Death signals from the B cell antigen receptor target mitochondria, activating necrotic and apoptotic death cascades in a murine B cell line, WEHI-231

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Abstract

B cell antigen receptor (BCR)-mediated cell death has been proposed as a mechanism for purging the immune repertoire of anti-self specificities during B cell differentiation in bone marrow. Mitochondrial alterations and activation of caspases are required for certain aspects of apoptotic cell death, but how the mitochondria and caspases contribute to BCR-mediated cell death is not well understood. In the present study, we used the mouse WEHI-231 B cell line to demonstrate that mitochondrial alterations and activation of caspases are indeed participants in BCR-mediated cell death. The peptide inhibitor of caspases, N-benzyloxy carbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), blocked cleavage of poly(ADP-ribose) polymerase and various manifestation of nuclear apoptosis such as nuclear fragmentation, hypodiploidy and DNA fragmentation, indicating that signals from the BCR induced the activation of caspases. In addition, z-VAD-fmk delayed apoptosis-associated changes in cellular reduction–oxidation potentials as determined by hypergeneration of superoxide anion, as well as exposure of phosphatidylserine residues in the outer plasma membrane. By contrast, although z-VAD-fmk retarded cytolysis, it was incapable of preventing disruption of the plasma membrane even under the same condition in which it completely blocked nuclear apoptosis. Mitochondrial membrane potential loss was also not blocked by z-VAD-fmk. Bongkrekic acid, a specific inhibitor of mitochondrial permeability transition pores, suppressed not only the mitochondrial membrane potential but also the change of plasma membrane permeability. Overexpression of Bcl-xL prevented mitochondrial dysfunction, nuclear apoptosis and membrane permeability cell death triggered by BCR signal transduction. These observations indicate that death signals from BCR may first cause mitochondrial alterations followed by activation of both necrotic and apoptotic cascades.

Introduction

Transmembrane signal transduction initiated by the B cell antigen receptor (BCR) is essential for various B cell activities, including cell activation, proliferation, anergy and deletion. The BCR complex is composed of a membrane Ig molecule non-covalently associated with two accessory molecules Igα and Igβ, which exist as disulfide linked heterodimers (1). Signaling through the BCR primarily drives mature B cells into proliferation leading to the expansion of antigen-specific clones. By contrast, immature lymphocytes usually undergo apoptosis rather than proliferation upon triggering through the BCR (2,3). This response is the foundation for negative selection, a process that ensures the generation of a self-tolerant immune repertoire during lymphocyte development (4).

Apoptosis is a basic feature of all nucleated mammalian cells, and is essential for normal development and tissue homeostasis (5). Loss of integrity of the cell membrane is an early event in necrosis while, in apoptosis, it is preceded by DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage, disassembly into membrane-
enclosed vesicles (apoptotic bodies) and plasma membrane phosphatidylserine (PS) transition. Recently, it has been demonstrated that the effectors of apoptosis are represented by a family of intracellular cysteine proteases known as caspases (6). A large body of genetic and biochemical evidence supports a cascade model for effector caspase activation (7). Death receptors can activate caspase cascades, causing an apoptotic demise of the cell within hours (8). In this situation, exemplified by the Fas and Fas ligand-induced death pathway, caspase inhibitors can prevent the cell death (9). However, in responses generated through other pro-apoptotic stimuli, caspase inhibitors do not always protect against cell death (10–15). Some pro-apoptotic stimuli are reported to induce mitochondrial alterations, including collapse of the mitochondrial inner transmembrane potential (Δψm), generation of reactive oxygen species (ROS) and release of cytochrome c, prior to activation of caspases (16). This mitochondrial dysfunction is also thought to cause another type of cell death, necrosis.

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 reaction and anti-tumor drug-induced cell death. To date, it is not certain whether cell death triggered by BCR cross-linking requires mitochondrial alterations and caspase activation. In order to clarify the molecular mechanism of BCR-mediated cell death, a mouse B cell lymphoma WEHI-231 cell has been used as a model for immature B cells (17,18) and for study of the mechanism of clonal selection (19). Death receptors can activate caspase cascades, causing DNA fragmentation (20). After 40 h of cell culture, the cells were collected and lysed in 0.5 ml of 0.6% SDS/10 mM EDTA containing 50 mg/ml propidium iodide. Samples were incubated overnight at 4°C. For each sample at least 1 × 10⁴ events were collected and analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Further analysis of flow cytometric data was performed using CellQuest software.

