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Inhibition of caspase activity induces a switch from apoptosis to necrosis

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Abstract The role of caspases in B lymphocyte cell death was investigated by using two broad spectrum inhibitors of the caspase family, Z-Asp-cmk and Z-VAD-fmk. They totally prevented spontaneous and drug-induced apoptosis and inhibited the CPP32/caspase-3-like activity exhibited by apoptotic cells. However, the suppression of apoptosis was not associated with a long-term increase of cell survival, but conversely, with a switch from apoptotic death to the necrotic form. These results strongly suggest that apoptosis and necrosis share common initiation pathways, the final issue being determined by the presence of an active caspase.

Key words: Caspase; Caspase inhibitor; Apoptosis; Necrosis; Dexamethasone; B lymphocyte

1. Introduction

Apoptosis, considered the physiological form of cell demise, as opposed to necrosis or accidental cell death, is an active process with distinct morphological and biochemical features [1–3]. Thus, apoptotic cells are characterized by condensed and fragmented nuclei, whereas necrotic cells present loss of plasma membrane integrity without apparent damage to nuclei. However, these two apparently opposite forms of cell death can be elicited by the same stimuli, according to their intensities [4,5], suggesting that initial common events could be shared by apoptosis and necrosis.

Apoptosis requires tightly regulated death pathways, including activation of cysteine proteases of the caspase family. These are characterized by the cleavage of specific substrates after an aspartic residue, and considered essential in the execution stage of the apoptotic process [6–10]. Caspase-3/CPP32-like proteases appear to be important in apoptosis of mammalian cells [11–14].

Peptide inhibitors, designed to mimic known sequences of caspase substrates, have been shown to suppress apoptosis in a number of cell culture systems, with the general assumption that inhibition of apoptosis would lead to cell survival. However, the long-term effect of caspase inhibition has rarely been investigated and contradictory results have been reported on whether the death process is completely prevented or only delayed [15–17]. It is also unknown whether the death occurring later is through necrosis or apoptosis.

The present study was undertaken to investigate the requirement for caspase activity in spontaneous and in drug-induced apoptosis in B lymphocytes. CPP32/caspase-3-like activity was essential in both cases. Surprisingly, while peptide caspase inhibitors totally inhibited apoptotic features, they did not improve long-term cell survival because they only deviated the apoptotic process to the necrotic form of cell death. The data suggest that caspase activity is determinant in the choice between apoptosis and necrosis.

2. Materials and methods

2.1. Materials

The caspase inhibitors Z-Asp-cmk and Z-VAD-fmk were purchased from Bachem Biochimie (France). The caspase substrates YVAD-pNA and DEVD-pNA were purchased from Alexis Biochemicals (Coger, France). The culture medium used throughout was RPMI 1640 (Biomedia, France) supplemented with 25 mM HEPES, 2 mM L-glutamine, standard antibiotics, 50 μM 2-mercaptoethanol and 8% heat-inactivated fetal calf serum (Biomedia, France). Dibutyryl-cAMP (dbcAMP), dexamethasone (DEX), 6-diamidino-2-phenylindole (DAPI), fluorescein diacetate (FDA), ethidium bromide (EtBr), hydrogen peroxide (H_2O_2) and the kit for lactate dehydrogenase analysis were purchased from Sigma (St. Louis, MO, USA).

2.2. B lymphocyte preparation and culture

Female C57BL/6 mice were purchased at 6–8 weeks of age (Iffa Credo, France). Mature B lymphocytes were purified as previously described [18]. Briefly, splenocytes ($3–4\times10^6$/ml in RPMI medium) were treated with anti-T cell monoclonal antibodies (Tebu, France) at 4°C for 60 min followed by incubation with guinea pig complement at 37°C for 60 min (Tebu, France). Cells were layered on top of a discontinuous Percoll gradient and cells at the 65–70% interface were recovered. B cells, at 5×10^5 cells/ml, were cultured in 24-well plates (Nunclon) in 6% CO_2 at 37°C. For cytometric analysis, cells were fixed in 70% ethanol and stored at −20°C.

