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Handling the Corpses: Apoptosis, Necrosis, Nucleosomes and (Quite Possibly) the Immunopathogenesis of SLE

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Abstract: Death happens. It is, in essence, part of life. Humans deal with death in a variety of different ways, but often by keeping it at arms' length. At the cellular level, there are many forms of death, part of the development of organs and tissues (apoptosis) and part of pathologic processes (necrosis). The former, as has been described in an earlier paper in this series, is designed to eliminate the corpse with no evidence that it was ever there. Clearance is usually swift and effective, avoiding inflammation and specific immune interventions or responses. However, there is gathering evidence that autoimmunity leading to systemic lupus erythematosus may be due to ineffective or improper clearance of apoptotic debris, making it proinflammatory and allowing it to become highly immunogenic. This formulation also suggests therapeutic options that have already been demonstrated effective in controlling models of human autoimmune disease. This article reviews some aspects of this theory and some of the molecular biologic features of necrosis, apoptosis, and other forms of cell death.

Key Words: nucleosomes, histones, apoptosis, HMGB1, phosphatidylserine, autoimmunity, SLE, necrosis

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INTRODUCTION—CELL DEATH OF MANY TYPES

As noted in a previous article in this series, necrosis is dangerous and apoptosis is neat. We will return to the former topic in depth in a bit. First, let us look more closely at cell death. A recent paper by Fink and Cookson puts a bit of a new spin on this. They describe a number of forms of cell death. Apoptosis you have previously met: mediated by a series of caspases (in case you were keeping score, initiated by caspases 2, 8, 9, and 10, with caspases 3, 6, and 7 doing the dirty work, the so-called "effector caspases"), apoptosis consists of a noninflammatory means of disposing of cells after they commit hara-kiri. The cells undergo nuclear and cytoplasmic condensation with formation of membrane-bound cellular fragments, called apoptotic bodies. These fragments express phosphatidylserine (PS) on their surface, a normal component of the inner leaflet of the cellular membrane (under normal circumstances well over 90% of PS is on the inner leaf, with small amounts cycling to the outer leaf under very tight control and rapid return, especially during cell activation). When expressed on the outer surface of a membrane, PS has immunosuppressive qualities. Apoptotic bodies are normally quietly taken up by phagocytes and are never heard from again. The process of uptake/disposal of apoptotic cells is called "efferocytosis" (from the Latin effere or effero meaning "to carry to the grave or to bury"; of note a very similar Latin root means to be wild or savage—sort of the flip side of efferocytosis, as we are discussing in this paper). The dendritic cells (DC) that ingest apoptotic debris are nonmature and tolerogenic (see previous article). The debris does not elicit the production of the cytokines implicated in DC maturation, so the DCs remain immature and tolerogenic. In older studies, this uptake was thought to suppress expression of inflammatory cytokines like TNFα, IL-1β, IL-12, and MIP-1α and up-regulate expression of suppressive cytokines like IL-10, TGF-β, and prostaglandin E2. Recent evidence suggests that the uptake of apoptotic cells, specifically of early apoptotic cells in vivo, is actually silent—the changes of cytokines noted above are an artifact of the in vitro studies, perhaps related to the persistence of late apoptotic cells. These properties are important in normal homeostatis; over one quarter of a trillion red blood cells die each day in our bodies—the likely signal for their removal is PS on the pock-marked surface of aging or damaged RBCs. Add that to the 100 million apoptotic polymorphonuclear cells being removed and you see that this safe "eat me, but do not make a fuss about it" signal is crucial.

Necrosis is actually the description of the postmortem appearance of cells having died a nonapoptotic accidental death. The end result is proinflammatory debris; by this I mean that necrotic debris invites and encourages local inflammation which, if well modulated, is part of the healing process. A recent example of pathologic outcomes is the finding that RNA released from necrotic synovial fluid cells can activate synovial fibroblasts, via their Toll-like receptor (TLR) 3 (the TLR that is activated by dsRNA) to produce interferon β, IL-6, and the chemokines CXCL10 and CCL5. Another recent study suggests

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that serum Dnase 1 and the plasminogen systems cooperate to penetrate necrotic cells and break down chromatin before it can elicit an antibody response; of note, mice deficient in Dnase 1 develop autoantibodies and autoimmunity. Other proinflammatory compounds are also released from necrotic cells—we will return to one of them, high-mobility group B (HMGB)1, later in this paper.

Necrotic cells' debris causes the maturation of DCs, making the DCs more able to elicit a subsequent immune response, precisely the desired outcome if you need to respond to a pathogen but potentially a double-edged sword, as it can cause much local damage. Of note, there is a phenomenon known as "secondary necrosis," where apoptotic bodies that have not been phagocytosed promptly undergo necrosis. Thus, delayed or inadequate clearance of apoptotic bodies and debris can lead to a necrotic mess, with inflammation, precisely what apoptosis is not supposed to be about!

