Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 1396–1400, February 1995
Medical Sciences

Recognition of oxidatively damaged and apoptotic cells by an oxidized low density lipoprotein receptor on mouse peritoneal macrophages: Role of membrane phosphatidylserine

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Contributed by Daniel Steinberg, October 10, 1994

ABSTRACT **We recently reported that oxidized low density lipoprotein (OxLDL), but not acetyl LDL (AcLDL), inhibited the binding and phagocytosis of nonopsonized, oxidatively damaged red blood cells (OxRBCs) by mouse peritoneal macrophages, implying the involvement of a "scavenger receptor" other than the AcLDL receptor. Numerous studies establish that loss of plasma membrane phospholipid asymmetry, which increases phosphatidylserine expression on the outer leaflet of the membrane, can play a key role in macrophage recognition of damaged and apoptotic cells. We report here that this recognition is in part attributable to the same mouse macrophage receptor that recognizes OxLDL. As described in an accompanying paper, this is a plasma membrane protein of 94–97 kDa. Phosphatidylserine liposomes show strong ligand binding to the same 94- to 97-kDa protein and this binding is inhibited by OxLDL but not by AcLDL. Inhibition of the RBC membrane phospholipid translocase by incubation with sodium vanadate caused a progressive increase in the appearance of phosphatidylserine on the cell surface and a parallel increase in the binding of these RBCs to macrophages, binding that was inhibited by OxLDL. Finally, OxLDL also inhibited the binding of sickled RBCs and apoptotic thymocytes to mouse macrophages. However, the latter was incomplete (≈50%), suggesting that other receptors are also involved. We suggest that the OxLDL receptor plays a significant role in recognition of damaged and apoptotic cells.**

Loss of plasma membrane phospholipid asymmetry is one of the mechanisms leading to macrophage recognition of damaged or aged red blood cells (RBCs) (1–3). Normally, very little, if any, phosphatidylserine (PS) is found on the external leaflet of the plasma membrane of the RBC and the maintenance of this asymmetry is an energy-requiring process attributed to an aminophospholipid translocase situated within the plasma membrane (reviewed in refs. 4 and 5). There is evidence that apoptotic thymocytes are also recognized in part by virtue of an increase in exposure of PS at the cell surface (6, 7). However, the nature of the macrophage receptor(s) involved has eluded definition. Sambrano *et al.* (8) recently presented evidence that the macrophage receptor recognizing oxidatively damaged RBC (OxRBC) might be the oxidatively modified low density lipoprotein (OxLDL) receptor. In this report, and in an accompanying paper by Ottnad *et al.* (9), we present evidence that a 94- to 97-kDa protein in the macrophage membrane, investigated as a possible receptor for uniquely recognizing OxLDL (10, 11), is at least in part responsible for recognizing PS-enriched plasma membranes.

Interest in the macrophage scavenger receptors originally stemmed from their role in the binding and internalization of modified forms of LDL, specifically acetyl LDL (AcLDL) (12) and OxLDL (10, 11, 13, 14). There is considerable evidence that oxidative modification of LDL in particular can play a critical role in atherogenesis (15–17), partly because it favors the formation of lipid-laden foam cells in the arterial intima. Uptake of OxLDL by macrophages occurs in part by way of the AcLDL receptor (10, 11, 13), which has been cloned and fully characterized by Krieger and coworkers (reviewed in ref. 18). However, as much as 30–70% of OxLDL uptake by mouse peritoneal macrophages is not competitively inhibited by AcLDL (10, 11, 13) and several lines of evidence point to the presence of at least one, and possibly more, additional "scavenger receptors" (a term used in this paper to include macrophage receptors recognizing AcLDL and/or OxLDL). While the functioning of these receptors in uptake of OxLDL may be of great importance in atherogenesis, it can hardly account for the persistence of these receptors in evolution. Because OxLDL is cytotoxic to endothelial cells (19) and could thus be prothrombotic even in young animals, we previously suggested this as a possible basis for persistence of the scavenger receptors in evolution (20). Recently, Abrams and coworkers (21) have demonstrated the presence of a receptor activity in *Drosophila* similar to that of the AcLDL receptor! Clearly this class of receptors must have an even more fundamental biological role. We recently suggested that their function might be to recognize damaged cells and scavenge them (8). We used OxRBCs as our first model and showed that these were bound and taken up via a receptor that also recognizes OxLDL (but not AcLDL). In an accompanying paper, Ottnad *et al.* (9) describe the partial purification and characterization of this OxLDL receptor from mouse peritoneal macrophages. Here we present evidence that the same receptor is involved in the binding and phagocytosis of oxidatively damaged and apoptotic cells, in part by recognizing an increase in expression of PS in the outer leaflet of the phospholipid bilayer of the target cells.

