Induction of histone H1.2 cytosolic release in chronic lymphocytic leukemia cells after genotoxic and non-genotoxic treatment

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ABSTRACT

Background
The aim of this study was to analyze whether in chronic lymphocytic leukemia the cytosolic release of histone H1.2, a new apoptogenic mechanism induced by DNA damage, was associated with the presence of genetic abnormalities and with the response to treatment.

Design and Methods
Primary tumoral chronic lymphocytic leukemia cells from 25 patients were investigated for histone H1.2 cytosolic release after treatment with genotoxic (fludarabine, mitoxantrone, etoposide, or X-ray radiation) and non-genotoxic (dexamethasone) agents. Cases were analyzed for the presence of poor-risk genetic alterations, particularly deletions at 17p13 and 11q22. Histone H1.2 release was correlated with the presence of genetic abnormalities and with the best clinical response obtained with standard treatments.

Results
DNA-damaging agents induced H1.2 release in a p53-dependent manner which was confirmed by the lack of H1.2 release in p53-deleted cases. Non-DNA-damaging agents induced release of H1.2 in both p53-deleted and non-deleted chronic lymphocytic leukemia cases. Moreover, nuclear H1.2 release was observed after genotoxic and non-genotoxic treatment independently of *ATM* function. From a clinical standpoint, the lack of histone H1.2 release correlated with resistance to genotoxic treatment.

Conclusions
In chronic lymphocytic leukemia cells, histone H1.2 traffic was dependent on the p53-status after genotoxic treatment, but could also be induced after treatments that acted independently of p53. By contrast, histone H1.2 release did not seem to be dependent on ATM function. Nuclear histone H1.2 release appears to be an important element in apoptosis induction in chronic lymphocytic leukemia, particularly in cases with abnormal p53 function resistant to conventional treatment.

Key words: chronic lymphocytic leukemia, histone H1.2, del17p, del11q, apoptosis

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Introduction

Chronic lymphocytic leukemia (CLL) is a lymphoproliferative malignancy resulting from the accumulation of mature CD5+ B-lymphocytes and represents 30% of the leukemias in the Western world.[1] The clinical course of CLL is highly heterogeneous. Some progress in therapy has been made but in spite of this CLL remains incurable.

Treatment of CLL, which had largely been based on the use of alkylating agents, is currently switching to purine analogs-based regimens with or without monoclonal antibodies.[2-6] These combinations induce higher response rates and a longer disease-free survival than alkylating agents or fludarabine alone.[3,4] However, a significant number of patients are refractory to therapy. The prognosis of these patients is very poor with a median survival of less than a year.[7-9] The most important molecular mechanism for treatment refractoriness in CLL is p53 dysfunction, a genetic abnormality that appears in 10% of cases.[7] The great majority of cytotoxic drugs like purine analogs and alkylators, act by inducing DNA double-strand breaks. This leads to p53 activation[10,11] and eventually to the activation of the mitochondrial pathway and caspase-dependent cell death.[10] In addition to p53, the ATM protein, a member of the PIKK family, is the main integrator of cellular response after DNA double-strand breaks (DSBs) and is responsible for the phosphorylation and stabilization of p53.[12] Inactivation of the ATM gene by deletion of 11q22.3-23.1 or by mutation occurs in a proportion of CLL patients with poor prognosis and is an alternative cause of p53 dysfunction.[13,14] In fact, it is not fully understood how apoptotic signals arising from DNA damage are transmitted to mitochondria which release apoptogenic factors into the cytoplasm that activate downstream destruction programs.

The release of histones, including internucleosomal H1 and core histones, into the cytoplasm during apoptosis is a well-known phenomena.[15-17] Konishi *et al.* recently reported new insights into the death signal transmission pathway.[18] They demonstrated the apoptogenic ability of histone H1.2 when this protein translocated from the nucleus into the cytosol after DNA double-strand breaks. H1.2 had the specific ability to induce cytochrome c release from mitochondria in a Bak-dependent manner leading to cell death. Interestingly, H1.2 translocation had been placed downstream from p53 after DNA damage.

Against this background, we analyzed whether histone H1.2 was released after DNA damage in primary tumoral CLL cells from cases with normal karyotype and with del(17p13) or del(11q23). In addition, this new apoptotic mechanism was also analyzed after treatments that act independently of p53 to provide a molecular basis for the use of such agents in CLL refractory to conventional treatments. The results showed that histone H1.2 could be an important protein for apoptosis induction in CLL, its releasing pattern correlating with 17p deletions and response to treatment.

