RESEARCH COMMUNICATION

prICE: a downstream target for ceramide-induced apoptosis and for the inhibitory action of Bcl-2

Miriam J. SMYTH*†‡, David K. PERRY‡§, Jiandi ZHANG||, Guy G. POIRIER¶, Yusuf A. HANNUN§ and Lina M. OBEID*†**

*Department of Medicine and Division of Geriatrics, Duke University Medical Center, Durham, NC 27710, U.S.A., †Geriatric Research, Education and Clinical Center, Department of Veterans Affairs Medical Center, Durham, NC, U.S.A., §Department of Medicine and Division of Hematology/Oncology, Duke University Medical Center, Durham, NC 27710, U.S.A., ||Department of Cell Biology, Duke University, Durham, NC 27710, U.S.A., and ¶Poly(ADP-Ribose) Metabolism Group, Laboratory of Molecular Endocrinology, Centre Hospitalier de l'Université Laval Research Center and Laval University, Sainte-Foy, Quebec G1V 4G2, Canada

The novel lipid second messenger, ceramide, specifically induced poly(ADP-ribose) polymerase cleavage through activation of the protease prICE. Over-expression of Bcl-2 inhibited ceramide-induced poly(ADP-ribose) polymerase proteolysis and protected

cells from ceramide-induced death. These data provide the first insight into the mechanism by which ceramide mediates apoptosis and suggest a mechanism by which Bcl-2 protects from cell death.

INTRODUCTION

Apoptosis is now recognized as an evolutionarily conserved and biochemically driven form of cell death with distinct morphological features [1]. Recent studies have shown that ceramide is a potent inducer of apoptosis [2]. Cellular levels of ceramide are regulated by tumour necrosis factor- α (TNF α), nerve growth factor, interleukin-1 β , dexamethasone, activators of FAS, chemotherapeutic agents and ionizing radiation (reviewed in [3]), though the mechanisms involved remain to be deciphered.

Activation of proteases has recently been postulated to be a general feature of apoptosis [4]. The following four proteins have been reported to be cleaved during apoptosis: fodrin [4], terminin [5], the protein component of the U1 small nuclear ribonucleoprotein [6] and poly(ADP-ribose) polymerase (PARP) [7]. PARP is a zinc-binding nuclear DNA-dependent enzyme that uses NAD⁺ as a substrate. At the onset of apoptosis, PARP is cleaved in human breast cancer cells, thymocytes and HL60 cells [7]. The protease prICE (Yama/CPP32 β /apopain), a mammalian homologue of the *Caenorhabditis elegans* death gene Ced-3, has been identified as the protease that cleaves PARP during apoptosis [8–11].

The key biochemical events that mediate apoptosis have not yet been elucidated. For example, despite much recent investigation of the role of activation of interleukin-1 β -converting enzyme (ICE)-like proteases in apoptosis, the pathways leading to this activation have not been identified. We, therefore, chose to investigate whether ceramide-induced cell death involves activation of prICE. We show that prICE is a downstream target of ceramide and that Bcl-2 inhibits both PARP proteolysis and ceramide-induced cell death. In this study, we provide important insight into the mechanisms of apoptosis by: (1) implicating specific proteolytic cleavage of PARP in the ceramide signalling pathway; (2) providing the first evidence of the signal transduction pathways leading to activation of prICE; and (3) presenting the first biochemical evidence for a role of Bcl-2 in inhibiting the PARP protease. In addition, our studies with Bcl-2 also establish the critical role of prICE in mediating ceramide-induced cell death.

MATERIALS AND METHODS

Cell culture

During routine maintenance, Molt-4 cells (ATCC), were cultured in RPMI 1640 supplemented with 10 % (v/v) fetal bovine serum (FBS) and maintained at 37 °C in a 5 % CO₂ incubator. During induction of apoptosis, cells in logarithmic growth were plated at 5 × 10⁵/ml in 2 % (v/v) FBS for 1–2 h before treatment. Molt-4 cells transfected with either vector alone or with vector expressing mouse *bcl-2* cDNA were maintained in RPMI medium as above, except for the addition of hygromycin at a concentration of 420 µg/ml.

Preparation of cell extracts

Cell extracts were prepared by suspending cells in ice-cold homogenizing buffer [20 mM Tris/HCl (pH 7.5), 250 mM sucrose, 10 mM EGTA (pH 7.4), 2 mM EDTA (pH 7.4), 1 mM PMSF and 0.02 % (w/v) leupeptin]. After sonication of the homogenates, an aliquot was removed for protein determination, and the remainder was mixed (1:3) with 4 × SDS sample buffer.

