Defects in the Disposal of Dying Cells Lead to Autoimmunity

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The fast and efficient uptake of dying cells is of main importance to prevent contact of the immune system with intracellular autoantigens. Insufficient clearance of the latter is discussed to drive the humoral autoimmune response in systemic lupus erythematosus. Many adaptor molecules and receptors are involved in the recognition of dying cells. In this paper we focus on the involvement of phosphatidylserine, glycooproteins, and complement and DNaseI in the clearance of apoptotic and necrotic cells, respectively. Furthermore, extracellular danger signals released from necrotic cells are discussed and the uptake process of primary necrotic cells is investigated in detail. Last but not least, the character and origin of clearance defects observed in some systemic lupus erythematosus patients is presented.

Introduction

In the past years, the importance of cell death and of the removal of the dying cells for the homeostasis in the body and for the maintenance of an intact immune status advanced in the awareness of the researchers. By now, two main forms of cell death are encountered in biology: apoptosis and necrosis.

Necrosis can be considered a form of violent cell death. Cells that may have not yet reached their full lifespan are hit by an external noxa that interrupts their vital functions or disrupts their physical integrity. The intracellular contents of necrotic cells are spilled into the microenvironment since these cells loose their membrane integrity very early [1]. Necrosis is often because of a drop of adenosine triphosphate (ATP), the influence of certain pathogens, and mechanical or oxidative stress.

In many cases cells are dying in a programmed way following the "Samurai law of biology": it is better to die than to be wrong [2–4]. Apoptosis is viewed as programmed cell death or cellular suicide [5]. In contrast to necrotic cells, apoptotic ones maintain their membrane integrity thereby preventing the release of intracellular components. They are usually cleared by macrophages through a noninflammatory pathway [6,7]. Apoptosis is characterized by specific morphologic changes of the dying cells including loss of membrane asymmetry, nuclear condensation, and DNA fragmentation.

Apoptotic cells are rarely found in vivo because of their rapid and efficient clearance by professional or even amateur phagocytes like fibroblasts. The early recognition of dying cells requires characteristic membrane surface changes that do not occur on normal cells. One such event is the exposure of phosphatidylserine (PS) in the outer leaflet of the plasma membrane associated with loss of phospholipid asymmetry [8,9]. Nevertheless, there is an enigma in the recognition and uptake of PS exposing cells, that is not yet resolved. There are also viable PS exposing cells (*eg*, activated B cells, monocytes), that are not phagocytosed. Therefore, we analyzed the binding of Annexin V (AxV), a protein that specifically binds to PS, to viable and dying monocytes, to understand this controversial feature of PS exposure.

Apoptotic cell engulfment and anti-inflammatory signaling are mediated through PS and through PS bridging proteins and their cognate receptors. Other early surface changes that might be involved in the removal of apoptotic cell are alterations of carbohydrates. Certain lectins such as C1q and the mannose-binding lectin have been described to bind and opsonize apoptotic cells and enhance their uptake. Nevertheless, the exact role of altered carbohydrates in the apoptotic clearance process is elusive. Lectins are, in general, carbohydrate binding proteins specific for certain sugar moieties or carbohydrate linkages [10]. They serve diverse functions in recognition, interaction, as receptors, and as adaptor molecules [11]. We analyzed the lectin binding of PI impermeable dying cells by flow cytometry and confocal microscopy to characterize modified sugar residues on cells undergoing apoptosis.

In addition, other adaptor molecules and receptors are involved in the recognition of dying cells [12–17]. In human systemic lupus erythematosus (SLE) decreased levels of serum DNaseI activities along with deficiencies in components of the classical complement pathway predis-

