

Intracellular pH is a Critical Element in Apoptosis Triggered by GM-CSF Deprivation in TF1 Cells

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ABSTRACT

Background: Hemopoietic cells require the constant presence of growth factors for survival *in vitro* and *in vivo*. Caspases have been known as central executors of apoptotic cell death. We have, therefore, investigated the pathways that regulate caspase activity and apoptosis using the CD34⁺ cell line, TF-1 which requires GM-CSF for survival. **Methods:** Apoptosis was measured by annexin V staining and mitochondrial membrane potential was measured by DiOC6 labelling. Intracellular pH was measured using pH sensitive fluorochrome, BCECF or SNARF-1, followed by flow cytometry analysis. Caspase activation was analyzed by PARP cleavage using anti-PARP antibody. **Results:** Removal of GM-CSF induced PARP cleavage, a hallmark of caspase activity, concomitant with pH_i acidification and a drop in mitochondrial potential. Treatment with ZVAD, a competitive inhibitor of caspases, partially rescued cell death without affecting pH_i acidification and the reduction of mitochondrial potential, suggesting that both these events act upstream of caspases. Overexpression of Bcl-2 prevented cell death induced by GM-CSF deprivation as well as pH_i acidification and the reduction in mitochondrial membrane potential. In parental cells maintained with GM-CSF, EIPA, a competitive inhibitor of Na⁺/H⁺ antiporter induced apoptosis, accompanied by a drastic reduction in mitochondrial potential. In contrast, EIPA induced apoptosis in Bcl-2 transfectants without causing mitochondrial membrane depolarization. **Conclusion:** Taken together, our results suggest that the regulation of H⁺ fluxes, either through a mitochondrion-dependent or independent pathway, is central to caspase activation and apoptosis. (*Immune Network* 2003;3(4):268-275)

Key Words: Apoptosis, caspases, intracellular pH, mitochondrial potential

Introduction

Apoptosis is a programmed cell death that serves as a major mechanism for the precise regulation of immune cell numbers (1) and a defence mechanism to remove unwanted and potentially dangerous cells such as self-reactive lymphocytes (2), virus-infected cells (3) and tumor cells (4). Since apoptosis plays critical role in regulation of immune system including tissue homeostasis and general cell turn-over, aberrant regulations of apoptosis lead to many immunological disorders such as autoimmune diseases (5), cancer and neurodegeneration (6).

Apoptosis was originally defined by morphological characteristics of dead or dying cells that exhibit

cytoplasmic blebbing, DNA fragmentation, chromatin condensation, and cell shrinkage (7). A number of biochemical changes have also been identified during induction of apoptosis including intracellular acidification and changes in mitochondria (8,9). A fall of the mitochondrial potential has been known to occur before the fragmentation of the DNA in oligonucleosomal fragment (10,11). Intracellular acidification has also been shown to be an early event that regulates caspase activation resulting in apoptosis (12). However, relationship between these events and the role of these events during apoptosis in relation to other apoptotic regulators have not been clarified.

Several intracellular regulators involved in apoptosis have been identified in *Caenorhabditis elegans* (13). Among them, caspases and Bcl-2 family are mostly well characterized. Cysteine protease of the IL-1 β -converting enzyme (ICE) family (caspases) has been reported as a homology to the *ced-3* gene product of the nematode, *C. elegans*, that is required for cell death during development (14). Bcl-2 has been known as

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mammalian homologue of *ced-9* in *C. elegans*, a negative regulator of apoptosis (15). Induction of apoptosis has been observed when caspases were overexpressed in their active form (16) and inhibition of apoptosis has been observed when caspases were specifically inhibited (17), indicating that caspase activation is essential process during apoptosis. In contrast, Bcl-2 has been shown to inhibit apoptosis induced by a wide variety of stimuli (18,19).

Apoptosis has been reported to occur during hematopoietic as well as neural cell development (4, 20). The tight control of proliferation, survival, and apoptosis of hematopoietic cells in bone marrow has a major role in allowing normal hematopoiesis. A variety of cytokines, including GM-CSF (granulocyte-colony stimulating factor) and IL-3 (interleukin-3), regulate viability, proliferation, differentiation, and function of hemopoietic cells (21). Hematopoietic cells undergo apoptotic cell death in the absence of growth factors, whereas an escape from this regulation may result in leukemogenesis. In hematopoietic cells, it has been shown that IL-3, GM-CSF and erythropoietin promote cell survival through retardation of DNA break down. GM-CSF has been shown to activate PKC in monocytic cell line, U937 which leads to cell survival (22). Furthermore, exposure of monocytes to GM-CSF results in prolonged activation of the Na^+/H^+ antiporter, which correlates with cell proliferation (23). As hematopoietic cells express both high and low affinity receptors of GM-CSF, GM-CSF require to maintain survival of hematopoietic cells in vitro (24).

