Selective involvement of caspase-3 in ceramide induced apoptosis in AK-5 tumor cells

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Abstract Ceramide, a product of sphingomyelin metabolism, is a novel lipid second messenger that mediates diverse cellular functions. The present study demonstrates the activation of caspase-3/CPP-32β, during apoptosis induced by cell permeable exogenous ceramides, in AK-5 tumor, a spontaneously regressing rat histiocytoma. The apoptotic events were suppressed by the caspase-3 specific tetrapeptide inhibitor DEVD-CHO but not by the caspase-1 inhibitor YVAD-CMK. In cells overexpressing Bcl-2, a significant decrease in cell death was observed after exogenous addition of ceramides. Furthermore the processing of caspase-3 to its active form upon apoptotic stimulus, and the subsequent cleavage of the substrate PARP, suggested a central role for caspase-3 in the ceramide mediated apoptosis in AK-5 tumor cells.

Key words: Ceramide; Caspase; AK-5; Apoptosis

1. Introduction

Apoptosis is a cell autonomous death process requiring the orchestrated participation of endogenous cellular enzymes for the dismantling of the cell and to maintain cellular homeostasis. Recent investigations have identified several ICE/CED-3 related proteases, called 'caspases' [1] which play a crucial role in the execution of apoptosis. These include caspase-2 (NEDD-2/ICH-1), caspase-3 (CPP32β/YAMA/Apopain), caspase-4 (TX/ICH-2/ECI_{rel}II), caspase-5 (ICE_{rel}III), caspase-6 (Mch-2), caspase-7 (Mch-3/CMH-1/ICE-LAP3), caspase-8 (FLICE/MACH/Mch-5), caspase-9 (ICE-LAP6/Mch-6) and caspase-10 (Mch-4) (reviewed in [2]). All the members of the caspase family are cysteine proteases that possess the unusual ability to cleave substrates after aspartate residues. These proteases are synthesized as precursors which, upon receiving the apoptotic signals, are processed to active enzymes that dismantle the cell in an apoptotic cascade.

Ceramide, a product of sphingomyelin hydrolysis (reviewed in [3]), is considered a second messenger with pleiotropic effects. Increased levels of ceramide have been correlated with cell cycle arrest [4], terminal differentiation [5], cellular senescence [6], and apoptotic processes [7]. Work by Flores et al. demonstrated a dual role for ceramide, both protective and inductive in apoptosis [8]. The mechanism by which ceramide induces such cellular effects is poorly understood. Intense interest has focused on the potential involvement of diverse signaling processes in the regulation of ceramide mediated apoptosis.

Work in our laboratory has been focused on dissecting the molecular events involved in the cell death of AK-5 tumor, a spontaneously regressing rat histiocytoma [9]. We have earlier reported that apoptosis in AK-5 cells is induced by NK cells [10] and an unidentified factor present in the serum from AK-5 tumor rejecting animals. Both in vivo [11] and in vitro [12] studies demonstrate the role of specific caspases in tumor cell apoptosis and their functional redundancy.

The present study shows the selective involvement of caspase-3 [13], in tumor cell apoptosis, upon induction with cell permeable exogenous ceramides, the C2-ceramide (natural) and the C8-ceramide (synthetic), which is confirmed by the cleavage of poly(ADP-ribose) polymerase [14], the key cellular substrate of caspase-3. Tumor cells overexpressing the Bcl-2 gene suppressed the apoptotic effects of ceramide. The addition of a caspase-3 specific peptide inhibitor blocked the activation of caspase-3 and the subsequent cell death, suggesting that the activation of caspase-3 plays an essential role in the process of apoptosis, in AK-5 tumor cells following ceramide treatment.

2. Materials and methods

2.1. Animals and tumor

AK-5 tumor was maintained as ascites in an inbred colony of Wistar rats by injecting 5×10^6 tumor cells i.p. Animals which had been injected with tumor cells (5×10^6) subcutaneously and had rejected the tumor were used as the source for the anti-AK-5 serum. We have used a single cell clone of AK-5 adapted to grow in vitro, called BC-8, in all the studies, to avoid ambiguity in results due to tumor heterogeneity [15]. BC-8 cells were grown in suspension in DMEM supplemented with 10% (v/v) heat inactivated fetal calf serum in an atmosphere of 95% air and 5% CO_2. Cell viability was assessed by the trypan blue dye exclusion assay. The serum from tumor rejecting animals (immune serum) was used as a specific inducer of apoptosis (known positive control).

