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Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: Evidence that oxidation-specific epitopes mediate macrophage recognition

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ABSTRACT **Apoptosis is recognized as important for normal cellular homeostasis in multicellular organisms. Although there have been great advances in our knowledge of the molecular events regulating apoptosis, much less is known about the receptors on phagocytes responsible for apoptotic cell recognition and phagocytosis or the ligands on apoptotic cells mediating such recognition. The observations that apoptotic cells are under increased oxidative stress and that oxidized low-density lipoprotein (OxLDL) competes with apoptotic cells for macrophage binding suggested the hypothesis that both OxLDL and apoptotic cells share oxidatively modified moieties on their surfaces that serve as ligands for macrophage recognition. To test this hypothesis, we used murine monoclonal autoantibodies that bind to oxidation-specific epitopes on OxLDL. In particular, antibodies EO6 and EO3 recognize oxidized phospholipids, including 1-palmitoyl 2-(5-oxovaleroyl) phosphatidylcholine (POVPC), and antibodies EO12 and EO14 recognize malondialdehyde-lysine, as in malondialdehyde-LDL. Using FACS analysis, we demonstrated that each of these EO antibodies bound to apoptotic cells but not to normal cells, whereas control IgM antibodies did not. Confocal microscopy demonstrated cell-surface expression of the oxidation-specific epitopes on apoptotic cells. Furthermore, each of these antibodies inhibited the phagocytosis of apoptotic cells by elicited peritoneal macrophages, as did OxLDL. In addition, an adduct of POVPC with BSA also effectively prevented phagocytosis. These data demonstrate that apoptotic cells express oxidation-specific epitopes—including oxidized phospholipids—on their cell surface, and that these serve as ligands for recognition and phagocytosis by elicited macrophages.**

The importance of apoptosis in the normal development of multicellular organisms and in the maintenance of cellular homeostasis is widely appreciated. Disruption of this process is associated with developmental abnormalities, cancer, and, most recently, atherogenesis. Compared with the explosive increase in our knowledge of the molecular events responsible for the regulation of apoptosis, relatively little is known about the processes responsible for clearance of apoptotic cells.

Apoptosis, or programmed cell death, is a morphologically and functionally distinct process of cell death by which unwanted cells are deleted from the body in a manner that for the most part is not associated with an inflammatory response (1–3). A critical stage for the recognition of apoptotic cells appears to involve the acquisition of cell-surface changes that result in engulfment by professional and semiprofessional phagocytes. On the one hand, the exact nature of the relevant changes on the surface of apoptotic cells responsible for this recognition is incompletely understood (4). Loss of membrane phospholipid asymmetry and consequent enhanced exposure of phosphatidylserine (PS) on the surface of apoptotic cells is generally thought to be responsible for phagocytosis by some macrophage populations (5, 6). In addition, changes in glycoprotein expression (7), loss of expression of glycosylphosphatidylinositol-linked protein antigens such as CD16 (8), and generation of a thrombospondin/CD36 binding site are all under study (9). On the other hand, the mechanisms by which professional phagocytes recognize apoptotic cells are also incompletely understood. These mechanisms are likely to be complex and may differ with differing cell types and subpopulations (4).

It is noteworthy that in some ways the cellular plasma membrane is similar to the surface of a low-density lipoprotein (LDL) particle in that both are composed mainly of phospholipids containing unsaturated fatty acids and protein. Oxidation of LDL leads to its enhanced uptake by various macrophage scavenger receptors. As shown previously (10, 11) and in the companion manuscript (12), the binding of oxidized LDL (OxLDL) to macrophages is inhibited either by the delipidated apoprotein B or by lipids extracted from OxLDL and reconstituted into microemulsions (12). Similarly, oxidation of the lipid or lipid-protein matrix of a plasma membrane is analogous to the oxidation of the LDL particle and might also be predicted to lead to recognition by macrophage receptors. In fact, several lines of evidence raise the possibility that cells undergoing apoptosis may present oxidatively modified moieties on their surface that are structurally analogous to moieties on the surface of the OxLDL particle. First, cells induced to undergo apoptosis by myriad stimuli generate reactive oxygen species that may induce membrane peroxidation (2, 13). Second, many of the macrophage receptors known to recognize OxLDL, e.g., scavenger receptor A (SR-A), CD36, CD68, SR-B1 (CLA-I), and LOX-I, also have been reported to bind apoptotic cells or PS liposomes (14–18). Third, as shown by Steinberg and colleagues (11–14), intact OxLDL, as well as lipid microemulsions prepared from OxLDL, can compete in part for binding of apoptotic cells to macrophages. These data suggest the hypothesis that both OxLDL and apoptotic cells have common ligands on their surfaces, consisting of oxidatively modified moieties that are recognized by common macrophage receptors. However, the exact nature of the ligands on apoptotic cells or on OxLDL responsible for such cross-competition has not been demonstrated.

