Trypsin-sensitive and Lipid-containing Sites of the Macrophage Extracellular Matrix Bind Apolipoprotein A-I and Participate in ABCA1-dependent Cholesterol Efflux*

A unique property of the extracellular matrix of J774 and THP-1 cells has been identified, which contributes to the ability of these cells to promote cholesterol efflux. We demonstrate high level apolipoprotein (apo) A-I binding to macrophage cells (THP-1 and J774) and to their extracellular matrix (ECM). However, high level apoA-I binding is not observed on fibroblasts, HepG2 cells, or U937 cells (a macrophage cell line that does not efflux cholesterol to apoA-I or bind apoA-I on their respective ECM). Binding to the ECM of THP-1 or J774 macrophages depends on the presence of apoA-I C-terminal helices and is markedly reduced with a mutant lacking residues 187–243 (apoA-IΔ187–243), suggesting that the hydrophobic C terminus forms a hydrophobic interaction with the ECM. ApoA-I binding is lost upon trypsin treatment or with Triton X-100, a preparation method that de-lipidates the ECM. However, binding is recovered with re-lipidation, and is preserved with ECM prepared using cytochalasin B, which conserves the endogenous phospholipid levels of the ECM. We also demonstrate that specific cholesterol efflux to apoA-I is much reduced in cells released from their native ECM, but fully restored when ECM-depleted cells are added back to ECM in the presence of apoA-I. The apoA-I-mediated efflux is deficient in plated or suspension U937 macrophages, but is restored to high levels when the suspension U937 cells are reconstituted with the ECM of J774 cells. The ECM-dependent activity was much reduced in the presence of glyburide, indicating participation of ABCA1 (ATP-binding cassette transporter 1) in the efflux mechanism. These studies establish a novel binding site for apoA-I on the macrophage ECM that may function together with ABCA1 in promoting cholesterol efflux.

Cholesterol efflux from arterial macrophages may provide a mechanism to reduce foam cell formation and to promote the regression of atherosclerosis. Cholesterol efflux to extracellular acceptors can occur either by an aqueous diffusion type mechanism that requires a gradient of cholesterol between the plasma membrane and the lipoprotein surface (1, 2) or by an energy-dependent process that results in the transfer of cholesterol and phospholipids to lipid-poor apolipoproteins. The human ATP-binding cassette transporter 1 (ABCA1) is a key protein in the removal of cellular lipids by lipid-poor apolipoproteins (predominantly apoA-I), as emphasized by the consequences of its defect in Tangier disease patients (3–6). ABCA1 resides in intracellular membranes, such as the Golgi, where it may play a role in shuttling phospholipids and possibly cholesterol between compartments and the cell surface (7). ABCA1 is also expressed on the cell surface consistent with a direct role in the binding of apoA-I to the cell surface (7, 56) or in the transport of cellular lipids across the plasma membrane to apolipoproteins (7, 9). Cross-linking studies have demonstrated a close association of apoA-I and ABCA1 on the cell surface (10, 56) that is dependent on a functional ABCA1 (11). SR-B1 (12, 13), or its human homologue CLA-1, has also been shown to bind apoA-I and HDL and participate in selective uptake of HDL-derived cholesteryl esters, but no role for SR-B1 in apoA-I-mediated efflux has been found. ApoA-I has been shown to interact with components of the extracellular matrix (ECM), although no link between the ECM and the mechanism of lipid efflux has been demonstrated. In vitro apoA-I binds in a concentration-dependent, saturable, and specific manner to fibronectin, one of the major components of the ECM (14, 15). Significant binding of apoA-I to collagen 1 has also been reported (16). Immunobiochemical studies have demonstrated co-localization of apoA-I with the ECM in atherosclerotic arteries (16, 17) and in liver biopsy specimens (16).

The ECM is a complex network of collagens, proteoglycans, laminin, fibronectin, and many other glycoproteins that compose a scaffold that fills extracellular spaces (18, 19). Many of the components of the ECM are attached to the cytoskeleton by transmembrane proteins or are involved in anchoring cells to surfaces (20, 21). Although the ECM is a minor component of most cell types, it has a crucial role in many cellular processes including cell growth, spreading and differentiation (18), and modulating the biological activities of growth factors (22, 23). There are also secreted proteins that reside on the ECM, such as heparin-binding growth factors (24, 25), which can modulate cellular responses and regulate cell growth (26, 27).

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The abbreviations used are: ABCA1, ATP-binding cassette transporter 1; apoA-I, apolipoprotein A-I; apoE, apolipoprotein E; BSA, bovine serum albumin; ECM, extracellular matrix; HDL, high density lipoprotein; LDL, low density lipoprotein; LRP, low density lipoprotein receptor-related protein; FBS, phosphate-buffered saline; POPC, 1-palmitoyl 2-oleoyl phosphatidylcholine; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PMA, phorbol 12-myristate 13-acetate; bFGF, basic fibroblast growth factor; TGFRβ1, transforming growth factor-β1; ACAT, acylCoA cholesterol acyltransferase.

