

EXTENDED REPORT

Extranuclear detection of histones and nucleosomes in activated human lymphoblasts as an early event in apoptosis

C Gabler, N Blank, T Hieronymus, M Schiller, J H M Berden, J R Kalden, H-M Lorenz

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See end of article for authors' affiliations

Correspondence to:
Dr H-M Lorenz, University of Erlangen-Nuremberg, Department of Medicine III, Institute for Clinical Immunology/Rheumatology, Krankenhausstr 12, 91054 Erlangen, Germany;
Hannes.Lorenz@med3.imed.uni-erlangen.de

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Objective: To evaluate the presence of histones and nucleosomes in cell lysates of freshly isolated peripheral blood mononuclear cells (PBMC), fully activated lymphoblasts, or lymphoblasts after induction of apoptosis.

Methods: Each histone class (H1, H2A, H2B, H3, and H4) was detected by western blot analysis with specific antibodies. Annexin V/propidium iodide (PI) staining was performed for each treatment to distinguish viable, early, and late apoptotic, and necrotic cells. DNA degradation was evaluated by PI staining in a hypotonic buffer.

Results: All five histones were detected in cell lysates of activated lymphoblasts in higher concentrations than in lysates of PBMC. An additional significant increase of H2A, H2B, H3, H4, and complete nucleosomes in cell lysates of lymphoblasts was found during interleukin (IL)2 deprivation for 8 or 24 hours. The content of these histones or nucleosomes in cell lysates decreased after delayed IL2 readdition. H1 content in cell lysates was not affected by IL2 deprivation or addition. Quantities of H2A, H2B, H3, and H4 in cell lysates correlated significantly with signs of early apoptosis. UV-B light exposure or etoposide treatment of lymphoblasts resulted in a distinct increase for each histone class in cell lysates compared with standard curves. No bands for post-translationally modified histones were detected in cell lysates in contrast with signals in nuclear preparations.

Conclusion: Extranuclear accumulation of histones and nucleosomes is an early event of apoptosis in human lymphoblasts. Dysregulation of early apoptosis might support the induction of autoimmunity against nuclear components.

Apoptosis is a programmed process for killing and eliminating cells in response to various stimuli.^{1–2} An important characteristic of apoptosis is the cleavage of chromatin by caspase activated DNase.³ The first step involves formation of high molecular size DNA fragments of 50–300 kb.⁴ The second step generates small, 180–200 bp, DNA fragments. This fragmentation occurs at the internucleosomal level and leads to the appearance of mono- and oligonucleosomes detected as a characteristic “DNA ladder” by agarose gel electrophoresis.⁵

At the centre of the nucleosome, two molecules each of histone H3 and H4 form a central (H3–H4)₂ tetramer flanked by two histone H2A–H2B dimers. Two superhelical turns of DNA are wound around the histone octamer to constitute the histone core particle. Adjacent nucleosome particles are linked like beads on a string of histone-free linker DNA, and a molecule of histone H1 is located at the point where DNA enters and exits the nucleosome.

Systemic lupus erythematosus (SLE) is a non-organ-specific autoimmune disease characterised by antibodies to a wide range of nuclear components.⁶ Antibodies to double stranded DNA (dsDNA), histones, and especially nucleosomes are rather specific markers of SLE. It has been reported that most patients with SLE develop autoantibodies to nucleosomes which include antibodies to whole nucleosomes, histone-DNA complexes, and free histones.^{7–8} Studies with sera or cells from patients with SLE have shown that serological or cellular reactivity can target all the different histones.^{9–12} Previous studies indicated that lymphocytes from patients with SLE showed a higher rate of apoptosis after 48–72 hours of cell culture than lymphocytes from patients with

rheumatoid arthritis or normal donors.^{13–14} The percentage of early apoptotic cells (annexin V (AxV) positive, propidium iodide (PI) negative) in peripheral blood was significantly higher in patients with SLE than in normal donors.¹⁵ Moreover, we have recently shown that SLE lymphoblasts are hyporesponsive to survival signals if they stem from patients with high inflammatory activity or a Th1 serotype (high serum levels of interleukin (IL) 12, interferon γ), leading to accelerated apoptosis.¹⁶ Furthermore, in vitro differentiated macrophages from patients with SLE showed a significantly reduced phagocytosis of apoptotic cells,¹⁷ leading to an accumulation of apoptotic cells.

To date, it is unclear which mechanisms lead to the loss of self tolerance in patients with SLE. An intriguing pathogenetic model favours a situation in which dysregulated apoptosis leads to the induction of autoimmunity against nuclear antigens associated with SLE.¹⁸ Our working hypothesis is that the extranuclear appearance of nuclear autoantigens during early apoptosis might contribute to the induction of autoimmunity against these nuclear constituents and, possibly, to the development of SLE.^{18–19}

We focused our research on histones and nucleosomes in cells derived from healthy normal donors in order to acquire

Abbreviations: AxV, annexin V; ELISA, enzyme linked immunosorbent assay; H1, H2A, H2B, H3, H4, histone 1, 2A, 2B, 3, 4; IL2, interleukin 2; med, medium; PBMC, peripheral blood mononuclear cells; PBST, phosphate buffered saline with 0.1% (v/v) Tween 20; PI, propidium iodide; R10, RPMI 1640 medium (R10) supplemented with 10% fetal calf serum, 4 mM L-glutamine, 10 mM HEPES buffer, 100 U/ml penicillin, 100 μ g/ml streptomycin; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SLE, systemic lupus erythematosus

better insights into the fate of these nuclear components during apoptosis. This study aimed at evaluating the content of each histone class (H1, H2A, H2B, H3, and H4) or complete nucleosomes in cell lysates from peripheral blood mononuclear cells (PBMC), activated lymphoblasts, or lymphoblasts after induction of apoptosis. Moreover, we wanted to test whether the appearance of histones in cell lysates correlates with markers of early apoptosis (AxV binding or DNA degradation) and whether histones derived from the nucleus or cell lysates differ in their post-translational modification.

