ARTEMIS Nuclease Facilitates Apoptotic Chromatin Cleavage

Sébastien Britton, Philippe Frit, Denis Biard, Bernard Salles, and Patrick Calsou

Abstract

One hallmark of apoptosis is DNA degradation that first appears as high molecular weight fragments followed by extensive internucleosomal fragmentation. During apoptosis, the DNA-dependent protein kinase (DNA-PK) is activated. DNA-PK is involved in the repair of DNA double-strand breaks (DSB) and its catalytic subunit is associated with the nuclease ARTEMIS. Here, we report that, on initiation of apoptosis in human cells by agents causing DNA DSB or by staurosporine or other agents, ARTEMIS binds to apoptotic chromatin together with DNA-PK and other DSB repair proteins. ARTEMIS recruitment to chromatin showed a time and dose dependency. It required DNA-PK protein kinase activity and was blocked by antagonizing the onset of apoptosis with a pan-caspase inhibitor or on overexpression of the antiapoptotic BCL2 protein. In the absence of ARTEMIS, no defect in caspase-3, poly(ADP-ribose) polymerase-1, and XRCC4 cleavage or in H2AX phosphorylation was observed and DNA-PK catalytic subunit was still phosphorylated on S2056 in response to staurosporine. However, DNA fragmentation, including high molecular weight fragmentation was delayed in ARTEMIS-deficient cells compared with cells expressing ARTEMIS. In addition, ARTEMIS enhanced the kinetics of apoptotic nuclease (25); this sequence maps to a S/MAR, which is sufficient to direct apoptotic cleavage when present in an artificially constructed DNA fragment. ARTEMIS facilitates the recruitment to chromatin of endonucleases including high molecular weight DNA (HMW) DNA (4) and DNA-PK catalytic subunit was still phosphorylated on S2056 in response to staurosporine. However, DNA fragmentation, including high molecular weight fragmentation was delayed in ARTEMIS-deficient cells compared with cells expressing ARTEMIS. In addition, ARTEMIS enhanced the kinetics of MLL gene cleavage at a breakage cluster breakpoint that is frequently translocated in acute or therapy-related leukemias. These results show a facilitating role for ARTEMIS at least in early, site-specific chromosome breakage during apoptosis.

Introduction

Apoptosis is one of the pathways of programmed cell death (1, 2). During this process, a family of cysteine proteases called caspases is activated that specifically degrades a large number of proteins leading to cell destruction (3). In addition, DNA is cut by a two-step process in which it is first cleaved into 50- to 300-kb fragments, termed high molecular weight (HMW) DNA (4) and then into fragments of low molecular weight. Although caspase-activated DNase activity is a key player in DNA fragmentation, more than 20 other nucleases can operate depending on the organism or tissue (5).

Because apoptosis produces DNA double-strand breaks (DSB), it is not surprising to find connections between this process and cellular pathways that repair damaged DNA. DNA-dependent protein kinase (DNA-PK) works in nonhomologous end joining (NHEJ), a major pathway for the repair of DSB. The DNA-PK complex includes the ring-shaped heterodimer KU, composed of KU70 and KU80, which binds the ends of the DNA break and then recruits and activates the serine/threonine kinase activity of the catalytic subunit DNA-PKcs, a phosphoinositide 3-kinase–related kinase (6, 7). A key step in the NHEJ reaction is DNA-PKcs auto-phosphorylation (8, 9).

One of DNA-PK functions is to regulate DNA end access to processing enzymes including the ARTEMIS nuclease. ARTEMIS was discovered as mutated gene in a group of patients associating radiosensitivity and severe immunodeficiency (10). It was subsequently characterized as a 5’-3’ exonuclease that interacts with DNA-PKcs. On phosphorylation and/or direct interaction with autophosphorylated DNA-PKcs, ARTEMIS acquires an endonuclease activity that acts on hairpin loops (11, 12). This endonuclease activity is responsible for opening hairpin-sealed coding ends during V(D)J recombination (12) and trimming of complex DNA ends caused by ionizing radiation or elastogenic drugs (13).

