EXTENDED REPORT

Time between onset of apoptosis and release of nucleosomes from apoptotic cells: putative implications for systemic lupus erythematosus

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Objective: To investigate the kinetics of nucleosome leakage from apoptotic cells in an in vitro system and extrapolate the results to autoimmune disease, in particular systemic lupus erythematosus.

Methods: A sensitive nucleosome enzyme linked immunosorbent assay (ELISA) was developed, using a monoclonal antibody (mAb) against histone 3 and an mAb against nucleosomes. Nucleosome release during apoptotic cell death was studied in Jurkat cells. AnnexinV binding (early apoptosis) and propidium iodide positivity (late apoptosis) of the cells were compared with nucleosome release at different times after apoptosis induction.

Results: Nucleosomes appeared in culture supernatant of Jurkat cells 24 to 48 hours after apoptosis induction, when the cells had been late apoptotic for more than 12 hours.

Conclusion: Nucleosomes are released from late apoptotic Jurkat cells, with a 12 hour delay from the appearance of AnnexinV binding cells. This result suggests that in vivo scavenger mechanisms have 12 hours to remove apoptotic material from the circulation.

E ukaryotic DNA is packed in chromatin. In inactive chromatin, the DNA is complexed to histones and forms nucleosomes. A nucleosome is an octamer of four pairs of histones 2A, 2B, 3, and 4, around which two superhelical turns of 146 bp dsDNA are wound. Histone 1 and a linker of 60 bp dsDNA connect the individual nucleosomes like beads on a string.

During apoptotic cell death, oligo- and mononucleosomes are generated by internucleosomal cleavage of chromatin.[1] The nucleosomes are then packed in apoptotic blebs along with other nuclear components.[2] In vivo, these blebs are released into the circulation and are cleared by various mechanisms, among which are direct complement binding, complement binding through C reactive protein, serum amyloid protein, and mannose binding lectin, and immunoglobulin G (IgG) binding, followed by phagocytosis and subsequent uptake by the liver.[3–9] Efficient clearance prevents the occurrence of nucleosomes in plasma.

In patients with systemic lupus erythematosus (SLE), nucleosomes have been found in the circulation and it is well established that they function as an autoantigenic target.[10–16] Several mechanisms have been proposed that might lead to the production of antinucleosome antibodies: bypass of tolerance, molecular mimicry between self antigens and bacterial antigens, and exposure to altered antigens or neoantigens.[17–20] The presence of somatic mutations and class switching suggests that production of antinucleosome antibodies is antigen driven and might be due to deficient clearance of apoptotic cells from the circulation.[21–28] If such cells circulate for long enough, they will eventually become late apoptotic and the nuclear membrane will become permeable. Intracellular and intranuclear components such as nucleosomes will be released which, upon contact with antigen presenting cells, can lead to formation of antinucleosome antibodies.[15 29–34]

We studied the leakage of nucleosomes from apoptotic Jurkat cells. For this purpose we developed a sandwich enzyme linked immunosorbent assay (ELISA) with monoclonal antinuclear antibodies for the measurement of nucleosomes, similar to nucleosome assays described by other groups.[11 13 26 35] We found that the time between induction of apoptosis and the presence of nucleosomes in the culture medium is 24 hours. This implies that clearance mechanisms in vivo have a generous time in which to remove apoptotic material from the circulation.

METHODS

Monoclonal antinuclear antibodies

The two antinuclear monoclonal antibodies (mAbs, CLB-ANA/58 and CLB-ANA/60) used in this study were taken from a large panel of mAbs, obtained by fusion of spleen cells of mice with spontaneous SLE ((NZB×W)F1) with the murine myeloma cell line SP2/0. The primary screening method used was an anti-dsDNA ELISA.[36] Hybridomas were grown in culture medium (Iscove modified Dulbecco's medium (IMDM), BioWhittaker Europe, Verviers, Belgium) supplemented with 2.5% fetal bovine serum (Bodinco BV, Alkmaar, The Netherlands), 50 µM β-mercaptoethanol, penicillin (100 IU/ml), streptomycin (100 µg/ml), and recombinant interleukin 6 (500 U/ml). Immunoglobulin was purified from the culture supernatant using a protein A Sepharose column (Pharmacia, Uppsala, Sweden) under high salt conditions (3 M NaCl, 1.5 M glycin-NaOH, pH 8.9).[37] After elution with 0.1 M sodium citrate pH 5, the immunoglobulin was dialysed against DNAse buffer (0.02 M Tris-HCl pH 7.5, 0.01 M CaCl$_2$, 0.01 M MgCl$_2$) and diluted to a final concentration of 0.5 mg/ml. DNAseI (Boehringer Mannheim, Mannheim, Germany) was added to a final concentration of 0.5 mg/ml followed by one hour's incubation at 37°C. DNAseI treated mAbs were directly applied on the second protein A column under high salt conditions[37] and eluted with 0.1 M sodium citrate pH 5. Monoclonal antibodies were dialysed against phosphate buffered saline (PBS) and kept at –20°C until use. Both mAbs were of the IgG2a κ subclass.