Design and Methods

Patients and Samples

Twenty-five patients (13 men, 12 women, median age 58 years) diagnosed with CLL according to the NCI-WG criteria[19] were included in the study. Peripheral-blood samples were taken after obtaining written informed consent and approval from the local Ethical Committee. Cases were selected according to the availability of a sufficient number of frozen samples and clinical data. The presence of chromosomal abnormalities was assessed by conventional cytogenetics and fluorescence *in situ* hybridization using specific probes (LSI p53/ LSI ATM and LSI D13S319 / LSI 13q34/ CEP 12 Multi-color Probe Sets, Vysis) and following the hierarchical classification previously described.[7] The main clinical and biologic variables and the response to standard treatments were recorded.

Isolation and treatment of cells

Isolation of peripheral blood CLL cells was performed by centrifugation on a Ficoll/Hypaque (Seromed, Berlin, Germany) gradient followed by cryopreservation in liquid nitrogen in the presence of 10% dimethyl sulphoxide (DMSO). CLL lymphocytes were thawed and cultured in RPMI medium supplemented with 10% fetal calf serum, 2 mmol/L glutamine and 1000 UI/mL penicillin/streptomycin, at 37°C in a humidified atmosphere containing 5% carbon dioxide. Mean cell purity of the samples was 96% (range 89-99%). Cells were treated 1 hr. after thawing under the following conditions: fludarabine monophosphate (Schering AG, Berlin, Germany) at 1 μg/mL, mitoxantrone (Lederle Lab., Hampshire, UK) at 0.5 μg/mL, etoposide (Bristol Myers, Princeton, NJ, USA) at 50 μM, dexamethasone (Merck, Darmstadt, Germany) at 13.2 μM. These doses have been proved to match the concentrations obtained in the clinical setting.[20] Cells were irradiated using a Cs^{137} source (25 Gy). After treatment, cells were harvested at 6, 12, and 24 hrs., depending on the experiments to be performed. Due to the low cell viability observed after 24 hrs., no further analysis was performed after that time point. In addition IL-4 (Sigma-Aldrich, Inc., USA) at 250 UI/mL was used in some experiments as well as the broad caspases inhibitor Z-VAD.fmk (N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) from Bachem (Bubendorf, Switzerland) at 200 μM.

Analysis of cell viability by Annexin V binding

Cell viability and apoptosis at different time points were assessed by annexin binding and propidium iodide (PI) DNA staining (human Annexin V-FITC kit, Bender MedSystems, Vienna, Austria) followed by flow cytometry analysis (FACScan; Becton Dickinson, Mountain View, CA, USA). Briefly, after washing $0.2\text{-}0.4\times10^6$ of cultured cells in 4 mL of binding buffer (10 mmol/L HEPES, pH 7.4, 2.5 mmol/L $CaCL_2$, 140 mmol/L NaCl), 0.5 μg/mL of Annexin-V fluorescein isothiocyanate (FITC) was added

and cells were incubated for 10 mins. in the dark. After a second wash with binding buffer, 5 μL of PI (20 μg/mL) was immediately added before the acquisition of up to 10,000 cells from each sample using the CELLquest software (Becton Dickinson). Thereafter, data were analyzed with the Paint-a-Gate Pro software (Becton Dickinson). For each case and time point, untreated samples cultured under the same conditions were used as controls.

Western Blot

The following primary antibodies were used in Western blot analysis: rabbit polyclonal anti-histone H1.2 (Abcam, UK), mouse monoclonal anti-PARP [C-2-10] (Abcam), mouse monoclonal anti-α-tubulin (Calbiochem, USA), rabbit polyclonal anti-GAPDH (Abcam), mouse monoclonal anti-p53 [BP53-12] (Abcam), polyclonal goat anti-ATM (Abcam) and rabbit polyclonal (phospho S1981) anti-ATM (Abcam).

Nuclear and cytosolic fractionation was performed in 10-20×10^6 cells per condition. Cells were resuspended at 4°C for 15 mins. with hypotonic buffer (10 mM Hepes, 10 mM KCL, 15 mM MgCl2, 1 mM DTT and proteases inhibitors) and 0.6% NP-40 was added immediately before centrifugation in order to obtain the cytosolic fraction. Equal amounts of cytosolic protein extracts were separated by electrophoresis on 6% or 12% polyacrylamide and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). Membranes were incubated with the previously described antibodies. Antibody binding was detected using secondary antibodies conjugated to horseradish peroxidase followed by chemiluminescence detection using ECL™ reagents (Amersham Pharmacia Biotech, UK). Image capture was performed with a Luminiscent Image Analyzer LAS-3000 (Fujifilm, Japan) using Image Reader software (Fujifilm).

For testing anti-histone H1.2 antibody specificity, blocking experiments with histone H1.2 peptide were performed in CLL samples. Briefly, anti-histone H1.2 was incubated during 2 hours at 37°C either with an equal amount of specific histone H1.2 peptide or PBS. After centrifugation during 15 minutes at 14,000 rpm and 4°C, supernatants were used as primary antibodies for membrane incubation according to the above conditions. In all the membranes, intensity of PARP was used as a control for nuclear contamination in the cytosolic fraction, and GAPDH or α-tubulin were used as a loading control. In addition, PARP cleavage was used as a hallmark of caspases activation and apoptosis.

