Apoptotic Cells with Oxidation-specific Epitopes Are Immunogenic and Proinflammatory

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Abstract

Oxidation of low density lipoprotein (LDL) generates a variety of oxidatively modified lipids and lipid-protein adducts that are immunogenic and proinflammatory, which in turn contribute to atherogenesis. Cells undergoing apoptosis also display oxidized moieties on their surface membranes, as determined by binding of oxidation-specific monoclonal antibodies. In the present paper, we demonstrated by mass spectrometry that in comparison with viable cells, membranes of cells undergoing apoptosis contain increased levels of biologically active oxidized phospholipids (OxPLs). Indeed, immunization of mice with syngeneic apoptotic cells induced high autoantibody titers to various oxidation-specific epitopes of oxidized LDL, including OxPLs containing phosphorylcholine, whereas immunization with viable thymocytes, primary necrotic thymocytes, or phosphate-buffered saline did not. Reciprocally, these antisera specifically bound to apoptotic cells through the recognition of oxidation-specific epitopes. Moreover, splenocyte cultures from mice immunized with apoptotic cells spontaneously released significant levels of T helper cell (Th) 1 and Th2 cytokines, whereas splenocytes from controls yielded only low levels. Finally, we demonstrated that the OxPLs of apoptotic cells activated endothelial cells to induce monocyte adhesion, a proinflammatory response that was abrogated by an antibody specific to oxidized phosphatidylcholine. These results suggest that apoptotic cell death generates oxidatively modified moieties, which can induce autoimmune responses and a local inflammatory response by recruiting monocytes via monocyte–endothelial cell interaction.

Key words: apoptosis • lipid peroxidation • autoimmunity • inflammation • oxidized LDL

Introduction

Apoptotic cells have been increasingly recognized as targets of autoantibodies that arise in a broad spectrum of autoimmune diseases. The evidence that apoptotic cells are targeted by autoantibodies was provided by studies showing that autoantibodies from humans and mice with systemic lupus erythematosus (SLE) recognize autoantigens that are prominently clustered in the surface blebs of apoptotic cells (1, 2). Several studies have also demonstrated that antiphospholipid antibodies (aPLs), which arise in a variety of autoimmune disorders such as the primary aPL syndrome and SLE, recognize the plasma membranes of apoptotic cells, but not viable cells (3, 4). In addition, mice immunized with syngeneic apoptotic cells have been shown to generate autoantibodies to apoptotic cells and cellular components, such as ssDNA and cardiolipin (5), implying that apoptotic cells not only serve as targets for antibodies but also provide autoantigens that trigger autoimmune responses. Moreover, evidence suggests that defective clearance and, hence, abnormal accumulation of apoptotic cells may contribute...
to such autoimmune response (6, 7). However, the exact molecular nature of the antigenic determinants or immunostimulatory molecules that induce such autoimmune response remains elusive.

Atherosclerosis is now considered a chronic inflammatory disease of the vascular wall; in addition, immune mechanisms modulate its progression (8, 9). Oxidized low density lipoprotein (OxLDL) has been demonstrated to play a key role in atherogenesis because of its proinflammatory and immunogenic properties (10, 11). Oxidation of LDL generates a variety of oxidatively modified lipids and lipid–protein adducts. For example, when polyunsaturated fatty acids in phospholipids of LDL undergo peroxidation, reactive decomposition products, such as the oxidized phospholipid (OxPL) backbone and malondialdehyde (MDA) are formed. These products can covalently modify protein and lipid moieties in LDL, leading to formation of neoself-epitopes, which we have termed “oxidation-specific” neoepitopes and demonstrated to be highly immunogenic (11). Antibodies to these oxidation-specific epitopes can be found in humans as well as in mice. We documented previously that hypercholesterolemic apolipoprotein E–deficient (apoE−/−) mice with atherosclerosis have robust antibody titers to OxLDL (12). Using copper Ox-LDL or MDA-modified LDL (MDA-LDL) as selecting antigens, we cloned monoclonal IgM autoantibodies to oxidation-specific epitopes of OxLDL from the spleens of naive apoE−/− mice, termed EO antibodies (13). Previous studies have shown that these monoclonal anti-OxLDL antibodies, such as the prototypic EO6, also recognize the plasma membranes of apoptotic cells but not viable cells (14), implying that some oxidation-specific epitopes on OxLDL are also present on membranes of cells undergoing apoptosis, which are known to be under increased oxidative stress. EO6 can block the uptake of OxLDL by macrophage scavenger receptors (15), and especially CD36 (16). Similarly, EO6 partially blocks the uptake of apoptotic cells by macrophages, implying that such OxPLs are ligands mediating apoptotic cell clearance as well (14). Indeed, Hazen et al. have demonstrated that a variety of phosphorylcholine (PC)-containing OxPLs are CD36 ligands (17).

