Fat(al) attraction: oxidized lipids act as "eat-me" signals

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Phagocytosis of apoptotic cell corpses is a conserved and well-regulated process and is required to maintain tissue homeostasis within an organism. Evidence suggests that apoptotic cell engulfment by macrophages is dependent upon the externalization of phosphatidylserine (PS) on the plasma membrane of the dying cell. Furthermore, oxidation of PS and other phospholipids may serve to facilitate cell corpse removal. However, our understanding of how these various lipid "eat-me" signals are recognized by macrophages has been limited. Using a combination of cellular and animal models, along with an array of biophysical methods, Hazen and his associates (Greenberg *et al.*, *J. Exp. Med.*, 2006, 203, 2613–2625; Li *et al.*, *Biochemistry*, 2007, 46, 5009–5017) have now identified the scavenger receptor CD36 as a putative receptor for oxidized PS on apoptotic cells; moreover, they have deduced the conformation of the oxidized lipid ligand that is recognized by this receptor, thus providing structural insight into how phagocytes recognize senescent or apoptotic cells.
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Monty Python, the famous and irreverent comedy team, released their final movie in the early 1980s, in which the meaning of life is explored through a series of humorous sketches from conception to death. In the current discourse, we will focus our attention on the meaning of death, and the process of programmed cell clearance, a term that is used to describe the mechanisms and consequences of phagocytic clearance of dying cells (Fadeel, 2003; Reddien and Horvitz, 2004). Apoptosis is an essential and physiological process that is required for the sculpting of organs during embryogenesis and the maintenance of tissue homeostasis in the adult organism. Apoptosis is followed under normal conditions by the swift disposal of cell corpses by neighboring cells or professional phagocytes (macrophages). Importantly, studies in recent years have indicated that phagocytic clearance of cells dying by apoptosis is much more than mere "waste disposal." Instead, the engulfment of dying cells by phagocytes may be considered to define the "meaning" of cell death (Savill and Fadok, 2000). Indeed, macrophage engulfment of apoptotic cells has been shown to modulate inflammatory responses, and dendritic cell uptake of cell corpses may play a role in the induction of immune tolerance. Moreover, when certain engulfment genes are deleted in mice, these animals develop profound autoimmune disease (Botto *et al.*, 1998; Hanayama *et al.*, 2004), indicating that faithful execution of apoptosis without subsequent clearance of apoptotic cells may have deleterious consequences.

During programmed cell clearance, dying cells expose specific recognition or "eat-me" signals on the cell surface, and these are in turn recognized by receptors on the engulfing cell. However, the main problem or paradox in this field of research is that there appears to be too