Immunofluorescence

For immunofluorescence techniques, treated CLL cells were washed and 0.5×106 cells in suspension were placed in a poly-L-lysine treated cover glass. Cells were fixed with 4% paraphormaldehyde and permeabilized with methanol. After 1 hr. of incubation with BSA 1% PBS, primary antibody anti-histone H1 (clone AE-4) (Upstate Biotechnology, USA) at 10 μg/mL was applied overnight. Fluorescein (FITC)-conjugated secondary antibody was used to detect anti-histone H1 antibody. PI nuclear staining was performed to improve H1 protein allocation. Images were acquired using a Leica TCS SL laser scanning confocal spectral microscope (Leica Microsystems Heidelberg GmbH, Manheim, Germany) with Argon and HeNe lasers attached to an inverted microscope. Image acquisition was performed at 12 and 24 hrs. after start of the treatment. As a control, histone H1 release was also evaluated in untreated cells at the same time points.

Results

Histone H1.2 release in CLL cells without poor-risk genetic abnormalities

Lymphocytes from 16 cases of CLL without relevant genetic abnormalities were analyzed for histone H1.2 release after genotoxic and non-genotoxic treatment. Main clinical and biologic features of CLL cases included in the present study are summarized in Table 1. Mean cell viability referred to the control at 24 hrs. was 22% (SD 11%) with FM, 37% (SD 17%) with etoposide, 31% (SD 18%) in irradiated cells and 56% (SD 16%) with dexamethasone treatment. Specificity of the anti-histone H1.2 antibody was determined in a blocking experiment (Figure 1A). In CLL cells, genotoxic treatment (fludarabine, mitoxantrone, FM, etoposide, or ionizing radiation) decreased cell viability and, as previously described, induced p53 stabilization. The presence of histone H1.2 was detected in the cytosol 6 hrs. after treatment with levels progressively increasing over 24 hrs. (Figure 1B). It is of note that the presence of histone H1.2 in the cytosol preceded the loss of cell viability and the cleavage of PARP protein.

CLL cells were treated with dexamethasone, an agent that does not cause direct DNA damage and exerts its proapoptotic effect in a p53-independent manner. The time-course pattern for nuclear H1.2 release into the cytoplasm was similar to that of CLL cells treated with genotoxic agents (Figure 1C). Treatment with dexamethasone did not stabilize p53. These results indicated that histone H1.2 can be released independently of DNA damage.

Histone H1.2 release was also observed after *ex-vivo* spontaneous programmed cell death, a process described in CLL cells as p53-independent[13] involving Bax and Bak conformational changes.[20] Twelve out of 16 CLL cases without poor-risk genetic abnormalities showed a slight release of histone H1.2 after 24 hrs. of culture, accompanied by PARP cleavage and lack of p53 stabilization. Cases releasing H1.2 spontaneously experienced higher loss of cell viability than cases without release of H1.2 (mean 77% *vs.* 84%; $p<0.05$). Spontaneous histone H1.2 release and subsequent apoptosis were prevented by the addition of IL-4 into the cell culture media in 3 cases (Figure 1D).

To examine whether histone H1.2 release occurred before caspase activation, some CLL cells were incubated with 200 μM Z-VAD.fmk prior to treatment with FM, DXM and 25 Gy (Figure 2). At 24 hrs. the effective inhibi-

Table 1. Patients' clinical and biologic characteristics.

	Gender	Age	Rai's stage at diagnosis	Response to treatment*	Survival (years)	ZAP-70 expression	FISH Findings	H1.2 Release	
								Genotoxics	DXM
CLL-3	F	75	2	PR	6.6+	High	normal	Yes	Yes
CLL-6	F	60	0	PR	7.8+	High	normal	Yes	Yes
CLL-13	M	74	2	PR	3.5	High	normal	Yes	ND
CLL-14	F	52	1	PR	3.2	High	normal	Yes	Yes
CLL-16	M	58	0	CR	10.5+	High	normal	Yes	Yes
CLL-10	F	74	0	F	10	Low	normal	Yes	Yes
CLL-25	M	56	0	CR	3.5+	Low	normal	Yes	Yes
CLL-12	M	47	1	PR	9.5	High	13q34: 6%	Yes	ND
CLL-4	M	73	0	–	2.5+	Low	13q34: 20%	Yes	Yes
CLL-1	F	41	0	PR	20	High	13q34: 86%	Yes	Yes
CLL-15	M	61	1	F	9.2	High	13q34: 96%	Yes	Yes
CLL-17	F	41	1	CR	7.8	High	13q34: 95%	Yes	ND
CLL-20	M	72	0	PR	13.2+	Low	13q34: 67%	Yes	Yes
CLL-23	M	45	4	PR	24	Low	13q34: 84%	Yes	Yes
CLL-24	M	62	0	CR	7.8+	Low	13q34: 91%	Yes	Yes
CLL-7	M	52	0	PR	9	High	13q34: 20%	Yes	Yes
CLL-7 bis*	–	–	–	–	–	High	17p13: 90%	No	Yes
CLL-2	F	72	2	F	1.0	Low	17p13: 98%	No	Yes
CLL-5	F	72	4	F	7.7	High	17p13: 78%	No	No
CLL-21	F	55	0	F	7.3	High	17p13: 95.9%	No	Yes
CLL-9	M	30	1	CR	5.6	High	17p13: 6%	Yes	ND
CLL-11	F	58	2	PR	26	High	17p13: 20% and 13q34: 96%	Yes	ND
CLL-22	M	57	0	CR	6.7+	High	11q22: 90%	Yes	Yes
CLL-8	F	66	0	CR	8.4+	High	11q22: 91% and CEP12:13%	Yes	Yes
CLL-18	M	53	2	CR	3.2+	Low	11q22: 87% and 13q34: 70%	Yes	Yes
CLL-19	F	53	1	PR	7.5	High	11q22: 87% and CEP12: 65%	Yes	ND