MATERIAL AND METHODS

Cell preparation

PBMC were immediately separated by Ficoll-Hypaque (Lymphoflot, Biotest AG, Dreieich, Germany) density gradient centrifugation from heparinised blood samples obtained from six healthy donors. They were suspended to 4×10^6 cells/ml in a modified RPMI 1640 medium (R10) supplemented with 10% fetal calf serum, 4 mM L-glutamine, 10 mM HEPES buffer, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Life Technologies, Eggenstein, Germany). For generation of lymphoblasts, freshly isolated PBMC were activated with 1 µg/ml phytohaemagglutinin (Sigma, Deisenhofen, Germany) and 500 IE/ml proleukin (EuroCetus, Frankfurt, Germany) for 5 days. On day 3, the medium was renewed and on day 5, cells were expanded with proleukin alone for the next 2 days. Cell culture was performed at 37°C and 5% CO₂ in a humidified atmosphere. Resulting lymphoblasts were >95% CD3+ with 60–80% CD4+ cells.²⁰

Cell treatment and induction of apoptosis

Expanded lymphoblasts were washed four times with Hanks's solution and resuspended in R10 to 4×10^6 cells/ml. This time is referred to as t₀ (starting point of the experiments).

All experiments were performed in 24 well tissue culture plates (Cellstar, greiner bio-one, Frickenhausen, Germany) at 37°C and 5% CO₂ in a humidified atmosphere. The extensively washed lymphoblasts (2×10^6 cells in 600 µl/well) were cultured for 8 or 24 hours without IL2. After 8 hours of IL2 deprivation, lymphoblasts were restimulated with 10 U/ml IL2 (Roche, Mannheim, Germany) for 1, 16, or 24 hours, respectively. For lymphoblasts after 24 hours of IL2 deprivation, IL2 stimulation was performed for 1 and 8 hours, respectively. As an additional control, cells were continuously cultured from t₀ for 24 hours in the presence of 10 U/ml IL2. Figure 1 shows the scheme of this experimental setting.

Alternatively, in some experiments apoptosis in lymphoblasts was induced by the following methods: UV-B light irradiation (312 nm) for 30 seconds or incubation in medium containing 250 µM etoposide (Sigma). Cells were incubated for 8 hours at 37°C in the absence or presence of 10 U/ml IL2.

For inhibition of protein neosynthesis, lymphoblasts were incubated in cycloheximide (0.1 or 1 µg/ml; Sigma) or actinomycin D (1 ng/ml; Sigma) for 24 hours before the cells were lysed as described.

Quantification of apoptosis

AxV-FITC and PI were used to distinguish viable, early, and late apoptotic, and necrotic cells by flow cytometry.²¹ Lymphoblasts (4×10^5) were treated as described above in 600 µl/well. Thereafter, 200 µl cell suspension was incubated for 30 minutes at 4°C in the dark with 500 µl AxV-PI labelling solution (10 ml Ringer Solution (Fresenius, Bad Homburg, Germany) containing 5 µl AxV-FLUOS (Roche) and 5 µl PI (10 mg/ml; Sigma)). Cells were analysed using an EPICS XL cytofluorometer (Coulter, Hiialeah, FL). Cells were

classified into the following four fractions²²: (a) viable cells (AxV^{neg}PI^{neg}) were impermeable for PI and also did not bind AxV; (b) early apoptotic cells (AxV^{pos}PI^{neg}) bound AxV and were PI impermeable; (c) late apoptotic or also called secondary necrotic cells (AxV^{pos}PI^{low}) bound AxV and were PI permeable with sub-G1 content; (d) primary necrotic cells (AxV^{pos}PI^{pos}) bound AxV and were PI permeable with G1 DNA content.

Furthermore, quantification of DNA degradation was performed by PI staining in hypotonic buffer.²³ Briefly, 4×10^5 cells were cultured under the conditions described above in 600 µl/well. Thereafter, 200 µl cell suspension was incubated with 500 µl staining solution containing 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100 (Sigma), and 2 µg/ml PI. After a minimum of 6 hours in the dark at 4°C, samples were analysed using an EPICS XL cytofluorometer.

Cell lysis and western blot analysis

PBMC or lymphoblasts were removed from each well and transferred into 1.5 ml microfuge tubes. Cells were centrifuged at 570 g for 5 minutes, supernatants were discarded and cell pellets were washed in 1 ml PBS. The resulting cell pellets were lysed for 1 hour at 4°C in 20 µl of a modified RIPA buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 1% (v/v) NP-40, 0.25% Na-deoxycholate, 1% (v/v) Triton X-100, 1 mM Na₃VO₄, 1 mM NaF, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. Lysates were centrifuged for 10 minutes (8000 g, 4°C) to pellet insoluble materials. Supernatants (specified as "cell lysates" throughout this paper) were carefully removed without destroying the pellet (containing the nucleus) and transferred into a new microfuge tube. A Bradford assay (Bio-Rad Protein assay, Biorad, Munich, Germany) was performed to estimate the protein content of the cell lysates. 40 µg of each sample was separated by one dimensional denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 18% (w/v) acrylamide/bisacrylamide (37.5:1)). The electrophoresed proteins were then transferred for 1 hour at 0.8 mA/cm² to nitrocellulose membranes (Hybond C+, Amersham Pharmacia, Freiburg, Germany) using a semidry blotter unit (NovaBlot, Amersham Pharmacia) according to the protocol of Kyhse-Andersen.²⁴ Membranes were air dried for 10 minutes and stained with Ponceau S (0.5% Ponceau S (Sigma), 1% acetic acid in water) to assess the quality of the transfer as well as to verify equal protein loading. To minimise unspecific antibody binding, nitrocellulose sheets were incubated for 1 hour in 5% milk powder in PBST (phosphate buffered saline (PBS) with 0.1% (v/v) Tween 20). For detection of histones we used the following specific monoclonal antibodies from cell culture supernatants: KM1 recognising H2B (15 kDa), KM2 recognising H2A (14 kDa) and H4 (10 kDa), MRA 12 recognising H1 (31–33 kDa), and LG 2.1. recognising H3 (17 kDa), respectively. As a positive histone control, we used 5 µg of a mixture containing all five histones (Roche) or 1/10 of the obtained nuclear pellet after cell lysis and centrifugation. Each monoclonal antibody was specific for the predicted histone and the size of the detected bands in cell lysates or nuclear preparations corresponded with the signals in the histone mixture (data not shown).

For western blot analysis, histone antibodies were used in a dilution of 1:8 in 1% milk powder in PBST. We used polyclonal rabbit antibodies directed against the following post-translational modifications: acetylated histone H4, phosphorylated histone H1 or H3 (all from Upstate Biotechnology, Lake Placid, NY). These antibodies were used in a concentration of 1 µg/ml in PBST with 1% milk powder.