2.3. Assessment of cell viability and cell death

Cells (10^6 cells/ml) were washed with PBS and then double-stained with FDA (1 μg/ml) and EtBr (10 μg/ml) for 5 min at 37°C. FDA, after being taken up by live cells, is cleaved by intracellular esterases, yielding green fluorescent fluorescein retained in the cytoplasm. EtBr, a non-specific DNA intercalating agent, is excluded by the plasma membrane of living cells and hence only dead cells exhibited orange-red fluorescent nuclei [19]. Quantitative analysis of each population was performed by counting more than 500 cells discriminated as viable (green fluorescence and unstained normal nucleus), apoptotic (red condensed or fragmented nucleus) and necrotic (red 'apparently normal' or patchy nucleus). Cell death was also assessed by measuring LDH release according to the manufacturer's instructions. LDH leakage was expressed as follows: 100×LDH in supernatants/(cytoplasmic LDH+LDH in supernatants). The results are presented as the mean of three experiments ± S.D.

2.4. Apoptosis determination by flow cytometry

Fixed cells (10^6 cells/ml) were washed twice with HBSS and then stained with DAPI (2.5 μg/ml) at 37°C for 30 min. DNA content was quantified by flow cytometric analysis performed on a PARTEC CA II flow cytometer (Chemunex France) equipped with a 100 W mercury lamp (type HBO). Fluorescence at 455 nm was recorded as a function of DNA content. Each histogram was generated until the analysis reached at least 15 000 cells. The percentage of apoptosis was determined from the sub-G1 events.

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Abbreviations: DEX, dexamethasone; dbcAMP, dibutyryl-cAMP; pNA, *para*-nitroaniline

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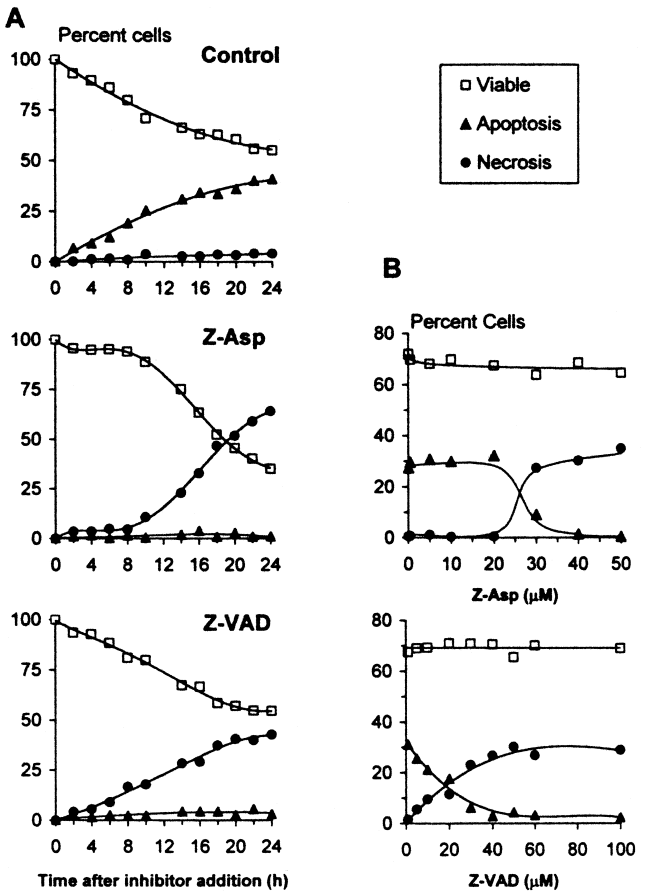

Fig. 1. Inhibition of apoptosis and induction of necrosis in B lymphocytes by caspase inhibitors. A: Time course of apoptosis: cells were incubated in the absence or the presence of Z-Asp-cmk (50 μM) and Z-VAD-fmk (100 μM), and the percentage of viable, apoptotic and necrotic cells was determined morphologically (see Section 2.3). B: Dose response of Z-Asp-cmk and Z-VAD-fmk measured after 16 h of culture.