The human myelo-erythroid CD34^+ leukemia cell line, TF-1 has been reported to require GM-CSF or IL-3 for cell survival and undergo apoptosis upon growth factor deprivation (25). In the present study, we examined the role of regulations in intracellular acidification and mitochondria during apoptosis induced by GM-CSF deprivation in using hematopoietic cells.

Materials and Methods

Cell lines and growth factors. The human CD34^+ hematopoietic cell line, TF1 cell that requires GM-CSF for cell survival was a kind gift from Dr. Thoshio Kitamura (DNAX, Seattle, WA, USA). The cells were cultured in IMDM (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (Gibco BRL) and 5 ng/ml GM-CSF. The cells were passaged every second day at 1.5×10^5 cells/ml. Purified recombinant GM-CSF was kindly provided by Dr. Steve Clark (Genetics Institute, Cambridge, MA, USA). Bcl-2 transfectant of TF1 was provided by Dr. Trang Hoang (IRCM, Montreal, QC, Canada).

Chemicals and antibodies. Mouse monoclonal anti-PARP

antibody, and rabbit polyclonal anti-caspase-3 antibody were kindly provided by Dr. Serkaly in University of Montreal, Canada. ZVAD (carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone) was purchased from Enzyme Systems Products (Livermore, CA, USA). DioC₆(₃) (rhodamine 123, 3,3'-dihexyl-oxacarbocyanine iodide) was purchased from Molecular Probe (Eugene, OR, USA). 5-(N-ethyl-N-isopropyl) amiloride (EIPA) was provided by Dr. Trang Hoang.

Apoptosis assay. Apoptosis levels were measured by flow cytometric analysis using annexinV staining (26). Cells were cultured in the presence or absence of GM-CSF. 5×10^5 cells were washed in PBS and resuspended in 400 μl of binding buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1.8 mM CaCl_2). FITC conjugated annexin V was added and incubated for 10 min at 4°C. Just before analysis, propidium iodide (PI) was added and the cells were analyzed by FACS (Becton Dickinson, Franklin Lakes, NJ, USA).

Measurement of mitochondrial membrane potential. Cells were cultured in the presence or absence of GM-CSF for 48 hrs. Cells were transferred to v bottom plate and centrifuged. 40 nM DiOC₆(₃) was added to the cell pellet and resuspended. The plate was incubated for 15 min at 37°C in dark condition. The cells were washed with PBS by incubating at 37°C for 30 min. Cells were collected by centrifugation and resuspended in PBS followed by analysis using FACS (Beckton Dickinson).

Intracellular pH monitoring. pH_i measurement was determined using pH sensitive fluorochrome, bis (carboxyethyl) carboxyfluorescein, BCECF (Molecular Probes) or 1-carboxy-seminaphthorhodafluor-1 (acethyl-methyl ester) (27), SNARF-1 (Molecular Probes) (12). Cells were loaded for 30 min at 37°C with 10 μM BCECF in sodium HEPES buffer. After washing, the cells were introduced in thermostated cuvette and the pH sensitive fluorescence was recorded continuously at 37°C. Variations in pH were monitored by on a SPEX model CM1T111 dual excitation spectrophotometer. The ratio of pH sensitive fluorescence (excitation wave length 500 nm) and pH insensitive fluorescence (excitation wave length 450 nm) allows the calculation of pH, that is independent of cell number and dye concentration. For SNARF staining, the ratio was analyzed at 575 nm and 620 nm by FACS.

Western blotting. TF1 cells were cultured in the presence or absence of GM-CSF. Cells were harvested and washed in PBS and lysed with sample buffer (62.5 mM Tris-HCl, pH 6.8, 6 M Urea, 10% glycerol, 2% SDS, 5% 2 ME, 0.00125% bromophenol blue) and boiled for 3 min. Lysates were subjected to 12% SDS-electrophoresis. Gels were transferred to nitrocellulose membrane (Amersham, Piscataway, NJ,

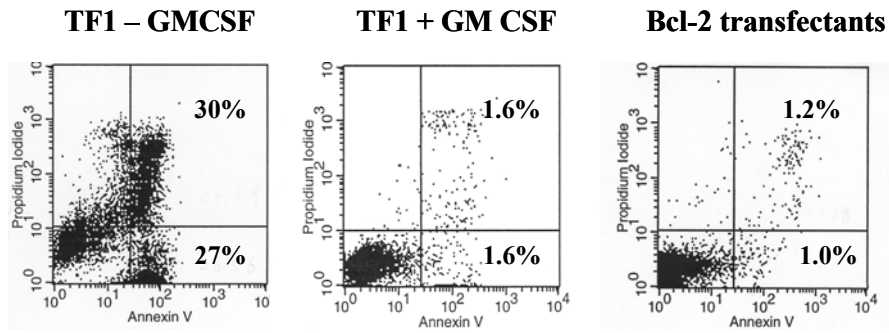


Figure 1. Annexin V and PI staining of apoptotic cells by GM-CSF deprivation in TF1 cells. TF1 cells were cultured for 48 hrs in the absence or presence of GM-CSF. Bcl-2 transfectants of TF1 were also cultured for 48 hrs in the absence of GM-CSF. Cells were collected and stained with annexin V-FITC and propidium iodide followed by FACS analysis. Apoptotic cells were represented as annexin-V positive and PI negative cells.