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as sphingomyelinase (24–27) and matrix metalloproteinases (28, 29) that play a role in atherogenesis, with the latter also likely involved in vascular wall remodeling and possibly thrombosis. ECM proteins can also play a role in certain types of cancer/tumor growth (30–32), multiple sclerosis (33, 34), and atherosclerosis (35, 36), highlighting the importance of understanding the function of the various components of the ECM. Additionally, the ECM has been demonstrated to play a role in binding of low density lipoprotein (LDL) (27) and in mediating the selective uptake of cholesteryl esters from LDL (38).

In monocyte-derived THP-1 macrophages, cholesterol translocation is initiated by binding of apoA-I to a cell surface site via its C-terminal domain (39). The C-terminal domain-dependent binding is macrophage-specific and has not been observed in other cell types. In this report we show that trypt-sin-sensitive sites of the macrophage ECM bind apoA-I via the C-terminal domain and represent the major binding site for apoA-I on the cell surface. We further characterize this site and provide evidence that it participates in apoA-I-mediated cholesterol efflux.

**EXPERIMENTAL PROCEDURES**

**Materials**—1-Palmitoyl 2-oleyl phosphatidylcholine (POPC) was obtained from Avanti Polar Lipids (Birmingham, AL). [1α,2α-3H]Cholesterol and [methyl-3H]-choline chloride were purchased from PerkinElmer Life Sciences. Na125I was obtained from Amersham Biosciences. Human skin fibroblasts (GM00038B) were obtained from the Coriell Institute for Medical Research (Camden, NJ). Human THP-1 monocytes (ATCC TIB-202) and mouse J774 macrophages were harvested in 12-well plates (8 wells/plate). 1 ml of Laemmli sample buffer (45) containing U937 cells were cholesterol-loaded as above following PMA-pretreated cells were labeled with [3H]cholesterol as described above.

**Preparation of the Mutant Proteins**—Wild-type apoA-I and a mutant lacking residues 187–243, apoA-1(187–243), both with the N-terminal extension Met-Arg-Gly-Ser-(His)6-Met, were expressed in a bacterial system as described previously (39, 40).

**Cell Culture**—Human skin fibroblasts were seeded in 12-well plates as previously described (39). Human THP-1 and U937 monocytes were grown in complete RPMI 1640 medium containing 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol, and 2 mM glutamine. To differentiate these cells into macrophages, the monocytes were seeded in 12-well plates at a density of 0.5–1.0 × 106 cells/well in the same medium containing 100 μg/ml doxorubicin (Sigma) and incubated for 72 h (THP-1 cells) or 7 days (U937 cells). Mouse J774 macrophage were seeded in 12-well plates and grown in complete DMEM (high glucose) containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Preparation of ECM**—ECM was prepared by the Triton X-100 or EDTA methods of Owensby et al. (41) or with cytochalasin B as described previously (42, 43). The purity of the fractions was assessed both microscopically for the absence of cells and particulate material and by Western blotting of the protein extracts with established markers of the ECM (fibronectin and laminin) and plasma membrane (TGFβRI and LRP).

To examine the relative amounts of 3H-labeled phospholipids and the effects of the different matrix preparation protocols on the matrix phospholipid content, J774 macrophages were labeled in DMEM containing 1% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and [3H]choline chloride (20 μCi/ml) for 48 h. The cells were then washed with warm DMEM to remove free choline chloride. Intact cells or ECM prepared by either Triton X-100 or cytosol/halogen B protocols were then solubilized overnight with 0.5 N NaOH. Phospholipids in the extracts were separated from free choline chloride by the method of Bligh and Dyer (44) prior to scintillation counting.

To determine the relative amounts of ECM produced by different cell types, the cells were labeled with [35S]cysteine/methionine. Briefly, the cells at 90–95% confluence were incubated for 1 h in cysteine/methionine-deficient EMEM. The medium was then changed and replaced with the same medium including 20 μCi of [35S]-PRO-MIX (Amersham Biosciences), containing [35S]-labeled cysteine and methionine, and incubated for 24 h. ECM was then prepared as described above and the associated radiolabel expressed as a percentage of the total cellular trichloroacetic acid-precipitable label.