**Best treatment reponse obtained with genotoxic therapies: chlorambucyl (5), 2 -cdA (2), fludarabine (2), FCM (13), CHOP (2); (+): alive. *CLL-7bis corresponds to the case CLL-7 after acquisition of 17p13 deletion.*

tion of caspases resulted in a reduced loss of cell viability and the absence of PARP cleavage. The presence of histone H1.2 release in all conditions could be observed. These results indicate that the release of histone H1.2 is an early event in the apoptotic pathway occurring upstream of the caspase activation.

Histone H1.2 release into the cytosol in CLL cells was also analyzed by immunofluorescence using an anti-histone H1 antibody, since the anti-H1.2 antibody did not work for immunofluorescence staining. In untreated CLL cells, histone H1 staining was observed in the nucleus. Genotoxic and dexamethasone treatment, as previously observed by Western blot, modified the intracellular distribution of histone H1, with this protein appearing in the cytosol with a diffuse fine staining (Figure 3).

Histone H1.2 release in CLL cases with del(17p13)

Tumoral cells from 4 patients with refractory CLL and deletion of 17p13 in 78%, 90%, 96% and 98% of the nuclei respectively were studied for H1.2 release (Table 1). In highly deleted cases, *p53* gene dysfunction was demonstrated by the lack of p53 protein expression after irradiation in 3 cases and by a constitutive p53 protein expression in the remaining 1 (CLL-21 and CLL-5 respectively in Figure 4B). Deficient p53 function was further confirmed by the lack of p21 expression after irradiation. Two additional cases of CLL with a low percentage of del(17p) (20% and 6%) were analyzed. These cases also exhibited a constitutive stabilization of p53 protein (CLL-11 and CLL-9 in Figure 4B) but, in contrast to cases with a high percentage of del(17p), p21 induction was observed after X-ray treatment.

In highly deleted CLL samples, mean cell viabilities referred to controls at 24 hrs. were 66% (SD 12%) with the FM combination, 77% (SD 7%) with etoposide, 48% (SD 20%) in irradiated cells, and 42% (SD 18%) with dexamethasone. After these treatments there was no release of histone H1.2 into the cytosol. By contrast, dexamethasone induced apoptosis in 17p deleted cases at similar levels to the wild-type cases (Figure 4A). Moreover, dexamethasone in del(17p) induced release of H1.2 into the cytosol (Figure 4C), indicating that histone H1.2 release was independent of p53 function when non-DNA damaging agents were used. Only 1 case did not present H1.2 release despite showing a reduced cell viability and PARP cleavage after DXM treatment. This patient failed to treatment with chlorambucil and prednisone. The 2 patients with a low percentage of del(17p) showed a pattern of histone H1.2 release similar to that observed in cases without 17p chromosomal abnormalities (data not shown), indicating that p53 function should be completely abrogated to avoid histone H1.2 release. Finally, spontaneous histone H1.2 release was observed in cases with a low percentage of del(17p), whereas spontaneous H1.2 release was not observed in highly deleted cases at 24 hrs.