pose for this disease. Others and we have suggested an important role of complement (mainly C1q) and DNaseI in the clearance process of dying cells and subcellular fragments [18–26]. Patients with primary antiphospholipid syndrome who have low levels of the complement components C3 and C4 and low CH 100 values also show immunologic alterations similar to those of SLE [27]. The fast and efficient uptake of dying cells is of main importance to prevent contact of the immune system with intracellular autoantigens. Insufficient clearance of the latter is discussed to drive the humoral autoimmune response in SLE [28–30]. We analyzed the phagocytosis potency of macrophages differentiated from CD34 positive stem cells derived from the peripheral blood from SLE patients and normal health donors (NHD) to investigate whether the clearance defect observed in some SLE patients is intrinsic. In addition, we tested sera of SLE patients, RA patients, and NHD in regard to their DNase and complement activity and their capability to degrade nuclei derived from necrotic cells. Furthermore, we examined factors responsible for the uptake of necrotic cells by human monocyte derived macrophages (HMDM). This paper deals with important features of dying cells and recognition molecules of the latter leading to their effective clearance and discusses clearance defects that might contribute to the induction and maintenance of autoimmunity.

Results and Discussion

Dying cells are naturally and rapidly cleared by phagocytes. The role of phosphatidylserine

As part of the apoptotic death program, cells undergo rapid surface changes such as modification of carbohydrates and exposure of anionic phospholipids, especially PS. An enigma exists with respect to the recognition and phagocytosis of PS exposing cells: viable PS exposing cells (*eg*, activated B cells [31,32], neutrophils in Barth Syndrome [33], or monocytes) are swallowed neither by amateur nor professional phagocytes. In contrast, apoptotic and necrotic PS exposing cells are efficiently taken up. What could be the reason for that? We suggest that dying cells have cell membranes with high lateral mobility of PS. We demonstrated that AxV, a specific PS-binding protein, binds to viable monocytes without co-operation whereas AxV binding to dying (apoptotic and necrotic) monocytes proceed in a cooperative manner (Appelt *et al*., In press). This suggests that AxV needs a critical density or clustering of PS molecules. It might also be that AxV needs a not yet defined cofactor that is only present on dying cells.

Changes of the glycoprotein composition in membranes of dying cells

By now the exact role of altered carbohydrates in the apoptotic clearance process is elusive. Carbohydrate binding proteins, the lectins, are discussed as players of the innate immune system. Binding of collectins, a family of collagenous calcium-dependent defense lectins in animals, to microorganisms may facilitate microbial clearance and also affects apoptotic cell clearance [34]. Galactose- and mannose-specific receptors are discussed to play an important role for the recognition of dying cells [35]. We selected and analyzed the lectin binding of PI impermeable, dying cells of 20 lectins by flow cytometry and confocal microscopy, to characterize modified sugar residues on cells undergoing apoptosis. We observed an increased binding of *Narcissus pseudonarcissus lectin*, *Griffonia simplificolia lectin II*, and *Ulex europaeus agglutinin I* on apoptotic cells in comparison with viable ones. According to their binding specificity we conclude that mannose-, N-acetylglucosamine- and fucose-containing epitopes are increasingly exposed on dying cells during the execution of apoptosis. We found that the exposition of these modified sugar moieties displays a delayed kinetic compared with PS. Therefore, the exposure of modified sugars may represent a back-up mechanism for clearance for cells that had escaped the early PS-dependent phagocytosis by macrophages. Lectins on macrophages may contribute in this way to the noninflammatory removal of immune complexes and abnormal cells.

Complement and DNaseI are involved in the clearance process of dying cells

Many defects are known with respect to the clearance of apoptotic cells, necrotic cells, and dying cell material, especially that of nuclear origin. Examples are low C-reactive protein (CRP) levels in patients with SLE [36], reduced activity and levels of DNaseI in serum [18], and complement defects [21,37–39]. The importance of complement in the scavenging process is becoming more and more evident. Complement activation by apoptotic cells, *eg*, endothelial cells, lymphocytes, and polymorphonuclear cells (PMN) was just recently reported by several groups independently [23,40,41]. Furthermore, an impaired uptake of apoptotic cells by human macrophages was to be observed in human serum depleted of specific complement components (C1q, C3) [20,42,43]. We also suggest an important role of complement and DNaseI in the clearance process of dying cells and subcellular fragments [26,44]. Disturbed clearance of nuclear DNA-protein complexes resulting from dying cells may initiate and propagate SLE [45]. Napirei *et al*. [46] showed that DNaseI deficient mice display classic symptoms of SLE. The absence or the reduction of extracellular DNaseI may, therefore, be a critical factor in the initiation of human SLE. This is further substantiated by decreased levels of DNaseI activity that had been observed in the sera of SLE patients. DNaseI, being the major serum nuclease, may be responsible for the degradation of chromatin accidentally released by inappropriately cleared dead cells [18,26,47,48].