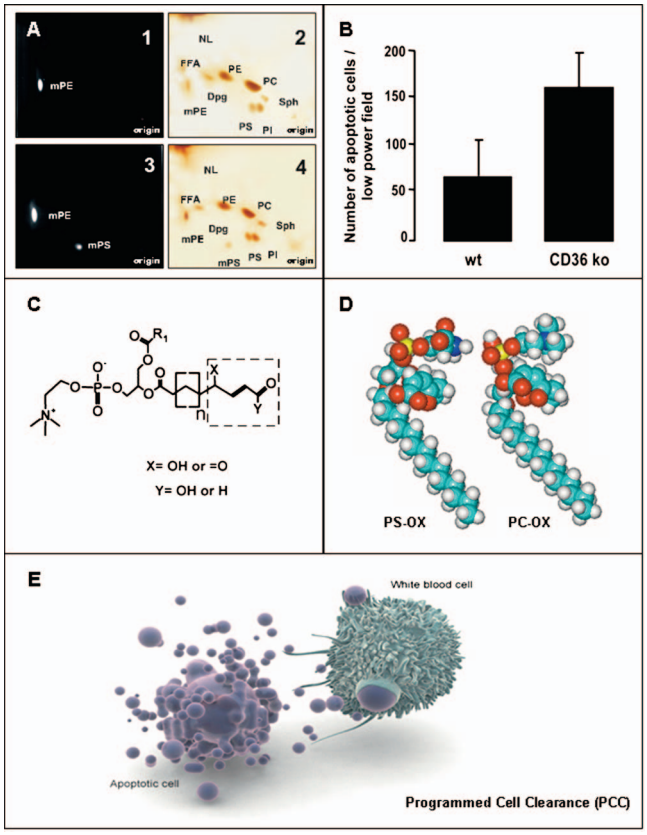

Figure 1. Programmed cell clearance: the importance of phospholipid oxidation and externalization. (A) Apoptotic Jurkat T cells display selective cell surface externalization of phosphatidylserine (PS). Two-dimensional high performance thin-layer chromatography of total lipid extracts from control (A1 and A2) and apoptotic Jurkat cells (A3 and A4). Chromatograms were visualized with a UV light source after treatment with fluorescamine, a cell-impermeable fluorescent reagent capable of reacting with primary amines (A1 and A3), or with a VIS light source after exposure to iodine vapors (A2 and A4). (mPE, fluorescamine-modified phosphatidylethanolamine; mPS, fluorescamine-modified PS). Figure reprinted from Kagan *et al.* (2002), with permission from The American Association of Immunologists. (B) Increased apoptotic cell accumulation in CD36 knockout (ko) mice compared to wild type mice (wt). Punch wounds were excised from the skin of wt and ko mice on day 8 after wound, and unengulfed apoptotic cell numbers were quantified using fluorescence microscopy. Figure reprinted from Greenberg *et al.* (2006), with permission from The Rockefeller University Press. (C) Structure of oxidized phoshatidylcholine (PC-OX). The length of the oxidized truncated *sn*-2 acyl chain varies depending on the parent (unoxidized) fatty acid precursor (n=2, 3, and 7 for docosahexanoic, arachidonic acid, and linoleic acid, respectively). The structural motif within the dashed box has been shown to confer high-affinity recognition through the macrophage scavenger receptor, CD36. Figure reprinted from Li *et al.* (2007), with permission from American Chemical Society Journals. (D) Molecular modeling of PC-OX and PS-OX. The conformation of PC-OX was established by *ab initio* energy minimization iteration using constraints derived from NOE of ^{1}H-NMR spectra published by Li *et al.* (2007). These constraints were removed in calculating the conformation of PS-OX. As can be seen from the figure, the two molecules are superimposable, with the conspicuous exception that the distance between the carboxyl carbon and the nitrogen atom of the phospholipid increases because of repulsion between the two charged carboxyl groups of PS-OX (gray=hydrogen; red=oxygen; cyan=carbon; blue=nitrogen; yellow=phosphorus). (E) Programmed cell clearance: a genetically programmed and evolutionarily conserved event underlying the rapid disposal of apoptotic cell corpses (Fadeel, 2003). Image reproduced courtesy of the U.S. National Library of Medicine.

many macrophage receptors, and too few proven ligands for these receptors; indeed, the only well-known and universal "eat-me" signal is phosphatidylserine (PS), an anionic phospholipid that is normally sequestered in the inner leaflet of the plasma membrane, but is flipped to the outer surface of the cell upon induction of apoptosis (Fadok *et al.*, 1992; Martin *et al.*, 1995) [Fig. 1(A)]. Macrophages and other phagocytes, on the other hand, are known to express a wide array of receptors for apoptotic cells, including several scavenger receptors, such as the class A scavenger receptor, CD36, macrosialin/CD68, and lectin-like oxidized low-density lipoprotein receptor-1, as well as the integrin receptors, $\alpha_V\beta_3$ and $\alpha_V\beta_5$, the endotoxin receptor, CD14, and the protein referred to as the PS receptor. In addition, there are numerous serum proteins that function as bridging molecules to facilitate the recognition and engulfment of apoptotic cell corpses through the binding both to the apoptotic prey and to the neighboring phagocyte. Some of these bridging proteins, including milk fat globule-epidermal growth factor-8 (MFG-E8) have been shown to interact with PS, and also with specific macrophage receptors (Hanayama *et al.*, 2002); however, it remains to be determined how such a wide

range of receptors and bridging proteins are utilized during cell clearance, and how specificity is conferred to the uptake process, when essentially only one ligand is known. One of the potential answers is that the "PS signal" for engulfment on apoptotic cells may in actual fact be comprised of several subcategories of signals that interact differently with different bridging molecules and macrophage receptors.

Recent studies have shown that PS is oxidized during apoptosis, and oxidized PS (PS-OX) was found to act as a recognition signal for macrophages (Kagan *et al.*, 2002). A related study showed that the exposition of oxidized phosphatidylcholine (PC-OX) may also serve as a recognition signal on "late" apoptotic cells, i.e., apoptotic cells that have started to loose their plasma membrane integrity (Chang *et al.*, 2002). Hazen and associates have gone one step further by identifying one specific class of PS oxidation products (and to a lesser extent, PC oxidation products) as a specific signal for the scavenger receptor, CD36 (Greenberg *et al.*, 2006). These studies are important not only because they are among the first to demonstrate a direct role for CD36 in apoptotic cell clearance *in vivo* (unengulfed cell corpses were found to accumulate in CD36 knockout mice relative to CD36 wild type mice) [Fig. 1(B)], but also because they suggest a solution to the conundrum referred to in the previous paragraph. Hence, different macrophage receptors may have very specific preferences for certain species of lipid oxidation products generated during apoptosis. In other words, these recent findings may help us to understand the apparent redundancy of engulfment receptors on macrophages and other phagocytes.