The dependence of histone H1.2 release on p53 status after DNA damage was further confirmed by the sequential analysis of a patient who acquired 80% of del(17p) during the course of the disease. At diagnosis, histone H1.2 was released into the cytoplasm after X-ray exposure or

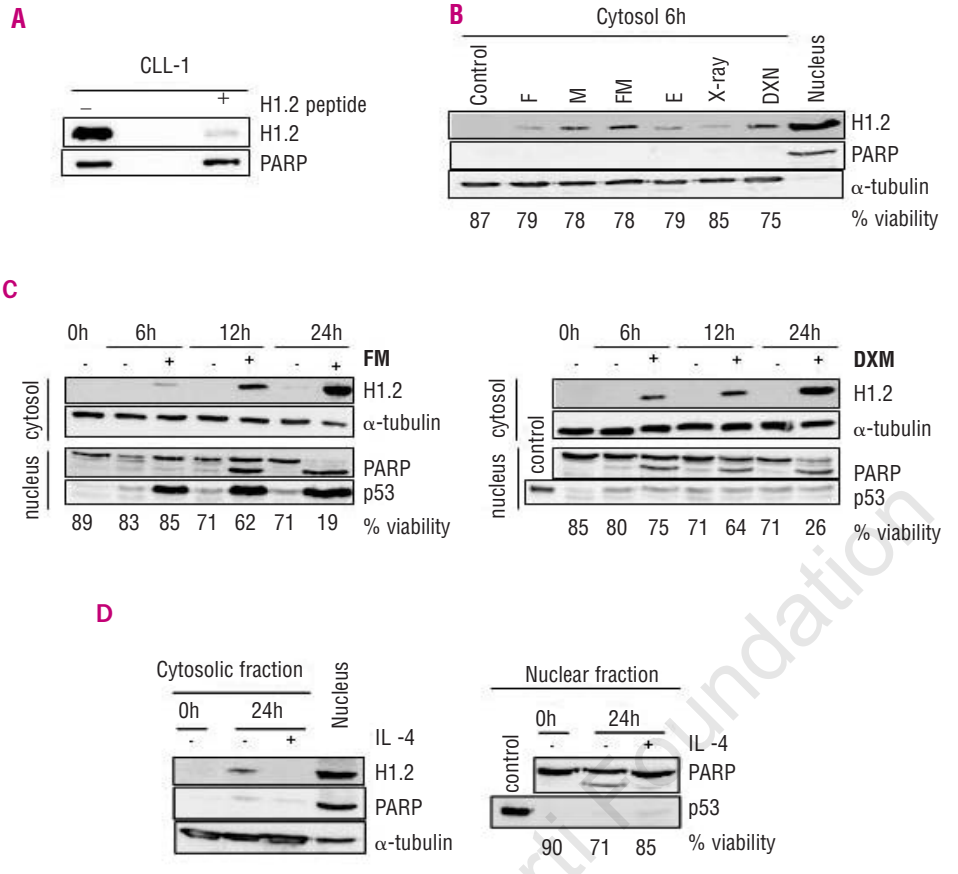

Figure 1. Cytosolic histone H1.2 release in CLL cells without genetic abnormalities. (A) *Blocking assay of histone H1.2* Nuclear extracts from a CLL case (CLL-1) were used to perform a blocking assay with a histone H1.2 synthetic peptide, showing that the anti-H1.2 antibody was specific against this protein. PARP nuclear staining was used as loading control. (B) *Histone H1.2 translocation in a representative case of CLL without poor risk genetic abnormalities*. H1.2 was detected in the cytosol after genotoxic and non-genotoxic treatment. PARP staining in the cytosol was used to exclude nuclear contamination, and α-tubulin as a loading control. A nuclear fraction from the same case was used for PARP and H1.2 control staining. (C) *Time course of histone H1.2 release after treatment with FM and dexamethasone*. Histone H1.2 was detected in the cytosol at 6 hours with FM and dexamethasone treatment, preceding PARP cleavage and loss of cell viability. H1.2 cytosolic release was increased over time reaching the highest level at last observation at 24 hrs. In contrast to FM, dexamethasone treatment released H1.2 without p53 stabilization. These results suggest that histone H1.2 release probably induces cell death in a caspase-dependent mechanism. (D) *H1.2 release induced by spontaneous programmed cell death*. A slight histone H1.2 release was observed in CLL cells after 24 hours in culture, with this phenomena being prevented by the addition of IL-4. PARP staining of the cytosol was used to exclude nuclear contamination, and a nuclear extract was used for a staining control of antibodies. Spontaneous apoptosis induced nuclear PARP cleavage but did not stabilize p53. F: fludarabine; M: mitoxantrone, E: etoposide, DXM: dexamethasone.

dexamethasone treatment, whereas after the acquisition of *p53 gene* deletion, only treatment with dexamethasone was able to induce H1.2 release (Figure 4D).

Histone H1.2 release in CLL cases with del(11q22)

The role of ATM activation after DNA damage in histone H1.2 release has not been established. CLL cells from 4 cases with monoallelic del(11q22), ranging from 87% to 91%, were analyzed for histone H1.2 translocation. To determine the functional status of the *atm* gene, ATM protein expression and phosphorylation at residue S1981 were assessed 1 hr. after X-ray exposure. All the samples with del (11q22) (cases CLL-22, CCL-8, CCL-18, and CCL-19) showed low amounts of ATM protein expression, whereas phosphorylation at residue S1981 was absent, thus confirming an impaired ATM function (shown in Figure 5A).