MATERIALS AND METHODS

Materials. $CuSO_4$, ascorbate, sodium orthovanadate, polyinosinic acid [poly(I)], dexamethasone, and fatty acid-free bovine serum albumin (BSA) were obtained from Sigma; acetic anhydride was from Aldrich; phospholipids were from Avanti Polar Lipids; glutaraldehyde, 25% in water, was from Fisher Scientific; *N*-{[6-(5-dimethylaminonaphthalene-1-sulfonyl)amino]hexanoyl}-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (dansyl-X-

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Abbreviations: LDL, low density lipoprotein; OxLDL, oxidatively modified LDL; AcLDL, acetyl LDL; RBC, red blood cell; OxRBC, oxidatively damaged RBC; SSRBC, RBC from sickle cell anemia patients; PS, phosphatidylserine; PC, phosphatidylcholine; C, cholesterol; dansyl-X-DHPE, *N*-{[6-(5-dimethylaminonaphthalene-1-sulfonyl)amino]hexanoyl}-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt; MDA, malondialdehyde.

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DHPE) was from Molecular Probes; ^{125}I-labeled annexin V (^{125}I-annexin V) was a generous gift from Jonathan Tait (University of Washington, Seattle).

Lipoproteins. Human LDL (d = 1.019–1.063) was isolated in EDTA (1 mg/ml) from fresh plasma by preparative ultracentrifugation (22). Protein was determined by the method of Lowry *et al.* (23). LDL (100 μg/ml) was oxidized by incubating overnight in phosphate-buffered saline (PBS) in the presence of 5 μM Cu^{2+}. The lysophosphatidylcholine (lysoPC) accumulating during oxidation was removed by incubating with 10 mg of fatty acid-free BSA per ml for 24 h and reisolating the LDL by preparative ultracentrifugation (d < 1.210). This was a necessary step because otherwise the lysoPC caused RBC lysis and also inhibited the effectiveness of OxLDL as a competitor. Parallel studies in this laboratory showed that addition of lysoPC can strongly inhibit macrophage uptake and degradation of OxLDL (Elke Ottnad, S.P., and D.S., unpublished results). Acetylation of LDL with acetic anhydride was as described by Basu *et al.* (12).

Liposomes. Liposomes were composed of brain PS, egg phosphatidylcholine (PC), and cholesterol (C) at a 1:1:1 molar ratio, or of PC and C at a 2:1 molar ratio. Lipids plus butylated hydroxytoluene (to give a final concentration of 20 μM when resuspended) were dried under nitrogen. The dried lipids were resuspended in 40 mM Tris·HCl/140 mM NaCl at a final total lipid concentration of 1.5 mM. The mixture was Vortex mixed and subsequently sonicated on ice under a nitrogen stream until clear (8–10 min). The liposome suspension was then centrifuged to remove large multilamellar liposomes and debris. No significant loss of phospholipid was observed. Liposomes were labeled for ligand blots by incorporating dansyl-X-DHPE at 2–8% of the total molar lipid concentration.