USA). Membranes were blocked with PBS containing 5% skim milk and 0.05% Tween 20 and washed. Then, the membrane was incubated with anti caspase antibody (1 : 1,000 in 5% skim milk) and PARP antibody (1 : 1,500 in 5% skim milk) at 4°C, overnight with shaking. The membrane was washed in PBS containing 0.5% Tween 20 and incubated with secondary antibody, anti rabbit HRP and anti mouse HRP for PARP. Detection was achieved by enhanced chemiluminescence (ECL, Amersham) and autoradiography.

Results

Caspase-3 activation by GM-CSF withdrawal. TF1 is a CD34⁺ hemopoietic cell line that requires the growth factor GM-CSF for cell survival. Apoptosis was induced by GM-CSF withdrawal in TF1 cells. The levels of apoptosis were measured using annexin V which binds to phosphatidylserine on apoptotic cells. As annexin V negative live cells undergo apoptotic cell death, apoptotic cells bind to annexin V and dead cells bind to propidium iodide (26). TF1 cells survived in the presence of GM-CSF, whereas annexin V positive apoptotic cells were induced in the absence of GM-CSF (Fig. 1). In contrast, apoptosis was inhibited in Bcl-2 transfectants of TF1 cultured even in the absence of GM-CSF indicating overexpression of Bcl-2 rescued apoptosis by GM-CSF withdrawal (Fig. 1). Since caspase-3 has been known as a central executor of apoptotic process in most of cells (28), we investigated caspase activation during apoptosis by Western blot analysis using polyclonal antibody against caspase 3 and PARP in TF1 cells. As shown in Fig. 2, p15 and p17, active forms of caspase 3 (cpp32) were detected in the cells cultured without GM-CSF. Furthermore, the activation of caspase was conformed by a cleavage of PARP, substrate of caspase-3. 116 kD of PARP was cleaved to 85 kD

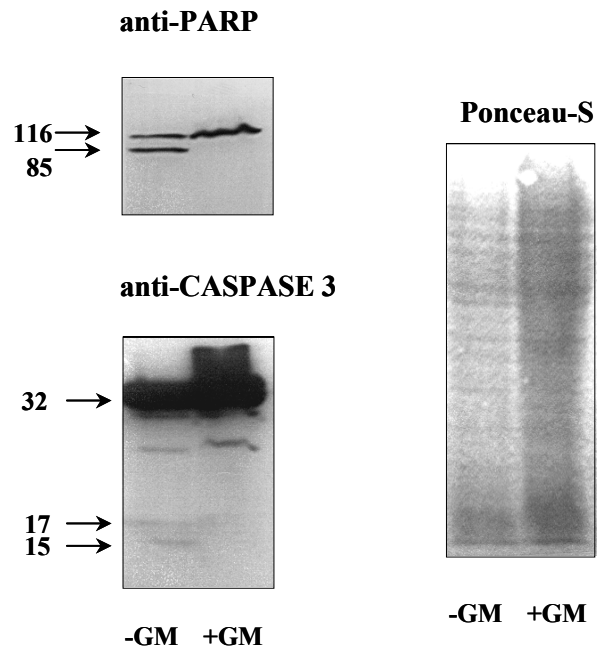


Figure 2. Immunoblot analysis of caspase-3 and PARP in TF1 cells. 1×10^6 of TF1 cells were cultured in the absence or presence of GM-CSF for 48 hr. Lysates of the cells were subjected to 12% SDS/PAGE and transferred to nitrocellulose membrane. Filters were incubated with polyclonal antibody against caspase-3 or PARP followed by incubation with goat anti rabbit IgG-HRP and anti mouse IgG-HRP, respectively. Immunoactivity was detected by ECL reaction. Quantification of protein was done by Ponceau-S staining. p85, the apoptotic fragment of PARP; p15 and p17, active form of caspase-3 (cpp32) were detected in the cells cultured without GM-CSF.

of apoptotic fragment in the cells cultured without GM-CSF indicating activation of caspase-3. These suggest that activation of caspase-3 involves the apoptosis of TF1 cells induced by growth factor deprivation.

