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The Binding *in Vitro* of Modified LDL to the Intermediate Filament Protein Vimentin

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Membrane-associated proteins with specific binding properties to modified LDL were investigated in J774 macrophages and Mono Mac 6 sr cells. Ligand blotting of membrane proteins revealed a 54-kDa protein which bound oxidized and acetylated but not native LDL. The 54-kDa protein, isolated by 2D-PAGE, was identified as vimentin. ^{125}I-AcLDL bound to purified vimentin and desmin in a saturable manner, with an approximate K_d of 1.7×10^{-7} M (89 μg/ml) and 8.0×10^{-8} M (41 μg/ml), respectively. Blots of vimentin mutant proteins with deletions in the positively charged N-terminal head domain showed that amino acids 26–39 are essential for the binding of AcLDL by vimentin. Taken together, our data indicate that vimentin binds modified LDL, but not native LDL, in a specific and saturable manner. Vimentin filaments extend throughout the cytoplasm as far as the inner surfaces of plasma and vesicular membranes. Vimentin may thus play a role in membrane-associated steps involved in the intracellular processing of oxidized LDL, contributing to its unregulated uptake and intracellular retention by cells of the atherogenic plaque.

Key Words: **vimentin; desmin; acetylated LDL; oxidized LDL; lipoprotein binding; ligand blotting.**

The physiological function of the intermediate filament (IF) protein vimentin remains a conundrum in cell biology research. Although a considerable body of information regarding its inter- and intracellular distribution, structure and biochemistry has accumulated, vimentin null or knockout mutations in transgenic mice have not yet provided evidence for a functional significance of this IF protein (1, 2). IF proteins are in physical contact with the cytoplasmic side of various membrane systems such as the plasma membrane, the mitochondrial membrane, the nuclear envelope, and membranes of various other vesicular structures (3). Previous work has shown that vimentin can associate with membranes either through protein-protein (4–7) or protein-lipid interactions (8–13). It has been proposed that vimentin may play a role in the positioning and movement of membrane-bound organelles and lipid droplets (14) and in the intracellular trafficking of lipoprotein-derived cholesterol (15).

In the present communication, we describe for the first time the specific and saturable binding of oxidized and acetylated but not native LDL to vimentin. Vimentin is an IF protein found in the mesenchymal cells of the vessel wall, endothelial cells, smooth muscle cells, macrophages and in atherosclerotic lesions (16–19). Thus, an interaction between modified lipoproteins and vimentin may participate in the unregulated uptake and intracellular accumulation of these lipoproteins in cells present in atherogenic lesions.

EXPERIMENTAL PROCEDURES

Cells and cell culture. Human monocytic cell line Mono Mac 6 was cultured in RPMI 1640 medium containing 10% FCS, insulin (9 μg/ml), oxaloacetate (1 mM), pyruvate (1 mM), penicillin (200 U/ml), streptomycin (200 μg/ml) and nonessential amino acids (1×). The medium was filtered through a Gambro 2000 column to eliminate LPS. J774 cells were obtained from American Type Tissue Culture Collection and were grown in RPMI 1640 medium supplemented with FCS (10%).

Lipoproteins. LDL was isolated from plasma of normolipidemic fasting subjects in the density interval of 1.019 to 1.063 by sequential ultracentrifugation. Ox LDL was prepared by incubating LDL (200 μg/ml) with 5 μM $CuSO_4$ in EDTA-free, O_2-saturated PBS for 20 h (20). Acetylation and iodination were performed as described by Basu *et al.* (21) and Bilheimer *et al.* (22), respectively. Protein concentrations were determined by the method of Markwell *et al.* (23). Liporoteins were filter sterilized (0.22 μm), stored at 4°C, and used within 1 month.

Abbreviations used: AcLDL, acetylated low-density lipoprotein; OxLDL, oxidized low-density lipoprotein; LDL, low-density lipoprotein; IF, intermediate filament; aa, amino acids.