2.5. Analysis of DNA fragmentation in agarose gels

DNA from 5×10^5 B lymphocytes was processed as previously described [20]. Briefly, after cell lysis, DNA was extracted and precipitated with 70% ethanol at −20°C overnight. Electrophoresis was performed in 1% agarose gel containing 1 μg/ml EtBr. DNA was visualized in UV light.

2.6. Determination of [^{3}H]thymidine incorporation

B lymphocytes (10^5) were cultured in 96-well microtiter plates in a 0.2 ml final volume per well in the presence of various stimuli. Proliferation was assessed on day 3 by pulsing cultures with 0.5 μCi of tritiated thymidine ([^{3}H]TdR, Amersham, Les Ulis, France) 6 h before harvesting. [^{3}H]TdR uptake was expressed as mean cpm for triplicate cultures.

2.7. Caspase assay

After 8 h culture in the presence or absence of drugs, as indicated, B lymphocytes (5×10^6) were washed and lysed in Triton X-100 buffer (0.5% Triton X-100, 2 mM EDTA, 1 mM PMSF) for 20 min on ice. Cell lysates were added with 0.5 ml of ICE buffer (100 mM HEPES-KOH pH 7.5, 10% sucrose, 10 mM DTT and 0.1% CHAPS) containing 100 μM DEVD-pNA or YVAD-pNA caspase substrates, and then incubated for 1 h and 10 h at 37°C. Enzyme-catalyzed release of pNA was monitored at 405 nm.

3. Results and discussion

3.1. Caspase inhibitors induce a shift from spontaneous apoptosis to necrosis

In order to determine the role of caspases in B lymphocyte apoptosis we tested the effect of Z-Asp-cmk and Z-VAD-fmk, two broad spectrum and irreversible inhibitors of the caspase family. Consistent with previous reports [21], B lymphocytes in vitro underwent a time-dependent cell death by apoptosis (Fig. 1A). Z-Asp-cmk (50 μM) and Z-VAD-fmk (100 μM) totally prevented the apoptotic process as assessed by morphological observation (Fig. 1B), cytometric analysis and laddering of DNA (Fig. 2A,B). However, the suppression of apoptosis was not associated with a long-term increase of cell viability, but conversely was linked to an induction of necrosis, as determined by counting cells after staining with FDA and EtBr (Fig. 1A and see Section 2.3). As can be seen in Fig. 1, the two peptides appeared to act in a different way since a threshold of more than 20 μM of Z-Asp-cmk was required to prevent apoptosis and to deviate the form of cell death, whereas Z-VAD-fmk, dose-dependently, inhibited the onset of apoptosis with a concomitant shift to necrosis. In addition, at their effective dose, Z-Asp-cmk, but not Z-VAD-fmk, led to a transitional increase of cell viability when compared to controls, accompanied by a delay of about 8 h in the switch to necrosis (Fig. 1A). Since long-term effects of both inhibitors were similar, we only present the results obtained with Z-VAD-fmk which has a higher specificity for the caspase family.

Cell death analysis, as evaluated by LDH leakage from dying cells, confirmed that Z-VAD-fmk did not prevent cell death since LDH release after 6 h and 24 h was not reduced when compared to controls (Fig. 2C). Cell survival was also

