B cell receptor-mediated nuclear fragmentation proceeds in WEHI 231 cells in the absence of detectable DEVDase and FRase activity

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Abstract Crosslinking of the WEHI 231 lymphoma B cell receptor (BCR) leads to growth arrest followed by apoptosis. In a study of the role of lysosomal cysteine proteinases in BCR-mediated apoptosis we provide evidence that commitment to apoptosis correlates with a time-dependent increase in caspase and cathepsin activities. We also show that activation of cathepsins is a caspase-independent process, and caspase cascade activation is independent of lysosomal endopeptidases. BCR-induced nuclear fragmentation was not prevented, but rather delayed in the absence of detectable caspase and cathepsin activities, suggesting that BCR-driven apoptosis of these cells may use an alternative proteolytic mechanism independent of caspases and cathepsins.

Key words: Caspase; Cathepsin; B cell receptor; Apoptosis; Inhibitor

1. Introduction

The B cell lymphoma WEHI 231 cell line has been used as a model to study immature B cell tolerance, based on its capacity to undergo growth arrest and programmed cell death on B cell receptor (BCR) crosslinking by anti-IgM antibodies [1,2]. Because of its very long time course, the mechanism of cell death induced by signal transduction from the BCR may be different from other mechanisms of apoptosis such as the Fas-mediated or anti-tumor drug-induced cell death [3]. Molecular mechanisms operating in BCR-mediated apoptosis are characterized by activation and processing of procaspase-7 [4]. The pathways leading to activation of the effector caspase cascade in BCR-mediated apoptosis are unclear and the relevance of the low levels of BCR-mediated caspase 9 on processing of procaspase 7 is under debate [4–6]. BCR crosslinking also induces calpain, a calcium-dependent protease, implying that calpain processes procaspase 7 [7,8]. Despite the characteristic time-dependent increases in caspase/DEVDase activity in anti-IgM-induced apoptosis in WEHI 231, it appears that such signalling can lead to the execution of cells independently of caspase activation, assigning an execution role to the lysosomal endopeptidase, cathepsin B [5]. Cathepsins, papain-like lysosomal proteinases, are also implicated in several other apoptotic pathways [9]. For instance, disruption of the lysosomal membrane with lysosome-directed oxidative stress or lysosomotropic detergents causes a release of lysosomal content into the cytoplasm and apoptotic dismiss of cells [10,11]. In tumor necrosis factor (TNF)-induced apoptosis of tumor fibrosarcoma cell WEHI-S and cervix carcinoma ME-180as cells, cathepsin B was assigned a dominant execution role; however, it appears to play a minor role in TNF-induced apoptosis in primary cells [12]. Furthermore, deletion of the cathepsin B gene in mice reduced TNF-α-associated hepatocyte apoptosis by inhibiting mitochondrial release of cytochrome *c* and the activation of caspases 9 and 3 [13]. A molecular mechanism of the involvement of cathepsin B in mitochondrial release of cytochrome *c* appears to be via cleavage of the Bcl-2 family member Bid, where truncated Bid triggers the intrinsic apoptosis pathway, by causing release of cytochrome *c* from mitochondria and subsequent caspase activation [14].

The aim of this study was to further elucidate the proteolytic events in BCR-mediated apoptosis of WEHI 231 cells. We investigated the relation of cell death progression to activation of cathepsins and caspases in order to identify any connection between the two processes. The present work reveals a concomitant but independent increase in caspase and cathepsin activities on surface IgM crosslinking. Inhibition of FRase as well as DEVDase activity failed to inhibit BCR-triggered cell death, implying this to be an execution process independent of cathepsins and caspases.