Specificity analysis of antinuclear antibodies

The Crithidia assay and HEp-2 assay were performed as described before, using an FITC conjugated goat antibody to mouse IgG (GM17-FITC, CLB, Amsterdam, The Netherlands).[38] [39] The antihistone ELISA was performed as described before,[37] with the following modifications: as a standard we used the culture supernatant of a specific antihistone mAb (CLB-ANA/92), which was defined as 1000 antihistone U/ml; all incubations were performed in high performance ELISA buffer (HPE buffer, CLB, Amsterdam, The Netherlands).

Histone immunoblotting was performed as follows: histones 1, 2A, 2B, 3, and 4 (Boehringer Mannheim, Mannheim, Germany) were run at 0.5 µg/lane on homogeneous 20% sodium dodecyl sulphate (SDS)-polyacrylamide (PAA) gels using the Pharmacia Phastgel system (Pharmacia, Uppsala, Sweden). Proteins were blotted onto nitrocellulose membranes (Protran, Schleicher&Schuell, Dassel, Germany) by heating the gels with the membranes on top (pre-wetted with PBS, pH 7.4) for 40 minutes at 70°C, followed by washing in PBS and drying. The blots were pre-wetted in PBS-0.1% (wt/vol) Tween 20 for 30 minutes before incubation. The mAbs CLB-ANA/58 and CLB-ANA/60 were diluted to a final concentration of 2.5 µg/ml in HPE buffer. Blots were incubated for 60 minutes, washed three times in PBS and then incubated with 10 µg/ml goat-antimouse immunoglobulin horseradish peroxidase (HRP) labelled antibodies (GM17-HRP, CLB, Amsterdam, The Netherlands) for 60 minutes in HPE buffer. After washing three times in PBS, blots were developed by incubation with 3,3′-diaminobenzidine tetrahydrochloride tablets (DAB tablets, Kem-En-Tec, Copenhagen, Denmark) and 0.03% hydrogen peroxide in distilled water for five minutes, followed by rinsing in distilled water and drying on air.

To visualise marker proteins and histones, extra gels were run simultaneously and silver stained using the Pharmacia Phastgelsystem Development Unit (Pharmacia, Uppsala, Sweden).

Histones

Histones were treated with DNaseI to remove any DNA contamination. Samples were diluted in DNase buffer to a final concentration of 0.5 mg/ml. DNaseI was added to a final concentration of 40 µg/ml, followed by one hour's incubation at 37°C. The reaction was stopped by adding sodium EDTA to a final concentration of 30 mmol/l.

Dimers of histones were made by mixing DNaseI treated histones at a final concentration of 25 µg/ml each in PBS, followed by 60 minutes' incubation at 4°C. For histone-dsDNA combinations, herring sperm dsDNA (Sigma, Zwijndrecht, The Netherlands) was added at a final concentration of 25 µg/ml before incubation at 4°C. Dimers were coated in different concentrations on microtitre plates using the antihistone ELISA protocol.

DNaseI treatment of Jurkat supernatant

Supernatant samples for the specificity experiments of the nucleosome ELISA were treated with DNaseI to degrade any nucleosomes present. Samples were diluted in DNAse buffer, and 40 µg/ml DNaseI was added. After one hour's incubation at 37°C sodium EDTA was added to a final concentration of 30 mmol/l. In negative controls sodium EDTA was added before addition of DNaseI.

