No littering: the clearance of dead cells and leaking cellular contents and possible pathological complications

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Abstract

Introduction

Apoptosis and necrosis differ in morphological, biochemical and immunological aspects, but both eventually result in engulfment of the dead cell or its remnants by a phagocyte. Apoptotic cells can be swallowed in one gulp due to the preserved impermeability of the plasma membrane. The cells may undergo secondary necrosis and lose plasma membrane integrity if clearance does not occur fast enough.

Conclusion

Secondary necrotic cells pose several problems for the body as follows: a) Necrotic cell clearance is pro-inflammatory due to danger signals leaking out of the cell. This is in strong contrast to the anti-inflammatory clearance of apoptotic cells. Exaggerated inflammation may cause tissue damage and exacerbate the course of various diseases. b) Chronic deficiencies in apoptotic cell clearance lead to an accumulation of late apoptotic and secondary necrotic cells. The persistent accessibility of otherwise occult intracellular antigens may challenge auto-tolerance and is a hallmark of systemic lupus erythematosus. c) Intracellular macromolecules that leak out of necrotic cells have to be cleared individually. In one gulp due to the preserved impermeability of the plasma membrane.

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promoting milieu. The cytokines secreted by macrophages after stimulation with apoptotic cells not only suppress or resolve inflammation, but also inhibit the development of adaptive immune responses. For example, IL-10 and TGF-β are known to strongly interfere with the antigen presenting abilities of monocytes. Interestingly, ‘eat me’ and ‘tolerate me’ signals are mediated independently, even though PS plays a key role in both. Engagement of the apoptotic cell is not a prerequisite for the induction of an anti-inflammatory response. The knockout of CD14, a protein required for the tethering of macrophages to apoptotic cells, impaired their phagocytic ability, but not the production of anti-inflammatory cytokines in response to apoptotic cells.

Necrosis

Necrosis is regarded as the accidental, non-physiological kind of cell death and differs from apoptosis in morphological, biochemical and immunological aspects. Its features are cytoplasmic swelling and in strong contrast to apoptosis, disintegration of the plasma membrane. This leads to the leakage of cellular content into the cells’ vicinity. Many intracellular molecules function as danger signals that attract phagocytes and induce an inflammatory cytokine profile in phagocytes employing various innate immune receptors. Among these, high mobility group box 1 protein (HMGB1) is particularly specific for the discrimination between necrotic and apoptotic cells. It is not released by apoptotic cells, because it is tightly bound to their chromatin and additionally is oxidised during apoptosis, neutralising its inflammatory effects. An inflammatory response to necrosis seems reasonable from a teleological perspective. Necrotic cell death is often caused by infections or toxic events and potentially requires the intervention by immune cells and/or the development of an adaptive immune response. In accordance with this, necrotic cells have been observed to actively produce and secrete the inflammatory cytokine IL-6. The ‘eat me’ signals displayed by necrotic cells and the processes involved in their engulfment are not as well understood as that of apoptotic ones. However, opsonisation with complement as well as PS externalisation seem to play a role. Exposed actin filaments were recently reported as an ‘eat me’ signal specific for necrotic cells.

Secondary necrosis

However, necrosis can also occur during the late phases of apoptotic cell death. Secondary necrosis is characterised by a loss of the plasma membrane integrity and cell swelling. It is the natural outcome of the apoptotic program in unicellular eukaryotes as well as in isolated cells from multicellular animals. Clearance is not the only possible fate of an apoptotic human cell in vivo, either. Secondary necrosis can occur if clearance is not completed in a timely fashion. The elaborate apoptotic cell clearance systems of multicellular animals certainly evolved under the pressure of secondary necrosis and its often deleterious effects in vivo, which will be discussed in the second part of this review. Morphologically, secondary necrotic cells combine features of apoptotic cells, like hypercondensed chromatin and nuclear fragmentation with features of primary necrosis, like cell swelling. The danger signals released by primary and secondary necrotic cells differ significantly. Secondary necrotic cells release less ATP, as it is consumed during apoptosis, but presumably higher levels of uric acid, owing to degradation of chromatin. HMGB1-nucleosome complexes as the result of internucleosomal deoxyribonucleic acid (DNA) cleavage during apoptosis and activated caspase-3 are reported to serve as specific danger signals from secondary necrotic cells.

Discussion

In this review, the authors have referenced some of their own studies. These referenced studies have been conducted in accordance with the Declaration of Helsinki (1964) and the protocols of these studies have been approved by the relevant ethics committees associated to the institutions in which they were performed. All human subjects, in these referenced studies, gave informed consent to participate in the studies.