2. Materials and methods

2.1. Cell culture

The WEHI 231 cell line was cultured in RPMI1640 (Sigma, Chemical Co., St. Louis, MO, USA) with 10% fetal calf serum (Gibco), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES and 50 μM 2-mercaptoethanol (Sigma), at 37°C, in humidified air with 5% CO_2. The surface IgM of WEHI 231 cells was crosslinked with 10 μg/ml of goat anti-IgM (Sigma). For inhibition studies cells were seeded in 6- or 24-well plates at 0.25×10^6 cells/ml in complete medium and (2*S*,3*S*)-*trans*-epoxysuccinyl-leucylamido-3-

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Abbreviations: Ac-DEVD.AMC, acetyl-Asp-Glu-Val-Asp-AMC; AFC, 7-amino-4-trifluoromethyl coumarin; AMC, 7-amino-4-methyl coumarin; DMSO, dimethylsulfoxide; fmk, fluoromethyl ketone; z-VAD.fmk, benzyloxycarbonyl-Val-Ala-Asp.fmk; z-FR.AFC, benzyloxycarbonyl-Phe-Arg-AFC; E-64d, (2*S*,3*S*)-*trans*-epoxysuccinyl-leucylamido-3-methyl-butane ethyl ester

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methyl-butane ethyl ester (E-64d; Peptide Institute, Osaka, Japan), or benzyloxycarbonyl-Val-Ala-Asp.fluoromethyl ketone (z-VAD.fmk; Bachem AG, Bubbendorf, Switzerland) added in adequate concentrations and $<0.5\%$ (v/v) dimethylsulfoxide (DMSO). The corresponding volume of solvent was added to the control cultures. Cells were incubated for up to 66 h.

2.2. Proliferation assay

Cells (1×10^4/well/100 μl) were plated in 96-well flat-bottom microtiter plates and cultured in the absence or presence of different concentrations of E-64d (Peptide Institute) for 23 h at 37°C, 5% CO_2. To determine the proliferative capacity, cells were exposed to MTS assay (CellTiter 96® AQ_{ueous} one solution cell proliferation assay; Promega, Madison, WI, USA). In brief, MTS reagent (10 μl/well) was added and the cells were incubated at 37°C for 1 h. The absorbance at 490 nm was measured with microplate reader (Bio-Rad, Laboratories Inc., Sunnyvale, CA, USA). Each experiment was performed in quadruplicate and repeated at least three times.

2.3. Flow cytometry analysis of the DNA content and cell cycle analysis

Apoptosis was evaluated by staining cellular DNA with propidium iodide (PI). Cells (10^5-10^6) were washed in phosphate-buffered saline and permeabilized with ice-cold methanol. They were then washed and treated with RNAse (20 μg/ml) for 30 min at 37°C. Cells were stained with PI solution and analyzed by fluorescence-activated cell sorting (FACS Calibur; Becton-Dickinson, San Jose, CA, USA). Data were evaluated using the CellQuest software. Apoptosis was determined as the percentage of cells located in the hypoploid sub-G_0/G_1 peak of the cell cycle.

2.4. Analysis of internucleosomal DNA fragmentation

The cleavage of DNA into nucleosomal fragments was analyzed by fractionation on agarose gel. Cells were lysed in 100 mM Tris–HCl buffer (pH 8.5) containing 5 mM EDTA, 0.2% sodium dodecyl sulfate, 200 mM NaCl, and 200 μg/ml proteinase K, and incubated overnight at 37°C. Nucleic acids were recovered with one volume of isopropanol, treated with RNaseA (50 μg/ml), and the solution equivalent to 1×10^5 cells was fractionated by electrophoresis on 1.8% agarose gel. The DNA bands were visualized by ethidium bromide staining and evaluated by comparison with a 100-bp DNA ladder standard.

2.5. Measurement of DEVDase activity

The activation of caspase zymogen in cell extracts was followed fluorometrically by monitoring 7-amino-4-trifluoromethyl coumarin (AFC) release from the Ac-DEVD.AFC substrate. Cell extracts were prepared by pelleting, lysing and sonicating cells in 0.1 M phosphate buffer pH 6.0 containing 0.1% Triton X-100. Cleared cell extracts (20 μg cellular proteins) were incubated in assay buffer (20 mM Pipes, 100 mM NaCl, 0.1% (w/v) CHAPS, 10% (w/v) sucrose, and 10 mM dithiothreitol (DTT), pH 7.2), and incubated at 37°C for 30 min. Activation was analyzed by adding the substrate Ac-DEVD.AFC (100 μM) in 96-well microplate format to a 37°C thermojacketed fluorescence microplate reader (LS-50 B, Luminescence Spectrometer, Perkin Elmer Inc., Wellesley, MA, USA). Free AFC was monitored at an excitation wavelength of 405 nm and an emission wavelength of 510 nm. The reaction was followed continuously for 60 min. Assays performed without cell lysate were used as background controls. DMSO, which was used as a solvent for the inhibitors, served as a negative control. Steady-state hydrolysis rates were obtained from the linear part of the curves. Data were expressed as increase in fluorescence as a function of time ($\Delta F/\Delta t$).

