Chapter 3

CELL CLEARANCE AND CANCER

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Abstract: Elimination of cells through apoptosis is crucial for tissue homeostasis. The process of cell removal is regulated by the expression of recognition signals on the dying cell and corresponding phagocytosis receptors on the engulfing cell; in addition, chemotactic factors emitted by apoptotic cell corpses serve to attract macrophages to the site of cell attrition. Cell clearance is thus an active, programmed process and as such is thought to play an important role in the resolution of inflammation and the prevention of autoimmune diseases. Moreover, recent studies suggest that engulfment of apoptotic cell remnants may result in lateral transfer of genetic information within a tumour cell population, and thus contribute to cancer progression. In addition, several recent studies have established a link between malignant transformation and autophagy, an alternative form of programmed cell death. Further elucidation of the mechanism of cell disposal (occurring either through apoptosis or autophagy/self-digestion) is expected to aid in our understanding of cancer development and may unveil novel targets of therapeutic intervention.

Key words: apoptosis, autophagy, cancer, horizontal gene transfer, phagocytosis

1. INTRODUCTION

Apoptosis is a process of cellular suicide that is essential for the sculpting of organs during embryogenesis and for the maintenance of homeostasis in adult tissues (Wyllie et al., 1980; Jacobson et al., 1997). Characteristic morphological features of apoptosis (a term derived from the Greek word describing the falling off of petals from a flower or leaves from a tree) are nuclear and cytoplasmic condensation with concomitant blebbing (zeiosis)

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of the plasma membrane, and fragmentation of the cell into so-called apoptotic bodies, containing structurally intact organelles as well as portions of the nucleus (Kerr et al., 1972). Studies in recent decades have provided a detailed characterization of the biochemical features of apoptosis, including the fragmentation of nuclear DNA, activation of cysteine proteases known as caspases, and externalization of recognition signals such as phosphatidyserine (PS) on the cell surface (for an excellent review, see Danial and Korsmeyer, 2004). Importantly, the caspase-driven dismantling of the cell ultimately results in the recognition, ingestion, and degradation of apoptotic bodies and cell remnants by neighboring phagocytes. The removal of dying cells prior to their necrotic disintegration requires phagocytosis receptors and recognition signals expressed on phagocyte and prey, respectively, and is thought to preclude tissue scarring and inflammation (Lauber et al., 2004; Fadeel, 2004).

An emerging theme in recent years is that tissue homeostasis is dependent not only on the degree of mitosis (cell proliferation) and apoptosis, but also on the balance between cell death and cell clearance. Consequently, a mismatch between apoptosis and cell disposal may contribute to disease pathogenesis (Savill and Fadok, 2000). For instance, although vast numbers of immature thymocytes die *in situ*, minimal evidence of apoptosis is seen in the thymus (Surh and Sprent, 1994), thus reflecting the efficient clearance of dying cells in this tissue under normal conditions. In contrast, mice treated with agonistic anti-Fas antibodies display fulminant liver destruction (Ogasawara et al., 1993), due most likely to massive death of Fas-expressing hepatocytes such that the phagocytic capacity of the liver is overwhelmed. Apoptosis can thus be viewed essentially as a mechanism of cell clearance; indeed, the term “programmed cell clearance” was recently introduced to underscore this notion and to emphasize that distinct molecular events govern the removal of apoptotic cell corpses (Fadeel, 2003). The current review aims to summarize the mechanisms of clearance of mammalian cells, as well as to discuss the functional significance of (defective) clearance of apoptotic cells in the context of inflammation, autoimmune disease, and cancer. Autophagy, a mode of cell-autonomous clearance that is distinct from apoptosis (Levine and Klionsky, 2004), and its role in the development of cancer, will also be considered.

2. MECHANISMS OF CELL CLEARANCE

2.1 Recognition signals

Cells undergoing apoptosis express recognition or "eat me" signals, including lipids, proteins, and modified sugar moieties, that facilitate recognition and ingestion by macrophages or neighboring cells (Savill et al., 2002; Lauber et al., 2004). The best-known "eat me" signal is the anionic phospholipid PS that translocates from the inner to the outer leaflet of the plasma membrane in a caspase-dependent manner (Fadok et al., 1992; Martin et al., 1995; Vanags et al., 1996). Early studies suggested that the simultaneous activation of a calcium-dependent phospholipid scramblase and inhibition of an ATP-dependent aminophospholipid translocase, which under normal conditions maintains the asymmetric distribution of PS in the plasma membrane, is required for PS externalization (Verhoven et al., 1995; Bratton et al., 1997). There is also evidence for the involvement of the ATP-binding cassette transporter ABC1 in the redistribution of PS, although its specific role during apoptosis remains to be determined (Hamon et al., 2000). More recent studies suggest that PS exposure is modulated by the level of intracellular ATP and transpires downstream of Bcl-2-regulated mitochondrial events, irrespective of the expression of the phospholipid scramblase (Gleiss et al., 2002; Uthaisang et al., 2003). In support of this notion, Blom and colleagues (2003) have shown that regional loss of mitochondrial membrane potential in hepatocytes is rapidly followed by externalization of PS at that specific site during apoptosis. These investigators proposed that a spatio-temporal relationship may exist between the drop in mitochondrial ATP production and inhibition of aminophospholipid translocation, thus yielding localized externalization of PS. Furthermore, Ricci et al. (2004) have demonstrated that disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain; importantly, the disruption of mitochondrial function and subsequent dissipation of intracellular levels of ATP was coupled with plasma membrane externalization of PS in this model. In addition, microinjection of the so-called apoptosis-inducing factor (AIF) into the cytoplasm of intact cells induces caspase-independent dissipation of the mitochondrial membrane potential and concomitant PS externalization (Susin et al., 1999). Taken together, the egress of PS during apoptosis appears to be a mitochondria-dependent event that is linked to a drop in ATP production within the cell. Furthermore, while PS externalization can be dissociated from other features of the apoptotic program (Zhuang et al., 1998; Uthaisang et al., 2003), we

and others have shown that this event is nonetheless essential for phagocytosis of apoptotic cells (Fadok et al., 2001; Kagan et al., 2002).

