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Review

Programmed cell clearance

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Abstract. Apoptosis, a physiological process of self-annihilation, is essential during development and for the maintenance of tissue homeostasis. Considerable efforts have been made in recent years to elucidate the molecular mechanisms that govern this mode of cellular demise; however, the subsequent recognition and removal of apoptotic corpses by neighboring phagocytes has received less attention. Nevertheless, macrophage engulfment of apoptotic cells is known to be important in the remodeling of tissues, and contributes to the resolution of inflammation through the removal of effete cells prior to the release of noxious cellular constituents. Moreover, apoptotic cells are a potential source of self-antigens, and clearance of cell corpses is thought to preclude the induction of autoimmune responses. The view is thus emerging that tissue homeostasis is dependent not only on the balance between mitosis and apoptosis, but also on the rate of apoptosis versus that of cell clearance. This review aims to discuss the mechanisms and consequences of macrophage recognition and disposal of apoptotic cells, a process which will be referred to as programmed cell clearance.

Keywords. Apoptosis; phagocytosis; macrophage; inflammation; autoimmune disease.

On Your Sleeve

You had on your sleeve
the last yellow butterfly
mistakenly roused
by the sun's mocking power.
When it noticed how vain that rousing had been,
it darted away from your hand
and interwove its flight with the falling leaves.

Harry Martinson

Introduction

Cell death is a normal part of animal development [1, 2]. Glücksmann [3], in his seminal report more than 50 years ago, cites numerous examples of naturally occurring cell death during vertebrate ontogeny, and emphasizes the importance of cell degeneration in the sculpting of tissues and organs. Similarly, Saunders [4], in a landmark description of death in embryonic systems, notes that 'abundant death, often cataclysmic in its onslaught, is a part of early development in many animals'. Physiological cell death is also seen during amphibian metamorphosis and in the deletion of larval components in butterflies and other insects [1]. Kerr and colleagues [5] highlighted the significance of controlled cell deletion in the maintenance of tissue homeostasis in the adult organism, and proposed that apoptosis (a term derived from the Greek for the falling off of petals from flowers, or leaves from trees) plays a 'complementary but opposite role to mitosis in the regulation of animal cell populations'. Numerous subsequent studies have served to delineate the underlying signaling pathways that regulate this mode of cellular demise [6, 7].

Apoptosis is a well-choreographed process. First, the cell undergoes nuclear and cytoplasmic condensation with

pulsation and blebbing of the plasma membrane, which have been likened to 'boiling of the cytoplasm' [8]. The cell then breaks up into membrane-bound fragments termed apoptotic bodies, containing intact organelles and portions of the nucleus. The final and frequently neglected stage of the apoptotic process is the removal of senescent cells by neighboring macrophages [9]. Importantly, this occurs prior to the disintegration of the dying cell and release of noxious intracellular constituents. Apoptosis is thus well suited to a role in tissue homeostasis since it can result in extensive deletion of cells in the absence of tissue disruption. By contrast, in necrosis or accidental cell death, there is irreversible swelling of the cytoplasm and organelles, and rupture of the plasma membrane with ensuing tissue scarring and inflammation. Necrotic cell debris is eventually ingested and degraded by phagocytes [8]. The current review focuses on the mechanisms and consequences of programmed cell clearance, i.e. on the mechanisms that govern the recognition and removal of effete cells, and on the outcome thereof, in health and disease.

The mechanism of cell clearance: receptor-ligand interactions, opsonins, chemotactic factors and more

