Carcinogenesis-relevant biological events in the pathophysiology of the efferocytosis phenomenon

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Abstract

The effective removal of cells undergoing programmed cell death, which is referred to as *efferocytosis*, prevents the leakage of intracellular contents into the surrounding tissue, which could lead to tissue damage and inflammation. Efferocytosis involves a coordinated orchestration of multiple steps that lead to a swift, coherent and immunologically silent removal of dying cells. The release of wound healing cytokines, which resolve inflammation and enhance tissue repair, is an important feature of efferocytosis. However, in addition to the healing cytokines released during efferocytosis, the immunosuppressive action of cytokines promotes the tumor microenvironment, enhances the motility of cancer cells and promotes the evasion of antitumor immunity. The aim of the present review was to comprehensively discuss the efferocytosis phenomenon, the important players associated with this process and their role in cancer-related biological events.

Introduction

Apoptosis or programmed cell death plays an essential role in the regulation of tissue growth and thus maintains tissue homeostasis. Cell overproduction, which occurs by mitotic activity during the embryonic and adult life, is counterpoised by programmed cell death.[1] Eliminating such apoptotic cells is critical for several processes in a multicellular organism such as development, tissue differentiation and response to injury.[2] However, the process of eliminating unwanted cells requires strict regulation, and any disruptions in this process may lead to a state of disease.[3] Thus, proper recognition and elimination of dying cells is important to prevent the disruption of the cell membrane integrity and consequent leakage of its contents into the surrounding tissue.[4]

The cytoplasmic contents of an apoptotic cell serve as a primordial *biohazard* that has the ability to wreak extensive tissue damage due to their cytotoxic nature and ability to initiate inappropriate inflammatory responses and eliminate autoantigens.[5,6] Certainly, negligence of the removal of apoptotic cells has been associated with the development of several chronic inflammatory and autoimmune diseases, including systemic lupus erythematosus, retinitis pigmentosa, cystic fibrosis, bronchiectasis, COPD, asthma, idiopathic pulmonary fibrosis, rheumatoid arthritis, glomerulonephritis and atherosclerosis.[7] Thus, it is crucial to identify and remove apoptotic cells with immunological stealth to maintain tissue homeostasis by counterbalancing cell death.[3]

This ability to effectively remove dying cells that have unique morphological features and downstream consequences has been termed *efferocytosis* by Decathelineau and Henson.[3] The present article sheds light on the significance of efferocytosis, the molecular mechanism involved and the consequences of ineffective efferocytosis in various pathological conditions including cancers, particularly oral cancers.

The term efferocytosis stems from the Latin word *efferre*, which translates *to take to the grave* or *to bury* and is the complex process by which apoptotic/dying cells are eliminated by phagocytic cells.[3] This swift and effective elimination of apoptotic cells is essential for creating space for biological cells and perpetuating the function of the tissue and, in turn, a healthy organism.[6,8,9] The dying cells that are not efficiently eliminated can be subjected to secondary necrosis and release of intracellular contents, leading to various pathologies.[5,9,10]

The identification of apoptotic cells in a tissue gives a snapshot of the harmony between the proliferation, apoptosis and clearance of apoptotic cells.[11] Elucidating the distinct steps involved in efferocytosis is crucial for increasing our knowledge about diseases related to inefficient clearance and for potentially manipulating efferocytosis for future therapeutic advantages.[9]

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Pathogenesis

Efferocytosis is largely carried out by certain specific cells such as macrophages and immature dendritic cells.[12] These professional cells express specific receptors for classical phagocytic opsonins. However, most eukaryotic cells are capable of performing latent and very primitive types of phagocytic activity. Such non-professional cells include epithelial cells,[13] endothelial cells[3] and fibroblasts.[13] The process of efferocytosis is very much similar to macropinocytosis. In response to certain stimuli, phagocytic cells engulf dying cells and fluid into a heterogenous vesicle referred to as an efferosome, which is approximately 0.2-2 micrometers in diameter. However, since the efferosome of macropinocytosis is not larger than 2 micrometers, it is not capable of entrapping a whole apoptotic cell. Therefore, the aggregation of lipid rafts at the interface of the apoptotic cell and the phagocyte during efferocytosis may serve to recruit and accumulate apoptotic-cell-receptor complexes and signaling molecules to promote a robust signal to drive particle engulfment, whereas pseudopod extension and uptake during phagocytic *zippering* is localized and driven by sequential ligand-receptor-binding events. Thus, it can be hypothesized that efferocytosis is a hybrid mechanism that includes both macropinocytosis as well as a specialized zippering mechanism.[3]