Evidence of selective oxidation of PS has been provided in several models of oxidative stress-induced apoptosis (for a review, see Kagan et al., 2003). Moreover, recent studies of non-oxidant-triggered apoptosis have yielded further evidence that oxidation of PS is selective and precedes its externalization (Kagan et al., 2002; Matsura et al., 2002; Koty et al., 2002). Moreover, we have shown, in collaboration with Kagan and his associates, that the expression of oxidized PS (PS-OX) in conjunction with its non-oxidized counterpart on the surface of apoptotic cells serves as a critical recognition signal for macrophages (Kagan et al., 2002; Arroyo et al., 2002). These findings were supported by the demonstration that enrichment of the plasma membrane of non-apoptotic cells with exogenous PS and/or PS-OX resulted in phagocytic removal of these cells. Moreover, antibodies to oxidized low-density lipoprotein (LDL) have been shown to bind to apoptotic cells and prevent their uptake by macrophages, thus providing further evidence for oxidation-specific epitopes on the surface of apoptotic cells (Chang et al., 1999). Indeed, C-reactive protein (CRP) was recently found to bind both oxidized LDL and "late" apoptotic cells (i.e. apoptotic cells that have undergone secondary necrosis due to prolonged *in vitro* culture) through recognition of a common ligand, oxidized phosphatidylcholine (PC-OX) (Chang et al., 2002). The mechanism of PS (and PC) oxidation remains to be clarified; however, recent data suggest that cytochrome *c* released from mitochondria into the cytosol may act as a catalyst that utilizes reactive oxygen species generated by disrupted mitochondrial electron transport for selective PS oxidation (Jiang et al., 2003; Fadeel et al., 2004a).

Recent proteomics studies have identified annexin I (also known as lipocortin I) as a novel recognition signal (Arur et al., 2003). Annexin I is thus recruited from the cytosol to the plasma membrane during apoptosis, and is required for efficient uptake of apoptotic cells by human umbilical vein endothelial cells, i.e. non-professional phagocytes. Moreover, annexin I, a PS-binding protein (Schlaepfer and Haigler, 1987), co-localizes with PS on the surface of apoptotic cells leading to the clustering of PS receptors on the phagocytic cell surface. Similarly, Fan et al. (2004) have reported that apoptotic Jurkat cells and primary T cells, but not apoptotic thymocytes, express annexin I on their cell surface. These investigators were also able to demonstrate constitutive annexin I and II expression on macrophages, and could show that antibodies to these annexins suppressed macrophage engulfment of apoptotic target cells. Hence, annexins may potentially serve as both ligand and receptor and thereby promote cell clearance, alone or in conjunction with PS or PS-OX, albeit in a cell type-specific manner.

Interestingly, macrophages derived from annexin I-deficient mice display anomalies in phagocytosis of zymosan particles (Hannon et al., 2003); whether annexin I-null cells also exhibit defects in engulfment of apoptotic targets remains to be determined.

Recent studies have identified detachment signals that contribute to the protection of viable cells from accidental clearance. Hence, CD47 (also known as integrin-associated protein) was shown to serve as a marker of "self" on viable erythrocytes; red blood cells that lack CD47 are rapidly cleared from the bloodstream by splenic red pulp macrophages (Oldenborg et al., 2000). Similarly, non-apoptotic neutrophils were reported to express CD31 (platelet-endothelial cell adhesion molecule 1, PECAM-1) (Brown et al., 2002). Apoptosis of neutrophils apparently disables CD31-mediated cell detachment from phagocytes, thereby promoting binding and engulfment of target cells. In line with the latter observations, a recent report has provided evidence that CD31 is required for efficient engulfment of apoptotic B lineage cells by bone marrow-derived macrophages (Dogusan et al., 2004). In addition, Tada et al. (2003) have shown that CD47 may act as a tethering molecule during apoptosis; concomitant externalization of PS, however, was essential for engulfment. The molecular mechanism underlying the switch from CD47- and CD31-mediated repulsion to adhesion remains an important area of investigation. Further studies are also required to assess whether an influx of calcium following CD31 engagement (O'Brien et al., 2001) can promote the calcium-dependent externalization of annexin I, as suggested by Orrenius et al. (2003).

2.2 Phagocytosis receptors

Macrophages utilize an array of phagocytosis receptors for engulfment of cell corpses. These include the so-called PS receptor (PSR), the class A scavenger receptor (SRA), CD36 (a class B scavenger receptor), CD68 (macrosialin; a class D scavenger receptor), the integrin receptors $\alpha_V\beta_3$ and $\alpha_V\beta_5$, the bacterial lipopolysaccharide (LPS) receptor CD14, and the calreticulin-CD91 receptor complex (Savill et al., 2002, and references therein). Tissue- and cell type-specific differences in receptor usage provides a partial explanation for this broad repertoire of phagocytosis receptors. For example, monocyte-derived macrophages and dendritic cells utilize the $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin receptor, respectively, for the uptake of cell corpses (Savill et al., 1990; Albert et al., 1998a). In addition, the engulfment process may require the serial engagement of distinct receptors, some of which are involved in the initial tethering of apoptotic cells and others in the subsequent stage of cytoskeletal rearrangement that is needed for ingestion of cells. Indeed, Hoffmann et al. (2001) have shown, using a surrogate

system consisting of erythrocytes coated with various protein ligands, anti-receptor antibodies, or phospholipids, that the majority of "eat me" signals mediate tethering (attachment) of erythrocytes to macrophages. Internalization of target cells, on the other hand, required the presence of PS as well as the PS-specific receptor, PSR. PSR was originally described to be a cell surface receptor that interacts in a stereospecific manner with externalized PS on apoptotic cells (Fadok et al., 2000); however, more recent findings call into question the membrane localization of this molecule (Cui et al., 2004; Cikala et al., 2004). Notwithstanding the issue of nuclear versus cell surface localization of PSR, a role for this molecule in the ingestion of cell corpses has been demonstrated in several mammalian model systems (Fadok et al., 2000; Todt et al., 2002; Hisatomi et al., 2003). Moreover, a PSR homolog was recently identified in C. elegans and shown to be an upstream receptor for the signaling pathway containing CED-2, CED-5, CED-10, and CED-12 (CED, cell death abnormal) proteins, and to play an important role in PS recognition during corpse clearance in the nematode (Wang et al., 2003). The contact site between the apoptotic cell and the phagocyte may thus be viewed as an "engulfment synapse", akin to the neural or immunological synapse, in which complex interactions between numerous ligands and receptors take place (Henson et al., 2001; Somersan and Bhardwaj, 2001). This model makes it feasible for signaling to proceed despite the low avidity of PS-PSR interactions (Fadok et al., 2000). In addition, an engulfment synapse allows for regulation and specificity in the uptake of dying cells. Indeed, because some cell types can transiently express PS upon activation (Dillon et al., 2000; Martin et al., 2000), the two-step process of tethering and engulfment may also serve to protect against accidental clearance of viable cells.

