



## Review

# Autoantigens as substrates for apoptotic proteases: implications for the pathogenesis of systemic autoimmune disease

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## Abstract

Systemic autoimmune diseases are a genetically complex, heterogeneous group of diseases in which the immune system targets a diverse, but highly specific group of intracellular autoantigens. The clustering and marked concentration of these molecules in the surface blebs of apoptotic cells, and their modification by apoptosis-specific proteolytic cleavage and/or phosphorylation at these sites, has focused attention on a unique apoptotic setting as the potential initiating stimulus for systemic autoimmunity. This apoptotic event is likely to (i) occur in a microenvironment containing high concentrations of the targeted antigens, (ii) be pro-immune in nature (e.g. viral infection), and (iii) allow suprathreshold concentrations of antigen with non-tolerized structure (either novel fragments, post-translational modifications, or complexes) to enter the class II processing pathway and initiate a primary immune response. Defective clearance or reduced anti-inflammatory consequences of apoptotic material may be important susceptibility factors in this group of diseases. Once the primary immune response to apoptotic antigens has been initiated, other apoptotic events (occurring in the course of homeostasis or damage) may stimulate the secondary immune response with less stringency, resulting in flares.

**Keywords:** apoptosis; autoimmunity; protease; autoantigen; caspase; granzyme B

**Abbreviations:** CTL, cytotoxic T lymphocytes; DFF, DNA fragmentation factor; ER, endoplasmic reticulum; NK cells, natural killers cells; PARP, poly(ADP-ribose)polymerase; PS, phosphatidylserine; SLE, systemic lupus erythematosus

## Autoantibodies: probes of the perturbed state

There is a growing consensus that the highly specific humoral immune response to autoantigens in systemic autoimmune diseases is T cell-dependent, and that flares of disease result when this primed immune system is rechallenged with self-antigen.<sup>1–3</sup> Although the mechanisms responsible for initiation and propagation of the immune response to specific autoantigens remain unclear,<sup>4,5</sup> autoantigens are unified by their selection by the immune system for an immune response. The targeted molecules must therefore have satisfied the stringent criteria for initiation of a primary immune response during disease initiation (i.e. presentation of suprathreshold concentrations of non-tolerized structure in a pro-immune context.<sup>6</sup>

Since tolerance is only induced to dominant determinants in autoantigens (which are generated and presented at suprathreshold concentrations during natural processing of whole protein antigens), a potential exists for T cell autoreactivity directed against cryptic determinants (which are generated at subthreshold concentrations).<sup>7,8</sup> The hypothesis that autoimmunity arises when usually cryptic determinants become visible to the immune system has received increased attention in the past several years. Indeed, several experimental systems have now clearly demonstrated circumstances which allow previously cryptic determinants to be presented.<sup>8</sup> These include novel autoantigen cleavage,<sup>9,10</sup> and altered autoantigen processing induced by high affinity ligand binding (e.g. to an antibody or receptor molecule).<sup>11–13</sup> We have proposed that autoantigens in systemic autoimmune diseases are unified by the fact that they satisfy the stringent criteria for initiation of a primary immune response during disease initiation.<sup>14,15</sup> The corollary of this proposal is that if the perturbed state can be recreated *in vitro*, these common features of autoantigens (similar alterations of concentration, distribution and structure) will be observed. We and others have therefore used high titer autoantibodies as probes of cell biology and biochemistry of autoantigens during different clinically-relevant perturbed states, to search for those circumstances in which autoantigens become clustered, concentrated and structurally modified.<sup>14–20</sup> This review highlights apoptosis as the potential perturbed state underlying the initiation and propagation of systemic autoimmune diseases, and focuses attention on the alterations in the cell biology and biochemistry that unify autoantigens during this form of cell death. The numerous current gaps in knowledge make parts of this review necessarily speculative. However, these gaps raise

