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IL-4 inhibits apoptosis and prevents mitochondrial damage without inducing the switch to necrosis observed with caspase inhibitors

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Abstract

We previously demonstrated that the broad-spectrum caspase inhibitor, zVAD-fmk, totally deviated apoptosis to necrosis in B lymphocytes. We report here that, in contrast with zVAD-fmk, IL-4 protected B cells from spontaneous and from dexamethasone-induced apoptosis and actually maintained cell viability. This was assessed by morphological and biochemical criteria and accompanied by the maintenance of mitochondrial transmembrane potential ($\Delta\Psi$m) and elevated glutathione (GSH) levels. Under these conditions, zVAD-fmk also totally inhibited apoptosis in thymocytes, but it partly preserved cell viability with a parallel increase in the percentage of cells exhibiting high $\Delta\Psi$m and elevated GSH levels. Nevertheless, non-rescued cells were deviated to necrosis. Therefore, the pathway leading to either apoptosis or necrosis appears to involve common mitochondrial dysfunctions which could not be reversed by caspase inhibition, suggesting that the pharmacological inhibition of cell death should occur at an earlier stage.

Keywords: apoptosis; necrosis; mitochondria; interleukin-4; dexamethasone; murine B cells

Abbreviations: DAPI, 6-diamidino-2-phenylindol; DEX, dexamethasone; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; EtBr, ethidium bromide; FDA, fluorescein diacetate; GSH, glutathione; H_2O_2, hydrogen peroxide; IL-4, interleukin-4; $\Delta\Psi$m, mitochondrial transmembrane potential; MCB, monochlorobimane; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethylketone

Introduction

Apoptosis, considered as the physiological form of cell demise, as opposed to necrosis or accidental cell death, is a fundamental feature of living cells.[1] Apoptosis is essential to normal development and tissue homeostasis and is thus critical to the function of the immune system.[2,3] Apoptotic cell death is characterized by morphological features, including reduction in cell volume, chromatin condensation, and nuclear DNA fragmentation, and is thus distinct from necrotic cell death.[3–6]

Apoptosis is linked to activation of cysteine proteases, with aspartate specificity, belonging to the family of caspases.[7,8] Since inappropriate or defective apoptosis is the cause of many diseases,[9] the therapeutic potential of caspase inhibition is being explored.[10]

In addition to caspase activation, mitochondria appear to play a key role in the control of apoptotic cell death.[11–13] However the sequence of changes that occur in the initial steps of apoptosis has not been clearly elucidated and, according to the experimental system used, the translocation of cytochrome c from mitochondria to cytosol[14] and/or induction of the mitochondrial permeability transition, which causes the loss of the mitochondrial membrane transmembrane potential ($\Delta\Psi$m), have been reported to be critical in the apoptotic pathway.[15,16]

Peptide inhibitors, designed to mimic known sequences of caspase substrates, are currently used to suppress apoptosis. Recently, the long-term consequences of caspase inhibition have been investigated leading to contradictory results on whether the death process is completely prevented, or only delayed[17–19] and even deviated to another form of cell death.[20–22]

Mature B lymphocytes undergo apoptosis *in vitro*,[23] a process that can be increased by a variety of treatments or regulated by inhibition of some signalling pathways and activation of several cell surface molecules, including IL-4 receptors.[24] IL-4 was initially recognized as a viability factor and a growth cofactor for B cells.[25] It has been observed to decrease apoptosis of cultured B splenocytes[23,26,27] and leukemia cells,[28] to prevent apoptosis induced by anti-Ig treatment[26,29] and to induce tolerance to anti-Fas mediated apoptosis.[30]

Recently, we found that the general caspase inhibitor, zVAD-fmk, effectively inhibited the main characteristic features of apoptosis while it failed to maintain cell viability, inducing a switch from apoptosis to necrosis in murine B lymphocytes undergoing spontaneous or drug-induced apoptosis.[21] A similar switch was observed in thymocytes undergoing drug-induced apoptosis.[31]

These findings, and the general observation that a given stimulus can induce either apoptosis or necrosis depending on the intensity of the insult, suggested that apoptotic and necrotic death share a common pathway, the final issue

being dependent on caspases. Consistent with its inability to prevent cell death, we observed here that zVAD-fmk was unable to prevent the loss of $\Delta\Psi$m and the reduction in GSH levels associated with apoptosis. In contrast, the anti-apoptotic interleukin, IL-4, could protect B cells from both apoptosis and from mitochondrial damage and led to an effective maintenance of cell viability. This indicated that the rescue from apoptosis should occur upstream of mitochondrial perturbations to avoid the commitment to another form of cell death.