Several of the aforementioned receptors (not only the PSR) bind PS on apoptotic cells, either directly or indirectly (via bridging molecules) (Savill et al., 2002; and see below). Importantly, macrophage scavenger receptors were originally identified based on their ability to bind chemically modified structures, such as acetylated or oxidized LDL, but not their unmodified counterparts (Steinbrecher, 1999). A common feature of these proteins is their ability to recognize a wide range of structurally unrelated ligands, including oxidized LDL, bacterial LPS and the anionic phospholipid PS, and this lack of specificity is consistent with the idea that scavenger receptors act as receptors for apoptotic cells (Platt et al., 1998). For instance, the class B scavenger receptor, CD36, is required for phagocytosis of apoptotic cells by various classes of macrophages and non-professional phagocytes (Fadok et al., 1998a; Shiratsuchi et al., 1999). Similarly, croquemort ("catcher of death"), a Drosophila melanogaster homolog of CD36, is required for the engulfment of apoptotic cells in the fruitfly (Franc et al., 1999), and CED-1,

a scavenger receptor-like molecule in C. elegans, was suggested to recognize a phospholipid ligand on the surface of apoptotic cell corpses in the nematode (Zhou et al., 2001). As discussed above, apoptotic cells express oxidation-specific epitopes, including PS-OX and PC-OX, on their cell surface, and it is tempting to suggest that scavenger receptors are specifically involved in the recognition of such oxidized phospolipid species on apoptotic cells. Indeed, we have shown that anti-PSR antibodies, but not anti-CD36 antibodies, are able to inhibit phagocytosis of Jurkat cells with exogenous PS integrated into their plasma membrane (Kagan et al., 2003). In contrast, both anti-PSR antibodies and anti-CD36 antibodies were effective in suppressing phagocytosis of target cells enriched with a combination of PS and PS-OX. These data, which imply that CD36 and PSR cooperate to recognize PS and its oxidized counterpart on the surface of apoptotic cells, are thus in concordance with the engulfment synapse model outlined above. Indeed, Fadok and colleagues (1998a) have previously demonstrated that CD36 contributes not only to $\alpha_V\beta_3$ integrin-dependent recognition, but also serves as a co-factor during PS-dependent clearance.

Recent studies have shown that members of the collectin family, such as the lung surfactant proteins A and D (SP-A and SP-D), mannose-binding lectin (MBL), and the collectin-like complement protein, C1q, facilitate the attachment of apoptotic cells to phagocytes (for a recent review, see Roos et al., 2004). Hence, C1q and MBL engagement of calreticulin and CD91 on the macrophage cell surface was shown to initiate macropinocytosis and uptake of apoptotic cells in vitro (Ogden et al., 2001). Similarly, SP-A and SP-D bind to apoptotic cells and drive apoptotic cell ingestion by phagocytes through a mechanism dependent on calreticulin and CD91 (Vandivier et al., 2002). Of note, several studies suggest that these opsonins, and related molecules such as the pentraxin, PTX3, recognize primarily “late” apoptotic cells and/or apoptotic debris (Rovere et al., 2000; Gaipl et al., 2001; Nauta et al., 2003); therefore, other mechanisms of cell disposal may prevail in normal clearance of early apoptotic cells (Roos et al., 2004). Furthermore, it is important to consider that collectins, pentraxins, and other molecules of the innate immune system, could trigger undesirable pro-inflammatory responses, thus precluding the “meaning” of cell death (Savill and Fadok, 2000). For comparison, CD14 induces pro-inflammatory responses upon recognition of bacterial LPS, yet mediates clearance of apoptotic cells without inciting inflammation (Gregory, 2000). Interestingly, SP-A and SP-D were recently shown to act in a dual manner, enhancing or suppressing inflammatory cytokine production depending on their orientation and on their specific binding partners (Gardai et al., 2003). Further studies are warranted to elucidate how opsonization of apoptotic cells, in the context of

a putative engulfment synapse between phagocyte and apoptotic prey, can facilitate clearance without triggering a deleterious inflammatory response.

2.3 Bridging molecules

As mentioned in the preceding sections, recognition signals such as PS may not bind directly to phagocytosis receptors on the macrophage, but rather indirectly via membrane-bound co-factors (such as annexin I) or via soluble bridging molecules (such as collectins or pentraxins). Indeed, the basic principle of PS recognition via bridging molecules holds true for a number of phagocytosis receptors, such as the $\alpha_V\beta_3$ integrin receptor and the receptor tyrosine kinase, Mer (Lauber et al., 2004). Hence, it was shown some 10 years ago that macrophages are able to recognize $\alpha_V\beta_3$ together with the soluble bridging protein, thrombospondin, and the co-receptor, CD36 (Savill et al., 1992). More recent studies show that milk fat globule epidermal growth factor 8 (MFG-E8), a protein that is secreted by certain classes of macrophages and DCs, serves as a molecular bridge between PS on the apoptotic cell and $\alpha_V\beta_3$ or $\alpha_V\beta_5$ on the phagocyte surface (Hanayama et al., 2002; Akakura et al., 2004; Miyasaka et al., 2004). Indeed, our recent *in vitro* data demonstrate that PS-OX may serve as a preferential ligand for MFG-E8 binding (Borisenko et al., 2004), suggesting that exposure of PS-OX during apoptosis could promote clearance of cell corpses at least in part through interaction with MFG-E8. Further studies are needed to determine whether other bridging molecules such as Del-1, protein S, and Gas6 also bind preferentially to PS-OX, as opposed to non-oxidized PS.

Hanayama and colleagues (2004a) have shown that developmental endothelial locus-1 (Del-1) is functionally homologous to MFG-E8, insofar as it binds to PS on apoptotic cells and $\alpha_V\beta_3$ on engulfing cells. Importantly, thioglycolate-elicited macrophages expressed MFG-E8, but not Del-1, whereas fetal liver and thymic (resident) macrophages expressed Del-1, but not MFG-E8. These findings suggest that these two bridging molecules may play non-redundant roles in the clearance of cell corpses. Furthermore, β2-glycoprotein I, a soluble serum protein, also binds PS and enhances phagocytosis of apoptotic targets through recognition of a phagocyte receptor that is distinct from CD36, CD68, and CD14 (Balasubramanian et al., 1997; Balasubramanian and Schroit, 1998). Of note, β2-glycoprotein I has also been reported to bind to annexin II on the surface of endothelial cells (Ma et al., 2000). The latter data suggest that constitutive expression of annexin II on the macrophage surface (discussed above) may link the engulfing cell to PS on apoptotic cells via the bridging molecule, β2-glycoprotein I; future experiments should address this possibility.

