

Fig. 3. Effect of proteoglycan depletion on the density of the secreted apoE-lipid particle. Control or proteoglycan-depleted J774E$^+$ macrophages were incubated in DMEM containing 0.1% BSA for 24 h. The medium was collected and subjected to KBr density-gradient ultracentrifugation, as described in Materials and Methods. ApoE in each fraction was detected and quantified as described in Materials and Methods.

depletion were labeled to equilibrium with [^{3}H]cholesterol, and the time course for the appearance of labeled cholesterol in the medium was monitored. The efflux of cholesterol was expressed as a percentage of total labeled cellular cholesterol present at the start of the efflux incubation. As shown in **Fig. 2**, there was increased cholesterol efflux from J774E$^+$ compared with J774E$^-$ cells in the proteoglycan-replete state as we have previously reported (23). Depletion of cellular proteoglycans significantly reduced the efflux of cholesterol from J774E$^+$, but not from J774E$^-$ cells. In summary, the results of the above experiments examining either cellular cholesterol mass, medium cholesterol mass, or cholesterol efflux kinetics indicate that reduction of cellular proteoglycans reduces cholesterol efflux from J774E$^+$, but not from J774E$^-$ macrophages. This result indicates that the decrease in cholesterol efflux after proteoglycan depletion of macrophages involves a proteoglycan-apoE interaction.

Proteoglycan depletion leads to secretion of a denser apoE-lipid particle from J774E$^+$ cells

We examined the density distribution of secreted apoE particles from J774E$^+$ cells compared with proteoglycan-depleted J774E$^+$ cells. J774E$^+$ cells (with intact proteoglycans) secreted an apoE particle with two major density peaks (**Fig. 3**). Depleting cellular proteoglycans prior to collection of the particles resulted in a shift in the distribution of the secreted apoE particle from the more buoyant to the denser peak (Fig. 3). This result indicated that proteoglycan depletion resulted in the release of more lipid-poor apoE particles from J774E$^+$ cells.

ApoE-lipid particles in the pericellular proteoglycan layer are denser and have a lower lipid-to-apoE ratio than secreted particles

We previously showed that a significant portion of cellular apoE is sequestered in a pericellular proteoglycan matrix. If release of lipid-poor particles in proteoglycan-depleted J774E$^+$ cells (shown in Fig. 3) was due to decreased retention of these particles on the cell surface, then apoE-lipid particles harvested from this pericellular proteoglycan layer should be denser and lipid-poor compared with secreted particles. We directly evaluated this prediction. J774E$^+$ cells were grown without proteoglycan depletion, and secreted and proteoglycan-associated cell surface apoE particles were collected (**Fig. 4**). ApoE collected from the medium demonstrated a prominent buoyant peak that was absent from the cell surface pool of apoE, consistent with our prediction. The basis for the difference in density between cell surface and secreted apoE particles was then further investigated; that is, we sought to confirm that differences in density were specifically due to changes in lipid-to-apoE ratio. Cellular apoE and lipids were labeled to equilibrium over 18 h in apoE-expressing cells. After labeling, secreted and cell surface apoE particles were harvested and isolated in the absence of detergents on protein G agarose beads coated with apoE antiserum as described in Materials and Methods. The purified apoE-lipid particles were then analyzed for labeled lipid and apoE content. The results of this analysis are shown in **Fig. 5**. The left panel of this figure shows the ratio of total lipid radioactivity to apoE radioactivity in isolated particles. This ratio was significantly lower in the cell surface fraction compared with the medium fraction. The right panel shows the results for cholesterol (separated and