Recognition signals

Numerous ligands, receptors and serum factors have been implicated in the recognition of apoptotic cells (fig. 1). Early reports suggested the presence of lectin-like molecules on the surface of macrophages that recognize changes in carbohydrates ('eat-me' signals) on apoptotic cells [10, 11]. Consistent with these observations are the more recent studies on asialoglycoprotein receptor-dependent macrophage ingestion of apoptotic cells in the liver [12] and the demonstration that peritoneal macrophages recognize modified sugar chains on the surface of virus-infected cells [13]. The most well-studied surface change during apoptosis is, however, the loss of plasma membrane phospholipid asymmetry and the concomitant externalization of phosphatidylserine (PS) [14–16]. PS exposure is modulated by extracellular calcium [17, 18] and mitochondrial ATP [19, 20], and is crucial for recognition and engulfment of apoptotic cells to occur [21, 22]. However, egress of PS has also been documented in cells undergoing necrosis [23], and transient PS exposure is seen in non-apoptotic cells [24, 25], thus indicating that additional surface alterations are required for the selective engulfment of apoptotic cell corpses. Importantly, the exposition of oxidation-specific epitopes may serve as auxiliary eat-me signals for macrophages [26, 27]. Indeed, recent studies have indicated that the externalization of oxidized PS is a critical event that serves to promote macrophage recognition of apoptotic cells [22, 28]. The spatial reorganization during apoptosis of membrane structures may also serve as an important determinant of programmed cell clearance. Hence, the characteristic membrane protrusions (blebs) on the surface of apoptotic cells have been shown to provide a context for the externalization of PS [29, 30], and recent studies suggest that the colocalization in discrete membrane patches of PS and annexin I, a protein that is recruited from the cytosol of apoptotic cells to the cell surface, is critical for programmed cell clearance [31].

Engulfment receptors

The first phagocytosis receptor to be identified, more than 10 years ago, was the vitronectin receptor, $\alpha_V\beta_3$ [32]. Since that time, numerous other receptors, including $\alpha_V\beta_5$ [33], CD14 [34], the so-called PS receptor (PSR) [35] and the scavenger receptors, SRA [36], CD36 [37], CD68 [38], and LOX-1 [39], have been implicated in the recognition of apoptotic cells. Several of these receptors bind PS on apoptotic cells, either directly or indirectly [9]. By contrast, CD14 was shown to mediate engulfment of apoptotic cells through its interaction with intercellular adhesion molecule-3 (ICAM-3), an eat-me signal on apoptotic cells that is distinct from PS [40]. Recent data also suggest that repulsive signals transmitted through cell surface molecules such as CD47 [41] or platelet endothelial cell adhesion molecule-1 (PECAM-1, also known as CD31) [42] may prevent phagocytosis of viable, non-apoptotic cells. The reason for the vast array of macrophage phagocytosis receptors is unclear, although cell- and tissue type-specific differences in receptor usage [43, 44] may provide a partial explanation. The trigger to cell death may also determine the efficiency with which cells doomed to die are ingested [45], suggesting that receptor usage may vary depending on the nature of the apoptotic 'meal'. It is also possible that the engulfment process requires the serial engagement of distinct receptors, some of which are involved in the initial tethering of apoptotic cells and others in the cytoskeletal rearrangement that is required for ingestion of cells [46]. Further support for the receptor cooperativity model was provided by Sambrano and colleagues [47], who reported that disruption of phospholipid asymmetry is sufficient for tethering of erythrocytes to macrophages, while additional oxidative changes are required for engulfment to occur. Moreover, the recent observation that apoptotic cells display both oxidized and non-oxidized PS on their surface [48] suggests that the serial or concomitant engagement of distinct, PS-binding receptors may be required for programmed cell clearance. Scavenger receptors, such as CD36 and CD68, are likely candidates for sensors of oxidized PS and other modified lipid moieties on the surface of dying cells. In keeping with the involvement of these receptors in the uptake of apoptotic cells in mammals [49], the scavenger receptor homologs,