2.6. Measurement of FRase and RRase activity

Cysteine-endopeptidases activities in cell extracts were tested against z-Arg-Arg.AMC (AMC, 7-amino-4-methyl coumarin), a substrate for cathepsin B, and z-Phe-Arg.AMC, which is hydrolyzed by a number of cathepsins such as L, B and S. Cell extracts were prepared as described above, and enzymatic activities were measured in 40 μg of whole cell extracts by adding a reaction buffer (100 mM phosphate buffer, pH 6.0, containing 250 mM sucrose, 1 mM EDTA and 5 mM DTT) and 100 μM z-Arg-Arg.AMC or 100 μM z-Phe-Arg.AMC as substrates. The intensity of fluorescence of the liberated AMC was then measured ($\lambda_{ex}=355$ nm, $\lambda_{em}=460$ nm). Release of the product was measured continuously for 60 min at 37°C and data evaluated as described above.

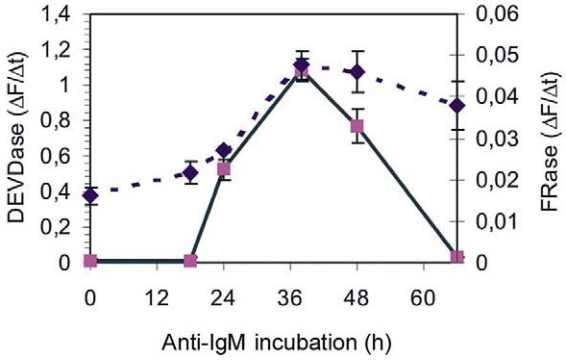

Fig. 1. Time-dependent activation of caspases and cathepsins in BCR-induced apoptosis. Cells (0.25×10^6 cells/ml) were cultured in the presence of 10 μg/ml of anti-IgM antibody for the times indicated. Cell extracts were analyzed for their proteolytic activity towards Ac-DEVD.AFC (DEVDase activity, solid line) and z-FR.AMC (FRase activity, dotted line). The increase in fluorescence as a function of time ($\Delta F/\Delta t$) denoted the amount of released AMC or AFC. Results are representative of multiple experiments.

3. Results

3.1. Activation of cathepsins is characteristic of apoptosis induced by surface IgM crosslinking on WEHI 231

Cathepsins are activated following BCR-mediated apoptosis, suggesting their involvement in the proteolytic machinery of programmed cell death. The kinetics of caspase and cathepsin activation were determined by incubating cells with anti-IgM antibodies for up to 66 h. Cells were harvested at different time points and the cell extracts examined for the ability to cleave specific substrates. Ac-DEVD.AFC was used as a specific substrate of caspase-3-like enzymes [15]. DEVDase activity started to be observed at 18 h and reached maximum levels at 38 h (Fig. 1). Similarly, the activity of cathepsins was evaluated in cell extracts against z-FR.AMC and z-RR.AMC substrates. Increases in the basic levels of FR-, and RR-like activity were detected 8 h after anti-IgM incubation, with maximum levels at 38 h, and a decline thereafter (Fig. 1). z-FR.AMC hydrolyzed three-fold faster than z-RR.AMC which is hydrolyzed by cathepsin B only (data not shown).

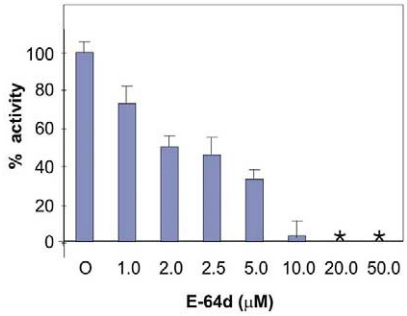

Fig. 2. Concentration-dependent inhibition of endogenous cathepsin activity. Cells (0.25×10^6 cells/ml) were incubated with various concentrations of E-64d for 30 min. Cell extracts were analyzed for enzymatic activity against z-FR.AMC synthetic substrate. The inhibitory potential of E-64d is expressed as the percentage of maximal FRase activity. All values represent means of triplicate determinations ± S.D. * denotes undetectable levels.