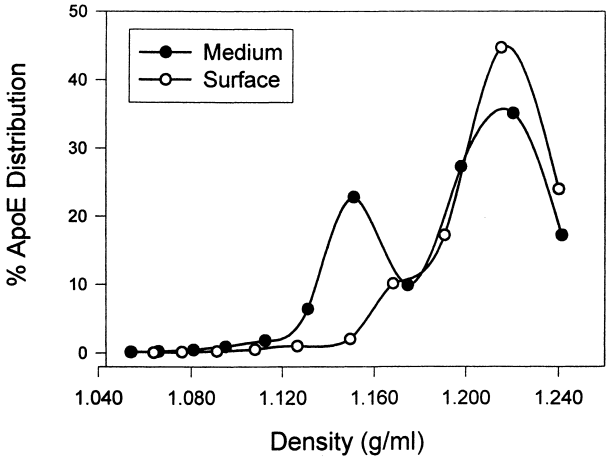

Fig. 4. Comparison of the density distribution between secreted and proteoglycan-associated cell surface apoE-lipid particles. Secreted apoE-lipid particles were collected in medium from J774E$^+$ cells with intact proteoglycans as described in the legend to Fig. 3. A pericellular proteoglycan-associated pool of apoE-lipid particles was harvested by treating proteoglycan-replete cells with medium containing heparinase (5 U/ml) for 2 h at 37°C. Medium and pericellular apoE particles were analyzed as described in the legend to Fig. 3.

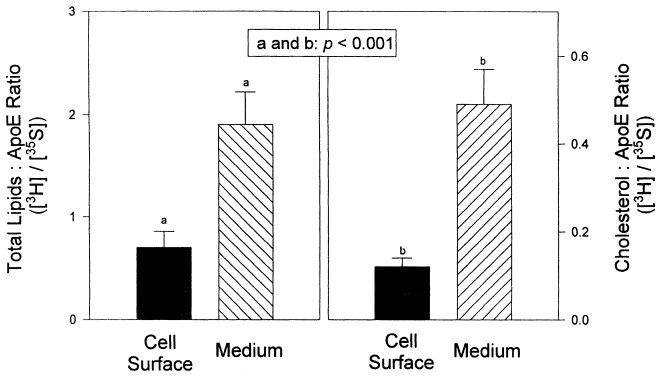

Fig. 5. Comparison of lipid to apoE and cholesterol to apoE ratios in secreted and proteoglycan-associated cell surface apoE-lipid particles. J774E$^+$ cells were incubated in DMEM containing 10% lipoprotein-deficient FCS for 8 h and subsequently labeled in medium containing [^{3}H]sodium acetate (40 μCi/ml) and [^{35}S]methionine (100 μCi/ml) for 18 h as described in Materials and Methods. The cells were then washed and incubated in 0.1% BSA containing DMEM for 1 h. This medium was then collected for subsequent analysis (medium fraction). The labeled apoE-lipid particles remaining in the pericellular proteoglycan-associated pool were harvested by heparinase treatment as described in the legend to Fig. 4 (surface fraction). Medium and cell surface apoE-lipid particles were recovered on protein G agarose beads coated with apoE antisera and analyzed for labeled lipid content as described in Materials and Methods. ApoE particles isolated by immunoaffinity were denatured and analyzed by SDS-PAGE as described in Materials and Methods. The apoE band was identified by phosphorimager, and radioactivity in apoE was quantitated as described in Materials and Methods. Data shown are mean ± SD from triplicate samples.

identified by TLC) to apoE (isolated on SDS-PAGE) ratios. Again, there is a significantly lower ratio in apoE particles harvested from the proteoglycan pericellular pool compared with those secreted into the medium.