Mer is another engulfment receptor that binds PS on apoptotic cells via a soluble bridging molecule, Gas6 (growth arrest-specific gene 6) (Nakano et al., 1997; Scott et al., 2001). Furthermore, protein S, a serum protein that is related to Gas6, was shown to facilitate clearance of cell corpses through binding of PS (Anderson et al., 2003). Interestingly, protein S also mediates binding of the complement-regulating protein, C4BP (C4b-binding protein), to apoptotic cells, and this C4BP-protein S complex was shown to inhibit phagocytosis of apoptotic cells (Webb et al., 2002; Kask et al., 2004). Although the C4BP-protein S complex and free protein S exert opposing effects on cell clearance, binding of the protein complex may nonetheless be beneficial to the cell since this will prevent further complement attack and subsequent necrosis (Kask et al., 2004). Similarly, binding of CRP to apoptotic cells protects these cells from assembly of terminal complement components (Gershov et al., 2000). Importantly, several studies have demonstrated that complement opsonization renders apoptotic cells more appetizing to macrophages; PS externalization has been shown to promote this process (Roos et al., 2004; Hart et al., 2004). Hence, both iC3b and C1q can bind to the surface of apoptotic cells, and facilitate macrophage engulfment of cell corpses (Takizawa et al., 1996; Korb and Ahearn, 1997; Mevorach et al., 1998; Ogden et al., 2001; Nauta et al., 2002). Opsonization of apoptotic cells with C3bi also facilitates clearance of dying cells by immature dendritic cells (DCs) (Verbovetski et al., 2002), and internalization of circulating apoptotic cells by splenic marginal zone DCs was shown to require complement receptors (Morelli et al., 2003). In addition, Taylor and colleagues (2000) have provided compelling *in vivo* evidence that complement plays a role in the phagocytic clearance of apoptotic cells by inflammatory macrophages; these investigators also found that defects in apoptotic cell clearance correlated with predisposition to autoimmune disease (discussed below).

2.4 Chemotactic factors

C. elegans lacks dedicated macrophages and dying cells are usually engulfed by neighboring cells (Robertson and Thomson, 1982). Engulfment by neighboring cells (i.e. non-professional phagocytes) also occurs in higher organisms, as evidenced in macrophage-less mice, null for the transcription factor, PU.1, in which the task of phagocytosis is taken over by mesenchymal neighbouring cells (Wood et al., 2000). However, such resident phagocytes are less mobile and have been shown to engulf with slower kinetics than professional phagocytes (Parnaik et al., 2000), perhaps because they fail to express the full repertoire of engulfment receptors and thus are unable to engage the “engulfment synapse”. In some specific

instances, cell clearance may occur through a cell-autonomous, non-apoptotic mechanism and not depend on phagocytosis, as suggested for chondrocytes that are embedded in extracellular matrix in cartilage tissue (Roach et al., 2004). Notwithstanding such alternative modes of cell clearance, it is evident that professional phagocytes (macrophages) are required for efficient disposal of dying cells in higher organisms. It is thus important to consider how (and where) apoptotic cells encounter macrophages. First, effete cells may traffic through the body until they reach the site of cell disposal; for instance, clearance of circulating neutrophils is effected by so-called Kupffer cells, resident (professional) macrophages of the liver (Shi et al., 2001). Similarly, chilling of blood platelets causes these cells to rapidly leave the circulation, and this was shown in an elegant series of experiments to occur through recognition by hepatic macrophage complement type 3 (CR3) receptors (Hoffmeister et al., 2003). However, since apoptotic cells in solid tissues are not always situated in close proximity to macrophages, it is reasonable to assume that the dying cell needs to emit soluble chemotactic signals in order to selectively recruit more phagocytes to the site of cell death. Indeed, recent studies have provided evidence for apoptosis-specific "come-and-get-me" signals (Grimsley and Ravichandran, 2003; Lauber et al., 2004).

Horino and colleagues (1998) 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. Subsequent studies revealed that intracellular transglutaminase activity was required for the generation of the monocyte chemotactic properties (Nishimura et al., 2001). Moreover, membranous vesicles (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* (Segundo et al., 1999). Of note, recent studies indicate that membrane vesicles and apoptotic blebs contain biologically active oxidized phospholipids (Huber et al., 2002; and V. Kagan, personal communication), suggesting that membrane-bound PS-OX and/or other modified phospholipid species could play a role not only as recognition signals, but also as chemotactic agents. Moreover, in addition to its role as a bridging molecule, thrombospondin derived from apoptotic cells may also act as a signal to recruit macrophages (Moodley et al., 2003).

Using an *in vitro* transmigration system, it was recently demonstrated that apoptotic cells secrete a chemotactic signal, in a caspase-3-dependent fashion (Lauber et al., 2003). This factor was identified as lysophosphatidylcholine (LPC), a phospholipid previously shown to be a chemoattractant for monocytes and T cells (Hoffman et al., 1982; McMurray

et al., 1993). Apoptotic vesicles could be excluded in this model system since neither filtration nor ultracentrifugation could abrogate the chemotactic activity of apoptotic cell supernatants (Lauber et al., 2003). Interestingly, the release of LPC during apoptosis was linked to caspase-3-mediated activation of the calcium-independent phospholipase A_2 ($iPLA_2$). Of note, Kim et al. (2002) have previously reported that $iPLA_2$ activation during apoptosis can promote cell surface exposure of LPC leading to the binding of natural IgM antibodies and subsequent complement opsonization of the dying cell. Taken together, these novel findings, which further emphasize the importance of phospholipid-dependent signaling in cell clearance (Fadeel, 2004), raise several interesting questions. For instance, does LPC act alone, or in concert with other co-factors or binding partners, such as vitamin D-binding protein (Homma et al., 1993), and is metabolism of LPC required for chemotaxis to occur (McMurray et al., 1993)? Moreover, are the chemotactic effects of secreted LPC receptor-mediated, and if so, which receptors (on which classes of macrophages) are involved; indeed, do DCs also respond to chemotactic signals emitted by apoptotic cell corpses?