In the present manuscript, we used murine monoclonal autoantibodies cloned from apoE-deficient mice that recognize spe-

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Abbreviations: LDL, low-density lipoprotein; OxLDL, oxidized LDL; OxPL, oxidized phospholipid; POVPC, 1-palmitoyl 2-(5-oxovaleroyl) phosphatidylcholine; MDA, malondialdehyde; PAEC, porcine aortic endothelial cells; PI, propidium iodide; PS, phosphatidylserine; FSC, forward light scatter; EO, apoE-deficient.

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cific oxidized phospholipids (OxPL)—present either as free lipid or as OxPL-protein adducts—to demonstrate the presence of these oxidized moieties on the surface of apoptotic cells but not normal cells. These antibodies inhibited the phagocytosis of apoptotic cells by macrophages, as did a specific oxidized phospholipid-protein adduct. In addition, we also demonstrate that other autoantibodies directed against another "oxidation-specific" epitope, a malondialdehyde (MDA)-lysine adduct, also bind to apoptotic cells and inhibit their phagocytosis by macrophages. These data demonstrate that OxPL and other oxidation-specific epitopes on the surface of apoptotic cells are ligands that mediate recognition and phagocytosis of apoptotic cells by elicited peritoneal macrophages.

MATERIALS AND METHODS

Cell Culture and Induction of Apoptosis. Porcine aortic endothelial cells (PAECs) from wild-type swine were obtained by collagenase treatment of aortic segments (19). Cells were cultured in medium 199 with 1× Earle's salt solution with 15% FCS in 95% air, 5% CO_2 humidified atmosphere. Apoptosis was induced during the sixth passage by culturing PAECs for 16 h in serum-free media (20) by using Teflon membrane-coated culture flasks (Sarstedt) to inhibit anchorage-dependent cell spreading (21).

Thymocytes were isolated from the thymus of 6- to 8-wk-old Swiss–Webster mice. To induce apoptosis, thymocytes were incubated with 1 μM dexamethasone in RPMI/10% FCS for 14 h as described (5, 22).

Determination of Apoptosis. Cell death by apoptosis was confirmed by visualization of internucleosomal DNA cleavage on agarose gels and by classic apoptotic morphology (23–25).

To quantitate the apoptotic cell population among apoptosis-induced cells, cells were fixed with cold 70% ethanol and then treated with RNase and propidium iodide (PI). The content of DNA per cell was estimated by flow cytometry (26).

Flow Cytometry. PAECs were harvested by 0.05% trypsin treatment and washed in ice-cold buffer A (PBS/0.1% BSA). Cells (1×10^6) were incubated with 50 μg/ml of the indicated EO antibody or isotype matched control mouse IgM (Sigma) in buffer A at 4°C for 20 min, washed, and then incubated for another 20 min with 10–20 μg/ml of fluorescein-conjugated $F(ab')_2$ fragments against mouse IgM (Jackson ImmunoResearch) in buffer A. Cells were washed, incubated for 10 min with 1 μg/ml of PI, and immediately analyzed by FACScan instrument (Becton Dickinson). Data from each experiment were captured on magneto optic discs and analyzed on a Power Macintosh 7600/130 computer by using CELLQUEST software.

Immunofluorescence and Confocal Microscopy. Untreated and apoptosis-induced PAECs were fixed with 80% methanol (5 min, 4°C) and spun down on glass slides by using cytospin. Cells were incubated with 10 μg/ml of primary antibody (EO6, EO14, and control IgM), and then slides were washed by dipping in PBS. Cells were stained with 10 μg/ml of fluorescein-conjugated $F(ab')_2$ fragments against mouse IgM, and subsequent staining of DNA with Hoechst dye (Sigma) to differentiate between normal and apoptotic cells by DNA fragmentation. Coverslips were mounted on glass slides with Vectashield (Vector Laboratories) before viewing and photography on a microscope (Nikon) equipped with phase contrast and epifluorescence optics. Confocal microscopy was performed on a scanning confocal microscopy system (model MRC1000; Bio-Rad) in the National Center for Microscopy and Imaging Research at University of California, San Diego.

Murine Monoclonal Antibodies for Oxidized LDL. Monoclonal autoantibodies directed against oxidation-specific epitopes of OxLDL were cloned from hybridomas generated from apo E-deficient mice that had not been immunized. They were termed EO monoclonal antibodies. Selected hybridomas binding to copper OxLDL or to MDA-LDL were cloned by limiting dilution, isotyped as IgM, and purified from ascites by FPLC as described (27). EO antibodies EO3 and EO6 bound to OxLDL and to the isolated lipid and isolated protein of OxLDL but not to native LDL or to the lipid of native LDL (10). In particular, these antibodies bind to OxPL including 1-palmitoyl 2- (5-oxovaleroyl) phosphatidylcholine (POVPC) present as either pure lipid or as a lipid-protein adduct, such as POVPC-BSA (10). EO antibodies EO12 and EO14 in contrast bound to MDA-LDL and similarly modified MDA-proteins but not to native LDL or native LDL lipids or protein (10, 27).