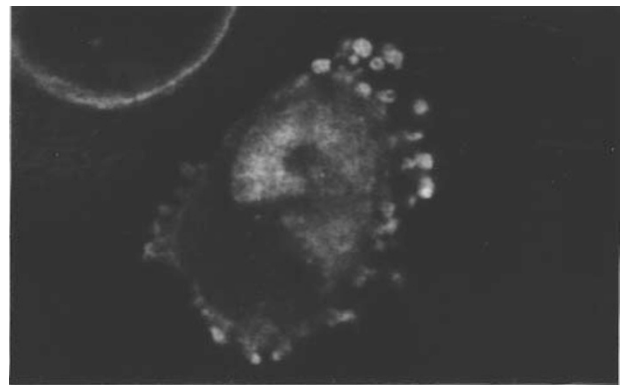
important questions about the normal immune consequences of different forms of apoptosis in tissues, and about whether defects in the signaling, execution and clearance phases of the apoptotic process are susceptibility factors for the development of systemic autoimmune disease.

### Lupus autoantigens undergo a striking redistribution during apoptosis, becoming clustered and concentrated in the surface blebs of apoptotic cells

Studies to delineate the potential perturbed states that might initiate systemic autoimmune diseases have been focused by a striking clinical observation in systemic lupus erythematosus (SLE): that ultraviolet irradiation has a marked propensity to induce flares of both systemic and skin disease in lupus patients.<sup>21</sup> In this regard, the epidermis appears to be an important target of the immunopathologic response in lupus, and constitutes an appropriate *in vitro* model with which to address the effects of flare-inducing stimuli (e.g. UVB).<sup>22</sup> Earlier studies demonstrated that normally intracellular autoantigens can be stained at the exterior surface of keratinocytes incubated *in vitro* for 20–24 h after irradiation with UVB, although the mechanism of this ‘redistribution’ of antigens was not determined.<sup>23,24</sup> To determine how this phenomenon might arise, we studied the subcellular distribution of lupus autoantigens at increasing times after UVB irradiation, in both intact and permeabilized cells.<sup>14</sup> Our initial studies made several observations: (i) UVB-irradiated keratinocytes undergo apoptosis, beginning a few hours after irradiation. Apoptotic keratinocytes manifest the classic morphologic hallmarks of this process, including prominent surface blebbing (early event), and nuclear condensation and fragmentation into apoptotic bodies (later event); (ii) Lupus autoantigens, which are not restricted to any specific subcellular compartment in control cells, are strikingly redistributed in apoptotic cells, such that they become clustered and concentrated within small surface blebs and apoptotic bodies (Figures 1 and 2). Thus, small surface blebs (which contain fragmented rough endoplasmic reticulum (ER); see Figure 1) are highly enriched in 52 kDa Ro, ribosomal autoantigens, as well as those autoantigens found within the ER lumen (e.g. calreticulin). This marked enrichment of autoantigens in small surface blebs is accompanied by a concomitant depletion from the cytosol. Nuclear autoantigens also undergo a striking redistribution and concentration during apoptosis (Figure 2). Thus, 60 kDa Ro, La, the snRNPs, Ku and poly(ADP-ribose)polymerase (PARP), which normally have a diffuse nuclear distribution, initially become concentrated as a rim around the condensing chromatin in early apoptosis. As apoptosis progresses and the nucleus becomes fragmented into multiple membrane-bound apoptotic bodies, nuclear autoantigens remain as a rim around the condensed chromatin.<sup>14,15</sup>

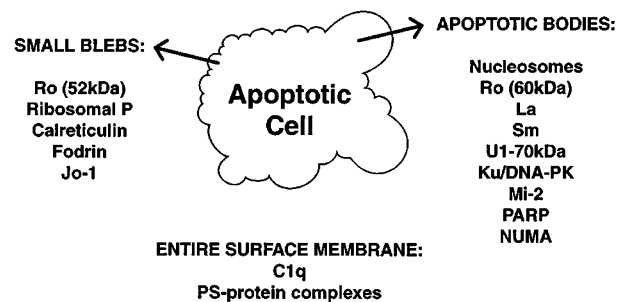
Interestingly, the surface of these apoptotic surface blebs also has the capacity to concentrate potential autoantigens (Figure 2). For example, phosphatidylserine (PS) becomes concentrated at this site early in the apoptotic process.<sup>16,25–27</sup> PS, normally restricted to the

inner surface of the plasma membrane bilayer, becomes rapidly redistributed early in apoptosis, and appears at the external membrane surface. This generates a procoagulant external cell surface which has the capacity to bind several autoantigenic PS-binding proteins, including  $\beta$ 2-glycoprotein-1 and annexin V.<sup>16,26,28,29</sup> The demonstration that these phospholipid-binding proteins decorate the surface of apoptotic cells strongly suggests that the immunogenic phospholipid-protein complexes form at the surface of apoptotic cells *in vivo*. This is further underscored by the observation that IgG purified from the plasma of patients with antiphospholipid syndrome binds to the surface of apoptotic cells, and inhibits its procoagulant activity.<sup>19</sup>