Of considerable interest, EO6 has been demonstrated recently to be identical to highly conserved T15 clonospecific natural antibodies specific for PC (18). These T15 antibodies recognize PC as a component of the capsular polysaccharide of microorganisms, such as Streptococcus pneumoniae, and participate in the innate immune response to microorganisms in mice (19). We have shown previously that EO6/T15 bind to OxPLs containing the PC headgroup, such as 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-PC (POVPC), but did not bind to native, nonoxidized phosphatidylcholines even though they contained the same PC moiety (15, 18, 20). Thus, the PC headgroup is a cryptic epitope of phosphatidylcholine in native LDL or viable cell membranes, and the PC epitope becomes accessible for binding by these anti-PC antibodies after oxidation-induced conformational changes occur in the phosphatidylcholine molecule in OxLDL or apoptotic cells. In point of fact, it is the PC head group of aldol condensates of POVPC dimers, trimers, or larger aggregates that appears to be the actual epitope of free POVPC (20).

In the present paper, we demonstrate that immunization of mice with syngeneic apoptotic cells induced humoral immune responses to oxidation-specific epitopes of OxLDL and apoptotic cells. In addition, we demonstrate that cells undergoing apoptosis contain increased levels of biologically active OxPLs and that these apoptotic cells activate endothelial cells to induce monocyte adhesion, which can be blocked by EO6. These data indicate that cells undergoing apoptosis generate oxidation-specific neoepitopes, including biologically active OxPLs, which in turn serve as dominant autoantigens as well as provide “proinflammatory” signals, mediating autoimmune and inflammatory responses.

Materials and Methods

Mice and Cell/Immunogen Preparation. All experimental protocols were approved by the Animal Subjects Committee at the University of California, San Diego. Thymocytes were isolated from 4–6-wk-old National Institutes of Health (NIH)/Swiss-Webster and C57BL/6 mice (Harlan Sprague Dawley, Inc.). To obtain apoptotic cells, thymocytes were incubated with 1 μM dexamethasone (Sigma-Aldrich) or 50 ng/ml PMA (Sigma-Aldrich) in RPMI 1640/10% FCS for 15 h. Under these conditions, ~80% of thymocytes have undergone apoptosis as judged by annexin V binding and among this population, ~60% of thymocytes express oxidation–specific epitopes on their surface membranes as determined by binding of EO6 or EO14, which are autoantibodies specific for OxLDL and MDA-LDL, respectively (13). The apoptotic thymocytes were harvested and washed twice with PBS before the use. For monocyte adhesion and chemokine assays, we used serum deprivation for 18 h or UV irradiation (20 mJ/cm2) to generate apoptotic thymocytes, which also express OxPLs, as determined by EO6 binding (unpublished data).

Lipid Extraction and Analysis by Mass Spectrometry. Total lipids were extracted from thymocytes by chloroform/methanol as described previously (15, 21). The OxPLs in these lipid extracts were analyzed by liquid chromatography multiple reaction monitoring (LC/MRM) as described previously (21). A detailed description of these procedures can be found in the online supplemental Materials and Methods (available at http://www.jem.org/cgi/content/full/jem.20031763/DC1).

Immunization Protocol. Three groups of 10-wk-old NIH/Swiss-Webster mice were immunized in the presence of adjuvant with the following: (a) PBS, (b) viable thymocytes, or (c) dexamethasone-treated thymocytes. 100 μl of 20 × 106 thymocytes in PBS or PBS alone was mixed with an equal volume of Freund’s adjuvant, and used to immunize each mouse. For primary immunization, immunogens with CFA were injected subcutaneously (LC/MRM) as described previously (21). A detailed description of these procedures can be found in the online supplemental Materials and Methods (available at http://www.jem.org/cgi/content/full/jem.20031763/DC1).

Four groups of 10-wk-old C57BL/6 mice were also immunized in the absence of adjuvant with the following: (a) PBS, (b) viable thymocytes, (c) dexamethasone-treated thymocytes, or (d) necrotic thymocytes. The primary necrotic thymocytes were generated by three cycles of freezing and thawing of initially viable cells. After the final thaw, the entire cell mixture was sus-
were incubated with 1 and then washed. For immunofluorescence microscopy study, cells were incubated with 15 ml of propidium iodide (PI) for 10 min and immediately analyzed for intracellular content of biologically active OxPLs including POVP, 1-palmitoyl-2-epoxyisoprostane-sn-glycero-3-PC (PEIPC), as well as by antibody EO14, which recognizes MDA-modified epitopes (14). These recent observations suggested that membranes of apoptotic cells contain apoptotic cell–associated molecular patterns (7) that are derived from phospholipid oxidation. To definitively identify PC-containing OxPLs in membranes of cells undergoing apoptosis, we analyzed lipid fractions extracted from normal and apoptotic thymocytes by LC/MRM. Consistent with the immunologic characterization, lipid fractions of dexamethasone or PMA-treated apoptotic thymocytes had a significantly increased content of biologically active OxPLs including POVP.
PEIPC, 1-palmitoyl-2-glutaroyl-sn-glycero-3-PC, and 1-palmitoyl-2-lyso-sn-glycero-3-PC (lyso-PC) compared with lipid fractions from viable thymocytes (Fig. 1). As we have shown recently that EO6 and EO14 recognize surface membrane determinants that newly arise on apoptotic cells (14), the current data indicate that apoptotic cells contain increased levels of these oxidized moieties, which may also have redistributed to facilitate their immune recognition.

