Changes in the protein pattern of H1 histones associated with apoptotic DNA fragmentation

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Characteristic steps in the course of cellular apoptosis are the induction of chromatin condensation and cleavage of the DNA, leading to the formation of oligomers of nucleosomes. Since the H1 histones represent functional elements that are essential for the generation of highly condensed chromatin structures, we analysed the total cellular H1 histones of five leukaemic and three solid human tumour cell lines, comparing the H1 pattern of exponentially growing cells with that of apoptotic cells. For the induction of apoptosis, cell lines were treated with the water-soluble camptothecin derivative, topotecan (a topoisomerase I inhibitor), or with an apoptosis-inducing monoclonal anti-CD95 (Fas/APO-1) antibody. Total histone H1 proteins were isolated by extraction with 5% perchloric acid and were analysed by means of capillary zone electrophoresis (CZE) separation. The identities of the peaks representing different histone H1 subtypes

on CZE electropherograms were confirmed by analysis of preparations (recombinant proteins purified from transformed yeast used as internal standards) mixed with each of the subtypes respectively. The progress of topotecan- or anti-CD95-induced cell death was monitored by flow cytometry analysis and also by agarose electrophoresis of fragmented DNA. During early apoptosis of three of these cell lines, we observed the induction of internucleosomal DNA cleavage and, simultaneously, a typical change in the histone H1 protein pattern, leading to an increase in the relative amounts of histone subtypes H1.4 and H1.5. Upon apoptosis induction, these changes were only observed in correlation with the occurrence of DNA fragmentation, thus possibly reflecting a prerequisite for DNA accessibility and/or endonuclease activity.

INTRODUCTION

The process of apoptosis is characterized by multiple intracellular alterations which finally lead to the degradation of the cell into apoptotic bodies, subcellular particles which can be removed by neighbouring cells. Many of these alterations have been studied in detail during the past years, e.g. induction of apoptosis by receptor-mediated activation of protease cascades [1], alterations in the cytoskeletal structure of cells undergoing apoptosis [2], altered gene expression [3] and specific patterns of DNA degradation [4]. The cleavage of DNA into nucleosome-size fragments of 180–200 bp was first demonstrated in glucocorticoid-induced cell death in thymocytes [5] and has rapidly become a biochemical hallmark of apoptosis [6]. Visualization of the characteristic DNA 'ladder' is now widely used for monitoring apoptosis in many experimental cell systems, including the one described here. Recently, a DNase has been described which seems to be responsible for internucleosomal cleavage, at least in some model systems [7]. However, the composition (i.e. the histones and other DNA-binding proteins, as well as the DNA sequences preferentially cleaved) of the nucleosomal fragments has not been clarified so far. On the other hand, knowledge about the DNA architecture in normally growing cells and about the histones which are involved in building and maintaining this structural entity has been increased significantly during the last few years. In the case of the human H1 histones, which are also called linker histones because of their role in (inter-)nucleosomal linker DNA organization, seven different subtypes have been

characterized and cloned, termed H1.1–H1.5, H1^o and H1t [8]. The mRNA and protein pattern of these highly similar subtypes vary between different tissues, e.g. H1t is restricted to the testis, whereas H1^o is only abundant in some differentiated tissues [9]. In this study we addressed the questions of whether, first, the structural change and cleavage of the DNA during apoptosis induction is associated with changes in the histone H1 pattern and, secondly, whether the linker histone composition of the DNA–protein complexes within the nucleus could be correlated to the apoptotic DNA fragmentation process. To investigate this, we used HL60 cells as a model system for the study of tumour-cell apoptosis. Programmed cell death can easily be induced and monitored within these cells since they are very sensitive to chemical apoptosis induction, e.g. by topoisomerase inhibition [10]. We also screened seven other human tumour cell lines for their histone H1 protein pattern as well as for their sensitivity towards apoptosis induction by topoisomerase I inhibition.

For the analysis of the cellular H1 subtype pattern it was necessary to establish a method to isolate and separate the histone H1 proteins present in human cell lines. Since these proteins have very similar isoelectric points and molecular masses, standard separation methods, i.e. PAGE or liquid chromatography, failed to resolve all subtypes. We therefore used capillary zone electrophoresis (CZE) for the separation of human H1 histones by modifying a method described by Lindner et al. [11,12] for the analysis of murine histone H1 subtypes.

CZE is a very sensitive technique for the separation and

Abbreviations used: Br-dUTP, brominated deoxyribonucleotide triphosphate; RT, room temperature; PCA, perchloric acid; CZE, capillary zone electrophoresis; PI, propidium iodide; RPMI, Roswell Park Memorial Institute medium; TUNEL, terminal deoxyribonucleotidyltransferase-mediated dUTP nick end-labelling.