Incubation for the primary antibody was performed overnight at 4°C. Membranes were washed six times for 10 minutes with PBST. A peroxidase conjugated

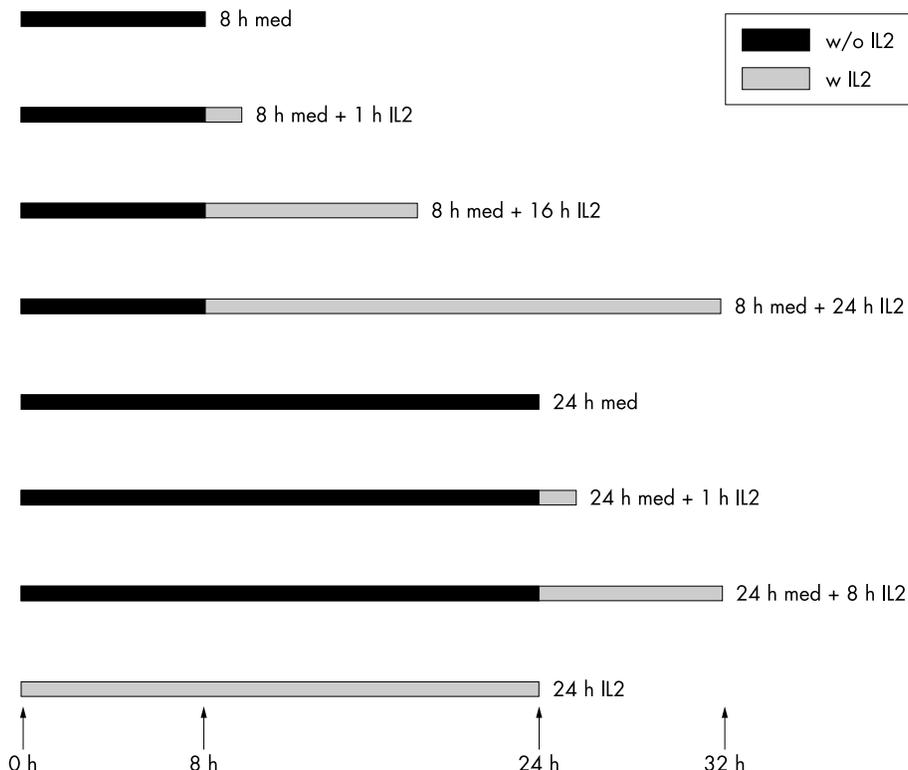


Figure 1 Experimental design showing times of IL2 withdrawal and readdition. Black bars represent the time of IL2 deprivation (med = medium R10); grey bars represent the time of the treatment with 10 U/ml IL2.

goat-antimouse or goat-antirabbit secondary antibody (Dianova, Hamburg, Germany) was diluted with 1% milk powder in PBST to 1:5000 or 1:2000, respectively. Membranes were incubated for 90 minutes at room temperature with the secondary antibody. Afterwards, membranes were washed another six times for 10 minutes with PBST. Immunoreactive proteins were visualised using enhanced chemiluminescence (Amersham Pharmacia) as described by the manufacturer. Histone contents in cell lysates were estimated by comparison with standard curves of purified histones (H1, H2A, H2B, H3, or H4; all from Roche) in a range of 0.05 µg/lane to 0.5 µg/lane on the same gel (fig 5).

Nucleosome enzyme linked immunosorbent assay (ELISA)

This assay (Cell Death Detection ELISA; Roche) is based on the sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones.

Lymphoblasts (2×10^6 cells in 600 µl/well) were cultured as described above for the IL2 withdrawal experiments. Cells were removed from each well, transferred into 1.5 ml microfuge tubes, and centrifuged at 570 *g* for 5 minutes. Supernatants were discarded and cell pellets were washed in 1 ml PBS. The resulting cell pellets were lysed for 30 minutes at room temperature in 500 µl of the supplied lysis buffer. The lysate was spun down at 8000 *g* for 10 minutes to pellet cell debris and nuclei. Cytoplasmic apoptotic nucleosomes in the supernatant were collected and stored at 4°C for not more than 24 hours. The protocol of the ELISA was performed as described by the manufacturer. Cell equivalents, 1600 per well, were analysed and measured at 405 nm against substrate solution as blank (reference wavelength 620 nm). Absorption values (calculated for 1600 cells) of each ELISA were standardised to lymphoblasts at t0 as the negative control (negative control = 1).

Densitometric and statistical analysis

Dried films were scanned using the MultiImage Light Cabinet (Alpha Innotech Corporation, San Leandro, CA). The scanned band intensities of the immunoreactive histones were estimated with the Alphamanager 1220 (Alpha Innotech Corporation) after subtraction of background. Background subtraction was individually checked for each lane. Furthermore, all data were compared with the visual impression of the band intensities. For comparison of band intensities between the various experiments, we attributed times with the smallest variation in the optical density of all experiments (usually after 8 or 24 hours of cytokine withdrawal) a relative value of 1. Thereafter, intensities of histone bands at the other times were calculated in relation to 1.

All data from western blot analysis, nucleosome ELISA, and quantification of apoptosis were analysed by the paired *t* test. These statistical calculations including regression analysis were performed with Instat (version 3.0, GraphPad Software, San Diego, CA).

RESULTS

Histone content in cell lysates from freshly harvested PBMC and expanded lymphoblasts

Cell lysates of PBMC or expanded lymphoblasts were evaluated for the presence of histones. We found only barely detectable amounts of histones H2A, H2B, H3, and H4 in lysates from PBMC (figs 2B–E). No signals for histone H1 were found in samples of six normal donors (fig 2A). We detected bands for histones H2A, H2B, H3, and H4 in cell lysates from expanded lymphoblasts at t0 (figs 2B–E). Signal intensities of the bands obtained were at least 20–30 times higher than those with freshly harvested PBMC as determined by densitometry. In contrast with PBMC, we also obtained signals for histone H1 in cell lysates from lymphoblasts at t0 (fig 2A).