Molecular mechanism of efferocytosis

The rapid, well-organized and immunologically silent removal of apoptotic cells involves a tight coordination of multiple steps. Ravichandran KS divided efferocytosis into four major steps: i) release of *find-me* signals by the apoptotic cells;[14] ii) specific recognition of the dying cell;[4] iii) engulfment and degradation of the dying cells;[15,16] iv) post-engulfment consequences.[5,6,8]

Release of find-me signals by the apoptotic cells

Genetic studies in *Caenorhabditis elegans* indicated that phagocytic cells identify and engulf apoptotic cells prior to the completion of cell death.[17,18] Ravichandran[19] proposed the concept that apoptotic cells release certain mediators known as *find-me* signals that attract phagocytes and cause rapid cell clearance. Several studies postulated the release of potential find-me signals by apoptotic cells, including lysophosphatidylcholine, fractalkine, sphingosine 1-phosphate (S1P) and nucleotides ATP and uridine 5'-triphosphate (UTP). Although these find-me signals have the ability to attract monocytes in vitro, only fractalkine and nucleotides have been shown to function as find-me signals *in vivo*.[20,21] These find-me signals are released from intact apoptotic cells, with no leakage of cellular contents, and are released in a caspase-dependent manner. The release of such find-me signals creates a chemotactic gradient that attracts the phagocytes; the range of these signals is determined by the tissue concentration of a given find-me molecule and its ability to degenerate. Depending on these factors, the find-me signals can attract phagocytes from local tissue (short-range find-me signals) or from the body circulation (long-range find-me signals). Thus far, only short-range find-me signals have been identified. The possibility of the release of multiple find-me signals from the same apoptotic cell and the synergistic effect of these signals needs to be explored.[9] It is well established that efferocytosis involves non-immunogenic clearance of apoptotic cells *via* monocytes in the absence of neutrophils and inflammation.[6,22] Bournazou *et al.*[23] proposed that apoptotic cells release certain Stop signals, such as lactoferrin, that intercept neutrophil recruitment.

Specific recognition of apoptotic cells

The professional or non-professional phagocytic cells recognize apoptotic cells through several ligands and receptors known as apoptotic cell-associated molecular patterns (ACAMPs). Phosphatidylserine (PS) is an important *eat-me* signal that is expressed on apoptotic cells.[24] PS is confined to the inner leaflet of the plasma membrane in a normal viable cell, and if any PS is exteriorized, it is flip-backed by the action of aminophospholipid translocases. Apoptosis leads to an increase in the PS flip-flop activity across the plasma membrane and the inactivation of translocases, thus causing PS to be permanently exposed on the outer leaflet.[25,26] The soluble bridging molecules and/or receptors present on phagocytic cells recognize these PS molecules on the apoptotic cell surface.

Calreticulin, a protein that is normally found in the endoplasmic reticulum, is present at increased levels and/or is redistributed on apoptotic cells and serves as another ligand for apoptotic cell recognition.[27] Glycosylated cell surface proteins and/or altered surface charge of an apoptotic cell have also been implicated as apoptotic ligands and signaling molecules.[28,29] A number of candidate receptors and groups of bridging molecules recognize the PS on the apoptotic cell surface. Some of these crucial receptors are T-cell immunoglobulin and mucin domain-containing molecules (TIM4 and TIM1), brain-specific angiogenesis inhibitor (BAI)-1 and stabilin-2.[30-32] The *PS receptor* was described in the past as a chief receptor in apoptotic cell recognition;[33] however, Bose *et al.*[34] and his fellow workers decline any participation of this receptor in efferocytosis.[35] CD36, a class B scavenger receptor, is another apoptotic cell receptor.[36,37] Some other possible apoptotic cell receptors include CD14,[38] class A scavenger receptor,[39] and CD68.[40] Milk fat globule EGF8 (MFG-E8)[41] and growth arrest-specific 6 (Gas6)[42] are some of the important PS-binding bridging molecules. The collectin protein family of the innate immune system, which includes mannose-binding lectin (MBL), C1q, surfactant protein (SP)-D and -A and adiponectin, facilitates efficient engulfment of the dying cell by acting as a bridging molecule between calreticulin and the phagocyte.