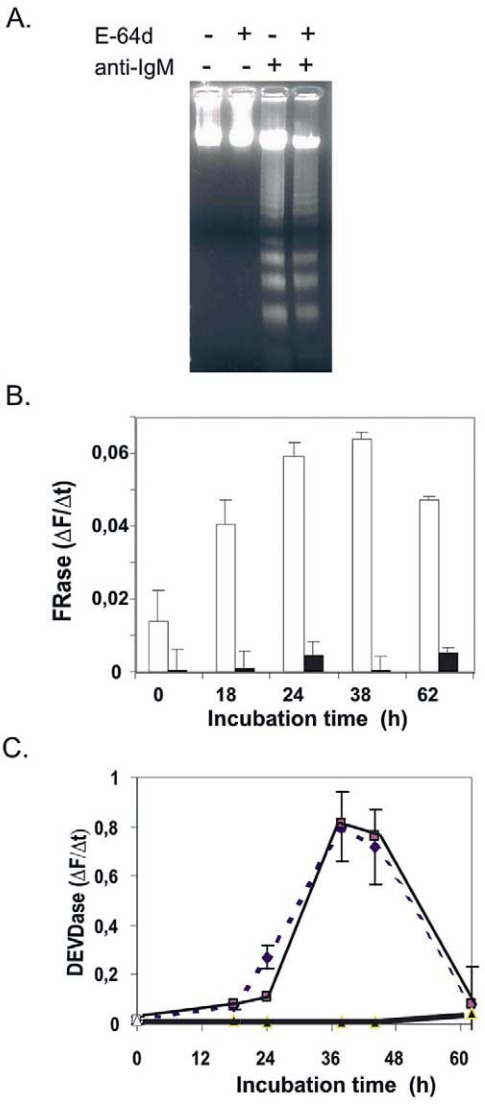

Fig. 3. Cysteine proteinase inhibitor E-64d does not affect BCR-induced cell death. Cells (5×10^5 cells/ml) were incubated alone or with E-64d (100 μM) for 1 h before addition of anti-IgM antibody (10 μg/ml), and harvested at the times indicated. A: DNA fragmentation assays. Cells were harvested for isolation of total DNA after 48 h of incubation. Electrophoresis was on 1.8% agarose gels. B: Enzyme activity assays. Cells were pretreated with (black bars) or without (open bars) 25 μM E-64d prior to anti-IgM incubation. Cells were harvested at indicated times and 40 μg of total cell proteins was analyzed for FRase activity. All values represent means of triplicate determinations ± S.D. C: Similarly, cells were treated with 50 μM E-64d (thick line bars), with 10 μg/ml anti-IgM (dotted line) or a combination of both (thin line). Twenty μg of total cell proteins was analyzed for DEVD-like activity. All values are means of triplicate determinations ± S.D. These results are representative of four independent experiments.

3.2. Inhibition of cathepsins does not relieve antigen receptor-mediated growth arrest or apoptosis in WEHI 231

The cell-permeable E-64d, which selectively inhibits cysteine endopeptidases (including cathepsins B and L, and calpains), was used to determine the concentration and time dependence of in vivo inhibition of cathepsin activity. However, incubation of cells for 30 min with E-64d at concentrations higher than 10 μM inhibited the total cellular FR- and RR-like activity (Fig. 2). E-64d was shown not to be cytotoxic or to have any effect on cell proliferation by MTT assays, at concentrations up to 150 μM (data not shown).