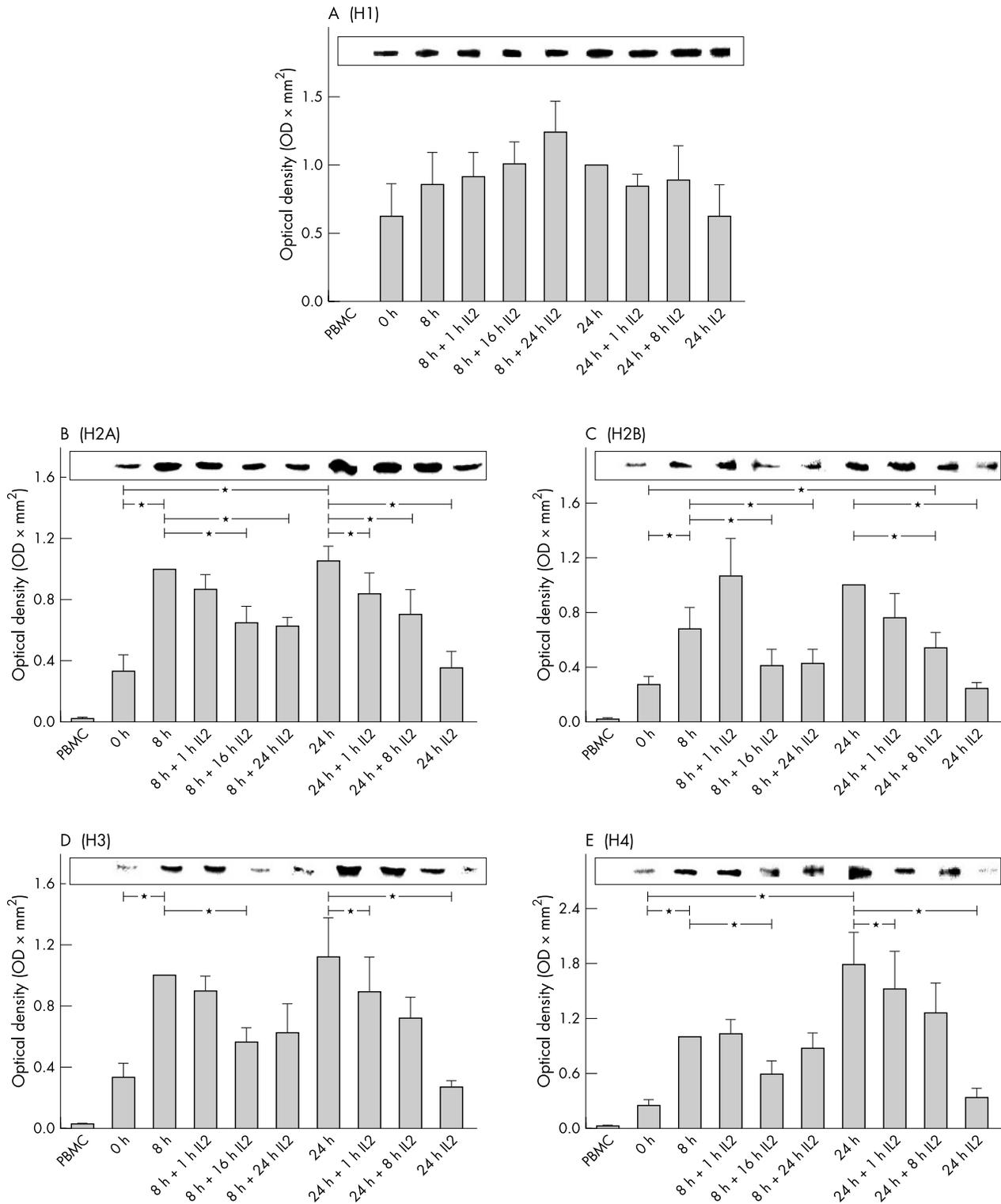


Figure 2 Densitometric analysis of histone H1 (A), H2A (B), H2B (C), H3 (D), and H4 (E) in cell lysates of PBMC (first column) and lymphoblasts (all other columns). Cells were lysed, 40 μ g of cell lysates were separated using SDS-PAGE as described in "Material and methods". Western blot was performed with monoclonal antibodies directed against distinct histones. Band intensities were scanned and analysed by densitometry. The corresponding integrated absorbance (mean (SEM); $n = 6$ normal healthy donors) is depicted in relation to the band intensity of the histone band at time 8 (B, D, E) or 24 hours (A, C) of cytokine withdrawal. A representative example of histone bands is illustrated. *Indicates significant difference ($p < 0.05$) between the different times. In addition, the amount of each histone class in cell lysates of PBMC was significantly lower ($p < 0.001$) than in cell lysates of lymphoblasts at all times.

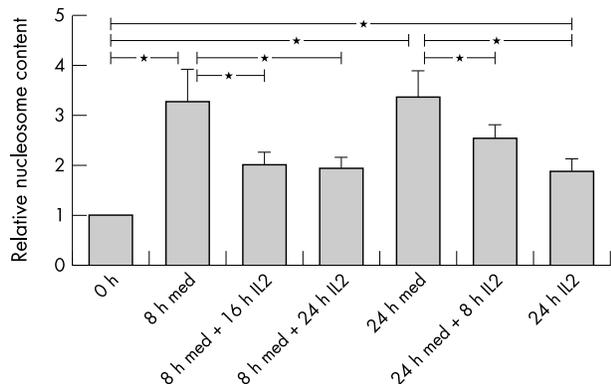


Figure 3 Relative contents of nucleosomes in cell lysates of apoptotic lymphoblasts compared with lymphoblasts at t0. Nucleosome ELISA was performed as described in "Material and methods". Absorption values (405 nm) were referred to lymphoblasts at t0 (value of 1). Data are mean (SEM) of six normal healthy donors. *Indicates significant difference ($p < 0.05$) between the different times.

Appearance of histones in cell lysates from lymphoblasts during IL2 deprivation

Figure 1 depicts the scheme of the experimental design with times of IL2 withdrawal and readdition. Western blot analysis of cell lysates after 8 hours of IL2 deprivation showed a threefold higher signal intensity for histones of the nucleosome (H2A, H2B, H3, and H4) than cell lysates of lymphoblasts at t0 (figs 2B–E). After 24 hours of IL2

withdrawal, contents of H2A and H3 in cell lysates were similar to the amounts after 8 hours of IL2 withdrawal, whereas H2B and H4 content in cell lysates further increased (figs 2B–E). Inhibition of protein neosynthesis through incubation of lymphoblasts in cycloheximide or medium containing actinomycin D did not prevent this increase (data not shown).

After 8 hours of IL2 deprivation and subsequent readdition of IL2 for 1 hour, the content of H2A, H2B, H3, and H4 did not change significantly. In contrast, after 16 or 24 hours of IL2 addition, the content of H2A, H2B, H3, and H4 in these cell lysates was significantly reduced compared with 8 hours of IL2 deprivation (figs 2B–E). The same effect was noted after 24 hours of IL2 withdrawal and IL2 readdition for 1 or 8 hours. Continuous IL2 treatment from t0 did not lead to increased concentrations of any histone derived from the nucleosome core in cell lysates (figs 2B–E). In contrast, IL2 deprivation as well as IL2 readdition did not obviously affect the amount of histone H1 in the cell lysates (fig 2A).