In addition, the *do not eat-me* signals that normally protect viable cells from inappropriate uptake into phagocytes may detach from the apoptotic cells. Gardai *et al.*[2,43] and Brown *et al.*[44] proposed that CD47 and CD31 constitute *do not eat-me* signals on the surface of normal cells that prevents their phagocytosis.[11]

Engulfment and degradation of the dying cell

After recognition of the apoptotic cell, its internalization occurs through cytoplasmic rearrangement of the engulfing cell.[11] Internalization signals seem to modulate the maturation of the phagosome, certain post-engulfment responses of the phagocyte and other essential biological outcomes. Based on studies in mammals and the simple nematode model, two partially redundant pathways are recognized as playing advanced roles in efferocytosis. In the first pathway, the proteins CED-2 (CrkII), CED-5 (Dock180) and CED-12 (ELMO) activate the protein CED-10 (Rac1)[45,46] downstream of BAI1 (a G-protein-coupled receptor)[31] and potentially integrins[47] in mammals. In the second pathway, the candidate receptor CED-1 (MEGF10/LRP1) binds to an unknown ligand on the apoptotic cell and signals, via its cytoplasmic tail, to the adaptor protein CED-6 (hCED-6/GULP),[48,49] whereas CED-7 (ABCA1) is thought to play a role in membrane dynamics.[15]

After internalization, the particle undergoes maturation through a series of acidified membrane-bound organelles, which are called phagosomes.[50] The degraded proteins from the target cells are cross-presented by MHC class 1 molecules and are tolerogenic. In phagosome maturation, the particle-containing phago-

some matures into an increasingly acidic membrane-bound structure, leading to the formation of an acidic phagolysosome. This acidic phagolysosome then fuses with lysosomes. Proteins that are required for phagosome maturation have been identified through recent genetic studies in *Drosophila*, *Dictyostelium* and *Caenorhabditis*. A pathway for maturation of apoptotic cell-containing phagosomes has been developed through studies in nematodes. During maturation, the proteins that are present on the intracellular face of the phagosomal membrane are altered. Upon internalization, the phagosome is coated with GTPase Rab5,[51] which is later replaced by Rab7[51,52] and then ultimately by the lysosomal marker LAMP-1.[53,54] The complex process of phagosome maturation requires a series of GEFs (Guanine Nucleotide Exchange Proteins),[15] GAPs (GTPase Activating Proteins) and effectors.[15] During the Rab7(+) stage, phagosome maturation is regulated by the HOPS complex, which is a Rab7 activator and effector.[15]

Post-engulfment consequences

Several consequences of the engulfment of apoptotic cells may also be influenced by signaling from the phagosome. First, the elimination of the apoptotic cells has been associated with increased release of pro-healing cytokines such as TGF-beta[55] and IL-10.[56] These cytokines reduce inflammation from the surrounding environment and thus assist in wound healing. Phagocytic receptors such as stabilin-2[32] and CD36[56] may alter the secretion of cytokines. Second, recent studies have shown that efferocytosis of dying cells leads to cholesterol efflux from the cell, which can be impaired by activation of the nuclear receptor LXR, thus leading to amplified transcription of the transporter ABCA1.[57] Third, the degraded protein constituents of the cell are cross-presented by MHC class 1 molecules[58,59] and are usually debarred from class 2 presentation.[60] The self-tolerance of immunity during efferocytosis is essential and has been attributed to apoptotic cell-associated antigen.[61] Potential specific pathways for DNA degradation in efferocytosis have been described in both nematode[62] and mouse models.[63] Efficient phagocytosis is thus important for the health of organism, and any disruptions in the process can lead to autoimmune diseases.[15]

Epigenetic regulation and efferocytosis

Over the last several decades, the status of gene expression has been widely associated with epigenetically controlled processes such as DNA methylation and covalent chromatin modifications including acetylation, phosphorylation, ubiquitination, sumoylation, and methylation of histones. Among covalent modification of histones, the well-accepted process of histone methylation is viewed as dynamic changes that are mediated by a set of dedicated enzymes such as histone methyltransferases and demethylases.[64-66] Among the class of histone methylation enzymes, the polycomb repressive complex has been reported to contain the H3K27 methyltransferase Ezh2 and to mediate dimethylation and trimethylation of H3K27 (H3K27me2/3). Another histone demethylase enzyme is the Jumonji domain-containing protein 3 (Jmjd3, also called as KDM6B), which has been reported to act as a H3K27 demethylase that catalyzes the demethylation of H3K27me2/3.[65]

Currently, there are growing views that epigenetic enzymes can act as important modulators of macrophages, which is central to the outcome of many inflammatory diseases.[67] Further, epigenetic-modifying enzymes have been suggested to be regulated by the metabolic signature of macrophages such as acetyl-coenzyme A, S-adenosylmethionine and α-ketoglutarate. It has been suggested that reprogramming intracellular metabolism pathways, including high glycolysis for M1 macrophages and high oxidative metabolism for M2 macrophages, can be crucial for the proper polarization and functions of activated macrophages.[68] A previous study supported the possible implications of epigenetic regulation in altering macrophage metabolism and macrophage activation and, thus, influencing the outcome of human disease.[69,70] Recently, Yildirim-Buharalioglu *et al.*[71] provided evidence to establish a direct link between IFN-γ- or IL-4-mediated upregulation of the histone demethylase, KDM6B, which facilitates macrophage polarization.