Normal Mice Immunized with Syngeneic Apoptotic Thymocytes Develop Autoantibodies against Oxidation-specific Epitopes. To determine whether these stress-induced oxidation-specific epitopes on apoptotic cells could induce specific immune responses, we immunized NIH/Swiss-Webster mice with syngeneic apoptotic thymocytes emulsified in Freund’s adjuvant. After the final boost, plasma was obtained and tested for antibody binding to several models of oxidation-specific epitopes, including copper OxLDL, POVPC-modified LDL (POVPC-LDL), and MDA-modified LDL (MDA-LDL). As seen in Fig. 2, all mice immunized with apoptotic cells developed high IgM and high IgG titers to oxidation-specific epitopes of OxLDL. Moreover, mice immunized with apoptotic cells also developed IgM (Fig. 2 A) and to a lesser extent IgG (Fig. 2 B) titers to PC-KLH, suggesting the induction of anti-PC antibodies, which represent a family of autoantibodies including the natural anti-PC antibody T15/EO6 (18, 25, 26). In contrast, mice immunized only with PBS in adjuvant or with viable thymocytes in adjuvant developed lower or undetectable antibody titers to these antigens, respectively (Fig. 2, A and B).

Using competition immunoassay studies, we demonstrated that the IgM binding of antisera from immunized mice to OxLDL was nearly completely inhibited by both OxLDL and MDA-LDL, but not native LDL, indicating high specificity of the induced antibodies for oxidation-specific epitopes (Fig. 3 A). PC-KLH also very efficiently inhibited the IgM binding of the antisera to OxLDL, whereas KLH alone did not, clearly demonstrating the specificity of some of these antibodies for PC (Fig. 3 A). Therefore, immunization with apoptotic cells induced antibodies that are highly specific for epitopes derived from phospholipid oxidation.

To compare the apparent binding affinities of the IgM and IgG of antisera to OxLDL, we performed competition immunoassays with these antisera using soluble OxLDL as a competitor, and determined the dissociation constants ($K_d$) of antibodies according to the Klotz method (23). The $K_d$ values for IgM and IgG binding were $1.5 \times 10^{-9}$ mol/l and $6.13 \times 10^{-9}$ mol/l, respectively (Fig. 3, B and C).

To further evaluate the immunogenicity of apoptotic cells, we also immunized C57BL/6 mice with syngeneic apoptotic thymocytes in PBS alone (i.e., without adjuvant), as well as with viable thymocytes, or primary necrotic thymocytes generated by repeated freeze-thaw cycles (Necrotic). Sera from NIH/Swiss-Webster and C57BL/6 mice were diluted at 1:400 and 1:250 in PBS with 2% BSA, respectively, and IgM and IgG bindings were measured as described in Materials and Methods. Data are expressed as relative light units (RLU) per 100 ms. Each point is the mean of triplicate determinations.