We tested sera of SLE patients in regard to their capability to degrade necrotic cell-derived chromatin. We found a significant correlation between the percentage of hypochrome nuclei and the total classic complement activity of

the sera (CH 100 values). Sera with CH 100 values lower and higher than 50 significantly differed in their degradation capability (4.2 ± 3.6 and 27.9 ± 7.9 [T-test: P = 0.000013], respectively). We also observed a strongly reduced degradation capacity of necrotic cells of sera from SLE patients with inherited deficiencies for C1q or C2.

Furthermore, we found a significant activity reduction of DNaseI in sera of rheumatoid arthritis (RA) and SLE patients in comparison with NHD. Most of the sera with a high activity reduction of DNaseI showed a strongly reduced degradation capacity of necrotic cell-derived chromatin. Most interestingly, SLE sera showed a strongly reduced degradation capacity of necrotic cell-derived chromatin in comparison with RA sera and NHD sera. Seven of 20 SLE sera led to a degradation of less than 20% after 1 day of incubation with necrotic cells. In contrast only one of 20 RA sera and none of the NHD sera led to degradation below 20% (Gaipl *et al*., Unpublished data). We conclude that an additional protection from chromatin implicated in the development of autoimmune disorders such as SLE can be achieved by the C1q and DNaseI dependent clearance of degraded chromatin.

Ligands and receptors involved in the uptake of primary necrotic cells

Many ligands, bridging molecules, receptors, and mechansims involved in the clearance of apoptotic cells have been described. The ligation of PS-receptor (PSR) by PS on apoptotic cell surfaces is considered important for signaling the anti-inflammatory uptake of dying cells that are tethered to phagocytes through various other receptors [8,49]. However, as only recently shown, the phagocytosis and clearance of apoptotic cells is normal in PSR deficient mice [50]. This observation suggests that a yet unknown receptor for PS exists that may act as primary PS recognition receptor. The PSR on phagyoctes may serve a dual role on the cell surface and in the nuclei, since the protein encoded by the PSR cDNA was found to be localized in the nuclei [51]. Further important recognition and uptake systems are described. The latter include collectin receptors, calreticulin / CD91, Fcγ-receptors, c-Mer, β_2-glycoprotein I receptor, integrins, lectins, CD14, ATP binding-cassette transporters, and scavenger receptors including CD36 [13,14,52–55]. The ligands of some of these receptors have been identified. The thrombospondin receptor (CD36) and the vitronectin receptor αvβ3 cooperate in binding thrombospondin, that interacts with apoptotic cells. Thereby, thrombospondin forms a "molecular bridge" between the phagocyte and the dying cell [16,56,57]. The interactions can be efficiently inhibited by monoclonal antibodies targeting the recognition and uptake machinery of the phagocytes [58]. By now little is known about the receptors and ligands involved in the uptake process of primary necrotic cells. We established a flow cytometric based phagocytosis assay to quantitatively monitor the uptake by HMDM of primary necrotic cells. Our results show, that interaction of PS that is exposed on the surface of apoptotic and on necrotic cells with HMDM, serves as a recognition signal for the rapid removal of primary necrotic cells (Gaipl *et al*., Unpublished data). In addition, the CD36/(αvβ3) / thrombospondin complex as well as CD14 and the complement component C1q contribute to the engulfment of primary necrotic cells. Therefore, at least some of the ligands, bridging molecules, receptors, and mechanisms involved in the uptake of apoptotic cells mediate also the clearance of primary necrotic cells generated by heat, methanol or ethanol treatment. These findings reveal that recognition and uptake mechanisms for apoptotic and necrotic cells are, at least partially, identical. The results may have important implications for the etiopathogenesis of autoimmune diseases such as SLE, where an impaired phagocytosis of dead cells appears to present an important step for breaking self tolerance.

