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Bcl-2 and Bcl-X_L Antagonize the Mitochondrial Dysfunction Preceding Nuclear Apoptosis Induced by Chemotherapeutic Agents[1]

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ABSTRACT

A number of apoptosis-inducing agents used in cancer therapy (etoposide, doxorubicin, 1-β-D-arabinofuranosylcytosine), as well as the pro-apoptotic second messenger ceramide, induce a disruption of the mitochondrial transmembrane potential ($\Delta\Psi_m$) that precedes nuclear DNA fragmentation. This effect has been observed in tumor cell lines of T-lymphoid, B-lymphoid, and myelomonocytic origin *in vitro*. Circulating tumor cells from patients receiving chemotherapy *in vivo* also demonstrate a $\Delta\Psi_m$ disruption after *in vitro* culture that precedes nuclear apoptosis. Transfection-enforced hyperexpression of the proto-oncogenes bcl-2 and bcl-X_L protects against chemotherapy-induced apoptosis, at both the level of the mitochondrial dysfunction preceding nuclear apoptosis and the level of late nuclear apoptotic events. Bcl-2-mediated inhibition of ceramide-induced $\Delta\Psi_m$ disruption is observed in normal as well as anucleate cells, indicating that bcl-2 acts on an extranuclear pathway of apoptosis. In contrast to Bcl-2 and Bcl-X_L, hyperexpression of the protease inhibitor cytokine response modifier A fails to protect tumor cells against chemotherapy-induced $\Delta\Psi_m$ disruption and apoptosis, although cytokine response modifier A does prevent the $\Delta\Psi_m$ collapse and posterior nuclear apoptosis triggered by cross-linking of Fas/Apo-1/CD95. In conclusion, $\Delta\Psi_m$ disruption seems to be an obligatory step of early (pre-nuclear) apoptosis, and $\Delta\Psi_m$ is stabilized by two members of the *bcl-2* gene family conferring resistance to chemotherapy.

INTRODUCTION

Genetically determined or acquired deficiencies in apoptosis regulation are involved in a number of different pathologies, including oncogenesis (1, 2). The involvement of apoptosis dysregulation in cancer development has first become clear for the proto-oncogene bcl-2, based on the observation that genetic translocation or transgenic manipulation causing overexpression of the apoptosis-inhibitory oncoprotein Bcl-2 may provoke the development of lymphomas (3–6). Today, several members of a growing multigene family of bcl-2 homologues, as well as a whole series of additional oncogenes (*e.g.*, c-*myc*) and anti-oncogenes (*e.g.*, p53), are thought to intervene in cancer development at two different levels. At the first level, mutations in the expression level and/or primary structure of apoptosis-regulatory genes may intervene in oncogenesis *ab initio* by favoring the persistence of mutated cells that, under normal circumstances, would be eliminated by apoptosis (2–6). At the second level, the Darwinian selection of cancer cells by adverse intrinsic conditions (limited trophic supply and oxygen shortage) and/or therapeutic agents (chemotherapy and radiotherapy) may favor the survival of proliferating cells that acquire an increasing resistance to apoptosis induction (7–9). Irrespective of the precise role of apoptosis-regulatory genes in cancer development, epidemiological data clearly indicate that the expression level of apoptosis-regulatory genes from the bcl-2 family is a prognostic marker for tumors arising from many different cell types (5, 6).

Apoptosis constitutes a physiological mechanism to eliminate superfluous or damaged cells. It involves the participation of cellular enzymes that act within the limits of a near-to-intact plasma membrane to cause a regular pattern of macromolecule degradation coupled to a stereotypic morphological appearance. Although many features of characteristic apoptosis concern the nucleus (chromatin condensation, endonuclease-mediated oligonucleosomal DNA degradation in mono- and oligomers of ~200 bp, and characteristic cleavage of nuclear proteins), it has become clear that cytoplasmic (non-nuclear) structures must be involved in apoptotic control. Thus, anucleate cells can be induced to undergo apoptosis (10, 11), and cytoplasmic structures (mitochondria and specific proteases) participate in death control (12–15). We and others have recently postulated that mitochondria must play a major role in the apoptotic process (12, 15–20). This idea is based on at least four observations: (*a*) in response to apoptosis-inducing stimuli, cells manifest a disruption of the inner transmembrane potential ($\Delta\Psi_m$)[3] that precedes the nuclear signs of apoptosis (15–18, 21, 22). In this sense, the $\Delta\Psi_m$ collapse constitutes an early (pre-nuclear) feature of apoptosis; (*b*) isolated mitochondria or soluble mitochondrial products can induce features of apoptosis in isolated nuclei in a cell-free reconstituted system of apoptosis (12, 13, 19, 20); (*c*) mitochondria recovered from apoptotic cells cause apoptosis of isolated nuclei *in vitro*, indicating that mitochondria can transfer apoptosis from one system to the other (19); and (*d*) prevention of $\Delta\Psi_m$ disruption by $\Delta\Psi_m$-stabilizing agents prevents apoptosis in cells (19).

These data have been obtained in physiological (that is receptor-mediated) systems of apoptosis induction (15–19, 21, 22). Here, we addressed the question of whether chemotherapeutic agents would trigger the same cascade of mitochondrial and nuclear events as that observed in physiological apoptosis. In addition, we determined which manifestations of chemotherapy-induced apoptosis would be prevented by the oncoproteins Bcl-2 and Bcl-X_L. Our results suggest that chemotherapy triggers the same sequence of events as that observed in receptor-induced apoptosis, indicating that the mitochondrial and nuclear features of apoptosis are undissociable. Moreover, it seems that both Bcl-2 and Bcl-X_L are able to prevent the early (mitochondrial) features of apoptosis.