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Preparation of cell membrane pellets. Between 2×10^8 to 6×10^8 Mono Mac 6 sr and J774 cells were washed twice in PBS, suspended for 30 min in a hypotonic lysis buffer (10 mM Tris–HCl, pH 7.4, containing EDTA 1 mM, PMSF 0.6 mM, aprotinin 0.5 μg/ml, leupeptin 0.5 μg/ml and pepstatin 0.7 μg/ml) and subsequently homogenized with 20 to 80 strokes in a Dounce homogenizer. After adjustment to 150 mM NaCl, the homogenates were centrifuged at 800*g* for 10 min. The supernatant was centrifuged at 10,000*g* for 15 min, and the resulting supernatant was centrifuged at 100,000*g* for 1 h to obtain a crude membrane pellet. The membrane pellet was stored at −80°C, when not used immediately.

Solubilization of membrane pellets. Pellets were solubilized by needle aspiration in solubilization buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl and 1 mM EDTA) containing 2% Chaps. Insoluble material was sedimented at 100,000*g* for 1 h. The supernatant was stored at −80°C, when not used immediately.

Purified proteins. The preparations of purified vimentin and desmin used were a gift from Professor P. Traub (Max Planck Institute of Cell Biology, Ladenburg, Germany) (24).

Gel electrophoresis and ligand blotting. Solubilized membrane pellets and purified proteins were separated under nonreducing conditions on 5–15 or 10% polyacrylamide slab gels as described by Laemmli (25). Proteins were electrotransferred (125 V, 1 1/2 h) onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad) in a Towbin buffer (25 mM Tris–HCl, 192 mM glycine, pH 8.3) without methanol but with 0.01% SDS. Nonspecific binding was blocked by incubation at 4°C overnight with 0.5% casein in 50 mM Tris–HCl, 90 mM NaCl, and 1 mM EDTA, pH 7.8. Membranes were then incubated with [^{125}I]lipoproteins (10–20 μg/ml; specific activity between 150 and 400 cpm/ng) for 4 h at room temperature and subsequently washed 8 times (15 min each) in the above blocking buffer. Membranes were air dried and exposed to Kodak X-Omat AR film for 4 to 12 h at −80°C.

2D-PAGE and double replica blotting. Membrane pellets were solubilized in sample buffer (6 M urea, 2 M thiourea, 2% Chaps, and 10 mM DTT) containing 30 g/liter Ultrodex (Pharmacia). Insoluble material was sedimented at 100,000*g* for 30 min. A Pharmacia Biotech Multiphor II Electrophoresis Unit was used to perform 2-D electrophoresis. Immobiline pH 4–7 linear strips ($110 \times 3 \times 0.5$ mm) were rehydrated with sample buffer overnight in a reswelling cassette (Pharmacia). Sample was loaded at the anodic (acidic) end. After focusing in the first dimension, IEF gels were equilibrated for 15 min in equilibration buffer (6 M urea, 2 M thiourea, 30% glycerol, 2% SDS, 0.003% bromphenol blue and 50 mM Tris–HCl, pH 8.8) with 100 mg dithiothreitol, followed by 15 min in equilibration buffer containing 480 mg iodacetamide. Equilibrated IEF gels were fixed with agarose at the top of 8–12% SDS–polyacrylamide gradient gels and the second dimension was run in a Protean II xi Cell (Bio-Rad). Replica blotting onto PVDF membranes was performed according to Johansson (26).

Calculation of K_d values. Binding affinity constants were determined by means of Scatchard plot analysis. The measured protein concentrations (23) and the estimated molecular weight of apolipoprotein B-100 of 513,000 daltons were used in these calculations.

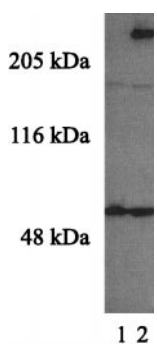

FIG. 1. Ligand blotting analysis of Mono Mac 6 sr and J774 membrane proteins. Crude membrane protein preparations from Mono Mac 6 sr (lane 1) and J774 cells (lane 2) were subjected to SDS–PAGE (400 μg protein per lane) followed by electroblotting onto PVDF membranes. The membrane strip was blocked with 0.5% casein and 250 μg/ml native LDL and incubated with 20 μg/ml ^{125}I-AcLDL. Bound radioactivity was visualized by autoradiography.