After comparison with a standard curve of commercially available histones at known quantities, the following mean contents of each nucleosomal histone in the cell lysates were found (per 2×10^6 cells): 0.2–0.3 μg at t0 or after 24 hours of continuous IL2 treatment and about 0.6 μg after 8 hours of IL2 deprivation. For the linker histone H1, we found contents in the lysates between 0.6 and 1.0 μg per 2×10^6 cells at all times.

A specific nucleosome ELISA was performed to reveal the relative content of complete nucleosomes in cell lysates during the course of IL2 withdrawal and IL2 readdition. We

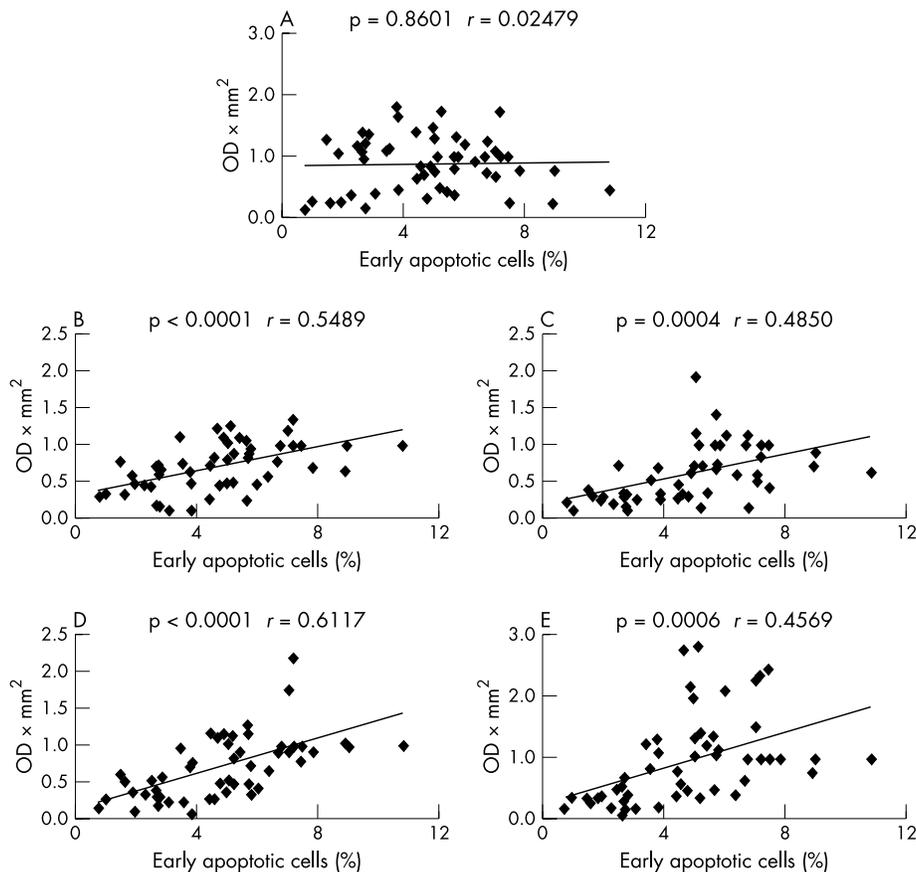


Figure 4 Correlation of integrated optical density ($\text{OD} \times \text{mm}^2$) of histone bands H1 (A), H2A (B), H2B (C), H3 (D), and H4 (E) with the percentage of early apoptotic cells. The normalised values of the band intensities for each histone after each treatment were correlated with the percentage of early apoptotic cells ($\text{AxV}^{\text{P}25}\text{PI}^{\text{neg}}$) after identical treatment within the identical experiment. The p values and the correlation coefficients r are given.

Table 1 Determination of apoptosis with annexin V/propidium iodide staining

	Viable cells (%)	Early apoptotic cells (%)	Late apoptotic cells (%)	Necrotic cells (%)
<i>IL2 deprivation</i>				
0 h	86.5 (3.0)	2.0 (1.2)	5.1 (0.9)	6.4 (1.3)
8 h med	81.7 (3.5)	7.8 (1.5)	6.0 (1.5)	4.5 (0.6)
8 h IL2	83.8 (2.0)	6.4 (1.4)	5.4 (1.1)	4.4 (0.7)
8 h+1 h IL2	82.0 (3.0)	6.6 (1.5)	6.4 (1.8)	5.0 (0.7)
8 h+16 h IL2	82.8 (3.0)	3.1 (1.0)	9.1 (2.6)	5.0 (1.0)
8 h+24 h IL2	81.9 (3.1)	3.8 (0.6)	8.9 (1.8)	5.4 (1.8)
24 h med	76.9 (3.6)	6.6 (0.7)	10.6 (2.9)	5.9 (1.6)
24 h+1 h IL2	77.6 (3.8)	5.8 (0.9)	10.8 (2.6)	5.8 (1.5)
24 h+8 h IL2	77.0 (3.8)	5.3 (0.2)	11.6 (2.7)	6.1 (1.5)
24 h IL2	84.6 (2.5)	2.9 (0.9)	8.1 (2.0)	4.4 (1.1)
<i>Induced apoptosis</i>				
8 h med	81.7 (3.5)	7.8 (1.5)	6.0 (1.5)	4.5 (0.6)
8 h IL2	83.8 (2.0)	6.4 (1.4)	5.4 (1.1)	4.4 (0.7)
UV	26.6 (7.5)	50.6 (5.9)	12.5 (3.5)	10.3 (2.3)
UV+IL2	28.5 (6.7)	50.8 (4.5)	10.8 (3.0)	9.9 (1.8)
Etoposide	24.1 (6.1)	52.7 (5.1)	12.1 (2.6)	11.1 (1.6)
Etoposide+IL2	29.4 (6.7)	48.2 (5.6)	11.5 (2.4)	10.9 (1.3)

Annexin V/PI staining of lymphoblasts. After generation of lymphoblasts, cells were cultured and stained with AxV/PI as described in "Material and methods" and fig 1. Each value represents the mean (SEM) of normal healthy donors (n=6)

found a threefold increase of the nucleosome content in lysates after 8 or 24 hours of IL2 withdrawal compared with t0 (fig 3). Furthermore, nucleosome content in the lysates decreased after readdition of IL2. The pattern of the nucleosome quantities was similar, but not identical, to that of nucleosomal histones in cell lysates (figs 2B–E). Continuous 24 hour incubation in medium containing IL2 increased nucleosome content in cell lysates twofold compared with t0 (fig 3).