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detection of proteins in nanolitre amounts of samples. However, because of the low sample volume it is almost impossible to collect fractions of individual peaks for preparative analysis. Thus, we expressed human H1 subtypes in yeast, purified these proteins and mixed them with the preparations from cell lines to identify individual subtypes within the preparations. By using these recombinant proteins as internal standards, we were able to analyse the H1 protein pattern of different tumour cell lines and to study the influence of apoptosis induction and DNA fragmentation on the relative amounts of all the different H1 subtypes within the pattern.

EXPERIMENTAL

Purification of histone H1 proteins from transformed yeast

Yeast cells were transformed with human H1 subtype genes and were induced to express the respective proteins, as described in detail elsewhere [13]. Yeast cell suspensions were centrifuged (10 min, 4 °C, 4000 *g*), cells were washed with 0.1 M Tris/SO₄, pH 9.2, and resuspended in PT buffer (1 M sorbitol/46 mM K₂HPO₄/4 mM KH₂PO₄/20 mM dithiothreitol). The cells were then incubated for 30 min at 37 °C, centrifuged (as above) and resuspended in buffer D (1 M sorbitol/1 mM EDTA) plus 2 mg/ml zymolyase. After a second incubation step (30 min, 37 °C) the cell suspension was centrifuged and washed once with buffer D. Yeast cells were then treated with perchloric acid (PCA) at a final concentration of 5%. One volume of fine glass beads was added and the cells were stirred for 2 × 30 s and then incubated for 2 h on ice. After a third stirring of the mixture, lysed cells were centrifuged for 20 min at 4 °C and 14000 *g*. The supernatant was stored on ice and the pellet was re-extracted with 5% PCA. The supernatants of both extractions were collected and centrifuged (10 min, 4 °C, 14000 *g*). Histones were precipitated from these supernatants by the addition of 7 vol. of acetone (−20 °C) and overnight incubation at −20 °C. Samples were centrifuged (30 min, 4 °C, 14000 *g*) and the pellet was washed once with cold acetone and vacuum-dried. The pellet was then solubilized in 10 mM HCl by repeated stirring and incubation on ice. H1 histones were precipitated by saturation of the 10 mM HCl solution with (NH₄)₂SO₄ (final concentration approx. 90% w/v) and incubation for 1 h on ice. The samples were centrifuged (20 min, 4 °C, 15000 *g*) and the pellets were resolubilized in 10 mM HCl. A third precipitation step followed (addition of trichloroacetic acid to a final concentration of 30%) to remove traces of (NH₄)₂SO₄. After incubation for 1 h on ice, samples were centrifuged (20 min, 4 °C, 15000 *g*) and the pellet was resolubilized in 10 mM HCl and analysed for purity by SDS/PAGE (results not shown) and CZE.

Purification of histone H1 proteins from human tumour cell lines

HL60 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum in a humidified air/CO₂ (19:1) atmosphere at 37 °C and treated with topoisomerase inhibitor or anti-CD95 antibody as indicated in the Figures. After the incubation, cell suspensions (usually 5 × 10⁷ cells per incubation) were centrifuged [5 min, 300 *g* at room temperature (RT)] and the cells were washed once with PBS. PCA (5%) was added to the cell pellets and cells were lysed and extracted by incubation for 1 h at RT under continuous stirring. Samples were centrifuged (30 min, 4 °C, 14000 *g*) and the supernatant was stored while the pellet was re-extracted once with 5% PCA. Supernatants were collected and centrifuged again. The acid-soluble proteins were precipitated by addition of 7 vol. of acetone (−20 °C) and overnight incubation at −20 °C.

After centrifugation (30 min, 4 °C, 14000 *g*), pellets were washed twice with cold acetone and vacuum-dried. The histones were then solubilized in 10 mM HCl and subjected to analysis by PAGE (results not shown) and CZE. The other cell lines were cultured in RPMI 1640 (U937, K562, Jurkat, Raji, HepG2), McCoy's 5A (Tera2) or minimal essential medium (HeLa) supplemented with 10% fetal bovine serum and treated as above, with the exception that adherent cells (HeLa, HepG2, Tera2) were removed from the cell culture flask by means of a rubber policeman before washing with PBS. All cell lines were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) or the European Collection of Cell Cultures (Salisbury, U.K.). Cell-culture reagents and media were purchased from Biochrom (Berlin, Germany). All biochemical reagents except topotecan (SmithKline Beecham) were from Sigma (München, Germany).

