Differential involvement of caspases in apoptosis of myeloid leukemic cells induced by chemotherapy versus growth factor withdrawal

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Abstract To assess the potential involvement of the caspase family in the IL-3-dependent murine myeloid leukemic cell line 32D, we studied the effect of bcl-2, crmA and three synthetic caspase inhibitors on apoptosis induced by chemotherapy or IL-3 withdrawal. Apoptosis induced by IL-3 deprivation or by ActD appears to be mediated by a crmA-insensitive pathway. Cell death by IL-3 withdrawal is inhibited by the caspase-inhibitor ZVAD-fmk, but not DEVD-fmk or YVAD-cmk. In contrast, DEVD-fmk as well as ZVAD-fmk protect 32D cells from ActD-induced apoptosis. These results indicate that different caspases are involved in apoptosis induced by growth factor withdrawal and by chemotherapy.

Key words: Caspase; Apoptosis; Myeloid leukemia

1. Introduction

The biochemical pathways leading to apoptosis remain poorly understood. However, the recent demonstration that the nematode death gene *ced-3* encodes a cysteine protease related to the mammalian interleukin-1-β converting enzyme (ICE) has led to the identification of a large family of cysteine proteases (caspases) which, on the basis of phylogenetic analysis, can be divided into three subfamilies: the CASP-1 subfamily (CASP-1, -4, -5, -11, -12), the CASP-3 subfamily (CASP-3, -6, -7, -8, -10) and a third family which only contains CASP-2 and CASP-9 [1–5]. Transient overexpression of each member of the human and murine caspase families induces apoptosis in mammalian cells. Convincing evidence for a functional role of the caspase family in Fas- and TNF-mediated apoptosis of eukaryotic cells has come from studies with the viral encoded caspase inhibitors crmA and baculovirus p35 and the use of peptide-based synthetic inhibitors of the caspase family [6–10]. In contrast, only few studies have been performed to analyze the role of the caspase family in apoptosis of growth factor-dependent leukemic cells [11].

The present studies show that apoptosis of murine IL-3-dependent myeloid leukemic cells (32D) induced by actinomycin D (ActD) or growth factor deprivation is mediated by a crmA-insensitive mechanism. In addition, we demonstrate that different caspases are involved in apoptotic death induced by either ActD or IL-3 deprivation.

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2. Materials and methods

The protease inhibitors ZVAD-fmk and DEVD-fmk were purchased from Enzyme Systems Products (Dublin, CA). Ac-YVAD-cmk from Calbiochem (Cambridge, MA). The substrates *N*-acetyl-YVAD-pNA and *N*-acetyl-DEVD-pNA were obtained from Biomol (Plymouth Meeting, PA). ActD and puromycin were obtained from Sigma Chemicals Co. (St. Louis, MO). The XTT test was purchased from Boehringer-Mannheim. Murine IL-3 was kindly provided by Sandoz (Vienna, Austria). FITC-labeled Annexin V was obtained from Bender and Co. (Vienna, Austria).

2.1. Cell culture and transfections

The IL-3-dependent murine leukemic cell line 32D was maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and 10% WEHI-3B cell-conditioned medium as a source of murine IL-3. CrmA-expressing 32D cells were generated by cotransfection of 8 μg of pCAGGS-CrmA [12] and 1.8 μg pBSpacdp (puromycin) by electroporation. Stable transfectants were selected in the presence of 1 μg/ml puromycin. Positive crmA clones were identified by Western blotting. The isolation and characterization of 32D+bcl-2 cells has been described previously [13].

2.2. Induction and analysis of apoptosis

In experiments analyzing apoptosis, cells were washed 3 times and resuspended at 5×10^5 cells/ml in the presence or absence of IL-3 (10 ng/ml) or ActD (1 μg/ml). When appropriate, 32D cells were pre-incubated for 3 h with caspase inhibitors. Viable cells were counted by a trypan blue exclusion assay. Cell survival was also determined with the XTT test. In parallel, apoptosis was quantified by flow cytometry using FITC–Annexin V (0.3 μg/ml) [14] and CELL-Quest software.