Cells. RBCs were washed three times with PBS, resuspended at 20% hematocrit in PBS containing 0.1% glucose, and stored at 4°C. OxRBCs (4% hematocrit in PBS) were prepared by incubating at 37°C for 90 min in the presence of 0.2 mM $CuSO_4$ plus 5 mM ascorbate as described (9). Aldehyde modification of a 4% hematocrit RBC preparation was accomplished by incubating with 1 mM glutaraldehyde in PBS for 1 h at 37°C. Unreacted aldehyde was removed by washing the cells three times with Tris buffer. Membrane phospholipid asymmetry was disrupted by treatment with 100 μM sodium orthovanadate (10% hematocrit in phosphate-buffered saline).

Resident mouse peritoneal macrophages were isolated as described (10) and plated in RPMI 1640 medium supplemented with 10% fetal bovine serum and gentamicin. After 4 h, nonadherent cells were removed by washing three times with PBS. Macrophages were used immediately after the washing step, since overnight incubation results in an acquired ability to recognize normal human RBCs via a sialic acid-dependent mechanism. The adherent macrophages were kept in Dulbecco's modified Eagle's medium for binding and phagocytosis experiments.

A single-cell suspension of thymocytes was obtained by disrupting mouse thymus tissue through a stainless steel wire mesh into RPMI 1640 medium. Thymocytes were washed and cultured as described by Fadok and coworkers (6, 7). Apoptosis was induced by culturing cells in the presence of 1 μM dexamethasone for 4 h.

RBCs (hematocrit 0.1%) were incubated with macrophages at 37°C for 1 h. After washing to remove unbound RBCs, the percentage of macrophages binding (and/or phagocytosing) one or more RBCs was determined as described (8). In addition, the total number of RBCs bound per 100 macrophages was determined where indicated. Macrophage-bound RBCs were removed by hypotonic lysis with 5 mM phosphate buffer and macrophages were fixed with methanol prior to determination of RBC phagocytosis.

Exposure of membrane PS was measured by ^{125}I-annexin V binding to RBCs as described by Tait and Gibson (24). Nonspecific binding was measured in the presence of 5 mM EDTA. Bound and free ^{125}I-annexin V were separated by centrifugation of samples through silicone oil.

Ligand Blot. Macrophage membranes were prepared and ligand blots were performed as described by Ottnad *et al.* (9). Nonspecific sites on nitrocellulose were blocked with 5% nonfat dry milk (Carnation) prepared in incubation buffer (50 mM Tris·HCl/140 mM NaCl/2 mM $CaCl_2$). Nitrocellulose membranes were then introduced to labeled ligand in 0.1% nonfat dry milk in incubation buffer for 1.5–2.0 h. Unbound ligand was removed by washing several times. Binding of ^{125}I-labeled ligand was detected by autoradiography and binding of dansyl-X-DHPE-labeled liposomes under a long-wave UV lamp. In some experiments, the detection of PS liposome binding at 37°C was amplified by probing the blot with ^{125}I-annexin V (in the same buffer used to measure binding) followed by autoradiography.

RESULTS

As shown in Fig. 1, PS-containing liposomes (PS/PC/C, 1:1:1) were fully as effective as OxLDL in inhibiting the binding of OxRBCs to mouse peritoneal macrophages. Liposomes containing only PC and C (PC/C, 2:1) and no PS failed to compete. These results are consonant with those of previous investigators showing that a loss of membrane phospholipid asymmetry of RBCs increases their binding to macrophages (1–3). However, there remains the possibility that recognition of OxRBCs by macrophages depends also on other changes in the membrane induced by oxidation (fatty acid and sterol peroxidation, conjugation of lipid fragments to protein, protein degradation, etc.). Sodium vanadate has been shown to specifically inhibit the RBC aminophospholipid translocase and thereby create cells expressing PS on their surface (25). We found that treatment of RBCs with vanadate for 4 h at 37°C, resulted in recognition of these cells by ≈40% of the macrophages screened (Fig. 1). PS liposomes and also OxLDL inhibited the binding of vanadate-treated RBCs but AcLDL, native LDL, and PC liposomes did not. To check whether vanadate might be inducing oxidative changes in addition to inhibiting the translocase, we looked for the formation of methemoglobin but found no difference between vanadate-treated and untreated cells (whereas cells treated with Cu^{2+}/ascorbic acid for 90 min showed a 4-fold increase in methemoglobin). Thus, it appears that the effect of vanadate may be predominantly due to its inhibition of the translocase.