3. CELL CLEARANCE AND DISEASE

3.1 Inflammation

Neutrophils are short-lived cells of the innate immune system that contain proteolytic enzymes, reactive oxygen species (ROS), and numerous other bactericidal factors. These cells therefore need to be removed prior to their disintegration as noxious contents would otherwise expel into the extracellular space and prolong inflammation (Savill, 1997; Fadeel and Kagan, 2003). Neutrophils cultured *ex vivo* undergo constitutive death within 24 hours, with typical hallmarks of apoptosis, and senescent neutrophils are believed to undergo apoptosis *in vivo* prior to their removal by macrophages. Indeed, Savill and associates (1989) have shown in pioneering studies that macrophages from acutely inflamed joints preferentially ingest apoptotic neutrophils, and histological evidence was presented for the *in situ* occurrence of this process. The recognition and disposal of intact senescent neutrophils may thus serve as a means to limit the degree of tissue injury and prevent chronic inflammation. Importantly, the process of cell clearance is not a passive event, but is thought to play an active role in the resolution of inflammation, through macrophage production of anti-inflammatory cytokines such as TGF-β and downregulation of pro-inflammatory mediators such as TNF-α (Voll et al., 1997; Fadok et al., 1998b; Byrne and Reen,

2002; Huynh et al., 2002). Furthermore, injection of apoptotic cells into the knee joint and uptake of these cells by synovial lining macrophages was recently shown to inhibit the onset of experimental arthritis in mice, suggesting an active role in the resolution of joint inflammation (van Lent et al., 2001).

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 ROS; the underlying genetic defect is a mutation in the NADPH oxidase (Roos et al., 1996). We have previously shown that ROS-dependent externalization of PS is defective in CGD neutrophils (Fadeel et al., 1998). We surmise that the absence of this crucial recognition signal (PS and/or PS-OX) 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 (Hampton et al., 2002). In addition, macrophages derived from CGD patients are compromised in their ability to produce anti-inflammatory mediators such as TGF-β upon ingestion of apoptotic targets (Brown et al., 2003). Early studies suggested that constitutive and Fas-triggered apoptosis of CGD neutrophils was impaired (Kasahara et al., 1997). However, subsequent examinations of CGD patients in several laboratories have shown that the execution of apoptosis is normal in CGD patient-derived neutrophils and monocytes (Fadeel et al., 1998; Yamamoto et al., 2002; Bernuth et al., 2004). The latter findings do not, however, rule out a role for oxidative stress in the modulation of apoptosis in neutrophils (Hampton et al., 1998; Fadeel and Kagan, 2003). Nevertheless, these data indicate that while NADPH oxidase-derived ROS are required for PS-mediated signaling during the disposal phase of cell death, ROS may not be crucial for the demolition phase of apoptosis in these cells.

3.2 Autoimmune disease

Apoptotic cells may serve as potential reservoirs of "self" antigens that might initiate and drive autoimmune responses (Rosen and Casciola-Rosen, 1999). Indeed, caspase-driven dismantling of cells may be potentially harmful as this generates neoantigens that become accessible on surface structures of apoptotic cells (Casciola-Rosen et al., 1994; Casciola-Rosen et al., 1995; Casiano et al., 1996). These findings suggest that autoimmune responses could result from an impairment of cell clearance. Several recent *in vivo* studies have provided evidence in support of this notion. Hence, mice

deficient for the complement component (and opsonizing factor) C1q have high titers of autoantibodies and systemic lupus erythematosus (SLE)-like glomerulonephritis with evidence of numerous unengulfed apoptotic bodies (Botto et al., 1998). A similar lupus-like syndrome has been described in mice with defects in the tyrosine kinase Mer, a phagocytosis receptor (Scott et al., 2001; Cohen et al., 2002). Interestingly, macrophages isolated from these animals were defective for ingestion of apoptotic targets, while tethering of target cells remained intact, thus providing further support for the two-step model of tethering and engulfment of cell corpses. Recent studies have shown that deletion of the bridging molecule, MFG-E8, also results in autoimmune disease and impaired uptake of apoptotic cells (Hanayama et al., 2004b). These mice developed enlargement of the spleen, with formation of numerous germinal centers, and suffered from glomerulonephritis as a result of autoantibody production. It will be of interest to learn whether deletion of the related bridging molecule, Del-1, elicits a similar phenotype.

Additional lessons come from two recent studies of PSR-deficient mice. In the first study, Li and colleagues (2003) report a dramatic phenotype in PSR-null animals, with fatal neonatal respiratory failure associated with a reduction in the number of airways and an accumulation of non-engulfed cells and cellular debris in the developing lung. These findings suggest that PSR may be of particular importance for clearance in the lung as opposed to other tissues; for comparison, C1q-deficient mice exhibit impaired clearance of cell corpses in the peritoneum and kidney, but not in the skin (Pickering et al., 2001). In addition, these investigators found that a proportion of PSR-defective mice also exhibited hyperplastic brain malformations due to an overproduction of cells within the brain. The latter phenotype might suggest the loss of a non-phagocytic function of PSR; alternatively, PSR-expressing macrophages in normal mice could be responsible for killing of other cell types. In this context, previous studies in *C. elegans* have shown that engulfment may, indeed, promote the execution phase of cell death (Reddien et al., 2001; Hoeppner et al., 2001). In fact, cell death-related nuclease (*crn*) genes that regulate DNA degradation in *C. elegans* have also been implicated in cell clearance (Parrish and Xue, 2003). In a second study, Kunisaki et al. (2004) generated a strain of PSR-null mice that also exhibited perinatal lethality, albeit with a severe block in definitive erythropoiesis and thymocyte development. This phenotype is reminiscent of that seen in animals deficient for DNase II, an endonuclease expressed in macrophages (Kawane et al., 2001; Kawane et al., 2003). In addition, clearance of apoptotic cells by macrophages was impaired in both liver and thymus of PSR-deficient embryos. Again, these studies suggest that cell clearance may be linked to the execution of cell death, as shown in the nematode.