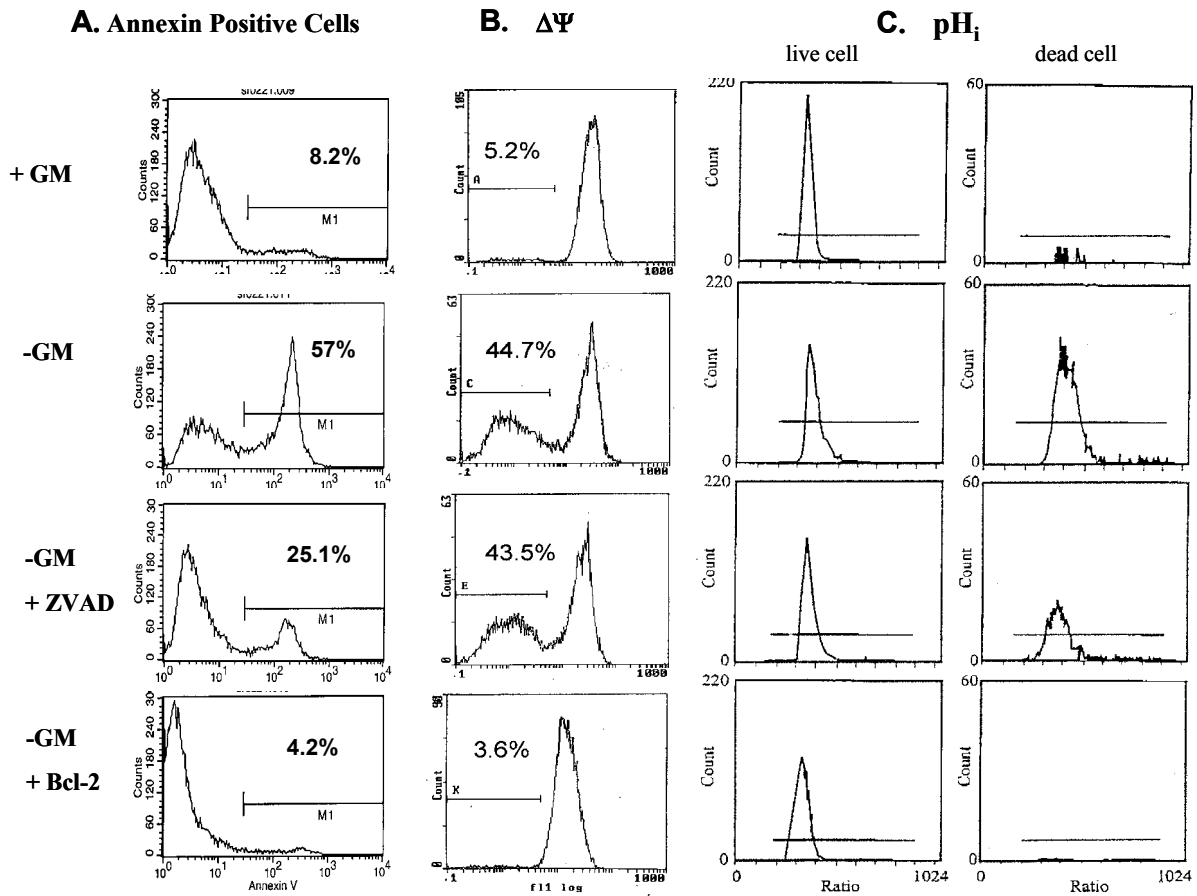


Figure 3. Effect of ZVAD and Bcl-2 on biochemical events of apoptosis. To examine the effect of Bcl-2, Bcl-2 transfectants of TF1 cells were employed. Cells were treated with caspase-3 inhibitor, ZVAD in the absence of GM-CSF for 30 hr. (A) Apoptotic cells were measured by annexin V and propidium iodide staining followed by FACS analysis. Apoptotic cells were represented as annexinV positive cells. (B) For measurement of mitochondrial potential, cells were stained with 40 nM of DiOC₆₍₃₎ and analyzed by flow cytometry. DiOC₆₍₃₎, cationic dye which can be retained by mitochondrial inner membrane. The percentages reflect the cells in which mitochondrial potentials are reduced. (C) Intracellular pH was determined using pH sensitive fluorochrome, bis (carboxyethyl) carboxyfluorescein, BCECF and analyzed by dual excitation spectro-photometer. The 575 nm/620 nm ratio represents intracellular pH (a higher ratio reflects decreased pH).