Pathological consequences of acute clearance deficiency

In vivo apoptotic cells become secondary necrotic if they are not cleared by phagocytes in time. The lack of apoptotic cell clearance may result from various causes. The clearance capacities of the phagocytic system can get overwhelmed if a large number of cells simultaneously undergo apoptosis. For example, administration of anti-Fas antibodies, which are specific inducers of apoptosis, caused massive secondary liver cell necrosis in mice. A potentially fatal outcome of such an overwhelming demand for clearance, the tumour lysis syndrome (TLS), will be discussed below. Intrinsic deficiencies in clearance-related proteins or direct damage to phagocytes can also cause an overload of the clearance system. Secondary necrosis seems to occur mainly in pathological situations. In areas of the body, where resident macrophages physiologically are sparse, amateur phagocytes function as a back-up system. They can remove physiological amounts of apoptotic cells before they undergo secondary necrosis.

The effects of secondary necrosis can be detrimental for the organism, because danger signals leaking out through a disrupted plasma membrane shift the immunological response from silent to inflammatory. Secondary necrosis of neutrophils is particularly dangerous because it may foster a vicious circuit of cell death and inflammation (Figure 1a).
Leaking cationic proteins and acidic granule contents not only directly cause tissue damage, but also affects clearing abilities of macrophages by the interaction with recognition receptors\textsuperscript{20,25}. Both effects can contribute to further overstraining of the clearance system. Occurrence of this vicious circle was observed in several studies. For instance, mice that had been depleted of alveolar macrophages showed no higher bacterial load during \textit{Streptococcus pneumoniae} infection, as the capsulated bacteria bind only poorly to alveolar macrophages. However, neutrophils that had been recruited at the beginning of the inflammation and became apoptotic during its course could not be cleared properly. This caused secondary necrosis of the neutrophils and leakage of harmful intracellular contents. Ultimately, perpetuated inflammation led to an increase of mortality\textsuperscript{26}. Another \textit{in vivo} example for the harmful effects of secondary necrosis is lipopolysaccharide (LPS)-induced lung injury in mice. It is associated with inflammation and tissue damage and therefore, necrosis might be expected to be the predominant kind of cell death in this model. However, necrosis did not occur immediately but subsequent to apoptosis. LPS stimulated the secretion of TNF-\(\alpha\), well known to cause apoptosis in a variety of cells and also to attract neutrophils. The excessive presence of apoptotic signals resulted in the overstraining of the clearance capacities. Apoptotic neutrophils that were not cleared underwent secondary necrosis, releasing their histotoxic contents and constituted the main source of damage in this model\textsuperscript{27}.

However, apoptotic cell clearance exerts more anti-inflammatory effects than the mere prevention of secondary necrosis. The anti-inflammatory cytokines released by macrophages confronted with apoptotic neutrophils are thought to play a key role in the resolution of inflammation (e.g., in ischemic heart disease)\textsuperscript{28}. Insufficient clearance and subsequent lack of pro-resolving cytokines is prone to lead to exaggerated inflammation and in this context, this lead to adverse remodelling or heart failure following myocardial infarctions.

**Chronic clearance deficiency and systemic lupus erythematosus**

Acute clearance deficiencies or overstraining of the clearance capacities lead to secondary necrosis and exaggerated inflammation, but not per se to auto-immunity. Accordingly, injection of high numbers of apoptotic cell caused persistent auto-immunity only in mice with a susceptible background\textsuperscript{29}. Chronic deficiencies of the clearance of apoptotic cells, however, are associated with auto-immunity and in particular with systemic lupus erythematosus (SLE)\textsuperscript{21}. SLE is a chronic auto-immune disease involving the clearance of apoptotic cells.

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**Figure 1:** Implications of acute or chronic clearance deficiency and accumulation of secondary necrotic cells.

a) Acute overload of the clearance capacities or otherwise affected clearance leads to secondary necrosis of apoptotic cells. This initiates a vicious circle of inflammation and cell death, especially if neutrophils undergo secondary necrosis. Secondary necrosis renders otherwise occult intracellular antigens accessible.

b) Chronic clearance deficiencies contribute to the break of self-tolerance and the maintenance of inflammation in SLE. Secondary necrosis renders otherwise occult intracellular antigens accessible.

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characterised by the production of auto-antibodies and systemic inflammation. Apoptotic cells have been shown to accumulate in the bone marrow of SLE patients and in the skin of patients with cutaneous lupus erythematosus following ultraviolet light (UV) lesions. In the latter case, secondary necrosis, auto-antibody binding and subsequent inflammatory uptake of dead cells, contribute to the photosensitivity that is one of the 11 classification criteria for SLE of the American College of Rheumatology. Deficient clearance mechanisms play key roles in aetiology and pathogenesis of SLE (Figure 1b).