However, the structure of the oxidized lipid ligand, and its specific mode of interaction with the corresponding scavenger receptor, remained to be determined. Indeed, it was particularly puzzling how a specific low-abundance molecular species of oxidized phosholipid can be recognized within a "sea" of fluid phase phospholipid in the plasma membrane. In a subsequent study, using solution nuclear Overhauser enhancement (NOE) of ^{1}H-NMR (nuclear magnetic resonance) spectroscopy data and associated modeling, Hazen and associates proceeded to determine the conformation of PC-OX within model bilayers (Li *et al.*, 2007). The membrane architecture posited in the classic "fluid mosaic" model (Singer and Nicolson, 1972) explains how individual phospholipids and proteins can diffuse throughout the two-dimensional surface of the membrane; however, as stated by Hazen and associates, this model does not readily explain how a macrophage receptor such as CD36 can identify apoptotic cell corpses via the presence of specific oxidized phospholipid species interspersed within the membrane bilayer. Previous studies suggested that oxidation products of PC possessing *sn*-2-esterified γ-hydroxy(or oxo)-α,β-unsaturated carbonyl-containing fatty acids have specific CD36-binding activity (Podrez *et al.*, 2002). Molecular modeling performed by Li *et al.* (2007) has now revealed an unusual conformation of PC-OX whereby the distal end of the oxidized *sn*-2 acyl chain protrudes into the aqueous phase, rather than pointing inward [Fig. 1(C)]. Interrogation of the NMR data thus shows that the terminal end of the *sn*-2 acyl chain of PC-OX and the polar headgroup are in close spatial proximity within membrane bilayers, supporting a model in which the oxidized *sn*-2 chain of PC-OX extends into the aqueous interface, as opposed to penetrating deep into the hydrophobic core. As Hazen and his colleagues have pointed out, the results also demonstrated a PC-OX conformation that possesses a distance of <5 Å between the proximal portions of both *sn*-1 and *sn*-2 acyl chains, but not between the mid and distal *sn*-1 chain and the mid or distal *sn*-2 chain (Li *et al.*, 2007). In other words, the proximal portion of the *sn*-2 acyl chain in PC-OX is markedly bent with the distal end protruding completely into the aqueous phase. These results thus suggest how oxidation of PC may produce a surface-accessible "eat-me" signal to facilitate macrophage recognition through CD36. Moreover, in analogy with these findings, one may also postulate that oxidation of PS will result in the exposition of a recognition motif for CD36. Indeed, our *ab initio* calculations suggest that both PS and PC may adopt a similar, albeit not identical molecular conformation upon oxidation, suggesting that both PC-OX and PS-OX could perhaps serve as recognition signals for CD36 [Fig. 1(D)]. Together, these observations provide new structural insight into the potential conformational alterations that occur during membrane phosholipid oxidation associated with apoptosis in general, and more specifically, for CD36-oxidized lipid ligand interactions during programmed cell clearance. Hence, although lipid peroxidation is often conceived of as an unavoidable and nonspecific process in aerobic respiring organisms, the oxidation of PC and/or PS that occurs during apoptosis and the reorientation of certain *sn*-2 fatty acids in the plasma membrane that follows from this oxidative lipid modification may ultimately generate a specific recognition signal for macrophages. In this context, it should be noted that nonenzymatic lipid peroxidation is an inherently random process yielding different phospholipid hydroperoxides as the primary products along with numerous secondary degradation products (Bayir *et al.*, 2007). Enzymatically catalyzed peroxidation, on the other hand, is phospholipid specific and produces a limited number of oxidation products. Elucidating the process—enzymatic or nonenzymatic—leading to accumulation of oxidized phospholipids with an *sn*-2 acyl chain that incorporates a terminal γ-hydroxy(or oxo)-α,β-unsaturated carbonyl that serves as a high-affinity ligand for CD36 (Podrez *et al.*, 2002; Greenberg *et al.*, 2006) thus remains an interesting and important challenge.