Results

IL-4 protects B cells from spontaneous and drug-induced apoptosis maintaining cell viability without inducing necrosis

Mature splenic B cells can be induced *in vitro* to undergo apoptosis and can be protected from induction of apoptosis by a number of biochemically distinct stimuli.[24] We previously observed that a broad-spectrum and irreversible inhibitor of caspases, zVAD-fmk (100 μM), effectively protected B cells from apoptosis but not from cell death; in fact zVAD-fmk totally deviated the apoptotic form of cell death to necrosis.[21] We therefore examined whether inhibition of apoptosis by IL-4 led to similar consequences on long term survival and cell death. We first defined its effect on spontaneous and on DEX-induced apoptosis. Cytometric analysis after DAPI staining showed that the time-dependent apoptosis of B lymphocytes (quantitated by sub-G1 DNA) which is occurring in culture was totally inhibited by zVAD-fmk and greatly reduced by IL-4 (Figure 1A). DEX-induced apoptosis of B cells was similarly inhibited by zVAD-fmk and reduced by IL-4. IL-4 and zVAD-fmk inhibitory effects on B cell apoptosis were confirmed by the electrophoretic pattern of DNA laddering (Figure 1B). Since activation of caspase-3-like proteases appears to be an essential step in the execution phase of apoptosis,[32] we examined whether the modulation of apoptosis was associated with activation of DEVD-specific caspases, as assessed by hydrolysis of the specific DEVD-pNA substrate (Figure 1C). Data show that caspase activation occurring in cultured B cells was increased by DEX, reduced by IL-4 and totally abrogated by the peptide inhibitor zVAD-fmk. Similarly, DEVDase activity of DEX-treated cells was reduced by IL-4 and totally inhibited by zVAD-fmk. Morphological observation of cells stained with FDA and EtBr (see 2.3) revealed that IL-4 actually protected B cells from cell death since it reduced the number of apoptotic cells with a corresponding increase of normal living cells. In contrast, zVAD-fmk treatment only deviated the form of cell death leading to a total disappearance of apoptotic cells with a corresponding appearance of necrotic cells (Figure 1D). It must be stressed that necrotic cells have G0/G1 DNA content and thus could not be distinguished from normal diploid cells by cytometric analysis.

IL-4, but not zVAD-fmk, increases B cell viability and maintains $\Delta\Psi$m and GSH levels

It has been proposed that mitochondrial perturbations constitute an early step in the apoptotic process. To gain insight into the mechanism of IL-4-induced protection against apoptosis, a cytometric analysis was performed to quantitate cell viability by FDA staining and to examine IL-4 effect on mitochondrial functions. $\Delta\Psi$m was measured by using the fluorochrome $DiOC_6(3)$ and the reduction of GSH levels, which is associated with $\Delta\Psi$m dissipation and uncoupling of the respiratory chain, was determined by means of MCB.

More than 93% B cells were recorded as viable at the initiation of cultures (T0), as determined by FDA staining, and they presented high $\Delta\Psi$m and elevated GSH levels (Figure 2). Cells treated with H_2O_2 (1 mM) were used as a standard for necrosis. More than 95% of H_2O_2-treated cells were recorded as necrotic by morphological observation (data not shown); they also exhibited a total loss of viability (FDA staining), had low $\Delta\Psi$m and GSH levels (Figure 2). After 8 h of culture in medium, the loss of cell viability was already important and affected about 25% of cells. A good correlation was observed between the number of viable cells and that of cells scored as positive for $\Delta\Psi$m and for elevated GSH levels (75, 77, and 76% respectively). Spontaneous death was essentially due to apoptosis, as determined by cytometric analysis (Figure 1A). In agreement with morphological observation, zVAD-fmk, which deviated apoptosis to necrosis, did not appear to modify cell viability, as determined by the three criteria. In contrast, IL-4 improved global cell viability (from 75–88%), and increased the percentage of positive cells for $\Delta\Psi$m (from 77–86%) and elevated GSH levels (from 76–84%). Dexamethasone, within 8 h, increased apoptosis to about 50% (Figure 1A) and its effect was associated with a parallel decrease in the number of viable cells and in the number of cells exhibiting normal $\Delta\Psi$m and elevated GSH levels (53 and 50% respectively, Figure 2). As observed in the case of spontaneous apoptosis, zVAD-fmk failed to improve global cell viability as assessed by FDA staining, the non-viable cells 'saved' from apoptosis being characterized as necrotic by morphology. It also did not appear to counteract DEX effects on the decline of $\Delta\Psi$m and on the reduction of GSH levels. In contrast, IL-4 increased cell viability (from 50–76%) with a parallel increase in the percentage of cells having normal $\Delta\Psi$m and high GSH levels (from 53–76% and from 50–73%, respectively).