We simultaneously quantified early and late apoptosis and necrosis at the various times (table 1). The data obtained from the AxV/PI staining were correlated with the relative optical densities of western blot bands for each histone. The content of H2A (fig 4B), H2B (fig 4C), H3 (fig 4D), or H4 (fig 4E) in cell lysates correlated significantly with the percentage of early apoptotic lymphoblasts (AxV^{pos}PI^{neg}). We found no correlation with the number of late apoptotic cells or necrotic cells. No correlation was noted between the content of histone H1 in cell lysates and the percentage of early or late apoptotic or necrotic cells (fig 4A for early apoptotic cells). In addition, we determined the percentage of cells with degraded DNA (table 2). The percentage of cells with degraded DNA increased after 8 hours of IL2 withdrawal compared with t0. A further increase was noted after 24 hours of IL2 deprivation. Readdition of IL2 did not affect the percentage of cells with subdiploid DNA content. Continuous incubation in medium containing IL2 from t0 for 24 hours resulted in a small increase of cells with degraded DNA (table 2). The content of H2A ($r = 0.4661$), H2B ($r = 0.5245$), H3 ($r = 0.5531$), or H4 ($r = 0.4579$) in cell lysates correlated significantly ($p < 0.002$) with the percentage of cells with subdiploid DNA content, but there was no correlation of DNA degradation with the content of H1 in cell lysates.

Appearance of histones in cell lysates during induction of apoptosis with UV light or etoposide in lymphoblasts

To expand our data to situations with stronger pro-apoptotic stimuli, we exposed activated lymphoblasts to UV-B light (an established risk factor for initiation of SLE flares) or incubated cells in medium containing etoposide. Eight hours after a 30 second UV-B light exposure or after 8 hours of etoposide treatment, percentages of apoptotic cells and

histone content in these cell lysates were significantly higher than with controls at t0 (tables 1 and 2; figs 5A–E). However, the increase of the signal intensities differed for each histone under these conditions. The greatest increase of signal intensity was seen for histones H2B (80-fold) and H4 (70-fold) compared with activated lymphoblasts at t0 (figs 5C and E). In contrast, the signal intensity for the two other core histones was less augmented in cell lysates: histone H3 (40-fold) and histone H2A (35-fold) (figs 5D and B).

Different antibodies show generally a distinct affinity or binding to their specific antigen. Therefore each antibody reveals a distinct dose proportionality of the obtained signal in western blot analysis. So we compared these cell lysates with a standard curve of known quantities of commercially available histones. In comparison with these standard curves, the following mean quantities were obtained for each nucleosomal histone after induced apoptosis with UV light exposure or etoposide treatment (per 2×10^6 cells):

Table 2 Determination of DNA degradation with PI-Triton staining

	Cells with a subdiploid DNA content (%)
<i>IL2 deprivation</i>	
0 h	8.0 (1.2)
8 h med	16.0 (1.5)
8 h IL2	12.9 (1.5)
8 h+1 h IL2	15.8 (1.4)
8 h+16 h IL2	15.1 (2.0)
8 h+24 h IL2	16.4 (2.0)
24 h med	21.8 (1.3)
24 h+1 h IL2	20.5 (1.3)
24 h+8 h IL-2	22.7 (1.9)
24 h IL2	12.8 (1.2)
<i>Induced apoptosis</i>	
8 h med	16.0 (1.5)
8 h IL2	12.9 (1.5)
UV	68.1 (3.6)
UV+IL2	64.4 (4.4)
Etoposide	70.3 (3.3)
Etoposide+IL2	63.9 (2.6)

PI staining in a hypotonic buffer. After generation of lymphoblasts, cells were cultured and stained as described in "Material and methods" and fig 1. Each value represents the mean (SEM) of normal healthy donors (n=6).