During apoptosis, DNA-PK is responsible for the phosphorylation of the histone variant H2AX concurrently with the initiation of DNA fragmentation. DNA-PKcs autoprophosphorylation arises early during apoptosis (14) before its caspase-3–dependent cleavage at later stages (15, 16). In addition, DNA-PKcs in complex with the PIDD protein was recently found to phosphorylate the nuclear caspase-2, a necessary step for caspase-2 cleavage and downstream regulation of the G2-M DNA damage checkpoint (17). Finally, in DT40 cells, KU, DNA-PKcs, or ARTEMIS deficiency impairs cleavage of caspases substrates and DNA fragmentation following treatment with a high dose of the DNA topoisomerase II inhibitor etoposide (18).

Several links have been established between apoptotic DNA fragmentation and chromosome translocations with leukemogenic potential. Recent studies have identified cellular intermediates and reversible apoptotic states with potential genomic instability (19); as a result, propagation of some chromosome translocations, such as those involving the MLL gene in acute and therapy-related leukemias, may rely on recovery from initial apoptotic DNA fragmentation induced by genotoxic or nongenotoxic stimuli (20–22). In addition, it has been proposed that HMW DNA fragmentation corresponds to a nucleolytic cleavage at scaffold/matrix attachment regions in chromosomes (23). Scaffold/matrix attachment regions are typically AT-rich DNA regions located at chromosomal bases of chromatin loops (24). Strikingly, the MLL gene contains a site susceptible to cleavage by various agents, including apoptotic nuclease (25); this sequence maps to a S/MAR, which is sufficient to direct apoptotic cleavage when present in an epissome (26). The cleavage at this site has been shown to occur concurrently with the HMW DNA fragmentation (20) and DNA-PKcs was found associated with the site of MLL apoptotic cleavage (27).

These various results support the idea that DNA-PK may direct ARTEMIS activity on the DNA ends generated during apoptotic DNA degradation.
DNA fragmentation. Here, we investigate this hypothesis and show that, by means of its relationship with DNA-PK, ARTEMIS plays a role in the early, site-specific chromosome breakage of apoptosis.

Materials and Methods

Chemicals and antibodies. Calicheamicin–γ1 was a gift from P.R. Hamann (Wyeth Research). Stauroporine (Sigma-Aldrich), NU7026, γ-VAD-fmk, and MG-132 (Calbiochem) were dissolved in DMSO. The antibodies used were mouse monoclonal anti-tag MYC (9E10; Clontech), anti-DNA-PKcs (18-2) and anti-UKU80 (S10B1; Neomarkers), anti-β-actin (AC-15; Ambion), anti-poly(ADP-ribose) polymerase-1 (PARP-1; 4C10-5; BD Pharmingen), anti-β-H2AX (JBW301; Upstate Biotechnology), anti-ATM (2C1; Genetex), anti-α-H2 (1H2; 2G2; gift of R. Losson, Institut de Génétique et de Biologie Moléculaire et Cellulaire), rabbit monoclonal anti–casepase-3 (8G10; Cell Signaling Technology), rabbit polyclonal anti-XRCC4 (produced at the University of Ulm), rabbit monoclonal anti–caspase-3 (8G10; Cell Signaling Technology), rabbit polyclonal anti–XRRCC4 (produced at the laboratory), anti–DNA ligase IV (Soretoc), anti–phospho-S2056-DNA-PKcs (gift of J. Chen, University of Texas Southwestern Medical Center), and goat anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (Jackson Immunoresearch Laboratories).

Plasmids. pcDNA1.1-MYC-HIS and pcDNA1.1-ARTEMIS-MYC-HIS were provided by J.P. de Villartay (INSERM). The pB650 and pBD743 vectors, coding for control short hairpin RNA and short hairpin RNA against DNA-PKcs, respectively, have been described previously (28). DNA sequences of all plasmids were verified (MilleGen).

Cell culture. Cells were grown in a 5% CO2 humidified incubator at 37°C. All culture media and antibiotics were from Invitrogen. Medium was supplemented with 10% FCS, 125 units/ml penicillin, and 125 µg/ml streptomycin. ARTEMIS-deficient and ARTEMIS-MYC-complemented fibroblasts (immortalized by SV40 large T antigen and telomerase), Guetel and Guetel-A, respectively (provided by J.P. de Villartay), were grown in RPMI 1640 Glutamax I MRC5-SV clones that stably express short hairpin RNA against DNA-PKcs (PK12) or control short hairpin RNA (C2) were grown in DMEM supplemented with 0.1 mg/ml hygromycin B; they correspond to clones stably carrying the episomal vector pB6D50 (C2) or pBD743 (PK12) as described in ref. 28. All experiments were done with nonconfluent, exponentially growing cells at low passages.