CZE of H1 histones

CZE was performed on a Perkin-Elmer/Applied Biosystems 270A-HT system. The separation conditions were optimized according to the method of Lindner et al. [12]. The capillary was made of fused silica: length 122 cm, inner diameter 50 µm. Separations were performed under the following conditions: temperature, 30 °C; voltage, 20 kV; current, approx. 35 µA; separation time, 50 min; sample injection by pressure; injection time, 2 s; sample protein concentration, approx. 0.5 mg/ml; absorbance detection at 200 nm. The separation buffer was 30 mM H₃PO₄/60 mM HClO₄/0.02% (w/v) hydroxypropyl-methylcellulose, pH 2.00, adjusted with triethylamine. Absorbance data were recorded and analysed by a PC-based Kontron Integration System.

Analysis of fragmented DNA by agarose-gel electrophoresis

For the preparation of fragmented DNA, cells were harvested and washed as described for the purification of histone H1 proteins. Cell pellets were then incubated on ice for 30 min in 450 µl of lysis buffer (0.2% Triton X-100 in 10 mM Tris/HCl/10 mM EDTA, pH 8.0). Samples were microcentrifuged (11600 *g* for 10 min at 4 °C) and the non-sedimentable DNA fragments of the supernatants were precipitated by addition of 450 µl of propan-2-ol and 100 µl of 10 M LiCl and incubation at −20 °C overnight. After microcentrifugation as above, the pellets were washed twice with ice-cold 70% (v/v) ethanol and vacuum-dried. The pellets were then resolubilized in 50 µl of TE buffer (10 mM Tris/HCl/1 mM EDTA, pH 8.0). Samples were extracted twice for DNA with chloroform/3-methylbutan-1-ol (24:1) and the purified DNA was again precipitated by the addition of 100 µl of ethanol and 15 µl of 3 M sodium acetate (pH 4.8) and incubation for at least 2 h at −70 °C. After microcentrifugation as above, pellets were vacuum-dried and solubilized in 30 µl of TE buffer. Extracted DNA was resolved in 1.2% agarose gels using a constant current of 70 mA and revealed by staining with ethidium bromide.

Analysis of apoptotic cells by dual-colour flow cytometry

For the analysis of apoptotic cells by dual-colour flow cytometry we used a kit (APO-BRDU[®]; Pharmingen, San Diego, CA, U.S.A.). Labelling of the cells was performed according to the manufacturer's instructions. Briefly, cells were suspended in 500 µl of PBS, then fixed by the addition of 5 ml of 1% (w/v) paraformaldehyde in PBS and incubation on ice for 15 min. The cells were then centrifuged (5 min, 4 °C, 300 *g*) and the super-

natants were discarded. Cells were washed twice in PBS, then resuspended in 500 μ l of PBS. Ice-cold 70 % (v/v) ethanol (5 ml) was added and cells were incubated for at least 30 min at 4 °C, then stored at -20 °C until further use. The cellular DNA was labelled by incubation of the fixed cells with terminal deoxyribonucleotidyltransferase and brominated deoxyribonucleotide triphosphate (Br-dUTP) as provided in the kit for 60 min at 37 °C. Cells were then washed twice and then incubated with an FITC-labelled anti-Br-dUTP monoclonal antibody as provided with the kit. Incubation with the antibody was for 30 min at RT in the dark. Total cellular DNA was labelled by the addition of 0.9 ml of staining buffer [PBS containing 0.1 mg/ml propidium iodide/0.1 % (v/v) Triton X-100/0.037 mg/ml EDTA/1 mg/ml RNaseA] and again incubation for 30 min in the dark. The cells were analysed in a flow cytometer (FACScan[™], Becton-Dickinson, San Jose, CA, U.S.A.). Flow cytometer settings were as recommended in the kit.

RNase protection analysis

RNase protection analysis of H1 histone subtype mRNAs was performed as described elsewhere [14]. Briefly, total RNA from HL60 cells was isolated using the Qiagen RNeasy kit (Hilden, Germany) according to the manufacturer's instructions. Equal amounts of RNA were used per assay. The RNA concentration was determined photometrically and both the quantity and integrity of the RNA preparations were checked by ethidium bromide staining of electrophoresis gels (results not shown). Template plasmids for RNase protection analysis were constructed by subcloning DNA fragments of human histone H1 subtype genes into Bluescript KS⁺. The inserted fragments contained the proximal part of the 5' flanking region and part of the coding region. For *in vitro* transcription, the plasmid templates were linearized with the appropriate restriction enzyme and the antisense transcript was synthesized using [α -³²P]UTP and T3 or T7 RNA polymerase respectively. The labelled full-length transcript was purified by separation in a denaturing 8 M urea/5 % (w/v) polyacrylamide gel. For protection analysis, the Ambion RPAII kit (Ambion, Austin, TX, U.S.A.) was used according to the instructions in the manufacturer's manual. Total RNA (10 μ g) from HL60 cells was hybridized with the ³²P-labelled antisense transcript at 45 °C for 16 h. The probes were then subjected to RNase digestion using the RNase A/RNase T1 mixture provided with the kit. After precipitation, protected fragments were separated in an 8 M urea/5 % (w/v) polyacrylamide gel and bands were detected by exposure to an autoradiography film for 24 h.