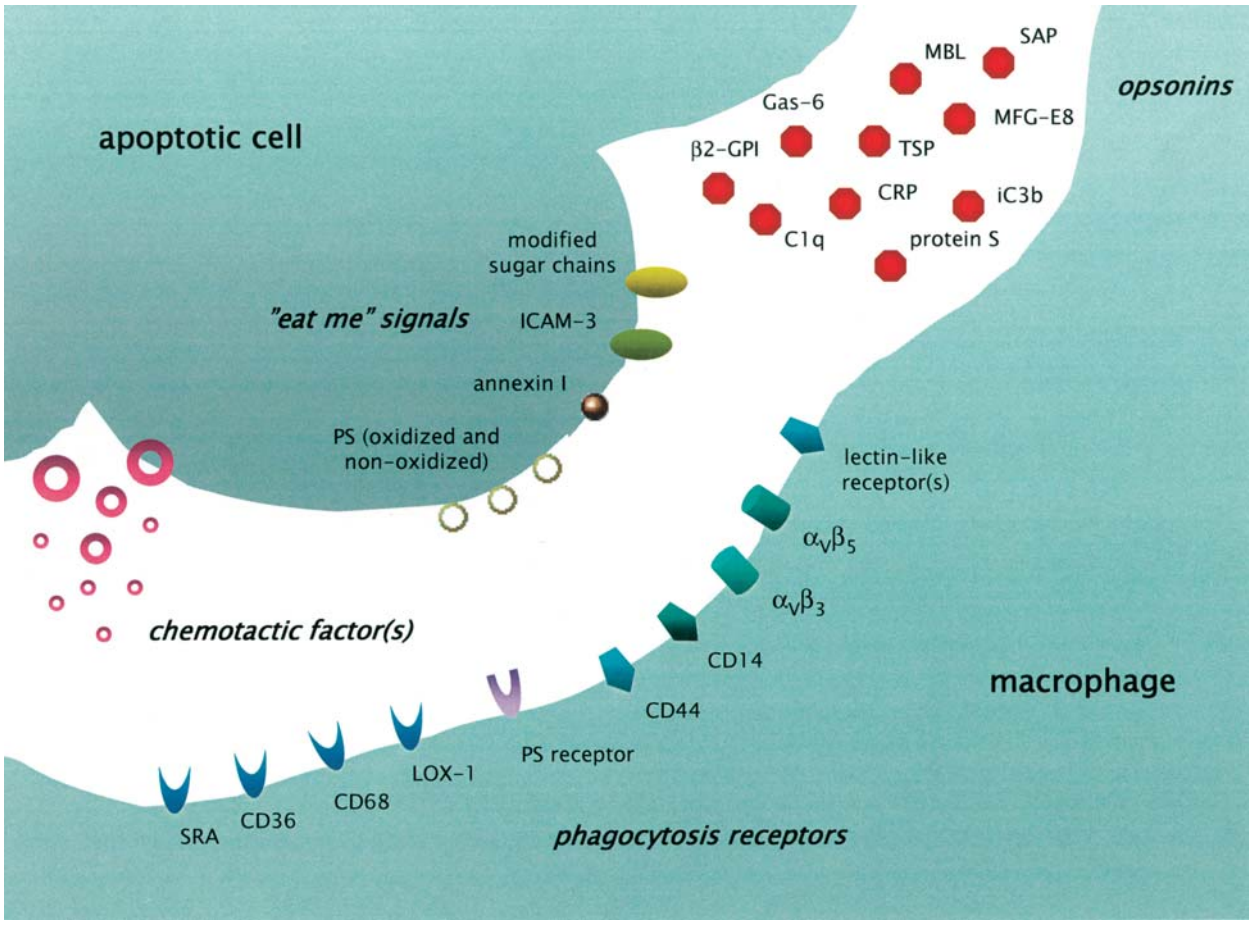

Figure 1. Programmed cell clearance: the mechanism of uptake of apoptotic cells. Dying cells expose 'eat-me' signals, including the aminophospholipid phosphatidylserine (PS), that interact with an array of receptors on the phagocyte surface, resulting in the tethering and ingestion of cell corpses. In addition, a number of bridging molecules (opsonins) have been shown to facilitate the uptake of apoptotic cells. Chemotactic factors that are released from the dying cell may also serve to attract neighboring phagocytes. β2-GPI, β2-glycoprotein I; CRP, C-reactive protein; Gas-6, growth arrest-specific gene-6; ICAM, intercellular adhesion molecule-3; iC3b, inactivated C3b; LOX-1; lectin-like oxidized low-density lipoprotein receptor-1; MBL, mannose-binding lectin; MFG-E8, milk-fat globule epidermal growth factor-8; SAP, serum amyloid P component; SRA, class A scavenger receptor; TSP, thrombospondin.

CED-1 (cell death abnormal-1) and croquemort ('catcher of death'), have been demonstrated to play a critical role in engulfment in *Caenorhabditis elegans* [50] and *Drosophila melanogaster* [51], respectively.

