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The putative role of apoptosis-modified histones for the induction of autoimmunity in Systemic Lupus Erythematosus

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Abstract

In recent years, it has become evident that Systemic Lupus Erythematosus (SLE) is a disease characterized by an array of autoantibodies directed against the native nucleosome, its DNA component and/or its histone component. Nuclear antigens are generated and released *in vivo* during apoptosis. A hallmark of apoptosis is the cleavage of chromatin by caspase-activated DNase. This fragmentation occurs at the internucleosomal level and leads to DNA ladder formation classically associated with apoptosis. Thus, dysregulation of DNA fragmentation might be directly linked to the induction of autoimmunity in SLE. In our studies, activated human lymphoblasts contain high amounts of core histones in their cell lysates after apoptosis induction. This accumulation correlated highly with markers of early apoptosis (Annexin V positive, propidium iodide negative), but not with markers of late apoptosis or necrosis. Interestingly, accumulation of core histones or nucleosomes in cell lysates was detected as early as 30 or 60 min after UV irradiation, whereas phosphatidylserine externalization occurred 2 hr after apoptosis induction. Our results suggest that extranuclear accumulation of core histones is a very early event in apoptosis, preceding the externalization of phagocytosis signals on the outer membrane surface of apoptotically dying lymphoblasts. The following review will discuss these results in a broader perspective which includes our hypothesis of how apoptosis dysregulation during early phases may contribute to the induction of autoimmunity against nuclear autoantigens as seen in SLE.

Keywords: Apoptosis; Autoantibody; Autoimmunity; Histone; Nucleosome; SLE

1. Introduction

Systemic Lupus Erythematosus (SLE) is characterized by the production of autoantibodies to multiple nuclear antigens, including antibodies directed against DNA and histones [1]. In recent years, it has become evident that the nucleosome, the fundamental unit of the chromatin, might play a key role in the pathogenesis of SLE [2]. A major mechanism by which nuclear antigens are generated and released *in vivo* is by the process of programmed cell death (apoptosis) [3].

2. Apoptosis and autoantibodies

We hypothesise that accelerated apoptosis of circulating lymphocytes occurs under certain circumstances in SLE patients. Consequently, this increased rate of apoptosis could lead to an overflow of the phagocytic system with apoptotic cell material, resulting in an increased amount of nuclear components, e.g. dsDNA, nucleosomes, or histones in the serum of these patients. This intracellular or nuclear material would be presented to and recognized as nonself antigens by immunocompetent cells, leading to the formation of autoantibodies against intracellular or nuclear particles. Antibodies to dsDNA or nucleosomes are observed in about 70% of lupus patients [2,4]. Therefore, these autoantibodies are considered as keymarkers of SLE.

It was shown in a study published by Arbuckle *et al.* [5] that 55% of SLE patients had already anti-dsDNA antibodies in their serum before diagnosis. In many patients the amount of anti-dsDNA antibodies fluctuates in parallel

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Abbreviations: AxV, annexin V; dsDNA, double-stranded DNA; H1, H2A, H2B, H3, H4, histones 1, 2A, 2B, 3, 4; IL-2, interleukin-2; Med, medium; PBMC, peripheral blood mononuclear cells; PI, propidium iodide; SLE, Systemic Lupus Erythematosus; UV, ultraviolet.

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with the clinical disease activity [6]. Furthermore, patients with a significant rise in anti-dsDNA antibodies at diagnosis were more likely to have renal disease than those without an increase in dsDNA antibody titers [5]. It is discussed that these autoantibodies bind to the glomerular basement membrane either alone or as antigen (dsDNA, nucleosome, histone)/antibody complexes, thereby causing inflammation and tissue damage in the kidney [7].

In this view, it was interesting that there was a correlation between SLE disease activity and the rates of lymphocyte apoptosis in culture [8]. This suggests that a higher apoptosis rate may lead to the production of autoantibodies, triggering disease activity.

3. General features of apoptosis

Apoptosis is characterized by morphological changes such as chromatin condensation, and a reduction of cell volume [9]. A hallmark of apoptosis is the cleavage of chromatin by caspase-activated DNase [10]. DNA fragmentation seems to occur in four steps [11]. DNA fragments of 1–2 Mbp were the first observed degradation products. These DNA fragments were gradually degraded into DNA fragments of 200–800 kb or smaller than 100 kb fragments upon further incubation. With increasing time, a characteristic DNA ladder appears with multiples of 180–200 bp DNA fragments [12].