Nucleosome ELISA

All incubations were carried out at room temperature in a volume of 100 µl/well. Plates were washed five times with PBS/ 0.02% (wt/vol) Tween 20 between incubations. Microtitre plates were coated overnight with 2 µg/ml mAb CLB-ANA/60 (antihistone 3) in 0.05 M $NaHCO_3$ pH 9.6. Samples were diluted in HPE buffer supplemented with 5% (vol/vol) normal mouse serum and incubated for 60 minutes at room temperature. Biotinylated mAb CLB-ANA/58 (0.5 µg/ml) in HPE was added
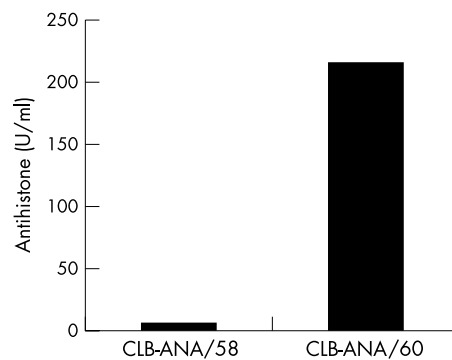

Figure 1 Antihistone activity of mAbs CLB-ANA/58 and CLB-ANA/60. The mAbs were tested by ELISA against a mixture of histones H1, H2A, H2B, H3, and H4. Units were calculated by comparison with a standard antihistone mAb, which was defined as 1000 antihistone U/ml.

for 60 minutes at room temperature. Poly-HRP labelled streptavidin (CLB, Amsterdam, The Netherlands) diluted 10 000 times in HPE buffer was added for 20 minutes. Plates were developed by addition of 100 µg/ml 3,3′,5,5′-tetramethylbenzidine (TMB, Merck, Darmstadt, Germany) and 0.003% hydrogen peroxide in 0.11 M sodium acetate buffer pH 5.5 for five minutes. Development was stopped by addition of 2 M H_2SO_4. Extinction was measured at 450 nm. As a standard we used culture supernatant of Jurkat cells (10^6 cells/ml), cultured for an additional week to obtain 100% apoptotic cells. One unit is the amount of nucleosomes released by approximately 100 Jurkat cells. The detection limit of the assay is 5 U/ml.

Induction of apoptosis

Apoptosis of Jurkat cells was induced by 200 µM etoposide or 5 µg/ml CD95 mAb CLB-CD95/15 (van Lopik T *et al*, unpublished data). Jurkat cells were grown in culture medium (IMDM, BioWhittaker Europe, Verviers, Belgium) supplemented with 5% fetal bovine serum (Bodinco BV, Alkmaar, The Netherlands), 50 µM β-mercaptoethanol, penicillin (100 IU/ml), and streptomycin (100 µg/ml). Before addition of etoposide, cells were washed three times in Earle's balanced salt solution (GibcoBRL Life Technologies, Paisley, Scotland) to remove the fetal bovine serum. Etoposide was added to a final concentration of 200 µmol/l in wells with 5×10^5 cells (volume 200 µl) and incubated for 24 hours. Cells and supernatant were carefully separated for flow cytometric analysis or ELISA measurement. Supernatant was centrifuged for 15 minutes at 13 000 *g* to make sure all cells and membranes were removed. Procedures for the incubation with mAb CLB-CD95/15 are the same as those of etoposide apoptosis induction, except this incubation is performed in the presence of 2.5% fetal bovine serum.

Flow cytometric analysis (FACS staining)

Cells were washed three times with HEPES buffer (9.2 mM HEPES, 0.15 M NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$ in distilled water, pH 7.4) and incubated for 30 minutes with FITC labelled AnnexinV (BenderMed, Vienna, Austria) diluted 1500 times in HEPES buffer. After three washes with HEPES buffer, cells were resuspended in HEPES buffer-0.5% (wt/vol) bovine serum albumin (Sigma, Zwijndrecht, The Netherlands). Propidium iodide was added to a final concentration of 500 ng/ml and samples were measured immediately (Becton-Dickinson FACScan).

RESULTS

Characterisation of mAbs CLB-ANA/58 and CLB-ANA/60

In earlier studies, supernatants of mAbs CLB-ANA/58 and CLB-ANA/60 were shown to be specific for histones or nucleosomes,
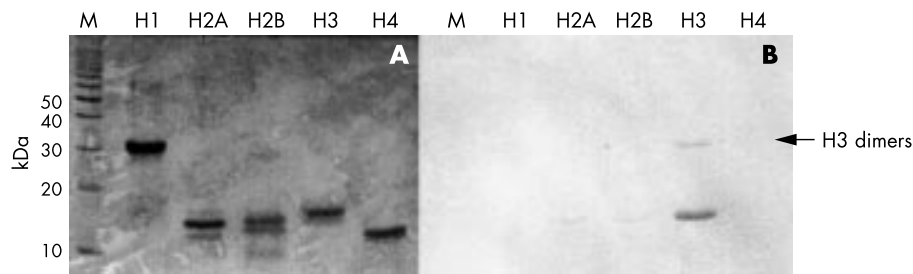
Figure 2 Epitope determination of mAb CLB-ANA/60 on histone immunoblot. (A) 20% SDS-PAA gel with histones, silver staining. (B) Histone immunoblot incubated with 2.5 µg/ml mAb CLB-ANA/60. Lane M, 100 kDa protein marker; lane 1, histone 1; lane 2, histone 2A; lane 3, histone 2B; lane 4, histone 3; lane 5, histone 4.