RESULTS

Purification of H1 histones from HL60 cells

The H1 histones of exponentially growing HL60 cells were extracted by lysis of the cells with 5 % PCA. This method was developed by Smith and Johns [15] and has previously been shown to be very effective for the enrichment of H1 proteins because of the remarkably high acid-solubility of the basic H1 histones. The histones were then precipitated by addition of ice-cold acetone, solubilized in 10 mM HCl and subjected to CZE. Preparation of H1 histones from cell lysates by selective PCA solubility yields highly enriched H1 preparations, which is demonstrated in Figure 1 (top). The purity of H1 preparations was also analysed by SDS/PAGE (results not shown) and by reversed-phase HPLC (results not shown).

Figure 1 CZE separation of H1 histones from HL60 cells

HL60 cells were harvested and H1 histones were prepared by PCA extraction as described in the Experimental section. H1 histones were then solubilized in 10 mM HCl and subjected to CZE using a fused-silica capillary with a total length of 122 cm and an inner diameter of 50 μ m. Absorbance data (E) were continuously recorded. The maximal peak absorbance of the representative sample shown was 0.023. Top: the entire electropherogram; bottom: the absorbance curve of the same electropherogram run between 38 and 45 min after injection. The histone H1 subtypes of the H1 pattern from HL60 cells are indicated (bottom). Peak assignment was done as described for Figure 2. The peak marked with an asterisk represents the high-mobility group protein HMG17 (see the Results section).

Identification of individual H1 subtypes in histone H1 preparations through addition of recombinant proteins

The identification of histone H1 subtypes was done by mixing of the preparations from cell lines with recombinant proteins purified from transformed yeast, which acted as internal standards. The advantage of the yeast expression system was that *Saccharomyces cerevisiae* lacks endogenous histone H1, thus we obtained pure preparations of these individual recombinant proteins by PCA extraction. The CZE analysis for recombinant human H1.2 is shown in Figure 2 (top left-hand graph). Mixing of e.g. H1.2 with the H1 preparation from human cell lines resulted in the selective increase of one single peak, indicating that this peak represents H1.2 within the mixed preparation. (Figure 2, top right-hand graph). These mixing experiments were done with all human recombinant subtypes, except H1t which is exclusively expressed in the testis. The analysis of the mixed preparations is also shown here for H1.3, H1.4 and H1.5. Analyses with mixed preparations for H1.1 and H1° (both subtypes absent from HL60 cells) are not shown in Figure 2 in the interest of clarity.

Analysis of the histone H1 pattern of HL60 cells

The characteristic H1 subtype pattern for HL60 cells after PCA extraction and CZE separation of the H1 histones is depicted in Figure 1 (bottom). H1.2 is the predominant subtype present in this cell line. The H1 pattern of HL60 cells is very similar to that of e.g. U937 cells, with the exception that HL60 cells express very little H1.3 protein. H1.1 protein is undetectable in this cell line. The expression of the replacement variant H1° depends on the

Figure 2 CZE electrophoretograms of recombinant human histone H1 subtypes alone and mixed with HL60 H1 preparations

To identify the individual subtypes within the pattern of H1 histones, each of the human H1 subtypes was expressed in yeast and purified as described (see the Experimental section). These proteins were then analyzed by CZE separation with running conditions as described in Figure 1, either alone (left side) or mixed with an aliquot of the histone H1 preparation shown in Figure 1, bottom, from HL60 cells (right side). All eight graphs show the absorbance profiles within a 4.0 min time period (absorbance is measured on the y-axis). Maximal absorbances at 200 nm were between 0.007 and 0.018.

type of cell line and on the cell-cycle distribution of the population [14]. The analysis revealed that H1^o protein was missing in the H1 preparation from HL60 cells. This was of course not surprising, since it has been previously demonstrated that these cells do not express H1^o mRNA [14].