In contrast to PSR-null mice, mice defective for tissue transglutaminase 2 (TGase2), a protein-crosslinking enzyme, show no major developmental abnormalities (De Laurenzi and Melino, 2001). However, Szondy and colleagues (2003) have recently uncovered a stress-inducible phagocytosis defect in these animals. Hence, clearance of apoptotic cells was found to be defective during the involution of the thymus elicited by dexamethasone, anti-CD3 antibody, or γ-irradiation, and in the liver after $PbNO_3$-induced hyperplasia. The TGase2 deficiency was associated with the development of splenomegaly, autoantibody production, and glomerulonephritis. Interestingly, the lack of TGase2 prevented the production of TGF-β by macrophages exposed to apoptotic cells, which, in turn, was required for the upregulation of TGase2 in the thymus and for efficient clearance of apoptotic bodies. These studies thus suggest extensive cross-talk between apoptotic target cells and macrophages, and further emphasize the importance of corpse clearance in the prevention of autoimmunity.

Defects in macrophage clearance of apoptotic β-cells could contribute to the initiation of insulin-dependent (autoimmune) diabetes. Peritoneal and bone marrow-derived macrophages from diabetes-prone NOD mice are defective for engulfment of apoptotic cell corpses (O'Brien et al., 2002). In addition, NOD macrophages react aberrantly to both necrotic and apoptotic cells, with secretion of inappropriately high amounts of pro-inflammatory cytokines, including TNF-α (Stoffels et al., 2004). However, treatment of NOD mice with a superoxide dismutase (SOD) mimetic results in a reversal of macrophage defects in phagocytosis and cytokine production (Haskins et al., 2003). The latter findings are of considerable interest and suggest that defects in corpse clearance are amenable to pharmacological intervention *in vivo*. Finally, recent studies have disclosed that macrophages from human SLE patients display an impairment in phagocytosis of apoptotic cell; it was suggested that persistently circulating apoptotic "waste" (debris) may serve as immunogen for the induction of autoreactive responses in these individuals (Herrmann et al., 1998).

3.3 Cancer

3.3.1 Tumor-host cell interactions

Tumor-associated macrophages (TAMs) represent a major component of host cell infiltrates in malignant tumors *in vivo* (Mantovani et al., 1992; Opdenakker and Van Damme, 1992; Klein and Mantovani, 1993). These cells exert pleiotropic functions and have a complex (sometimes symbiotic) relationship to the neoplastic cells of the tumor; hence, TAMs have been

suggested to affect tumor growth by influencing the proliferation of cancer cells, and promoting vascularization, but they can also kill cancer cells directly and/or elicit tumor-destructive reactions in collaboration with other components of the immune system. Early studies on the macrophage content of tumors suggested that infiltration of macrophages is associated with an effective immune response of the host to the tumor (Eccles and Alexander, 1974). However, other investigators have provided evidence that the macrophage content of tumors is regulated by tumor-derived chemotactic factors (Bottazzi et al., 1983; Graves et al., 1989). In addition, a recent study has shown that the infiltration rate of macrophages in malignant lymphoma depends on the rate of proliferation of the tumor; of interest, the degree of phagocytosis of apoptotic cells was reduced in malignant lymphoma as compared to normal lymphoid tissue (Hermann et al., 1998). These investigators proposed that the lack of efficient clearance of apoptotic tumor cells in certain lymphoma sub-entities might be due to a defective expression of recognition ("eat me") signals, rather that an over-burdening of phagocytes by a high cellular turnover in these tumors.

A classic example of macrophage infiltration in tumors is the "starry sky" appearance of Burkitt lymphoma (BL), representing scattered macrophages that have engaged in phagocytosis of cell debris among proliferating lymphoma cells. Fujita et al. (2004) have provided evidence that Epstein-Barr virus (EBV)-infected BL cells in the so-called lytic viral cycle, which eventually lapse into cell death, are phagocytosed prior to their rupture by macrophages that have migrated into the parenchyma of the tumor. This mechanism of cell clearance, which constitutes an example of a beneficial interaction between host and tumor cells, might serve to prevent EBV from spreading beyond infected lymphoma cells. In contrast, tumors may sometimes subvert the host response and take advantage of apoptotic cell effects on macrophages. Reiter and colleagues (1999) have found that exposure of macrophages to apoptotic tumor cells results in impairment of macrophage-mediated tumor defense *in vitro* and supports tumor cell growth. These findings could have implications for cancer treatment since chemotherapy-induced apoptosis of cancer cells (discussed below) might lead to a suppression of local anti-tumor reactions.

Another important class of tumor-infiltrating immune cells are the dendritic cells. DCs are antigen-presenting cells whose primary function is to monitor the environment for "danger" signals and transduce these signals to T cells. DCs are also capable of engulfing apoptotic cells, and can present antigen derived from ingested cell corpses in an MHC class I-restricted manner (Albert et al., 1998b). The question of whether recognition of apoptotic "self" induces a tolerogenic or immunogenic response remains controversial (Rovere et al., 1998; Ronchetti et al., 1999; Sauter et al., 2000;

Steinman et al., 2000; Scheffer et al., 2003). Nevertheless, the outcome of apoptotic cell engulfment by DCs has significant implications for vaccine strategies and immunotherapeutic approaches to cancer (Savill et al., 2002). In a recent *in vivo* study, Goldszmid et al. (2003) showed that DCs loaded with cells undergoing apoptosis are able to prime melanoma-specific helper and cytotoxic T cells and provide long-term protection against a poorly immunogenic tumor in mice. In addition, enforced recruitment of intratumoral DCs has been shown to suppress tumor growth, thus providing additional evidence that DCs can be manipulated *in vivo* to boost anti-tumor immunity (Fushimi et al., 2000). Indeed, recent results obtained in two different murine models support the notion that the number of tumor-associated DCs as well as the tumor milieu determines the ability of tumor-bearing hosts to mount an effective immune response (Furumoto et al., 2004).

3.3.2 Horizontal gene transfer

Horizontal gene transfer has been described in bacteria and fungi and is thought to play an important role in the generation of resistance to antibiotics as well as to the adaptation of microorganisms to new environments (Jain et al., 2002). The range and frequencies of horizontal gene transfer in higher organisms are often constrained by selective barriers (Kurland et al., 2003). However, *in vitro* transfer of DNA from bacteria to somatic cells has been demonstrated (Darji et al., 1997). Moreover, recent studies from two laboratories have shown that tumor cells are able to engulf apoptotic bodies and re-utilize the salvaged DNA, suggesting that horizontal (or lateral) transfer of genetic information between somatic cells may, indeed, occur. Propagation of intact genes through phagocytosis of apoptotic cells is counterintuitive, given the fact that the DNA of apoptotic cells is degraded into oligonucleosomal fragments (Wyllie, 1980). Nevertheless, Holmgren et al. (1999) observed that co-cultivation of cell lines containing integrated copies of EBV resulted in rapid uptake and transfer of EBV DNA as well as genomic DNA to the nucleus of the engulfing cell. In a subsequent study, these investigators provided evidence for horizontal transfer of oncogenes and cellular transformation upon uptake of apoptotic bodies (Bergsmedh et al., 2001). Importantly, no transformation was detected in the engulfing cell when apoptotic bodies were cultured with recipient cells harboring intact p53, indicating that p53 may protect normal cells from incorporation of "foreign" DNA. Indeed, p53 activation of the *p21* (*Cip1/Waf1*) gene was shown to block the propagation of horizontally transferred DNA in normal cells (Bergsmedh et al., 2002). In contrast, feeding apoptotic bodies derived from a rat fibrosarcoma to p21-deficient murine embryonic fibroblasts