Transfections. Transfections of MRC5-SV clones by plasmid DNA were done with the JetPEI reagent (Polyplus-Transfection) according to the manufacturer's instructions. In each experiment, cells were treated 24 h after transfection.

Recruitment analysis by biochemical fractionation. To isolate nuclear soluble or chromatin subfractions from Guetel or MRC5-SV cells, a detergent-based extraction procedure was used as reported (29). For whole-cell extracts, the cells were resuspended in 200 µl lysis buffer as reported (29), boiled, cooled down, and sonicated on ice. Protein concentration was determined by the Bradford assay (Bio-Rad).

Immunoblotting. Aliquots of soluble or chromatin fractions, derived from equivalent cell numbers, or whole-cell extract samples with the same amount of proteins were added to loading buffer, boiled, and loaded on SDS-PAGE. After migration, proteins were transferred to nitrocellulose membranes (Millipore). Immunoblots were visualized by enhanced chemiluminescence (Yelen).

Oligonucleosomal DNA fragmentation analysis. Cells (2.5 × 10⁴) were used per point. After treatment, cells were scraped, centrifuged at 1,500 rpm for 10 min at 4°C, washed with cold PBS, and centrifuged at 4,500 rpm for 5 min at 4°C. Pellets were resuspended in 250 µl of 10 mmol/L Tris-HCl (pH 8)-1 mmol/L EDTA (TE), added to 250 µl lysis buffer [5 mmol/L Tris-HCl (pH 8), 20 mmol/L EDTA, 0.5% Triton X-100], vortexed, and incubated 30 min at 4°C. Then, unfragmented DNA was removed by centrifugation at 15,000 × g for 15 min at 4°C. DNA was precipitated by ethanol/NaCl. Pellet was resuspended and incubated for 30 min at 37°C in 500 µl TE buffer supplemented with 0.5 mg/ml RNase A. Proteins were removed by adding 500 µl biophenol/chloroform/soyamin alcohol mix (25:2:4; I; Biosolve) followed by vortexing and centrifuging 5,000 × g for 3 min at 4°C. DNA was precipitated by ethanol/NaCl. After drying, the pellet was resuspended in TE buffer with 0.5% SDS. Samples were separated on TBE 1 × 1.8% agarose gel. After staining with SYBR Gold (Invitrogen), gels were visualized using a Typhoon Trio (GE Healthcare) with excitation and emission filter at 488 and 526 nm, respectively.

HMW DNA fragmentation analysis by pulse-field gel electrophoresis. Cells (10⁶) were scraped on ice, centrifuged at 1,500 rpm for 10 min at 4°C, washed in PBS, and centrifuged at 2,000 × g for 3 min at 4°C. Pellet was resuspended in 100 µl agarose buffer [150 mmol/L NaCl, 2 mmol/L KH₂PO₄/KOH (pH 6.8), 1 mmol/L EDTA, 5 mmol/L MgCl₂], mixed with 100 µl molten (60°C) 1% low melting agarose (Bio-Rad) in agarose buffer, and cast in a precooled (4°C) insert mold. The resulting plugs were placed in 24-well plates, washed in proteinase buffer [10 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 9.5), 25 mmol/L EDTA, 1% N-lauroylsarcosine (Sigma-Aldrich)], and incubated for 48 h at 25°C under gentle shaking in buffer supplemented with 0.2 mg/ml protease K. Plugs were washed three times in 1 ml TE buffer at 4°C under gentle shaking and stored in 50 ml sodium/ EDTA (pH 8) at 4°C until loading on a TBE 0.5 × 1% agarose gel. Gels were run in a pulse-field gel electrophoresis system (CHEF-DR III; Bio-Rad) for 20 h at 6 V/cm with 120° switch angle and 50 and 90 s for initial and final switch times, respectively. Gels were stained with SYBR Gold (Invitrogen) and visualized on a UV transilluminator (Gel Doc XR; Bio-Rad). Acquisition was done with the QuantityOne software (Bio-Rad) and the fraction of DNA released was calculated with ImageJ (NIH); the fraction of HMW DNA fragments corresponds to the intensity of the DNA signal in the 50 to 300 kb range over the total signal.