Our results indicate the involvement of cathepsins in anti-IgM-induced cell death as suggested by their activation kinetics which, similar to those of the caspases, accompany apoptosis. This prompted us to examine whether inhibition of endogenous cathepsin activity could modulate this process. Here we present evidence that inhibition of cathepsin activity did not protect cells from anti-IgM-induced apoptosis. Pretreatment of WEHI 231 cells with up to 150 μM of E-64d for 1 h prior to and following anti-IgM treatment, had no effect on the cell cycle profile (see below, Fig. 5A, E-64d row). Growth arrest was observed by accumulation of cells in G_0/G_1 phase and a decrease in the S phase of the cell cycle at 24 h posttreatment, and apoptosis was determined as the fraction of cells in the subG_0 peak observed at 48 h. The effect of E-64d on the anti-IgM-induced apoptotic process was also examined by looking for internucleosomal DNA fragmentation. Substantial DNA laddering was clearly detectable following 48 h of anti-IgM treatment and was not protected by preincubating the cells with 100 μM E64d (Fig. 3A).

Further evidence that the apoptotic process progresses in the absence of detectable levels of cathepsin activity comes from the kinetic studies of DEVDase activation during the course of apoptotic induction. Incubation of WEHI 231 cells with 25 μM E-64d for 1 h prior to and following IgM cross-linking completely inhibited endogenous FRase activity throughout a 66-h experiment, but did not affect the time course and level of DEVDase activation (Fig. 3B).

3.3. BCR-mediated increase in cathepsin activity is caspase independent

Concentration-dependent inhibition of cathepsin/FRase activity by z-VAD.fmk has been determined in resting, but not in apoptotic cells. WEHI 231 cells were incubated with 25 or 150 μM of z-VAD.fmk for 1 h and their extracts examined for FRase activity. Basic levels of cathepsin activity were only moderately affected by low concentrations (25 μM) but almost completely inhibited by 150 μM z-VAD.fmk. To examine the

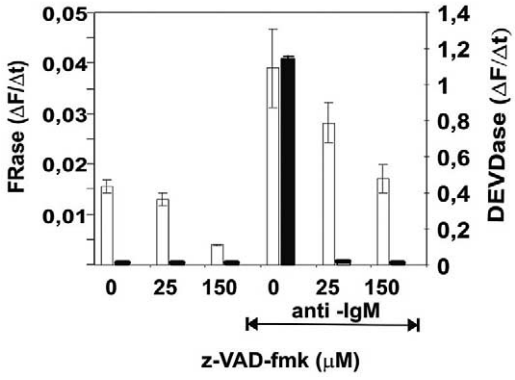

Fig. 4. Effects of fmk peptide inhibitors on the cathepsin activity in resting and IgM-treated WEHI 231 cells. WEHI 231 cells (5×10^5 cells/ml) were treated with z-VAD.fmk for 1 h and harvested for lysis or incubated with anti-IgM for 38 h and then lysed. Samples were analyzed for FRase and DEVDase activity by incubating 40 or 20μg of cell extracts with z-FR.AFC (benzyloxycarbonyl-Phe-Arg-AFC), or Ac-DEVD.AMC (acetyl-Asp-Glu-Val-Asp-AMC), respectively. Activity was measured with the fluorogenic substrates as described. Data are expressed as increase in fluorescence as a function of time ($\Delta F/\Delta t$).