As shown in Fig. 2, during vanadate treatment there was a progressive, almost linear, increase in exposure of PS on the

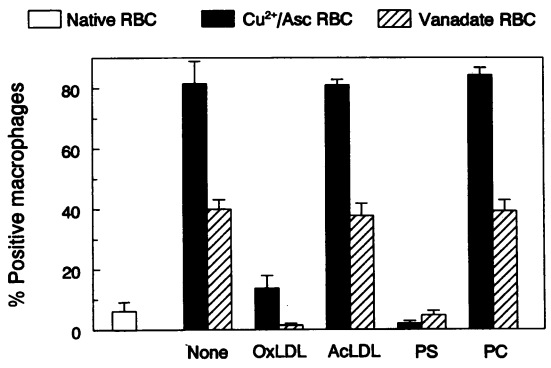

FIG. 1. Binding to mouse peritoneal macrophages of oxidized RBCs (solid bars) and vanadate-treated RBCs (hatched bars) and extent of inhibition by several competing ligands. OxLDL and AcLDL were added to a final concentration of 100 μg/ml. Liposomes were added at a total lipid final concentration of 1 mM. Values represent percentage of macrophages having bound one or more RBCs and are expressed as mean ± SD of four determinations. Asc, ascorbic acid.

RBCs and a parallel increase in the binding of those RBCs to macrophages, compatible with the proposition that it is the progressive increase in PS that accounts for macrophage binding. However, it should be noted that there was no phagocytosis. Even after vanadate treatment of RBCs for 24 h, which increased annexin V binding to values similar to those seen with OxRBCs (30,000 molecules of annexin V/RBC), phagocytosis of vanadate-treated RBCs was significantly less than that of OxRBCs. The reason for this dissociation is not known.

Fadok and coworkers (6, 7) have shown that macrophage recognition of apoptotic neutrophils and thymocytes can be mediated by a number of different receptors. These include the vitronectin receptor, CD36, a lectin-like receptor, and, of course, the implied PS receptor. We tested for the potential involvement of some of these in the recognition of OxRBCs. We found that the peptide RGDS, which effectively inhibits vitronectin receptor-dependent binding, had no effect and that basic amino acids (lysine and arginine), which have been shown to disrupt the CD36/thrombospondin-dependent binding, had no effect either.

Macrophage binding of sickle cell RBCs (SSRBCs) has been shown to be inhibited by PS liposomes, presumably mediated by a putative but still undefined PS receptor (26). As shown in Table 1, OxLDL was almost as effective as PS liposomes in inhibiting the macrophage binding of SSRBCs, compatible with the possibility that the OxLDL receptor is the putative PS receptor. Binding of SSRBCs was also inhibited strongly by poly(I) but not by PC liposomes or by AcLDL.

To determine whether the OxLDL receptor might play a role in recognition of apoptotic cells, we treated mouse thymocytes with dexamethasone and tested the ability of OxLDL to compete with their binding. As shown in Table 1, there was significant inhibition. However, even at high concentrations of OxLDL the inhibition did not exceed 50%, whereas OxLDL can almost completely inhibit the binding of OxRBCs. It is noteworthy that PS liposomes inhibited thymocyte binding nearly as effectively as they inhibited OxRBC binding.