**Aetiology**

In germinal centres of lymph nodes, B-cells (BC) undergo maturation and proliferation. This leads to a high rate of apoptosis and only a small fraction of the initially generated immature BC get selected for survival. In a subgroup of patients with SLE, the resident tingible body macrophages are considerably reduced. These specialised phagocytes are responsible for the clearance of apoptotic BC. In these patients, apoptotic cell-derived material is bound to follicular dendritic cells instead and might, in turn, provide survival signals for BC specifically recognising (nuclear) auto-antigens. This pathological process persistently challenges self-tolerance, causes humoral autoimmunity and ultimately, clinical overt disease.

**Pathogenesis**

Clearance deficiency and subsequent secondary necrosis result in persistent accessibility of otherwise occult intracellular antigens. Auto-antibodies opsonise these dying cells, resulting in Fc receptor-mediated uptake by neutrophils and monocytes. This process is accompanied by the release of inflammatory cytokines. Nucleic acid leaking from secondary necrotic cells can form immune complexes (IC) with certain anti-nuclear auto-antibodies. DNA-antibody complexes, but not nucleic acid or auto-antibodies alone, induce high interferon alpha (IFN-α) production by plasmacytoid dendritic cells. Exerting various immune-modulatory effects, type I interferons reportedly play a critical role in the pathogenesis of SLE. FC receptor-mediated phagocytosis of nucleic acid containing IC and subsequent stimulation of endosomal toll-like receptor (TLR) 9 seem to be important for the induction of IFN-α production. Alternatively, undigested nucleic acid from overloaded lysosomes might leak into the cytosol and cause IFN-α production via TLR independent pathways. Of note, clearance deficiency might also occur as a consequence of a flare of the disease, for example, by complement consumption due to chronic inflammation.

**Disposal of leaking cellular components**

Apoptotic cells can be swallowed in one gulp by macrophages. Clearance of primary or secondary necrotic cells poses a greater challenge to the immune system, because intracellular content leaks into the cellular surrounding. Some of these cellular components are known to have important signalling functions. However, all waste needs to be disposed in the end. This employs a plethora of mechanisms, which we have summarised below (Figure 2).

**DNA**

DNA released from necrotic cells is, in contrast to RNA, biochemically stable and can circulate within the bloodstream. In non-pathogenic situations, DNA needs to be disposed of rapidly. In non-pathogenic situations, DNA needs to be disposed of rapidly. In non-pathogenic situations, DNA needs to be disposed of rapidly. In non-pathogenic situations, DNA needs to be disposed of rapidly. In non-pathogenic situations, DNA needs to be disposed of rapidly. In non-pathogenic situations, DNA needs to be disposed of rapidly. In non-pathogenic situations, DNA needs to be disposed of rapidly.

**Figure 2:** Mechanisms and signals involved in clearance of dead cells and disposal of cellular remnants. A plethora of mechanisms and signals contribute to clearance and disposal. Depending on the context, they occur in or the fate they meet, the involved molecules can exert diverse functions.
for DNAse I digestion. Oposinisation of chromatin from necrotic cells by the complement component C1q was shown to promote DNAse I digestion as well. For efficient uptake of degraded chromatin by monocyte-derived phagocytes, C1q was required. Oposinisation and predigestion may help phagocytes to recognise and take up DNA debris. The importance of these mechanisms in vivo is highlighted by the fact that deficiencies in the classical pathway of complement and in DNAse I are well-known predisposing factors for the development of SLE. One of the hallmarks of SLE, in turn, is anti-dsDNA auto-antibodies, which may be generated in response to residual chromatin debris in the germinal centres of lymph nodes (see above).

**Neutrophil extracellular traps**

Neutrophil extracellular traps (NETs) are a special type of chromatin that require specific disposal. NETs are composed of DNA, histones, granular enzymes and anti-microbial proteins. They are ejected by neutrophils in response to strong phagocytic stimuli in a process referred to as NETosis. This morphologically unique cell death is characterised by the production of reactive oxygen species, conversion of chromatin to NETs and ultimately, plasma membrane rupture. It is regarded an 'emergency response', as the release of neutrophil intracellular components tends to increase inflammatory tissue damage. The knowledge with regard to the disposal of NETs is still scarce, although a dependency on DNAse I was recently reported. In contrast to its function in degradation of chromatin released from necrotic cells, C1q inhibits DNAse I if deposited on NETs in high concentrations. This situation presumably occurs at local sites of C1q production only, as physiological concentrations of C1q in normal serum do not inhibit NET degradation. NETs do not bind the established dead cells' opsonins C3b, galectin-9, and C-reactive protein (CRP). Taken together, NETs are probably treated neither as usual 'chromatin waste' nor as 'dead cells' by the immune system, but dealt with in a specific way still to be elucidated. NETs are increasingly appreciated as a source of auto-antigens in auto-immune diseases, such as SLE, rheumatoid arthritis and vasculitis. Deficiencies in NET disposal could initiate a vicious circuit, as it was shown that NETs form IC with anti-dsDNA antibodies and IC, in turn, can trigger NET ejection.