**Figure 2.** Binding of immune sera to oxidatively modified epitopes. Chemiluminescent immunoassay for the binding of IgM (A) and IgG (B) from pooled immune sera of NIH/Swiss-Webster mice to indicated antigens. NIH/Swiss-Webster mice were immunized in the presence of adjuvant with the following: PBS, viable, normal thymocytes (Viable), and dexamethasone-treated apoptotic thymocytes (Apoptotic). (C) Binding of IgM from pooled immune sera of C57BL/6 mice immunized in the absence of adjuvant with the following: PBS, viable, normal thymocytes (Viable), dexamethasone treated apoptotic thymocytes (Apoptotic), or suspension of primary necrotic thymocytes generated by repeated freeze-thaw cycles (Necrotic). Sera from NIH/Swiss-Webster and C57BL/6 mice were diluted at 1:400 and 1:250 in PBS with 2% BSA, respectively, and IgM and IgG bindings were measured as described in Materials and Methods. Data are expressed as relative light units (RLU) per 100 ms. Each point is the mean of triplicate determinations.
Immune Sera Bind to Apoptotic Thymocytes via Oxidation-specific Epitopes. Next, we characterized the binding and specificity of IgM and IgG from antisera to apoptotic cells. Using deconvolution microscopy, we demonstrated specific binding of the immune sera to apoptotic cells (Fig. 4A). Using immune sera from NIH/Swiss-Webster mice, we noted both IgM and IgG binding to syngeneic apoptotic cells seen as surface staining in a specific discrete pattern (Fig. 4A, green), consistent with membrane blebbing that occurs with apoptosis (Fig. 4A). The condensed nuclei characteristic of apoptotic cells were detected by Hoechst DNA staining (Fig. 4A, blue). Preimmune sera did not stain the apoptotic cells (Fig. 4B), and immune sera did not show reactivity with viable thymocytes (Fig. 4C). Moreover, flow cytometry analyses demonstrated that the binding of these immune sera (IgM) was especially pronounced in apoptotic thymocytes that were highly positive for PI staining, indicating a later stage of apoptosis (Fig. 5A, R3). Because apoptotic cells generate OxPLs in their membranes (Fig. 1), we tested whether the binding of immune sera to apoptotic cells was in part due to binding to oxidation-specific epitopes. We performed competition studies of the IgM binding to apoptotic cells by flow cytometry and determined the intensity of binding of the antisera after preabsorption with indicated antigens. IgM binding of immune sera to the gated cells (Fig. 5A, R3) was strongly decreased by absorption with either OxLDL or MDA-LDL, but not by native LDL, suggesting the importance of these epitopes for the immunoreactivity of apoptotic cells (Fig. 5C). Moreover, preabsorption of immune sera with both OxLDL and MDA-LDL together led to an even stronger and dose-dependent (~70%) inhibition of the binding to apoptotic cells (Fig. 5C). Thus, a large part of the apoptotic cell–reactive IgM antibodies induced by immunization with apoptotic cells bind via oxidation-specific epitopes. In similar studies, IgG binding to apoptotic cells was also decreased by preabsorption of immune sera with either OxLDL or MDA-LDL by 50%, and both OxLDL and MDA-LDL together showed similar inhibition (~60%) as was seen for the IgM binding to apoptotic cells (unpublished data).

Splenocyte Cultures from Immunized Mice Secrete Both Th1 and Th2 Cytokines. Because NIH/Swiss-Webster mice immunized with apoptotic cells in the presence of adjuvant developed high titers of IgG antibodies to oxidation-specific epitopes, we tested whether cellular immune responses were induced in these mice as well. Therefore, we determined the cytokine secretion of splenocyte cultures obtained from all immunized NIH/Swiss-Webster mice.

In the initial studies, splenocytes were cultured in the absence or presence of syngeneic apoptotic or viable thymocytes to assess antigen-specific cytokine secretion. After 72 h of incubation, cytokine levels were measured in culture supernatants. Surprisingly, we found high cytokine se-
creatin in the culture supernatants of immunized mice, but not naïve mice, even in the absence of any added antigenic stimuli. We speculated that splenocytes themselves could undergo apoptosis during the in vitro culture for 72 h, which in turn could provide autoantigenic stimuli. Indeed, splenocytes cultured for 72 h had definite evidence of apoptosis, which was detected by DNA electrophoresis documenting the typical ladder pattern of DNA fragmentation (unpublished data). Indeed, a similar degree of apoptosis was found in the splenocyte cultures obtained from all three experimental groups.

As shown in Fig. 6, splenocyte cultures from mice immunized with apoptotic cells with OxPLs showed only very low levels of cytokines in the absence of any added antigen. In sharp contrast, splenocyte cultures from mice immunized with PBS or viable thymocytes (which also contained adjuvant) showed both Th1 (IFN-\(\gamma\)) and Th2 (IL-5, IL-10, and IL-13) cytokine release in the absence of added antigen. Additional control cultures were stimulated with plate-bound anti-CD3 to assess maximal nonspecific secretion of cytokines, which yielded similar results among splenocyte cultures of all three groups (unpublished data). These findings clearly demonstrate that the resulting cytokine secretion was specific (with the possible exception of IFN-\(\gamma\), which may be related in part to the adjuvant) for the immunization with apoptotic cells, which induced strong cellular immune responses after immunization in the presence of adjuvant.

**Apoptotic Cells with Oxidation-specific Epitopes Induce Monocyte Adhesion to Endothelial Cells.** Monocytes are the primary cells that are localized to chronic inflammatory lesions, including atherosclerosis. Initially, monocytes roll along the vascular endothelium where they become activated by soluble or surface-bound chemokines. The activated monocytes adhere firmly to the endothelium and transmigrate through the endothelial cell monolayer. This occurs by the expression of adhesion molecules on overlying endothelial cells induced by inflammatory stimuli. Ox-LDL and OxPLs, such as POVPC and PEIPC, have been shown to be capable of activating endothelial cells, leading to monocyte-endothelial cell interaction (21, 27, 28).