Preparation of POVPC-Modified BSA. POVPC was prepared as described in the companion paper (12). It was dried onto a glass tube (final 2 mM) and incubated with 1 mg/ml BSA at 37°C for 4 h. Then, $NaCNBH_3$ was added (final concentration 10 mM), and the mixture was further incubated overnight at 37°C. After incubation, the unbound POVPC was removed by extensive dialysis with PBS. Measurement of the number of free epsilon-amino groups conjugated indicated that 41% of lysine groups of BSA were derivatized.

Phagocytosis Assay. Mouse peritoneal macrophages were isolated from female Swiss–Webster mice by peritoneal lavage 2 days after the injection of thioglycollate. Macrophages were plated in 24-well plates at 2×10^6 cells/well and nonadherent cells were removed after 1 h. The macrophage monolayers were cultured overnight in complete medium (DMEM containing 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin/streptomycin) before use in the phagocytosis assay.

Dexamethasone-treated thymocytes were suspended in buffer A (10^8 cells/ml) and labeled with 5 μM final concentration of Calcein AM (Molecular Probes) for 15 min at 37°C. This dye was selected because preliminary studies demonstrated that compared with other dyes it had significantly lower dye transfer to other cells. Afterward, cells were washed twice with 15 ml of ice-cold buffer A and resuspended in DMEM containing 10% FCS.

To assess the phagocytosis of apoptotic cells by macrophages, 2×10^6 labeled thymocytes were added to macrophage-containing wells in the absence or presence of competitors (EO antibody, OxLDL or POVPC-BSA) and then incubated for 80 min at 37°C. Wells were washed five times with ice-cold PBS and treated with 0.25% trypsin/0.02% EDTA for 20 min at 22°C to remove bound thymocytes. Macrophages in each well were harvested by scraping with a rubber policeman, washed with buffer A, and fixed with 2% paraformaldehyde. The fluorescence of 10,000 macrophages harvested from each well was analyzed with flow cytometry by a size-gating technique that included macrophages but excluded unbound labeled thymocytes, as described in *Results* (e.g., macrophages are much larger in size than thymocytes). In these experiments, we did not find any evidence that at the concentrations used OxLDL, POVPC-BSA, or the various antibodies caused any toxicity to the macrophages, as judged by changes in visual inspection as well as measurements by flow cytometry.

RESULTS

Induction of Apoptosis in PAECs. Culture of PAECs in serum-free medium with anchorage inhibition for 16 h produced many floating blebbed cells (20). Apoptotic cells can be recognized and distinguished from normal or necrotic cells by using flow cytometry analysis of cellular DNA (23). The DNA-binding fluorochrome, PI, identified a distinct hypodiploid cell population typical of apoptotic cells (23), which we have designated "Ao" cells (Fig. 1). Untreated PAECs show normal diploid DNA content characteristic of G_o/G_1 (Fig. 1*A*), whereas 51% of PAECs exposed to serum deprivation displayed DNA content below G_o/G_1 (Ao) (Fig. 1*B*), implying that about half of the serum-deprived PAECs underwent apoptosis. The serum-deprived PAECs were also analyzed according to parameters of forward light scatter (FSC), which reflects cell size, and PI fluorescence. Whereas normal PAECs were found in the lower right quadrant (Fig. 1*C*), serum-deprived PAECs were divided

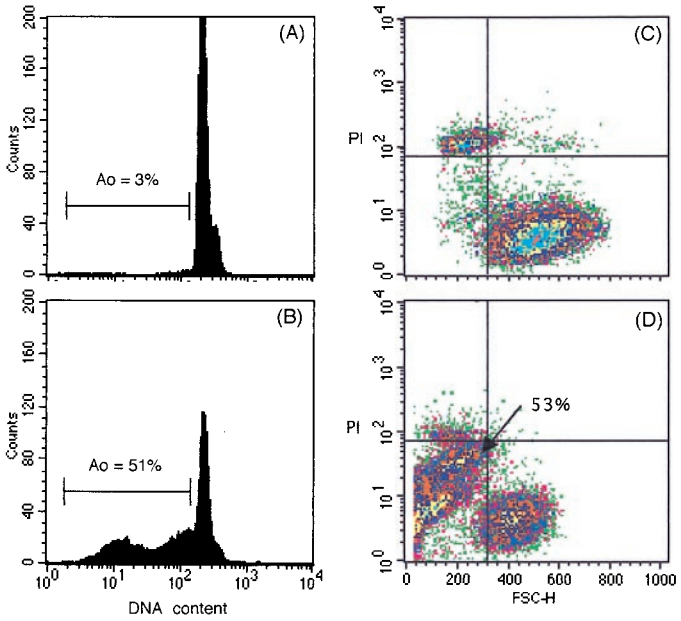

FIG. 1. FACS analysis of PAECs: DNA content was measured by DNA staining. (*A*) Normal PAECs show mostly diploid DNA. (*B*) Apoptosis-induced PAECs show that DNA content of 51% of PAECs are below G_o/G_1 (Ao). Morphologic change of (*C*) normal PAECs and (*D*) apoptosis-induced PAEC were analyzed with X parameter of FSC and Y parameter of PI fluorescence. (*E*) Agarose gel electrophoresis of DNA obtained from normal PAECs (lane 2) and apoptosis-induced PAECs (lane 3). The migration positions of 100-bp marker DNA are indicated (*Left*, lane 1). Apoptosis-induced PAECs show the characteristic ladder pattern of fragmented DNA.