MILLBrcr cleavage analysis by Southern blot. For each point, 2 × 10⁸ cells were scraped, centrifuged at 1,500 rpm for 10 min at 4°C, and washed in PBS. For genomic DNA purification, the Wizard Genomic DNA Purification kit (Promega) was used according to the manufacturer's instructions. Twenty micrograms of the DNA were digested for 5 h at 37°C by 80 units EcoRI and 10 µg were loaded on a large TAE 1 × 1% agarose gel cast with 0.5 µg/ml ethidium bromide. Gels were run at 100 V and the homogeneity of DNA between each condition was checked with UV transilluminator. DNA was denatured by two incubations of gels for 20 min in 0.5 mol/L NaOH/1.5 mol/L NaCl neutralized by incubation two times for 20 min in 0.5 mol/L Tris-HCl (pH 7.2)/1.5 mol/L NaCl, and transferred overnight in semidynd conditions against 20 × SSC on a nitrocellulose membrane (Genescreen Plus; Perkin-Elmer). The probe corresponds to a 399-bp genomic DNA region of mixed-lineage leukemia breakage cluster region (MLLbcr; location 11q23, 21921379-21921777). This region was cloned in the pUC-SV40-tsVP1(290T)-Cla vector kindly provided by L. Wiesmüller (University of Ulm). A PCR product corresponding to the region of interest was used as matrix for the Megaprime DNA labeling system (GE Healthcare) according to the manufacturer's instructions. For incubation with the probe, membranes were washed for 1 h at 55°C in CHURCH buffer [0.5 mol/L Na₂HPO₄ (pH 7.2), 7% SDS, 1% bovine serum albumin, 1 mmol/L EDTA], incubated overnight at 55°C with the radioactive probe, and washed twice for 30 min at 55°C in 0.2× SSC-0.1% SDS. A phosphorimager cassette was used to recover the signal. The percentage of MLLbcr cleavage was quantified using ImageJ (NIH): the cleavage of the EcoRI fragments carrying the MLLbcr (4,400 bp) releases two bands (1,157 and 3,257 bp) and the smaller carries the probe recognition site. The percent of cleavage of MLLbcr corresponds to the intensity of the 1,157-bp fragment divided by the signal intensity for the two bands.

Results

ARTEMIS is recruited onto damaged chromatin during apoptosis. Guetel-A fibroblasts express a MYC-tagged ARTEMIS construct after stable retroviral transduction of Guetel ARTEMIS-deficient cells (30). Here, apoptosis was induced by cell treatment with staurosporine, a potent inducer in most cell types (31). To assess in situ the recruitment of proteins to apoptotic chromatin, we used a detergent-based cellular fractionation protocol that we have reported previously for the mobilization of NHEJ proteins to DSB in cells (29). Briefly, nuclei of cells treated or not with
staurosporine were extracted with a Triton X-100–containing buffer, the clarified cell extract supernatant (S1) and the insoluble chromatin fraction (P2) were collected, and proteins were detected by immunoblotting. Under these conditions, the whole NHEJ repair machinery is recovered in the P2 fraction after cell treatment with potent DSB inducers such as neocarzinostatin or calicheamicin-γ1 (29, 32, 33). Figure 1A shows the immunoblot analysis following SDS-PAGE of cell-equivalent aliquots of the S1 and P2 fractions after cell treatment with increasing doses of staurosporine or a dose of calicheamicin-γ1 as control. Cell apoptosis can be monitored by PARP-1 cleavage (34) and DSB formation by γ-H2AX accumulation (35). Whereas a dose-dependent PARP-1 cleavage and H2AX phosphorylation occurred after exposure to staurosporine (Fig. 1A), a dose-dependent mobilization of both ARTEMIS and DNA-PK (DNA-PKcs plus KU) to the chromatin fraction was also observed. In a time-course experiment (Fig. 1B), γ-H2AX signal was detected between 2 and 3 h, concomitantly to ARTEMIS, KU, and DNA-PKcs proteins retention in the chromatin fraction and in close synchrony with the appearance of PARP-1 fragments in the S1 fraction. After 4 h, ARTEMIS still accumulated on the apoptotic chromatin together with KU80, DNA-PKcs, and γ-H2AX up to the 8 h time point. DNA-PKcs and the XRCC4 protein belonging to the ligation complex in NHEJ are targets for apoptotic proteases (15, 16, 36). Accordingly, fragments of both proteins were detectable after 3 to 4 h; interestingly, DNA-PK recruitment to chromatin occurs before its cleavage. However, no cleavage of ARTEMIS protein was observed during apoptosis. Although endogenous ARTEMIS was difficult to detect in most of the cell types analyzed, we could observe loading onto chromatin of the native ARTEMIS protein together with DNA-PK during staurosporine-induced apoptosis in the human osteosarcoma U2OS cell line (Fig. 1C). This shows that this phenomenon is not restricted to the recombinant ARTEMIS form expressed in Guetel-A cells.