To determine whether OxPLs that are present in the context of apoptotic cells (14) may also exert such proinflammatory properties, we tested the ability of the apoptotic cells to activate PAECs, as assessed by the ability of PAECs to bind monocytes. Preincubation of PAECs with apoptotic thymocytes led to the subsequent enhanced adhesion of THP-1 cells, a human monocyte-like cell line frequently used in these studies (24, 29), to the confluent layer of PAECs (Fig. 7 A). In contrast, preincubation of viable thymocytes did not lead to activation of the PAECs (unpublished data). Furthermore, the monocyte adhesion induced by apoptotic thymocytes was almost abolished by the inclusion of EO6 during the preincubation, which
binds specifically to PC-containing OxPLs; however, note that EO6 does not bind to lyso-PC (20). EO6 did not inhibit LPS-induced monocyte adhesion, implying that OxPLs that were bound by EO6, such as POVPC or PEIPC, were responsible for this effect. We also used HCAECs in a similar study. As shown in Fig. 7 B, we observed a significant ability of apoptotic cells to activate HCAECs to bind THP-1 monocytes, and again viable thymocytes did not.

To directly address the mechanism by which apoptotic cells induce monocyte–endothelial cell interaction, we tested the expression of adhesion molecules on HCAECs using flow cytometry. We did not observe enhanced expression of VCAM-1 and CS-1 fibronectin on the stimulated endothelial cells (10). However, we did observe HCAECs to secrete a significantly higher level of IL-8 when they were incubated with apoptotic cells compared with that in the absence of stimulant or in the presence of normal, viable cells (Fig. 8). Recently, IL-8 has been reported to mediate firm monocyte adhesion to endothelial cells even under flow conditions (30, 31). Furthermore, the ability of PC-containing OxPLs to induce expression of IL-8 in endothelial cells has been documented (21).

Discussion

Because the oxidative modification of LDL renders it immunogenic and induces robust autoantibody responses to oxidized lipids and/or lipid-protein adducts (11), and because apoptotic cells display such oxidized moieties on their plasma membranes (14), we investigated whether oxidation-specific epitopes of apoptotic cells could also give rise to similar autoimmune responses. Cell membranes contain phospholipids with polyunsaturated fatty acids that would be susceptible to oxidation when cells undergo apoptosis. Indeed, a substantial body of evidence suggests that cells undergoing apoptosis are under increased oxidative stress, and that reactive oxygen species and/or OxPLs mediate an apoptotic program and recognition of dying cells by phagocytes (32–34).

In the present paper, we demonstrate directly that apoptotic cells have an increased content of biologically active OxPLs and that apoptotic cells bearing such oxidatively modified moieties are immunogenic. Indeed, immunization of mice with syngeneic apoptotic cells induced high titters of autoantibodies to various oxidation-specific epitopes of OxLDL, whereas immunization with neither viable, normal cells nor primary necrotic cells did (Fig. 2). Thus, the process of apoptosis is requisite for the generation of immunostimulatory, oxidation-specific neoepitopes that induce such autoimmune responses. Reciprocally, we also demonstrate that immune sera bind to the cells at later stages of apoptosis, in large part through the recognition of oxidation-specific epitopes, implying that these epitopes can also serve as antigenic determinants for the recognition of apoptotic cells by autoantibodies. Although it has been demonstrated previously that syngeneic apoptotic cells can be immunogenic under appropriate conditions, our data demonstrate that, remarkably, up to 70% of the IgM from
such murine sera (and ~60% of IgG) are directed at oxidation-specific epitopes of apoptotic cells. These data demonstrate that such oxidation-specific epitopes are immunodominant neoepitopes, just as they are when LDL undergoes oxidative modification.

Apoptosis is a physiological form of cell death responsible for removal of unwanted cells in diverse physiological and pathological processes (7). Normally, apoptotic cells are cleared swiftly by phagocytes without inciting inflammatory or immune responses. However, under certain conditions, the pathological accumulation of increased numbers of apoptotic cells may incite inflammatory and immune responses (6, 7, 35). Indeed, an increased rate of formation, and/or a defective clearance of apoptotic cells has been postulated as a potential mechanism that may be responsible for autoimmune responses (7, 36). The systemic exposure of apoptotic cells in mice by immunization with syngeneic apoptotic cells, but not viable cells, has been shown to initiate autoantibodies to apoptotic cells or autoantigens, such as ssDNA and cardiolipin (4, 5). Moreover, studies have demonstrated that humans and mice with C1q deficiency, or mice lacking a potential mechanism that may be responsible for autoimmunity (6, 7, 35). Indeed, an increased rate of formation, and/or a defective clearance of apoptotic cells has been postulated as a potential mechanism that may be responsible for autoimmune responses (7, 36). The systemic exposure of apoptotic cells in mice by immunization with syngeneic apoptotic cells, but not viable cells, has been shown to initiate autoantibodies to apoptotic cells or autoantigens, such as ssDNA and cardiolipin (4, 5). Moreover, studies have demonstrated that humans and mice with C1q deficiency, or mice lacking a potential mechanism that may be responsible for autoimmunity (6, 7, 35).