Bridging molecules (opsonins)

Early work indicated that macrophage engulfment of apoptotic thymocytes depends on the presence of a heat-labile factor in serum [8]. Subsequent studies have confirmed that serum factors may, in fact, serve as 'bridging' molecules, or molecular liaisons, between phagocytes and apoptotic cells. Hence, thrombospondin is known to bind to $\alpha_V\beta_3$ on the macrophage surface and to poorly characterized structures on the surface of apoptotic cells [37, 52]. Similarly, Nagata and his group [53] have shown that MFG-E8 (milk-fat globule epidermal growth factor-8, also known as lactadherin) binds to $\alpha_V\beta_3$ on macrophages, and to externalized PS on the surface of apoptotic cells, thereby potentiating phagocytosis. A number of other PS-binding proteins, including β2-glycoprotein I [54] and protein S [55], may act as a bridge between phagocytes and their prey. In addition, recent studies have shown that several molecules of the innate immune system, including complement components [56, 57] and pentraxins [58–61], may act as opsonins that stimulate macrophage ingestion of apoptotic cells. The involvement of molecules of the innate immune system in programmed cell clearance indicates that the recognition of 'unwanted' (i.e. apoptotic) self is a primitive function that shares certain features with the mechanism of non-self recognition. Of note, CD14, another element of the innate immune system, induces proinflammatory responses upon recognition of bacterial lipopolysaccharide, yet mediates clearance of apoptotic cells without inciting inflammation [62]. The reason for these divergent out-

comes of CD14 ligation is unclear, but may depend on the differential engagement of macrophage coreceptors by microbes and cell corpses, respectively, as well as on the cytokine milieu and, hence, the degree of macrophage activation.

Cytoskeletal rearrangements

Gräper observed, some 90 years ago, that physiological elimination of cells during the shrinkage of organs involves the engulfment of dying cells by 'sister cells', i.e. neighboring cells endowed with phagocytic abilities [63]. Numerous cells, including fibroblasts, endothelial cells, Sertoli cells, renal mesangial cells and immature dendritic cells have since been identified as amateur (nonprofessional) phagocytes [64]. Amateur phagocytes fulfill an important backup function, as evidenced in macrophage-less (PU.1-deficient) mice, in which the task of phagocytosis of apoptotic cells in the developing footplate is assumed by neighboring mesenchymal cells [65]. However, these cells are reluctant undertakers, in the sense that they are poorly phagocytic and respond slowly; in contrast, professional phagocytes (macrophages) are motile and can infiltrate tissues, and possess a high phagocytic capacity [66, 67]. *C. elegans* lacks dedicated macrophages [68] and is a suitable model for the elucidation of nonprofessional (or semiprofessional) phagocytic responses. Seven genes have been characterized to date that regulate the clearance of cell corpses in the nematode (fig. 2). CED-2, CED-5 and CED-10, homologs of mammalian CrkII, DOCK180 and Rac-1, respectively, are involved in cytoskeletal reorganization during engulfment

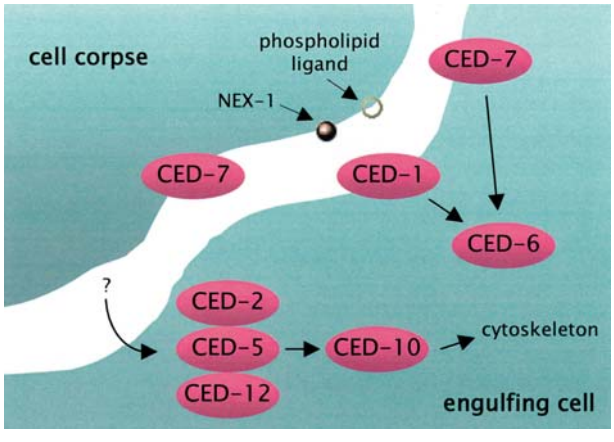

Figure 2. Programmed cell clearance in the nematode. Genetic analyses have defined seven genes that regulate corpse removal in *C. elegans*. CED-2, CED-5, CED-10 and CED-12 are involved in the cytoskeletal rearrangement that occurs upon engulfment of effete cells, as well as in the migration of so-called distal tip cells. CED-1 is a scavenger receptor-like molecule, and is thought to recognize a phospholipid ligand, alone or in conjunction with NEX-1, on the surface of cell corpses. CED-6 and CED-7 act in the same pathway as CED-1. CED, cell death abnormal; NEX, annexin.

