RAPID COMMUNICATION

Activation of the CPP32 Protease in Apoptosis Induced by 1-β-D-Arabinofuranosylcytosine and Other DNA-Damaging Agents

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The response of human myeloid leukemia cells to treatment with 1-β-arabinofuranosylcytosine (ara-C) includes the induction of apoptosis. Ara-C induced apoptosis is associated with proteolytic cleavage of poly(ADP-ribose) polymerase (PARP) and protein kinase C (PKC) δ. However, the signals involved in this response are unknown. The present studies show that ara-C treatment of U-937 cells is associated with induction of a protease activity that cleaves the tetrapeptides Ac-DEVD-pNA and Ac-DMQD-pNA found at the cleavage sites of PARP and PKCδ, respectively. The ara-C–induced protease activity was sensitive to overexpression of the anti-apoptotic protein Bcl-xL and the baculovirus protein p35. By contrast, overexpression of the cowpox virus protein CrmA blocked apoptosis induced by engagement of the Fas receptor but not that induced by ara-C. CrmA overexpression also had no detectable effect on ara-C–induced cleavage of PKCδ.

The results further show that ara-C induces activation of the CPP32 protease by a CrmA-insensitive and p35-sensitive mechanism. Similar results were obtained with cisplatinum, etoposide, and camptothecin. These findings indicate that ara-C and other DNA-damaging agents activate a CrmA-sensitive apoptotic pathway involving CPP32 and that these signals differ from those associated with apoptosis induced by the Fas receptor.

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MATERIALS AND METHODS

Cell culture. Human U-937 myeloid leukemia cells (American Type Culture Collection, Rockville, MD) were grown as described.11 Stable transfectants of U-937 cells expressing CrmA were selected after transfecting pcDNA3 and pEF1-CrmA12 by electroporation (Gene Pulser; Bio-Rad, Hercules, CA; 0.25 V, 960 μF). To generate a p35-expressing cell line, U-937 cells were similarly transfected using pEF1 containing neo (designated pEF2) or pEF2-p35.11 Transfectants were selected in the presence of 400 μg/mL geneticin sulfate. CrmA and p35 expression was confirmed by Northern blotting. The cells were treated with 10 μmol/L ara-C (Sigma Chemical

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Co. St Louis, MO), 3 μg/mL etoposide (Bristol-Myers Squibb Co. Princeton, NJ), 100 μmol/L cisplatinum (Sigma), 0.5 ng/mL 9-nitrocamptothecin (Stehlin Foundation, Houston, TX), and 5 μg/mL anti-Fas mouse monoclonal antibody.  

**Analysis of DNA fragmentation.** Cells were suspended in 50 μL of 5 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA, and 0.5 mg/mL proteinase K (Sigma). After incubation at 50°C for 6 hours, the samples were mixed with 50 μL of 10 mmol/L EDTA (pH 8.0) containing 1% (wt/vol) low melting point agarose and 40% sucrose at 70°C. The DNA was separated in 2% agarose gels/TAE (40 mmol/L Tris-acetate and 1.0 mmol/L EDTA, pH 8.0) buffer at 35 V for 16 hours. After treating with RNase, the gels were visualized by UV illumination after ethidium bromide staining.

**Immunoblot analysis.** Preparation of cell lysates for PARP and anti-PCK6 immunoblot analysis was performed as described using C-2-10 anti-PARP monoclonal antibody and anti-PCK6 monoclonal antibody. Lysates were also prepared by suspending cells in lysis buffer (50 mmol/L Tris, pH 7.6, 150 mmol/mL NaCl, 1 mmol/L phenylmethylsulfonfluoride, 1 mmol/L sodium vanadate, 1 mmol/L DTT, 10 mmol/L sodium fluoride, 10 μg/mL each of leupeptin and aprotonin, and 1% Brij-96). The lysates were cleared by centrifugation and subjected to electrophoresis in an sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins were then transferred to nitrocellulose, blocked with 5% dry milk in phosphate-buffered saline containing 0.1% Tween 20, and probed with anti-CPP32 polyclonal antibody (previously purified and described). Antibody complexes were visualized by chemiluminescence (ECL detection system; Amersham, Arlington Heights, IL).

**Protein expression and purification.** CrmA contained an N-terminal polyhistidine linker and was expressed as described. The cell lysate was applied to a NiSO₄-charged Hi-Trap column (Pharmacia Biotech Inc., Piscataway, NJ) and the protein was eluted with buffer A (50 mmol/L HEPES, pH 7.5, 10% glycerol, 0.2 mol/L NaCl, 200 mmol/L imidazole). Fractions containing CrmA as judged by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were pooled and dialyzed at 4°C against 20 mmol/L Tris, pH 7.5. The sample was applied to a Mono Q column (Pharmacia), equilibrated with buffer B (20 mmol/L Tris, pH 7.5, 5 mmol/L dithiothreitol [DTT]), and eluted with a gradient to buffer C (buffer B plus 300 mmol/L NaCl). p35 was purified as described.