There are several additional peaks within the histone H1 pattern. One of them (indicated with an asterisk in Figure 1, bottom) represents the high-mobility group protein HMG17 [16]. Evidence for the identity of this peak with HMG17 was provided by separation of the PCA extract by HPLC and analysis of the collected fractions by PAGE. The other peaks presumably arise from covalently modified H1 histones and not other HMG proteins or histones, since they were also present in the histone H1 fraction eluting from the HPLC separation as a

single peak (results not shown). By dephosphorylation assays using different phosphatases (P-Ser/P-Thr as well as P-Tyr phosphatases) we could exclude the possibility of phosphorylated H1 proteins being parts of the unidentified peaks within the CZE electrophoretogram (results not shown). However, several other covalent modifications of H1 histones [e.g. (ADP-ribosyl)ation, acetylation, methylation] have been described in the literature [17].

Histone H1 protein pattern of different human tumour cell lines

We then analysed four other leukaemia or lymphoma cell lines, U937, Jurkat, K562 and Raji, and three cell lines established from solid tumours, namely from teratoma (Tera2), hepatoma (HepG2) and cervix carcinoma (HeLa) cells for their pattern of totally extractable H1 proteins. The results are listed in Table 1. It is shown that, independent of their origin, but according to their pattern of H1 histones, these tumour cell lines can be divided into three subgroups, with the first group expressing all six somatic H1 histones, the second group lacking H1.1 and the third group possessing neither H1.1 nor H1^o. Within this third group, the HL60 cells are significantly different from the three other cell lines in that they have only trace amounts of H1.3 protein. In the two cell lines of the first group, Raji and Tera2, we found histone H1.5 to be the predominant linker histone, whereas in all other untreated cell lines we have tested, H1.2 dominated within the pattern.

Treatment of HL60 cells with topotecan

To induce apoptosis, HL60 cells were cultured in the presence of 150 ng/ml topotecan, a water-soluble camptothecin derivative which inhibits topoisomerase I [18]. The process of topotecan-induced apoptosis was monitored by phase-contrast microscopy (results not shown), by analysis of DNA fragmentation (Figure 3) and by double staining of the cellular DNA with propidium iodide and by the TUNEL method [19] and subsequent flow cytometry analysis (Figure 4A). At different times after treatment, the cells were lysed in 5% PCA and the H1 histones were subsequently extracted, separated by CZE separation and analysed (Figure 4B). As shown, the pattern of histone H1 subtypes changed in HL60 cells within the first 2 h of incubation with topotecan. In relation to the amount of H1.2, there was a significant increase in the amounts of unmodified histones H1.4 and H1.5 in HL60 cells. This change in the H1 subtype pattern did not reflect an increase in total amounts of H1 histones during apoptosis. Rather, the overall amounts of extractable H1 histones per 5×10^7 cells, as determined by PAGE, Coomassie staining and densitometric quantification of the bands representing histone H1, remained constant throughout the incubation period (results not shown), indicating that the changes we observed must be due to a decrease in either some of the known unmodified H1 subtypes (e.g. H1.2) or some modified forms of H1 histones. One candidate peak for such a modified form of H1.4 and/or H1.5, which is detected by its 200 nm absorption at approx. 43 min of the CZE separation electropherogram, decreases at the same time as H1.4 and H1.5 increase. The identity of this peak has not yet been clarified.

Treatment of HL60 cells with anti-CD95 antibody

To exclude the possibility that the changes in the histone H1 pattern were due to a direct effect of topoisomerase inhibition on DNA topology, rather than correlated with apoptosis, we treated the cells with an activating antibody (IgM) against the apoptosis-

Table 1 Distribution of histone H1 subtypes in human tumour cell lines

Cells were cultured and H1 histones were prepared from exponentially growing cells as explained in the Experimental section. The pattern of H1 histones was then analysed by CZE using a 122 cm capillary as described. The histone H1 subtypes were identified by mixing of the H1 preparations from cell lines with individual recombinant H1 proteins as described in Figure 2. The absorbance peaks at 200 nm, representing the histone H1 subtypes, were analysed and quantified by means of computer-aided integration. The relative amounts of H1 subtype proteins are given as percentages of total identified histone H1 proteins within the pattern. Results are given as means $\pm$ S.D. from quantifications of three independent experiments. n.d., not detectable.