**Detection of nuclear change**

To assess the percentage of cells undergoing DNA fragmentation, DNA content per cell was determined (19). After culture, cells were washed with PBS and resuspended in 200 μl hypotonic buffer (0.1% sodium citrate, 0.1% Triton-X and 20 μg/ml RNase A) containing 50 mg/ml propidium iodide. Samples were incubated overnight at 4°C. For each sample at least 1 × 10⁴ events were collected and analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Further analysis of flow cytometric data was performed using CellQuest software.

**Western blot analysis of poly (ADP-ribose) polymerase (PARP) cleavage**

After culture, ~2 × 10⁵ cells were pelleted and dissolved in SDS–PAGE sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 10% glycerol, 1% mercaptoethanol and 0.004% bromophenol blue). Cell lysates were subjected to 7.5% SDS–PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The filters were then blocked with 5% non-fat milk in TBST (10 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.05% Tween 20) for 1 h and incubated with polyclonal rabbit anti-PARP antibodies (BIOMOL, Plymouth Meeting, PA) at a dilution of 1:5000 in 5% non-fat milk TBST for 1 h at room temperature. After three washes with TBST, filters were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham, San Clemente, CA) for 1 h. After three washes, signals were visualized with the ECL system (Amersham) according to the manufacturer’s protocol.

**Plasma membrane PS transition**

After cell culture, binding buffer (HEPES-buffered saline solution supplemented with 2.5 mM CaCl₂, pH 7.4) was added to the cell suspension at the concentration of 4 μg/ml each. The samples were examined by fluorescence microscopy.

**Methods**

**Cell culture**

WEHI-231 cells (kindly provided by T. Tsubata) were cultured at 37°C in a humidified atmosphere with 5% CO₂ in RPMI 1640 containing 5 × 10⁻⁵ M 2-mercaptoethanol, supplemented with 10% heat-inactivated FCS (Gibco/BRL, Grand Island, NY). N-benzyloxy carbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) (Kamiya Biochemical, Seattle, WA) was dissolved in DMSO and added at the onset of culture at various concentrations. As a control, the same volume of DMSO was added. Anti-IgM antibodies [F(ab)²] fragment; ICN Pharmaceuticals/Cappel, Aurora, OH) (10 μg/ml) were added 1 h after the addition of z-VAD-fmk. Bongkrekic acid (BA) was kindly provided by H. Terada and Y. Shinohara (Tokushima University, Japan). BA was added to the cell culture 30 h after anti-IgM stimulation to a final concentration of 50 μM, and the cells were incubated further for 1 h at 37°C and then analyzed.
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Analysis for membrane permeability cell death

Cells were stained with 1 µg/ml of propidium iodide in PBS, containing 2% FCS/0.05% NaN3 for 5 min on ice and the percentage of propidium iodide-positive (dead) and -negative (live) cells was determined with a FACScan (Becton Dickinson) flow cytometer. Analysis of flow cytometric data was performed using CellQuest software.

Δψm and ROS measurement

After cell culture, cells were incubated for 15 min at 37°C with 3,3'-dihexyloxacarbocynine iodide [DiOC6(3); Sigma] (40 nM) for Δψm quantification and dihydroethidium (DE; Sigma) (2 µM) for determination of superoxide anion generation, followed by FACScan analysis.

Transfection of mouse bcl-xL

The murine bcl-xL cDNA was isolated from a murine mRNA by RT-PCR amplification using primers corresponding to sequences in the bcl-xL coding region (5' -CCCTATATATATTGCTCGAG-3') and (5' -GAAGAAGTCCCTGAGTTCATCAGTG-3'). A band of appropriate size (0.8 kb) was subcloned into pHBluescriptSK(+) (Stratagene, La Jolla, CA). To generate an 8 amino acid FLAG tag, FLAG sequences were attached to the N-terminus of the murine Bcl-xL protein by PCR. The 5' primer (5' -AGAGACATCCCTGACATCAGTTCATCAGTG-3') incorporated a FLAG tag and a consensus translation initiator site into Bcl-xL. The 3' primer (5' -GGAGGAGATGTTCATCAGTTCAG-3') incorporated the natural bcl-xL stop codon. Amplification of FLAG-bcl-xL was performed by PCR through 35 cycles (1 min at 94°C, 1 min at 56°C, 1 min at 72°C). The amplified band was subcloned into pHBluescriptSK(+) and the authenticity of FLAG-bcl-xL was confirmed by sequencing. The insert was digested with EcoRI and subcloned into the pCXN-2 expression vector (21) (kindly provided by J. Miyazaki).