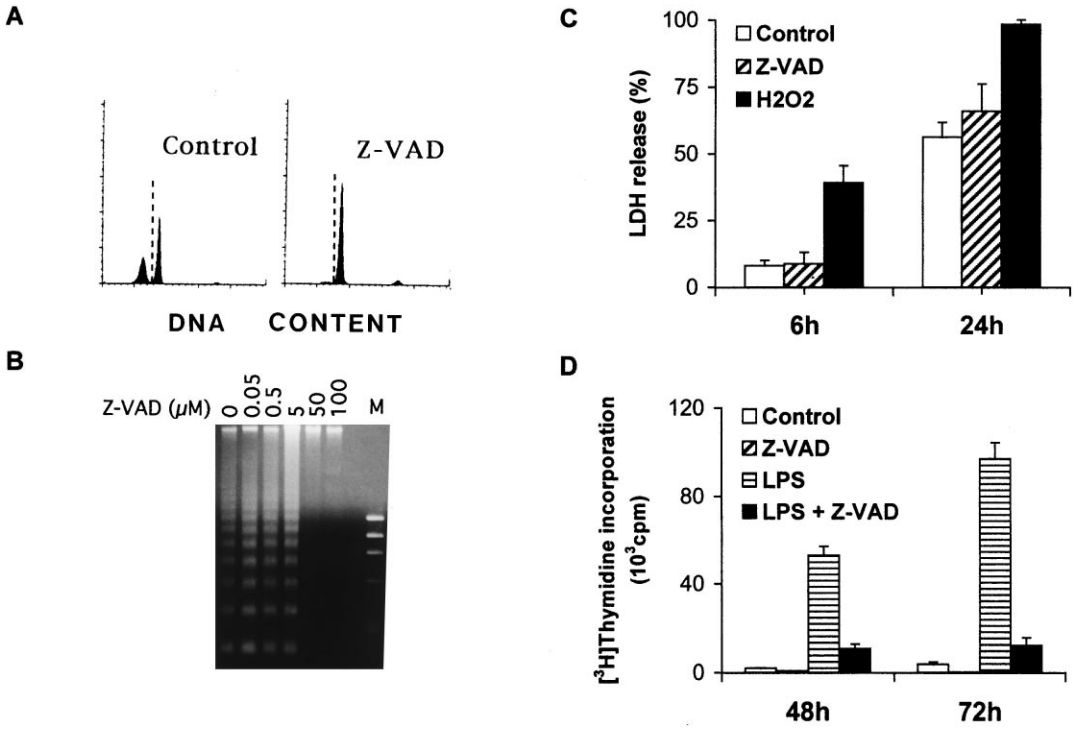

Fig. 2. Prevention of apoptosis without improvement of cell viability by caspase inhibition. A: Cytogram showing the disappearance of the sub-G1 apoptotic peak, to the left of the dotted line, in Z-VAD-fmk-treated cells. B: Agarose gel electrophoresis of total DNA from B lymphocytes cultured for 24 h in the absence or the presence of increasing doses of Z-VAD-fmk. Lane M: ladder DNA markers. C: Cell death as measured by LDH leakage from dead cells; H_2O_2 (1 mM) was used as a positive control of necrosis. D: Cell growth as determined by thymidine incorporation on days 2 and 3 by cells cultured in the absence or the presence of LPS with or without Z-VAD-fmk (100 μM); results are expressed as the mean of three distinct experiments ± S.D.

assessed by measuring cell growth. Z-VAD-fmk did not improve thymidine uptake by B lymphocytes and greatly impaired LPS-induced B cell proliferation on days two and three (Fig. 2D). This is in agreement with data showing that the proliferation potential of growth factor-dependent cell lines [17], of fibroblasts [16] and of Ramos BL cells [22] could not be restored by inhibiting apoptosis through caspase inhibition. On the other hand, repression of staurosporine-induced apoptosis and of Fas-mediated apoptosis has been reported to be associated with maintenance of viability and proliferation [15,23].