Dying cells release extracellular danger signals

Through the constant interplay of cellular and extracellular components, the microenvironment of tissues directs immune responses. "Danger signals" released by dying cells are constituents of inflammatory environments. Recently, it has been shown that primary necrotic cells release the inflammatory high mobility group B1 (HMGB1) protein that is "frozen" on the chromatin of apoptotic cells and remains immobilized even under conditions of secondary necrosis [59,60]. During apoptotic cell death, HMGB1 gets tightly attached to the hypoacetylated chromatin/histones and is not released, thereby preventing inflammation [59,61]. However, in conditions with impaired clearance of apoptotic cells, nucleosomes with tightly attached HMGB1, acting as an adjuvant, may contribute to break T cell tolerance towards histones and other chromatin associated proteins. We detected HMGB1 in polyethylene glycol-precipitated immunocomplexes from SLE patients (Unpublished data). The circulating DNA/nucleosomes contain HMGB1 and, therefore, may be predominantly derived from late apoptotic cells (secondary necrotic) rather than from (primary) necrotic cells. Since primary and secondary necrosis involve the display of different inflammatory signals, the fine-tuning of responses against dying cells is of major importance.

Besides HMGB1, extracellular ATP has emerged as an important regulator of inflammatory and immune responses. It can affect the functions of various cells through activation of P2 purinoceptors [62] ATP can be released by regulated exocytosis, traumatic cell lysis, or passive leakage from damaged cells. Therefore, most likely, the extracellular ATP concentration is raised during tissue injury or inflammation [63].

The secretory phospholipase A2 (sPLA2) IIA is another relevant secretory protein that floats freely in the serum and strongly binds PS. sPLA2 IIA is not able to hydrolyze efficiently the phospholipids of the outer membrane leaflet of normal intact cells. Thus, sPLA2 IIA is only able to

react with phospholipids in the outer leaflet of normal cells if they have undergone a flip flop from the inner leaflet as in apoptosis or necrosis. After interaction with sPLA2 IIA, cells are left with an increased proportion of lysophospholipids like lysophosphatidylcholine (lyso-PC) in the outer membrane leaflet. This modification disturbs the packing of the phospholipids and generates binding sites for the pentraxin CRP in the outer leaflet [64]. Once bound, CRP induces complement activation through the classical pathway, that in turn triggers the influx of neutrophils, decorates the surface of the ligand with opsonizing complement fragments, and enhances phagocytosis of the cells that have bound CRP and complement [65]. In addition to the membrane of intact injured cells, CRP also binds to membranes and nuclear constituents of necrotic cells. Several nuclear constituents, including histones, small nuclear ribonucleoproteins and ribonucleoprotein particles have been shown to bind CRP in a calcium-dependent fashion [66]. Deposition of CRP to nuclei of necrotic cells at sites of inflammation has been observed while CRP does not cross the plasma membrane of apoptotic cells. This might be considered another reasonable mechanism of fine tuning the differentiation between apoptosis and necrosis.

PTX3, being the prototypic long pentraxin, is structurally related to, but distinct from CRP and serum amyloid P component (SAP). PTX3 binds to necrotic cells to a lesser extent then to apoptotic cells. Human DC failed to internalize dying cells in the presence of PTX3, while they macropinocytosed particulate substrates [67]. These results suggest that PTX3 sequesters cell remnants from antigen-presenting cells, possibly to prevent the onset of autoimmune reactions in inflamed tissues.