Now, some readers are saying "Wait—necrotic cells reveal PS as well, don't they? Why isn't the PS suppressing inflammation?" Well, there may not be as much PS revealed in necrosis as in apoptosis. Current thinking is that cytoplasmic enzymes, e.g., elastase, may cleave the PS receptor on the phagocyte or that there may be cytoplasmic PS binding molecules that block the PS receptor; the final answer is not yet in.

According to Fink and Cookson, there are other ways that cells can die. Autophagy is degradation of cells and their components within dying cells in "autophagocytic vacuoles." These are also noninflammatory, i.e., when phagocytes encounter these vacuoles, ingestion does not lead to an inflammatory reaction. Oncosis (from the Greek "onkos" meaning swelling) represents a prelethal pathway to cell death with swelling of the cells and their organelles, accompanied by (or caused by) increases in membrane permeability. All of this seems to be related to change in intracellular calcium, perhaps due to enzyme-catalyzed changes. Cells infected with rotavirus and *Pseudomonas aeruginosa* may undergo oncosis. Whatever the underlying mechanisms, the end result is the liberation of very proinflammatory materials—not good for the surrounding tissues. Also not good for the neighborhood is a process known as pyroptosis ("pyro" for fire or fever). This can be seen in cells infected with *Shigella* and *Salmonella* species, which activate caspase 1, the same enzyme that processes IL-1β and IL-18. The end result is inflammation and tissue damage.

CLEARANCE OF APOPTOTIC CELLS—MORE THAN JUST PHOSPHATIDYLSERINE

So, there are a number of ways for cells to die. Why should rheumatologists know this? Recall that apoptotic cells are meant to be eaten and destroyed with no fanfare. Apoptotic cells express a number of molecules, e.g., PS, on the surface of the resulting bodies that encourage uptake by phagocytes. Many of these apoptotic membrane markers are identified by circulating proteins, like β2 glycoprotein I, thrombospondin, the pentraxins [serum amyloid P (SAP) component and CRP] and the connectins [C1q and mannose binding protein]. These molecules constitute "bridging molecules," spanning the gap between the apoptotic body and the receptor on the phagocyte. These and perhaps other circulating proteins, as well as molecules within the apoptotic body membrane, are recognized by a number of receptors on the surface of phagocytes. The list of these receptors is as yet incomplete; nonetheless it is already long: SAP binds to the Fcγ receptors CD16, CD32, and CD64; PS binds to a specific PS receptor; the collectins (specifically C1q—a recent study suggests this mechanism is defective in SLE patients) bind to calreticulin and CD91; CD36 (the thrombospondin receptor), CD68 (the receptor for oxidized LDL), CD14, SR-A (scavenger receptor), and other lectin receptors may be part of the process. Of note, autoantibodies to many of these opsonins of apoptotic structures, including C1q, β2 glycoprotein I, annexin V, CRP and SAP, are found in the serum of patients with lupus.

Another interesting player in the process is the phagocyte surface molecule Mer, a member of the Axl/Mer/Tyro3 receptor tyrosine kinase family, which seems to be required (and in fact may be crucial) for removal of apoptotic thymocytes (details not yet worked out). Uptake of apoptotic cells is probably a cooperative phenomenon; early studies suggested PS exposure might be sufficient to induce phagocytosis, but now it seems clear that interaction with multiple phagocyte receptors is necessary.

Of note, there is evidence that one of the statins (lovastatin) enhances efferocytosis in a lung model, suggesting to the authors that statins might be useful in cystic fibrosis, bronchiectasis, and COPD.

Cellular defects have been described as well. Tas et al found that macrophages from patients with SLE and with RA have defective adhesion to plastic but only lupus macrophages have impaired uptake of apoptotic cells; of note, the binding of lupus macrophages, like that of rheumatoid macrophages, is normal. In a study by Bijl et al, defective uptake of apoptotic cells was found to correlate with low C1q, C4, and C3 levels.

DEFECTIVE CLEARANCE AND ITS CONSEQUENCES

Clearance of apoptotic cells is important to eliminate a variety of structures, e.g., nucleosomes (nucleosomal histones [that pack DNA] are immunogenic, requiring T cell intervention, i.e., these are T cell dependent antigens). As noted, under normal conditions apoptotic bodies/cells are ingested by nonmature, tolerogenic DCs that process and present the material to T cells. There is mounting evidence that there may be a defect in clearance of apoptotic cells in lupus patients, which may help explain how autoimmunity to many nuclear antigens occurs. A recent study documented an accumulation of apoptotic cells in the ultraviolet-exposed skin of patients with cutaneous lupus. Apoptotic cells not rapidly removed may undergo secondary necrosis, with release of inflammatory mediators and autoantigens. Possibly complicating the picture, autoantibodies might bind to apoptotic cells, aiding in phagocytic uptake and inflammatory mediator release.