As published previously (14), a dose- and time-dependent phosphorylation of DNA-PKcs was found on the S2056 autophosphorylation site (Fig. 1A and B). The ARTEMIS fraction retained on the

![Figure 1](image_url)

**Figure 1.** ARTEMIS mobilization on chromatin during staurosporine-induced apoptosis. Western blot analysis of subcellular protein fractions (W, whole-cell extract; P2, chromatin fraction; S1, detergent-soluble fraction). A, Guetel-A cells were treated with the indicated dose of staurosporine (Stauro) for 3 h or, as a control, with the indicated dose of calicheamicin-γ1 (Cali) during 1 h. B, Guetel-A cells were treated with 0.5 μmol/L staurosporine for the indicated time. C, U2OS cells were treated with 0.5 μmol/L staurosporine for 4 h. As a control of anti-ARTEMIS antibody specificty, whole cell extracts from ARTEMIS deficient (G−) or ARTEMIS complemented (G+) Guetel cells were loaded on the same gel. D, Guetel-A cells were treated with 10 nmol/L calicheamicin-γ1 during 1 h. Cells were then postincubated in fresh medium for the indicated time.
Figure 2. DNA and caspase dependency of ARTEMIS mobilization in chromatin during apoptosis. Western blot analysis of subcellular protein fractions (P1 and P2, successive extraction-resistant fractions; P3, DNase-resistant fraction). A, Guetel-A cells were treated with 1 μM staurosporine for 6 h. B, Guetel-A cells were treated with 1 μM staurosporine for 6 h. When indicated, the cells were pretreated for 1 h with 50 μM z-VAD-fmk before staurosporine treatment.

Figure 3. Role of DNA-PKcs in ARTEMIS recruitment to chromatin during apoptosis. Western blot analysis of subcellular protein fractions. A, MRC5-SV cells stably expressing control or anti–DNA-PKcs short hairpin RNA were transfected with ARTEMIS expressing or empty vectors and treated with 1 μM staurosporine for 6 h. B, Guetel-A cells were treated with 0.5 μM staurosporine for 5 h or with 10 μM/L calicheamicin-γ1 for 1 h with a postincubation of 4 h. When indicated, cells were pretreated with 20 μM/L NU7026 for 1 h before staurosporine treatment.

chromatin in Guetel-A cells was clearly retarded in SDS-PAGE after calicheamicin-γ1 but not staurosporine treatment (Fig. 1A; see also Fig. 3B) for which no slower form was detected even at late time points (Fig. 2B). Under these conditions, this shift of ARTEMIS has been shown elsewhere to correspond to a DNA-PKcs–dependent hyperphosphorylation state (32). This indicates that although ARTEMIS together with DNA-PK is mobilized to chromatin containing genotoxic or apoptotic DSBs, it is not similarly hyperphosphorylated in both cases.

The mobilization of ARTEMIS to cell nuclei in response to staurosporine was also observed by immunocytolocalization after in situ extraction (Supplementary Fig. S1A and B). A similar recruitment of ARTEMIS and DNA-PK was observed with other apoptosis-inducing agents (Supplementary Fig. S2; data not shown). Recently, it has been shown that apoptotic doses of etoposide in DT40 cells induce the loading of DNA-PKcs onto chromatin (18). We found that ARTEMIS was also strongly recruited on apoptotic chromatin at late time points after cells treatment with a high dose of calicheamicin-γ1, leading even to a progressive depletion of ARTEMIS from the soluble protein fraction (Fig. 1D).