**Figure 1** Ro is highly enriched in small blebs at the surface of early apoptotic keratinocytes. Irradiated keratinocytes were stained with a serum recognizing 52 kDa and 60 kDa Ro, and examined by confocal immunofluorescence microscopy. A single apoptotic cell is seen in this field; the partial visualization of the adjacent non-apoptotic cell results from their situation in a different plane. The small surface blebs are highly enriched in 52 kDa Ro, and a concomitant depletion of Ro from the cytosol is noted. No significant changes in distribution of nuclear Ro (60 kDa) is noted at this early time-point

### Clustering of Autoantigens in Apoptotic Surface Structures



**Figure 2** Clustering of autoantigens in apoptotic surface structures. Autoantigens targeted in systemic autoimmune diseases are clustered and concentrated in surface structures of apoptotic cells. Small blebs contain molecules associated with endoplasmic reticulum and membrane skeleton, apoptotic bodies are enriched in nuclear autoantigens, and the entire surface membrane of the apoptotic cell provides a surface to which phospholipid-binding proteins (e.g.  $\beta$ 2-glycoprotein-1, annexin V) or C1q attach

It is also of interest that the surface blebs of apoptotic keratinocytes bind C1q,<sup>30</sup> whose collagen-like domains are the frequent (~47%) target of a high titer autoantibody response in patients with SLE.<sup>31</sup> The exact binding partner for C1q at the apoptotic surface is not yet known, but recent studies suggest that C1q binding to apoptotic cells may have a critical function in clearance of apoptotic cells in some microenvironments.<sup>32</sup> Interestingly, C1q deficiency is strongly associated with the development of SLE in both humans and mice.<sup>33</sup> In the C1q-null mouse, Walport and colleagues noted a marked accumulation of apoptotic cells in the kidney, suggesting that clearance of apoptotic cells is defective in this microenvironment,<sup>34</sup> and focusing attention on clearance of apoptotic cells as an important potential defect underlying the development of systemic autoimmunity.

### **Susceptibility to efficient cleavage by caspases unifies a subgroup of systemic disease autoantigens**

Proteolysis plays an important mechanistic role in the apoptotic pathway, accomplished through the specific cleavage of a limited number of downstream substrates.<sup>35</sup> This apoptosis-specific proteolysis is catalyzed by a unique family of cysteine proteases (called caspases, for cysteine proteases that cleave after aspartic acid), that have an absolute requirement for aspartic acid in the substrate P<sub>1</sub> position.<sup>36</sup> Since initiation of the primary immune response requires that non-tolerized structure be generated, we were intrigued by the observation that the first proteolytic victims of the caspases discovered in apoptosis were poly(ADP-ribose)polymerase (PARP) and lamins,<sup>37</sup> both of which are autoantigens targeted in systemic autoimmune diseases.<sup>38,39</sup> We therefore addressed whether other autoantigens were similarly cleaved by caspases during apoptosis. Using Western blotting of lysates of control and apoptotic cells, we detected a group of >20 autoantigens that were recognized by a high titer autoantibody response, and which were cleaved early in apoptosis.<sup>15,18,19</sup> The cleaved molecules included U1-70 kDa, DNA-PK<sub>cs</sub>, NuMA, as well as several other molecules that remain to be identified. Cleavage of these molecules occurred early during apoptosis, and was inhibited by caspase inhibitors, implicating the involvement of a caspase either directly or upstream of these cleavage events. Subsequent studies demonstrated that U1-70 kDa and DNA-PK<sub>cs</sub> are indeed directly cleaved by caspase-3, with efficiencies similar to that previously observed for PARP.<sup>19</sup> Several other groups have demonstrated that other systemic disease autoantigens are similarly cleaved during apoptosis, including topoisomerase I, NOR-90, fodrin, and hnRNP C1/C2.<sup>20,40–42</sup>