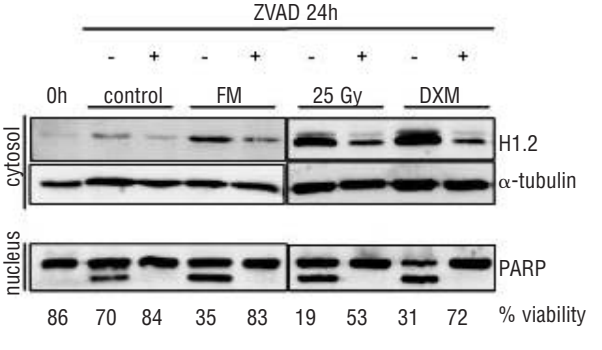

Figure 2. H1.2 release precedes caspases activation. CLL cells from a representative case were preincubated with or without 200 μM Z-VAD.fmk and FM, 25 Gy or DXM were applied. Cytosolic and nuclear fractions at 24 hrs. are shown along with cell viability obtained after annexin V and PI staining. Z-VAD.fmk treatment abrogated PARP cleavage and loss of cell viability whereas histone H1.2 release was present even in untreated cells.

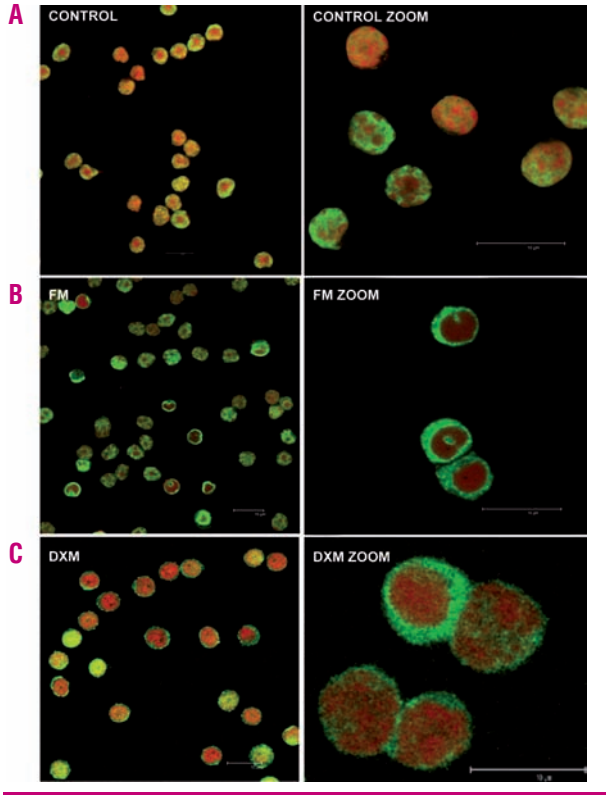

Figure 3. Immunofluorescence analysis of H1 release in the cytosol. (A) Control CLL cells at 12 hrs. Histone H1 had a patched nuclear distribution colocalized with iodur propidium staining; (B) and (C) CLL cells at 12 hrs. after FM and DXM treatment. Histone H1 presented a peri-nuclear distribution and an apparent cytosolic staining.

Clinically, all the cases with del(11q22) responded to treatment (Table 1).

In cases with del(11q22), cell viability at 24 hrs. after treatment was similar to that obtained in cases without poor-risk genetic abnormalities: 31% (SD 12%) with FM, 36% (SD 17%) with etoposide, 32% (SD 13%) with 25 Gy, and 53% (SD 9%) with dexamethasone treatment (Figure 4A). In contrast to p53 deleted cases, CLL cells with impaired ATM function had histone H1.2 shuttle from the nucleus to the cytoplasm after genotoxic treatment. Moreover, dexamethasone treatment also results in the release of histone H1.2 into the cytoplasm at levels similar to genotoxic treatment (Figure 5B). In addition, spontaneous H1.2 release was observed. This is similar to wild-type patients and is in contrast to highly deleted 17p cases. Together, these results suggest that histone H1.2 release after treatment with DNA-damaging agents or dexamethasone is not dependent on the ATM protein function.

Discussion

It has recently been demonstrated that histone H1.2 plays an important role in transmitting apoptotic signals from the nucleus to the mitochondria following DNA double-strand breaks. Following this, we showed that in primary CLL cells nuclear H1.2 was released to the cytoplasm after different treatment modalities. To our knowledge, apart from the recent description of this apoptotic mechanism in thymocytes as well as in different tumoral cell lines (MEFs, MCF7 and HCT 116),[18] this is the first evidence of nuclear H1.2 translocation in primary tumoral cells.