**Binding of 125I-apoA-I and 125I-apoA-I(187–243) to Cells and ECM**—Macrophages, fibroblasts, or ECM prepared from these cells were washed three times with DMEM-BSA and then incubated at 4°C with [125I]-apolipoprotein (1 × 106 cpm; representing ~0.2 μg of protein) in DMEM containing 1% fatty acid-free BSA for 2 h. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled apoA-I. The cells or ECM were washed three times with cold PBS containing 1.7 mM CaCl2 solubilized overnight in 0.5 N NaOH, and counted in a γ counter. Radioactivity and protein content of the cell extracts were determined, and the amounts of [125I]-apoA-I or [125I]-apoA-I(187–243) bound to cells were expressed as a function of cell protein. Binding of the apolipoprotein to ECM was expressed as percentage bound. In some experiments ECM, prepared by the Triton X-100 method described above, was pre-incubated at 37°C for 30 min with DMEM-BSA containing Liposyn II or POPC micelles. Unbound lipid was removed by washing the wells three times with DMEM-BSA. The ECM was then incubated with radiolabeled apolipoproteins in DMEM containing 1% BSA for 2 h at 37°C. The ECM was washed three times with PBS and solubilized overnight in 0.5 N NaOH and then counted in a γ counter.

**Reconstituting Suspension Cells with ECM and Its Effect on Cholesterol Efflux**—Cholesterol-loaded and labeled suspension cells were prepared as follows. J774 cells were sent into T-175 culture flasks and cholesterol-loaded in DMEM containing 1% FBS, the Sandoz ACAT inhibitor 58-035 (5 μg/ml), and [3H]cholesterol (20 μCi/ml final), which had been pre-incubated with acetylated LDL (75 μg/ml final) for 30 min at 37°C. After labeling for 48 h, the cells were rinsed twice with warm DMEM-BSA and allowed to equilibrate for 3 h. Suspension cells were prepared from these flasks by the method of Owensby et al. (41). Briefly, the cells were washed with warm PBS and then incubated with 3 mM EDTA in PBS for 5 min at 37°C to disrupt the interactions between the cells and the culture flask. Tapping the side of the culture flask then detached the cells. The suspension cells were washed twice with DMEM-BSA (1300 rpm/ Sorval RT 6000D centrifuge). Finally, the cells were re-suspended in DMEM-BSA to a concentration of 0.4 mg of cell protein/ml. Suspension U937 cells were cholesterol-loaded as above following PMA-induced differentiation to macrophages, and some preparations were labeled with [3H]cholesterol as described above.

The ECM used for reconstitution studies was prepared in 12-well culture plates from unlabeled J774 or U937 cells by the cytochalasin B method described above. ECM or empty well controls were then pre-incubated with 50 μg of apoA-I or HDL in 0.5 ml of DMEM-BSA for 30 min at 37°C to allow binding of apoA-I to the ECM. Cholesterol-labeled suspension cells (0.5 ml representing 0.2 mg of cell protein) were added to the wells to initiate cholesterol efflux. The incubation was continued for 3 h, after which time the entire reaction volume of 1 ml was collected and centrifuged at 2000 rpm for 5 min (Eppendorf 5415C centrifuge) to pellet the cells. A 0.9-ml sample of the supernatant was removed and centrifuged at 15,000 rpm for 15 min to pellet any remaining particulate matter. Finally, a 0.8-ml aliquot of the final supernatant was counted.

To determine cholesterol efflux from attached cells, J774 macrophages or U937 macrophages were seeded in 12-well culture plates and cholesterol-loaded for 48 h and equilibrated as described above. There was ~0.2 mg of cell protein/well. After washing, efflux was initiated with the addition of 50 μg of apoA-I or HDL. Medium was removed after 3 h and processed as described above.

**Electrophoresis and Western Blotting**—ECM was prepared as described above from J774 macrophages grown in 12-well culture plates (8 plates/matrix preparation). 1 ml of Laemmli sample buffer (45) containing SDS (2%) and 2-mercaptoethanol (5%, w/v) was transferred sequentially from well to well at 25°C, allowing a 1-min solubilization period per well. Whole cell extracts were prepared by adding 1 ml of Laemmli sample buffer to a single well representing ~0.2 mg of protein. The samples were heated at 95°C for 5 min, and the cell or ECM proteins...
were separated by SDS-polyacrylamide gel electrophoresis (10% separating gel; 4% stacking gel) and then transferred to nitrocellulose membranes (0.45 μm, Bio-Rad). PBS containing powdered milk (3%, w/v) was used to block the nitrocellulose membranes and to dilute antibody stocks. The nitrocellulose membranes were incubated with gentle rocking for 1 h at 25 °C with goat antiserum specific for fibronectin (Santa Cruz; sc-9535) or rabbit antiserum specific for TGFβR1 (Santa Cruz; sc-399), laminin (Sigma; L-9393), or human LRP or human ABCA1 (Novus Biologicals). This was followed by an 1-h incubation with the appropriate secondary antibody coupled to horseradish peroxidase. Peroxidase activity was detected using the Roche Molecular Biochemicals chemiluminescence method.