Based on their sensitivity to cleavage by caspases, lupus autoantigens segregate into two groups. In general, those autoantigens that are efficiently cleaved by the caspases during apoptosis are infrequent targets (0.2–5% of patients) of a high titer autoantibody response in SLE. Many of the frequent (>15% of patients) targets of high titer autoantibodies in SLE (particularly ribonucleoprotein [Ro, La, Sm] and deoxyribonucleoprotein antigens) are

not cleaved during apoptosis. Thus, while susceptibility to efficient cleavage by a caspase is a frequent feature of simple protein autoantigens, it is not a universal feature of all autoantigens. This imperfect, though striking correlation of susceptibility to caspase cleavage and status as an autoantigen require that caution be exercised in ascribing an essential role of caspase cleavage for defining molecules as autoantigens, since it suggests that additional (partially overlapping) properties might be relevant. For example, (i) autoantigens that are cleaved by caspases during apoptosis might also be cleaved by other proteases during specific forms of apoptosis in unique tissues/microenvironments; (ii) autoantigens that are not cleaved by caspases or other proteases might undergo other apoptosis-specific structural modifications (e.g. formation or alteration of novel protein-protein or protein-nucleic acid complexes). Understanding whether and how the structure of uncleaved autoantigens might be altered during apoptosis is an important focus for future studies.

It is important to note that autoantigen clustering and cleavage occurs in almost all forms of apoptosis described to date, which often occur in non-immune contexts.<sup>18–20,43,44</sup> The marked frequency of apoptosis in normal development and homeostasis, coupled with the infrequency of systemic autoimmunity in the population, strongly suggest that only a very restricted subset of apoptotic events (e.g. those occurring in a pro-immune setting), in individuals that are genetically predisposed to allowing the generation of novel autoantigen structure (e.g. from abnormalities in the clearance and degradation of apoptotic material in tissues), will initiate a self-sustaining autoimmune response. In this regard, it is of great interest that intravenous immunization of non-autoimmune mice with  $\gamma$ -irradiated, syngeneic apoptotic thymocytes results in the transient production of low levels of autoantibodies (anticardiolipin and anti-ssDNA), and some associated renal immunopathology.<sup>45</sup> It will be of interest to define whether unique forms of apoptotic cell death, and defects in the clearance of apoptotic material might alter the response to immunization with apoptotic material in this model.

### **Novel autoantigen fragments are produced during cytotoxic lymphocyte-induced target cell apoptosis**

One form of apoptosis that frequently occurs in a pro-immune setting is the death of virally-infected target cells, induced by cytotoxic T lymphocytes (CTL). CTLs use several pathways to induce target cell apoptosis, including Fas-ligation and granule exocytosis.<sup>46</sup> Granzyme B, a serine protease found in the cytoplasmic granules of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells, has a similar substrate specificity to the caspases, in its requirement for aspartic acid in the substrate P<sub>1</sub> position.<sup>47</sup> Granzyme B also has a similar tetrapeptide specificity to the group III caspases (requiring Ile/Val in P<sub>4</sub> and Glu in P<sub>3</sub>).<sup>48</sup> Recent studies have shown that granzyme B plays an important role in inducing apoptotic changes in target cells during granule exocytosis induced cytotoxicity,<sup>49,50</sup> partly by catalyzing the cleavage and activation of several caspases.<sup>51</sup> Granzyme B also initiates

caspase-independent pathways which contribute to target cell death, although the nature of these additional pathways remains undefined.<sup>52,53</sup> One candidate process is the direct proteolysis of death substrates by granzyme B. In recent studies, we demonstrated that DNA-PKcs and NuMA are directly and efficiently cleaved by granzyme B, both *in vitro* and in cells undergoing granule-induced cytotoxicity.<sup>54</sup> Granzyme B-mediated cleavage of these substrates generates unique fragments not generated during any other form of apoptosis studied to date. Efficient cleavage by granzyme B (with the generation of distinct fragments) has recently been observed for numerous other systemic disease autoantigens, including several that are not cleaved by caspases (Casciola-Rosen *et al*, unpublished results). This striking common feature of many autoantigens, together with their clustering at the same site in apoptotic cells, focuses attention on the process of granule-induced apoptosis as a potential initiator of autoimmunity in the susceptible host. It will be extremely important to evaluate whether the immunogenicity of these novel forms of autoantigen is indeed increased over uncleaved forms of these molecules.