[69, 70]. CED-12 also belongs to the CED-2/CED-5/CED-10 signaling pathway [71, 72], and ELMO (engulfment and cell motility), its mammalian counterpart, was shown to form a complex with CrkII and DOCK180 that results in activation of Rac-1 [73]. CED-1, the human SREC (scavenger receptor from endothelial cells) homolog, and CED-7, a homolog of the ABC (ATP-binding cassette) transporters, work in concert to mediate phospholipid- and/or annexin-dependent engulfment of cell corpses [31, 50, 74]. Finally, CED-6, and its mammalian homolog GULP (engulfment adaptor protein), was shown to act downstream of CED-1 and CED-7 [75–78]. Recent studies have provided further insight into the coupling of receptor-ligand interactions and cytoskeletal signaling in mammalian systems. Hence, Albert and colleagues [79] have shown that engagement of the $\alpha_V\beta_5$ integrin receptor on the surface of human phagocytic cells triggers the recruitment of the CrkII/DOCK180/Rac-1 molecular complex. Moreover, PS-dependent recognition through the PSR induces membrane ruffling, a process dependent on PSR-mediated activation of Rac-1 and the related Rho family GTPase, Cdc42 [46]. The Wiskott-Aldrich syndrome (WAS) protein (WASp) is activated by Cdc42 and stimulates actin polymerization [80], and recent studies revealed an impairment in phagocytosis of apoptotic cells by macrophages derived from WASp-deficient mice [81]. In addition to rearrangements of the actin cytoskeleton, large amounts of membrane are needed at the macrophage cell surface to form the phagosome. Interestingly, Gagnon and colleagues [82] have shown that the phagosomal membrane is derived, in part, from the endoplasmic reticulum (ER). This observation suggests that the ER, through its fusion with the plasma membrane, may provide a source of molecules involved in the engulfment of cell corpses. Indeed, cell surface exposure of calreticulin, a protein normally present in the ER, has been shown to mediate macropinocytosis and uptake of apoptotic cells by human monocyte-derived macrophages [57].

Chemotactic factors

When apoptosis occurs on a large scale, as in certain phases of embryogenesis, hordes of phagocytes appear on the scene; a classical example is the programmed cell clearance that takes place in the interdigital zones [4, 63]. However, the nature of the signals that trigger the migration of macrophages to the site of apoptosis has remained poorly understood. Horino and colleagues [83] have provided evidence that a cross-linked homodimer of S19 ribosomal protein can function as a chemotactic factor in the recruitment of monocytes from the circulation to apoptotic lesions. Moreover, blebs derived from apoptotic germinal center B cells were shown to be chemotactic for monocytes in vitro, and it was hypothesized that a gradient of apoptotic blebs released from dying B cells

may attract macrophages in vivo [84]. More recent studies suggest that in addition to its role as a bridging molecule [37], thrombospondin derived from apoptotic cells may act as a signal to recruit macrophages [52]. Detailed assessment of in vivo models of physiological cell death, such as the involuting mammary or pituitary glands [85, 86], is likely to provide further information on the mechanism of macrophage recruitment to apoptotic lesions.

The meaning of cell clearance: engulfment of apoptotic cells in development and disease

Macrophage responses

The consequences of programmed cell clearance, or its failure, are manifold (fig. 3). Hence, the removal of apoptotic cells is thought to play an active role in the resolution of inflammation, through macrophage production of antiinflammatory cytokines and downregulation of proinflammatory cytokine production [87–89]. In addition, efficient clearance of apoptotic cells may be important to prevent inadvertent immune responses to self-antigens (discussed below). Macrophages are also important in tissue remodeling in vivo, not only as scavengers of apoptotic debris, but also through the active induction of apoptosis, as seen during ocular tissue remodeling [90, 91]. Degradation of DNA into oligonucleosomal fragments is a hallmark of apoptosis, and the endonuclease responsible for this event is termed CAD (caspase-activated DNase) [92]. Macrophages have been shown to provide an auxiliary mode of DNA fragmentation upon engulfment of the dying cell [93]. Mice deficient for the macrophage-specific endonuclease, DNase II, are anemic and die before birth [94, 95], and it was concluded

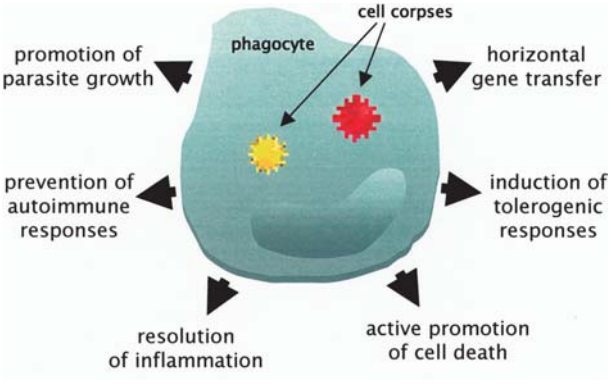

Figure 3. The consequences of programmed cell clearance. Macrophage engulfment of apoptotic cells is not a passive event, but results in numerous effects on the immune system. Dead cells that are ingested by phagocytes (macrophages or dendritic cells) are thus 'gone, but not forgotten' [142]. In addition, pathogens and tumor cells can, in some instances, subvert macrophage responses to apoptotic cells. Active promotion of the execution phase of cell death is seen in *C. elegans*, and has yet to be demonstrated in a mammalian system.