Binding of Cy3-ApoA-I and Immunofluorescence Microscopy—The ECM prepared by the cytochalasin B method for J774 or U937 macrophages plated on coverslip dishes (glass-bottomed microwell dishes from MakTek Corp., Ashland, MA) was blocked in PBS containing 0.2% BSA for 30 min, then incubated with 0.4 μg/ml Cy3-labeled apoA-I for 2 h at 37 °C. The ECM was washed three times in PBS and incubated overnight at 4 °C with a mouse or a rabbit antibody directed toward fibronectin or laminin (1:200). This was followed by incubation with anti-mouse or anti-rabbit antibody-conjugated fluorescein IgG (1:200) and detected with an Olympus IX50 Microscope. Images were captured and saved using Winview.

Other Procedures—Lipoproteins were prepared from the plasma of normal subjects as described by Rall et al. (46). Acetylated LDL was prepared as described by Basu et al. (47). HDL was iodinated by the iodine monochloride method (48). Iodination of lipid-free apoA-I and apoA-IΔ(187–243) was performed by the IODOBEAD method (Pierce). Cy3 labeling of apolipoprotein A-I was carried out by the recommended procedure of the manufacturer (Amersham Biosciences product specification) and purified by size exclusion chromatography. The protein concentration of lipoproteins was determined by the Markwell-Lowry method (49). Cell protein was determined by the method of Lowry et al. (50) with BSA as standard.

RESULTS

Binding of Lipid-free ApoA-I to the Macrophage ECM Requires the C-terminal Domain of ApoA-I—We have previously demonstrated that an apoA-I deletion mutant lacking residues 187–243 of the C-terminal domain, herein referred to as Δ(187–243), exhibits both reduced cell surface binding and reduced ability to promote lipid efflux to THP-1 macrophages (39). Fig. 1 (panel A) demonstrates the variable binding of apoA-I and apoA-IΔ(187–243) to several macrophage cell lines and human fibroblasts at 4 °C. In agreement with our previous findings, full-length apoA-I bound efficiently to THP-1 macrophages whereas apoA-IΔ(187–243) did not. A similar pattern was observed in J774 macrophages. In contrast, both apoA-I and apoA-IΔ(187–243) bound poorly to human fibroblasts and U937 macrophages. Binding of apoA-I and apoA-IΔ(187–243) to the ECM from these cell lines prepared by treatment with Triton X-100 and re-lipidation with Liposyn II was also evaluated. The amount of protein in ECM represents a small proportion of the total cellular protein and therefore cannot be accurately assessed by conventional protein assays. To correct for potential variations in the amounts of ECM, and hence apoA-I binding, associated with these cell lines, we labeled the cells in the presence of [35S]cysteine/methionine for 24 h prior to preparing the ECM. ApoA-I binding to the ECM was then expressed as a function of [35S]cysteine/methionine incorporation into the ECM. ApoA-I binding to the ECM was then measured by the IODOBEAD method (Pierce). [35S]cysteine/methionine incorporation into the ECM represented 0.29 ± 0.02% of the total cellular counts, respectively. The pattern of binding to each ECM was very similar to that observed for the corresponding cells, with full-length apoA-I binding efficiently to the ECM of THP-1 and J774 macrophage cell lines, but not the ECM prepared from fibroblasts or U937 macrophages. In contrast, apoA-IΔ(187–243) bound poorly to the ECM of all cell types. This study indicates that apoA-I binds much better to the ECM of J774 and THP-1 macrophages and requires the presence of the C-terminal domain.

We also examined the ECM fractions by Western blotting to ensure both the presence of matrix components and absence of plasma membrane contamination. Fibronectin and laminin are well-defined components of the ECM and were visible in homogenates of J774 cells and in ECM prepared from these cells (Fig. 2). In these experiments the ECM was prepared from cell culture dishes representing a 96-fold greater surface area than the cell fractions. In contrast, the small 85-kDa subunit of LRP and re-lipidated with Liposyn II as described under "Experimental Procedures." The cell or ECM preparations were then washed twice with DMEM containing 1% fatty acid-free BSA and then incubated for 2 h at 4 °C with 5 × 10^5 cpm/ml, specific activity = 6000–8000 cpm/ng). To determine nonspecific binding, a 100-fold excess of unlabeled ligand was added to replicate wells. After binding the wells were washed three times with PBS and solubilized overnight in 0.5 n NaOH and then counted in a γ counter. Results represent the mean and S.E. of three separate determinations. In panel B binding of apoA-I and apoA-IΔ(187–243) is corrected for the amounts of [35S]-ECM protein produced by the different cell lines.