Cell size, as evaluated by forward light scatter, appeared to be decreased in apoptotic cells, as compared with living cells (positive cells for FDA staining and $\Delta\Psi$m). However, forward light scatter could not be used as a criteria for necrosis in dead cells (dim cells for FDA staining and $\Delta\Psi$m assessment) since necrotic H_2O_2-treated cells did not exhibit a clear and homogeneous cell size increase.

To investigate whether the respective effects of IL-4 and zVAD-fmk were transient or long-lasting, cultures were measured after 24 h (Figure 3). By that time about 40% of B cells were characterized as apoptotic by cytometric analysis of DNA content, and less than 5% necrotic cells could be morphologically detected (see legend under Figure 3). As previously observed at 8 h, IL-4 decreased both spontaneous and DEX-induced apoptosis with a corresponding increase of cell viability (Figure 3A) and a parallel increase in the percentage of cells positive for $\Delta\Psi$m (Figure 3B) and GSH levels

(Figure 3C). In contrast, zVAD-fmk was almost devoid of any effect on global cell viability, on $\Delta\Psi m$ and on GSH levels (Figure 3A–C), the loss of apoptotic cells being compensated by the appearance of an equivalent percentage of necrotic cells. Again, necrotic cells (>95% in H_2O_2-treated cells) appeared as non-viable (FDA staining and morphology) and non-apoptotic (cytometric analysis of DNA content and Figure 3A) and were recorded as negative for $\Delta\Psi m$ (Figure 3B) and GSH levels (Figure 3C).