The defect in removal of apoptotic cells may reside in abnormalities of any of the molecules that bind to apoptotic materials, e.g., CRP, SAP, C1q, β2 glycoprotein I, or the

receptors that engage these bridging molecules, e.g., Fcγ receptors. It is probably not an "all or none" phenomenon. Perhaps the problem is one of capacity: an increase in "apoptotic load," e.g., ultraviolet light exposure or infection, perhaps exacerbated by the release of endogenous or exogenous adjuvants exceeding the diminished capacity of that individual, e.g., a defect in bridging molecules or receptors. In either event, the previously immunologically "silent" apoptotic materials become immunogenic, with dire consequences. Endogenous adjuvants can bind to TLR on DCs and activate them, resulting in loss of tolerance; recent studies suggest uric acid crystals are a potent endogenous adjuvant! Recent studies suggest that endogenous adjuvant activity is found within the RNA components of Ro 60 and Sm/RNP.

Decreased clearance of apoptotic cells probably leads to persistence of apoptotic cells and secondary necrosis, allowing persistence of autoantigens in a proinflammatory milieu, which favors maturation of DCs. These autoantigens are then available for processing with the possible release of cryptic immunogenic epitopes. Of note, many lupus-related autoantigens are posttranslationally modified during apoptosis, many cleaved by caspases, and many epitopes are the result of cleavage by granzyme B, a proapoptotic protease. In a caspase-dependent manner, apoptotic cells release DNA (necrotic cells also release DNA, but it is often digested); DNA-anti-DNA immune complexes can be bound by TLR (via CpG sequences) to elicit the release of α interferon, which also promotes the maturation of DCs. Immune complexes containing small RNAs, like Ro, can crosslink TLR3, further driving DC maturation and autoimmunity. So, uptake of autoantigens by mature DC changes a tolerogenic effect into an autoimmune response. Whatever the mechanism, the combination of endogenous adjuvant with autoantigen persistence (a "two-hit phenomenon" if you will) may be at the root of autoimmunity.

Speaking of tolerance . . . a recent study by Patel et al shows that apoptotic and necrotic cells induce distinct signals in the ingesting cells, the latter being proinflammatory and the former not. In fact, apoptotic cells may induce tolerance. The authors advance the premise that apoptotic cells may not be capable of causing autoimmunity in and of themselves. The debate is wide open as of now.

HIGH MOBILITY GROUP BOX 1

HMGB1 was identified in the 1970s. It is an abundant (over 1,000,000 copies per cell) 30 kD protein, very highly conserved (99% identity in mammals) protein that binds (with no discernible sequence specificity) to the minor groove of DNA. This allows bending of the double helix, enabling a variety of transcription factors to bind to DNA, including p53, NFκB, recombinase activating genes (RAG), and steroid hormone receptors. By binding transiently to chromatin, HMGB1 helps control gene transcription. The protein contains 2 DNA-binding domains ("HMG boxes," "A" and "B") with a negatively charged C terminus. The 2 boxes are similar in structure, although they bear only 20% amino acid identity.

That is part one of the story: the crucial role of HMGB1 in normal cell function. Part two: HMGB1 is also found on the extracellular surface of the cell membrane. When cells die, there is a lot of debris with which to deal. As you know, apoptosis makes of the cell a nice neat package; necrotic cells are a bit more of a mess, leaking all sorts of cell contents, including HMGB1. In the extracellular milieu, HMGB1 is very proinflammatory and recruits macrophages and monocytes (chemoattractant activity) to the site of tissue damage and stimulates them to secrete proinflammatory cytokines like IL 1β, TNFα, and IL6, aiding in clearance of debris and in mounting a defense against pathogens. The cytokine-like activity of HMGB1 resides in box B, whereas box A is actually a specific antagonist, decreasing macrophage secretion of cytokines—both yin and yang in one molecule!

HMGB1 is also actively secreted in prepackaged secretory lysosomes by monocytes and macrophages and by dendritic cells after cell stimulation by cytokines like IL1, TNFα, IL18, and IFNγ. HMGB1 then stimulates monocytes to secrete more cytokines like IL1 and TNFα, inducing a multiplier effect, if you will. The effects of HMGB1 are delayed compared with the more classic inflammation mediators, like LPS.