Growth factor withdrawal triggers apoptosis concomitant with a reduction in mitochondrial potential and intracellular acidification. The dissipation of mitochondrial transmembrane potential ($\Delta\Psi$) (10,30) and intracellular acidification have been suggested as early events of the cell death process (12). We observed changes in mitochondrial potential using DiOC₆₍₃₎ and intracellular pH using pH sensitive fluorochrome, BCECF during the apoptosis induced by deprivation of GM-CSF. As shown in Fig. 3A, apoptosis represented as annexin V positive cells was induced concomitant with reduction of mitochondrial potential and pH_i acidification in TF1 cells cultured in the absence of GM-CSF. In order to examine the roles of caspase on mitochondrial potential intracellular acidification during apoptosis, cells were treated with ZVAD, ICE (interleukin-1 β converting enzyme family) inhibitor,

in the absence of GM-CSF. ZVAD partially recovered apoptotic cells in the absence of GM-CSF, however had no effect on reduction of mitochondrial potential and intracellular acidification induced by GM-CSF withdrawal (Fig. 3B, 3C). On the other hand, overexpression of Bcl-2 rescued cell survival and restored the mitochondrial membrane potential and intracellular acidification. These data imply that caspase-3 acts downstream of mitochondrial depolarization and intracellular acidification act upstream of caspases and down stream of Bcl-2.

Na⁺/H⁺ antiporter inhibitor, EIPA induce apoptosis. In order to further dissect the role of mitochondrion and Bcl-2 in regulating pH_i and caspase activation, we prevent H⁺ influx through Na⁺/H⁺ antiporter using EIPA, a competitive inhibitor of Na⁺/H⁺ antiporter. EIPA induced apoptosis in a dose dependent

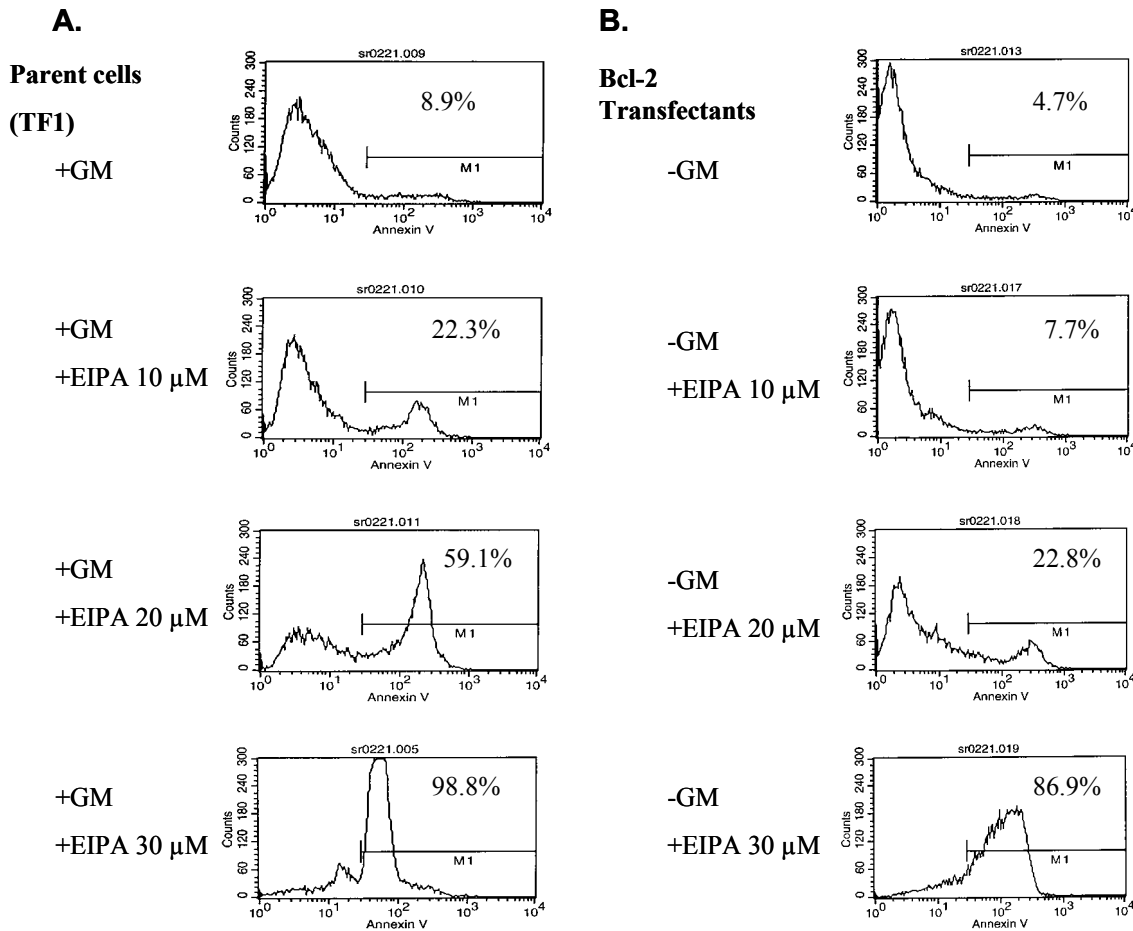


Figure 4. Effect of antiporter inhibitor, EIPA on apoptosis in TF1 and its Bcl-2 transfectants. Parental cells (TF1) (A) and Bcl-2 transfectant (B) were treated with different dose of EIPA in the absence or presence of GM-CSF for 40 hrs. The cells were collected and stained with annexin V. FACS analysis was performed and apoptotic cells were represented as annexin-V positive cells.