that macrophages are required for the destruction of nuclear DNA that occurs during erythropoiesis [94]. Furthermore, thymic development is perturbed in mouse embryos deficient for both CAD and DNase II [96], thus providing additional support for the role of macrophage-driven dismantling of cells during development. Interestingly, recent studies in *C. elegans* suggest that phagocytosis may actively promote the execution of cell death. Hence, cells expressing a partial loss-of-function mutation of the *ced-3* 'killer' gene appeared to be poised between life and death, and could recover completely if engulfment was prevented [97, 98].

Control of inflammation

The accumulation and persistence of leukocytes, including polymorphonuclear granulocytes or neutrophils, is a characteristic feature of chronic inflammation [99]. Importantly, neutrophils, replete with potentially deleterious contents, need to be removed prior to their lysis, as contents would otherwise expel into the extracellular milieu and perpetuate inflammation. Studies in recent years have suggested that apoptosis and subsequent macrophage ingestion (i.e. programmed cell clearance) of neutrophils may aid in the resolution of the inflammatory response [100, 101]. Chronic granulomatous disease (CGD) is a rare hereditary condition characterized by severe recurrent bacterial and fungal infections, and an inability of neutrophils and other phagocytes to generate reactive oxygen species (ROS); the underlying genetic defect is a mutation in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [102]. Importantly, ROS-dependent externalization of PS is defective in CGD neutrophils [103]. The absence of this crucial recognition signal on the surface of neutrophils may disrupt the clearance of cells in vivo, thus contributing to the formation of inflammatory granulomas and tissue destruction evidenced in these patients. Indeed, an increased accumulation of neutrophils was observed in peritoneal exudates of NADPH oxidase-defective mice injected with heat-inactivated bacteria, indicative of a clearance defect in this model of CGD [104]. In addition, macrophages derived from CGD patients are compromised in their ability to produce anti-inflammatory mediators upon ingestion of apoptotic targets [105]. The latter findings thus provide further support for the notion that defects in programmed cell clearance may contribute to persistence of inflammation in CGD. Cystic fibrosis (CF) is a chronic inflammatory condition characterized by a massive influx of cells into the airways and release of intracellular proteases, including neutrophil elastase [106]. Vandivier and colleagues [107] have provided evidence for elastase-mediated cleavage of the PSR on macrophages and ensuing disruption of programmed cell clearance in the airways of CF patients. The latter findings suggest that the sustained inflammation ev-

idenced in these patients may result, at least in part, from defective clearance of inflammatory cells. Elastase released from damaged neutrophils is also known to stimulate macrophage secretion of proinflammatory cytokines [108]. Similarly, HMGB-1 (high-mobility group box chromosomal protein-1), an abundant chromosomal architectural protein, is released from necrotic, but not apoptotic cells, and stimulates macrophage production of proinflammatory mediators [109]. Furthermore, necrotic, but not apoptotic, cells release heat shock proteins, including gp96, which deliver maturation signals to dendritic cells [110]. CD91 is a receptor for gp96, and has been proposed to act as a sensor for necrotic cell death [111], just as the PSR, in conjunction with other phagocytic receptors, may act as a sensor for apoptotic cell death [35].