Proteoglycan depletion of J774E$^-$ cells does not reduce efflux to exogenously added apoE

The above experiments demonstrate that apoE in a pericellular proteoglycan pool is important for facilitating the cholesterol efflux associated with endogenously expressed apoE in macrophages. Because this interaction occurred on the cell surface, we next determined whether apoE added exogenously to J774E$^-$ cells would be similarly affected by proteoglycan depletion. Proteoglycan depletion in the absence of exogenous apoE has no effect on sterol efflux from J774E$^-$ cells (Fig. 2 and Table 2). We previously showed that addition of exogenous apoE at 0.5 and 1.0 μg/ml significantly enhances sterol efflux from J774E$^-$ cells with intact proteoglycans (23). For the current evaluation, we added exogenous apoE at 2 μg/ml to J774E$^-$ cells with intact and depleted proteoglycans. We also examined J774E$^-$ cells with basal levels of cholesterol and after cholesterol loading. The results of a representative experiment are shown in **Table 3**. A portion of the cell cultures were harvested prior to addition of apoE for measurement of total cell cholesterol mass. ApoE (2 μg/ml) was then added to all remaining cultures and the cholesterol in the medium was measured after 24 h. Pro-

TABLE 3. Proteoglycan depletion of J774E$^-$ cells does not reduce cholesterol efflux to exogenously added apoE

	J774E$^-$ Basal Cholesterol		J774E$^-$ Cholesterol Loaded	
Total Cholesterol	Control	Proteoglycan-Depleted	Control	Proteoglycan-Depleted
	μg/mg cell protein			
Cell	22.1 ± 2.30	24.7 ± 4.00	52.8 ± 2.52	48.81 ± 2.73
Medium	0.54 ± 0.10	0.75 ± 0.10	1.0 ± 0.27	1.22 ± 0.22

J774E$^-$ cells were grown in 10% FCS with or without 1 mM β-DX. For cholesterol-loaded cells, acetylated LDL at 80 μg/ml was also included. After 48 h, the medium was aspirated, the cell layer was washed, and cultures previously treated with β-DX were incubated with 10 U/ml heparinase for 2 h at 37°C in 0.2% BSA. Control cells received 0.2% BSA for 2 h at 37°C alone. A portion of cell cultures from each group was harvested for measurement of total cell cholesterol. The remainder of the cell layers were extensively washed and 0.1% BSA containing 2 μg/ml apoE was added. After 24 h, the cell medium was recovered, and cholesterol mass in the medium was measured. Values shown are the mean ± SD from triplicate samples.

teoglycan depletion did not reduce sterol efflux from cells with either basal or expanded cholesterol stores to exogenous apoE. In fact, efflux to exogenous apoE tended to be somewhat greater from proteoglycan-depleted cells. These results indicate that reduction of apoE-associated sterol efflux from macrophages after proteoglycan depletion is specific for endogenously synthesized apoE.

DISCUSSION

We previously reported that interrupting proteoglycan synthesis, or removing proteoglycans from the cell surface, markedly reduces apoE retention in a cell surface pool and increases its release from the cell layer (25). We also previously showed that at steady state, as much as 8–10% of total cellular apoE is in a cell surface pool, and that this pool serves as precursor for secreted apoE (29). We also made the unexpected observation that there appears to be a cell surface fraction of apoE with a long retention time, and that it undergoes recycling to the Golgi prior to secretion (29). We previously considered multiple mechanisms by which endogenous synthesis/secretion of apoE could enhance cholesterol efflux utilizing mechanisms distinct and somewhat more effective than those utilized by exogenous addition of lipid-free apoE (23). Included in our considerations was the possibility that a cell surface pool, efficiently formed by endogenous expression of apoE, could be involved. The studies in this article establish a role for proteoglycan-apoE interaction in facilitating cholesterol efflux; with specific importance of the proteoglycan-associated cell surface pool of apoE.