into two distinct populations (Fig. 1*D*). One population remained in the right lower quadrant characterized by normal range of cell size and negative PI staining. This population was regarded as normal and/or at very early stage of apoptosis with minimal morphologic change. The other population, in the left lower quadrant, was characterized by reduced cell size and was further subdivided into dim and bright PI staining. We regard those cells as apoptotic cells and bodies, and the percentage of the total cells in this population, i.e., 53%, matched that of hypodiploid cells ("Ao" in Fig. 1*B*). Also, DNA gel electrophoresis demonstrated the characteristic "ladder" pattern of DNA fragmentation in multiples of 100 bp (Fig. 1*E*).

EO Antibodies Recognize Apoptotic Cells and Bodies but Not Normal PAECs. Because OxLDL is known to compete for macrophage binding to apoptotic cells (14), we hypothesized that oxidation-specific epitopes would be present on apoptotic cells. To test this idea, we examined the ability of the oxidation-specific IgM EO antibodies to bind to apoptotic PAECs showing dim and bright PI staining. We used (*i*) EO3 and EO6, selected for binding to OxLDL and in particular to oxidized-phospholipid epitopes, and (*ii*) EO12 and EO14, selected for their ability to bind to MDA-lysine epitopes, as found on MDA-LDL. As shown in Fig. 2*A*, the binding of EO6 and EO14 to apoptosis-induced PAECs showed a significant shift to the right compared with nonimmune control mouse IgM. We also analyzed EO6 and EO14 binding to these cells according to FSC and PI intensity (Fig. 2*B*). Fig. 2*C* demonstrates the binding of EO6 to cells in regions 1, 2, and 3 of Fig. 2*B*. Note that EO6 did not bind at all to normal cells (R1) but bound extensively to cells in the dim (R2) and bright (R3) PI staining cells (Fig. 2*C*), reflecting increasing stages of apoptosis. Fig. 2*D* reflects a similar pattern for the binding of EO14. Also by combining EO6 binding and DNA content measurement, we confirmed that EO6 selectively recognized "Ao cells," but not normal cells (data not shown). Similar data were also demonstrated for antibodies EO3 and EO12.

EO Antibodies Bind to Apoptotic Cell Membrane. To demonstrate morphologically that the oxidation-specific EO antibodies bound specifically to apoptotic PAEC, an immunofluorescence study was done. Fig. 3 demonstrates that EO6 bound to an apoptotic cell and apoptotic bodies with characteristic fragmented and condensed nuclei, as demonstrated by correlation between Hoechst DNA staining (Fig. 3*A*) and EO6 binding, as detected by surface FITC staining (Fig. 3*B*). We also performed immunofluorescence confocal microscopy (Fig. 3 *C–F*). EO6 and EO14 bound to the surface membrane of the apoptotic cells and bodies in a specific punctuate pattern (Fig. 3 *C* and *D*). In contrast, only a diffuse nonspecific staining or autofluorescence was observed when normal PAEC were stained with EO6 (Fig. 3*E*) or when apoptotic PAEC were stained with nonimmune control murine IgM (Fig. 3*F*). These findings directly demonstrate that apoptotic cells display oxidation-specific epitopes on their cellular membrane.

Phagocytosis of Apoptotic Thymocytes Is Inhibited by EO Antibodies, OxLDL, and POVPC-BSA. OxLDL inhibits the macrophage binding of apoptotic cells (14), and EO antibodies EO3 and EO6 inhibit the uptake of OxLDL by macrophages (10). To