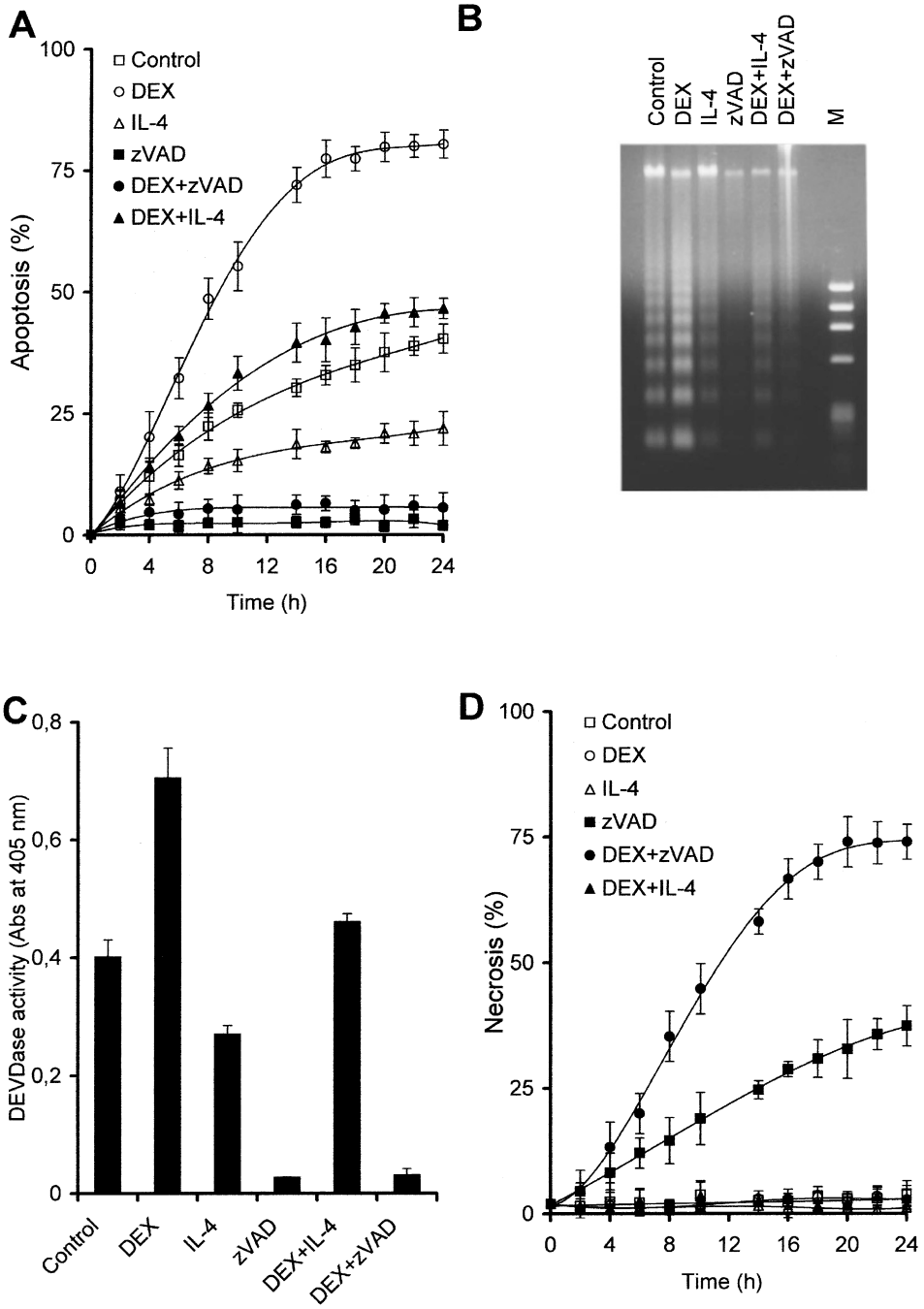

Figure 1 Comparative effects of zVAD-fmk and IL-4 on B lymphocyte apoptosis and necrosis. B lymphocytes (5×10^5 cells ml^{-1}) were incubated for various times, with or without dexamethasone (DEX, 5 nM), in the absence or the presence of zVAD-fmk (100 μM) or IL-4 (100 U/ml) as indicated. (**A**) Apoptosis was determined using cytometry and the DNA probe DAPI as the frequency of the sub-G1 cell population. (**B**) Agarose gel electrophoresis of DNA extracted from B lymphocytes cultured for 24 h in the absence or the presence of zVAD-fmk or IL-4 with or without DEX, lane M: ϕX174RF/*Hae*III digest used as DNA size marker. (**C**) Caspase-3-like activity in B cells (5×10^6) cultured for 8 h with or without DEX in the presence or the absence of zVAD-fmk or IL-4 was assessed by hydrolysis of DEVD-pNA substrate measured after 10 h at 405 nm. (**D**) Quantitation of necrosis was performed by counting cells cultured in the conditions indicated for (**A**) after double staining with FDA and EtBr as described in Materials and Methods

zVAD-fmk inhibits apoptosis in thymocytes with a progressive shift to necrosis

To determine whether these findings could be extended to another cell type, thymocytes undergoing spontaneous or DEX-induced apoptosis were cultured in the presence or in the absence of IL-4 or zVAD-fmk and analyzed by morphological examination and flow cytometry for global cell viability, mitochondrial functions and analysis of DNA content. IL-4 was devoid of any effect on the apoptosis of thymocytes and inhibition of caspases by zVAD-fmk initially led, after 6 h, to a rescue from apoptosis with an increase of cell viability (data not shown). After 24 h the percentage of thymocytes undergoing spontaneous apoptosis was of 44%, as estimated by sub-G1 DNA content, and more than 50% cells were considered as viable according to FDA staining, high $\Delta\Psi$m and elevated GSH levels (Figure 4A–C). In the presence of zVAD-fmk, the number of viable cells and cells with high $\Delta\Psi$m and elevated GSH levels was increased to about 80%, but the number of apoptotic cells was less than 5%, thus suggesting the presence of 15% non-apoptotic dead cells. Indeed, these dead cells were identified as necrotic by morphologic examination (data not shown). Dexamethasone increased apoptosis to almost 90%, as determined by DNA content with a parallel loss of FDA stainability and in the percentage of cells positive for $\Delta\Psi$m and GSH levels. As expected, zVAD-fmk totally abrogated DEX-induced apoptosis but, in contrast with results obtained in B cells, it led to a partial rescue since it prevented the loss of $\Delta\Psi$m and the reduction of GSH levels in about 50% of cells which were recorded as viable by FDA staining. Morphological examination revealed that the remaining non-viable and non-apoptotic cells were necrotic (40%, data not shown). Only necrotic cells were detected, according to the four criteria, in H_2O_2 (1 mM)-treated thymocytes.

Discussion

Caspase activation is essential in the apoptotic process but its inhibition is not always synonymous with increased cell life. Accordingly, the present study indicates that zVAD-fmk failed to restore cell life of B lymphocytes since it could only deviate the organized cell death process to necrosis, whereas similarly treated thymocytes could partly be saved. In contrast, IL-4 could effectively protect B cells from apoptosis and preserve cell viability at a premitochondrial stage.