Therefore, we speculate that in conditions that lead to defective clearance of apoptotic cells and advancement to later stages of apoptosis, novel antigenic determinants are generated, which in turn stimulate such autoimmune responses. Consistent with this, autoantibodies found in patients with SLE and aPL syndrome have been shown to recognize apoptotic cells as well as OxLDL (1–3, 41). In collaboration with others, we demonstrated enhanced titers of autoantibodies to oxidation-specific epitopes of OxLDL in patients with SLE (42), even in young children who have no evidence for clinical cardiovascular disease (43). However, the precise nature of the antigenic determinants of apoptotic cells that lead to such autoimmunity has not yet been clearly elucidated. Several studies have suggested that the autoantigens inducing autoimmune response during apoptosis might be structurally modified proteins, generated as a result of proteolytic cleavage and/or phosphorylation (36). Furthermore, earlier studies in our laboratory have shown that many so-called aPLs are in fact directed against OxPL and/or oxidized lipid-protein adducts, but not against non-OxPLs (44–46). These observations support our demonstration that oxidative modification serves to generate antigenic determinants. Another group also has shown that monoclonal antcardiolipin antibodies established from the (NZWxBXSB)F1 mouse model of aPL syndrome cross-reacted with OxLDL (47), and we too have made similar observations (unpublished data). Thus, our present studies provide direct evidence that as a consequence of apoptosis, oxidative modification of lipid components generates neo-self-oxidation–specific epitopes, which in turn lead to an autoimmune response and serve as antigenic determinants targeted by the autoantibodies.

As noted in the Introduction, EO6 is a classic natural antibody specific to PC, and we have demonstrated previously that they bind specifically to apoptotic cells, but not to viable cells (14, 18). In mice, such natural anti-PC antibodies provide a first line of innate-like immune defense against a variety of microbial infections, such as S. pneumoniae (19), and mice deficient in T15 antibodies are uniquely susceptible to S. pneumoniae infection (48). Similar antibodies have been demonstrated in the plasma of healthy humans as well (49, 50). Because these germline encoded antibodies are naturally selected and are present within the first week of life even in mice grown in germ-free conditions (51), we have postulated that they are positively selected by PC exposed by oxidized phosphatidylcholine present on apoptotic cells and/or oxidized lipoproteins (18). In the present paper, we directly demonstrate that apoptotic cells can stimulate such anti-PC antibodies (Figs. 2 and 3). Therefore, we propose that oxidative modification of phosphatidylcholine in the membranes of cells undergoing apoptosis reveals PC as a natural antigenic determinant, which in turn stimulates B cell immune responses to PC. Furthermore, these data suggest that apoptotic cells bearing PC might play a role in the ontogeny of anti-PC antibodies.

The functional capacity of the immune system represents a sophisticated balance between the defense from invading infectious pathogens, and the avoidance of autoreactivity that can damage the host. Certainly, some B-1 cell–derived IgM antibodies may exert adverse effects (52). However, it is now also generally accepted that certain aspects of autoimmunity may occur in, and perhaps even contribute to, health (53). Several studies suggest that the recognition of IgM anti–PC antibodies to apoptotic cells is important in clearance of apoptotic cells as well as immune modulation. One consequence that occurs as a result of induction of apoptosis is the generation of enhanced oxidative mechanisms, resulting in enhanced content of oxidized phosphatidylserine (34). These likely form the important recognition signals for binding to the phosphatidylserine receptor on macrophages (34). We now add the generation of oxidized phosphatidylcholines in apoptotic cells as well. The presence of such PC-containing OxPLs are known ligands mediating CD36 binding (16, 17) and explains why antibody EO6 inhibits binding and uptake of apoptotic cells by macrophages (14).

In addition, our studies demonstrated the enhanced generation of lyso-PC (Fig. 1). The latter may occur as oxidized phosphatidylcholines contain oxidized sn-2 fatty acids that become preferred substrates for several PLA$_2$-type enzymes, which lead to lyso-PC generation. A recent paper by Kim et al. has shown that human natural anti–PC IgM antibodies from sera of normal individuals bind to late apoptotic cells through recognition of lyso-PC. Subsequently, their binding recruited C1q on the cell surface leading to C3 activation (54), which in turn would enhance clearance of opsonized apoptotic cells (55). Moreover, because macrophages or dendritic cells phagocytosing apoptotic cells op-
sonized with C3 produce immunosuppressive cytokines, complement activation mediated by these IgM antibodies may play a crucial role in modulating the immune response (56, 57). In our paper, we directly demonstrate another potential protective capacity of such natural IgM anti-PC antibodies, such as EO6, which have the capacity to block proinflammatory properties (Fig. 7) and, thus, may represent another conserved arc of innate immunity that is recruited to dampen inflammatory responses. In a similar manner, EO6 binds to apoptotic cell blebs and abrogates some of their proinflammatory properties (58). Therefore, the possible scenario would be that these anti-PC antibodies stimulated by PC exposed in the context of apoptotic cells may confer protection to the host from more devastating inflammatory and autoimmune responses by promoting the clearance of dying cells and also by modulating these responses.