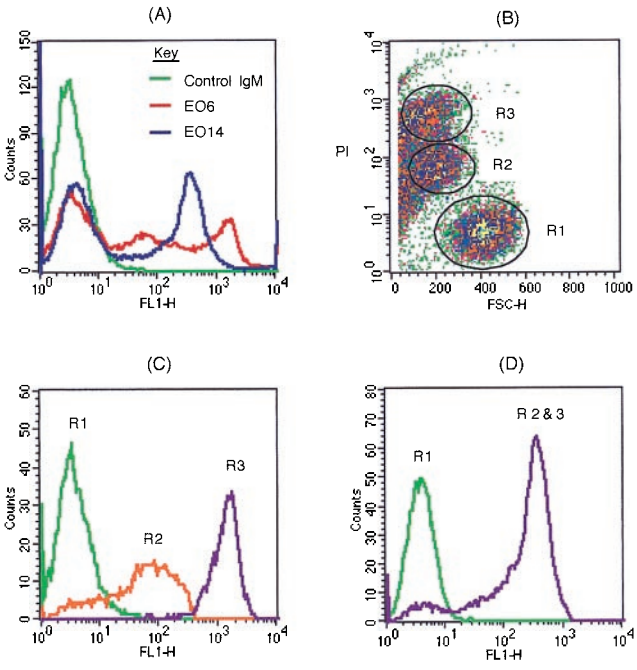

FIG. 2. FACS analysis of EO antibody and control IgM binding to apoptosis-induced PAECs. (*A*) Binding of EO6 and EO14 to the PAECs shows two and one peak shifts, respectively, above the fluorescence of control IgM. (*B*) Apoptosis-induced PAECs were gated into three populations according to PI intensity and FSC; normal (R1), dim (R2), and bright PI staining (R3). (*C*) EO6 and (*D*) EO14 staining according to three gated regions shown in *B*. Note that EO6 and EO14 do not bind to cells in region1 (R1) but bind to cells in dim PI staining region (R2) and bright PI staining region (R3).

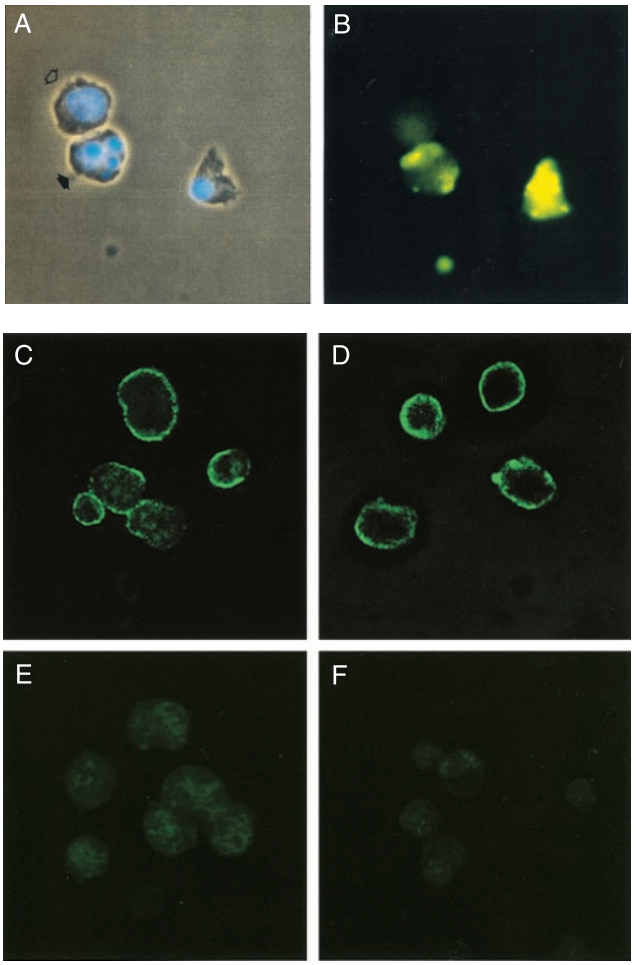

FIG. 3. Immunofluorescence microscopy of apoptosis-induced PAECs. (*A*) DNA staining with Hoechst dye showed normal cell with single nucleus (open arrow) and apoptotic cell with characteristic fragmented nuclei (closed arrow). Note apoptotic body below. (*B*) Binding of EO6 to the cells in the same field observed under a fluorescence microscope. The upper normal cell showed diffuse nonspecific fluorescence, whereas the apoptotic cells and body showed specific punctuate fluorescence. Confocal microscopy of apoptosis-induced PAECs: (*C*) EO6 and (*D*) EO14 bound to surface of apoptotic cells but not to (*E*) normal PAEC. (*F*) Apoptotic PAEC stained with control mouse IgM showed nonspecific fluorescence.

test whether the oxidatively modified moieties on apoptotic cell membranes could play a role as ligands for phagocytic clearance by elicited macrophages, we tested the ability of these antibodies to inhibit uptake of apoptotic cells. To assess macrophage phagocytosis, we developed a method that was objective and more quantitative than assays using visual counting of macrophages under the microscope. For these assays, thymocytes were chosen as the apoptotic cell, because their smaller size (Fig. 4*A*) makes them easily distinguishable from macrophages (Fig. 4*B*) by flow cytometry analysis. In preliminary studies, we showed that apoptotic thymocytes also were recognized by EO antibodies, whereas normal thymocytes were not (data not shown).