Another characteristic feature of cells undergoing apoptosis is the loss of phospholipid asymmetry in the plasma membrane with exposure of phosphatidylserine on the outer membrane leaflet [13]. It was shown that annexin V (AxV) preferentially binds to phosphatidylserine [14]. Propidium iodide (PI) staining is widely used to discriminate between living cells, which exclude this DNA dye, from dead cells, which are permeable to it. When using a double staining with AxV and PI by flow cytometry, four different fractions of cells with different properties are visible [15]: (1) viable cells ($AxV^{neg}PI^{neg}$) are impermeable for PI as well as do not bind AxV; (2) early apoptotic cells ($AxV^{pos}PI^{neg}$) bind AxV and are PI impermeable; (3) late apoptotic or also called secondary necrotic cells ($AxV^{pos}PI^{low}$) bind AxV and are PI permeable with a sub-G1 DNA content; (4) primary necrotic cells ($AxV^{pos}PI^{pos}$) bind AxV and are PI permeable with G1 DNA content.

It was observed that cells whose membranes which bind AxV contain fragmented DNA. After induction of apoptosis in B-cell lines and freshly isolated germinal center B-cells, cells which bound AxV also showed nuclear condensation [16]. This was accompanied by the appearance of DNA fragments. Similar results were published by Bacso *et al.* [17]. In the Jurkat T-cell line, these authors reported that AxV binding gradually increased 2 hr after apoptosis induction and a strong increase was observed after 4 hr. They also detected a weak increase of damaged DNA after 2 hr and a significant augmentation 4 hr after apoptosis induction compared to cells before apoptosis induction. This suggests that the increase of DNA damage in apoptotic cells occurs somewhat later than the increase of AxV binding. However, on the single cell level most of the AxV positive, PI negative cells contained damaged DNA.

All these results show that the expression of the phagocytosis signal on the membrane surface and the DNA degradation occur at very early timepoints during apoptosis.

4. Release of histones and nucleosomes during early apoptosis

The nucleosome is composed of a core particle with a tetramer of each two molecules histones H3 and H4 in the center flanked by two histones H2A–H2B dimers. Two superhelical turns of DNA are wound around this histone octamer. The linker histone H1 is associated with the nucleosome core particle at the entry and exit points of the DNA. DNA fragmentation occurs at the internucleosomal level and leads to DNA ladder formation classically associated with apoptosis [12].

We recently reported that only very weak signals of the nucleosomal histones (H2A, H2B, H3, and H4) were detected by Western Blot analysis in cell lysates of freshly isolated human PBMC [18]. However, no bands for the linker histone H1 were detected by this method. In contrast, activated human lymphoblasts showed elevated amounts of all five histones in cell lysates compared to PBMC. Furthermore, we observed an increased amount of the core histones H2A, H2B, H3, and H4 in cell lysates after 8 or 24 hr IL-2 withdrawal compared to the starting point ($t0$). Signal intensity of these histones in cell lysates decreased after re-addition of IL-2 to the lymphoblasts after IL-2 withdrawal. However, the content of the linker histone H1 in cell lysates was not affected by IL-2 deprivation or addition. The content of H2A, H2B, H3, or H4 in cell lysates correlated significantly ($P < 0.001$ and $r > 0.52$) with the percentages of early apoptotic lymphoblasts (AxV positive, PI negative). We did not find any correlation to the number of late apoptotic cells or necrotic cells.

UV (ultraviolet) light exposure and etoposide treatment resulted in an increase of all histones in cell lysates compared to activated lymphoblasts at $t0$ [19]. The greatest increase of 60–100 times was observed for the histones H2B and H4, whereas histones H3 and H2A were augmented 30–50-fold. In contrast to these core histones, the content of linker histone H1 in cell lysates was only 2-fold augmented. Furthermore, this increase of histone amount in cell lysates was accompanied by a considerable increase of early apoptotic cells, but no significant change of late apoptotic cells or necrotic cells was observed. The percentages of cells with subdiploid DNA contents increased also to similar amounts obtained with AxV/PI staining.

In a time course experiment, a continuous increase of the relative amount of histones and nucleosomes was observed in cell lysates after UV irradiation compared to lymphoblasts before UV light exposure [20]. An increase of the nucleosomal histones in cell lysates was detected even 30 or 60 min after UV light exposure. However, no difference between the percentage of early apoptotic cells (AxV positive, PI negative) was observed between *t*0, 30, and 60 min (about 2%). The first increase in the percentage of early apoptotic cells was noted after 2 hr to about 7%. Moreover, the increase of the relative ratios for each histone class differed during the first 2 hr after UV light exposure: the relative increase of signal intensities were higher for the histones H2B and H4 compared to the histones H2A or H3 highlighting the specificity of these observations.