Activated apoptotic caspases are required for the recruitment of ARTEMIS and DNA-PK to chromatin during apoptosis. To examine the effect of DNA removal from the detergent-insoluble fraction of apoptotic cells, staurosporine-treated cells were extracted and the insoluble fraction P2 was treated with DNase I, leading to a P3 pellet (Fig. 2A). DNA digestion released KU, DNA-PKcs, and ARTEMIS, whereas the heterochromatin HP-1 protein was still abundant. DNA-PK and ARTEMIS have been shown to bind to chromatin in response to genotoxic DSBs (29, 32) and these results show that they also bind to chromatin concomitantly to apoptotic DSB formation.

One major event in apoptotic DNA fragmentation is the activation of caspase-activated DNase following the caspase-3–dependent cleavage of its inhibitor ICAD (5). When cells were treated by staurosporine in the presence of z-VAD-fmk, a pan-caspase inhibitor (Fig. 2B), PARP-1 and caspase-3 were no longer

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cleaved (top) and neither DNA-PK nor ARTEMIS was recovered in the chromatin fraction after staurosporine treatment (bottom). We also observed a strong reduction in ARTEMIS retention on chromatin following inhibition of apoptosis induced by staurosporine on overexpression of BCL2 in Guetel-A cells compared with control cells (Supplementary Fig. S3). These results imply a role for apoptotic caspases activation in the recruitment of ARTEMIS and DNA-PK to chromatin during apoptosis.

DNA-PKcs is required for ARTEMIS recruitment onto apoptotic chromatin. To determine the involvement of DNA-PKcs in the recruitment of ARTEMIS on apoptotic chromatin, we used previously described MRC5 human cells in which DNA-PKcs gene expression was stably silenced by pEBV RNA vector (28). As shown in Fig. 3A, the MRC5-shPKcs clone exhibited a strong reduction in the expression of DNA-PKcs compared with the control MRC5-Vec clone. Subsequently, both clones were transfected with the MYC-tagged ARTEMIS expressing vector. Forty-eight hours later, clones were treated with staurosporine and the recruitment of ARTEMIS to the insoluble chromatin fraction was assessed. Although ARTEMIS was equally expressed in both cases, it was only recruited to the chromatin fraction after treatment with staurosporine in DNA-PKcs–proficient cells (Fig. 3A). These results indicate that DNA-PKcs protein is necessary for ARTEMIS recruitment to apoptotic DSBs in chromatin. Indeed, DNA-PKcs and ARTEMIS still interact during apoptosis as observed in a coimmunoprecipitation experiment (data not shown).

Then, Guetel-A cells were pretreated or not with the DNA-PKcs inhibitor NU7026 before treatment with staurosporine and analysis of γH2AX protein fraction by Western blot (Fig. 3B). The γH2AX signal decreased when staurosporine and NU7026 were combined. Because DNA-PKcs is primarily responsible for H2AX phosphorylation during apoptosis (14), this result indicates efficient DNA-PKcs inhibition by NU7026. On DNA-PKcs inhibition, ARTEMIS was no longer detectable in the P2 fraction of cells treated with staurosporine, although the mobilization of Ku80 and DNA-PKcs to the apoptotic chromatin was preserved. Thus, the stabilization of ARTEMIS on apoptotic DSBs is dependent on the kinase activity of DNA-PK. Furthermore, apoptosis was induced in Guetel cells expressing a form of ARTEMIS mutated for endonuclease activity although still able to interact with DNA-PKcs (37). Strikingly, the mutant protein was not stably associated with apoptotic chromatin in contrast to DNA-PK (Supplementary Fig. S4). Together, these results suggest that activation of the ARTEMIS endonuclease activity is necessary for its association with broken DNA.

As published previously (32), ARTEMIS was hyperphosphorylated in the P2 fraction and migrated as a retarded form in SDS-PAGE after genotoxic treatment (Fig. 3B). Interestingly, no such shift was noticed in staurosporine-treated cells, indicating that ARTEMIS is not similarly hyperphosphorylated when mobilized on chromatin containing genotoxic or apoptotic DSBs.

ARTEMIS enhances DNA fragmentation during apoptosis. Recently, an ARTEMIS deficiency in DT40 cells was found to impair...
apoptosis induced by etoposide (18). To seek for a potential role of ARTEMIS in apoptosis in human cells, Guetel and Guetel-A fibroblasts, deficient or proficient for ARTEMIS, respectively, were treated with staurosporine and the time course of apoptosis was followed. No defect in PARP-1, caspase-3, and XRCC4 cleavage or in H2AX phosphorylation was observed in Guetel cells. DNA-PKcs, in contrast, was still phosphorylated on S2056 in response to staurosporine in the absence of ARTEMIS (Fig. 4A).