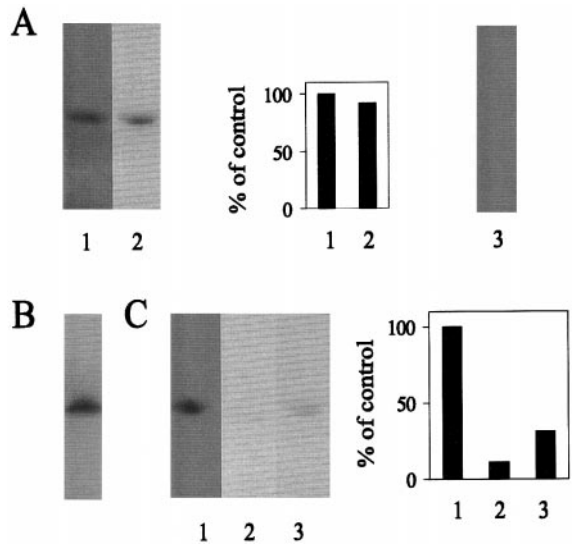

FIG. 2. Ligand blotting of the 54-kDa protein with modified and native LDL. (A) ^{125}I-AcLDL and ^{125}I-LDL ligand blots: crude membrane lysates from Mono Mac 6 sr cells were subjected to SDS–PAGE (200 μg protein per lane), followed by electroblotting onto PVDF membranes. PVDF membrane strips were incubated with 10 μg/ml ^{125}I-labeled lipoproteins (^{125}I-AcLDL, lane 1; ^{125}I-LDL, lane 3). In lane 2, PVDF membrane strips were incubated with 10 μg/ml ^{125}I-AcLDL in the presence of a 25-fold excess of LDL. Bound radioactivity was visualized by autoradiography, and the 54-kDa region was excised for counting. Background radioactivity was determined by counting an excision of a blot sample of equal size from the 45 kDa region and subtracted from all experimental values. (B) ^{125}I-OxLDL ligand blot: crude membrane protein lysate from Mono Mac 6 sr was subjected to SDS–PAGE (400 μg protein) followed by electroblotting onto PVDF membranes. The membrane strip was blocked with 0.5% casein and incubated with 10 μg/ml ^{125}I-OxLDL. Bound radioactivity was visualized by autoradiography. (C) Competition for binding of ^{125}I-AcLDL to 54 kDa protein by unlabeled AcLDL and OxLDL: Crude membrane lysates from Mono Mac 6 sr cells were subjected to SDS–PAGE (200 μg protein per lane) followed by electroblotting onto PVDF membranes. PVDF membrane strips were incubated with 10 μg/ml ^{125}I-AcLDL in the absence (lane 1) and presence of a 25-fold excess of AcLDL (lane 2) and OxLDL (lane 3). Binding values were calculated as described for A.

RESULTS

As demonstrated in Fig. 1, ^{125}I-AcLDL bound to a 54-kDa protein in membrane preparations from murine J774 macrophage and Mono Mac 6 sr cells. In addition, ^{125}I-AcLDL binding to the 260-kDa protein corresponding to the SR-AI/AII was observed in the

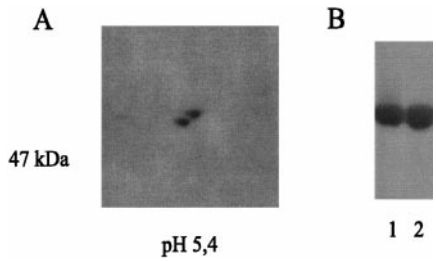

FIG. 3. Identification of the 54 kDa ^{125}I-AcLDL binding protein as vimentin. (A) Crude membrane pellet from Mono Mac 6 sr cells was solubilized and loaded (600 μg) onto the focusing gel at the cathode. 1, dimension: IPG (pH 4–7); 2, dimension: SDS 8–12% gradient gel. By double replica blotting two replica PVDF membranes were produced. One was used for ligand blotting with 10 μg/ml ^{125}I-AcLDL, the other was Coomassie stained for N-terminal sequencing. (B) Purified mouse vimentin (lane 1) and purified recombinant mouse vimentin (lane 2) were subjected to SDS–PAGE (40 μg protein per lane) followed by electroblotting onto PVDF membranes. The membrane strip was incubated with 20 μg/ml ^{125}I-AcLDL.

membrane preparations from the J774 cells but not in those of Mono Mac 6 sr cells. We have previously shown the absence of SR-AI/AII in this cell line (27, 28).