It was recently reported that histones were also found in cell lysates obtained from apoptotic Jurkat cells and U937 cells [21]. However, H1 was only detected in cell lysates of Jurkat cells, but the signal was absent or only very weak in U937. Other studies showed that Jurkat cells and human-activated lymphocytes accumulate nucleosomes in the cytoplasm during apoptosis [22,23]. However, not all cell lines generate nucleosomal fragments [24] or a sub-G1 population [25] during apoptosis. Furthermore, it is important to note that accumulation of histones is not a necessary consequence of apoptosis-related DNA fragmentation. Wu *et al.* [26] showed that not all cell lines release histones from nucleosomes during DNA fragmentation and apoptosis. It was demonstrated that cells can contain fragmented DNA, but their cell lysates did not contain histones. Thus, the authors convincingly provided evidence that histone release and DNA fragmentation can be clearly separated in two independent processes in their tumor cell lines.

Because H2A, H2B, H3, and H4 form the core structure of the nucleosome and bind to DNA, they are normally insoluble in non-ionic detergents like Triton X-100. Consequently, their detection in apoptotic cell lysates prepared in detergent containing buffer indicates that they have been released or separated from chromatin in form of cleaved nucleosomes or as free histones. All the detected histones are likely to represent cytoplasmic histones/nucleosomes since the lysis conditions would not solubilize nuclear intact material, which would therefore be present in the pellet rather than in the supernatant after centrifugation of the lysates. This is further supported by the different kinetics of the various histones appearing in cell lysates, especially after stronger induction of apoptosis with etoposide or UV light exposure and by the fact that cell lysates from PBMC only contained very low amounts of histones. Furthermore, the nuclear membrane and nuclear pore complexes remain intact throughout apoptosis [27]. These findings support the notion that the appearance of histones in cell lysates is not attributed to detergent mediated nuclear membrane degradation. All these arguments suggest that the detected histones in the cell lysates were localized in the cytoplasm.

It was reported that human lymphocytes release nucleosomes into the culture medium during the process of apoptosis [8]. There was a strong correlation between the amount of apoptosis and the nucleosomes in cell culture supernatants. Furthermore, nucleosomes were also detected in cell culture supernatants of apoptotic Jurkat cells [28]. These cells showed first an increased AxV binding and before these cells later on were also PI permeable. There was a strong correlation of nucleosome appearance in the medium to signs of late apoptosis (AxV binding, PI permeable). The nucleosome release from late apoptotic Jurkat cells was 12 hr delayed to the appearance of AxV binding cells.

These data support the concept that even at early timepoints of apoptosis small nucleosomal fragments are present in the cytoplasm of the dying cell. Our results indicate that DNA degradation measured by a nucleosome ELISA was observed earlier than AxV binding [29]. In contrast, DNA degradation (sub-G1 peak) measured by PI staining in a hypotonic buffer was only detected after 3–3.5 hr after apoptosis induction (unpublished results). This suggests that different processes must occur in the cell nucleus. First small nucleosomal fragments might be released from the nucleus before DNA is degraded into large fragments, which are subsequently cut into smaller fragments forming the DNA ladder.

5. SLE pathogenesis and apoptosis

The plasma of SLE patients contains larger amounts of circulating DNA than the plasma of healthy individuals [30–32]. This circulating DNA is present in the form of oligonucleosomal DNA fragments (180–200 bp) complexed with histones [33]. These DNA fragments were present as nucleosomes (dsDNA and histones) in the plasma [34]. It was noted that serum from SLE patients contained significantly higher amounts of nucleosomes compared to normal healthy donors [34]. In murine models of SLE, it was demonstrated that the initial immune response is directed against nucleosomes and spreads later to its individual components dsDNA and histones [35,36]. Thus, it seems that SLE is a disease characterized by an array of autoantibodies directed against the native nucleosome (nucleosome-specific antibodies), its DNA component (bona fide anti-ds-DNA antibodies), and its histone component (bona fide antihistone-specific antibodies) which together compose the large family of antinucleosome antibodies [36].

Previous studies revealed that lymphocytes from SLE patients showed a higher rate of apoptosis after 48–72 hr of cell culture compared to lymphocytes from rheumatoid arthritis patients or normal donors [8,37]. The percentage of early apoptotic cells (AxV positive, PI negative) in

peripheral blood was significantly higher in SLE patients compared to normal donors [38]. All these observations might explain the higher plasma levels of DNA and nucleosomes in SLE patients compared to normal healthy donors [30–32,34].