### Defects in clearance and degradation of the apoptotic corpse in tissues may be an important defect underlying SLE

Recent studies have emphasized that apoptotic cells are not immunologically inert, but rather have either positive or negative immune effects, depending on the antigen-presenting cell with which they interact.<sup>55-57</sup> For example, human dendritic cells (but not macrophages) efficiently present antigen derived from apoptotic (but not non-apoptotic) cells, stimulating class I-restricted CTLs.<sup>56</sup> When macrophages are present together with dendritic cells in this assay, the presentation of apoptotic material is markedly inhibited. Several potential mechanisms might account for this observation: (i) phagocytosis and degradation of apoptotic cells by macrophages is highly efficient,<sup>57</sup> thereby minimizing access of apoptotic material to dendritic cells; and (ii) phagocytosis of apoptotic cells by macrophages induces the production of anti-inflammatory cytokines (e.g. TGF- $\beta$ 1), and inhibits the production of several proinflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$ ).<sup>58,59</sup> It is possible that an abnormality in any of these pathways that are involved with rapid clearance of apoptotic material, or expression of its anti-inflammatory activities, may initiate autoimmunity. The recent observation of increased numbers of uncleared apoptotic cells in the kidneys of an SLE-susceptible C1q-null mouse reinforces that abnormal clearance of apoptotic cells may play a role in the pathogenesis of SLE in this animal.<sup>34</sup> Although there is at present no data that directly addresses whether, and by what mechanism, impaired clearance of apoptotic cells may initiate or exacerbate the autoimmune state *in vivo*, delayed clearance might change (i) the compartmentalization of autoantigens (allowing leakage of autoantigen during secondary necrosis, and access of soluble molecules to efficient macropinocytotic and endocytic antigen capture by dendritic cells), or (ii) provide apoptotic cells and membrane-bound fragments access to different (pro-immune) populations of antigen presenting cells, from which they are normally excluded.<sup>56</sup>

While a recent study in humans has demonstrated that the clearance of apoptotic lymphocytes and fragments by macrophages is impaired in some patients with SLE, there was significant heterogeneity in the level of impairment among patients, which did not appear to correlate with disease activity or therapy.<sup>60</sup> It remains unclear whether (i) this phenomenon results from abnormalities in recognition, binding or phagocytosis of apoptotic cells by SLE macrophages; (ii) the phagocytosis of all types of apoptotic cell are similarly affected; and (iii) the degree of impairment in a particular patient varies with disease activity. Recent advances in understanding the molecular basis for recognition and phagocytosis of apoptotic cells<sup>29,57,61</sup> will begin to allow the molecular basis of impaired clearance of apoptotic material in patients with autoimmune diseases to be defined. The marked heterogeneity of the degree of impairment observed in different patients predicts significant complexity in this population, resulting from the contribution of multiple different clearance pathways. Furthermore, understanding the immunologic consequences of apoptosis is likely to be similarly complex, since both the form of apoptotic cell, the phenotype and physiologic state of the antigen-presenting cells, and the relevant tissue microenvironment may contribute.

### Stress-induced phosphorylation of autoantigens during apoptosis: indicator of the perturbed state?

Recent studies by Utz *et al* have demonstrated that several autoantigens targeted in SLE are specifically phosphorylated by a family of stress-activated protein kinases during apoptosis.<sup>62,63</sup> Interestingly, several autoantibodies preferentially recognize the phosphorylated forms of their autoantigens,<sup>64</sup> suggesting that these modifications were present during initiation of the primary immune response. These studies focus attention on defining those perturbing stimuli which lead to autoantigen phosphorylation and initiate apoptosis, and on addressing whether phosphorylation of autoantigens during apoptosis alters the immunogenicity of these molecules.