2.2. Ceramide treatment

AK-5 cells (2×10^6) in DMEM-FCS were treated with C2-ceramide isolated from bovine brain (30 μM final concentration) or C8-ceramide (Calbiochem) (40 μM concentration) for 8 h, at 37°C in a CO_2 incubator. The cells were washed and used for analysis.

2.3. Fluorescence microscopy

The normal and apoptotic cells were washed with PBS and fixed in 80% methanol. Cells were washed twice with PBS and stained with propidium iodide reagent (propidium iodide, Calbiochem, 50 μg/ml in 0.1% sodium citrate containing 0.1% Triton X-100) and observed under a fluorescence microscope (Nikon Optiphot).

2.4. DNA extraction and electrophoresis

Cells were washed and suspended in citrate phosphate buffer, and the fragmented DNA was extracted following the previously published procedure [16]. Fragmented DNA with the loading buffer was electrophoresed on 0.8% agarose gel at 2 V/cm for 16 h. The DNA in the gel was visualized under UV after staining with 5 μg/ml ethidium bromide.

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2.5. Western analysis

Samples were electrophoresed on a 10% SDS-polyacrylamide gel for 3 h at 30 mA. Separated proteins were transferred to a nitrocellulose membrane (Hybond C; Amersham Corp.) and probed with an appropriate dilution of the primary antibody for PARP and CPP32 respectively. The immune complex was detected using alkaline phosphatase conjugated anti-rabbit antibody.

2.6. Fluorogenic assays

The activities of ICE-like and CPP32-like proteases were assayed as described earlier [17]. Briefly, 10^6 cells were lysed in lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 10 mM Tris-HCl, pH 8, 2 mM DTT, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin) for 15 min at 4°C followed by centrifugation (20 000 × *g*) for 5 min. ICE-like and CPP32-like activities were detected in the supernatants by measuring the proteolytic cleavage of the fluorogenic substrates YVAD-AMC and DEVD-AMC [18] respectively in assay buffer (100 mM HEPES, 10% sucrose, 0.1% CHAPS, 2 mM DTT, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

2.7. Inhibitor competition experiments

Specificity for caspase-3-like activity was demonstrated by inhibition with DEVD-CHO, a specific tetrapeptide inhibitor for caspase-3-like proteases [19]. Aliquots containing 50 μg of protein were preincubated with increasing concentrations of DEVD-CHO for 30 min, followed by the addition of the fluorescent substrate and further incubation for 1 h at 30°C and the caspase-3 activity was determined.

3. Results and discussion

3.1. Ceramide induces apoptosis in AK-5 cells

Cell permeable analogues of ceramides were able to induce typical morphological changes of apoptosis in a time dependent manner, such as cell shrinkage, membrane blebbing and ruffling, chromatin condensation, formation of apoptotic bodies, as assessed by propidium iodide staining (Fig. 1). C2-ceramide induced apoptosis at 30 μM concentration and was more potent and induced high levels of apoptosis within 7–8 h, whereas C8-ceramide, used at 40–50 μM concentration, required longer incubation times. At higher concentrations, the apoptotic effect of ceramide declined, and cells showed morphological features of necrosis. The specificity of ceramides was confirmed using dihydroceramide, a structural ana-