It is important to mention that dsDNA is not immunogenic *per se* [39]. However, these authors reported that plasma nucleic acids from SLE patients was immunogenic in contrast to dsDNA. The mechanism of the immune response is not completely understood. It is interesting to speculate that plasma nucleosomes in SLE are derived from apoptotic cells. Alterations occurring during apoptosis [40,41] might render these nucleosomes immunogenic. We have indeed found that histones in cell lysates of human lymphoblasts were deacetylated (H4) or dephosphorylated (H1 and H3) in contrast to nuclear-derived histones which were acetylated or phosphorylated [42].

It was observed that *in vitro* cultured PBMC from a subset of SLE patients contained higher amounts of cell debris compared to PBMC from normal healthy donors [43]. This finding was accompanied by a lower ratio of macrophages from SLE PBMC containing phagocytosed apoptotic nuclei compared to macrophages from normal donors. In addition, in a sub-group of SLE patients, apoptotic cells were not properly cleared by macrophages within the germinal center of the lymph node [44]. This apoptotic material from SLE patients was directly associated with the surface of follicular dendritic cells. Consequently, nuclear apoptosis-derived antigen was bound to potential antigen presenting cells, may trigger the immune reaction and provide survival signals for autoreactive B-cells.

These events (dysregulated apoptosis and/or phagocytosis) might contribute to the development of SLE [7,45].

6. The possible role of histones for the induction of autoimmunity in SLE

Apoptosis is a physiological form of cell death required to ensure that the rate of division is balanced by the rate of cell death in multicellular organisms. As mentioned above, a stereotypical pattern of morphologic changes occurs after the initiating signal, including cytoplasmatic shrinkage, chromatin condensation and DNA cleavage at the internucleosomal sites. In the last step, this apoptotic cellular material is packed in membrane-capsuled blebs before budding from the cell as apoptotic bodies [9]. These blebs contain autoantigens as nucleosomal DNA, Ro, La, and small ribonucleoproteins [46]. Under normal circumstances, these apoptotic bodies are rapidly phagocytosed by neighboring cells and resident phagocytes. During apoptotic breakdown, many nuclear constituents are post-translationally modified, possibly altering antigenicity. Therefore, it is not surprising that failure to appropriately achieve programmed cell death and to clear apoptotic cell fragments is discussed as a key pathogenetic factor leading to autoimmunity.

Various studies have demonstrated an increased apoptosis of *in vitro* cultured blood mononuclear cells of SLE patients. The increased rate of apoptosis of PBMC from SLE patients could be diminished by feeding the cultures with IL-2 [37]. Interestingly, lymphocytes of SLE patients with a bacterial infection die via apoptosis in much higher quantities [37]. This might link to the clinical observation that a flare of SLE frequently follows an infectious episode [47,48].

During the accelerated induction of apoptosis in SLE cells, caspases will be activated and the membrane potential for mitochondria decreased. Through the caspase pathway phosphatidylserine is externalized from the inner membrane to the outer membrane as signal for phagocytic removal of the dying cell without induction of an inflammatory response. Especially during these early phases of apoptosis, a dysregulation would be deleterious in a way that failure to appropriately clean the organism from apoptotic cells might expose neighboring antigen presenting cells to nuclear neoepitopes. At these very early phases of apoptosis, we found free histones or nucleosome-complexed histones already released from the nucleus. This occurred earlier than phosphatidylserine expression on the cell surface. As shown, nucleosomes might be released into the medium as well. Thus, the increased levels of nucleosomes in SLE sera might stem from dying cells. However, it is important to note that this is not specific for SLE sera but is also seen in sera from patients under dialysis or during sepsis. Whether the reduced DNase I concentration in SLE sera contribute to the development of autoimmunity against apoptotic cells and thereby to the pathogenesis of the disease itself (as seen in DNase I knockout mice [49–51]) is unclear to date. *In vitro*, apoptotic material can indeed activate T-cells and B-cells: co-incubation of autologous apoptotic material with freshly isolated PBMC led to the expansion of histone-specific T-cell clones which could provide help to B-cells for the formation of dsDNA antibodies [52]. In similar experiments, there were nucleosome-specific T-cells detected in SLE patients as well [53].

Whether this *in vitro* scenario is also central for an *in vivo* situation in SLE is investigated by many groups right now.

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