To further examine the possibility that OxLDL and PS share a common receptor, cross-competition studies with both ligands were performed. Nishikawa and coworkers (27) have previously shown that OxLDL inhibits the binding of PS-containing liposomes to mouse peritoneal macrophages. Our studies confirm these findings and also show the reciprocal competition in that PS (but not PC liposomes) inhibited ^{125}I-OxLDL binding and/or uptake (Table 2). In the studies of

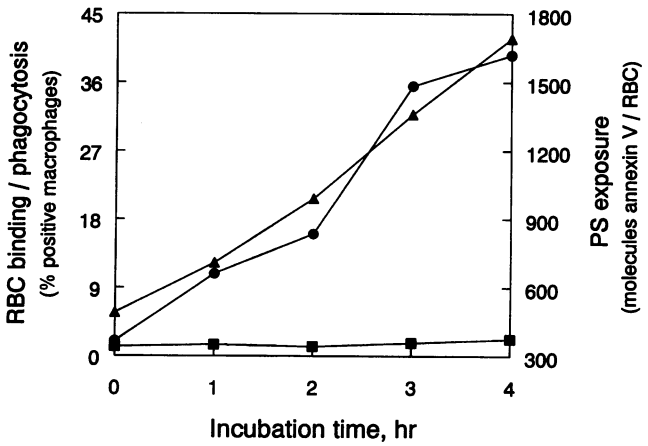

FIG. 2. Vanadate treatment (100 μM) induces parallel increases in ^{125}I-annexin V binding to RBCs (exposure of PS) (●) and in binding of those RBCs to macrophages (▲) but without an increase in phagocytosis (■). Each data point represents mean of four determinations of RBC binding and three determinations of ^{125}I-annexin V-binding.

Table 1. Effects of various competitors on binding of RBCs from a patient with sickle cell anemia (SSRBCs) and of apoptotic thymocytes to mouse peritoneal macrophages

Competitor	% inhibition	
	SSRBCs	Apoptotic thymocytes
None	0	0
OxLDL	60	47
AcLDL	2	4
Native LDL	0	10
PS liposomes	86	84
PC liposomes	0	6
Poly(I)	67	62

SSRBCs were added to macrophages at a final concentration of 0.1% hematocrit. Thymocytes were added at a concentration of 4×10^6 cells per ml. Lipoproteins were added at 100 μg/ml, liposomes were added at 0.1 mM total lipid, and poly(I) was added at 50 μg/ml.

Nishikawa *et al.*, the inhibitory effect of AcLDL on PS liposome binding was found to be significant but not as great as with OxLDL. We on the other hand found no effect of AcLDL on OxRBCs or vanadate-treated RBC binding as long as the AcLDL preparation was properly protected against oxidative damage. Lee *et al.* (28) found no binding of PS liposomes to CHO cells expressing the type I or type II AcLDL receptor, supporting our findings that recognition of OxRBCs or PS liposomes occurs via a distinct OxLDL receptor.

Using ligand blotting techniques, Ottnad *et al.* (9) have shown that OxLDL binds to a 94- to 97-kDa macrophage membrane protein. Binding to this protein was inhibited by both OxLDL and PS liposomes but not by AcLDL. We now demonstrate (Fig. 3) that PS liposomes bind to a 94- to 97-kDa protein in crude mouse peritoneal macrophage membranes (lane B), which corresponds to the OxLDL-binding protein (lane A). Control PC/C liposomes showed no affinity for the 94- to 97-kDa band. Binding of PS liposomes to this protein was inhibited by unlabeled OxLDL (lane C) but not by AcLDL (data not shown).

DISCUSSION

The data presented in this paper, together with the data of Sambrano *et al.* (8) and those of Ottnad *et al.* (9) in an accompanying paper, strongly support the conclusion that macrophage recognition of OxRBCs and/or SSRBCs, which depends to a greater extent on loss of membrane phospholipid asymmetry (26), is attributable in large part to the same 94- to 97-kDa receptor that recognizes OxLDL. We present evidence here that the same may be true for recognition of apoptotic thymocytes, again previously shown by Fadok *et al.* (6, 7) to be recognized in part by virtue of PS on the outer leaflet. However, the competition by OxLDL in this latter case was less than complete. Indeed, additional mechanisms have been shown to operate in the recognition of apoptotic thymocytes by other cell types, as discussed below.