FIG. 1. Binding of apoA-I and apoA-IΔ(187–243) to cells (upper panel) and ECM (lower panel). ECM in 12-well plates was prepared from cells labeled with [35S]cysteine/methionine by the Triton X-100 method and re-lipidated with Liposyn II as described under "Experimental Procedures." The cell or ECM preparations were then washed twice with DMEM containing 1% fatty acid-free BSA and then incubated for 2 h at 4 °C with [35S]-apoA-I or apoA-IΔ(187–243) (5 × 10^5 cpm/ml, specific activity = 6000–8000 cpm/ng). To determine nonspecific binding, a 100-fold excess of unlabeled ligand was added to replicate wells. After binding the wells were washed three times with PBS and solubilized overnight in 0.5 n NaOH and then counted in a γ counter. Results represent the mean and S.E. of three separate determinations. In panel B binding of apoA-I and apoA-IΔ(187–243) is corrected for the amounts of [35S]-ECM protein produced by the different cell lines.
cytochalasin B treatment.

Panels A and B represent the filipin stains of J774 cells and the corresponding ECM prepared by cytochalasin B treatment as described under “Experimental Procedures.” Panels C and D are the immunostains of fibronectin and laminin, respectively, on the J774 ECM.

Panels E and F illustrate the binding of Cy3-apoA-I to the plated J774 and to the ECM underneath the cells, as seen after removal of the cells by cytochalasin B treatment.

bronectin or laminin and fluorescein-labeled anti-mouse or anti-rabbit immunoglobulin. Both fibronectin and laminin stains formed clear and overlapping but distinct footprints of the cells (Fig. 3, C and D) compatible with their secretion by the cells and their attachment to the coverslip dish underneath the J774 cells. For the ECM binding site for apoA-I to be physiologically functional, it should be accessible when cells are adherent on their matrices. To demonstrate that this is the case at least in vitro, Cy3-apoA-I was allowed to bind to plated J774 cells and the unbound protein washed away. The cells were then released by cytochalasin B treatment, and apoA-I bound to the

cells and that remaining associated with ECM were compared (Fig. 3, E and F). In agreement with results obtained with 125I-apoA-I (data not shown), a lower but highly significant Cy3-apoA-I binding remained associated with the ECM, which demonstrated its accessibility underneath the cells.

Co-localization experiments of Cy3-labeled apoA-I and the matrix elements indicated that apoA-I binding to the ECM coincided most closely with fibronectin (Fig. 4, A and B) and to a lesser extent with laminin (Fig. 4, C and D). The same results were observed independently of the order of addition (data not shown). However, the intensities of the signals for Cy3-apoA-I and for fibronectin were not always proportional, which suggested another intervening parameter in the co-localization and possibly in the binding of apoA-I to a fibronectin-linked complex. Finally, we showed that the binding of Cy3-apoA-I to the ECM is reversible and could be competed off by purified recombinant (Fig. 5, A and B) or plasma (data not shown) apoA-I, demonstrating the specificity of binding.

The ECM Binding Site Requires Lipids for Optimal Binding of ApoA-I—Preparation of the ECM by the Triton X-100 method would be expected to severely disrupt the lipid environment of the ECM, which could decrease the binding of apoA-I. Consequently, we have pre-incubated the Triton X-100-derived ECM of J774 macrophages with either POPC micelles or Liposyn II at 37 °C prior to the addition of radiolabeled apoA-I. Lipid pre-incubations produced a concentration-dependent increase in the binding of apoA-I to the macrophage ECM (Fig. 6, left panel). Liposyn II and POPC micelles both increased binding maximally by 20-fold at concentrations between 0.1 and 1.0 mg/ml. In contrast, lipid incubations did not significantly affect binding of apoA-I to the ECM of human fibroblasts (data not shown). ApoA-I binding to blank wells was also increased by lipid pre-incubations (e.g. 300 cpm/well in the absence of lipids and 2400 cpm/well in the presence of 1 mg/ml Liposyn II). For this reason blank well controls were run simultaneously with test samples and the resulting background counts subtracted from the data points of Fig. 6 and subsequent figures.

The binding of apoA-I and apoA-IΔ(187–243) to ECM prepared using cytochalasin B, a method that does not require the use of detergents, was also examined. In agreement with our previous studies (42), ECM prepared from J774 macrophage by
The cytochalasin B method was free of adherent cells when examined microscopically, and plasma membrane markers LRP and TGFβRI were not detected in Western blots of this material (data not shown). Fig. 6 (right panel) demonstrates that binding of apoA-I to the cytochalasin B-derived ECM is much greater than to the Triton X-100 ECM that has not been re-lipidated. Liposyn II treatment of the cytochalasin B ECM further increased apoA-I binding to levels observed for the lipid-saturated Triton X-100 matrix. In agreement with ECM prepared by the Triton X-100 method, apoA-I (187–243) did not bind to ECM prepared by the cytochalasin B method (data not shown).