2.3. Protease assays

After stimulation, 32D cells were washed and resuspended in ice-cold lysis buffer (50 mM HEPES (pH 7.5), 1 mM EDTA, 5 mM DTT, 0.5% CHAPS, 10 μg/ml pefablock and 5 μg/ml leupeptin). After centrifugation at $10\,000\times g$ for 10 min at 4°C, supernatant was used for the caspase assay. Protease assays included 90 μl of reaction buffer (100 mM HEPES (pH 7.5), 10% sucrose, 0.1% CHAPS, 10 mM DTT), 10 μl of cell lysate (25 μg protein) and 50 μl of substrate (100 μM final concentration of YVAD-pNA or DEVD-pNA). Samples were incubated at 30°C for 30 min in a microtiter plate reader. Enzyme-catalyzed release of *p*-nitroanilide was monitored at 405 nm. Under these conditions, conversion of the substrates was linear in time and amount of protein.

3. Results and discussion

To determine whether apoptosis in 32D cells is dependent on caspases, we generated 32D clones which stably overexpress crmA. In parallel we also analyzed the effect of bcl-2 overexpression, because the latter has been shown to regulate apoptosis and caspase activity in many cell lines [15]. Apoptotic cell death was induced either by IL-3 deprivation or by ActD treatment and analyzed as described in Section 2. CrmA did not inhibit apoptosis induced by IL-3 deprivation or by ActD treatment (Table 1). In contrast, 32D+bcl-2 cells were markedly protected against cell death under both conditions.

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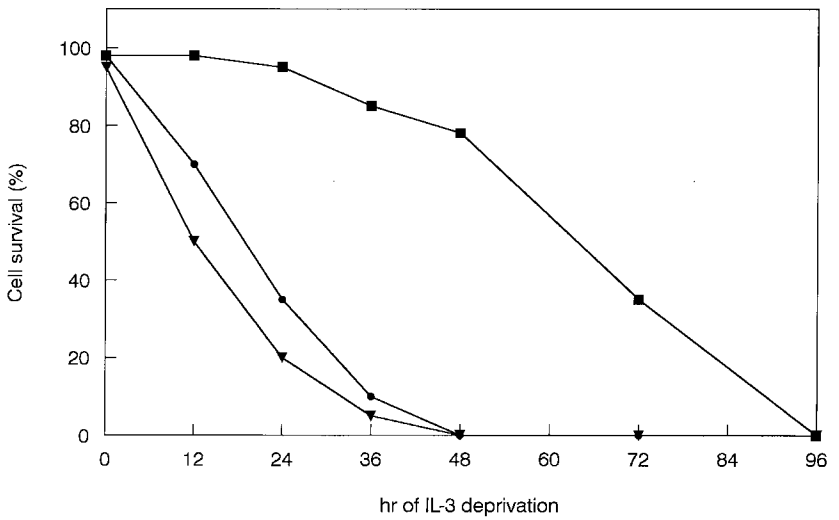

Fig. 1. Time kinetics of apoptosis induced by IL-3 deprivation in 32D cells expressing either crmA (▼), bcl-2 (■) or control vector (●). Cells were IL-3 deprived for various time periods. Cell viability was determined by trypan blue exclusion and is expressed as the % trypan blue-negative cells compared to cells cultured in IL-3-supplemented medium.