**Protease assays.** Protease activity was measured using tetrapeptide p-nitroanilide (pNA) substrates in a colorimetric assay. The assays were performed in 96-well plates by incubating 20 μL of cell lysates with 180 μL of reaction buffer (100 mmol/L HEPES, pH 7.5, 20% vol/vol glycerol, 5 mmol/L DTT, and 0.5 mmol/L EDTA) containing 100 μmol/L of peptidic substrate. The assays included Acetyl-Tyr-Val-Ala-Asp-pNA (Ac-YVAD-pNA), Ac-Asp-Glu-Val-Asp-pNA (Ac-DEVD-pNA), or Ac-Asp-Met-Gln-Asp-pNA (Ac-DMQD-pNA; California Peptide Research Inc., Napa, CA). Absorbance at 405 nm was monitored at 37°C for 30 minutes using a microtitr plate reader (Molecular Devices Inc., Sunnyvale, CA). In certain experiments, the cell lysate was diluted in assay buffer and first incubated with varying amounts of inhibitors at room temperature for 30 minutes.

**RESULTS**

Previous studies have shown that ara-C and other DNA-damaging agents induce proteolytic activation of PKCζ. To assess the potential involvement of ICE/Ced 3-like proteases, we assayed lysates from ara-C-treated U-937 cells for their ability to cleave tetrapeptide substrates. A low level of protease activity towards Ac-YVAD-pNA, a substrate that contains the amino acid sequence of a pro-interleukin-1β cleavage site, was detectable in untreated and ara-C–treated cells (Fig 1A). By contrast, ara-C treatment was associated with increases in Ac-DEVD-pNA cleaving activity that were detectable at 4 hours and maximal at 6 hours (Fig 1A). The kinetics of Ac-DMQD-pNA cleavage were similar but less pronounced (Fig 1A). These findings of protease activation coincided with the appearance of internucleosomal DNA fragmentation at 6 hours of ara-C treatment (Fig 1B).

Because ara-C incorporates into DNA and induces DNA strand breaks, we asked whether treatment with other agents that damage DNA is also associated with increases in ICE-like protease activity. Cisplatinum induces DNA intrastrand cross-links. Etoposide induces DNA strand breaks as a result of forming a complex with topoisomerase II and the DNA 5′-terminus. Camptothecin stabilizes topoisomerase I/DNA complexes and converts single-strand nicks to irreversible double-strand breaks. Treatment of cells with cisplatinum, etoposide, and camptothecin was associated with increases in Ac-DEVD-pNA cleaving activity (Fig 2A). Similar results were obtained for cleavage activity toward Ac-
Other studies have shown that the antiapoptotic effects of the cowpox virus protein CrmA and baculovirus protein p35 are mediated through the direct inhibition of ICE-like proteases. To determine whether these viral proteins inhibit apoptosis induced by ara-C and other agents, cells were prepared that stably overexpress CrmA or p35. Treatment of U-937 and U-937/CrmA cells with ara-C was associated with internucleosomal DNA fragmentation (Fig 4A). By contrast, overexpression of p35 in U-937/p35 cells resulted in inhibition of ara-C–induced DNA cleavage (Fig 4A). Similar findings were obtained with etoposide (Fig 4B), cisplatinum, and camptothecin (data not shown). Previous work has shown that CrmA blocks apoptosis induced by engagement of the Fas receptor. Whereas treatment of U-937 cells with ara-C induced DNA cleavage, whereas there was little if any effect of these agents on cleavage of Ac-YVAD-pNA (data not shown). Treatment with cisplatinum, etoposide, and camptothecin was also associated with internucleosomal DNA fragmentation (Fig 2B). These findings indicated that diverse DNA-damaging agents induce ICE-like protease activity in association with induction of apoptosis.