WEHI-231 cells were transfected by electroporation with the pCXN-2 containing FLAG-tagged mouse bcl-xL or pCXN-2 plasmid as a control, using a Bio-Rad Gene Pulser with a capacitance extender (250 V, 960 µF). Stable transfectants were selected for the acquisition of neomycin resistance by growth in the presence of G418 (1 mg/ml). Expression of Bcl-xL was confirmed by Western blot analysis using anti-FLAG mAb M5 (Eastman Kodak, New Haven, CT).

Results

Z-VAD-fmk blocks PARP cleavage during cell death in WEHI-231 cells

To determine the contributions of caspases to B cell apoptosis induced by cross-linking of the BCR, PARP cleavage in WEHI-231 cells was examined. PARP is cleaved by several caspases (22,23) and its cleavage has been considered an apoptotic hallmark. Intact PARP is ~110 kDa, but concomitant with the onset of cell death, PARP is cleaved by caspases to the 85 kDa apoptotic fragment. After treatment of WEHI-231 cells with anti-IgM, cells were collected at 12 h intervals. Cleavage of endogenous PARP to 85 kDa fragments was detected 24 h after the treatment (Fig. 1A). Z-VAD-fmk, containing aspartic acid at the P1 site, is a broad spectrum potent and irreversible inhibitor of caspases. Z-VAD-fmk is membrane permeable and non-toxic to cells. It has been shown to block various apoptotic death processes in intact cells, but does not block the aspartic acid-selective serine protease granzyme B (10,24,25). WEHI-231 cells were first incubated for 1 h with or without z-VAD-fmk and then cultured in the presence of anti-IgM antibodies for 36 h. Cells were harvested and PARP cleavage was examined. Cleavage of PARP to the 85 kDa apoptotic fragment was blocked by pretreatment with z-VAD-fmk in a dose-dependent manner (Fig. 1B). These data suggest that caspases may participate in the signal cascades for apoptosis from the BCR in WEHI-231 cells.

Z-VAD-fmk blocks nuclear apoptosis after cross-linking of the BCR on WEHI-231 cells

During BCR-mediated cell death, many events occur including DNA fragmentation, cell shrinkage, protein cleavage, transition of plasma membrane PS and increased membrane permeability (17,18,26). However, it is uncertain whether all of these events are induced by caspases. After cross-linking of the BCR, DNA fragmentation was examined by a gel electrophoresis assay (Fig. 1C) and by flow cytometry (Fig. 1D and E) respectively. In WEHI-231 cells, the DNA fragmentation accompanying BCR-induced apoptosis was prevented by pretreatment with z-VAD-fmk in a dose-dependent manner (Fig. 1C). The increase in the frequency of subdiploid cells in WEHI-231 cells after BCR cross-linking was also inhibited by the pretreatment with z-VAD-fmk (Fig. 1D and E). Upon BCR cross-linking, nuclei in WEHI-231 cells were degraded into several fragments, but z-VAD-fmk dramatically blocked these morphological manifestations of apoptosis (Fig. 1F and G). These data suggest that the DNA fragmentation and degradation of nuclei induced in WEHI-231 cells by BCR cross-linking are mainly due to the activation of caspases.

Z-VAD-fmk blocks membrane PS transition but not membrane permeability death

Twenty-four hours after cross-linking of the BCR, PS transition was observed by staining with Annexin-V and was partially prevented by pretreatment with z-VAD-fmk (Fig. 2A). Therefore, PS transition on plasma membrane is in part at least under control of caspases. In contrast, although z-VAD-fmk treatment retarded cytolysis, it was incapable of preventing disruption of plasma membrane events under the same conditions where nuclear fragmentation was completely blocked (Fig. 2B), indicating that the membrane permeability cell death is not directly associated with caspase activity.