Inhibiting proteoglycan synthesis reduced cholesterol efflux only from apoE-expressing macrophages. Additional studies confirmed that inhibiting synthesis of proteoglycans leads to the secretion of a denser apoE-lipid particle from J774E$^+$ macrophages. In addition, direct evaluation of apoE-lipid particles in the pericellular proteoglycan pool indicates that they are denser than secreted apoE-lipid particles. Direct examination of total lipid-to-apoE ratio or sterol-to-apoE ratio indicates that both of these are higher in secreted apoE-lipid particles compared with pericellular proteoglycan-associated apoE-lipid particles. These observations, in conjunction with our previous findings that cell surface apoE is the precursor pool for secreted apoE, suggest that nascent apoE arrives at the cell surface in a relatively lipid-poor state and acquires lipid while retained in a proteoglycan pool on the macrophage cell surface. This efflux mechanism could help to explain the greater efficacy of endogenous apoE expression compared with exogenous apoE addition for producing efflux from macrophages (23). It could be predicted that endogenous synthesis and secretion of apoE would more effectively lead to formation and maintenance of a cell surface pool. For example, endogenously synthesized apoE may be more efficiently sequestered at the cell surface due to an increased exposure to low affinity binding sites at the cell surface during passage through the pericellular space.

There are several mechanisms one could consider for how a proteoglycan-bound cell surface pool of apoE could facilitate cholesterol efflux. ApoE held in proximity to the plasma membrane by proteoglycan binding could facilitate passive desorption of lipid from the plasma membrane. Alternatively, binding by plasma membrane proteoglycans could facilitate the presentation of apoE to specific cell surface sites responsible for enhancing cholesterol efflux (30). In this regard, scavenger receptor BI (SR-BI) or ABC-1 could be involved (31–36). Both proteins are expressed on the macrophage cell surface and each has been shown to be important for the flux of free cholesterol across the plasma membrane. A direct interaction of cell surface apoE with ABC-1 or SR-BI may not be required; only the close proximity of apoE as a sterol acceptor may be needed. Finally, the recycling of proteoglycan-bound apoE through intracellular membrane compartments including the Golgi could facilitate lipid acquisition by apoE (29). These issues will require additional investigation.

The observations in this article were facilitated by the use of a stably transfected macrophage line that produces physiologic levels of apoE. The fact that depletion of proteoglycan had no effect on cholesterol efflux in nonexpressing cells indicates that the results in apoE-expressing cells were not due to nonspecific changes resulting from the treatment to deplete macrophages of proteoglycans, or to proteoglycan depletion itself. The results of our studies establish a new role for cell surface apoE in facilitating the cholesterol efflux associated with endogenous expression of apoE. Other important functions can also be considered. The matrix of the vessel wall has assumed increasing importance as the understanding of atherogenesis develops (37–39). Proteoglycans provide important binding sites for growth factors and cytokines, and the presence of apoE could potentially modulate retention of these factors on the macrophage cell surface. We previously showed that apoE on the macrophage cell surface modulates the interaction of cells with HDL (24). It was also recently shown that apoE in a pericellular proteoglycan matrix modulates the metabolism of apoE-containing triglyceride-rich lipoproteins by macrophages (40, 41).

In summary, in these studies, we established one mechanism by which endogenous expression of apoE facilitates effective cholesterol efflux from macrophages. Reduction of apoE in a proteoglycan pool suppresses sterol efflux. Further, the specific retention of endogenously synthesized macrophage apoE in a proteoglycan-associated cell surface pool appears to play a critical role in the enhanced cholesterol efflux produced by endogenous apoE expression in the macrophage. Our data further indicate that endogenously synthesized apoE arrives at the cell surface in a more lipid-poor state than when it is eventually released from the cell surface. We previously showed that cell surface apoE serves as the precursor for secreted apoE, and it is possible that changes in apoE conformation, which attend lipidation, play a role in its release from proteoglycan binding sites on the cell surface. Although a proximate source of lipid available to cell surface apoE resides in the plasma membrane, recent obser-

vations demonstrating recycling of cell surface apoE suggest that acquisition of lipid from intracellular membranes must also be considered and investigated.