Thymocytes were labeled with the fluorescent dye Calcein AM as described in *Methods*. Fig. 4*C* shows the intensity of Calcein AM fluorescence for the labeled thymocytes and Fig. 4*D,* that for the autofluorescence of isolated macrophages. To measure macrophage uptake, labeled apoptotic thymocytes were incubated with macrophages for 80 min at 37°C, and then the bound thymocytes were removed by trypsin treatment as described in *Methods*. Then, by analyzing Calcein AM fluorescence in gated region R4 (which excludes thymocytes), it was possible to calculate the percentage of macrophages that had phagocytosed

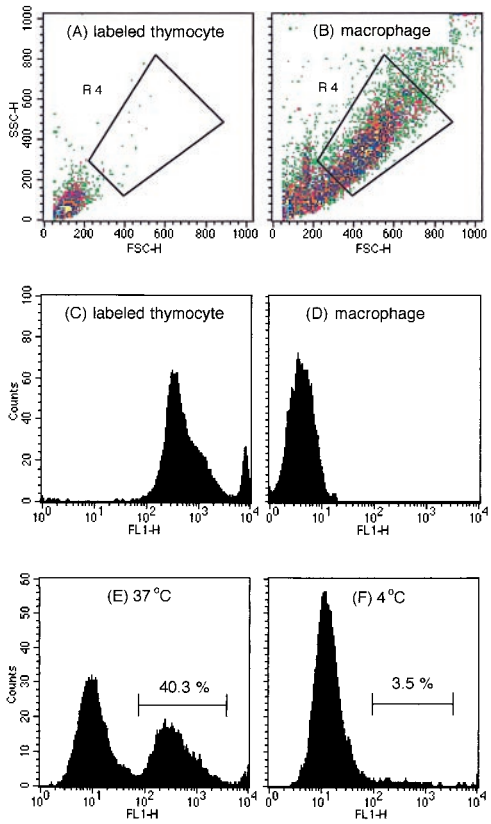

FIG. 4. Phagocytosis assay by using flow cytometry. (*A*) Fluorescence of calcein-AM labeled apoptotic thymocytes and (*B*) autofluorescence of macrophages by parameter of FSC and SSC (side scatter, which represent granularity). Fluorescence of (*C*) calcein-AM-labeled apoptotic thymocytes and (*D*) macrophages. Region 4 (R4) was gated to analyze calcein-AM fluorescence of engulfed thymocytes by macrophages. Gated macrophages were analyzed after the macrophages were incubated with calcein-AM labeled apoptotic thymocytes at (*E*) 37°C and (*F*) 4°C and then trypsinized to remove bound thymocytes.

labeled thymocytes. In the example shown (Fig. 4*E*), 40.3% of the macrophages contained apoptotic thymocytes. To validate that the fluorescence observed represented phagocytosis, the experiment was repeated at 4°C. As shown in Fig. 4*F*, fluorescence was reduced by more than 90%. Furthermore, only rare macrophages showed bound thymocytes when examined under the microscope.

To test the ability of the antibodies to inhibit uptake, we repeated the phagocytosis experiment in the absence and presence of various EO antibodies and OxLDL. In different experiments there was variation in the percent of macrophages that took up apoptotic cells in the absence of competitors. A representative competition experiment is shown in Fig. 5 *A–D*. In the absence of added antibody, 41.6% of macrophages took up thymocytes. EO6 reduced this to 26.1%, EO12 to 25.7%, and 100 μg/ml of OxLDL to 4.4%. Fig. 6*A* summarizes results of multiple experiments demonstrating the ability of the oxidation-specific antibodies to inhibit uptake. Note that at the concentrations of antibodies used, the inhibition produced by the combination of EO6 and EO12 was greater than that with either used alone. Note also that nonimmune murine IgM did not inhibit uptake, indicating that the inhibition by EO6 and EO12 is indeed attributable to their recognition of their specific epitopes on the cells. Fig. 6*B* demonstrates the ability of OxLDL to inhibit apoptotic thymocyte uptake in a dose-dependent manner. For any given concentration of OxLDL, the percentage inhibition of phagocytosis appeared greater than that previously reported for inhibition of binding (11, 14). Antibodies EO3 and EO6 bind to an adduct of OxPL, POVPC, with BSA. To further test the hypothesis that POVPC was a ligand on apoptotic thymocytes that mediated macrophage

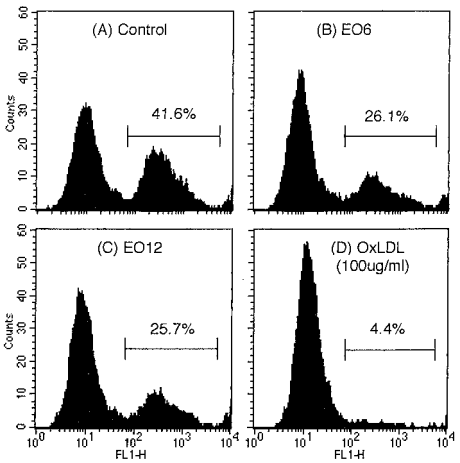

FIG. 5. Inhibition of phagocytosis of labeled apoptotic thymocytes. (*A*) Demonstrates the percentage of macrophages that have phagocytosed labeled apoptotic thymocytes in the absence of competitor, (*B*) apoptotic thymocytes preincubated with 1 mg/ml EO6, (*C*) 1 mg/ml of EO12, or (*D*) 100 μg/ml of OxLDL.

recognition, we tested the ability of POVPC-BSA to inhibit the uptake of apoptotic thymocytes (Fig. 6*C*). POVPC-BSA competed effectively for phagocytosis of apoptotic thymocytes, whereas native BSA did not.