Z-VAD-fmk does not prevent mitochondrial membrane potential collapse during cell death after cross-linking of BCR

Several cell-free systems have implicated mitochondria as being necessary for apoptosis (27). Superoxide anion generation and a fall in Δψm have been noted to be early events in several models of cell death (28,29). A reduction in Δψm was noted 24–30 h after IgM cross-linking of WEHI-231 cells...
Fig. 1. PARP cleavage, DNA fragmentation and nuclear fragmentation are blocked by the caspase inhibitor z-VAD-fmk in BCR-induced apoptosis of WEHI-231 cells. (A) WEHI-231 cells (2 x 10^5) were treated with 10 µg/ml of anti-IgM antibody and apoptotic degradation of PARP was analyzed by immunoblot at indicated times. p110 and p85 indicate full-length and cleaved PARP respectively. (B) Prior to the stimulation with anti-IgM (10 µg/ml) for 36 h, WEHI-231 cells were treated with z-VAD-fmk at the concentration of 25, 50, 100 and 200 µM (lanes 5–8) or DMSO as a control for each (lanes 1–4). (C) WEHI-231 cells were cultured with anti-IgM (10 µg/ml) after pretreatment for 1 h with z-VAD-fmk or DMSO as a control. At 40 h after the culture, DNA was extracted and electrophoresed on 2% agarose gel. Z-VAD-fmk was used at the concentration of 25, 50, 100 and 200 µM (lanes 2–5), and equal volumes of DMSO were used as controls (lanes 7–10). Lanes 1 and 6 are non-pretreated controls. (D and E) Cells were lysed in hypotonic buffer containing propidium iodide and nuclear DNA contents were analyzed by FACScan cytometer. (D) Representative data after 36 h treatment of anti-IgM with pretreatment of z-VAD-fmk (200 µM) or DMSO control. The percentage indicates appearance of the cells with decreased DNA content in the subdiploid area. (E) Time course of formation of subdiploid cells. For nuclear staining, cells were treated with anti-IgM (10 µg/ml) with pretreatment of (G) z-VAD-fmk (200 µM) or (F) DMSO. After 40 h culture, cells were stained with acridine orange and ethidium bromide, and examined by fluorescence microscopy. Note the diffuse staining of z-VAD-fmk-treated cells in (G) as contrast with the fragmented nuclei in (F). The results presented in this figure are representative of two or three independent experiments.
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Fig. 2. z-VAD-fmk retards the membrane PS transition and ROS generation, but not membrane permeability, cell death and Δψm collapse in BCR-induced apoptosis of WEHI-231 cells. WEHI-231 cells pretreated with z-VAD-fmk or DMSO were cultured with anti-IgM (10 µg/ml). Cells were stained with Annexin-V (A) or propidium iodide (B) and analyzed by FACScan cytometer. (C–F) For quantitation of Δψm and superoxide anion generation, cells were subjected to simultaneous staining with the Δψm-sensitive dye DiOC6(3) and DE. DE is non-fluorescent, but reacts with superoxide anion to form the fluorescent product ethidium. Numbers refer to the percentage of cells encountered in each quadrant. The experiments were performed twice with similar results.

Mitochondrial changes occur prior to the increase in plasma membrane permeability

To elucidate the relationship of the mitochondrial change and plasma membrane integrity loss, we measured their kinetics (Fig. 3). The cells were stimulated with anti-IgM and the percentage of dying cells were measured in three ways, i.e., Δψm collapse, plasma membrane integrity loss and hypodiploidy. Although the background level of each assay was different, the mitochondrial change and hypodiploidy preceded the increase in plasma membrane permeability. The kinetics of hypodiploidy appear similar to that of Δψm.

Bcl-xL blocks nuclear apoptosis and PS transition as well as the increase in membrane permeability increment

Bcl-xL is not expressed in WEHI-231 cells (30), but exogenously expressed Bcl-xL effectively blocks BCR-mediated apoptosis (20,31–33). WEHI-231 cells stably transfected with bcl-xL cDNA were treated with 10 µg/ml of anti-IgM for 24–48 h. DNA fragmentation, PARP cleavage and PS transition induced by BCR cross-linking were all blocked by the overexpression of Bcl-xL (Fig. 4A–C). Plasma membrane permeability death, Δψm collapse and generation of superoxide anion were also blocked by Bcl-xL (Fig. 4D and E). These results suggest that both plasma membrane permeability increment and nuclear apoptotic changes are closely linked to the alteration of mitochondrial function.