or both, rather than for DNA.[33] To analyse these mAbs in greater detail, we first tested their reactivity against a mixture of histones in the histone ELISA (fig 1). Monoclonal antibody CLB-ANA/60 was found to react very well, while mAb CLB-ANA/58 only generated a minor signal. Neither mAb reacted with dsDNA in the Crithidia assay, whereas both mAbs reacted equally well on nuclei of HEp-2 cells in an immunofluorescence test (results not shown). Taken together, these results suggest that mAb CLB-ANA/60 is specific for histones and mAb CLB-ANA/58 for other nuclear components. When tested on immunoblot, mAb CLB-ANA/58 did not react with histones, whereas mAb CLB-ANA/60 seemed to react with histones 2A, 2B, and 3. Careful examination of the blot, however, showed that the signal found in lanes 2 and 3 was due to contamination of the histone 2A and 2B preparations with histone 3 (fig 2A and B). For further experiments, the contamination with histone 3 was removed from the histone preparations using a mAb CLB-ANA/60 affinity column. Indeed mAb CLB-ANA/60 did not show any reaction with the histone 2A and 2B preparations after this purification (results not shown).

To test whether mAb CLB-ANA/58 was an antinucleosome antibody, the epitope was determined in an antihistone ELISA using different combinations of histones and DNA as coat. Biotinylated mAb CLB-ANA/58 was applied directly to the coat. The epitope recognised by mAb CLB-ANA/58 is a combined epitope of histone 2A, histone 2B, and dsDNA (fig 3). No reaction was seen with histones 3 and 4 either alone or as dimers (not shown).

Validation of nucleosome ELISA
Combination of mAbs CLB-ANA/58 and CLB-ANA/60 in a sandwich ELISA leads to an ELISA in which complexes of histone 2A, 2B, 3, and DNA (nucleosomes) can be measured in culture supernatant and plasma.
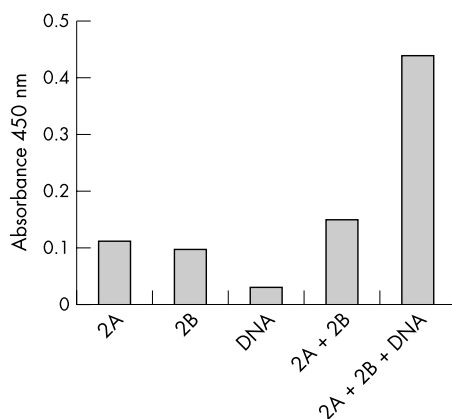
To determine the specificity of the nucleosome ELISA, the supernatant of late apoptotic Jurkat cells was used as a sample. Apoptosis was induced by incubation for 24 hours with 200 µM etoposide, which results in 100% late apoptotic cells (confirmed by AnnexinV and propidium iodide (PI) FACS staining, results not shown), and release of nucleosomes in the culture supernatant. Figure 4 shows that the nucleosome ELISA was indeed able to detect nucleosomes in this supernatant. When the supernatant was treated with DNAseI to degrade the nucleosomes, the signal was inhibited completely.
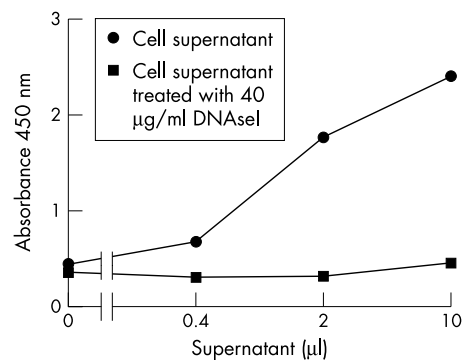