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REFERENCES

1. Mazzone, T. 1996. Apolipoprotein E secretion by macrophages: its potential physiological functions. *Curr. Opin. Lipidol.* **7:** 303–307.
2. Chait, A., P-H. Iverius, and J. D. Brunzell. 1982. Lipoprotein lipase secretion by human monocyte-derived macrophages. *J. Clin. Invest.* **69:** 490–493.
3. Rosenfeld, M. E., S. Butler, V. A. Ord, B. A. Lipton, C. A. Dyer, L. K. Curtiss, W. Palinski, and J. L. Witztum. 1993. Abundant expression of apoprotein E by macrophages in human and rabbit atherosclerotic lesions. *Arterioscler. Thromb.* **13:** 1382–1389.
4. O'Brien, K. D., S. S. Deeb, M. Ferguson, T. O. McDonald, M. D. Allen, C. E. Alpers, and A. Chait. 1994. Apolipoprotein E localization in human coronary atherosclerotic plaques by in situ hybridization and immunohistochemistry and comparison with lipoprotein lipase. *Amer. J. Path.* **144:** 538–548.
5. Goldberg, I. 1996. Lipoprotein lipase and lipolysis: central roles in lipoprotein metabolism and atherogenesis. *J. Lipid Res.* **37:** 693–707.
6. Semenkovich, C. F., T. Coleman, and A. Daugherty. 1998. Effects of heterozygous lipoprotein lipase deficiency on diet-induced atherosclerosis in mice. *J. Lipid Res.* **39:** 1141–1151.
7. Kashyap, V. S., S. Santamarina-Fojo, D. R. Brown, C. L. Parrott, D. Applebaum-Bowden, S. Meyn, G. Talley, B. Paigen, N. Meada, and H. B. Brewer. 1995. Apolipoprotein E deficiency in mice: gene replacement and prevention of atherosclerosis using adenovirus vectors. *J. Clin. Invest.* **96:** 1612–1620.
8. Babaev, V. R., S. Fazio, L. A. Gleaves, K. J. Carter, C. F. Semenkovich, and M. F. Linton. 1999. Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in vivo. *J. Clin. Invest.* **103:** 1697–1705.
9. Linton, M. F., V. R. Babaev, L. A. Gleaves, and S. Fazio. 1999. A direct role for the macrophage low density lipoprotein receptor in atherosclerotic lesion formation. *J. Biol. Chem.* **274:** 19204–19210.
10. Shimano, H., J. Ohsuga, M. Shimada, Y. Namba, T. Gotoda, K. Harada, M. Katsuki, Y. Yazaki, and N. Yamada. 1995. Inhibition of diet induced atheroma formation in transgenic mice expressing apolipoprotein E in the arterial wall. *J. Clin. Invest.* **95:** 469–476.
11. Fazio, S., V. R. Babaev, A. B. Murray, A. H. Hasty, K. J. Carter, L. A. Gleaves, J. B. Atkinson, and M. F. Linton. 1997. Increased atherosclerosis in mice reconstituted with apolipoprotein E null macrophages. *Proc. Natl. Acad. Sci. USA.* **94:** 4647–4652.
12. Hasty, A. H., M. F. Linton, S. J. Brandt, V. R. Babaev, L. A. Gleaves, and S. Fazio. 1999. Retroviral gene therapy in apoE-deficient mice: apoE expression in the artery wall reduces early foam cell lesion formation. *Circulation.* **99:** 2571–2576.
13. Linton, M. F., J. B. Atkinson, and S. Fazio. 1995. Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation. *Science.* **267:** 1034–1037.
14. Boisvert, W. A., and L. K. Curtiss. 1999. Elimination of macrophage-specific apolipoprotein E reduces diet-induced atherosclerosis in C57BL/6J male mice. *J. Lipid Res.* **40:** 806–813.
15. Bellosta, S., R. W. Mahley, D. A. Sanan, J. Murata, D. L. Newland, J. M. Taylor, and R. E. Pitas. 1995. Macrophage-specific expression of human apolipoprotein E reduces atherosclerosis in hypercholesterolemic apolipoprotein E-null mice. *J. Clin. Invest.* **96:** 2170–2179.
16. Boisvert, W. A., J. Spangenberg, and L. Curtiss. 1995. Treatment of sever hypercholesterolemia in apolipoprotein E-deficient mice by bone marrow transplantation. *J. Clin. Invest.* **96:** 1118–1124.
17. Plump, A. S., J. D. Smith, T. Hayek, K. Aalto-Setala, A. Walsh, J. G. Verstuyft, E. M. Rubin, and J. L. Breslow. 1992. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell.* **71:** 343–353.
18. Zhang, S. H., R. L. Reddick, B. Burkey, and N. Maeda. 1994. Diet-induced atherosclerosis in mice heterozygous and homozygous for apolipoprotein E gene disruption. *J. Clin. Invest.* **94:** 937–945.
19. Kruth, H. S., S. I. Skarlatos, P. M. Gaynor, and W. Gamble. 1994. Production of cholesterol-enriched nascent high density lipoproteins by human monocyte-derived macrophages is a mechanism that contributes to macrophage cholesterol efflux. *J. Biol. Chem.* **269:** 24511–24518.