**RNA**

In the case of mRNA and other RNA species, disposal is simplified by the high abundance of RNAses in the extracellular space. RNA may function as a local immune-stimulatory signal after stabilisation by accessory proteins and can fuel systemic inflammation in SLE as a part of IC. In general, however, it is quickly broken down into nucleotides. Free nucleotides are then decomposed into purine and pyrimidine bases. Pyrimidines are recycled as amino acids, whereas purines are converted by xanthine oxidase in the liver from hypoxanthine and xanthine into UA. The latter is excreted with the urine. The same fate awaits extracellular ATP, which is known to be converted via several steps to adenosine by extracellular enzymes.

**Uric acid**

The absolute necessity for functional and immediate disposal of cellular components is illustrated by the purine breakdown product UA. If the capacities for its renal excretion are overloaded, i.e., after massive cell death, this substance has a strong pathogenic potential. Soluble UA has pro-inflammatory effects and stimulates antigen-presentation and T-cell development. As soon as UA levels exceed the solubility limit of approximately 6.8 mg/dl, it precipitates as monosodium urate crystals (MSU). If taken up by monocytes, MSU induces release of TNF-α, IL-6, and IL-8 and of the NALP3 inflammasome-dependent pro-inflammatory cytokines IL-1 and IL-18. Inflammation activation is achieved by reducing intracellular potassium concentrations to values below 90 mM. However, no potassium is lost from the cytoplasm. Instead, cellular contents are diluted by massive water influx resulting from the increased osmolarity caused by breakdown of MSU into UA and sodium ions. MSU were also shown to be highly potent triggers of NETosis. NETosis may facilitate phagocytic MSU disposal by immobilisation of the crystals. The utilisation of such a rather drastic and potentially harmful mechanism for the disposal of MSU is well justified. If MSU crystals precipitate in tissues, they lead to severe conditions such as gout. In the kidney, they may even obstruct renal vessels, contributing to acute renal injury. The role of MSU in sepsis is still elusive.

A 'maximum credible accident' scenario: tumour lysis syndrome

Massive apoptosis can overexert the clearance mechanisms for apoptotic cells, which then tend to become secondary necrotic. Simultaneous lysis of a whole lot of cells, in turn, can overwhelm clearance, back-up and disposal mechanisms for leaked cellular content. Such a situation can occur in the human body upon any cytotoxic therapy of tumours, when contents of lysed cells enter the circulation. This is characterised by hyperuricaemia, hyperkalaemia, hyperphosphataemia, and hypocalcaemia and is commonly referred to as TLS. Disturbance of the electrolyte balance can lead to cardiac dysrhythmia, which is by definition one of the hallmarks of TLS. It may also contribute to the occurrence of acute renal failure (ARF). In addition to electrolyte abnormalities, multiple causes for ARF in TLS have

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been proposed. UA plays a central role either in MSU obstructing renal vessels or as a soluble, pro-inflammatory and anti-angiogenic signal. Furthermore, microemboli containing nuclear and cytoplasmic remnants of lysed tumour cells were found to obstruct blood vessels during murine TLS. The presence of similar structures was proposed for human disease; here renal embolism may lead to a decreased excretion capacity for toxic components, including UA and electrolytes.

**Conclusion**

Whether it comes in gulpable packages or leaks out into puddles, all waste has to be sorted and disposed off. The dogma behind modern source segregation strategies holds true not only for the household, but also in the human body, that is each piece of waste has to be submitted to its appropriate route of recycling or disposal, otherwise accumulation of harmful trash will render the environment hostile to life.

**Abbreviations list**

ARF, acute renal failure; ATP, adenosine triphosphate; BC, B-cells; DNA, deoxyribonucleic acid; HMBG1, high mobility group box 1 protein; IC, immune complexes; IFN-α, interferon alpha; IL, interleukin; LPS, lipopolysaccharide; MSU, monosodium urate crystals; NET, neutrophil extracellular trap; PGE2, prostaglandin E2; PS, phosphatidyserine; RNA, ribonucleic acid; SLE, systemic lupus erythematosus; TLR, toll-like receptor; TLS, tumour lysis syndrome; TGF-β, transforming growth factor beta; TNF-α, tumour necrosis factor alpha; UA, uric acid.

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