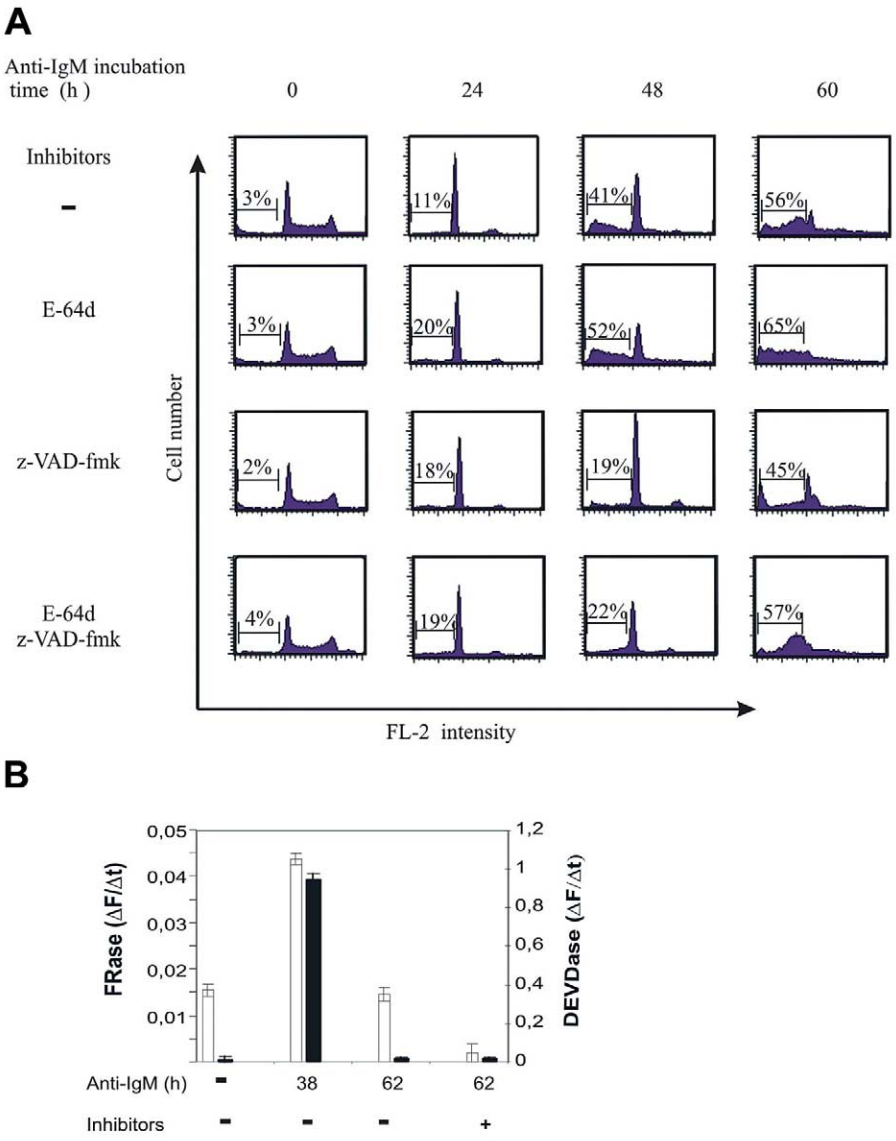

Fig. 5. Effect of caspase and cathepsin inhibition on BCR-induced nuclear fragmentation. A: Cells (0.25×10^6 cells/ml) were incubated alone or with z-VAD.fmk (100 μM), E-64d (100 μM), or a combination of both inhibitors for 1 h before addition of anti-IgM antibody (10 μg/ml). Cells were collected at the indicated times, pellets were permeabilized and stained with PI for cell cycle analysis. Apoptosis corresponds to the amount of fragmented DNA in the hypoploid subG$_0$/G$_1$ peak of the cell cycle. Values are expressed in percentages. B: Cells (7×10^5 cells/ml) were pretreated, with or without a combination of z-VAD.fmk and E-64d (100 μM, each), for 1 h and incubated with anti-IgM for 38 or 62 h and then lysed. Samples were analyzed for DEVDase and FRase activity as described. Data are expressed as increase in fluorescence as a function of time ($\Delta F/\Delta t$). Results are representative of at least three independent experiments.

inhibiting effects of z-VAD.fmk on BCR-mediated increases in cathepsin activity, cells were preincubated with 25 or 150 μM z-VAD.fmk for 1 h prior to the addition of anti-IgM antibodies, and harvested 38 h later. Substantial increases in cathepsin B activity were detected in samples incubated even in the presence of 150 μM z-VAD.fmk. DEVDase activity was completely blocked throughout the time course of the experiment. These results indicate that the BCR-mediated activation of cathepsins is independent of the presence of active caspases (Fig. 4).

3.4. BCR-mediated nuclear fragmentation proceeds in the absence of RRase and DEVDase activity

Nuclear fragmentation and morphological changes were observed to take place, even in the absence of detectable DEVDase activity. Cells preincubated with z-VAD.fmk inhibitor and treated with anti-IgM as above were harvested for FACS analysis at different time intervals. High concentrations (150 μM) of z-VAD.fmk led to a decrease in the fraction of cells in the subG$_0$ peak observed at 48 h after BCR ligation to 19%, compared to 41% in non-pretreated cells. Nevertheless, at longer incubation periods, such as 60 h, a substantial proportion (45%) of the cells was detected in the subG$_0$ population (Fig. 5A, z-VAD.fmk row). Thus, z-VAD.fmk did not rescue cells from BCR-mediated nuclear fragmentation but had rather delayed its onset.