The 54-kDa protein also bound OxLDL but not native LDL. Furthermore, AcLDL binding was competed for by AcLDL and OxLDL but not by native LDL (Fig. 2).

The 54-kDa protein was isolated by isoelectric focusing/SDS two-dimensional polyacrylamide gel electrophoresis. The protein was identified as vimentin by means of N-terminal sequencing from a PVDF membrane after double replica blotting. The 9 N-terminal amino acids of the protein corresponding to the lower molecular weight spot shown in Fig. 3 were determined. The sequence was 100% homologous to amino acids (aa) 16–24 of human vimentin. Thus, this spot represents a degradation product of the upper, higher molecular weight, N-terminally blocked full-length vimentin. The identification of vimentin as an AcLDL binding protein was confirmed by the binding of ^{125}I-AcLDL to purified and recombinant mouse vimentin (Fig. 3).

The binding characteristics of ^{125}I-AcLDL to purified mouse vimentin and purified hamster desmin, an IF protein closely related to vimentin, were determined by incubating blots of these proteins with increasing concentrations of ^{125}I-AcLDL, followed by excision and direct counting of the radioactivity. ^{125}I-AcLDL bound to vimentin and desmin in an saturable manner, with an approximate K_d of 1.7×10^{-7} M (89 μg/ml) and 8.0×10^{-8} M (41 μg/ml), respectively (Fig. 4).

As shown in Fig. 5, mutated vimentin protein in which two of the six arginines in the 39 N-terminal amino acids are replaced by glycine, as well as the vimentin deletion protein Δ1–13, in which three of the six arginines are deleted, still bind ^{125}I-acLDL in ligand blots. Notably, the deletion protein Δ1–17 shows less binding compared to Δ1–13, although no further arginine residue is deleted. Furthermore, AcLDL binding requires the presence of aa 26–39, containing only two of the 12 basic arginine residues situated in the N-terminal head domain of vimentin. The aa 22–29 of desmin show part homology to aa 31–38 of vimentin. Interestingly, this stretch of amino acids contains a number of hydroxy side chains which could serve to form hydrogen bonds potentially involved in the specific binding of modified LDL.

DISCUSSION

The present paper demonstrates for the first time specific and saturable binding *in vitro* of acetylated and oxidized but not native LDL to the IF protein vimentin. A saturable binding of ^{125}I-AcLDL to the related muscle cell IF protein desmin is also shown. The electrostatic attraction between the N-terminal arginines of vimentin and the negatively charged modified LDL appears

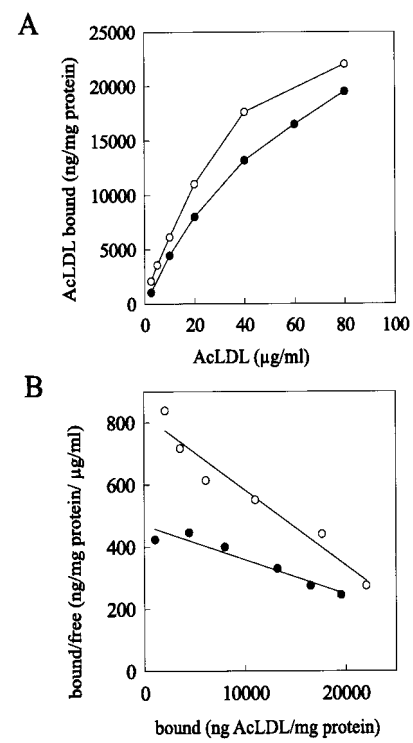

FIG. 4. Saturation and Scatchard analysis of ^{125}I-AcLDL binding to vimentin (●) and desmin (○). (A) Purified mouse vimentin and hamster desmin were subjected to SDS–PAGE (10 μg protein per lane) followed by electroblotting onto PVDF membranes. PVDF membrane strips were incubated with increasing concentrations of ^{125}I-Ac-LDL. The vimentin region was excised to quantify the radioactivity bound to the strip. Background radioactivity was determined by counting a blot sample of equal size from the 40-kDa region and subtracted from all values. (B) The dissociation constant (K_d) was approximated from the diagram by the method of Scatchard. Data show the means of two experiments.