Two nuclease-dependent processes attack chromat in apoptosis: DNA laddering by extensive internucleosomal fragmentation follows an earlier cleavage that generates HMW fragments (4). The time course of internucleosomal DNA fragmentation over a 6 h period after staurosporine treatment was analyzed in Guetel and Guetel-A cells (Fig. 4B). DNA ladders were detected at 2 h post-treatment and increased until 6 h in both lines. However, for the early 2, 3, and 4 h time points, ARTEMIS-proficient cells showed higher mobility DNA ladders in the agarose gel corresponding to a larger amount of low molecular weight DNA species. The kinetics of HMW fragmentation, which precedes DNA laddering, was then analyzed in ARTEMIS-proficient and ARTEMIS-deficient cells. A striking 2 h lag before the onset of HMW DNA fragmentation was observed after staurosporine treatment in Guetel cells compared with their ARTEMIS-proficient counterparts (Fig. 4C and D). This likely indicates that the delay in DNA laddering observed in ARTEMIS-deficient cells was related to a defect in the initial HMW DNA fragmentation step.

**Figure 5.** Apoptotic cleavage of the MLLbcr locus. Cells were treated with 1 μmol/L staurosporine for the indicated time. Genomic DNA was purified, digested by EcoRI, and analyzed by Southern blot with a probe directed against the MLL breakage cluster region (bcr). Mean ± SD of four independent experiments of the percent of the MLLbcr fragment band over the total signal for the MLLbcr probe for Guetel-A cells (squares) or Guetel cells (diamonds). Gray lines, linear regressions for the 0 to 4 h time points (R² = 0.987 and 0.984; slope ± SD: 7.847 ± 0.888 and 11.93 ± 1.510 for Guetel and Guetel-A cells, respectively).

These results support the follow mode for ARTEMIS activity during apoptosis (Supplementary Fig. S5). DNA-PK is early recruited to the DSBs that are initiated in scaffold/matrix attachment regions; a minor fraction of the DNA-PK nuclear pool hardly detectable under our conditions might already be present in these regions, because DNA-PK has been shown to bind to matrix attachment sequences (40). Once bound to DSBs, DNA-PK subsequently phosphorylates H2AX around the apoptotic breaks (14) and activates the endonuclease activity of ARTEMIS; this likely occurs via autophosphorylation (11) as suggested by the detection of S2056 autophosphorylation of full-length DNA-PKcs in apoptotic chromatin (Fig. 1A and B; Supplementary Figs. S6 and S7). In addition, negative torsional stress tends to produce single-strand DNA at scaffold/matrix attachment regions (41), which potentially favors the formation of secondary DNA structures. Therefore, preferred substrates for ARTEMIS endonuclease activity, such as hairpins or stem loops (12), might be abundant in these regions. In this way, the DNA-PKcs-dependent recruitment and activation of ARTEMIS to chromatin could amplify the rate of the region-specific HMW DNA fragmentation. However, our results cannot allow to exclude a role for ARTEMIS in global apoptosis fragmentation or site-specific cleavage.

Some translocations with leukemogenic potential are associated with recovery from initial apoptotic DNA fragmentation (20–22). The fact that ARTEMIS contributes to DNA fragmentation at early stages when apoptosis reversion is still possible suggests that ARTEMIS could play a role in such translocations. The precise molecular mechanism involved in the erroneous resealing of the translocation breakpoints is unknown. Specific chromatin structural elements have been mapped in the breakpoint regions of the genes concerned and many sequence rearrangements have been found at the junctions (38). DNA-PK–dependent NHEJ is less error-prone than previously thought and more error-prone alternative end joining mechanisms have emerged (42, 43). The fact that ARTEMIS favors MLLbcr cleavage would seem to indicate a detrimental protranslocation activity. However, as shown here, NHEJ proteins are recruited early on the apoptotic chromatin and...
performed at late time points (8 h) as both full-length and apoptotic protein fragments. Hence, it is possible that the NHEJ apparatus might counteract the activity of more error-prone alternative joining pathways either via its accurate repair activity or by sterically hindering DNA ends by these proteins. Thus, NHEJ could possibly antagonize the potential occurrence of growth advantageous translocations in some rare revertant apoptotic cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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