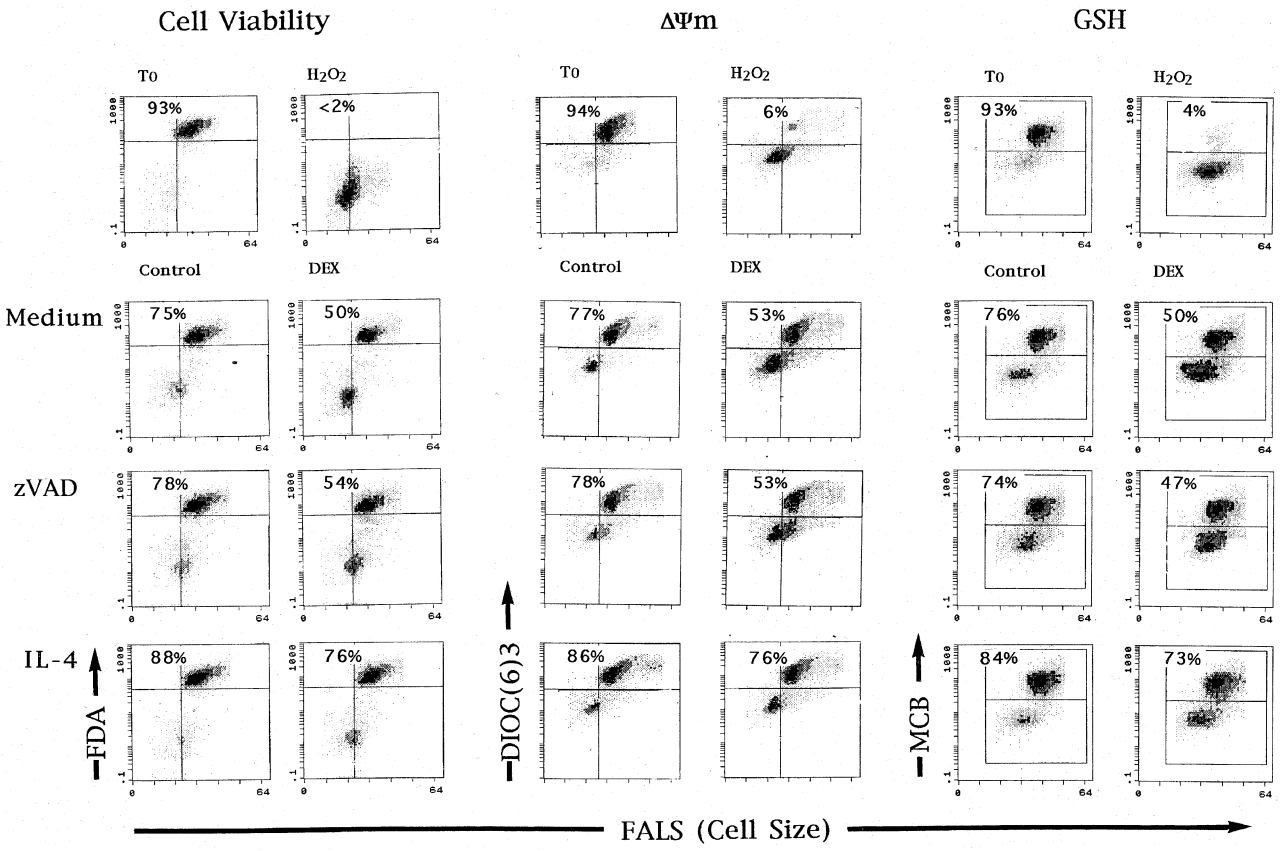

Figure 2 Multiparametric flow cytometric analysis of viability markers. B lymphocytes at the initiation of cultures (T0) or cultured in the presence of H_2O_2 (1 mM) were used as standards. B cells were cultured 8 h without (control) or with dexamethasone (DEX) in medium or in the presence of zVAD-fmk or IL-4, as indicated. Global cell viability was assessed by using FDA staining, mitochondrial potential ($\Delta\Psi$m) by the cell permeant probe $DiOC_6(3)$, and reduced glutathione levels (GSH) by the fluorochrome MCB (presented on ordinates, using a logarithmic scale), as described in Materials and methods. The forward angle light scatter is presented on abcissa (FALS on a linear scale). The percentage of cells scored as positive in each case, and delineated by an horizontal line, is presented on cytograms. These data are representative of at least three individual experiments