Thus, HMGB1 seems capable of performing 2 vital functions: within the nucleus, activation of gene expression and outside the cell helping orchestrate late aspects of the activation of inflammation and calling forward cells involved in damage repair. But if you think about it, what better marker of the utter and complete disorganization and death of a cell could there be than the release of a uniquely nuclear protein? And so HMGB1 serves as a good signal of nonapoptotic cell death, and in that role it enhances inflammation.

Membrane-bound HMGB1 has also been called "amphoterin"; it is also involved in mediation of neurite outgrowth, smooth muscle cell chemotaxis, and tumor cell metastasis. This version of the molecule is subtly different from that liberated by necrotic cells (the secreted form has had lysine residues acetylated, which allows packing into the lysosomes; this difference in structure may impart different characteristics to the 2 molecular forms).

HMGB1 has been implicated in the pathogenesis of septic shock, specifically the reaction to endotoxemia. Early in the syndrome caused by LPS there is secretion of IL1 and TNFα. Any of these 3 stimulants can cause mononuclear cells to secrete HMGB1, but this occurs many hours (18 to 24 hours) later, in marked contrast to the appearance of the other cytokines within minutes. So, what does HMGB1 do to its targets (macrophages, monocytes, DCs, and endothelial cells), aside from make them secrete proinflammatory cytokines? The glib answer is "plenty": it increases expression of genes encoding inflammatory factors in neutrophils; enhances maturation of antigens and the ability to render antigens immunogenic in and the T_H1 polarization of DCs; up-regulates adhesion molecules in endothelial cells; enhances transendothelial migration of monocytes, vascular smooth muscle cells, and vessel-associated stem cells (mesangioblasts); and increases proliferation of mesangioblasts. HMGB1 binds to a receptor known as RAGE (I love these evocative acronyms!), which stands for "receptor for advanced glycation end products." RAGE is a member of the Ig superfamily. Engagement of RAGE enhances adhesion to endothelial cells, promoting leukocyte recruitment.

Animals engineered to not respond to HMGB1 (either ineffective or absent receptor or no production of HMGB1)

are not normal phenotypically. Necrotic cells from *Hmgb1*$^{1/1}$ animals do not induce inflammation. Blockade of HMGB1 in vivo prevents leukocyte recruitment to a model of acute hepatic necrosis. Deletion of RAGE protects against lethal outcomes of sepsis. Of note, HMGB1 can still induce inflammation even if RAGE is blocked, suggesting that HMGB1 has other receptors. Two candidate receptors are the TLR2 and TLR4, which may recognize HMGB1 epitopes not bound by RAGE. This variety of receptors may help explain the many effects of HMGB1.

Perhaps HMGB1's most important effect is in maturing DCs, in large measure due to the B box. HMGB1 may be a main signal to DC that there is danger—necrotic cells might, after all, derive from infection. HMGB1 acts as an adjuvant—an endogenous activator of the immune response. This may, however, work to the detriment of some patients: Elevated levels of HMGB1 correlate with poor prognosis in sepsis. Of importance to us as rheumatologists (and why I have waxed on and on about HMGB1) is that HMGB1 is found in RA synovial tissue and fluid; injection of HMGB1 causes arthritis in mouse models and, on the contrary, blocking HMGB1 can suppress collagen-induced arthritis.

In addition, some tumors, e.g., colorectal, breast and prostate, overexpress HMGB1; higher expression correlates with greater metastatic potential; blockade of HMGB1 effects, either by interference with RAGE or HMGB1 function, suppresses tumor growth and metastasis. One theory is that HMGB1 stimulates infiltrating leukocytes to produce growth factors that enhance tumor cell growth and function. Thus, there is reason to hope that control of HMGB1 signaling may be of therapeutic use in malignancy as well as in autoimmunity.

CONCLUSION

The build-up or inability to clear apoptotic cells may lead to maturation of DCs, changing them from tolerogenic to immunogenic, and may lead to the production of autoantibodies. The precise physiological role of apoptotic bodies within immunologic function is not clear, but there is evidence suggesting that they may play a role in the maintenance of tolerance and/or the breaking of tolerance—not clear yet, but the answers may be forthcoming soon.

HMGB1 has been implicated in this change of DC phenotype and in physiologic and pathologic inflammation, but the entire truth is probably much more complicated with multiple parallel pathways—additive? synergistic? But for every *yin* there is always a *yang*, so the biotech industry will not be long in seeking to employ some of these mechanisms in pursuit of effective new treatments for autoimmune diseases.

So, we have seen that if not dealt with properly, the contents of dead cells can cause significant damage. Even normally innocuous apoptotic cells can elicit inflammation and nonsalutary immune responses if the cells are not cleared rapidly and effectively. Endogenous adjuvants can turn usually benign processes into autoimmune disasters. And it all hinges on knowing how to deal with death (of the cellular kind).

While I thought that I was learning how to live, I have been learning how to die.

Leonardo da Vinci

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