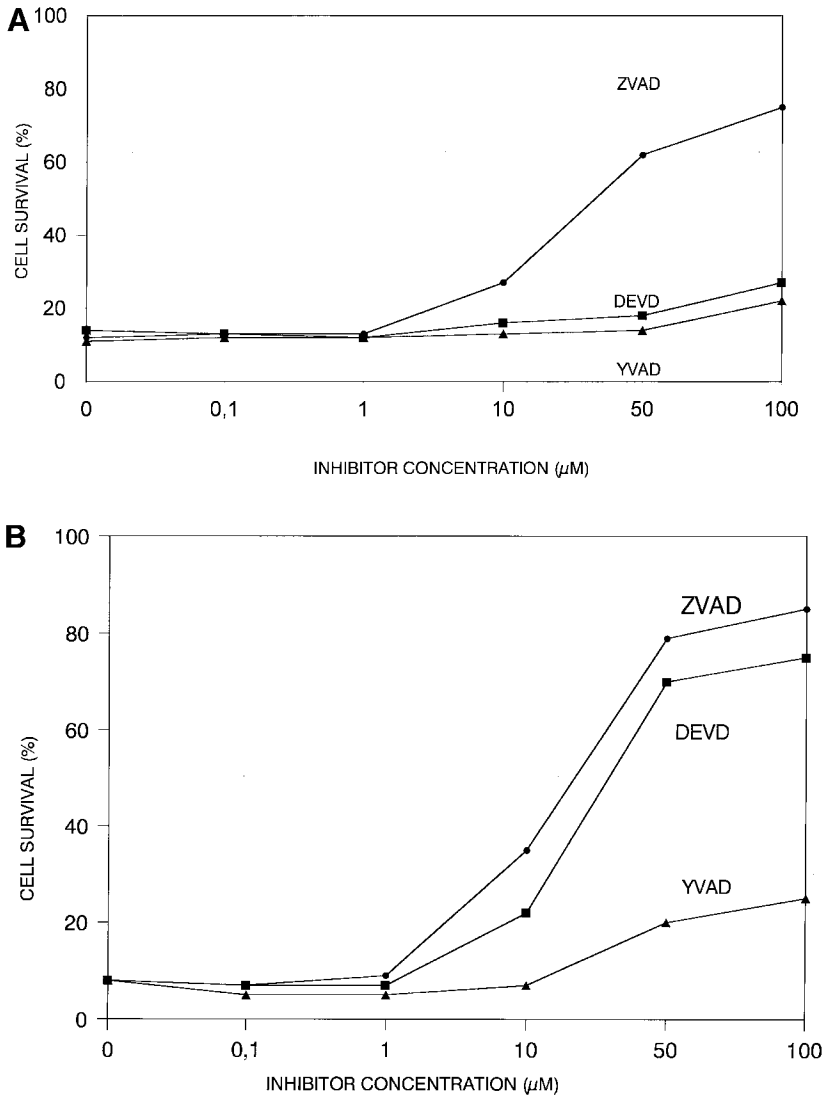

Fig. 2. Dose–response curve of various caspase inhibitors on apoptotic death induced by IL-3 deprivation (A) or ActD treatment (B). Cell viability was determined by XTT test. Experiments were performed in triplicate and % survival is expressed as % of 32D cells kept in IL-3-supplemented medium. ZVAD-fmk (●); DEVD-fmk (■); YVAD-cmk (▲).

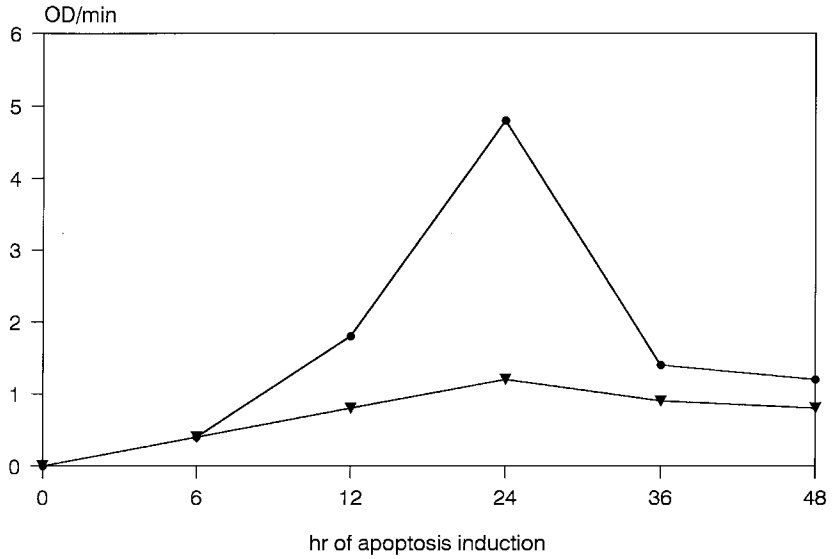

Fig. 3. Effect of IL-3 deprivation and ActD on caspase activation. Lysates from 32D cells deprived of IL-3 (▼) or treated with ActD (●) for several time periods were assayed for protease activity towards DEVD-pNA. Data shown are from a single experiment and are representative of three independent experiments.

