Role of Apoptotic Nuclease Caspase-Activated DNase in Etoposide-Induced Treatment-Related Acute Myelogenous Leukemia

Eszter S. Hars, Yi Lisa Lyu, Chao-Po Lin, and Leroy F. Liu

Department of Pharmacology, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey

Abstract

Etoposide-induced treatment-related acute myelogenous leukemia (t-AML) is characterized by rearrangements of the mixed lineage leukemia (MLL) gene with one of its >50 partner genes, most probably as a consequence of etoposide-induced DNA double-strand breaks (DSBs). Recent studies have shown that etoposide-induced DSBs occur predominantly within the breakpoint cluster region (bcr) of the MLL gene. However, bcr-specific DSBs induced by etoposide are not topoisomerase II–linked but the result of apoptotic nuclease–mediated DNA cleavage. Here, we test the involvement of caspase-activated DNase (CAD) and other apoptotic components in etoposide-induced gene rearrangements using two methods. First, we measured the effect of etoposide on the integration frequency of a transfected plasmid. Etoposide strongly stimulated plasmid integration in CAD cDNA–complemented mouse embryonic fibroblasts (MEFs) but not in CAD knockout (KO) MEFs. Consistently, down-regulation of ICAD (inhibitor of CAD, also known as DFF40), are responsible for etoposide-induced cleavage within the MLL locus. Etoposide stimulated MLL fusion product formation in CAD cDNA–complemented MEFs but not in CAD KO MEFs. Together, these results suggest that CAD and other apoptotic components may play an important role in etoposide-induced t-AML. (Cancer Res 2006; 66(18): 8975-9)

Introduction

Etoposide (VP-16), a topoisomerase II (Topo II) inhibitor, is an effective anticancer drug currently used for the treatment of a wide range of cancers. However, about 2% to 12% of treated patients develop treatment-related (secondary) acute myelogenous leukemia (t-AML; ref. 1). The most common genetic change in etoposide-induced t-AML is translocation at chromosome band 11q23. Most translocation junctions cluster within an 8.3-kb region [termed breakpoint cluster region (bcr)] of the mixed lineage leukemia or myeloid-lymphoid leukemia (MLL) gene (1).

A major mechanism for gene rearrangements is believed to result from the imprecise repair of DSBs, presumably through nonhomologous DNA end-joining (NHEJ). Etoposide is known to induce DNA double-strand breaks (DSBs) in the form of Topo II-linked DSBs (2). Etoposide-induced t-AML has been proposed to be a direct consequence of Topo II-linked DSBs within MLL bcr (3). However, a subsequent study argues for an indirect role of Topo II-linked DSBs, implicating apoptotic components (4). This study shows (a) that, in addition to etoposide (a Topo II-directed drug), camptothecin (a topoisomerase I-directed drug), 5-fluorouracil and methotrexate (antimetabolites), and vinblastine (a microtubule inhibitor) also induce the same site-specific cleavage within the MLL bcr, (b) that etoposide-induced DSBs within MLL bcr are not protein (Topo II) linked, and (c) that DSBs within MLL bcr can be abolished by the caspase inhibitor z-DEVD-FMK. These studies suggest that apoptotic nucleases, such as caspase-activated DNase (CAD; also known as DFF40), are responsible for etoposide-induced cleavage within the MLL bcr and thus implicate the apoptotic nuclease CAD in etoposide-induced t-AML.

CAD is the major apoptotic nuclease responsible for both high molecular weight and internucleosomal DNA cleavage (5). CAD is synthesized in its inactive form complexed with its inhibitor (ICAD, also known as DFF45). ICAD not only acts as the inhibitor of CAD but is also required for proper folding of CAD during translation (6). Thus, cells that lack ICAD do not express functional CAD. CAD activation requires cleavage of ICAD primarily by caspase-3 and the dissociation of the CAD-ICAD complex (7). Although CAD (or ICAD)-deficient cells do not exhibit DNA fragmentation, they still undergo apoptotic cell death.

In this study, we have tested the hypothesis that the apoptotic nuclease CAD is involved in etoposide-induced gene rearrangements. Our results show for the first time that etoposide-induced gene rearrangements involve the apoptotic nuclease CAD.