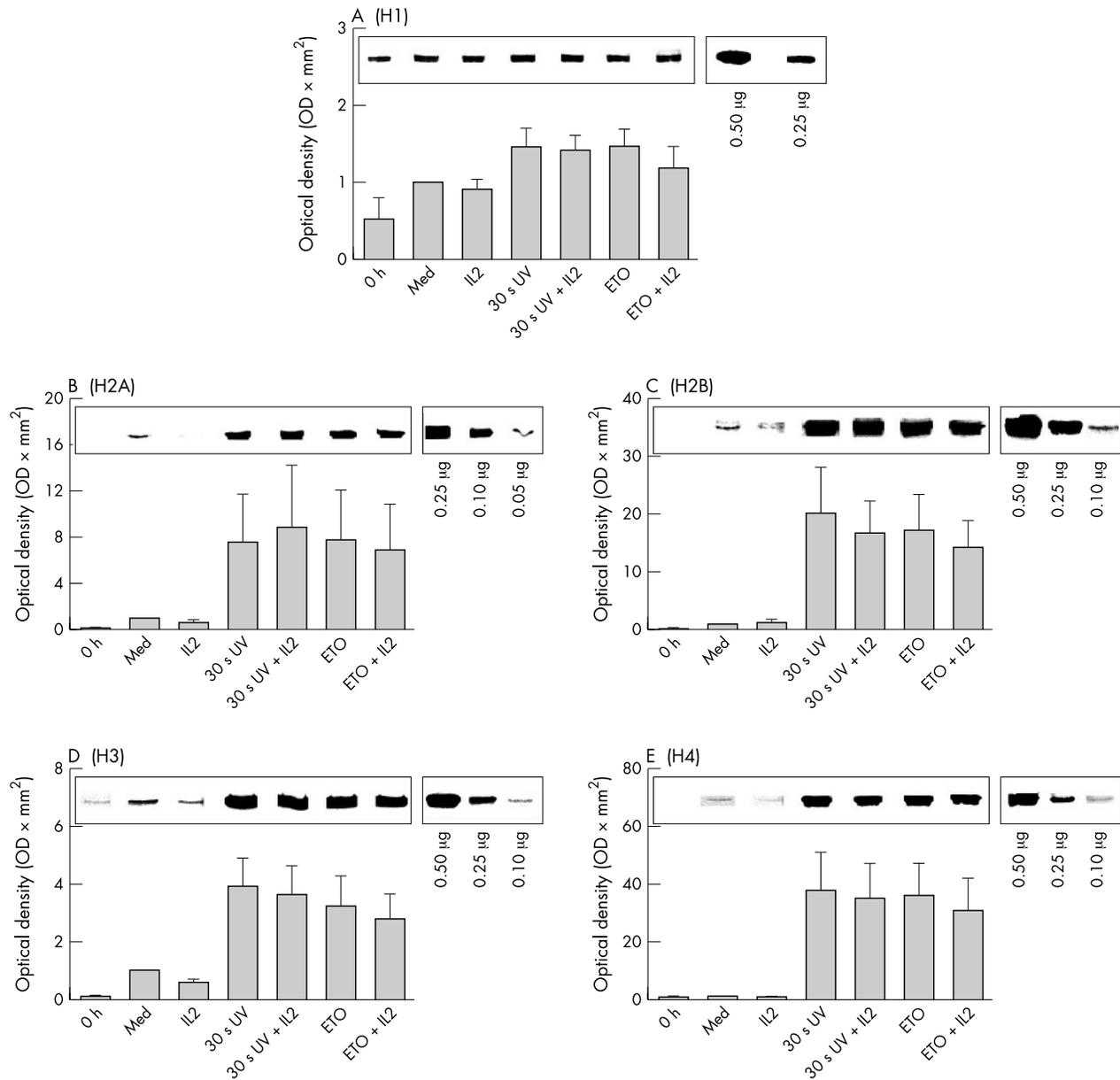


Figure 5 Densitometric analysis of histone H1 (A), H2A (B), H2B (C), H3 (D), and H4 (E) in cell lysates of lymphoblasts after induced apoptosis. Cells were lysed, and 40 μg of cell lysates was separated using SDS-PAGE as described in "Material and methods". Western blot was performed with monoclonal antibodies directed against distinct histones. Band intensities were scanned and analysed by densitometry. The corresponding integrated absorbance (mean (SEM); $n=6$ normal healthy donors) is depicted in relation to the band intensity of the histone band at 8 hours of cytokine withdrawal (8 hours med). A representative example of histone bands is illustrated. ETO, etoposide.

0.75–1.0 μg for H2A, 1.2–2.5 μg for H2B, 2.4–4.0 μg for H3, and 2.1–3.2 μg for H4 (fig 5). The contents for each histone at t_0 were 0.2–0.3 μg .

In contrast, we only observed a very moderate increase of H1 after UV-B light exposure or treatment with etoposide compared with lymphoblasts at t_0 (contents in the lysates about 1.0 μg per 2×10^6 cells) (fig 5A). Addition of IL2 to cells exposed to UV-B light or treated with etoposide had no obvious diminishing effect on histone accumulation or percentages of apoptotic cells.

Different post-translational modifications of nuclear histones compared with histones in cell lysates

We wanted to evaluate whether histones in cell lysates and nucleus derived histones differed in their acetylation or phosphorylation status. Nuclear preparations consisted of the

pellet after cell lysis and subsequent centrifugation. Western blot analysis showed that the nucleus derived histone H4 was acetylated (fig 6A), whereas the nucleus derived histones H1 (fig 6B) and H3 were phosphorylated (fig 6C). In contrast, we did not detect any signals in cell lysates using acetylation- or phosphorylation-specific antibodies, respectively (figs 6A–C). To ensure that nuclear samples contained the same amount of these histones as cell lysates, we diluted nuclear samples to a level where we obtained similar band intensities in nuclear samples and cell lysates with antibodies recognising H1 (MRA 12), H3 (LG2.1.), or H4 (KM2). When aliquots of the same samples were used, signals for acetylated H4 were only seen in the nuclear samples, but not in the cell lysates (figs 6A–C). However, as in cell lysates no signals for phosphorylated H1 or H3 were detected in diluted nuclear samples.

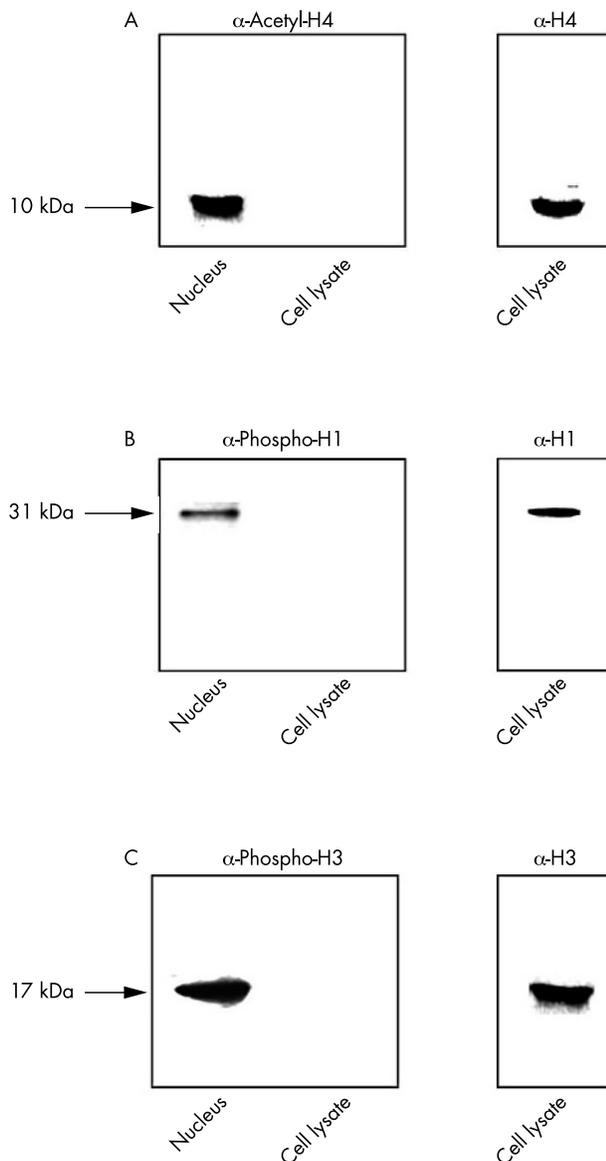


Figure 6 Western blots for the detection of the following post-translationally modified histones: (A) acetylated H4; (B) phosphorylated H1; and (c) phosphorylated H3. Cells were lysed and 40 μ g of cell lysates or 1/10 of the nuclear preparation from 2×10^6 lymphoblasts was separated using SDS-PAGE as described in "Material and methods". Western blot was performed with antibodies directed against the distinct post-translational modifications of the specific histone. As controls, the identical cell lysates were separated in a different gel. Western blot was performed with monoclonal antibodies directed against H4 (A; KM2), H1 (B; MRA12), or H3 (C; LG 2.1.).

DISCUSSION

This study shows that histones and nucleosomes appear in cell lysates from activated lymphoblasts during early apoptosis. Our results show different accumulation patterns for histones of the nucleosome (H2A, H2B, H3, and H4) as opposed to histone H1 of the linker region between the nucleosomes, highlighting the specificity of this phenomenon. Early apoptosis detected by AxV/PI staining correlated significantly with the accumulation of histones H2A, H2B, H3, and H4 in the cell lysates, but not with H1.