Alternately, it should be pointed out that autoimmune responses to PC and other oxidation-specific epitopes could bring harmful outcomes, in particular in the presence of other proinflammatory stimuli. As shown in Fig. 2 B, immunization of apoptotic cells in the presence of Freund’s adjuvant, a potent proinflammatory stimulant, induced high titers of IgG to oxidation-specific epitopes, but such IgG immune responses were not observed in the absence of the adjuvant. Were such IgG immune responses to occur in vivo as a consequence of inflammatory stimuli, this could lead to the generation of IgG responses, engagement of IgG immune complexes to macrophage Fcγ receptors, and the production of inflammatory cytokines. This phenomenon may play a role in the pathogenesis of autoimmune diseases when the defective clearance, and hence the pathological accumulation of apoptotic cells, occur coupled with various proinflammatory stimuli, such as infectious agents or toxic oxidized lipids (i.e., as found in the atherosclerotic plaque). Indeed, it was recently shown that mice with impaired clearance of apoptotic cells due to Fas ligand deficiency, when crossed into apoE−/− mice, developed accelerated atherosclerosis and attendant autoimmune phenomenon, including enhanced autoantibody titers to epitopes found in OxLDL (59).

Atherosclerosis is now considered a chronic inflammatory disease of the vascular wall (8, 60) and there is a substantial body of evidence that OxPLs in OxLDL induce proinflammatory responses promoting the atherogenic process (10). PC-containing OxPLs activate endothelial cells to recruit monocytes into the vascular wall and stimulate them by regulating inflammatory gene expression (10, 61) OxLDL with such OxPLs accumulate in atherosclerotic lesions (13, 27). Furthermore, there is also a marked accumulation of apoptotic cells in atherosclerotic lesions (62). In the present paper, we demonstrate directly that the ability of apoptotic cells to activate endothelial cells to tightly bind monocytes is completely abrogated by EO6. This implies that the enhanced content of biologically active oxidized phosphatidylcholines in apoptotic cells activates endothelial cells to induce adhesion and recruitment of monocytes. Adhesion of monocytes to endothelial cells is a crucial, rate-limiting step in the pathogenesis of atherosclerosis in particular, and an initial process for the recruitment of macrophages into damaged tissues and inflammatory lesions in general. In this paper, we demonstrated further that such monocyte–endothelial interaction can be mediated in part through IL-8. IL-8 is a CXC chemokine produced by endothelial cells that has recently been reported to mediate firm adhesion of monocytes to endothelial cells, even under flow conditions (30, 63). Oxidized PAPC (1-palmitoyl-2-arachidonoyl-sn-glycerol-3-phosphorylcholine), and specifically isomers of PEIPC as well as POVPC (products readily bound by EO6), are known to be present in the atherosclerotic lesion and can induce such IL-8 expression (21). In support of an important role of IL-8 is the observation that deletion of the IL-8 receptor in LDLR−/− mice markedly decreases atherosclerosis (64).

Consistent with our finding, recent studies have demonstrated that apoptotic cells have the ability to provoke an inflammatory response by unidentified proinflammatory mediators (65, 66), suggesting that under certain conditions, apoptotic cells themselves may provide endogenous “danger signals” triggering an inflammatory response. Lauber et al. have shown recently that apoptotic cells release lyso-PC to serve as a chemotactic signal that induces attraction of monocytes to apoptotic cells, presumably to ensure their efficient removal (67). Lyso-PC released from OxLDL has been demonstrated already to be a potent chemotactic factor for monocytes and T cells (68, 69). Because EO6 does not bind to lyso-PC (20), yet abrogated the ability of apoptotic cells to induce firm monocyte adhesion to endothelial cells (Fig. 7), our findings suggest that oxidized phosphatidylcholines present on apoptotic cells are also prominent mediators that lead to recruitment of inflammatory cells. Thus, they too serve as endogenous inflammatory danger signals, ensuring an ensuing host response that could play an important role in limiting the danger posed by the accumulated dying cells.

It is tempting to postulate that oxidative modification of lipids in cells that occur during apoptosis is a novel mechanism by which apoptotic cells could promote autoimmune and proinflammatory responses. Alternately, such responses may be protective by enhancing the clearance of apoptotic cells and/or repression of such proinflammatory response. However, they could also participate in the pathology of a variety of diseases in which the abnormal accumulation of such cells occurs, for instance, in the atherosclerotic lesion (62) and other chronic inflammatory diseases, including autoimmune disorders such as SLE (7).