Materials and Methods

Cell Lines and Reagents

Transformed wild-type (WT), CAD−, and CAD′ mouse embryonic fibroblasts (MEFs; ref. 8) were kindly provided by Dr. Shigekazu Nagata (Department of Genetics, Osaka University Medical School, Osaka, Japan). HT29 and HT29.C1.16E cells were obtained from Dr. Christian Laboisse (Institut National des Sciences de la Santé et de la Recherche Médicale U539, Nantes, France; ref. 8). The pUCSV-BSD plasmid was obtained from Dr. Makoto Kimura (Laboratory for Remediation Research, Plant Science Center, RIKEN, Saitama, Japan). All cells were cultured in DMEM supplemented with 10% FCS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L glutamine in a humidified incubator with 5% CO2 at 37°C.

Apoptotic DNA Fragmentation

The amount of DNA fragments released from chromosomes during apoptosis was measured as described previously (9). Briefly, cells were lysed with chilled lysis buffer [10 mmol/L Tris (pH 8.0), 10 mmol/L EDTA, 0.2% (w/v) Triton X-100], incubated on ice, and centrifuged. DNA fragments were extracted from the supernatant with phenol/chloroform and precipitated with ethanol. DNA was resuspended in Tris-EDTA buffer and digested with...
RNase A for 2 hours at 37°C. DNA fragments were resolved by electrophoresis in 1.5% agarose gel and stained with ethidium bromide.

**Measurement of Plasmid Integration Frequency**

**MEFs.** Cells (4 × 10⁵) were seeded. The next day, cells were transfected with EcoRI-linearized pUCSV-BSD plasmid (containing the blasticidine resistance gene), with the use of the Cellfectin (Invitrogen, Carlsbad, CA) transfection reagent (0.1 μg DNA + 2 μL Cellfectin). Etoposide was added at the same time. After 6 hours, cells were rinsed briefly and trypsinized. A small aliquot was removed, reseeded into fresh medium, and grown without the selection agent for survival determination. After 4 to 5 days, colonies were stained with methylene blue (Sigma, St. Louis, MO) solution (50 μg/mL in 50% methanol). The rest of the cells were reseeded into fresh medium followed by the addition of the selection agent blasticidine (3 μg/mL; Sigma) 24 hours later. Cells were replenished with selection medium once after 4 to 5 days. After 10 days, colonies were stained and counted.

Where indicated, the pan-caspase inhibitor z-VAD-FMK (100 μmol/L; R&D Systems, Inc., Minneapolis, MN) was added at the time of transfection and etoposide treatment. After 6 hours, cells were rinsed briefly and incubated with z-VAD-FMK for 4 more hours. Cells were trypsinized and processed as described above.

**HT29 cells.** HT29 or HT29.C1.16E cells (5 × 10⁶) were seeded. The next day, cells were transfected with XhoI-linearized pMAMneo plasmid DNA (containing the neomycin resistance gene) using the Cellfectin transfection reagent. Transfection and etoposide treatment lasted for 4 hours. Cells were trypsinized and processed as described above, except that G418 was used instead of blasticidine as the selection agent.

All treatments were done in three or four replicates and repeated at least once independently showing similar results. To calculate the plasmid integration frequency for each treatment, we divided the number of resistant colonies by the number of survival colonies (grown without selection, two per replicate). The average and SE were calculated from all ratios, normalized to “no-drug treatment” (1), and plotted. To determine the survival fraction, we calculated the average and SE of all survival colony numbers, normalized to “no-drug treatment” (100%), and plotted.

**Long-Template Inverse PCR**

Long-template inverse PCR was done following the published protocol (10) with some minor modifications. Cells were harvested with lysis solution [50 mmol/L Tris-HCl (pH 8.0), 100 mmol/L EDTA, 0.5% SDS] containing proteinase K (50 μg/mL) and incubated overnight at 55°C. Chromosomal DNA was extracted with phenol and then with phenol/chloroform/isoamyl alcohol, ethanol precipitated, and washed with 70% ethanol. After phosphatase treatment, NcoI digestion, and intramolecular ligation at high dilution, DNA was purified and concentrated using PCR purification columns (Qiagen, Valencia, CA). DNA (250 ng) was used as template for the PCR amplification. For quantification, the number of fusion product bands (between 4 and 10.4 kb; ref. 10) was counted. MLL fusion product frequencies represent the average of two sets of PCRs. Each set consisted of 8 to 14 replicates. The average number of fusion products was normalized to no-drug treatment and plotted. Error bars represent the SD of the two average values.