Cell fractionation

After treatment with C₆-ceramide or vehicle for 6 h, cells were pelleted by centrifugation, rinsed with PBS, resuspended in cell-free buffer [10 mM Hepes (pH 7.4), 100 mM KCl, 3 mM NaCl, 4 mM MgCl₂, 0.2 µM CaCl₂ and 1 mM dithiothreitol] and lysed by nitrogen cavitation (10 min at 3105 kPa); the post-100 000 g supernatant (cytosol) was recovered after centrifugation.

Electrophoresis and Western blotting of proteins

Samples containing protein from 3 × 10⁵ cells were analysed by Western-blot analysis as previously described [12]. A rabbit polyclonal antiserum raised against a peptide contained in the automodification domain of PARP [7] was employed at a dilution of 1:2000, and a horseradish peroxidase-conjugated goat anti-

Abbreviations used: PARP, poly(ADP-ribose) polymerase; TNF α , tumour necrosis factor- α ; ICE, interleukin-1 β -converting enzyme; FBS, fetal bovine serum.

† These two authors contributed equally to this work.

** To whom correspondence should be addressed, at Duke University Medical Center, Department of Medicine, Box 3345, Durham, NC 27710, U.S.A.

rabbit secondary antibody (Bio-Rad) was used at a dilution of 1:5000. Detection of signal was by enhanced chemiluminescence (Amersham).

Reagents

YVAD-chloromethane (YVAD-chloromethyl ketone) was purchased from Bachem Bioscience. RPMI 1640 and FBS were from Life Technologies. All other reagents were from Sigma.

RESULTS AND DISCUSSION

In order to determine if the signal transduction pathway activated by ceramide leads to proteolysis of PARP, Molt-4 cells were induced to undergo apoptosis by treatment with C_6 -ceramide ($20 \mu\text{M}$). Separation of extracted proteins by SDS/PAGE, followed by staining with Coomassie Brilliant Blue, showed that the bulk of the cellular polypeptides remained intact (results not shown). However, Western blotting with epitope-specific polyclonal antibodies against PARP indicated that C_6 -ceramide ($20 \mu\text{M}$) induced early (1–6 h) cleavage of the 116 kDa PARP polypeptide to the apoptosis-specific 85 kDa fragment (Figure 1). This cleavage was identical with that seen with etoposide (Figure 1), a previously established inducer of PARP proteolysis through activation of pRICE [7]. Subsequent time-course and dose-dependence studies established that, following treatment with C_6 -ceramide ($20 \mu\text{M}$), generation of the 85 kDa fragment was first evident after 4 h of treatment. Treatment with higher doses was more rapidly effective whereas lower doses ($10 \mu\text{M}$) exhibited decreased efficacy (results not shown). These results provide the first evidence implicating apoptosis-specific proteolytic cleavage of PARP in the mechanism of action of ceramide on cell death.

As ceramide is an amphiphilic lipid analogue that may have non-specific activities, it was important to establish its specificity

Figure 1 Effects of C_6 -ceramide on proteolytic cleavage of PARP

Molt-4 cells were treated with C_6 -ceramide ($20 \mu\text{M}$) for 0–12 h or with etoposide ($68 \mu\text{M}$) for 4 h. Total cellular homogenate was analysed by SDS/PAGE, followed by Western blotting using PARP-specific antibodies, as described in the Materials and methods section.

Figure 2 Ceramide is a specific inducer of proteolytic cleavage of PARP

Cells were treated for 6 h with ethanol vehicle (control), C_2 -ceramide ($10 \mu\text{M}$), C_2 -dihydroceramide (DHC $_2$ -ceramide; $10 \mu\text{M}$), C_6 -ceramide ($20 \mu\text{M}$), C_6 -dihydroceramide (DHC $_6$ -ceramide; $10 \mu\text{M}$) and dioctanoylglycerol (diC $_8$; $10 \mu\text{M}$). Total cellular homogenate was analysed by SDS/PAGE, followed by Western blotting using PARP-specific antibodies.