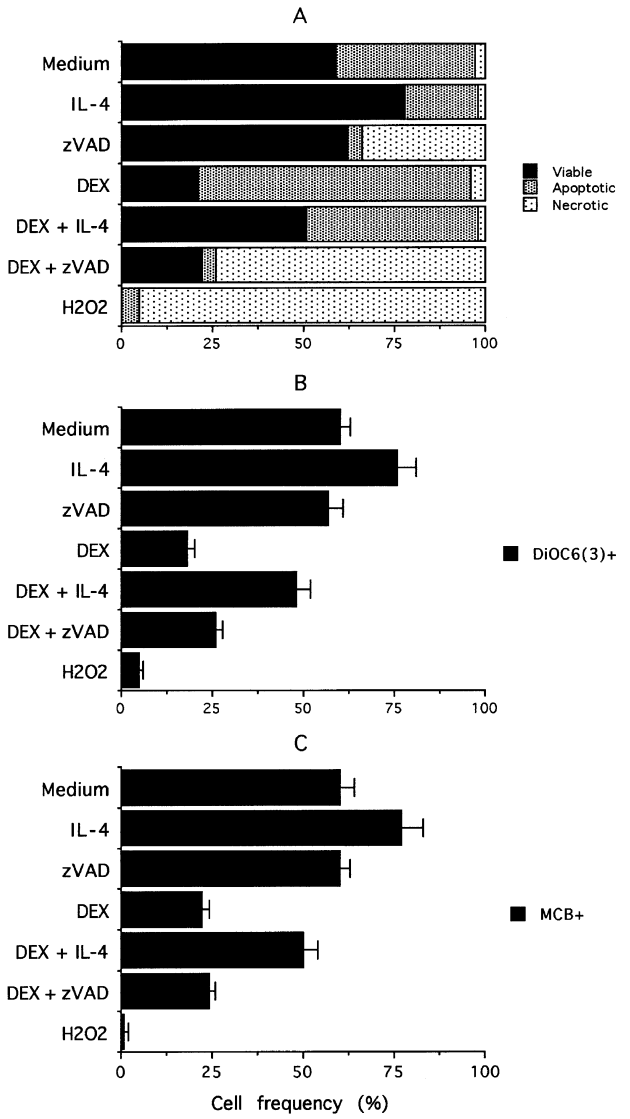

Figure 3 Influence of zVAD-fmk and IL-4 on the progression to apoptosis or to necrosis of B lymphocytes cultured 24 h with or without DEX. (**A**) The percentage of viable cells and of apoptotic cells was determined by cytometric analysis after staining with DAPI and FDA respectively. The difference between the percentage of dead cells (FDA negative) and that of apoptotic cells (sub-G1 DNA content) was due to the presence of necrotic cells as confirmed by morphological examination and quantitated as described in Materials and methods. (**B**) Percentage of cells presenting high $\Delta\Psi$m and scored as positive by cytometric analysis of $DiOC_6(3)$. (**C**) Percentage of cells presenting high GSH levels as determined by cytometric analysis of MCB. H_2O_2-treated cells were used as a standard for necrosis

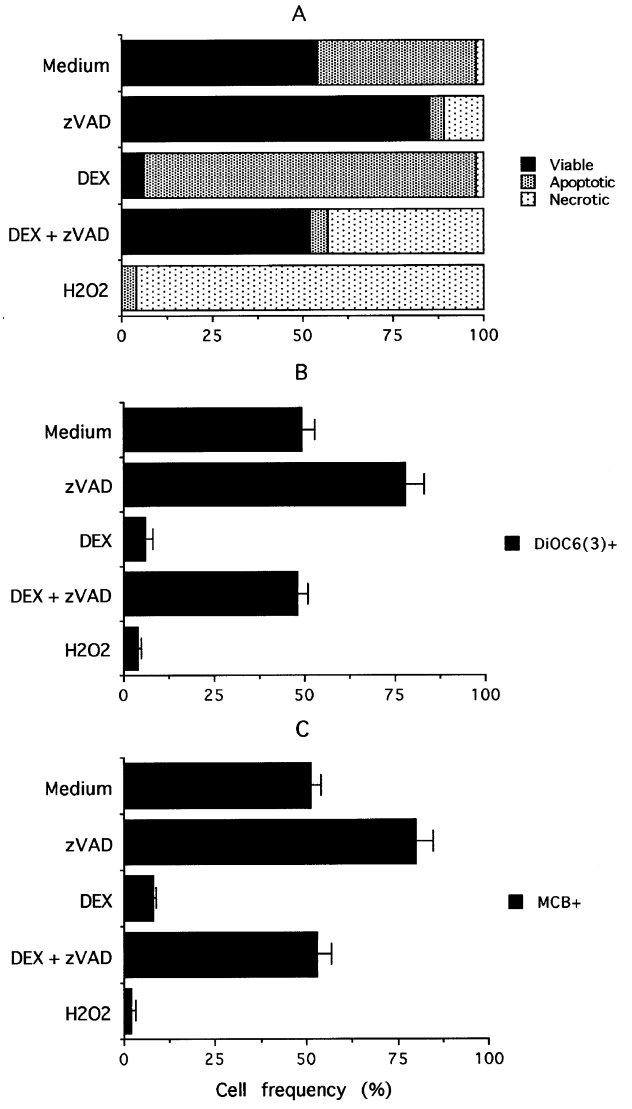

Figure 4 Influence of zVAD-fmk on the progression to apoptosis or to necrosis of thymocytes. Cells were cultured as described for B cells in Figure 3 and analyses were performed at 24 h