**Results**

To test whether CAD is involved in etoposide-induced gene rearrangements, we compared the frequencies of etoposide-induced plasmid integration in a pair of transformed MEFs derived from a CAD knockout (KO) mouse, CAD⁻ MEFs (CAD KO MEFs), and CAD⁺ MEFs (CAD⁺ MEFs with reintroduced CAD cDNA; ref. 5). As shown in Fig. 1A, etoposide induced DNA fragmentation (evidenced by the amount of released apoptotic DNA fragments) in CAD⁺ MEFs, but not in CAD⁻ MEFs, consistent with the lack of

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**Figure 1.** CAD dependence of etoposide-induced plasmid integration. A. MEFs (WT, CAD⁻, and CAD⁺) were treated with increasing concentrations of etoposide for 24 hours. Apoptotic DNA fragmentation assay was done as described in Materials and Methods. B and C. CAD⁻ and CAD⁺ MEFs were treated with etoposide for 6 hours. Integration frequencies (B) and survival (C) were measured as described in Materials and Methods. Points, average of the replicates; bars, SE.
CAD activity. Etoposide is known to stimulate plasmid integration in a variety of model systems as monitored by counting resistant colonies (11). As shown in Fig. 1B, etoposide indeed strongly stimulated plasmid integration in CAD+ MEFs but not in CAD− MEFs. For example, at 1 μmol/L etoposide concentration, the average integration frequency was 7.5-fold (range, 3-17; n = 3) higher in CAD+ MEFs than in CAD− MEFs. The integration frequency in CAD+ MEFs was higher than in CAD− MEFs at all toxicity levels. Both cell lines were sensitive to etoposide, although the CAD− to a lesser extent (Fig. 1C). The difference in integration frequency between the CAD+ and CAD− MEFs was not due to a difference in cell cycle distribution or the levels of Topo II isozymes as revealed by fluorescence-activated cell sorting (FACS) and Western blotting analysis (data not shown).

Another strategy to inhibit CAD function is to down-regulate ICAD. The cell line HT29.C1.16E, a clonal derivative of the colorectal cancer cell line HT29, does not express ICAD during exponential growth and thus does not show any CAD activity. As shown in Fig. 2A, etoposide induced DNA fragmentation in HT29, but not in HT29.C1.16E, consistent with the lack of CAD activity in HT29.C1.16E (8). Paralleling the results above, etoposide strongly stimulated plasmid integration in HT29 but not in HT29.C1.16E (Fig. 2B). For example, at 10 μmol/L etoposide concentration, the average integration frequency was ~25-fold (range, 10-53; n = 3) higher in HT29 than in HT29.C1.16E cells. Plasmid integration frequency in HT29 was higher than in HT29.C1.16E at all toxicity levels. Both cell lines were sensitive to etoposide, although HT29.C1.16E to a lesser extent (Fig. 2C). The difference in integration frequency was not due to a difference in cell cycle distribution or the levels of Topo II isozymes as revealed by FACS and Western blotting analysis (data not shown).

MLL bcr cleavage and gene rearrangements have been attributed previously to apoptotic signaling (12, 13). Both the breaks in MLL bcr and the expression of MLL fusion products can be suppressed by caspase inhibition (14). To test whether caspases also play a role in etoposide-induced gene rearrangements, we measured etoposide-induced plasmid integration in WT MEFs in the presence or absence of the pan-caspase inhibitor z-VAD-FMK. As shown in Fig. 3A, etoposide stimulated plasmid integration to a relatively small extent, reflecting the low level of CAD activity in WT MEFs.

**Figure 2.** ICAD dependence of etoposide-induced plasmid integration. A, HT29 and HT29.C1.16E cells were treated with etoposide for 5 hours, rinsed, and harvested 48 hours later. Apoptotic DNA fragmentation assay was done as described in Materials and Methods. B and C, HT29 and HT29.C1.16E cells (lacking ICAD) were treated with etoposide for 6 hours. Integration frequencies (B) and survival (C) were measured as described in Materials and Methods. Points, average of the replicates; bars, SE.

**Figure 3.** Caspase dependence of etoposide-induced plasmid integration. WT MEFs were treated with etoposide for 6 hours in the presence or absence of the pan-caspase inhibitor z-VAD-FMK (100 μmol/L). Integration frequencies (A) and survival (B) were measured as described in Materials and Methods. Points, average of the replicates; bars, SE.
(see also released apoptotic DNA fragments on etoposide treatment in Fig. 1A; ref. 5). Nevertheless, the caspase inhibitor z-VAD-FMK reduced etoposide (0.3 μmol/L)–induced integration by ~2-fold (range, 1.5-2.1; n = 2). Caspase inhibition also slightly increased survival (Fig. 3B).