Table 2. Inhibition by several competing ligands of ^{125}I-OxLDL association with mouse peritoneal macrophages

Competitor	% inhibition
None	0
OxLDL	81
AcLDL	38
PS liposomes	55
PC liposomes	20

Cells were incubated at 37°C for 1 h in Dulbecco's modified Eagle's medium with 10 μg of ^{125}I-OxLDL per ml and the indicated competitors. OxLDL and AcLDL were added at 150 μg/ml and PS and PC liposomes were added at 0.1 mM total lipid.

Certain noxious stimuli can induce either necrosis or apoptosis depending on the concentration of the agent and the duration of the exposure (29). Is there a commonality with respect to how necrotic and apoptotic cells become targeted for macrophage scavenging? An attractive final common pathway is the aminophospholipid translocase enzyme. It is situated at the surface of the cell and might be one of the earliest enzymes to undergo oxidative damage when a cell is "under attack." Since it is an ATP-requiring enzyme, any damage that compromises energy supply would tend also to lead to loss of phospholipid asymmetry. Some of the genes involved in the apoptosis "program" have been identified (30) but many have not. There is evidence, however, that free radical generation and changes in redox state accompany apoptosis (31). Thus, it may not be unreasonable to propose that inactivation of the membrane aminophospholipid translocase may be an early step in that program, signaling macrophages that a cell is scheduled for removal. If so, both oxidative damage and apoptosis would "mark" the damaged cell in the same way.

Both OxLDL and AcLDL are ligands for the AcLDL receptors types I and II (32). However, it appears that this receptor does not bind damaged cells or at least is not as effective in binding such cells. For example, Ottnad *et al.* (9) found no binding of oxidatively damaged RBCs to transfected cells expressing the type II receptor for AcLDL. In the present studies, OxLDL (but again not AcLDL) interfered with the macrophage binding of oxidatively damaged and apoptotic cells. These findings imply that the AcLDL receptor by itself is not able to bind oxidized or apoptotic cells but this does not rule out the possibility that it might play a role in concert with other membrane proteins. Nishikawa *et al.* (27) reported that both OxLDL and AcLDL inhibited the binding of PS liposomes to mouse peritoneal macrophages. OxLDL inhibited almost 100% but AcLDL inhibited only ≈50%, leading them to suggest that two different "scavenger receptors" might be involved. We, on the other hand, found no effect of AcLDL on the binding of oxidatively damaged RBCs or of vanadate-treated RBCs. Our findings are consonant with those of Lee and coworkers (28), who found no binding of PS liposomes to CHO cells expressing the type I or type II AcLDL receptor.

Scavenger receptors are known to bind a wide variety of negatively charged ligands. As demonstrated here, OxRBC binding appears to depend on recognition via a receptor sensitive to both PS and OxLDL. How are PS liposomes and OxLDL alike? Sambrano *et al.* (8) showed that treatment of RBCs with glutaraldehyde or malondialdehyde (MDA) caused them to bind to macrophages and that this binding was inhibited by OxLDL and by poly(I) but not by polycytidylic acid. We have recently found that PS liposomes also inhibit recognition of glutaraldehyde-treated RBCs (data not shown).† At first glance, it is difficult to see how the effects of glutaraldehyde and MDA can be fit into a unified mechanism with exposure of PS as the endpoint. One possibility is that treatment with these cross-linking aldehydes inactivates the aminophospholipid translocase. Another possibility is that these aldehydes react with phospholipid amino groups, producing a more negatively charged membrane surface that mimics a PS-rich membrane. In either case, an array of negatively charged groups such as those found in OxLDL could mediate binding to the receptor. This is supported by the ability of poly(I) to inhibit binding of both OxRBCs and glutaraldehyde-treated RBCs.