A possible conclusion from the above experiments could be that cathepsins act as an alternative execution pathway and are perhaps responsible for the final caspase-independent execution. To examine this possibility we inhibited both classes

of cysteine proteinases by incubating WEHI 231 with a combination of 100 μM E-64d and 150 μM z-VAD.fmk. The effect of inhibition was observed by determining the percentage of cells in the subG$_0$ fraction of the cell cycle. While this fraction was decreased in cells incubated for 48 h and 60 h with z-VAD.fmk, as compared to non-inhibited samples, no additional effect was observed in cells incubated with a combination of the two inhibitors, z-VAD.fmk and E-64d (Fig. 5A, E-64D+z-VAD.fmk row). DEVDase and FRase activities were suppressed throughout the time course of the experiment (Fig. 5B). The failure of both caspase and cathepsin inhibitors to rescue WEHI 231 from BCR-mediated apoptosis suggests that the antigen receptor-driven apoptosis of these cells may use an alternative mechanism independent of caspases and cathepsins.

4. Discussion

Control of the apoptotic process plays a selective role in the course of B cell maturation and is important for understanding B cell function. Transmembrane signalling initiated by the BCR is essential for various B cell activities including cell activation, proliferation, anergy and deletion [16,17]. Our results have provided evidence that commitment of these cells to antigen receptor-driven apoptosis correlates with time-dependent increases in caspase and cathepsin activities. Such a correlation does not, however, necessarily imply a direct causal connection.

We further show that while the increases in anti-IgM-induced cathepsin activity suggested their involvement in the apoptotic process, inhibition of the papain-like lysosomal cysteine proteases (cathepsins) and calpains by E-64d was not sufficient to alter the BCR-mediated apoptotic process or the induced DEVDase activity and its time course. Time-dependent increases in caspase activity are a hallmark of BCR-mediated apoptosis and caspase-7 has been assigned a major execution role in this process [3,4]. However, some other reports indicate that BCR-mediated execution of WEHI 231 cells can also proceed in a caspase-independent manner [5]. Both apparently opposing statements are based on studies of caspase function using the pan-caspase inhibitor z-VAD.fmk at different concentrations. While the classical death receptor-mediated apoptosis pathways are completely blocked by 1 μM zVAD.fmk [18], several recent studies delineating the role of caspases in apoptotic process have been conducted with concentrations of z-VAD.fmk up to 200 μM. Chloromethyl ketone and fmk peptide inhibitors of caspases such as z-VAD.fmk can also strongly inhibit cathepsins [18,19,20]. Such inhibition was observed at concentrations of z-VAD.fmk higher than 10 μM [21], although it somewhat depends on the cell type used [20]. It seems that the major reason for this crossreactivity is the high reactivity of the halomethylketones towards the active site Cys, since the secondary and tertiary structures of caspases do not resemble those of cathepsins, and the active site catalytic machinery is different [20]. Our results support these data showing that halomethyl ketone peptide inhibitors of caspases can at higher concentrations inhibit the basic levels of cathepsin activity in resting cells.

We report herein that blocking DEVDase activity by pan-caspase inhibitor z-VAD.fmk is not sufficient to rescue cells from the BCR-mediated cell death but that it only delays its onset. Furthermore, despite the non-specific inhibition of cathepsins by this inhibitor in resting cells, activity of cathepsins increased in cells undergoing apoptosis, implying a possible alternative proteolytic pathway. This possibility was, however, neglected based on results showing that inhibiting both classes of proteases by preincubating cells with a combination of z-VAD.fmk and E64d exhibited none of the additional protective effects. BCR-mediated apoptosis progressed in the absence of detectable DEVDase and FRase activities. The failure of caspase and cathepsin inhibitors to rescue the WEHI 231 immature B cells from antigen receptor-driven apoptosis suggests the involvement of yet another proteolytic pathway.

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