resulted in focus formation *in vitro* and tumor growth *in vivo* in a SCID mouse model. In an independent study, de la Taille and colleagues (1999) demonstrated that prostate cancer cells exchange and propagate drug resistance genes *in vitro* through the engulfment of apoptotic bodies; these investigators termed this phenomenon "apoptotic conversion."

These data suggest that transfer of oncogenes through the engulfment of apoptotic cell remnants may transpire within a tumor cell population, thus providing a novel mechanism for the propagation of genetic instability and/or diversity in tumors. Such events may be of particular relevance in cases of high frequencies of apoptosis within a tumor, such as conditions of hypoxia or restricted angiogenic support (Holmgren et al., 1995; Graeber et al., 1996). In addition, the aforementioned experimental findings may have clinical implications, since treatment of tumors with chemotherapeutic agents or radiation therapy induces apoptosis of cancer cells, thus likely enhancing the horizontal gene transfer effects. Indeed, horizontal passage of genetic defects (eg. defects in the apoptosis machinery of a cell) may accelerate the acquisition of drug resistance in cancer cells. Horizontal gene transfer could thus be considered as a means of adaptation of the tumor to its environment, analogous to the adaptation of microorganisms to changes in growth conditions. Further studies are required to determine the *in vivo* role of horizontal transfer, or apoptotic conversion, of engulfed DNA, and the putative role of this event in chemoresistance.

3.3.3 The "buried alive" hypothesis

The observation that anti-neoplastic drugs may trigger apoptosis of cancer cells was first reported by Searle et al. (1975). Today, we know that a wide variety of chemotherapeutic agents induce apoptosis in tumor cells (Makin and Dive, 2001). However, classic chemotherapeutic agents are associated with tumor cell resistance, toxicity due to bystander effects, and occasionally secondary neoplasia. We have recently presented a provocative hypothesis that enforced phagocytosis in the absence of a death signal may serve as an efficient means of deleting cancer cells without the associated bystander effects observed during conventional treatment (Fadeel et al., 2004b). In other words, we propose that cancer cells may be "buried alive" upon exposition of appropriate macrophage recognition signals.

Schroit and colleagues (1985) have shown that red blood cells containing an exogenous PS analogue in their plasma membrane are rapidly cleared from the peripheral circulation of syngeneic mice, suggesting that viable cells can be engulfed if they express appropriate "eat me" signals. Moreover, our *in vitro* studies have provided further impetus for this proposal. For instance, *N*-ethylmaleimide (NEM) treatment of the BL cell line, Raji, not

only triggers PS oxidation and externalization, but also results in the efficient engulfment of these cells by macrophages, despite the absence of other indices of apoptosis, such as caspase activation and DNA fragmentation (Kagan et al., 2002). Furthermore, enrichment of the plasma membrane of non-apoptotic tumor cells of lymphoid (Raji, Jurkat) and myeloid (HL-60) origin with exogenous PS and/or PS-OX suffices to induce macrophage engulfment of these cells. In line with these findings, earlier studies have shown that undifferentiated murine erythroleukemic cells and certain tumorigenic cell lines of human origin express increased amounts of PS on the cell surface while in a viable state; PS expression correlated with recognition and tethering by macrophages (Connor et al., 1989; Utsugi et al., 1991). Moreover, transfection of the phagocytosis receptor CD36 into human melanoma cells was reported to confer an increased capacity to ingest apoptotic cells, comparable to that exhibited by professional phagocytes (macrophages) (Ren et al., 1995). Taken together, these data support our hypothesis that the expression of recognition signals (such as PS and/or PS-OX) on target cells, as well as the enforced expression of their cognate receptors on phagocytic cells, could enhance clearance of "un-dead" cancer cells. Indeed, one could take this a step further and propose a model whereby the concomitant induction of phagocytic receptors and recognition signals in cancer cells might result in self-engulfment of the tumor, i.e. cancer clearance by the cancer itself (Fadeel et al., 2004b). Recent *in vitro* studies indicate that cancer cells are capable of devouring their homotypic, apoptotic neighbors; interestingly, the efficiency of engulfment was determined, in part, by the dying cells themselves (Simamura et al., 2001; Wiegand et al., 2001). Future studies are needed to determine whether the "buried alive" hypothesis also holds true for *in vivo* models of tumor clearance.

3.3.4 Autophagy (self-digestion)

A tremendous amount of information has accumulated in the last decades regarding the molecular mechanisms that govern apoptotic cell death. However, programmed cell death (PCD) does not always occur by apoptosis (Schwartz et al., 1993; Sperandio et al., 2000). Indeed, alternative types of PCD and cell clearance were classified some 30 years ago, based on their respective morphologies (Schweichel and Merker, 1973). Hence, type I PCD corresponds to "classical" apoptosis and is usually caspase-dependent; apoptotic cells are swiftly removed by neighboring cells or macrophages prior to the rupture of the plasma membrane, as discussed above. Type II PCD or autophagic cell death, on the other hand, refers to self-digestion of cellular components through the lysosomal system of the same cell; in other words, these cells are essentially "cannabilized" from inside. Type III PCD

is defined as non-lysosomal cellular degradation, and will not be discussed further in the present review. Importantly, cells undergoing autophagy seem to contain the machinery that is needed both to activate cell death and to degrade the dying cell (a process that occurs largely in the engulfing cell during apoptosis). Moreover, while apoptosis typically affects scattered single cells, autophagy is often observed when groups of contiguous cells or entire tissues die (Wyllie et al., 1980; Baehrecke, 2002). However, both forms of PCD appear to play a role during normal development; furthermore, recent studies indicate that these two types of PCD may be intricately connected also at the molecular level (Cohen et al., 2002).