Cell line	Histone subtype ...	H1.1	H1.2	H1.3	H1.4	H1.5	H1°
Raji		4.2 $\pm$ 0.7	17.2 $\pm$ 2.6	11.3 $\pm$ 1.2	25.6 $\pm$ 0.4	35.3 $\pm$ 4.5	6.4 $\pm$ 0.6
Tera2		6.7 $\pm$ 1.3	25.8 $\pm$ 3.7	12.3 $\pm$ 1.6	25.4 $\pm$ 1.9	27.4 $\pm$ 2.0	2.4 $\pm$ 1.1
HepG2		n.d.	41.7 $\pm$ 2.5	n.d.	47.6 $\pm$ 0.5	6.3 $\pm$ 0.8	4.4 $\pm$ 1.3
HeLa		n.d.	48.0 $\pm$ 2.2	6.2 $\pm$ 0.8	26.1 $\pm$ 1.1	16.9 $\pm$ 2.6	2.8 $\pm$ 1.0
K562		n.d.	64.2 $\pm$ 6.4	5.3 $\pm$ 1.6	20.0 $\pm$ 3.8	10.5 $\pm$ 1.4	n.d.
Jurkat		n.d.	44.3 $\pm$ 0.6	19.4 $\pm$ 0.3	22.9 $\pm$ 1.9	13.4 $\pm$ 1.6	n.d.
U937		n.d.	64.1 $\pm$ 3.2	7.6 $\pm$ 1.1	23.3 $\pm$ 3.5	5.0 $\pm$ 0.8	n.d.
HL60		n.d.	72.7 $\pm$ 3.9	0.6 $\pm$ 0.2	18.5 $\pm$ 2.0	8.2 $\pm$ 1.9	n.d.

Figure 3 Analysis of DNA fragmentation after topotecan treatment

Exponentially growing HL60 cells were treated with topotecan at a final concentration of 150 ng/ml (328 nM) for the times indicated. After termination of the incubation, cells were harvested and samples (approx. 5×10^7 cells) were further treated for isolation of fragmented DNA. An aliquot of DNA corresponding to 5×10^6 cells was separated on a 1.2% agarose gel at a constant current of 70 mA for 4 h, then stained with ethidium bromide. The DNA size marker (indicated with an M; purchased from Gibco-BRL, Karlsruhe, Germany) consisted of fragments between 250 and 3500 bp in multiples of 250 bp and an additional fragment of 5000 bp. The bands corresponding to the 1000 and 5000 bp fragments respectively were brighter than the others to provide internal orientation.

mediating receptor CD95 (Fas/APO-1) for 18 h. Then we analysed the H1 histone pattern and the DNA fragmentation as described above. The results are shown in Figures 5 and 6. Whereas 50 ng/ml anti-CD95 weakly induced DNA fragmentation and had a moderate effect on the pattern of H1 histones, 100 ng/ml of the antibody were equally as effective as topotecan, both in inducing DNA fragmentation (Figure 6) and in changing the H1 pattern (Figure 5). These results confirm that when DNA fragmentation is induced via triggering of receptor-mediated apoptosis, similar changes in the histone H1 pattern can be observed as in cells that were treated with the topoisomerase I inhibitor.

Interestingly, there is a remarkable difference in the oligomer size between the topotecan-treated cells and the cells treated with anti-CD95 antibody, with smaller fragments being observed after topotecan treatment. We investigated the time course of this cleavage and observed a decrease in the oligomer size as a

function of topotecan treatment time, whereas the oligomer size remained constant upon prolonged treatment with anti-CD95 antibody (results not shown). The reason for this observation is not yet clear. However, apoptosis induction by topotecan treatment may lead to the activation of an exonuclease, which progressively cuts off the ends of the oligomers, in addition to the action of the internucleosomally cleaving DNase.

Correlation between apoptotic DNA fragmentation and increase in unmodified H1.4

We then tested whether the changes we observed in the pattern of H1 histones of HL60 cells could also be shown in other types of tumour cell lines when treated with a topoisomerase inhibitor to induce apoptosis. In apoptotic U937 and Jurkat cells respectively, a similar change in the pattern of H1 histones could be observed. The time course of induction of DNA fragmentation observed in these two cell lines was similar to what we observed in HL60 cells (results not shown). Surprisingly, among all the other cell lines that we had previously analysed for their pattern of H1 histones (see Table 1), only Jurkat and U937 cells were sensitive to apoptosis induction by topoisomerase I inhibition. Although HeLa cells showed a diminished cell proliferation and an increase in the amount of dead cells after 24 h of topotecan treatment, we were unable to demonstrate nucleosomal DNA fragmentation within these cells. Accordingly, no changes in the pattern of H1 histones could be observed after topoisomerase treatment in the resistant cell lines (Table 2). We conclude that the increases in H1.4 and H1.5 are correlated with the occurrence of internucleosomal DNA cleavage.