BA inhibits the anti-IgM-induced Δψm disruption and plasma membrane permeability death in WEHI-231 cells

We used BA treatment of WEHI-231 in order to confirm that mitochondrial function is closely related to the cell death triggered by IgM receptor cross-linking. BA is a specific ligand of the adenine nucleotide translocator, located in the inner mitochondrial membrane (34). The adenine nucleotide translocator is included in the structures of the mitochondrial inner membrane that may participate in the formation of mitochondrial permeability transition (PT) pores. BA is known to affect the molecular conformation of the adenine nucleotide translocators, thereby reducing the probability of PT pore gating. Since long exposure to BA was itself toxic to
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Fig. 4. Bcl-xL expression in WEHI-231 cells blocks PARP cleavage, DNA fragmentation, PS transition, membrane permeability cell death and mitochondrial alterations induced by BCR cross-linking. (A) Cell lysates were prepared after 36 h treatment with anti-IgM (10 µg/ml) and PARP cleavage was visualized by Western blotting. Lanes 1 and 2 are two independent mock clones, and lanes 3 and 4 are two independent clones that express Bcl-xL. The expression of Bcl-xL was previously confirmed by Western blotting. (B) After 40 h stimulation with anti-IgM (10 µg/ml), total DNA was separated on agarose gels and visualized as described in Methods. Lanes 1/2 and 3/4 indicate mock control clones and Bcl-xL expressing clones respectively. Annexin-V binding (C) and propidium iodide staining (D) assay were performed as in Fig. 2. Open symbols show two independent mock control clones and closed symbols show two independent Bcl-xL expressing clones respectively. (E) For quantitation of Δψm and superoxide anion generation, cells were subjected to simultaneous staining with DiOC6(3) and DE, and analyzed as described before. The experiments were on two separate occasions with identical results.

Fig. 5. BA suppresses Δψm collapse and membrane permeability death. BA was added to the cell culture 30 h after anti-IgM stimulation (10 µg/ml) to the final concentration of 50 µM and the cells were incubated further for 1 h at 37°C. The cells were stained with DiOC6(3) to detect Δψm collapse or propidium iodide to detect plasma membrane permeability, and analyzed by FACScan. Closed and open symbols show the samples treated with or without BA respectively.

WeHI-231 cells (data not shown), we incubated the dying WEHI-231 cells (30 h after anti-IgM stimulation) for 1 h with BA. BA suppressed both the Δψm collapse and increase in plasma membrane permeability (Fig. 5). These data indicate that the change of plasma membrane permeability occurs after Δψm disruption.

Discussion

Previous study indicated that caspases contribute to nuclear changes after cross-linking of the BCR in mouse CH31 and human Ramos B cell lines (26,35,36), and that mitochondrial dysfunction occurs in WEHI-231 cells during BCR-induced apoptosis (28). However, the relationship between caspase activity, mitochondrial dysfunction and plasma membrane alterations in BCR-induced cell death is not certain. In the present study, involvement of mitochondrial alteration and activation of caspases were confirmed in cell death events induced by BCR cross-linking in WEHI-231 cells. We also clarified the order of these events, in that mitochondrial dysfunction precedes the necrotic and apoptotic changes induced by BCR cross-linking.