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mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. J. Biol. Chem. 272:13597–13607.


Lipid Extraction and Analysis by Mass Spectrometry. Total lipids were extracted from thymocytes by chloroform/methanol as described previously (1). In brief, viable or apoptotic cell pellets (~10⁶) were treated with 1 ml of methanol containing 0.3 mM BHT (Sigma-Aldrich) and 3 mM PMSF (Sigma-Aldrich), and 2 ml of methanol/4 ml of chloroform were added. After vortexing, the mixtures were kept on ice for 1 h, 6 ml of distilled H₂O was added, and the mixtures were centrifuged at 1,800 g for 10 min. The chloroform phase was collected and dried under nitro, and DMPC internal standard was added to the samples. For each sample, three separate extractions were performed. Data were pooled from two experiments. To test recoveries for this extraction method, known quantities of the individual lipids to be analyzed were separately extracted and 80–90% recovery was confirmed.

The levels of oxidized phospholipids were analyzed by liquid chromatography multiple reaction monitoring (LC/MRM) as described previously (2). In brief, dried lipid extracts containing internal standard were redissolved in 200 μl MeOH/H₂O (98:2) and loaded onto a PrepSep-C18 extraction column previously activated with 3 ml MeOH/H₂O (98:2). The columns were eluted with 2 ml of MeOH/H₂O (98:2) and 10 ml of MeOH. The pooled eluate was analyzed by quantitative LC/MRM analysis. For this analysis, samples were injected onto a C8 column (betasil 250 × 2 mm, 5 μm; Keystone Scientific) equilibrated in 80% MeOH containing 1 mM ammonium acetate. The column was eluted at 0.4 ml/min with a linear gradient to 100% MeOH containing 1 mM ammonium acetate over 45 min. The column effluent was split, and ~20% of the sample was directed to the Ionspray mass spectrometer source and the rest was collected for additional analyses including inorganic phosphate measurement. Argon containing 10% nitrogen was used as collision gas (instrumental CGT setting of 110) with an orifice voltage of 100. The ion currents for the transition of each preselected parent ion (m/z 782 [PAPC], 496.2 [lysoPC], 594.3 [POVPC], 610.3 [PGPC], 678.5 [DMPC], and 828.5 [PEIPC]) to the corresponding most abundant MS/MS fragment ion (in all cases this was the ion for phosphocholine ([OP(OH)₂OCH₂CH₂N⁺{CH₃}]⁺ at m/z 184.1) were recorded. Retention times for each phospholipid were determined using synthetic phospholipids (PAPC, lysoPC, POVPC, PGPC, and DMPC) or bioactive oxidized phospholipids isolated from Ox-PAPC (PEIPC). Instrument-supplied software was used for the extraction of MRM traces from each dataset. The amount of each phospholipid was calculated from the peak areas using calibration curves constructed with a fixed amount of DMPC and varying amounts of the phospholipids.

Chemiluminescent Immunoassay. Copper oxidized LDL (OxLDL), MDA-LDL, and POVPC-LDL were prepared as described previously (1). Phosphorylcholine-conjugated keyhole limpet hemocyanine (PC-KLH) and KLH were obtained from Biosearch Technologies Inc. In this assay, 5 μg/ml of antigen in dilution buffer (50 mmol/L PBS, pH 7.5, containing 0.27 mmol/L EDTA, 0.02% NaN₃) was added to wells of a 96-well white, round-bottomed MicroFlur microtitration plate (Dynex Technologies) and incubated overnight at 4°C. Plates were washed three times with washing buffer (PBS containing 0.27 mmol/L EDTA, 0.02% NaN₃) using an automated plate washer. Sera from NIH/Swiss-Webster or C57BL/6 mice were diluted at 1:400 and 1:250, respectively, in dilution buffer containing 2% BSA and incubated in each well for 1 h at room temperature. After three washes, bound antibodies were detected by 1 h incubation of 50 μl/well of an alkaline phosphatase-labeled goat anti–mouse IgM or IgG (μ-chain or γ-chain specific, respectively; Sigma–Aldrich) diluted in 2% BSA/TBS according to the supplier’s specification. After three washes, 25 μl of a 50% solution of Lumi-Phos 530 (Luminogen Inc.) were incubated with each well for 1–2 h at room temperature in the dark. Luminescence was determined using a Dynatech luminometer (Dynex Technologies). Antibody binding was measured as relative light units (RLUs) measured over 100 ms. All measurements of antibody binding to each antigen were done in a single assay, and each plasma sample was assayed in triplicate. The intra-assay coefficient of variation for these assays was 6–10%.

References