Role in autoimmune disease

Apoptosis dysregulation has been implicated in the pathogenesis of autoimmune disease in numerous studies. Hence, evidence has accrued for a role of 'too much' apoptosis in the effector (i.e. tissue destruction) phase in insulin-dependent diabetes, multiple sclerosis and other organ-specific autoimmune diseases, as well as for 'too little' apoptosis (of autoreactive B and T cells) in the initiation phase of systemic autoimmune conditions, such as SLE (systemic lupus erythematosus) and ALPS (autoimmune lymphoproliferative syndrome) [112, 113]. In addition, disruption of programmed cell clearance may trigger undesirable immune responses to self. Intracellular autoantigens are known to cluster on surface blebs of apoptotic cells [114]; moreoever, caspase- and granzyme B-mediated cleavage of autoantigens occurs during apoptosis, and may yield cryptic epitopes to which autoimmune responses are targeted [115–118]. C1q, the first component of complement, binds specifically to surface blebs of apoptotic cells [119], and has been implicated in the clearance of apoptotic cells [57, 120]. Interestingly, mice deficient for C1q have high titers of autoantibodies and SLE-like glomerulonephritis with evidence of numerous unengulfed apoptotic bodies [121]. Macrophages isolated from mice that lack a functional Mer receptor tyrosine kinase are also deficient in the clearance of apoptotic cells and display high titers of nuclear autoantibodies [122]. Taken together, these observations lend weight to the hypothesis that autoimmune disease can result from impairment of programmed cell clearance. Of note, macrophages from C1q-deficient humans with SLE-like disease also show a defect in the phagocytic uptake of apoptotic cells; this defect could be corrected in vitro by exogenous C1q protein [123]. Furthermore, macrophages from SLE patients display an impairment in phagocytosis of apoptotic cells, and it was suggested that persistently circulating 'apoptotic waste' may serve as immunogen for the induction of autoreactive responses in these individuals [124].

Immune modulation

Dendritic cells are antigen-presenting cells whose primary function is to monitor the environment for 'danger' signals and transduce these signals to T cells. Dendritic cells are also capable of engulfing apoptotic cells, albeit not as efficiently as professional phagocytes [125]; moreover, dendritic cells can present antigen derived from ingested cell corpses in a major histocompatibility complex (MHC) class I-restricted manner [126]. However, data concerning the effect of apoptotic cell ingestion on dendritic cells are conflicting. Some investigators have shown that co-culture with necrotic tumor cells, but not apoptotic cells, induces dendritic cell maturation [127, 128], and the uptake of apoptotic cells by dendritic cell was thus proposed to elicit tolerance to self-antigens [129]. However, others have reported that maturation of dendritic cells ensues following exposure to apoptotic debris [130]. The latter data thus suggest that the capture of apoptotic cells by antigen-presenting cells may, under particular circumstances, evoke immune responses. The question of whether recognition of 'unwanted' (apoptotic) self induces a tolerogenic or immunogenic response has significant implications for vaccine strategies and immunotherapeutic approaches to cancer [131, 132]. Future studies will need to assess the cell-intrinsic and/or environmental factors that determine whether apoptotic cell ingestion by dendritic cells favors or prevents the induction of an immune response [133]. Nevertheless, one can conclude from the aforementioned data that the process of programmed cell clearance exerts numerous effects on the immune system. In other words, apoptosis, and the subsequent removal of cell corpses, is not always 'silent' or immunologically inert [134]. In addition, intracellular pathogens such as *Trypanosoma cruzi* can subvert macrophage responses. Hence, the uptake of apoptotic cells by infected macrophages can enhance parasite growth through macrophage production of TGF-β, a 'trypanosoma-growth factor' [135]. Tumors may also take advantage of apoptotic cell effects on macrophages [136]. Moreover, Holmgren and his group [137] have provided evidence for the 'horizontal' transfer of oncogenes through phagocytosis of apoptotic bodies. Programmed cell clearance may thus constitute a mechanism for the propagation of genetic instability and/or diversity within a tumor cell population.

Concluding remarks

The view has evolved in recent years that human disease may result not only from excessive or inadvertent execu-

tion of cell death, but also from a mismatch between death and the clearance of cell corpses. Further dissection of the process of programmed cell clearance may thus yield novel strategies for therapeutic intervention in numerous diseases, including chronic inflammation, autoimmune conditions and cancer. Candidate approaches, such as CD36 gene transfer to amateur phagocytes [138] and ligation of macrophage CD44 [139], are beginning to emerge; moreover, granulocyte-macrophage colony-stimulating factor (GM-CSF) administration to carcinoma patients was recently shown to promote macrophage ingestion of apoptotic tumor cells [140], thus demonstrating that programmed cell clearance is amenable to pharmacological intervention in vivo. An important aim for future studies is to decipher the signals that determine the outcome of macrophage disposal of apoptotic versus necrotic cells, and cell corpses versus invading pathogens, respectively. Further development of relevant in vivo models [120, 141] is also needed, and will be useful for testing therapeutic strategies directed toward the modulation of programmed cell clearance.

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