Phospholipid oxidation products occur not only on the surface of senescent or apoptotic cells, but also accumulate in atherosclerotic lesions, and circulate in oxidized low-density lipoproteins. Therefore, as pointed out by Li *et al.*

(2007), the structural delineation of the oxidized lipid ligand for CD36 also provides a platform for the rational development of potential strategies for blocking macrophage recognition of oxidized lipoproteins and foam cell production, a crucial event in the formation of atherosclerotic lesions. However, therapeutic targeting of CD36-dependent recognition of oxidized lipoproteins needs to be considered with caution, since the inadvertent prevention of the normal process of apoptotic cell clearance could potentially trigger an autoimmune response. Furthermore, using liquid chromatography-electrospray ionization tandem mass spectrometry, Hazen and his associates have recently extended their observations to demonstrate that PC-OX accumulates in the plasma of hyperlipidemic mice at concentrations up to 40-fold higher than those found in normolipidemic mice, and that structurally defined oxidized lipid species are also found in substantial amounts in the plasma of humans with low levels of high-density lipoprotein (Podrez *et al.*, 2007). These investigators also showed that various specific forms of PC-OX (but not structural analogs lacking CD36-binding activity) bound to human and mouse platelets, and that this binding resulted in platelet activation, thereby promoting a pro-thrombotic phenotype. In sum, these studies suggest that physiologically relevant PC oxidation products are involved in CD36-dependent signaling pathways, and are likely to play an important role in dyslipidemic conditions associated with oxidative stress, such as atherosclerosis and the metabolic syndrome.

CONCLUSIONS AND FUTURE PERSPECTIVES

Programmed cell clearance is a fundamental physiological process, and recent *in vitro* and *in vivo* studies have begun to shed some light on the underlying molecular signaling events. The recent demonstration (Kagan *et al.*, 2002; Chang *et al.*, 2002) that oxidized lipids may act as "eat-me" signals for macrophages, and the novel finding that the scavenger receptor, CD36, is a specific receptor for such oxidized lipid ligands on apoptotic cell corpses (Greenberg *et al.*, 2006) suggest that lipid-dependent signaling plays an important role in tissue homeostasis. Moreover, the recent elucidation of the conformation of the conserved oxidized lipid signal within the membrane bilayer (Li *et al.*, 2007) has provided insight into the structural determinants of the lipid-scavenger receptor interactions that occur during programmed cell clearance. Indeed, these studies have shown that a biological problem (programmed cell clearance) can be dissected using numerous methodologies, ranging from cell biological and animal studies to biophysical and modeling techniques [Fig. 1(E)]. Moreover, these lipid-receptor interactions may be important not only for professional macrophages, but also for other specialized phagocytes, including retinal pigment epithelial cells. These cells ingest more material over a lifetime than any other cell type in the body (reviewed in Erwig and Henson, 2007), and recent studies have shown that light-induced oxidation of photoreceptor outer segment phospholipids generates ligands for CD36-mediated phagocytosis by retinal pigment epithelium (Sun *et al.*, 2006).

Notwithstanding these recent advances, there are several outstanding questions. For instance, it remains to be determined how much of the lipid oxidation product (PC/PS-OX) is formed under physiologically relevant conditions, where recognition of dying cells takes place. Indeed, does lipid oxidation play a more prominent role in apoptotic cell clearance at sites where lipid peroxidation is enhanced, such as during inflammation? Which other classes of lipid oxidation species and/or recognition motifs are involved in the clearance of cell corpses, and to which specific macrophage receptors do these different lipid ligands bind? Are other accessory factors (such as the PS-binding protein, annexin I) (Arur *et al.*, 2003) involved in organizing the oxidized lipids into domains in the plasma membrane of apoptotic cells? Furthermore, previous studies have shown that the so-called bridging molecule, MFG-E8 binds preferentially to PS-OX, and with much lower affinity to nonoxidized PS (Borisenko *et al.*, 2004), but what is the role of the other bridging molecules, and to what specific apoptotic cell ligands do they bind? (in fact, one may speculate that scavenger receptors are the only receptors that are able to recognize different classes of oxidized lipids, whereas all other engulfment receptors recognize PS indirectly, via bridging molecules).

One question that is of particular interest is whether lipid peroxidation plays a conserved role in programmed cell clearance in other species, such as the worm or the fruit fly. Previous studies in *Drosophila melanogaster* have shown that Croquemort, a CD36 homolog, is expressed on macrophages, and is essential for efficient phagocytosis of apoptotic corpses in the *Drosophila* embryo (Franc *et al.*, 1999). Of course, this begs the question whether PS and/or PS-OX act as "eat-me" signals in the fruit fly. Similarly, a scavenger receptor-like molecule, CED-1, has been implicated in programmed cell clearance in the nematode, *Caenorhabditis elegans* (Zhou *et al.*, 2001), but the ligand for this receptor was not identified. On the other hand, our recent studies have provided first evidence of phospholipid scramblase-dependent PS externalization in the worm (Wang *et al.*, 2007), and it will be of considerable interest to determine whether PS oxidation also occurs during apoptosis in *C. elegans*. We predict that the powerful genetic tools provided by model organisms such as the fruit fly and the worm will allow us to dissect the molecular genetic pathways that control not only phagocyte recognition of lipid signals, but also of the mechanism(s) underlying the lipid peroxidation process during apoptosis.

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