Figure 4 Validation of the nucleosome ELISA using Jurkat cell supernatant incubated 24 hours with 200 µM etoposide. 100% of the cells were late apoptotic.
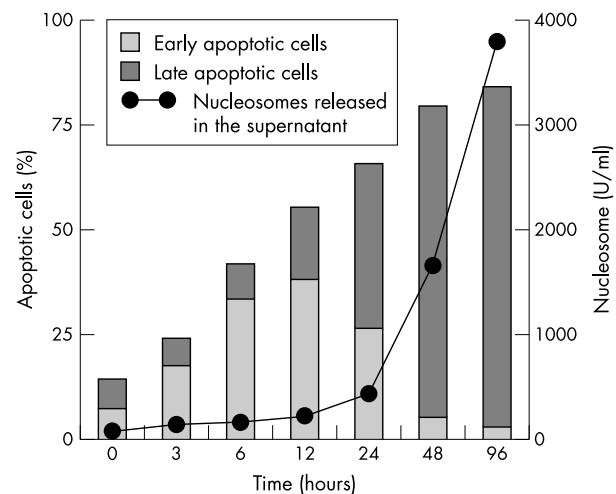

Figure 5 Determination of the time between apoptosis induction and nucleosome release. Apoptosis was induced by addition of 5 µg/ml anti-Fas. Apoptosis was measured by AnnexinV (early apoptosis) and PI (late apoptosis) FACS staining, nucleosomes were measured by ELISA.

Figure 3 Epitope determination of mAb CLB-ANA/58. Test of mAb on several combinations of histones or DNA, or both.

Determination of time span between apoptosis induction and nucleosome release

The time between apoptosis induction and release of intracellular components such as nucleosomes was studied in Jurkat cells. Apoptosis was induced by adding 5 µg/ml CD95 mAb and supernatant, and cell samples were taken after 0, 3, 6, 12, 24, 48, and 96 hours at 37°C. Apoptosis was determined by Annexin V and PI FACS staining. After 12 hours 40% of the cells were early apoptotic and 20% were late apoptotic (fig 5). Nucleosome measurement in the supernatant showed that despite the large percentage of apoptotic cells after 12 hours, nucleosomes were only released after 24–48 hours of incubation with anti-CD95 (fig 5). The control samples without CD95 mAb showed <10% apoptotic cells after 96 hours of incubation (not shown). Similar results were obtained when etoposide was used to induce apoptosis.

DISCUSSION

The mAbs used to develop the nucleosome ELISA have been described before by Kramers et al.[33] CLB-ANA/58 (DNA32 in ref 33) was found to be directed against H2A/H2B/DNA. CLB-ANA/60 (DNA34) was shown to react with H3, H4, and DNA. Yet our findings suggest that this mAb is specific for H3 alone. The reactivity to H4 must have been due to contamination of commercially available histone preparations.

When developing an ELISA for nucleosomes with mAbs extreme caution is required when purifying these antibodies. Monoclonal antibodies directed against nuclear antigens will become complexed to their antigen, derived from dying cells in hybridoma culture.[37 40] Even minor contamination of antibodies with their antigen results in an unacceptably high background in a sandwich ELISA. We decided, based on earlier purification experiments, to use a high concentration of DNAseI and two high salt protein A purifications. With this purification method no traces of nuclear components complexed to antibodies were found in our preparations (not shown).

The nucleosome ELISA with mAbs CLB-ANA/58 and CLB-ANA/60 was used to study nucleosome release during apoptotic cell death. After induction of apoptosis in Jurkat T cells with anti-CD95 most of the cells became late apoptotic within 12 hours. To our surprise it took such late apoptotic cells another 12 hours to release nucleosomes in the supernatant. Extrapolation to the situation in vivo suggests that apoptotic cells can circulate about 24 hours before nuclear material is exposed to the immune system. Therefore, to avoid an immune response, the body has a 24 hour period in which to remove dying cells.

Healthy people remove apoptotic cells efficiently and those cells will therefore not persist in the circulation for longer than 24 hours. When the clearance of apoptotic material is defective, however, released intracellular apoptotic material can cause immune responses and autoimmune phenomena. Mutations in the scavenger machinery indeed lead to SLE or SLE-like syndromes.[24 41] Antinuclear antibodies and nuclear antigens are found in the circulation of patients with SLE.[11 24 42–46]

In extrapolating the results we found in our Jurkat in vitro model to autoimmunity and especially to SLE, we suggest that 24 hours is probably sufficient for removal of apoptotic material, even when part of the scavenger machinery is not functional as a result of mutation. Problems will arise when large quantities of apoptotic cells are generated; a functional defect in the clearance mechanism will then become the limiting factor. This hypothesis is supported by the fact that SLE often exacerbates on exposure to ultraviolet light or after infection.[47 48] We are currently investigating if we can find support for our hypothesis by measuring nucleosomes and antinuclear antibodies in longitudinal series of SLE plasma and correlating the results with disease activity.

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