The varied binding of apoA-I to the Triton X-100- and cytochalasin B-derived ECM could reflect the levels of endogenous phospholipids in these preparations. To test this hypothesis, the phosphatidylcholine pool of J774 macrophages was labeled by including 3H-choline chloride in the growth medium. ECM prepared by the cytochalasin B method accounted for 2.3% of the labeled total cellular pool of phosphatidylcholine. As expected, label associated with the Triton X-100 ECM was much reduced and represented only 0.3% of the total cellular pool. When cells were incubated with 35S-cysteine/methionine, we found equivalent recoveries of label in the different ECM preparations (0.31 ± 0.09% in the cytochalasin B-prepared ECM and 0.3 ± 0.04% in the Triton X-100-prepared ECM). This suggests further that the observed differences in apoA-I binding likely reflects the lipid, rather than protein, content of the ECM preparations.

ECM prepared by cytochalasin B treatment on cells plated in coverslip dishes was also treated by phospholipase B to directly evaluate the contribution of matrix phospholipids to apoA-I binding. Phospholipase treatment did not alter the fibronectin signal (data not shown), but eliminated almost entirely Cy3-apoA-I binding (Fig. 7, A and B). This establishes that endogenous phospholipids of the ECM contribute to apoA-I binding.

**The ECM Binding Site for ApoA-I Is Sensitive to Trypsin**—Treatment of the Triton X-100-prepared ECM with trypsin before incubation with Liposyn II efficiently abrogated the subsequent binding of apoA-I (Table I). Inclusion of a 100-fold excess of soybean trypsin inhibitor to the wash steps following the trypsin incubation and to the subsequent incubation with 125I-apoA-I did not reduce the effects of trypsin (data not shown). This indicates that the reduced binding in response to trypsin was not the result of trypsin carry-over to the apoA-I incubation. In contrast, incubations with trypsin reduced the ECM phosphatidylcholine content by only 25% and total ECM protein by 16.5% (Table I). Incubations of the ECM with heparinase (3 units/ml) or chondroitinase ABC (1.5 units/ml) did not affect the binding of apoA-I to the ECM, indicating that macrophage glycosaminoglycans were not involved (data not shown). The trypsin sensitivity suggests the involvement of an ECM protein component in apoA-I binding. The limited effect of trypsin on phospholipid content and its elimination of apoA-I binding indicate that the lipids do not support binding in the absence of the native ECM proteins. To evaluate a possible protection of the apolipoprotein binding site by lipids, the ECM prepared by Triton X-100 treatment of J774 were treated with Liposyn II before trypsin digestion. However, this was not the case as the lipitated matrix remained sensitive to trypsin (data not shown).

**Cholesterol Efflux from Attached Cells and Suspension Cells**—To determine whether the ECM and the apoA-I binding site might be involved in lipid efflux, we compared cholesterol efflux from attached cells and suspension J774 macrophages (matrix-depleted), which were or were not reconstituted with ECM for the duration of the efflux assay. J774 macrophages were cholesterol-loaded and labeled and then released from the ECM with EDTA as described by Owensby et al. (41). Suspensions cells prepared by this method were 98% viable by trypan blue exclusion at the time of preparation and at the termination of the efflux assay. The suspension cells were added back to either empty wells or ECM that had been pre-incubated at 37 °C with apoA-I for 30 min prior to the addition of the cells. Cycloheximide (1 mM) was included in the assay mixtures of both attached and suspension cells to inhibit the synthesis of new ECM components during the course of the efflux assay. Diffusional cholesterol efflux to HDL was the same in attached cells and suspension cells (Fig. 8) and was unaffected by the presence of ECM. In contrast, efflux to apoA-I was significantly reduced (p < 0.001) in suspension cells without ECM, representing only 29.3% of that observed in attached cells. Reconstituting suspension cells with ECM, prepared by the cytocha-
ApoA-I-mediated efflux was observed (data not shown). Inclusion of apoA-I and HDL was determined using identical conditions. The cell medium was removed and cleared of cellular material by centrifugation at 3000 g for 5 min at 4 °C. Then, the cell-free supernatant was solubilized and counted for 3H radioactivity. All data are the mean and S.E. of three separate determinations.

**FIG. 8.** ApoA-I- and HDL-mediated cholesterol efflux from attached, ECM-depleted, and ECM-reconstituted J774 cells. J774 cells grown in T-175 flasks or in 12-well culture plates were loaded with [3H]cholesterol. Suspension cells were prepared with EDTA from the cholesterol-loading step in contrast did not repair cholesterol efflux to apoA-I (Fig. 9). Inclusion of a 100-fold excess of unlabeled apoA-I. To determine the effects of proteolysis on ECM phospholipid or protein content, the cells were grown in the presence of [3H]choline (10 μCi/well) or [35S]cysteine/methionine (10 μCi/well) for 24 h as described under “Experimental Procedures.” The trypsin treatment was performed as described above. The trypsin treatment was performed as described above. The trypsin treatment was performed as described above.