DISCUSSION

The swift identification and engulfment of apoptotic cells by professional and semiprofessional phagocytes is vital for the maintenance of normal development and the prevention of inflammation and disease. Recent studies have developed a detailed understanding of the complex molecular and cellular events involved in programmed cell death. However, much less is known about the docking molecules and receptors on macrophages responsible for recognition and phagocytosis, and even less is known about the molecules on the apoptotic cells that

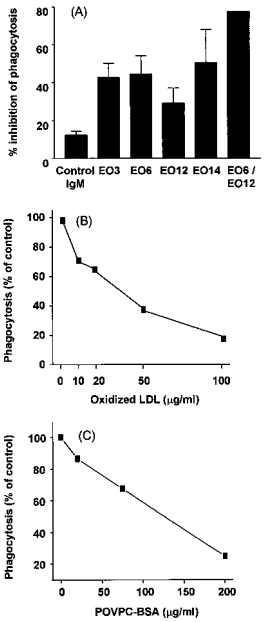

FIG. 6. Competition for macrophage phagocytosis of apoptotic cells. (*A*) Shown is a summary bar graph of the percentage inhibition of phagocytosis of apoptotic cells. Each value is mean ± SD of one to four separate experiments. (*B*) OxLDL dose-dependently inhibited the phagocytosis of apoptotic thymocytes. (*C*) POVPC-BSA dose-dependently inhibited the phagocytosis of apoptotic thymocytes.

mediate such recognition (4, 28). A number of changes on the surface of apoptotic cells have been implicated, including PS exposure, changes in carbohydrates, and expression of molecules interacting with a thrombospondin-mediated recognition pathway. As outlined in the *Introduction*, several lines of evidence suggested that apoptotic cells, which are known to be under conditions of enhanced oxidative stress, express oxidatively modified moieties on their surface that might mediate macrophage recognition and phagocytosis. Among these data were the observations that OxLDL and microemulsions of the lipids of OxLDL inhibited macrophage binding of apoptotic cells (11, 14). In this manuscript, we demonstrate that oxidation-specific monoclonal antibodies, originally identified by their ability to bind to OxLDL and to inhibit the uptake of OxLDL by macrophages, also bind to apoptotic cells and inhibit their phagocytosis by elicited mouse peritoneal macrophages.

In earlier studies, our laboratory made the observation that apoE-deficient (EO) mice, which have extensive hypercholesterolemia and associated atherosclerosis, have exceedingly high autoantibody titers to various epitopes of OxLDL (27). Monoclonal autoantibodies to OxLDL were cloned from hybridomas generated from EO mice that had not been exogenously immunized and were termed "EO" monoclonal antibodies. Selected hybridomas binding to OxLDL or to MDA-LDL were cloned and isotyped as IgM. EO antibodies EO3 and EO6 bound to OxLDL and in particular to OxPL including POVPC present as either pure lipid or as a lipid-protein adduct (e.g., POVPC-BSA) (10). The formation of OxPL-apoB adducts in OxLDL has previously been demonstrated by chemical techniques (29). EO antibodies EO12 and EO14, in contrast, bound to MDA-LDL and similarly modified MDA-proteins (10, 27). Furthermore, the EO-autoantibodies that bound to OxPL, e.g., EO6, inhibited binding and degradation of Ox-LDL by macrophages by up to 91% (10). EO6 also inhibited the binding of microemulsions made from the lipids of OxLDL (10, 12) as well as the modified apoB of OxLDL (12). Similarly, POVPC-BSA directly inhibited macrophage uptake of OxLDL as well as binding of the lipids and modified apoB of OxLDL (10, 12). These data suggested that OxPL, as well as OxPL-apoB adducts, represent one class of ligands on OxLDL that mediates binding and uptake by macrophage scavenger receptors (10). These same autoantibodies immunostain atherosclerotic lesions, and similar autoantibodies to epitopes of Ox-LDL are present in sera of normal and atherosclerotic animals and humans (27).