Similar results were obtained when we quantified apoptotic death on the FACScan using Annexin V [14]. A 50% increase in Annexin V-positive cells was observed after IL-3 deprivation for 24 h in empty vector or crmA-transfected 32D cells. However, no marked change in Annexin V-positive cells was detected in 32D+bcl-2 cells (data not shown). Analysis of the time kinetics of IL-3 withdrawal induced apoptosis revealed that the protective effect of bcl-2 expression reflects a delay of apoptosis rather than a complete inhibition of apoptosis (Fig. 1). The finding that crmA has no effect on apoptosis in 32D cells induced by chemotherapy is consistent with studies from Datta et al. who recently reported that crmA does not inhibit apoptosis induced by ara-C and other chemotherapeutic agents in the myeloid leukemic cell line U937 [16].

Because crmA only inhibits specific members of the caspase family, several cell permeable synthetic caspase inhibitors were used to study the possible role of crmA-insensitive caspases in apoptosis of 32D cells. YVAD-cmk is a specific CASP-1 inhibitor, while DEVD-fmk is known to be an inhibitor of the CASP-3-like proteases. ZVAD-fmk has been described as a more general caspase inhibitor [3,17]. Fig. 2A shows that apoptosis induced by growth factor deprivation for 48 h is inhibited by increasing concentrations of ZVAD-fmk, but not by DEVD-fmk or YVAD-cmk. In contrast, ActD-induced cell death in 32D cells was prevented by ZVAD-fmk as well as DEVD-fmk but not YVAD-cmk (Fig. 2B). These data demonstrate that apoptosis induced by ActD treatment and by IL-3 deprivation is differentially mediated by DEVD-sensitive and DEVD-insensitive mechanisms, respectively.

Table 1
Effect of CrmA or Bcl-2 expression on apoptosis induced by IL-3 deprivation or ActD treatment in 32D cells

	% Survival	
Transfectant	−IL-3	+ActD
Empty vector	19	16
CrmA	18	15
Bcl-2	67	65

32D clones were IL-3 deprived for 24 h or treated with 1 μg/ml ActD for 18 h. Cell viability was determined by a XTT test. % Survival is expressed as % of the value obtained for cells growing in IL-3 supplemented medium (100%). Experiments were performed in triplicate. The SD was less than 10%. Similar results were obtained with independent cell clones expressing either crmA or bcl-2.

Caspase activity was subsequently assayed in lysates made from 32D cells that were treated with ActD or deprived of IL-3 for different time periods. CASP-1- and CASP-3-like activity was measured on the specific tetrapeptide substrates, YVAD-pNA and DEVD-pNA respectively. No clear protease activity towards YVDA-pNA was detected in lysates prepared from cells that were induced to undergo apoptosis by IL-3 deprivation or ActD treatment (data not shown). In contrast, ActD treatment was associated with a marked increase in DEVD-pNA cleavage activity compared to apoptosis induction by IL-3 withdrawal which only marginally stimulated the cleavage of DEVD-pNA (Fig. 3). These findings of protease activation are consistent with the results obtained with the synthetic inhibitors as described above.

In conclusion, the data in this study demonstrate that members of the caspase family are involved in apoptosis of growth factor dependent myeloid leukemic cells. A crmA-insensitive mechanism is apparently involved in apoptosis induced by chemotherapy or by growth factor withdrawal in 32D cells. This is in contrast to Fas- and TNF-induced apoptosis, which occurs through a crmA-sensitive step [6]. Different capsases are activated during apoptosis induced by growth factor withdrawal or by chemotherapy, since members of the CASP-3-like proteases are clearly involved in apoptosis mediated by chemotherapy but not by IL-3 deprivation. CASP-1 is not activated upon any of the apoptotic stimuli. Further experiments to elucidate the mechanism of this stimulus-dependent caspase activation are in progress.

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