Histone H1.2 and H1.4 mRNA expression after topotecan treatment

To investigate whether the significant increase in unmodified histone H1.4 during apoptotic DNA fragmentation could be due to an enhancement of H1.4 gene transcription, we treated HL60 cells with 100 ng/ml anti-CD95 antibody for different time periods up to 24 h and subsequently analysed the amounts of histone H1.2 and H1.4 mRNA during apoptosis by means of RNase protection analysis (Figure 7). The results show that throughout this time after treatment the relative amounts of these mRNAs remained unchanged, although DNA fragmentation was induced and the relative amount of H1.4 protein increased within the same time (see Figure 5 and 6). It can therefore be excluded that the increase in H1.4 we observed in

Figure 4 Analysis of HL60 H1 protein patterns after topotecan treatment

Exponentially growing HL60 cells were treated with topotecan as described in the legend to Figure 3. After termination of the incubation, cells were harvested and parallel samples (approx. 5×10^7 cells each) were further treated for flow cytometric analysis of apoptotic cells (**A**) and histone H1 purification (**B**) as described in detail in the Experimental section. (**A**) The results of flow-cytometry analysis of topotecan-treated cells double-stained with the TUNEL reaction (for the visualization of DNA fragments) and with propidium iodide (for the visualization of total cellular DNA) as described in detail in the Experimental section. The fluorescence intensity for PI staining of individual cells is shown in the x-axis direction in linear mode, whereas the fluorescence intensity for FITC staining (TUNEL-labelled cells) is depicted in the y-axis direction in log mode. The two cell populations, designated as non-apoptotic and apoptotic respectively, are discriminated by a threshold FITC fluorescence signal height of 55, as marked with a horizontal line. The discriminator for intact cells was set at a PI fluorescence signal height of 110, as marked with a vertical line. The percentages of apoptotic cells with respect to total intact cells, as quantified by these discriminators, were 10.3, 11.7 and 50.7% for control cells and cells treated with topotecan for 2 and 8 h respectively. (**B**) CZE absorbance patterns of H1 histones from topotecan-treated cells, over a 4 min time period of a 50 min electrophoretic separation, from a single representative experiment (left). Right: the areas under the peaks identified as histone H1 subtypes within the corresponding CZE separations have been quantified from three independent experiments and are depicted as percentages $\pm$ S.D. of total identified H1 histones within the H1 preparation. Only the CZE electropherograms and quantifications of the time points 0 h (Control), 2 h and 24 h are shown.

Figure 5 Effect of anti-CD95 antibody treatment on HL60 histone H1 pattern

HL60 cells were treated with topotecan, as indicated, or with 50 or 100 ng/ml of an activating anti-CD95 monoclonal antibody (IgM, clone CH11; purchased from Biomol, Hamburg, Germany) for 18 h. Four minute time periods of the CZE separation graphs of the purified H1 histone fractions from the respective treatments are shown. E stands for absorbance.

correlation with apoptotic DNA fragmentation could be due to an enhancement in the transcriptional rate.

DISCUSSION

The H1 histones comprise seven subtypes, termed H1.1–H1.5, H1° and H1t [20], of which the proteins H1.2 and H1.4 are predominant in most human cell lines [14]. The main functional role of these proteins is to interact with linker DNA [21], thereby organizing higher-order structures and the condensation of chromatin [22]. There is also recent evidence that H1 histones can act as basal repressors of transcription [23]. The so-called linker

Figure 6 Effect of anti-CD95 antibody treatment on internucleosomal DNA cleavage

HL60 cells were treated with topotecan, as described in the legend to Figure 5, and DNAs were prepared as described in the text. Aliquots of internucleosomally cleaved DNA corresponding to 5×10^6 cells were separated on an 1.2% agarose gel and visualized by ethidium bromide staining. Marker bands are as in Figure 3.

Table 2 Correlation between DNA fragmentation and increase in histone H1.4 protein amounts

Comparison of the entire results obtained with the eight cell lines analysed in the present study with regard to induction of internucleosomal DNA fragmentation or increase in the histone H1 subtype H1.4 as a result of treatment with 150 ng/ml of the topoisomerase I inhibitor topotecan.