The molecular mechanism by which IL-4 protects B cells from apoptosis appears to be complex and is under study. A recent report has defined two separate mechanisms of action, one that is dependent on the insulin receptor substrate/phosphatidylinositol-3-kinase (IRS/PI3K) pathway and one that is independent of this IRS/PI3K-dependent pathway.[33] Two different pathways were also found, downstream of IL-4 receptor triggering. Thus IL-4 was shown to prevent, or to act independently of, the down-modulation of a 16 kDa Bax-associated protein ('P16') which occurs during *in vitro* induction of apoptosis in mature B cells. It was suggested that the 'P16' down-modulation, and thus protection from apoptosis by IL-4, was an early event which did not require caspase activation.[34] In agreement with an hypothesis on a rapid effect of IL-4, the present results indicate that IL-4 acted at an early stage, upstream of mitochondrial alterations, as shown by the maintenance of $\Delta\Psi$m, and led to an actual abrogation of cell death. IL-4 inhibited also the reduction in GSH levels observed in cells undergoing spontaneous or drug-induced apoptosis. This is in agreement with previous findings showing that a decrease in intracellular GSH precedes the onset of apoptosis in thymocytes.[35,36] It has been reported that inhibition of proteasomes, which act at a premitochondrial step, was effective in restoring cell viability, and thus

the deviation towards another form of cell death.[37] The possibility that IL-4 would inhibit proteasome activation is an hypothesis that is being explored.

Consistent with our previous findings on the total switch from apoptosis to necrosis upon treatment with zVAD-fmk,[21] cytometric evaluation of global cell viability by FDA staining indicated that general caspase inhibition failed to protect B cells against the commitment to cell death. In addition the multiparametric cytometric analysis, performed on an important number of cells, clearly showed that dead cells (FDA negative) were not apoptotic (sub-G1 DNA content) and found necrotic by other criteria[21] and by morphology. Consistent with its inability to maintain viability, zVAD-fmk was unable to counteract mitochondrial alterations (according to $\Delta\Psi$m and GSH levels) exhibited by apoptotic cells. It must be noted that a loss of $\Delta\Psi$m and low GSH levels were similarly observed in H_2O_2-induced necrotic cells and, thus, were not specific for apoptotic cell death.

It is becoming evident that caspase inhibition is not sufficient to avoid the commitment to death.[15] However, zVAD-fmk was also shown to block the reduction of $\Delta\Psi$m in a number of systems, including death receptor-induced apoptosis.[32,38,39] In another experimental system, zVAD-fmk led either to rescue from apoptosis or to another form of cell death according to the stimuli used to trigger apoptosis. Thus, pretreatment of Jurkat cells with zVAD-fmk inhibited anti-CD95 mAb triggered apoptosis, but not drug-induced apoptosis, with actual cell survival and maintenance of clonogenic potential, suggesting caspase-dependent or caspase-independent commitment to cell death, according to circumstances.[40] This could provide an explanation for the opposite results concerning the consequences of caspase inhibition. However, in the present study, zVAD-fmk partly protected thymocytes from spontaneous and from dexamethasone-induced apoptosis while it totally failed to prevent the commitment to cell death in similarly treated B cells. This suggests that the signaling pathway to death decision is also cell specific.

In agreement with recent findings showing that caspase inhibitors were unable to fully restore cell viability in thymocytes, rather leading to necrosis,[31] we observed that zVAD-fmk led partly to deviation from apoptosis to necrosis and partly to actual survival. The reason why a population of thymocytes was protected from cell death for a longer time is at present not well understood. Since those thymocytes which were saved from apoptosis presented normal $\Delta\Psi$m and high GSH levels, it must be admitted that caspases, which can be activated as a consequence of mitochondrial dysfunction, could themselves contribute to mitochondrial failure. A recent study on the specific role played by each caspase in the protease cascade involved in the execution of Fas-induced cell death in Jurkat cells indicated that a caspase, distinct from caspase-3 and -6, leads to the disruption of $\Delta\Psi$m.[41] However, in a cell-free system, caspase-1, -2, -3, -4, -6 were reported to cause the loss of $\Delta\Psi$m in isolated mitochondria.[42]It thus appears that the mechanisms involved in the control of cell death greatly depend on experimental conditions.