dent manner in parental cells maintained with GM-CSF (Fig. 4A). In addition, treatment with EIPA also induced apoptosis in Bcl-2 transfectants in a dose dependant manner (Fig. 4B), indicating EIPA prevents the effect of GM-CSF and Bcl-2 in suppressing apoptosis. Effect of EIPA on mitochondrial potential was also investigated. In parental cells maintained with GM-CSF, EIPA induced apoptosis accompanied by drastic reduction in mitochondrial potential (Fig. 5A). In contrast, EIPA did not affect mitochondrial potential in Bcl-2 transfectant, indicating that inhibition of the Na^+/H^+ antiporter in Bcl-2 transfectant induces apoptosis in the absence of mitochondrial membrane depolarization (Fig. 5B). These data suggest that apoptosis by inhibition of Na^+/H^+ antiporter might occur in a mitochondria dependant or independent pathway.

pHi acidification in apoptosis is rescued by ectopic expression of Bcl-2. The changes in intracellular pH were measured in parental cells and Bcl-2 transfectant using

SNARF-1. Cell viability was also examined using annexin V staining with the same cells. As shown in Fig. 6, EIPA decreased intracellular pH in the presence of GM-CSF to the similar level of pH_i observed in parental cells cultured without GM-CSF. In contrast, pH_i acidification by GM-CSF was rescued by overexpression of Bcl-2. However, EIPA induced intracellular acidification in Bcl-2 transfectant. In addition, cell viability was correlated with intracellular acidification in both parental cells and Bcl-2 transfectant, suggesting that the regulation of pH_i is crucial for the suppression of apoptosis by GM-CSF or Bcl-2.

Discussion

In an attempt to assess the role of cytoplasmic acidification and regulation of mitochondrial membrane potential during apoptosis. Apoptosis was triggered by growth factor deprivation using hemopoietic cell line, TF1 that requires GM-CSF for cell survival. We