Recent studies reveal convincing evidence to support the involvement of epigenetic players in modulating the necessary signaling events that promote macrophage polarization and inflammation.[64,72,73] Among such epigenetic players, JMJD3, a JmjC family histone demethylase, has been shown to be crucial for M2 polarization and anti-inflammatory macrophage polarization by regulating JMJD3 expression, which supports epigenetic therapeutic strategies in human diseases including obesity [64,66]. Satoh *et al.*[74] suggested that Jumonji domain-containing protein 3 (Jmjd3) can perform H3K27 demethylation, which requires the regulation of M2 macrophage polarization and development leading to anti-helminth host responses. In additional evidence linked to the anti-inflammatory abilities of macrophages, Van den Bossche *et al.*[75] indicated that inhibition of histone deacetylases (HDACs), particularly HDAC3, can be linked to the inflammatory response mechanisms of macrophages and may be a novel strategy for improving atherogenic macrophage activities.

Noda *et al.*[76] reported that inhibition of histone deacetylase (HDAC) using small molecule inhibitors such as trichostatin A (TSA) can result in the reduction of efferocytosis, which is somewhat similar to the effects caused by cigarette smoke extract (CSE). This study confirmed that smoking can hamper efferocytosis via disruption of HDAC/Rac/CD9 pathways. The sphingosine-1 phosphate receptor 5 (S1PR5) has been linked to the induction of alveolar macrophages to phagocytose apoptotic cells. There is a report on the potential epigenetic-based decreased DNA methylation in the target gene S1PR5 in COPD patients that was found to be responsible for higher expression in healthy individuals.[77]

Efferocytosis and carcinogenesis

Phagocytic engulfment of apoptotic cells, in addition to cytokine modulation that targets immune suppression, ensures that efferocytosis does not induce inflammation and tissue damage.[78] Further, on the role of efferocytosis in maintaining tissue homeostasis, studies have shown that efferocytosis may lead to the development of a more malignant tumor microenvironment and tumor progression.[79,80] It is well established that impaired clearance of dying cells encourages disease states; however, unimpaired efferocytosis can promote cancer. Cytokines associated with wound healing and immune suppression promote the tumor microenvironment, enhance tumor cell motility and facilitate evasion of anti-tumor immunity. In addition, the overexpression of several receptors and ligands involved in the process of efferocytosis has been demonstrated to play a specific role in tumorigenesis.[79,81,82] Overexpression of Tyro-3, Axl, MerTK and their PS-binding ligands Gas6 and ProS1 has been linked with several cancer types and the tumor microenvironment.[81,83] Activation of Axl through PI3K/Akt and MAPK/ERK signaling pathways aids in the proliferation and survival of tumor cells.[81,82] Activation and/or overexpression of MerTK in tumor cells boosts oncogenic signal-

ing pathways such as JAK/STAT, PI3K/Akt, Src/FAK and MAPK/ERK, which contributes to tumor cell survival, proliferation and metastasis.[80,81] Efferocytosis triggers the release of wound healing and immunosuppressive cytokines such as IL-10, IL-13, IL-4 and TGF-beta1 while suppressing pro-inflammatory cytokines such as IL-12 and IFN-gamma. Along with expression of MerTK within the macrophages of the tumor microenvironment, these events support immune suppression and tumor metastasis.[81,84]

Efferocytosis and carcinogenesis-relevant receptors

The receptor tyrosine kinase family of cell surface receptors have been well-established as playing a crucial role in transmitting growth and proliferation signals from the outside to the inside of cancer cells. Among several classes of receptor tyrosine kinase proteins, Tyro3, Axl, and MerTK (collectively TAM receptors) have been described as three homologous receptor tyrosine kinases with the affinity to bind vitamin K-dependent endogenous ligands, Protein S (ProS) and growth arrest-specific factor 6 (Gas6).[80,85] Further, accumulating evidence suggests that these TAM receptors and their ligands establish signals to promote clearance of apoptotic cells, which is phosphatidylserine (PS)-mediated-based signals. Additionally, these TAM receptors have been linked to the issue of chemoresistance and have been shown to be overexpressed in several types of tumor. In one study, Kasikara *et al.*[85] revealed an indirect link between TAM receptors, apoptosis and efferocytosis and suggested the use of anti-PD-L1 and anti-Tam receptor could promote apoptosis and resolve the issue of chemoresistance. Nguyen *et al.*[80] also suggested overexpression of MERTK, a member of TAM receptor in epithelial cancer, and knockdown of MERTJ could hinder apoptotic cell clearance.