Figure 3 Effect of the protease inhibitors on proteolytic cleavage of PARP

Top: lysates were prepared from control cells, from cells treated with C_6 -ceramide ($20 \mu\text{M}$), iodoacetamide (10 mM), or ceramide and iodoacetamide. After a 1 h pretreatment with iodoacetamide, cells were treated with C_6 -ceramide for 6 h. Bottom: purified bovine PARP (25 ng) was incubated for 1 h at 37°C with cytosol ($10 \mu\text{g}$) from Molt-4 cells treated with vehicle (lane 1) and with C_6 -ceramide ($20 \mu\text{M}$) for 6 h (lane 2). YVAD-chloromethane (YVAD-CMK) (10 and 100 nM) was preincubated with the active cytosol for 5 min, and the PARP substrate was then added and incubated for 1 h at 37°C . PARP proteolysis was analysed by Western blotting.

of action. This was approached by treating the cells with dihydroceramide (a closely related structural analogue of ceramide) and dioctanoylglycerol (a cell-permeable analogue of endogenous diacylglycerols). Whereas C_2 -ceramide and C_6 -ceramide induced proteolytic cleavage of PARP after 6 h of treatment, this apoptosis-specific cleavage was not induced by C_2 -dihydroceramide, C_6 -dihydroceramide or dioctanoylglycerol (Figure 2). In contrast with C_2 - and C_6 -ceramide, the dihydroceramides and diacylglycerols also fail to induce the apoptotic phenotype, as determined by DNA laddering and flow cytometric analyses [3]. Dihydroceramide differs structurally from ceramide only in the absence of the double bond between carbons 4 and 5 of the sphingoid backbone. Both ceramide and dihydroceramide show similar kinetics of uptake and minimal metabolism [13]. The differences in ability to cleave PARP are, therefore, not the result of differential delivery, and presumably reflect the ability of ceramide to transduce specifically an apoptotic signal that activates a protease in the cell-death pathway that, in turn, cleaves PARP.

Cleavage of PARP to the apoptosis-specific 85 kDa fragment results from activation of a distinct protease (pRICE) whose activity is defined by substrate recognition and inhibitor sensitivity [7,8]. As the definitive apoptosis-specific PARP fragment is produced *in vivo* after treatment with etoposide [8], the effects of protease inhibitors on the proteolytic cleavage of PARP induced by etoposide and C_6 -ceramide were investigated. Iodoacetamide (10 mM), a cysteine-protease inhibitor, prevented cleavage of PARP induced by etoposide (results not shown) and by C_6 -ceramide (Figure 3, top). *N*-ethylmaleimide was also

Figure 4 Bcl-2 inhibits ceramide-induced PARP proteolysis in intact cells

Molt-4 cells (mock-transfected or transfected with the mouse *bcl-2* gene) were treated with C_6 -ceramide (5, 10 or 20 μ M) for 6 h followed by analysis of total cellular homogenate by SDS/PAGE and Western blotting using PARP-specific antibodies, as described in the legend to Figure 1.

effective at inhibiting PARP cleavage induced by C_6 -ceramide (results not shown). In contrast, the serine-protease inhibitors 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one and 7-amino-1-chloro-3-L-tosylamidoheptan-2-one failed to prevent cleavage of PARP, as did the cysteine-protease inhibitor *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (results not shown).

As prICE was previously shown to be inhibited by the specific YVAD-chloromethane [8], we next investigated the ability of YVAD-chloromethane to inhibit cleavage of purified bovine PARP [14] in an assay *in vitro*. Purified PARP (25 ng) was added to cytosol (10 μ g) from Molt-4 cells treated with vehicle (control cytosol) and incubated for 1 h at 37 °C. Cleavage of PARP to the apoptosis-specific fragment was not detected, indicating that the control cytosol does not contain the activated protease (Figure 3, bottom, lane 1). In contrast, cytosol from cells treated with C_6 -ceramide (20 μ M) for 6 h (active cytosol) contained the activated protease, which was capable of cleaving PARP (Figure 3, bottom, lane 2). Having established that this protease specifically cleaved PARP, we examined the effect of YVAD-chloromethane on PARP cleavage in this system. Following preincubation of the inhibitor (10 and 100 nM) with the active cytosol for 5 min, the PARP substrate (25 ng) was added and incubated for 1 h at 37 °C. Some reversal of PARP cleavage was seen with 10 nM YVAD-chloromethane (Figure 3, bottom, lane 3), and complete inhibition of cleavage was seen with 100 nM (Figure 3, bottom, lane 4). The results of these inhibitor studies are consistent with the established spectrum of sensitivity of prICE and confirm that ceramide cleaves PARP through activation of prICE.