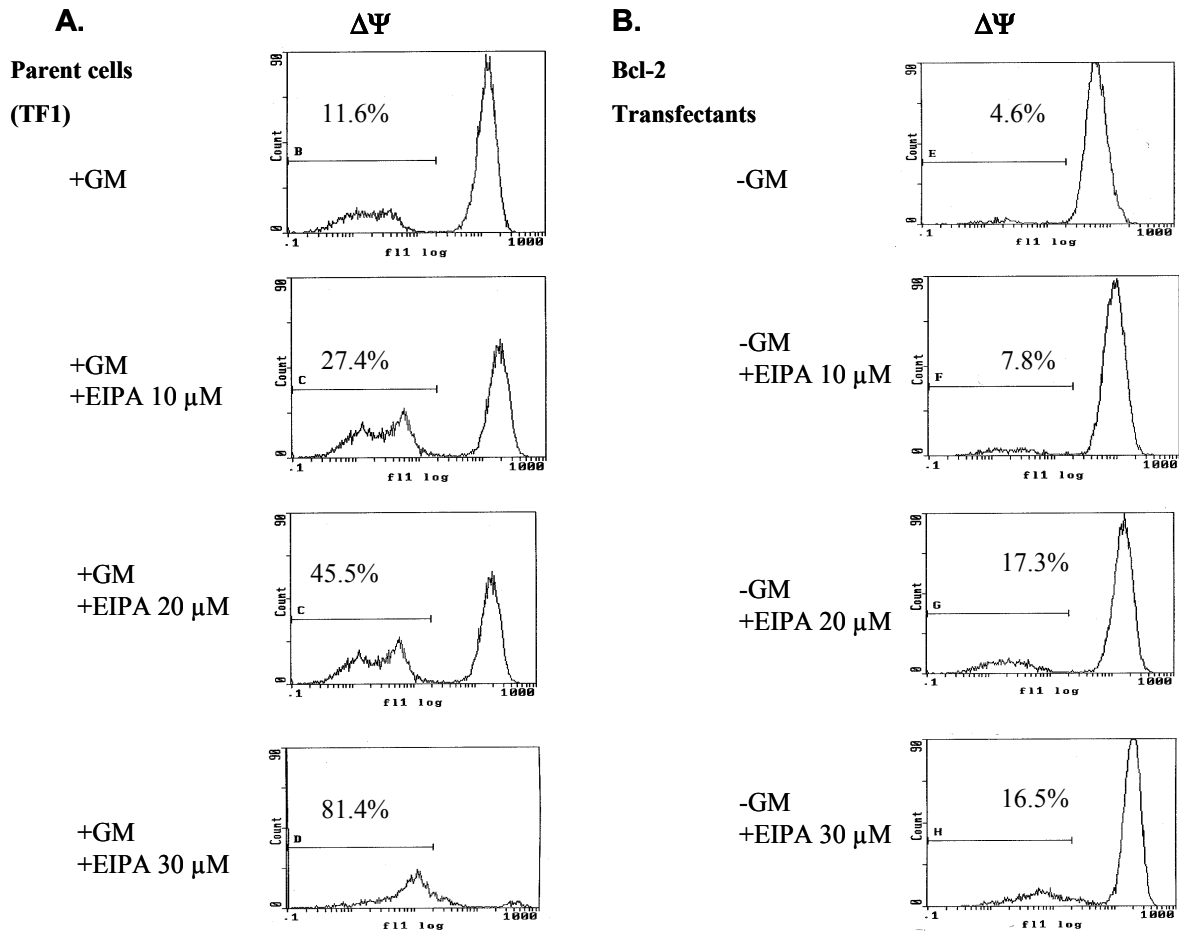


Figure 5. Effect of EIPA on mitochondrial potential maintained by GM-CSF and Bcl-2 over expression. Parental cells (TF1) (A) and Bcl-2 transfectants (B) were treated with different dose of EIPA in the absence or presence of GM-CSF for 40 hrs. Mitochondrial potential was measured using DiOC₆(₃) as described in Materials and Methods. FACS analysis was performed and reduction of mitochondrial potential was represented as the number of DiOC₆(₃) negative cells.

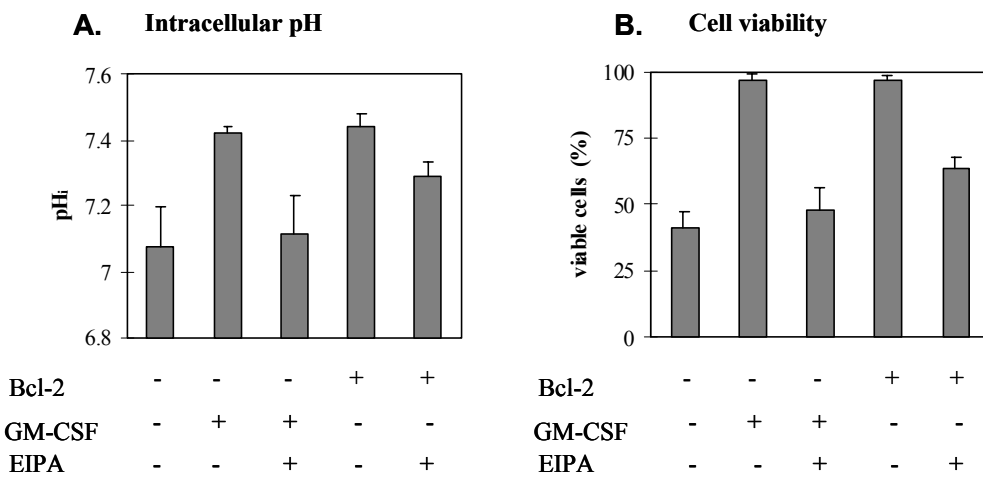


Figure 6. Effect of EIPA and Bcl-2 on intracellular pH and cell viability. Parental cells (TF1) (A) and Bcl-2 transfectants (B) were treated with antiporter inhibitor, EIPA 20μM for 40 hrs. The cells were performed annexin V staining and measured pHi as described in Materials and Methods. Cell viability was represented as % of annexinV negative viable cells. Intracellular pH was measured using SNARF-1. pH was estimated from standard curves generated in parallel using cells suspended in buffers of known pH.