To test the hypothesis that cells undergoing apoptosis expressed oxidatively modified lipids or lipid-protein adducts on their surface membrane and that these moieties would be ligands for macrophage phagocytosis, we tested whether the same EO antibodies would recognize apoptotic cells and block their uptake by macrophages. The data demonstrate that all of these oxidation-specific monoclonal autoantibodies showed specific binding to the surface of apoptotic cells, but not to the surface of normal cells. Nonimmune IgM did not bind to apoptotic cells. Furthermore, these antibodies inhibited macrophage uptake of apoptotic cells profoundly, whereas the control IgM did not. In addition, a specific OxPL-protein adduct, POVPC-BSA, inhibited apoptotic cell uptake by 90%, similar to its effect on inhibiting macrophage uptake of OxLDL (10). Similarly, antibodies to MDA-LDL also inhibited uptake, implying that MDA (a breakdown product of lipid peroxidation) forms apparent adducts with cell-surface proteins or lipids and also acts as another type of oxidation-specific ligand mediating macrophage uptake. These data, when combined with the observation that OxLDL was also a strong competitor, strongly suggest that oxidized lipids and/or oxidized lipid-protein adducts on the surface of apoptotic cells, presumably derived from membrane peroxidation that occurs during apoptosis, are ligands for phagocytosis of apoptotic cells. Furthermore, generation of such oxidatively modified moi-

eties may be a general mechanism that marks the surface of OxLDL, apoptotic cells, and even necrotic cells for phagocytosis.

The data demonstrating that the OxPL-specific monoclonal antibodies can inhibit apoptotic cell uptake and that POVPC-BSA is also a highly effective competitor directly support a major role for OxPL and/or OxPL-protein adducts as ligands for macrophage uptake. PS exposure on the surface of apoptotic cells has also been shown to be associated with enhanced recognition by various macrophage preparations (5, 6). In those studies, the involvement of PS has been inferred by the demonstration of enhanced surface exposure and by inhibition of phagocytosis by PS-containing liposomes. It is interesting to note that the majority of PS found in cell membranes is unsaturated. Furthermore, although not usually stated, it is likely that most of the PS liposomes prepared by using commercially obtained PS also contain unsaturated fatty acids. Such liposomes could easily undergo lipid peroxidation during preparation, especially because lipids are routinely lyophilized on a glass surface and presumably exposed to air before reconstitution. However, in some experiments, PS liposomes in which all fatty acids were saturated were still capable of inhibiting binding of apoptotic cells (11). Furthermore, it is possible that the same conditions leading to the exposure of PS on the cell surface may also be associated with the simultaneous generation and colocalized exposure of oxidized moieties, such as oxidized PS, oxidized phosphatidylcholine, and/or adducts of these with associated membrane proteins. Obviously further study will be needed to define the actual epitopes on apoptotic cells to which these antibodies bind and the relationship of these epitopes to PS exposure. In addition, it will be of considerable interest to define the macrophage scavenger receptors responsible for the oxidized-moiety-mediated phagocytosis of apoptotic cells.

The fact that apoptosis was overlooked for many years suggests the efficiency of the removal pathway (4). Evidence of enhanced appearance of apoptotic cells therefore suggests a major alteration in the kinetics of apoptotic cell generation and removal. Recently evidence has accumulated that there is an enhanced accumulation of apoptotic cells in atherosclerotic lesions, transplant arterial disease, and restenosis (30–33). The reasons for this are not clear because macrophages, which are professional phagocytes, are present in abundance in most atherosclerotic lesions. OxLDL, which is present in such lesions, can induce apoptosis in arterial cell types, possibly as a result of different oxidized lipids and sterols. Our observation that OxLDL can compete with macrophages for the uptake of apoptotic cells might also explain in part the accumulation of apoptotic cells. Similarly, IgM autoantibodies to oxidation-specific epitopes might also prevent macrophage binding. On the other hand, IgG autoantibodies of a similar specificity might actually promote such uptake by Fc-receptor mediated pathways. Other oxidatively modified lipids and proteins in the lesion, such as matrix proteins, might also act as competitors. In turn, the accumulation of apoptotic cells might be expected to lead to necrosis and release of proinflammatory and toxic contents, such as oxidized lipid moieties, which could lead to further destabilization of the plaque and ultimately to clinical events. Furthermore, the formation of oxidation-specific ligands on the surface of apoptotic cells, such as OxPL, results in the generation of immunogenic neoepitopes, many of which are in common with those found on OxLDL. Sera from patients with the antiphospholipid antibody syndrome (APS) have been shown to bind to apoptotic cells (34). Because we have shown that many of the autoantibodies in APS sera bind to OxPL and not native phospholipid (35, 36), we suggest that many of the antiphospholipid antibodies that bind to apoptotic cells are in fact directed to OxPL epitopes. This conclusion is consonant with the recent report by Mevorach *et al.* (37) showing that intravenous injection of syngeneic apoptotic thymocytes into mice elicited production of anticardiolipin antibodies.

In summary, these data demonstrate that when cells undergo apoptosis, they express oxidation-specific epitopes on their cell surface, including OxPL and/or OxPL-protein adducts, and that these serve as ligands for macrophage recognition and phagocytosis. The nature of these and other oxidation-specific epitopes and the receptors on macrophages to which they bind are currently under investigation.

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