### DISCUSSION

In this work we describe a novel-binding site for apoA-I located on the macrophage ECM that is involved in ABCA1-mediated cholesterol efflux. Efficient binding to the ECM requires both the presence of the C-terminal domain of apoA-I (Fig. 1) and that of phospholipids at a trypsin-sensitive site (Fig. 6 and Table I). The ECM binds apoA-I poorly, if at all (Fig. 1), suggesting that this defect could explain the inability of these cells to efflux cholesterol to apoA-I. To test this possibility we prepared cholesterol-loaded and labeled U937 suspension cells by the EDTA method. These cells were then added to the J774 ECM that had been pre-incubated with 50 μg of apoA-I. Fig. 10 demonstrates that reconstitution of suspension U937 macrophages with an active ECM binding site prompted a dramatic increase in cholesterol efflux to apoA-I (right panel) to levels observed for HDL-mediated cholesterol efflux from U937 macrophages attached to tissue culture plastic via their own ECM (left panel). In some experiments the ECM was washed twice following the apoA-I pre-incubation to remove unbound material. Therefore, in Fig. 10 (right panel), the no-wash condition includes 50 μg of apoA-I that is distributed between non-adsorbed apoA-I and apoA-I that is bound to the ECM. The two-wash condition reflects only apoA-I that is bound to the ECM and, from estimates of our binding analysis, represents −30 ng of apoA-I protein. Despite the very large difference in the amount of apoA-I present in the no-wash and two-wash conditions, similar levels of cholesterol efflux to apoA-I were observed, indicating that ECM-bound apoA-I is important for cholesterol efflux in macrophages whereas non-bound apoA-I is not. Glyburide inhibited the ECM-dependent efflux by greater than 90%, implicating the involvement of ABCA1 in this process (Fig. 10). Finally, when cholesterol-labeled U937 suspension cells were reconstituted with their own matrix, which had been pre-incubated with apoA-I, efflux to apoA-I remained defective as it was in the original plated cells (data not shown).

### FIG. 9.

U937 macrophages do not efflux cholesterol to apoA-I. U937 monocytes were seeded into 12-well plates, differentiated for 7 days with PMA, and loaded with [3H]cholesterol as described under “Experimental Procedures.” PMA or the Sandoz ACAT inhibitor (58-035) was either included or not during the cholesterol-loading step. Efflux was initiated with the addition of 50 μg of apoA-I or HDL and further incubated for 3 h. Medium samples were then collected, cleared of cellular debris and counted for [3H]radioactivity. All data are the mean and S.E. of three separate determinations.
and specifically their interaction with ABCA1 itself. This pathway of cellular lipid efflux has been shown to proceed via a two-step mechanism. Phospholipid efflux can be independently inhibited by inhibitors of ABCA1, such as glyburide, and by mutations in ABCA1 (52), whereas cholesterol efflux can be specifically inhibited by okadaic acid or vanadate (53). Studies with human smooth muscle and endothelial cells have shown that, whereas smooth muscle cells mediate both phospholipid and cholesterol efflux, endothelial cells mediate only cholesterol efflux unless the acceptor is apoA-I previously lipidated by incubation with smooth muscle cells (53). Thus, phospholipid efflux is the initial step in apoA-I-mediated lipid efflux, which is dependent on ABCA1 activity.

Many studies prior to the discovery of ABCA1-mediated efflux had established that the initial binding of apoA-I to the cell surface controlled a sequence of events leading to lipid and particularly cholesterol transfer from internal compartments to efflux-competent sites on the cell surface (54, 55), leading to the concept of an apoA-I binding site linked to a signaling pathway. Chemical cross-linking has shown that apoA-I forms a complex with ABCA1 in cells stimulated with CAMP (56) or in cells overexpressing ABCA1 (52). Although this seemed to establish direct binding of apoA-I, other observations have shown that apoA-I-ABCA1 interactions required a functional ABC transporter (11, 52), and furthermore the diffusion parameters of membrane-bound apoA-I indicate interaction with membrane lipids rather than proteins (11). We propose that apoA-I binding to a lipidated ECM site allows secondary interactions with lipids on the membrane and with ABCA1 that are dependent on its phospholipid flippase activity. Preliminary studies indicate that the ECM accounts for a major proportion (Fig. 3) of endogenously bound apoA-I, which express ABCA1 and lack an active ECM binding site, do not efflux cholesterol to apoA-I (Fig. 9) unless reconstituted with the J774 ECM, which contains an active binding site for apoA-I (Fig. 10). These studies indicate a clear correlation between the abilities of apoA-I to bind the ECM and to promote lipid efflux. Our studies also indicate that the ECM-bound apoA-I mediates cholesterol efflux, whereas unbound apoA-I does not promote any additional efflux (Fig. 10). This finding is supported by previous studies in macrophage documenting the sensitivity of apoA-I-mediated efflux to mild trypsinization of cell surface proteins (61, 62). Our studies would predict that the trypsin effect may also occur at the level of the ECM. In contrast, fibroblasts do not exhibit an ECM apoA-I binding site (Fig. 1) and continue to efflux cholesterol to HDL following mild incubations with trypsin (63).