In accordance with the results obtained by Konishi *et al.*[18], we observed histone H1.2 release after etoposide and X-ray irradiation, two treatments that induce DNA DSBs and p53 activation. In addition, although it has been hypothesized that only DNA DSBs are able to induce histone H1.2 release,[18] we observed histone H1.2 release after fludarabine and mitoxantrone, two agents that induced direct DNA damage by single or double-strand breaks. The observation that histone H1.2 release preceded PARP cleavage suggested that histone H1.2 induced a caspase-dependent mechanism of cell death.[18] In addition, the presence of histone H1.2 after caspase inhibition by Z-VAD.fmk confirmed that this release occurs upstream to caspase activation.

The dependence of H1.2 release on p53 status after genotoxic treatment was further confirmed in CLL cases with a high percentage of p53 deletion in which treatment with drugs that induce DNA damage did not cause loss of cell viability and H1.2 release. These results identified histone H1.2 as another important mediator of p53-induced apoptosis, probably acting at the same level as BH3-only proteins in inducing cytochrome c release.[21] In fact, there is experimental evidence suggesting the presence of other elements different from Puma or Noxa that have redundant functions.[21,22]

From a clinical standpoint, 17p deleted cases are usually refractory to conventional therapies and show a very poor survival.[7] It is noteworthy that, in CLL, response to treatment depends on the degree of p53 deletion. This has been demonstrated in clinical trials where patients with a less than 20% cell deletion showed a better therapeutic response than highly deleted cases.[23] Given this, cases from our series with a low degree of p53 deletion displayed a loss of cell viability and a histone H1.2 release pattern similar to that observed in non-deleted cases. These results underline the need for complete p53 dysfunction to abrogate H1.2 release with important implications for treatment.

Another observation was that treatment of CLL cells by two p53-independent inducers of apoptosis[13,22,24] also provoked histone H1.2 release from nucleus to cytoplasm. Importantly, histone H1.2 release and cytotoxicity after dexamethasone treatment were similar in both p53 deleted and non-deleted cases. It is well known that dexamethasone induces apoptosis independently of p53,[25-28] although the ultimate apoptotic mechanism of this drug is not completely known. Our results emphasize that histone H1.2 could be an essential apoptotic signal induced by agents that act independently of p53.

DNA damage mobilizes an intricate signaling network by activating the ATM protein kinase which is responsible for p53 activation. Lymphoproliferative disorders that have acquired ATM mutations usually have a poor clinical out-

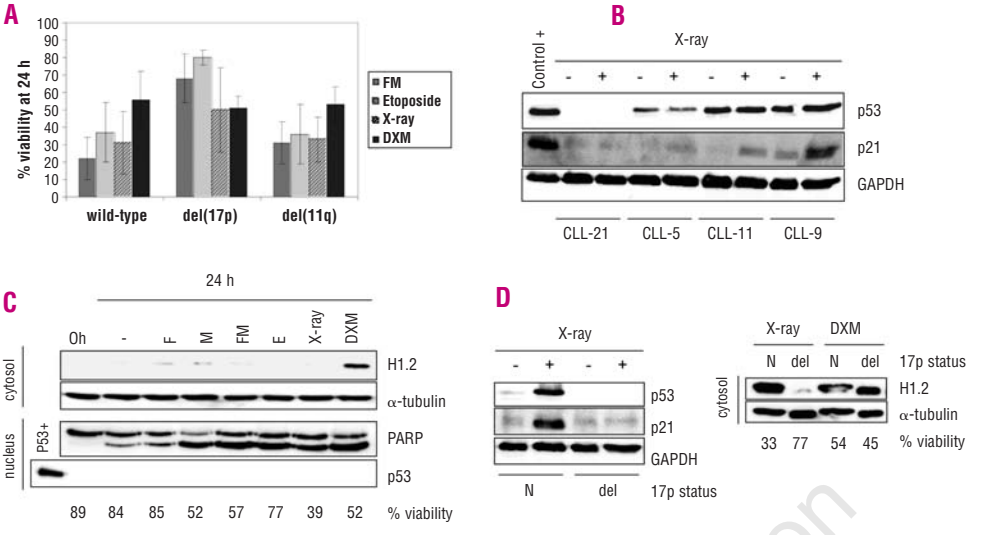

Figure 4. Pattern of histone H1.2 release in p53 deleted CLL cases. (A) *Cell viability according to genetic abnormalities referred to the control at 24 hours*. Cell viability (mean in bars, standard deviation in lines) after different treatments in cases without 17p or 11q abnormalities ("wild-type"), del17p13, or del11q22. Genotoxic treatment induced less cytotoxic effect in del17p cases than wild-type or del11q cases, whereas dexamethasone had a similar effect in all three groups. (B) *p53 stabilization and p21 upregulation*. Highly deleted cases (CLL-21 and CLL-5) and cases with low 17p deletion (CLL-11 and 9) were analyzed after X-ray exposure. Whilst absence or abnormal p53 stabilization were observed in all cases, p21 protein was upregulated in cases with low 17p deletion. (C) *Histone H1.2 release in a highly 17p deleted case (CLL-2) at 24 hours*. Only treatment with dexamethasone induced H1.2 release. In contrast, and in spite of PARP cleavage and a cytotoxic effect, genotoxic treatment did not induce H1.2 release. (D) *Sequential analysis of a case (CLL-7) acquiring a 17p deletion*. At diagnosis, X-ray treatment induced a normal p53/p21 function at diagnosis, which disappeared after del 17p acquisition. At diagnosis, X-ray treatment induced histone H1.2 release, which become absent after p53 impairment. Importantly, H1.2 release induced by DXM was not affected by the acquisition of a 17p deletion. Abbreviations: F, fludarabine; M, mitoxantrone; E, etoposide; N, normal; del, deleted.