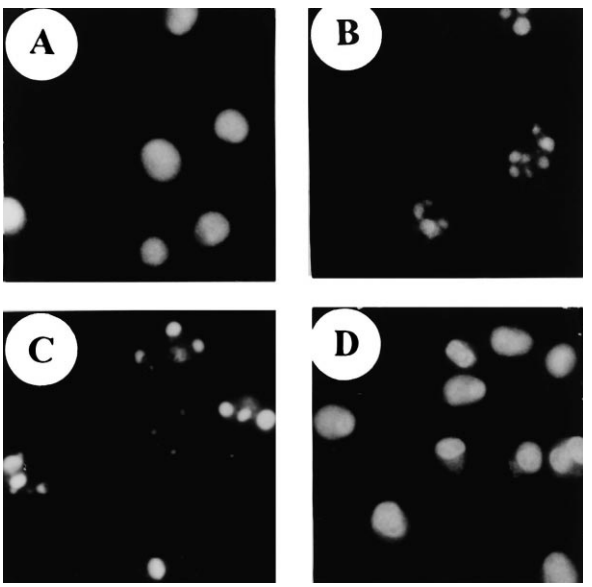

Fig. 1. Propidium iodide staining of AK-5 cells, after treatment with (B) 30 μM C2-Cer, (C) 50 μM C8-Cer, (D) 75 μM dihydroceramide; A: control BC-8 cells.

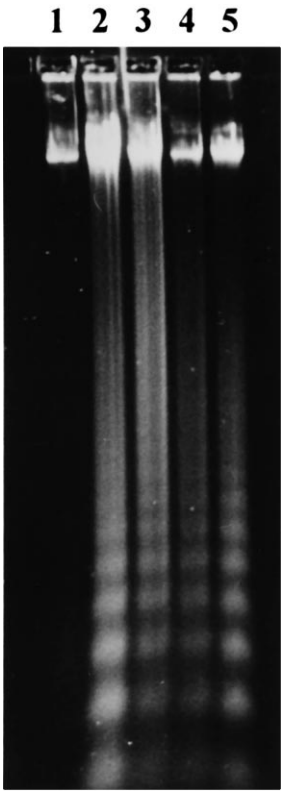

Fig. 2. Internucleosomal DNA cleavage of BC-8 cells as observed on 0.8% agarose gel stained with ethidium bromide. BC-8 cells treated with 1 μM dexamethasone (lane 2), 10% immune serum (lane 3), 30 μM C2-Cer (lane 4), 50 μM C8-Cer (lane 5). Lane 1 represents control cells. Dexamethasone and immune serum are known positive controls.

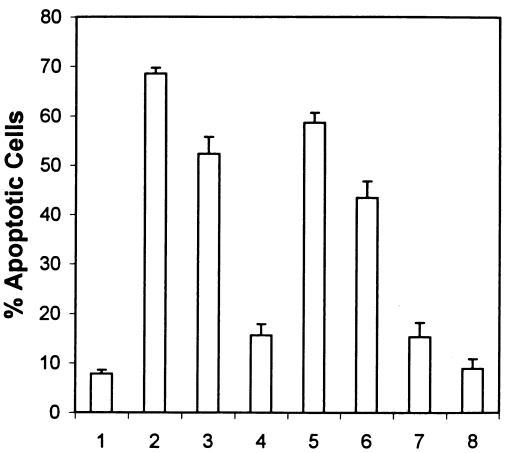

Fig. 3. Quantitative determination of apoptosis in BC-8 cells. Apoptosis was determined after staining cells with propidium iodide and flow cytometry. (1) Control cells, (2) cells treated with 30 μM C2-Cer, (3) cells pretreated with 200 μM YVAD-CMK for 1 h followed by treatment with C2-Cer, (4) cells pretreated with 50 μM DEVD-CHO for 1 h followed by treatment with C2-Cer, (5) cells treated with 50 μM C8-Cer, (6) cells pretreated with 200 μM YVAD-CMK for 1 h followed by treatment with C8-Cer, (7) cells pretreated with 50 μM DEVD-CHO for 1 h followed by treatment with C8-Cer, (8) Bcl-2 gene transfected AK-5 cells treated with 30 μM C2-Cer. Bars represent S.E.M.