20. Zhang, W-Y., P. M. Gaynor, and H. S. Kruth. 1996. Apolipoprotein E produced by human monocyte-derived macrophages mediates cholesterol efflux that occurs in the absence of added cholesterol acceptors. *J. Biol. Chem.* **271:** 28641–28646.
21. Mazzone, T., and C. Reardon. 1994. Expression of heterologous human apolipoprotein E by J774 macrophages enhances cholesterol efflux to HDL_3. *J. Lipid Res.* **35:** 1345–1353.
22. Smith, J. D., M. Miyata, M. Ginsberg, C. Grigaux, E. Shmookler, and A. S. Plump. 1996. Cyclic AMP induces apolipoprotein E binding activity and promotes cholesterol efflux from a macrophage cell line to apolipoprotein acceptors. *J. Biol. Chem.* **271:** 30647–30655.
23. Lin, C-Y., H. Duan, and T. Mazzone. 1999. Apolipoprotein E-dependent cholesterol efflux from macrophages: kinetics study and divergent mechanisms for endogenous versus exogenous apolipoprotein E. *J. Lipid Res.* **40:** 1618–1626.
24. Lin, C-Y., M. Lucas, and T. Mazzone. 1998. Endogenous apoE expression modulates HDL_3 binding to macrophages. *J. Lipid Res.* **39:** 293–301.
25. Lucas, M., and T. Mazzone. 1996. Cell surface proteoglycans modulate net synthesis and secretion of macrophage apolipoprotein E. *J. Biol. Chem.* **271:** 13454–13460.
26. Obunike, J. C., S. Pillarisetti, L. Paka, Y. Kako, M. J. Butteri, Y-Y. Ho, W. D. Wagner, N. Yamada, T. Mazzone, R. J. Deckelbaum, and I. J. Goldberg. 2000. The heparin-binding proteins apolipoprotein E and lipoprotein lipase enhance cellular proteoglycan production. *Arterioscler. Thromb. Vasc. Biol.* **20:** 111–118.
27. Mazzone, T., L. Pustelnikas, and C. A. Reardon. 1992. Post-translational regulation of macrophage apoprotein E production. *J. Biol. Chem.* **267:** 1081–1087.
28. Lucas, M., P-H. Iverius, D. Strickland, and T. Mazzone. 1997. Lipoprotein lipase reduces secretion of apolipoprotein E from macrophages. *J. Biol. Chem.* **272:** 13000–13005.
29. Zhao, Y., and T. Mazzone. 2000. Transport and processing of endogenously synthesized apoE on the macrophage cell surface. *J. Biol. Chem.* **275:** 4759–4765.
30. Takahashi, Y., and J. D. Smith. 1999. Cholesterol efflux to apolipoprotein AI involves endocytosis and resecretion in a calcium-dependent pathway. *Proc. Natl. Acad. Sci. USA.* **96:** 11358–11363.
31. Krieger, M. 1999. Charting the fate of the "good cholesterol": identification and characterization of the high-density lipoprotein receptor SR-BI. *Annu. Rev. Biochem.* **68:** 523–558.
32. Hirano, K-I., S. Yamashita, Y. Nakagawa, T. Ohya, F. Matsuura, K. Tsukamoto, Y. Okamoto, A. Matsuyama, K. Matsumoto, J-I. Miyagawa, and Y. Matsuzawa. 1999. Expression of human scavenger receptor class B type I in cultured human monocyte-derived macrophages and atherosclerotic lesions. *Circ. Res.* **85:** 108–116.
33. Kellner-Weibel, G., M. de la Llera-Moya, M. A. Connelly, G. Stoudt, A. E. Christian, M. P. Haynes, D. L. Williams, and G. H. Rothblat. 2000. Expression of scavenger BI in COS-7 cells alters cholesterol content and distribution. *Biochemistry.* **39:** 221–229.
34. Langmann, T., J. Kluken, M. Reil, G. Leibisch, M-F. Luciani, G. Chimini, W. E. Kaminski, and G. Schmitz. 1999. Molecular cloning of the human ATP-binding cassette transporter 1 (hABC1): evidence for sterol-dependent regulation in macrophages. *Biochem. Biosphys. Res. Comm.* **257:** 29–33.
35. Orso, E., C. Broccardo, W. E. Kaminski, A. Bottcher, G. Liebisch, W. Drobnik, A. Gotz, O. Chambenoit, W. Diederich, T. Langmann, T. Spruss, M-F. Luciani, G. Rothe, K. J. Lackner, G. Chimini, and G. Schmitz. 2000. Transport of lipids from Golgi to plasma membrane is defective in Tangier disease patients and Abc1-deficient mice. *Nat. Genet.* **24:** 192–196.
36. Lawn, R. M., D. P. Wade, M. R. Garvin, X. Wang, K. Schwartz, J. G. Porter, J. J. Seilhamer, A. M. Vaughan, and J. F. Oram. 1999. The Tangier disease gene product ABC1 control the cellular apolipoprotein-mediated lipid removal pathway. *J. Clin. Invest.* **104:** R25–R31.
37. Olin, K. L., S. Potter-Perigo, H. R. Barrett, T. N. Wight, and A. Chait. 1999. Lipoprotein lipase enhances the binding of native and oxidized low density lipoproteins to versican and biglycan syn-