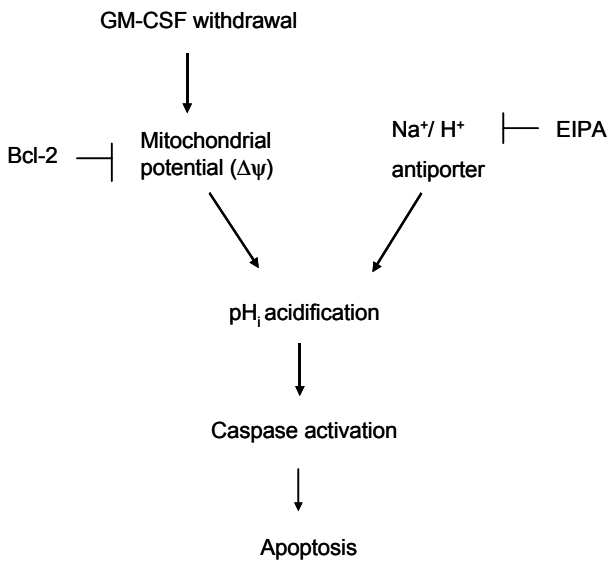


Figure 7. Schematic model of apoptosis triggered by GM-CSF deprivation in hematopoietic cell line, TF1. TF1 cells undergo apoptosis through mitochondrial membrane depolarization which can be blocked by Bcl-2, which is followed by intracellular acidification and caspase activation in the absence of GM-CSF. On the other hand, inhibition of Na^+/H^+ antiporter can also induce apoptosis through intracellular acidification followed by caspase activation.

investigated activation of caspase-3 that has emerged as a central executor of apoptosis. Caspases are synthesized as inactive precursors that require proteolytic conversion to become active protease. For instance, during apoptosis triggered by TCR cross-linking in T lymphocytes, the 32 kD caspase proenzyme is first cleaved to release fragment of 12 and 20 kD. Removal of the 3 kD propeptide form p20 generates the p17 form associated with caspase-3 activity and apoptosis (29). Activation of caspase-3 accompanied by a cleavage of the substrate for caspase-3, Poly (ADP-ribose) polymerase (PARP), which cleaved to 80 kD of apoptotic fragment by caspase-3 during apoptosis (30). Our results showing p15 and p17, activated forms of caspase 3 (cpp32) and 85 kDa of cleavage fragment of PARP indicate activation of caspase-3 during the apoptosis.

During the apoptosis induced by GM-CSF withdrawal in TF1 cells, intracellular acidification and reduction of mitochondrial potential were observed concomitantly with caspase activation. Competitive inhibitor of caspase-3, ZVAD partially recovered apoptosis without affecting mitochondrial potential and intracellular pH. If caspase activation is a prerequisite for intracellular acidification or reduction of mitochondrial potential to occur, then inhibitor of caspase should inhibit acidification or reduction of mitochondrial potential. In our result, ZVAD has no

effect on intracellular acidification and reduction of mitochondrial potential, suggesting that caspase activation is not prerequisite, that is, caspase act downstream of both events during apoptosis triggered by growth factor deprivation. On the other hand, ectopic expression Bcl-2 completely rescued cell death in the absence of GM-CSF with preventing from reduction of mitochondrial potential and intracellular acidification. These are supported by report that Bcl-2 family members are upstream of caspase activation (through their association with CED-4), regulate pH_i and prevent mitochondrial depolarization (12).

The mitochondria have been known to play an essential role in the apoptotic cell death by releasing various apoptotic proteins including cytochrome c into the cytoplasm. In the process of apoptosis, release of cytochrome c into the cytoplasm activates death promoting caspase, which in turn cleave a set of cellular proteins and promote the death program (9, 31). It has been shown that Bcl-2 family of proteins regulate mitochondrial changes during apoptosis (32) and they can directly control mitochondrial membrane permeability, mainly through regulation of formation of apoptotic proteinconducting pores in the outer mitochondrial membrane (31). It has also been suggested that intracellular acidification results from a change in the set point of Na^+/H^+ antiporter which might result from its dephosphorylation (33). In order to further dissect the role of the mitochondrion and of Bcl-2 in regulating pH_i and caspase activation, we prevented H^+ fluxes using 5-(N-ethyl-N-isopropyl) amiloride (EIPA) that specifically block Na^+/H^+ antiporter. In parental cells maintained with GM-CSF, EIPA induced apoptosis, accompanied by a drastic reduction in mitochondrial potential, whereas EIPA induces apoptosis in Bcl-2 transfectants without causing mitochondrial membrane depolarization. These suggest that intracellular acidification by inhibition of Na^+/H^+ antiporter might occur mitochondria dependant or independent pathway. These results are in agreement with hypothesis that at least two principle pathways for apoptosis; one requiring the participation of mitochondria, which activate caspase by releasing cytochrome c and another in which mitochondria are bypassed and caspases are activated directly (34,35).

Taken together, our results, as summarized in Fig. 7, show that mitochondrial membrane depolarization followed by intracellular acidification leads to apoptosis through caspase activation in TF1 cells when GM-CSF is deprived. In addition, inhibition of Na^+/H^+ antiporter can induce apoptosis through intracellular acidification followed by caspase activation without causing mitochondrial depolarization. These suggest that the regulation of H^+ fluxes, either

through a mitochondrion-dependent or independent pathway, is central to caspase activation and apoptosis induced by growth factor deprivation.

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