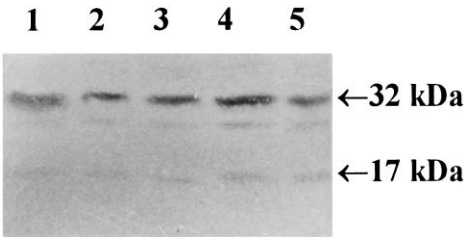

Fig. 4. Immunoblot showing the proteolytic processing of caspase-3/CPP32 by exogenous ceramides. Processing of the 32 kDa proform of caspase-3 to the 17 kDa active form by 1 μM dexamethasone (lane 2), 10% immune serum (lane 3), 30 μM C2-Cer (lane 4), and 50 μM C8-Cer (lane 5). Lane 1 represents untreated BC-8 cells. The faint extra band present in all the lanes is of a non-specific nature.

logue of ceramide, which lacks the C4–C5 *trans* double bond in the sphingolipid backbone, and failed to induce apoptosis (Fig. 1D) [20].

The fragmentation of DNA and formation of a characteristic ladder pattern, a hallmark of apoptosis [21], was confirmed by agarose gel electrophoresis of the fragmented DNA. A characteristic ladder pattern was visible after 6 h of treatment with ceramides, which was absent in uninduced cells (Fig. 2). The pattern produced was similar to that obtained from BC-8 cells treated with serum factor, a known trigger of apoptosis.

The role of caspases in ceramide induced apoptosis was assessed using caspase specific tetrapeptide inhibitors. Cells were pretreated with YVAD-CMK and DEVD-CHO, non-cleavable derivatives of the natural recognition site for caspase-1 and caspase-3-like proteases respectively [19], prior to the treatment with ceramide, and analyzed by flow cytometry. DEVD-CHO treated cells showed a marked reduction in apoptotic morphology whereas YVAD-CMK was ineffective (Fig. 3). This suggests a specific role for DEVD dependent protease in ceramide induced apoptosis. Cells overexpressing the Bcl-2 gene, a mammalian homolog of Ced-9 in *Caenorhabditis elegans* [22], were resistant to apoptosis induced by ceramides. This suggests that Bcl-2 is downregulating a key event in the apoptotic cascade, prior to the caspase activation.

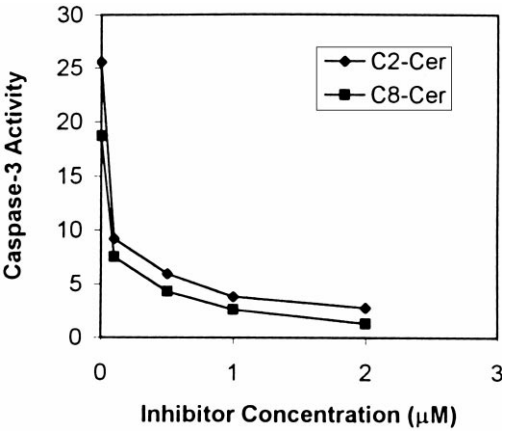

Fig. 6. Inhibition of DEVD dependent protease activity in ceramide treated cell extracts pretreated with various concentrations of the caspase-3 inhibitor DEVD-CHO. Caspase activity is expressed as relative fluorescence units.

3.2. Processing of caspase-3

Caspase-3 is synthesized in the cells as a 32 kDa proenzyme, and upon receiving a specific apoptotic stimulus, it is processed to an active form of 17 kDa. Immunoblotting experiments showed the specific processing of caspase-3 in ceramide treated cells using a monoclonal antibody for caspase-3, p17 subunit (Fig. 4). A marginal level of caspase-3 processing was observed in the untreated cells. The signal corresponding to the processed form of 17 kDa appears to be fainter probably due to the fact that not all cells in any induced culture are undergoing apoptosis at one time point because of heterogeneity in cell cycle events. Further, since caspase-3 acts in an autocatalytic mode, a lower concentration of the active enzyme may be sufficient to trigger the downstream cascade of apoptotic events.