	Internucleosomal DNA fragmentation	Histone H1.4 increase
HL60	++	++
U937	++	++
Jurkat	++	++
K562	—	—
HeLa	—	—
HepG2	—	—
Raji	—	—
Tera2	—	—

histones constitute a family of closely related proteins that have very few differences in their amino acid sequence, and hence in their physicochemical and immunochemical properties. It is therefore difficult to separate these proteins by means of standard biochemical methods. On the other hand, one important step in the course of apoptosis is the condensation of chromatin, forming a characteristic 'half-moon' at the nuclear periphery, as shown in several cellular model systems [24]. Thus we suggest that the H1 histones are essential for the generation of these condensed

Figure 7 RNase protection analysis of histone H1.2 and H1.4 mRNAs after treatment with anti-CD95 antibody

HL60 cells were treated with anti-CD95 antibody at a final concentration of 100 ng/ml for the time periods indicated. Total RNAs of HL60 cells were isolated and equal amounts of RNA were hybridized with 32 P-labelled antisense transcripts for H1.2 and H1.4 respectively, as described in the Experimental section. The probes were subjected to RNase digestion and the protected fragments were isolated and resolved in an 8 M urea/5% (w/v) polyacrylamide gel, then visualized by autoradiography. The process of DNA fragmentation was monitored by isolation and visualization of fragmented DNA from parallel incubations (results not shown).

structures and, in addition, for the initiation of the subsequent internucleosomal cleavage of DNA, which is a hallmark of, although not essential for, the process of programmed cell death [25]. Moreover, H1 histones have been assigned for specific gene repression and the regulation of cellular differentiation [26]. We were therefore interested in a comparative analysis of the complete histone H1 protein pattern of normally growing versus apoptotic human tumour cells. Here we describe the purification and analysis of the protein pattern of the H1 histone subtypes of eight different human tumour cell lines, of which five are leukemia or lymphoma cell lines and three are established from solid human tumour tissues.

A comparison of the patterns from these cell lines revealed several regularities, which led us to a classification of the patterns into three groups. Within the first group, all six somatic histones are present, although in different amounts. The histones H1.5 and H1.4 dominate the pattern, whereas the proteins H1.2 and H1.3 play a minor role. The protein expression of H1.1 and H1 $^{\circ}$ is generally very low. The two cell lines of the second group, HepG2 and HeLa, have in common that they lack H1.1 protein and that, in these cells, H1.2 is now the subtype dominating the pattern, followed by H1.4. Remarkably, HepG2 was the only cell line that we have analysed so far that lacks H1.3 expression. The third group of cells comprises solely haematopoietic cell lines. Within these cells, H1.1 and H1 $^{\circ}$ are both undetectable. In addition, the amount of H1.3 in HL60 cells is very low. Thus, it is conceivable that cell lines with even less complex histone H1 patterns will be found.

The cells were then induced to undergo apoptosis by topoisomerase I inhibition or activation of the CD95 (Fas/APO-1) receptor. Subsequently, the histone H1 pattern and the time-course of internucleosomal DNA fragmentation were monitored. We found that the pattern of H1 histones extractable from three of the human leukaemic cell lines changed when cells underwent apoptosis and DNA was fragmented into nucleosomal units. Since these changes were correlated with the observation of DNA fragmentation, and since the mRNA levels of the two prominent histone H1 subtypes H1.2 and H1.4 were unchanged upon apoptosis induction (see Figure 7), it seemed likely that the

differential H1 histone pattern reflected changes in the post-translational modification state of distinct subtypes, rather than in the protein amount of some of the H1 histones. This hypothesis is further supported by the observation that a peak appearing shortly after histone H1.2 (at approx. 43 min in Figure 1, bottom) decreases at the same time as the peaks representing H1.4 and H1.5 increase. This, until now unidentified, peak could represent a modified form of the subtype H1.4 and/or H1.5, and the pattern change that we observe could thus result from a selective de-modification of some of the H1 histones. This possibility is currently under investigation.

The selective increase of only two of the four histone H1 subtypes present in HL60 cells after induction of DNA cleavage supports the hypothesis that the DNase responsible for the apoptotic internucleosomal DNA fragmentation (presumably caspase-activated DNase, which was recently described by Enari et al. [7]), does not cleave the DNA randomly. Instead, there may be a recognition signal for DNase action on the sites that are preferentially cleaved. This signal could be encoded by the distinct subtypes of H1 histones and/or their modification status. At the sites where these linker histones are bound to the DNA, the internucleosomal cleavage site could be more accessible for the DNase due to slight topological changes.

We show here that there is a very strong correlation between the occurrence of internucleosomal DNA fragmentation in the course of apoptosis of human leukaemic cells and changes in the total protein pattern of H1 histone subtypes that could be isolated from these cells (Table 2). The identities of the key players in this scenario, namely the substrate specificity of caspase-activated DNase or a related DNase, as well as the putative (de)modifications of those H1 histones increasing in quantity upon initiation of DNA cleavage, will have to be clarified in order to elucidate the putative function of H1 histones as signalling molecules for the process of internucleosomal DNA fragmentation during apoptosis.

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