Altogether, this study extends our previous findings by demonstrating that mitochondrial alterations are common and irreversible events in the commitment to cell death, whether the issue is necrosis or caspase-dependent apoptosis, and that IL-4 by countering early mitochondrial dysfunctions provides an anti-apoptotic and real survival signal.

Materials and methods

Materials

The culture medium used throughout was RPMI 1640 supplemented with 25 mM HEPES, 2 mM L-glutamine, standard antibiotics, 50 μM 2-mercaptoethanol and 8% heat-inactivated fetal calf serum (Biomedia, France). The caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethylketone (zVAD-fmk) was from Bachem Biochimie (France). The caspase substrate DEVD-pNA was from Alexis Biochemicals (Coger, France). Dexamethasone (DEX), 6-diamidino-2-phenylindol (DAPI), fluorescein diacetate (FDA), ethidium bromide (EtBr), hydrogen peroxide (H_2O_2) were from Sigma (St Louis, MO, USA). 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] and monochlorobimane (MCB) were from Molecular Probes (Eugene, OR, USA).

Cell 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.[27] Briefly, splenocytes ($3-4\times10^6$ ml^{-1}) were treated with anti-Thy 1.2 (CD90) monoclonal antibodies at 4°C for 30 min followed by incubation with Low-Tox rabbit complement at 37°C for 30 min (Cedarlane, Canada). Cells recovered at the 55–70% interface of a discontinuous Percoll gradient were characterized as B lymphocytes by IgM expression. Thymocytes were obtained by dilaceration of thymus. Cells, at 5×10^5 cells ml^{-1} in RPMI medium, were cultured in the presence or absence of stimuli as indicated in 24-well plates in 6% CO_2 at 37°C. For cytometric analysis, cells were fixed in 70% ethanol and kept at −20°C until analysis of DNA content.

Cell-staining for morphologic analysis

Cells (10^6 cells/0.1 ml) were washed with PBS and then stained with FDA (1 μg/ml) and EtBr (10 μg/ml) for 5 min at 37°C. FDA is cleaved into green fluorescein by intracellular esterases present in living cells, while EtBr, a non-specific DNA intercaling agent, is taken up by dead cells.[43] Quantitative analysis of each population was performed by counting more than 500 cells discriminated as viable cells (green fluorescence and nonstained normal nucleus), apoptotic cells (red condensed or fragmented nucleus) and necrotic cells (red 'apparently normal' or patchy nucleus). The results are presented as the mean of three experiments.

Analysis of DNA fragmentation in agarose gels

DNA from 5×10^5 cells was extracted as previously described.[44] 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 ethidium bromide. DNA was visualized in UV light. ϕX174RF/*Hae*III digest was used as DNA size marker.

Caspase assay

Cells (5×10^6) were cultured for 8 h in the presence or absence of drugs, as indicated and then 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 caspase substrate, and then incubated for 8 h at 37°C. Enzyme-catalyzed release of *p*-nitroanilide was monitored at 405 nm.

Flow cytometryic analysis

Fixed cells (5×10^5 cells ml^{-1}) were washed twice with HBSS and then stained with DAPI (2.5 μg/ml) at 37°C for 20 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 10^4 cells. The percentage of apoptosis was determined from the sub-G1 events.

Mitochondrial transmembrane potential ($\Delta\Psi$m) and intracellular reduced gluthatione (GSH) levels were measured as previously described.[45] $\Delta\Psi$m was determined by retention of $DiOC_6(3)$, a cell permeant, cationic lipophilic fluorochrome which specifically accumulates into mitochondria according to $\Delta\Psi$m. Cell staining was performed by treating cells (5×10^5 ml^{-1}) with $DiOC_6(3)$ (0.1 μM) for 20 min at 37°C. GSH levels were assessed using a specific cell permeant probe, MCB, which forms a fluorescent compound upon interaction with GSH, cells were treated with MCB (50 μM) for 20 min at 37°C. Flow cytometric measurements were performed on an ELITE ESP flow cytometer (Coulter France). Fluorescence excitation was obtained through the blue line (488 nm) of an argon ion laser operating at 15 mW. Green fluorescence of $DiOC_6(3)$ was collected with a 525 nm band pass filter. A 100 mW UV excitation (356 nm) from a laser (INOVA 305, coherent) was used for quantification of [GSH/MCB] fluorescence integrated above 457 nm. Analyses were performed on 10^4 cells in list mode. Light scatter values were measured on a linear scale of 64 channels and fluorescence intensity on a logarithmic scale of four decades of log.

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