The Bcl-XI protein, like Bcl-2, functions as an inhibitor of apoptosis. U-937 cells transfected with the neo-containing vector (U-937/neo) responded to ara-C with increases in Ac-DEVD-pNA and Ac-DMQD-pNA cleaving activity (Fig 3A and data not shown). By contrast, the ara-C-induced protease activity was completely blocked by overexpression of Bcl-XI (Fig 3A and data not shown). The absence of protease activation after ara-C treatment was also associated with a Bcl-XI–mediated block in internucleosomal DNA fragmentation (Fig 3B). These results indicate that Bcl-XI functions upstream to the activation of proteases associated with ara-C–induced apoptosis.
ROLE OF CPP32 IN DNA DAMAGE-INDUCED APOPTOSIS

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Ara-C-induced protease activation is insensitive to CrmA but sensitive to p35. Lysates from U-937/CrmA cells, but not in U-937/p35 cells (Fig 5A). Similar results were obtained for induction of Ac-DMQD-pNA cleaving activity (Fig 5B). To confirm insensitivity of the ara-C-induced protease activity to CrmA, we added recombinant CrmA to lysates of ara-C-treated cells. There was little inhibition of ara-C-induced Ac-DEVD-pNA (Fig 5C) or Ac-DMQD-pNA (Fig 5D) cleaving activity at CrmA concentrations of up to 10 μmol/L. However, the addition of recombinant p35 to similar concentrations completely blocked cleavage of both tetrapeptide substrates (Figs 5C and D).

The findings that ara-C induces Ac-DEVD-pNA and Ac-DMQD-pNA cleaving activity prompted an analysis of PARP and PKCδ cleavage. Treatment of U-937 and U-937/CrmA cells with ara-C for 4 to 6 hours resulted in the cleavage of PARP to an 85-kD fragment (Fig 6). By contrast,
which purified CPP32 was added (upper panel) or anti-Ich-1 (lower panel). Large subunits (p19/p17) of CPP32 are indicated by the two arrows in the upper panel.

Fig 7. Activation of CPP32 and/or CPP32-like enzyme(s) by ara-C. (A) Lysates from ara-C-treated U-937 cells (○) or untreated cells to which purified CPP32 was added (□) were tested for inhibition by Ac-DEVD-cho using Ac-DEVD-pNA as substrate. (B) Cell lysates were subjected to immunoblot analysis with anti-CPP32 (upper panel) or anti-Ich-1 (lower panel). Large subunits (p19/p17) of CPP32 are indicated by the two arrows in the upper panel.

there was no detectable PARP cleavage in ara-C-treated U-937/p35 cells (Fig 6). The kinetics of PKCδ cleavage to the 40-kD catalytic fragment were similar to those found for PARP (Fig 6). Moreover, ara-C-induced PKCδ cleavage was unaffected by overexpression of CrmA and inhibited in U-937/p35 cells (Fig 6).

Proteolytic cleavage of Ac-DEVD-pNA and PARP has been attributed to CPP32.18 PKCδ is also cleaved by CPP32 in vitro (unpublished data). To test the contribution of CPP32 to ara-C-induced protease activity in cell lysates, we compared inhibition of ara-C-induced protease activity in lysates to that of control cell lysates to which a similar total activity of purified CPP32 was added (Fig 7A). The observation that the protease activity of treated cell lysates is about fivefold more sensitive to Ac-DEVD-cho inhibition than is purified CPP32 (Fig 7A) suggests that the induced activity is due to CPP32 and/or CPP32-like enzyme(s). To more directly assess activation of CPP32, we assayed lysates of ara-C-treated cells for cleavage of the proenzyme to its active subunit,16,28 by Western blotting. Treatment of U-937 and U-937/CrmA cells with ara-C was associated with CPP32 activation, whereas there was no apparent cleavage of the CPP32 proenzyme in ara-C-treated U-937/p35 cells (Fig 7B). As a control, ara-C treatment had no detectable effect on Ich-1L levels (Fig 7B). These results indicate that ara-C induces apoptosis in association with activation of CPP32.

DISCUSSION

Recent studies have shown that the stress-activated protein kinase (SAPK) is involved in the induction of apoptosis by ionizing radiation (IR), tumor necrosis factor, and growth factor withdrawal.42-47 SAPK is activated in cells treated with diverse types of DNA damaging agents, such as IR, alkylating agents, and topoisomerase inhibitors.43-48 The cellular response to ara-C also includes activation of SAPK and downstream signals that include induction of c-jun gene transcription.46,49,50 The finding that activation of c-jun expression occurs temporarily with induction of apoptosis in ara-C-treated cells has supported a potential relationship between these events.6 However, recent work has suggested that a block in induction of c-jun expression has little effect on ara-C-induced apoptosis.21 Thus, activation of the c-jun gene may be a response to ara-C-induced apoptosis or, alternatively, SAPK activation may function upstream to both c-jun expression and apoptosis. Other studies have shown that overexpression of Bcl-2 or Bcl-XL blocks ara-C-induced apoptosis.10 However, the finding that these antiapoptotic proteins have no effect on activation of SAPK or c-jun expression by ara-C suggests that Bcl-2 or Bcl-XL may function downstream of these events or in an independent pathway.