### Model of SLE

The studies presented above have focused attention on unique forms of apoptosis as a candidate process that initiates and propagates systemic autoimmune diseases. In the genetically susceptible individual (e.g. someone who has a defect in the ability to efficiently phagocytose and degrade apoptotic cells and debris, or to mount an adequate anti-inflammatory response upon ingestion of apoptotic material), the confluence of several forces allows the generation of suprathreshold concentrations of non-tolerized structure in the presence of co-stimulatory signals (Figure 3A), and the access of this material to the MCH class II pathway of a population of antigen-presenting cells that efficiently initiate a primary immune response. The low frequency of this form of autoimmunity in the population likely reflects this need to simultaneously satisfy several very stringent criteria to initiate the primary immune response. The molecules targeted are

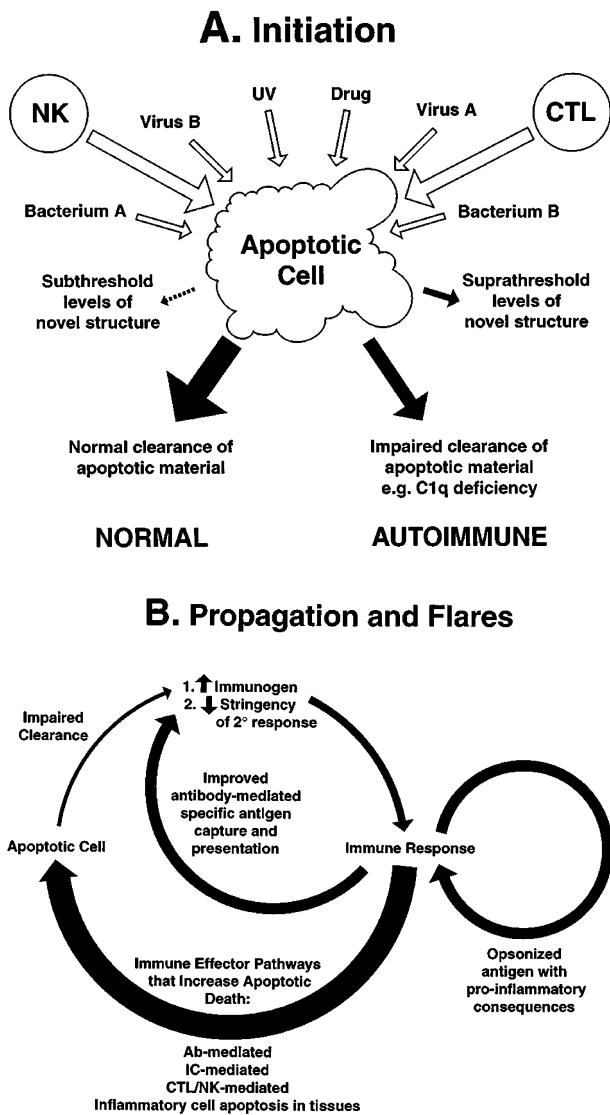


Figure 3 Model of Systemic Autoimmune Disease. See text for details

unified by their susceptibility to modification during the perturbing process, likely revealing previously cryptic structure. Available data demonstrates that many (but not all) autoantigens are specifically cleaved by caspases during apoptosis. Furthermore, a similar (but not identical) subset of autoantigens is also directly cleaved by granzyme B, generating unique fragments not observed during any other forms of apoptosis studied to date. Once primary immunization has occurred, the repeated generation of apoptotic material (e.g. during sun exposure, viral infection, drug exposure) might efficiently rechallenge the primed immune system (the stringency of this secondary response being significantly lower than that of the primary response) (Figure 3B). Furthermore, the effector pathways activated by the primed immune system include several which themselves generate loads of apoptotic material (e.g. cellular cytotoxicity, myelomonocytic cell recruitment and apoptosis).<sup>57</sup> The

opsonization of apoptotic material by antiphospholipid and anti- $\beta$ 2-glycoprotein I antibodies,<sup>65,66</sup> may both increase the efficiency of apoptotic antigen capture, as well as induce the production of pro- rather than anti-inflammatory cytokines, potentially further driving the immune response. This capacity for immune-driven autoamplification may be one of the critical principles underlying severe systemic autoimmune disease (Figure 3B).

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