thesis by cultured arterial smooth muscle cell. *J. Biol. Chem.* **274:** 34629–34636.
38. Miao, H-Q., R. Ishai-Michaeli, R. Atzmon, T. Peretz, and I. Vlodavsky. 1996. Sulfate moieties in the subendothelial extracellular matrix are involved in basic fibroblast growth factor sequestration, dimerization, and stimulation of cell proliferation. *J. Biol. Chem.* **271:** 4879–4886.
39. Evanko, S. P., E. W. Raines, R. Ross, L. I. Gold, and T. N. Wight. 1998. Proteoglycan distribution in lesions of atherosclerosis depends on lesion severity, structural characteristics, and the proximity of platelet-derived growth factor and transforming growth factor-β. *Amer. J. Path.* **152:** 533–546.
40. Al-Haideri, M., I. J. Goldberg, N. F. Galeano, A. Gleeson, T. Vogel, M. Gorecki, S. L. Sturley, and R. J. Deckelbaum. 1997. Heparan sulfate proteoglycan-mediated uptake of apolipoprotein E-triglyceride-rich lipoprotein particles: a major pathway at physiological particle concentrations. *Biochemistry.* **36:** 12766–12772.
41. Ho, Y-Y., M. Al-Haideri, T. Mazzone, T. Vogel, J. F. Presley, S. L. Sturley, and R. J. Deckelbaum. 2000. Endogenous expressed apolipoprotein E has different effects on cell lipid. *Biochemistry.* **39:** 4746–4754.