Although certain insights are available regarding events associated with ara-C-induced apoptosis, little is known about the potential involvement of cysteine proteases in the response to treatment with ara-C or other DNA-damaging agents. The finding that overexpression of ICE or Ced-3 in mammalian cells induces apoptosis has indicated that members of the ICE/Ced-3 family contribute to this form of cell death.15 Multiple ICE/Ced-3 homologs have now been identified, although their roles in inducing apoptosis are unclear. Recent studies have shown the sequential activation of ICE-like and then CPP32-like proteases in Fas-mediated apoptosis.52 In the present report, there was no apparent induction of an ICE-like activity (as determined by Ac-YVAD-pNA cleavage) in ara-C-treated cells. However, the finding that ara-C treatment is associated with Ac-DEVD-pNA cleavage supports induction of a CPP32-like activity. PARP is cleaved by Ced-3, CPP32, and ICE-LAP3.17,18,23,53 Moreover, PKCδ is cleaved by CPP32, but not ICE, Ich-1, Ich-2, Mch2, Mch3, or ICEeIII (unpublished data). The findings that PARP and PKCδ are cleaved in ara-C-treated cells thus provided further support for activation of a CPP32-like protease. Indeed, direct assessment of CPP32 proenzyme cleavage to its functional subunits confirmed activation of this protease in ara-C-induced apoptosis. Recent studies have shown that Bcl-2 and Bcl-XL block staurosporine-induced, but not Fas-induced, activation of CPP32 and apoptosis.54
The finding that Bcl-X<sub>L</sub> blocks ara-C–induced CPP32 activity and apoptosis further indicates that this antiapoptotic protein functions upstream to both events.

Further evidence for involvement of CPP32 activity in ara-C–induced apoptosis is derived from studies in cells that overexpress CrmA and p35. CrmA is a poxvirus serpin that inhibits ICE by direct binding. Several studies have shown that CrmA inhibits Fas- and tumor necrosis factor-induced apoptosis. CrmA also blocks apoptosis induced by cytotoxic T cells and growth factor withdrawal. Although the present studies also show that CrmA blocks Fas-mediated apoptosis, there was little if any effect of CrmA on apoptosis in ara-C–treated cells. Similar results were obtained in U-937/CrmA cells treated with cisplatinum, etoposide, and camptothecin. These findings suggest that DNA-damaging agents activate a CrmA-insensitive pathway that is distinct from those involved in a number of other models of CrmA-sensitive apoptosis. By contrast, ara-C–induced protease activity and apoptosis were sensitive to overexpression of the baculovirus p35 protein. p35 inhibits apoptosis in cells from diverse species and blocks the proteolytic activity of ICE, Ced-3, CPP32, Ich-1, and Ich-2. The finding that the addition of recombinant p35 to lysates of ara-C–treated cells blocks Ac-DEVD-pNA cleaving activity is thus consistent with activation of CPP32. Moreover, because CrmA has no effect on CPP32 at concentrations up to 10 μM, the activation of CPP32 in ara-C–treated cells is in concert with involvement of a CrmA-insensitive, p35-sensitive pathway.

The available evidence suggests that apoptosis may occur as a result of activating an amplifiable protease cascade. The cytosolic fraction of apoptotic osteosarcoma cells induces apoptosis in isolated nuclei by a mechanism that is blocked by an inhibitor of CPP32. However, the finding that CPP32 alone fails to induce nuclear apoptosis suggests that this protease may be necessary but not sufficient. Alternatively, cytosolic substrates of CPP32 may be critical for induction of apoptosis in isolated nuclei. In addition to PARP, recent work has shown that PKCδ is proteolytically cleaved and thereby activated in cells induced to undergo apoptosis by DNA-damaging agents and other signals. Loss of PARP function could contribute to inhibition of DNA repair or endonuclease activation. Although the functional significance of PKCδ activation remains unknown, CPP32 is thus far the only known protease to proteolytically activate this kinase. Moreover, CPP32-mediated cleavage of PKCδ provides a potential link between activation of cysteine protease and kinase cascades. With regard to potential signals upstream to CPP32, we show that p35 inhibits ara-C–induced Ac-DEVD-pNA cleaving activity in cells and in vitro and that p35 blocks cleavage of CPP32 to its active subunits. Our recent studies have identified a novel aspartate-specific cysteine protease, designated Mch4, that is inhibited by the tetrapeptide aldehyde Ac-DEVD-cho and cleaves CPP32 to the active subunits. Further studies are now needed to determine whether Mch4 is sensitive to p35 and whether this new protease is activated by ara-C and other DNA-damaging agents.

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