The mechanisms for recognizing and internalizing OxRBCs may be more complex, however. Unlike vanadate-treated RBCs, glutaraldehyde-modified RBCs and OxRBCs are readily phagocytosed. It is possible that phagocytosis of these RBCs depends on additional modifications such as protein crosslinking. An additional receptor that recognizes such modifications may cooperate with the 94- to 97-kDa OxLDL/PS receptor to facilitate internalization. Preliminary studies in this laboratory show that the macrophage line RAW 264.7 expresses the 94- to 97-kDa membrane protein as described by Ottnad *et al.* (9) and binds OxLDL and PS liposomes to about the same extent as do mouse peritoneal macrophages. However, the RAW line failed to bind OxRBCs. The implication is that binding and phagocytosis of OxRBC depends on an additional receptor or coprotein not needed for the binding and uptake of PS liposomes or OxLDL. Further studies are needed to elucidate the receptor system(s) responsible for OxRBC recognition and phagocytosis by macrophages.

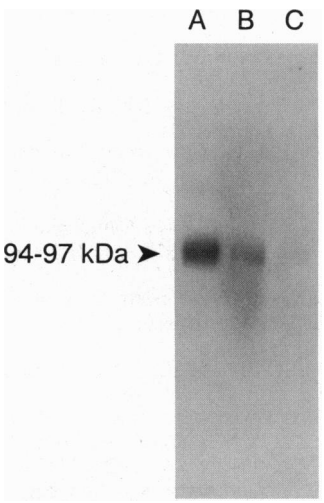

FIG. 3. Ligand blot of ^{125}I-OxLDL and PS liposomes to membrane preparations from mouse peritoneal macrophages. PS liposomes were added to membranes and binding was detected with ^{125}I-annexin V. Binding of PS liposomes occurred at a single prominent band (lane B) similar to that of ^{125}I-OxLDL (lane A) at 94–97 kDa. Addition of excess unlabeled OxLDL (130 μg/ml) inhibited binding of PS liposomes to the membranes (lane C).

Finally, we would stress that recognition of membrane PS by the OxLDL receptor is almost certainly not the only mechanism by which macrophages recognize and scavenge damaged cells. For example, Fadok *et al.* (7) have shown that the phagocytosis of apoptotic thymocytes by mouse peritoneal macrophages is inhibited by PS liposomes but that phagocytosis by mouse bone marrow macrophages is not. The latter is mediated by the vitronectin receptor, an $\alpha_v\beta_3$ integrin, which uses the RGDS sequence rather than PS as its recognition signal. The vitronectin receptor is also involved in recognition of apoptotic neutrophils but it requires cooperation with CD36 and thrombospondin (34). OxLDL has been shown to also bind to CD36 (35), which has been resolved from the 94- to 97-kDa oxidized LDL receptor (9). The possibility of cooperative interactions between CD36 and the 94- to 97-kDa oxidized LDL receptor should be explored.

We acknowledge our indebtedness to Dr. Jonathan F. Tait (University of Washington, Seattle) for his advice and his gift of ^{125}I-annexin V. This research was supported by the National Heart, Lung and Blood Institute (Grant 2P50 HL14197-22 SCOR on Arteriosclerosis) and by the Sam and Rose Stein Institute for Research on Aging. This work was done during the tenure of a research fellowship from the American Heart Association, California Affiliate, in partial fulfillment of the Ph.D. degree in the Biomedical Sciences Graduate Program, University of California, San Diego (G.R.S.).

†Pradhan *et al.* failed to find any effect of MDA treatment on RBC membrane phospholipid asymmetry (33), but they did not use concentrations any higher than 0.08 mM, whereas Sambrano *et al.* (8) used MDA at 10 mM.

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