In order to determine the role of prICE in mediating ceramide-induced death, the effect of Bcl-2 on PARP cleavage was investigated. Bcl-2 is known to inhibit apoptosis in response to a wide spectrum of agents, including chemotherapeutic agents, TNF α , ionizing radiation and activation of the FAS receptor [15,16]. Recently, overexpression of Bcl-2 has been shown to protect human T lymphoblastoid cell lines [17] and ALL-697 leukaemia cells [18] from ceramide-induced cell death. Bcl-2, however, did not interfere with ceramide formation in response to the chemotherapeutic agent vincristine, suggesting that Bcl-2 acts downstream of ceramide [18]. Although the mechanism of action of Bcl-2 is presently unknown, insight is provided by studies in *C. elegans*. In this organism, apoptotic cell death involves the action of several genes, including *ced-3* and *ced-9*. Ced-9 is the *C. elegans* homologue of Bcl-2, and Ced-3 is a homologue of prICE and related proteases (reviewed in [19]). Genetically, Ced-9 inhibits cell death mediated by Ced-3. It was,

therefore, important to determine whether Bcl-2 would inhibit ceramide-mediated activation of prICE.

Molt-4 cells stably expressing mouse Bcl-2 or control vector were utilized. After treatment of these cells with C_6 -ceramide (5, 10 or 20 μ M) for 6 h, cells expressing the *bcl-2* gene were unable to undergo the specific proteolytic cleavage of PARP in response to ceramide (Figure 4). These results provide the first biochemical evidence that Bcl-2 interferes with activation of prICE in intact cells, thus confirming and extending the genetic evidence from *C. elegans*. The mature forms of the ICE-like proteases are derived from precursors by proteolytic cleavage at Asp residues. Ongoing studies are now investigating the role of ceramide in the conversion of pro-prICE into prICE.

The demonstration that Bcl-2 inhibited activation of prICE in intact cells led to investigation of the importance of prICE in ceramide-mediated cell death. In cells treated with C_6 -ceramide (3–10 μ M) over a 24 h period, expression of Bcl-2 inhibited cell death, as measured by flow cytometric analyses and Trypan Blue studies (results not shown) [18]. These results establish that interference with PARP proteolysis is accompanied by inhibition of ceramide-induced death. Therefore activation of prICE is critical for ceramide-mediated apoptosis. As *ced-3* is the genetic target for the action of Ced-9, these results implicate prICE as a major mediator of programmed cell death.

These studies delineate an apoptosis-mediating pathway in which ceramide acts as a second messenger causing downstream activation of prICE by a mechanism that is inhibited by Bcl-2. Specifically, the data presented here provide important insights into signal transduction during the apoptotic program by: (1) implicating prICE in the ceramide signalling pathway and in the mechanism of ceramide-induced cell death; (2) providing the first evidence of a signal transduction pathway mediating proteolysis of PARP; and (3) establishing Bcl-2 as an inhibitor of the PARP protease. The results with Bcl-2 also corroborate the specificity of action of ceramide by establishing that PARP proteolysis is not a result of lipid-induced non-specific damage to cells.

The insight gained here into deciphering the mechanism of action of ceramide in transducing the apoptotic signal makes a very important contribution. Previously, ceramide has been shown to mediate the effects of TNF α in inducing apoptosis [2]. Additionally, endogenous ceramide levels have been shown to increase in response to a number of apoptotic agents (reviewed in [3]). Ceramide has also recently been shown to induce cleavage of fodrin during apoptosis [4], with possible implications for apoptosis-specific membrane blebbing. However, the exact role of ceramide as a mediator of cell death and its mechanism of action have remained undefined.

Ongoing studies are seeking to establish the mechanism by which ceramide mediates the proteolytic cleavage of PARP. Several candidate direct targets for ceramide have been proposed, including a protein phosphatase [20], a proline-directed protein kinase [21] and an isoform of protein kinase C (ζ) [22]. As addition of exogenous ceramide has no effect on sphingosine levels in this cell system [23], it appears that the effect of ceramide on PARP cleavage is not mediated through sphingosine. In order to investigate the role of the ceramide-activated protein phosphatase in mediating PARP cleavage, we utilized the phosphatase inhibitor okadaic acid. At concentrations between 3 and 10 nM, okadaic acid caused a partial reversal of PARP cleavage after treatment with 20 μ M C_6 -ceramide (results not shown). This implicates a potential role for ceramide-activated protein phosphatase in mediating ceramide-induced PARP cleavage. Ongoing studies, investigating the mechanism by which ceramide activates prICE and defining the role of Bcl-2 in this cascade, are expected to yield novel insights into signalling pathways in apoptosis.

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