3.3. Fluorogenic enzyme assays

The activation of specific caspases was also assessed using fluorogenic peptide substrates for ICE and CPP-32 respectively. An increase in DEVD dependent protease activity was readily detectable within 1 h of induction of apoptosis,

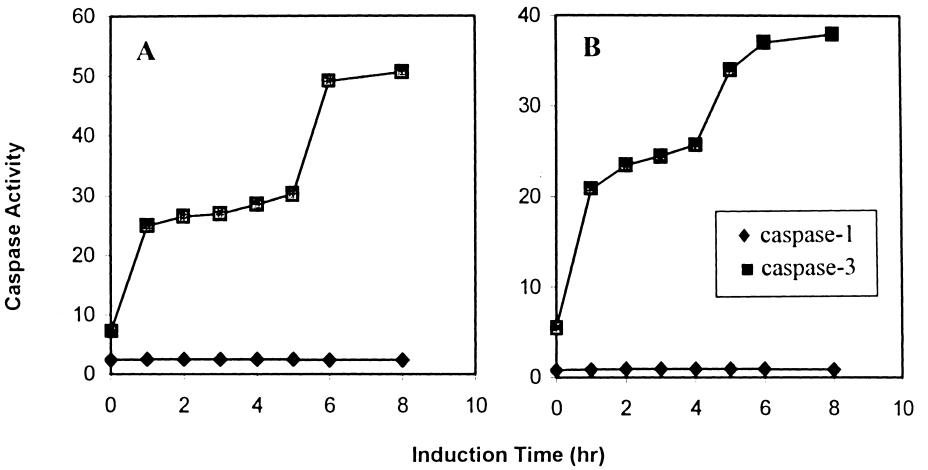

Fig. 5. Determination of DEVD dependent and YVAD dependent protease activities in cells treated with (A) 30 μM C2-Cer and (B) 50 μM C8-Cer for different time intervals. Caspase activity is expressed as relative fluorescence units.

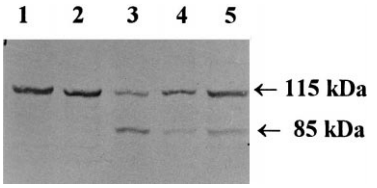

Fig. 7. Immunoblot showing the cleavage of PARP to the 85 kDa fragment upon treatment of cells with immune serum (lane 3), 30 μM C2-Cer (lane 4), 50 μM C8-Cer (lane 5). Lanes 1 and 2 represent untreated control cells and cells treated with dihydroceramide, respectively.

which is several fold higher in the induced extract than in the uninduced one (Fig. 5), whereas there was no detectable YVAD dependent protease activity. The low level of DEVD-AMC cleavage activity in untreated cells may be a consequence of the low percentage of apoptotic cells in the normal cell population. These results indicate that DEVD dependent protease activity is responsible for apoptotic characteristics induced by the ceramides. The lack of involvement of caspase-1 in the induction of apoptosis is not surprising, given the fact that caspase-1 knockout mice do not show a general defect in apoptosis [23,24] whereas disruption of the caspase-3 gene in knockout mice affects the morphogenetic cell death in the central nervous system, leading to abnormal brain development [25].

We next examined whether the activation of caspase-3 is inhibited by DEVD-CHO, its specific inhibitor. In our experiments pretreatment of the extracts with 1 μM DEVD-CHO totally blocked AMC liberation, and about 50% inhibition was obtained with a dose of 10 nM (Fig. 6).

3.4. Cleavage of PARP

In order to confirm the involvement of caspase-3-like protease in the induction of apoptosis in AK-5 tumor cells, the cleavage of the DNA repair enzyme PARP [14] and endogenous substrate of caspase-3 was monitored. Immunoblotting with a polyclonal antibody for PARP showed the specific cleavage of PARP to its signature fragment of 85 kDa (Fig. 7). Proteolytic cleavage of PARP precludes the catalytic domain of PARP from being recruited to the sites of DNA damage and presumably disables PARP from coordinating subsequent genome maintenance.

It is known that in the family of caspases, caspase-3 has the highest sequence homology and substrate specificity to Ced-3, the prototype cell death gene in nematodes [13]. It is also activated by a variety of agents that induce apoptosis [26,27], which was also proved in a cell-free system [19].

Overall these studies have established that caspase-3 is a key player during ceramide induced apoptosis in AK-5 tumor cells, and inhibition of caspase-3 activity blocks apoptosis. Furthermore, the results suggest that caspase-1 does not play a significant role, as is true in other systems. This implies that the mechanisms leading to the activation of distinct caspases are highly specific and redundant. The AK-5 tumor model may provide insight into the tissue specific expression of caspases in tumors and their regulatory mechanisms.

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