The broad spectrum caspase inhibitor, z-VAD-fmk, prevented PARP cleavage, nuclear fragmentation, DNA fragmentation and hypodiploidy of WEHI-231 cells (Fig. 1). PARP is one of the substrates of caspases, especially caspase-3 (22,23). In living cells, caspase-activated DNase (CAD) exists as an inactive complex with inhibitor of CAD (ICAD) or by guest on May 1, 2016 http://intimm.oxfordjournals.org/ Downloaded from
mitochondria, Apaf-1 and dATP, indicating that proteases, and alteration of cellular reduction–oxidation release of proteins that trigger activation of caspase family transport, oxidative phosphorylation, ATP production and changes in mitochondria, such as disruption of electron important in controlling cell life and death (16,51). The tern of Bcl-2 family proteins was different in B and T cells, of Bcl-2 family proteins may promote apoptosis through such as Bcl-2 family proteins or caspases. For example, some different molecules that account for cell death machinery, certain. The discrepancy may be due to the presence of WEHI-231 death and the previous data on thymocytes is not ing that caspases are considered to be indispensable for cell death triggered by four independent input pathways, inhibitors do not always protect against cell death. In the apoptotic signal of WEHI-231 cells after BCR cross-linking, the activation of the initiator caspases seems to be closely related to the mitochondrial alterations as discussed below. After cross-linking of the BCR on WEHI-231 cells, PS transition to the cell surface and plasma membrane permeability increase was preceded by Δψm collapse and ROS generation (Fig. 5). Δψm collapse indicates the opening of PT pores (46) and ROS production mainly indicates the decrease in coupling efficiency of the electron transport chain in mitochondria. Thus, both mitochondrial dysfunction and plasma membrane alterations occur after cross-linking of BCR on WEHI-231 cells. Among these changes after cross-linking of the BCR on WEHI-231 cells, PS transition and ROS production were partially blocked by z-VAD-fmk, indicating an intricate relationship between caspase activation and PS transition or ROS production. However, z-VAD-fmk was incapable of preventing collapse of Δψm and disruption of the plasma membrane even under conditions in which nuclear changes were completely blocked. These data suggest that mitochondrial dysfunction and plasma membrane permeability loss occurs independent of caspase activity. These findings are different from those reported for thymocytes. In thymocytes, z-VAD-fmk was a potent and complete inhibitor of both apoptotic nuclear damage and cell permeability death triggered by four independent input pathways, corticosteroid, DNA damage by irradiation or etoposide, immobilized anti-Fas and immobilized anti-CD3 (10), indicating that caspases are considered to be indispensable for cell death in thymocytes. An explanation of the difference between WEHI-231 death and the previous data on thymocytes is not certain. The discrepancy may be due to the presence of different molecules that account for cell death machinery, such as Bcl-2 family proteins or caspases. For example, some of the Bcl-2 family proteins are cleaved by caspases and inactivated during apoptosis, and the cleaved fragments of Bcl-2 family proteins may promote apoptosis through mitochondrial change (47–49). However, the expression pattern of Bcl-2 family proteins was different in B and T cells, and also dependent on their differentiation stages (50) The available data suggest that mitochondrial alteration is important in controlling cell life and death (16,51). The changes in mitochondria, such as disruption of electron transport, oxidative phosphorylation, ATP production and release of proteins that trigger activation of caspase family proteases, and alteration of cellular reduction–oxidation potential culminate in cell death. The study of cell death kinetics in WEHI-231 cells indicated that the mitochondrial changes preceded the plasma membrane permeability alterations in BCR-induced cell death (Fig. 3). To confirm the relationship of mitochondrial dysfunction and plasma membrane permeability, BA, an inhibitor of a PT pores, was used. BA suppressed not only Δψm collapse but also the plasma membrane permeability death. Additionally, we showed that Bcl-xL could block mitochondrial dysfunction, plasma membrane alteration and nuclear changes. These findings illustrate a critical involvement of mitochondria prior to the plasma membrane and caspase activation causing nuclear changes in cell death induced by BCR cross-linking.

Once mitochondrial dysfunction occurs in WEHI-231 cells, it is considered to induce commitment of the cell to die by either an apoptotic mechanism involving Apaf-1-mediated caspase activation or through another caspase-independent necrotic process, e.g. due to collapse of electron transport, resulting in a variety of harmful events including generation of oxygen free radicals and decreased production of ATP. Both of those apoptotic and necrotic changes in WEHI-231 cells may be triggered and controlled by mitochondria during cell death after BCR cross-linking.

In conclusion, the results described in this paper suggest that mitochondrial alteration may be a rate-limiting step in the BCR-mediated death signal. After the mitochondrial alterations, both necrotic and apoptotic cascades proceed.

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Abbreviation
BA bongkrekic acid
BCR B cell antigen receptor
CAD caspase-activated DNase
DE dihydroethidium
Δψm mitochondrial membrane potential
DOCP(3) 3,3’-dihexyloxacarbocyanine iodide
ICAD inhibitor of CAD
PARP poly (ADP-ribose) polymerase
PS phosphatidylserine
PT permeability transition
ROS reactive oxygen species
z-VAD-fmk N-benzyloxy carbonyl-Val-Ala-Asp-fluoromethylketone

References
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32 Mcl-2 is activated during apoptosis and cleaves the death substrate lamin A. J. Biol. Chem. 271:16443.


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