come.[7,29,30] These cases, however, have a less aggressive clinical course and retain a capacity for apoptosis after DNA damage compared with cases with p53 dysfunction.[13] The relationship between ATM and histone H1.2 traffic has not yet been addressed. Here, we analyzed histone H1.2 release in cases with del(11q22) and abnormal ATM function. Our results disclosed a similar pattern of H1.2 release and loss of cell viability in both deleted and non-deleted ATM cases, suggesting that this apoptotic signal is independent of ATM functionality. These results are in agreement with the fact that, although the ATM and p53 pathways overlap, in some CLL cases they are not congruent,[31] showing that it is still unclear why ATM- and p53-deficient CLL cases respond differently to DNA damage. It is also possible that other members of the DNA-damage protein response family PIKK, such as ATR, may be involved in this apoptotic response. However, the ATR protein apparently does not play an important role in CLL cells[32] and it has recently been proved that its recruitment is regulated by ATM.[33]

A number of other findings in our study deserve comment, in particular, regarding the observation that p53 deleted cases experienced some degree of loss of viability but no release of histone H1.2 after DNA damage. These results support the concept that histone release into the cytoplasm was not merely a consequence of DNA fragmentation, and agree with the fact that apoptosis could be detected in apoptotic cells that did not present DNA frag-

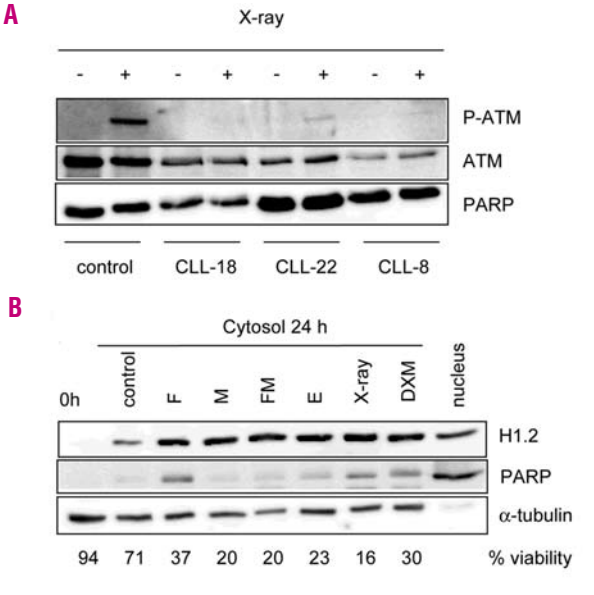

Figure 5. Pattern of histone H1.2 cytosolic release in ATM deleted cases. (A) ATM function was impaired in cases with del(11q22) as shown by the low ATM protein expression and lack of significant ATM phosphorylation in S1981 residue after 1hr. radiation (cases CLL-22, CLL-18, CLL-8 are shown). (B) Histone H1.2 release in a representative case with del11q (CLL-18). Histone H1.2 was released into the cytosol after genotoxic and non-genotoxic treatment. PARP staining was used to quantify nuclear contamination. α-tubulin was used as a loading control. F: fludarabine; M: mitoxantrone; E: etoposide; DXM: dexamethasone.

mentation.[17]

To summarize, histone H1.2 release into the cytoplasm in CLL cells could represent a novel and common step in apoptosis induced by different stimuli. Importantly, this new apoptotic effector is abrogated when using DNA damaging agents in highly deleted 17p13 cases. This helps to explain resistance mechanisms in CLL. Finally, p53-independent apoptotic stimuli were able to induce histone H1.2 release in CLL cells. These findings should be taken into consideration when designing new therapeutical strategies aimed at overcoming p53-related drug resistance.

Authorship and Disclosures

FB was the principal investigator for the article, responsible for the conception and design of the study. EG contributed to the design and performed the laboratory assays. FB and EG wrote the paper. MC contributed to the interpretation of results and in drafting the article. AM collected clinical data and contributed to laboratory assays. EC and MB also contributed to laboratory assays and interpretation of results. EM contributed to the revision and scientific review of the manuscript. The authors reported no potential conflicts of interest.

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