There are reports on the use of monoclonal antibodies such as GMAB1 and GMAB2 to neutralize Gas6 ligand, which binds to the AXL receptor and is involved in blocking apoptosis and cancer growth in pancreatic ductal adenocarcinoma (PDAC).[86,87] Kirane *et al.*[88] also emphasized another approach by using warfarin, which has been shown to control Gas6-mediated activation of Axl in PDAC and may block plasticity and metastasis. In line with the potential therapeutic interference of TAM receptors, Kimani *et al.*[89] reported on the small molecule inhibitors (RU-301 and RU-302) for their affinity to target the extracellular domain of Axl at the interface of the Ig-1 ectodomain of Axl and the Lg-1 of Gas6.

Efferocytosis, tumor microenvironment and tumor progression

Two major types of macrophages have been identified with other polarized states.[90] *Classically activated* M1 macrophages are activated by inflammatory mediators such as GM-CSF and IFN-gamma. This activation promotes M1 polarization, causes the release of Th1 pro-inflammatory cytokines such as CXCL19 and CXCL10, IL-12, IFN-gamma, plays a role in antigen presentation and encourages an anti-tumor response.[91] However, M2 macrophages, more specifically M2c polarized subtypes, are involved in efferocytosis.[92] M2 macrophages release Th2 cytokines such as IL-10, IL-13, IL-4, TGF-beta1, CCL17, CCL22 and CCL24 and trigger anti-inflammatory responses and pro-tumorigenic activity.[91] M2 polarized macrophages are typically associated with cancer, have been found to promote cell growth and recruitment through the production of IL-6, TNF-alpha, IL-23 and may promote tumor development through immunosuppressive effects via the release of TGF-beta and IL-10.[93]

Evasion and suppression of the host immune system play a crucial role in malignant tumor progression.[94] Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells that maintain normal tissue homeostasis and function in response to infection or stress.[95,96] These cells promote tumor vascularization[96] and alter immune mechanisms such as antigen presentation by dendritic cells[97] and T-cell activation.[93,97] IL-10 and VEGF released during efferocytosis influences development and MDSC regulation, which suggests the role of these cytokines in the immunosuppressive effects of MDSCs.[98]

Efferocytosis and oral cancers

The occurrence of efferocytosis in OSCC is a well-reported phenomenon. Various studies have well characterized the ability of non-phagocytic cells, such as epithelial cells, to perform efferocytosis. Sarode[99] reported evidence of apoptotic cancer cells being engulfed by malignant cells of OSCC. The epithelial tumor cells revealed signs of efferocytosis in that the nuclei were pushed to the cell periphery and the presence of a clear halo surrounding the ingested apoptotic tumor cell, suggesting the formation of efferosome. Evidence of both the partial and complete engulfment of apoptotic cancer cells has been reported.[99]

Future direction and conclusions

Future experimental approaches should test the hypothesis to show whether components from chewing tobacco and other biological and non-biological potential carcinogens can modulate macrophage-mediated efferocytosis in premalignant and malignant lesion tissues. Previous studies reported that cigarette smoking components and certain toxins secreted from bacteria can obstruct macrophage-mediated efferocytosis and can cause severe physiological disturbances.[76,100] On the other hand, there are potential scopes for future studies in using pharmacological inhibitors to inhibit specific growth receptors such as Tyro3, Axl, and MerTK (TAM), which comprise a unique family of receptor tyrosine kinases that may disrupt the efferocytosis signaling pathway.

Efferocytosis is an evolutionarily conserved phagocytic process that protects against immunity to self-antigens. As such, it is important to have a thorough understanding of the innate and adaptive immune responses. Unchecked cellular proliferation and/or uncurbed inflammation gives rise to disease pathogenesis. Failed or unattempted efferocytosis leads to tissue damage and disease progression. Additionally, the role of efferocytosis in establishing the tumor microenvironment, tumor development and metastasis should not be neglected. The use of cancer chemotherapy in the treatment of malignancies leads to overall cell death, resulting in efferocytosis. In this manner, the immune system may contribute to the recurrence and metastatic process of cancer progression. Therefore, it is important to thoroughly understand the process of efferocytosis and the immune response to discover innovative modalities for cancer prevention, treatment and cure.

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