3.2. *Drug-induced apoptosis is inhibited by Z-VAD-fmk and shifted to necrosis*

The switch from apoptosis to necrosis was also observed when apoptosis was increased by drug treatment of B lymphocytes. Consistent with previous reports [24,25], an important time-dependent increase of apoptosis was observed in B cells cultured in the presence of DEX or dbcAMP (Fig. 3A). The presence of Z-VAD-fmk inhibited apoptosis, as demonstrated by electrophoresis of DNA (Fig. 3B). To further investigate whether the shift to necrosis was a consequence of caspase inhibition and was not associated with an intrinsic activity of the drug itself, the time of addition of Z-VAD-fmk was delayed relative to that of drugs. Fig. 3C shows that pretreatment (−4 h) with Z-VAD-fmk did not influence the intensity of the switch between apoptosis and necrosis observed when cells were cultured for 24 h in the presence of Z-VAD-fmk and drugs added simultaneously at T_0 (0). In contrast, when Z-VAD-fmk addition was delayed for 4 h, 6 h or 8 h, the apoptotic process being already triggered in some cells, those cells committed to apoptosis could no longer be deviated to the necrotic form of cell death. The deviation to necrosis was thus progressively lost.

The present data indicate that, in B lymphocytes, once the signaling pathway leading to caspase activation has been initiated, its inhibition leads to necrosis. These results are in apparent contradiction to the general assumption that inhibition of caspase would rescue cells from death. For example, Z-VAD-fmk appeared to increase cell survival in Ramos BL cells [22], in B-CLL [26], and in myeloid leukemic cell lines [27], supporting a difference of the susceptibility to death between cell lines or transformed cells and normal primary cells. This could be linked to higher levels of ATP expressed by the latter, since recent findings have demonstrated the essential role of appropriate energy supply in the execution phase of apoptosis. Accordingly, ATP depletion could shift the death mechanism to necrosis [28,29]. Recently, a switch from apoptosis to necrosis has been observed in thymocytes cultured in the presence of inducers of permeability transition and caspase inhibitors; it was suggested that alteration of mitochondrial functions could constitute a common event in both forms of cell death, the choice being determined by a caspase event [30]. The present findings argue for such a hypothesis and clearly demonstrate that specific inhibition of potentially active caspase counteracts only the form of cell death but not the death itself.