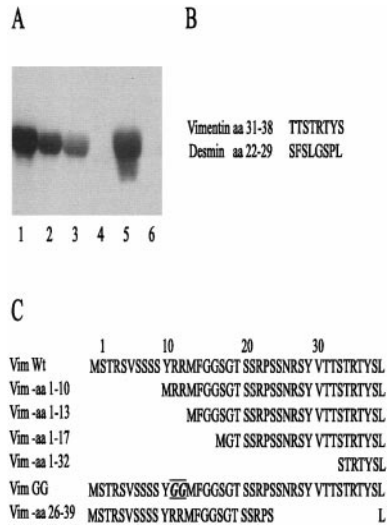

FIG. 5. Determination of the AcLDL-binding region of vimentin. (A) Vimentin mutant proteins with N-terminal deletions or mutation (C) were subjected to SDS–PAGE (20 μg protein per lane) (lane 1, Δ1–10; lane 2, Δ1–13; lane 3, Δ1–17; lane 4, Δ1–32; lane 5, $R_{11}R_{12}\Rightarrow G_{11}G_{12}$; lane 6, Δ26–39;) followed by electroblotting onto PVDF membranes. The membrane strip was incubated with 20 μg/ml ^{125}I-AcLDL. (B) aa 31–38 in vimentin show part homology to aa 22–29 of desmin.

not to be essential. Rather, a sequence of amino acids, in part homologous in vimentin and desmin and containing a number of hydroxy side chains which could form hydrogen bonds, are more likely essential for the specific binding described.

A number of observations support the hypothesis that an interaction between vimentin and modified LDL may play a role in atherogenesis. Firstly, analogous to our findings, a number of lipoprotein receptor proteins such as SR-AI/AII, CD68, LOX-1, and the LDL receptor (29–32) have been shown to bind their ligands in ligand blots. Secondly, vimentin and desmin are the two IFs located in mesenchymal cells of the vessel wall, the site where modified LDL is believed to play a pivotal role in the development of the atherosclerotic plaque. Interestingly, the content of both IF proteins in the vessel wall has been shown to increase in atherosclerosis. In a rabbit model of diet-induced atherosclerosis, morphological changes in arterial smooth muscle cells correlated with changes in IF protein expression. Concomitantly, an increase in total IF protein content, with vimentin increasing in thoracic aorta and desmin in pulmonary artery, was observed (17). An increase in vimentin mRNA expression in atherosclerotic plaques of aortic vessels from swine with dietary-induced vascular lesions has also been reported (33). Furthermore, vimentin is strongly expressed in foam cells (34) and smooth muscle cells (19, 35) of human atheromatous plaques.

Vimentin is found throughout the cell, from the nucleic to the plasma membrane. A role for vimentin in some stage of uptake, intracellular transport and retention of modified LDL, where modified LDL follows a pathway distinct from the one for native LDL, is thus conceivable. Several mechanisms of interaction between vimentin and modified LDL can be postulated. Using GFP vimentin, it has been shown that vimentin is dynamic in nature. Movements of vimentin filaments include both extension and retraction and are dependent on the presence of intact microtubules. Vimentin filament fragments are also dynamic and can be visualized moving at the leading edge of migrating cells (36). The reorganization of vimentin, resulting in its assembly into diffuse, non-filamentous aggregates seen just beneath the cap in lymphocyte immunoglobulin capping (37), may play a role in immunoglobulin-mediated uptake of immunogenic material such as oxidized LDL. Furthermore, the N-terminal head domain of vimentin can also penetrate lipid vesicles (38) and interact with the hydrophobic core of lipid bilayers (13). An interaction of vimentin with Ox LDL, which unlike native LDL is not degraded and accumulates in intracellular compartments (39, 40), is thus conceivable. In summary, we show a specific, saturable binding of modified LDL to vimentin. We propose that this novel interaction between vimentin and modified LDL, at or beneath the cell surface or in vesicles, could take part in some aspect of the not yet fully elucidated processing of atherogenic lipoproteins.

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