To analyze the role of CAD in etoposide-induced translocations at the MLL bcr locus specifically, we compared MLL fusion product frequencies between CAD− MEFs and CAD+ MEFs using long-template inverse PCR (10). This semiquantitative method is designed to detect single translocation events among a large number of WT (germ-line) MLL loci. Figure 4B is a representative gel picture showing the formation of MLL fusion gene products as revealed by long-template inverse PCR. The results were quantified and shown in Fig. 4A. Consistently with the plasmid integration assay, etoposide induced significantly more MLL fusion products in CAD+ MEFs than in CAD− MEFs (Fig. 4A).

Discussion

Our results are most consistent with a model for etoposide-induced gene rearrangement shown in Fig. 4C. In this model, etoposide induces DNA damage (Topo II-linked DSBs) somewhere in the chromosomes. Etoposide-induced DNA damage then activates the apoptotic signaling pathway, which leads to activation of caspase-3 followed by ICAD cleavage and activation of CAD. Activated CAD then cleaves DNA within the MLL bcr as well as within MLL partner genes. The repair of the DSB within the MLL bcr through NHEJ results in rearrangement of the MLL gene with one of its >50 partner genes and hence t-AML.

The role of apoptotic nuclease in genome instability is a surprising finding. It is generally thought that apoptotic nucleases, such as CAD, play a role late in the apoptotic cell death program. Our results suggest that a population of apoptotic cells with activated CAD may escape cell death and survive with a few DSBs (15). A possible consequence of this escape is gene rearrangements. It should be pointed out that our results do not rule out the direct involvement of Topo II-mediated DSBs in DNA sequence rearrangements. In fact, we have shown that caspase inhibition has only a small effect on plasmid integration in WT MEFs that express low levels of CAD (Fig. 3). Apparently, the CAD-dependent sequence rearrangements may play a particularly important role only in cells that express high levels of CAD (e.g., hematopoietic cells).

CAD is responsible for both high molecular weight DNA and internucleosomal DNA fragmentations (5). High molecular weight DNA fragmentation has been shown to precede internucleosomal DNA fragmentation (16). CAD-mediated site-specific cleavage

Figure 4. CAD dependence of etoposide-induced MLL fusion product formation. A, CAD− MEFs (white columns) and CAD+ MEFs (gray columns) were treated with etoposide (100 μmol/L) for 6 hours and processed for long-template inverse PCR analysis as described in Materials and Methods. Columns, average number of MLL fusion products in two sets of PCRs, normalized to no-drug treatment; bars, SD. B, a representative gel picture showing the MLL fusion gene products revealed by long-template inverse PCR. C, a model for etoposide-induced MLL gene rearrangements mediated by the apoptotic nuclease CAD. White box, the MLL gene, with the bcr region (gray highlight); black box, a hypothetical fusion partner on another chromosome; star, etoposide-induced DNA damage (Topo II poisoning); wavy arrows, DSB introduced by CAD; dotted line arrow, chromosome translocation.
within *MLL* bcr is likely to be the result of high molecular weight DNA fragmentation occurring at limited sites on chromosomes. Although the molecular mechanism for CAD-mediated high molecular weight DNA fragmentation is unclear, it seems possible that CAD-mediated cleavages occur at sites of DNase I hypersensitivity, which reflect altered chromatin structure (17). Indeed, a DNase I-hypersensitive site has been mapped to *MLL* bcr. Interestingly, in addition to a DNase I-hypersensitive site, *MLL* bcr contains several scaffold attachment regions and Topo II consensus sites (18). Thus, it seems likely that CAD-mediated DNA cleavage within *MLL* bcr is not sequence specific but is the result of altered chromatin structure in this region. Alternatively, CAD could be recruited to *MLL* bcr by Topo II (19).

Our current study could implicate apoptotic nuclease CAD as a potential mechanism for etoposide-induced genome instability and t-AML. Recent studies have shown that apoptosis is not the major mechanism of cell killing by etoposide (20). Consequently, the use of CAD or caspase inhibitors during etoposide chemotherapy could possibly reduce the incidence of t-AML without compromising etoposide efficacy. Clearly, further studies are necessary to establish the potential role of apoptosis in genome instability.

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