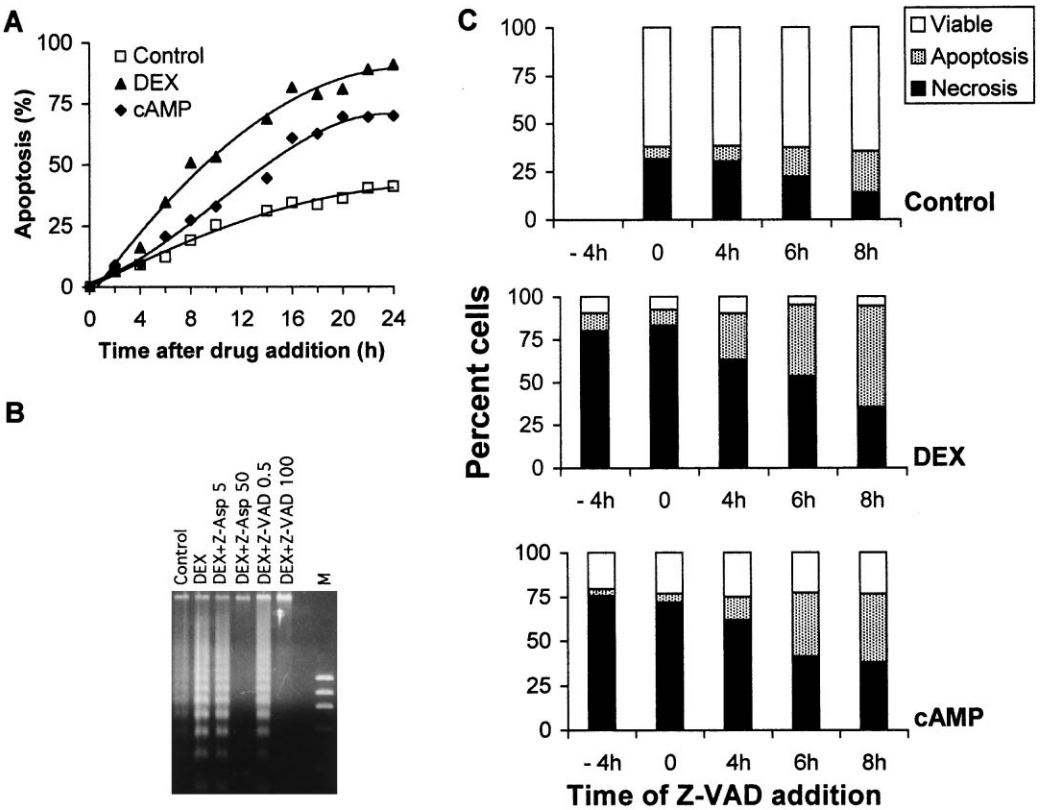

Fig. 3. Time-dependent switch from apoptosis to necrosis. B lymphocytes were cultured in the presence or the absence of dexamethasone (DEX, 5 nM) or dbcAMP (cAMP, 100 μM). A: Time course of apoptosis induction. B: Electrophoresis of DNA from B lymphocytes cultured for 24 h in the absence or the presence of DEX with or without Z-Asp-cmk and Z-VAD-fmk, concentrations indicated as μM. C: Frequency of viable, apoptotic or necrotic cells was assessed 24 h after culturing in the presence or the absence of DEX or cAMP, whereas Z-VAD-fmk (100 μM) was added before (−4 h), simultaneously (0), or after (4 h, 6 h, 8 h) the initiation of cultures.

3.3. *Caspase-3-like protease is activated during spontaneous and drug-induced apoptosis*

To characterize the type of caspase involved in B lymphocyte apoptosis, the presence of ICE/caspase-1-like or CPP32/caspase-3-like activity in cytoplasmic extracts was investigated, by measuring the cleavage of their respective substrates, YVAD-pNA or DEVD-pNA. Apoptotic cells were totally devoid of ICE/caspase-1-like activity (data not shown). In contrast, Fig. 4 shows that spontaneous as well as DEX- and dbcAMP-induced apoptotic cells cleaved the CPP32/caspase-3-like DEVD-pNA substrate. In every case, this activity was abrogated by the two peptide inhibitors Z-Asp-cmk and Z-VAD-fmk. CPP32/caspase-3 activation has been implicated in the apoptosis of Ramos BL B cell line [22] and in that of B-chronic lymphocytic leukemia cells [26]. The present study demonstrates, for the first time, its implication in the apoptosis of normal primary B lymphocytes, supporting the importance of CPP32/caspase-3-like proteases in the B cell lineage.

In conclusion, although necrosis and apoptosis have long been considered unrelated phenomena, recent reports suggest that the two forms of death might not be so distinct as initially thought. However, the signaling pathway leading to the choice between life and death or between the two forms of cell death is still unknown. The present data suggest that the commitment to apoptotic or to necrotic cell death is a common event that could involve caspase expression as well, the final decision being linked to the capacity of caspases to exert their activity.

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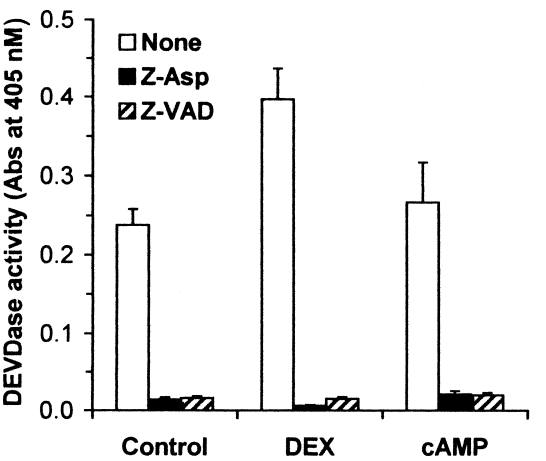

Fig. 4. Caspase-3-like activity in apoptotic cells. B lymphocytes were cultured for 8 h in the presence or the absence of drugs, with or without caspase inhibitors. Hydrolysis of DEVD-pNA substrate was measured after 10 h at 405 nm.

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