Intrinsic clearance defects in some systemic lupus erythematosus patients

As mentioned before, apoptotic cells are usually cleared in the early phases of apoptosis. Effective clearance of dying cells induces neither inflammation nor immune responses [7,68]. If apoptosis progresses, the cells can enter the stage of secondary necrosis. During necrotic as well as apoptotic cell death autoantigens are cleaved or otherwise modified [69–72]. These modifications may render cryptic epitopes immune dominant [73,74]. Dendritic cells may then acquire modified autoantigens like apoptotic nuclei and chromatin and consequently autoreactive T cells can be activated. Impaired clearance functions for dying cells may explain accumulation of apoptotic cells, and subsequently of secondary necrotic cells in various tissues of SLE patients [28–30,75]. Increased levels of DNA and nucleosomes that have been observed in some SLE patients [76,77] are most likely because of secondary necrotic cells that are not able to retain this material. Furthermore, it was recently shown that an extranuclear accumulation of histones and nucleosomes is an early event of apoptosis in human lymphoblasts. Dysregulation of early apoptosis might lead to an overload of autoantigens (and in particular of nucleosomes) in circulation or in target tissues [78] and support the induction of autoimmunity against nuclear components [79]. We showed that in a subgroup of patients with SLE apoptotic cells accumulated in the germinal centers of the lymph nodes. The numbers of tingible body macrophages usually containing engulfed apoptotic nuclei were significantly reduced in these patients. In contrast to all controls, apoptotic material was observed associated with the surfaces of follicular dendritic cells. This observation described for the first time the accumulation of free apoptotic cells in germinal centers of the lymph nodes in humans with SLE [30].

The next step was to investigate whether the impaired clearance observed in certain SLE patients is an intrinsic defect. We analyzed the phagocytosis potency of macrophages differentiated from CD34 positive stem cells derived from the peripheral blood from SLE patients and NHD, respectively. SLE and NHD-derived stem cells showed similar proliferation in vitro. However, the differentiation into macrophages was reduced in SLE stem cell cultures. Much less macrophages differentiated from CD34 positive stem cells. Furthermore, the macrophages of SLE patients showed a different morphology: they are smaller and have less differentiated "catching arms." Most of the SLE stem cell-derived macrophages also showed reduced phagocytic capacity and died early. Taken together, in certain SLE patients an intrinsic defect of particle phagocytosis and dying cell clearance was found. Factors responsible for the bad differentiation rate are currently under investigation. Cytotoxic effects of ATP in the culture may play a role. In contrast to fibroblast-like cells, hematopoietic cells, and macrophages form under the influence of ATP4- (the fully ionized form of ATP) lesions in their plasma membranes [80]. Hemopoietic cells are highly sensitive to the cytotoxic effects of ATP and its derivates [81]. Nearly all cells have binding sites for nucleosides and nucleotides, the purinoceptors (P2 receptors). The latter are discussed to be potentially involved in the apoptotic process [82].

Heterogeneous clearance defects in some systemic lupus erythematosus patients

The next question was to investigate whether the clearance defects in SLE patients is heterogeneous. We examined the uptake of various beads and dying cells by phagocytes of SLE patients and NHD. In whole blood, granulocytes of certain SLE patients show reduced uptake of beads. Fifty percent of the SLE patients' PMN showed a reduced phagocytosis of albumin-coated beads. However, the uptake of IgG-opsonized beads was only impaired in approximately 20% of the patients' PMN. We found that macrophages and/or granulocytes of some SLE patients showed a strongly reduced uptake of albumin beads, polyglobuline beads, apoptotic and necrotic cells as well as degraded chromatin. Very interestingly, phagocytes from different SLE patients showed in part different phagocytic defects.

Conclusions

Efficient phagocytic clearance of dying cells is extremely important in many biologic processes. The presented data provide evidence that the early recognition of dying cells requires characteristic membrane surface changes that do not occur on viable cells, like altered carbohydrate compositions and high lateral mobility of PS. Evidence is accumulating that complement proteins, besides others, opsonize apoptotic cells, [23] leading to phagocytosis mediated by well-defined “old-fashioned” receptors like those for complement [83]. We observed a strongly reduced degradation capacity of necrotic cells of sera from SLE patients with inherited deficiencies for C1q or C2 or low CH100 values. We showed that similar mechanisms as for the phagocytosis of apoptotic cells seem to be involved in the uptake of primary necrotic cells. Regarding the “late phase clearance,” the complement component C1q and a serum DNase, namely DnaseI, are the main players. We further conclude that the defective clearance of dying cells in a subgroup of SLE patients seems to be an intrinsic defect since less macrophages differentiated from stem cells and some of the generated phagocytes also showed reduced uptake efficiency. Furthermore, the impaired clearance capacities of granulocytes from some SLE patients could play an important role in the development of autoimmunity. Others and we conclude that altered mechanisms for clearance of dying material represent a central pathogenic process in the development and acceleration of autoimmune diseases like SLE [29,44,84].

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