Recent data suggest a role for autophagy in cancer development (for a recent review, see Gozuacik and Kimchi, 2004). Liang et al. (1999) have shown that *beclin 1* (the mammalian homolog of the yeast autophagy gene, *Apg6*) triggers autophagy in human breast carcinoma cells; this autophagy-promoting activity of beclin 1 was associated with inhibition of cellular proliferation *in vitro*, and with tumorigenesis in nude mice. To investigate whether *beclin 1* acts as a tumor suppressor and whether loss of *beclin 1* would contribute to an increased incidence of cancer, these investigators, and another independent laboratory, recently generated *beclin 1*-deficient mice (Qu et al., 2003; Yue et al., 2003). These studies demonstrate that loss of *beclin 1* is associated with a reduction in autophagic vacuole formation, that beclin 1-mediated regulation of autophagy is required for normal development, and that animals with reduced levels of beclin 1 display a pronounced increase in hematopoietic and other malignancies. Hence, *beclin 1* acts as a tumor suppressor gene, and autophagic cell death (self-digestion) appears to be an important means to prevent cellular transformation (Edinger and Thompson, 2003).

Some cancer cells respond to chemotherapeutic agents and irradiation by undergoing autophagy, rather than apoptosis (Bursch et al., 2000; Kanzawa et al., 2003; Paglin et al., 2003). These data thus indicate the potential utility of autophagic cell death induction in the treatment of cancer (Okada and Mak, 2004). However, it is important to consider the recent observation that caspase inhibition can trigger beclin 1-dependent, autophagic cell death (Yu et al., 2004). These findings indicate that therapeutic caspase inhibition could have the untoward effect of exacerbating cell death by activating an alternative program of cell degradation. Further studies are thus needed to investigate the putative "molecular switches" between type I PCD and type II PCD, and the role of such molecules in cancer development. Moreover, the design of cancer therapies capable of targeting the machinery of both apoptotic and autophagic cell death may be particularly advantageous.

4. CONCLUDING REMARKS

An appreciation of the molecular mechanisms that regulate the attraction, recognition, and degradation of apoptotic cells has emerged in recent years (Figure 1). The importance of this programmed cell clearance for the resolution of inflammation and the prevention of autoimmune responses is also evident. However, there is a paucity of studies on the mechanisms and consequences of clearance of cancer cells; in particular, our understanding of cancer cell clearance *in vivo* remains limited. Exciting recent studies show that horizontal transfer of genetic information may occur through engulfment of apoptotic bodies within a tumor population (so-called apoptotic conversion). However, more mechanistic studies of cancer cell clearance are required, and may unveil novel targets for cancer treatment. Another emerging theme is that cell clearance is not a passive event. Macrophages play a crucial role in the remodeling of tissues, not only as scavengers of apoptotic debris, but also through the active induction of apoptosis in certain tissues (Lang and Bishop, 1993). Moreover, even the dying cell itself may contribute to tissue homeostasis by promoting compensatory proliferation in response to cell death (Huh et al., 2004). The latter findings thus suggest that dying cells may communicate cell fate or behaviour instructions to their neighbors. Finally, evidence has accrued for alternative, non-apoptotic forms of cell death, including autophagy (self-digestion), so-called paraptosis, and necrosis-like PCD (Leist and Jäättelä, 2001). Future studies should focus on the molecular regulation of these divergent modes of cell demise, and on the harnessing of these signaling pathways for therapeutic purposes in the treatment of cancer and other diseases.

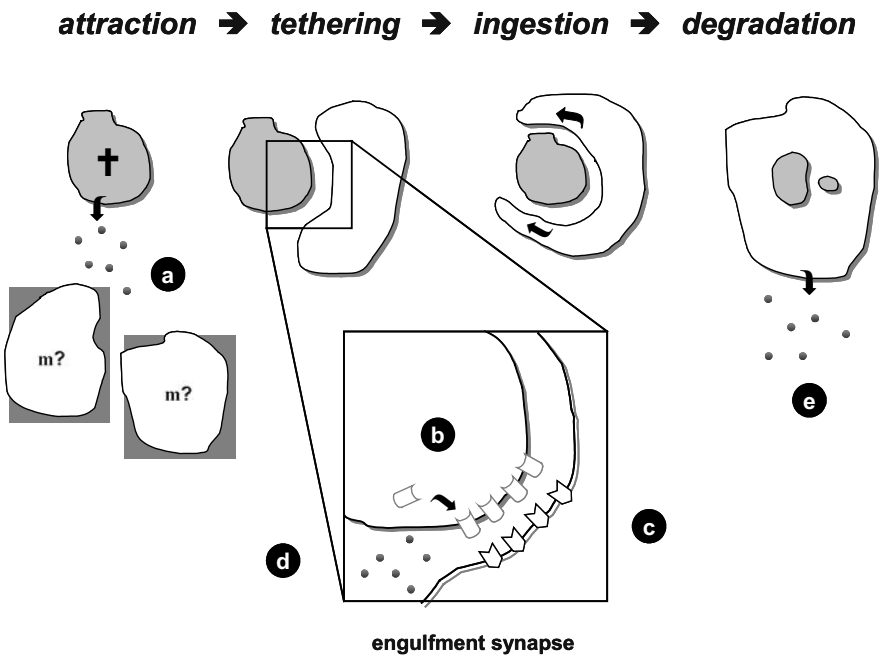

Figure 1. Programmed cell clearance.Mammalian cells dying by apoptosis emit chemotactic factors (a) that serve to recruit macrophages to the site of cell attrition. The engulfment of apoptotic cells by phagocytes is regulated by numerous recognition signals (b) on the apoptotic cell corpse and corresponding receptors (c) on the engulfing cell; soluble bridging molecules (d) serve to facilitate this recognition process. In addition, repulsion signals on the apoptotic cell are disabled, thus preventing the active rejection of phagocytes. Recent studies suggest that recognition involves an initial step of tethering (attachment) with subsequent ingestion of cell corpses; the engagement of multiple receptor-ligand pairs within the so-called engulfment synapse ensures the specific interaction between phagocyte and apoptotic prey. Following ingestion and degradation of apoptotic cells, macrophages produce cytokines (e) that contribute to the resolution of inflammation and thus precludes scarring of the surrounding tissue. Consult text for details.

We call it a grain of sand,
but it calls itself neither grain nor sand.
It does fine without a name,
whether general, particular,
permanent, passing,
incorrect, or apt.

(Wislawa Szymborska)

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