The trypsin sensitivity of apoA-I binding and the requirement for lipids may indicate the involvement of lipid-binding proteins of the ECM. Collagen, type I, and fibronectin have been reported to bind apoA-I (14, 15), but there is no evidence that these proteins also bind lipids. Our own preliminary data indicate that the binding of apoA-I to fibronectin, but not collagen I, can be increased by 3-fold after incubation with POPC micelles.3 Our fluorescence microscopy experiments demonstrate significant co-localization of Cy3-apoA-I binding and fibronectin, which provides supporting evidence for a link between apoA-I binding site and this matrix protein (Fig. 3).

However, variations in the relative intensities of the two signals suggest the existence of an intervening parameter in their association, which we propose might be the lipids required for binding. This does not rule out the possibility of another intermediary molecule between lipids and fibronectin. The phospholipid-binding annexins are also potential candidates for the apoA-I-binding site on the ECM. Brownawell and Creutz (64) have documented the calcium-dependent binding of apoA-I to annexin I and annexin VII by Western ligand blotting following SDS-PAGE. Although this method would be expected to minimize any contributions from associated phospholipids, SDS may have provided a lipid-mimetic conformation for apoA-I. Annexins I–V have been described in J774 macrophage (65), and it is well documented that annexin V is a key component of the chondrocyte ECM (66, 67).

Our studies indicate that as much as 2.3% of endogenously labeled cellular choline-containing phospholipids are found on the macrophage ECM, and the elimination of apoA-I binding by phospholipase B treatment provides direct evidence for phospholipid involvement in the ECM binding site (Fig. 7). There are reports that describe the association of lipids with the ECM and the origin of ECM lipids in other cell types. Matrix vesicles, for example, are membrane-enclosed vesicles of the chondrocyte ECM that accumulate calcium, with the assistance of annexin V, during the initiation of mineral formation in growing bone (66, 67). Matrix vesicles are enriched in cholesterol, free fatty acids, sphingomyelin, glycolipids, lysophospholipids, and phosphatidylserine (68). The composition is like that of the

**Fig. 10.** An active ECM binding site promotes apoA-I-mediated cholesterol efflux in U937 macrophages. U937 macrophages were cholesterol-loaded and labeled as described under “Experimental Procedures.” Suspension U937 macrophages (right panel) were released with 3 mM EDTA (5 min at 37°C), pelleted (1500 rpm/5 min), and washed twice at 4°C with DMEM-BSA. The cells were added to ECM, prepared by the cytoskeleton B method from J774 macrophages, grown in 12-well plates, that had been pre-incubated with 50 µg apoA-I. In some wells unbound apo-A-I was removed by washing the wells twice with DMEM-BSA. The cells and ECM combinations were incubated for 3 h at 37°C. Cholesterol efflux from attached U937 macrophages (left panel) was performed as described under “Experimental Procedures.” In both attached and suspension cell studies, the medium was collected, cleared of cells, and counted. Glyburide was added to the cell suspensions just before addition to the ECM. All data are the mean and S.E. of three separate determinations.
plasma membrane of cells and supports electron microscopy evidence that the vesicles arise by budding from microvilli of the chondrocyte surface membrane (69). The lipid fraction of the macrophage ECM could then serve both as a hydrophobic tether for apoA-I and as a rapidly effluxing pool of cellular lipids, provided this pool is dynamic and readily replaced. As an alternative to matrix vesicular transport, we suggest that the ECM and its cellular anchors may provide the vertical interactions between certain membrane constituents, such as raft domains, that facilitate their recruitment and eventual interactions with the lipid efflux pathway.

What would be the benefits of initiating apoA-I-dependent lipid efflux from the ECM rather than the plasma membrane? ECM stability and long half-lives of ECM components, such as collagen and fibronectin (70, 71) or ECM-bound proteins (72) would provide a stable environment for bound and aggregated low density lipoproteins (38). In this system the subendothelial ECM provides a protected environment for apoA-I in close proximity to circulating inhibitors (78). The ECM binding site could therefore provide for the selective uptake of cholesteryl esters by macrophages, thereby forming a nascent HDL particle (80).

With regard to cholesterol efflux, interactions between cell surface ABCA1 and the stable ECM binding site, as suggested by the sensitivity of ECM-mediated efflux to glyburide, can promote the efficient removal of cellular lipids to apoA-I. When sufficient lipiddation is achieved, a nascent apoA-I-containing lipoprotein is released,

and facilitate the solubilization of a portion of the plasma membrane, thereby forming a nascent HDL particle (80).

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