Because H2A, H2B, H3, and H4 form the core structure of the nucleosome and are bound 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 the form of cleaved nucleosomes or as free histones. All detected histone bands are likely to represent cytoplasmic histones/nucleosomes because the lysis conditions would not solubilise intact nuclear 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. In addition, cell lysates from PBMC only contained very low amounts of histones. It is important to note that the nuclear membrane and nuclear pore complexes remain intact most of the time during apoptosis, even though the nuclear lamina is solubilised.⁴ But we cannot completely exclude the possibility that the nuclear membrane will be disrupted during cell lysis using detergents, especially in apoptotic cells. However, we found a different release pattern of the linker H1 versus the core histones during the phase of early apoptosis. Therefore, it seems unlikely that histones diffuse non-specifically through a disrupted membrane into the cytoplasm. If they did, we would expect a similar increase of all histones in the cell lysates, which is not the case. These findings support the view that the appearance of histones in cell lysates is not attributed to artificial nuclear membrane degradation.

The increase of histones in cell lysates was not affected by blockade of protein neosynthesis, supporting the concept that histones do indeed derive from the nucleus and have not been neosynthesised in the cytoplasm. The histones and nucleosomes may be actively exported or diffuse passively through the nuclear pores, but this is to date unknown.

We also detected complete nucleosomes in cell lysates of activated lymphoblasts at all times after IL2 withdrawal. This implies that DNA is present in the cytoplasm because we used a specific antibody against DNA for the detection of nucleosomes. The pattern of the nucleosome quantities was similar to, but not identical with, the scheme of accumulation of nucleosomal histones in cell lysates. Continuous incubation of lymphoblasts in medium containing IL2 prevented an increase of the histone contents in cell lysates, whereas nucleosomes were detected in higher quantities than at t0. This theoretical contradiction might be explained by differences in release characteristics of nucleosomes versus histones between early and late apoptotic cells. After IL2 deprivation, we found different patterns of accumulation in cell lysates for each histone class. This was much more striking after exposure of lymphoblasts to UV-B light or treatment with etoposide. A 10–20-fold (compared with t0) increase of histones H3 or H4 in cell lysates was found, which was much less evident for H2A and H2B. Owing to this unbalanced appearance of the different histone classes, we can conclude that nucleosomes in cell lysates cannot be the only source for the detected histones, and that uncomplexed histones may accumulate in the cytoplasm coming from the nucleus during early apoptosis. This view is supported by the observation that nucleosomes fall apart during apoptosis releasing "free" histones and DNA particles.²⁵ In addition, the normal assembly of nucleosomes may be inhibited in these early phases of apoptosis, resulting in the accumulation of free histones. Alternatively, H2A and H2B may be more efficiently degraded in the cytoplasm or secreted from the cells than H3 and H4. But this possibility is not substantiated by any reported data, and we found no breakdown products of histones in our cell lysates.

It was recently reported that histones were found in cell lysates obtained from apoptotic, non-physiological tumour cells like Jurkat cells and U937 cells.²⁶ However, H1 was only detected in cell lysates of Jurkat cells, but not in U937. Other

studies showed that Jurkat cells and human activated lymphocytes accumulate nucleosomes in the cytoplasm during apoptosis.^{27, 28} However, not all cell lines generate nucleosomal fragments during apoptosis.²⁹ It is important to note that accumulation of histones is not a necessary consequence of DNA fragmentation related to apoptosis. Wu *et al* showed that not all cell lines release histones from nucleosomes during DNA fragmentation and apoptosis.²⁵ Based on their experiments, the authors convincingly showed that histone release and DNA fragmentation can be clearly separated into two independent processes in their tumour cell lines. As these experiments were performed with tumour cells which are obviously altered in their regulation of apoptosis, we employed primary human cells for our studies. Our data for the first time provide evidence that cytoplasmic accumulation of histones and nucleosomes in physiological cells is an early event in apoptosis, occurring in parallel with the initial phagocytosis signals.

It has been suggested that nuclear components are altered during apoptosis.^{30, 31} This could cause structural alterations leading to improved recognition as autoantigens, thereby contributing to the pathogenesis of autoimmune diseases like SLE. Our results suggest that cytoplasmic histone H4 generated by apoptosis is structurally different from nuclear histone H4. The fact that apoptosis related histones can indeed be antigenic has been well documented by our previous work¹²: we initiated an immune response directed against histones in lymphocytes which had been stimulated in vitro with autologous apoptotic cells or commercially available, unmodified histones. In further support for our hypothesis that post-apoptotic modifications of cellular structures occur during apoptosis, Casciola-Rosen *et al* have shown that proteases like granzyme B can cleave autoantigens in the process of apoptosis.³⁰

Addition of the cytokine IL2 serves as an important survival factor, preventing apoptosis in activated lymphoblasts in vivo and in vitro.^{14, 32, 33} As shown in this study, IL2 also lowers extranuclear histone contents in cell lysates. This fact cannot be easily explained. Histones and nucleosomes may be reintroduced into the nucleus, but this alternative is not substantiated by any data and is hard to imagine. It is more likely that histones are released or secreted from the cells. As mentioned earlier, degradation is unlikely because we have never observed breakdown products of histones in our western blot detection.

This hypothetical situation might be meaningful for the pathogenesis of SLE: known SLE autoantigens like histones or nucleosomes are located outside the nucleus in apoptotically dying, IL2 deprived lymphoblasts and might thus be more easily accessible for immunocompetent cells. In an earlier paper,¹⁶ employing the identical cellular model, we demonstrated that activated SLE lymphoblasts are hyporesponsive to the γ c chain cytokines (like IL2), leading to accelerated apoptosis. Our data support the concept that activated lymphoblasts, much more than quiescent lymphocytes or PBMC, are a source for histones or nucleosomes as autoantigens in systemic autoimmune diseases. An early release of histones and nucleosomes from nuclei of apoptotically dying lymphocytes suggests that dysregulation of early apoptosis might be decisive for the induction of autoimmunity against nuclear autoantigens in SLE, a hypothesis we are currently following in our work.

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Authors' affiliations

C Gabler, N Blank, T Hieronymus, M Schiller, J R Kalden, H-M Lorenz, Department of Medicine III, Institute for Clinical Immunology and Rheumatology, University of Erlangen-Nuremberg, Erlangen, Germany
J H M Berden, Division of Nephrology, University Medical Centre, Nijmegen, The Netherlands

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