MATERIALS AND METHODS

Cell Lines, Culture Conditions, and Apoptosis Induction. U937 myelomonocytic cells were obtained from the American Type Culture Collection. 2B4.11 T-cell hybridoma cell lines stably transfected with an SFFV.neo vector

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[3] The abbreviations used are: $\Delta\Psi_m$, mitochondrial transmembrane potential; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; Eth, ethidium; HE, hydroethidine; ROS, reactive oxygen species; CrmA, cytokine response modifier A; ara-C, 1-β-D-arabinofuranosylcytosine; TNF, tumor necrosis factor; CHX, cycloheximide; ICE, interleukin-1β converting enzyme.

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containing the human *bcl-2* gene or the neomycin resistance gene only (23) were kindly provided by Jonathan Ashwell (NIH, Bethesda, MD). WEHI-231 cell lines transfected with the human *Bcl-2* gene (24) were a gift from Carlos Martínez-A. (National Center of Biotechnology, Madrid, Spain), and cells transfected with the human $Bcl\text{-}X_L$ gene (25) were obtained from Gerry Klaus (National Institute for Medical Research, London, United Kingdom). Human CEM-C7.H2 lymphoma cells (26) were transfected with a β-actin STneo B vector containing the cowpox virus serpin crmA cDNA (27) or with the neomycin-resistance-conferring vector only. Three different sublines expressing crmA mRNA and protein were obtained and yielded similar functional results.[4] Data are shown for one of these sublines (C7.H2/D1.2/2E8). All cell lines were cultured in RPMI 1640 supplemented with L-glutamine, penicillin, streptomycin, and 10% FCS. Cells were cultured in the presence of several distinct apoptosis inducers: doxorubicin (1 μg/ml; Pharmacia), etoposide (10 μg/ml; Pierre Fabre), ara-C (10 μg/ml; Upjohn), recombinant TNF-α (4 ng/ml; Research Diagnostics, Flanders, NJ) plus CHX (0.5 μg/ml, Sigma), cyclosporin A (10 μM; Sandoz), C_2 ceramide (50 μM; Sigma), the Fas-cross-linking IgM monoclonal antibody CH-11 (100 ng/ml; TCS Biologicals; Bucks, United Kingdom), or γ-irradiation (10 Gy). After the indicated interval, cells were recovered and tested for apoptosis-associated features.

Patients. Peripheral blood cells were recovered from patients with different lymphomas or leukemias: (*a*) acute myeloid leukemia type 1 (AML1; type 2 according to the French-American-British criteria; $CD13^+CD33^+$; male, 39 years); (*b*) acute myeloid leukemia type 2 (AML2; $CD13^+CD33^+CD34^+$; male, 43 years); and (*c*) mantle cell lymphoma with circulating tumor cells (MCL; phenotype of circulating cells, $CD19^+CD20^+CD5^+CD10^-CD23^-$; male, 62 years; Refs. 28 and 29). Peripheral blood leukocytes containing >80% tumor cells were obtained before chemotherapy as well as after initiation of standard protocols of i.v. chemotherapy for AML1 (100 mg/m^2/day ara-C via continuous perfusion; 100 mg/m^2 etoposide on days 1, 3, and 5; and 50 mg/m^2 daunorubicin on days 1–3), AML2 (100 mg/m^2/day ara-C and 50 mg/m^2 daunorubicin on day 2), and MCL (60 mg/m^2/day prednisolone, 1.5 mg/m/day vincristin, 750 mg/m^2/day cyclophosphamide, and 50 mg/m^2/day doxorubicin). Cells were recovered by Ficoll gradients, frozen in the presence of DMSO (10% final concentration) at −80°C after standard procedures, and analyzed simultaneously after culture in complete medium during the indicated incubation periods, followed by cytofluorometric evaluation of apoptotic parameters.

Anuclear Cells. Cells were enucluated following published procedures (10). Briefly, cells (30×10^6 cells in 3-ml medium) were cultured for 45 min in the presence of 10 μg/ml cytochalasin B (Sigma), followed by centrifugation on a discontinuous Ficoll density gradient (2 ml of 25%, 2 ml of 17%, 0.5 ml of 16%, 0.5 ml of 15%, and 2 ml of 12.5% Ficoll in medium containing 10 μg/ml cytochalasin B; pre-equilibrated for 6 h at 37°C) in a pre-warmed Beckmann SW41 rotor at 25,000 rpm at 30°C. Anucleate cells were recovered from the interface between 15 and 17% Ficoll layers. Control stainings with vital dyes (propidium iodine and trypan blue) and the membrane-permeable DNA/RNA-specific dye acridine orange revealed >97% viability in this fraction containing less than <5% nucleate cells (data not shown).

Cytofluorometric Analysis of Apoptosis-associated Changes. The cationic lipophilic fluorochrome $DiOC_6(3)$ (Molecular Probes) was used to measure the $\Delta\Psi_m$. Cells were incubated at 37°C for 15 min in the presence of $DiOC_6(3)$ (40 nM; Ref. 22), followed by immediate analysis of fluorochrome incorporation in an Epics Profile II cytofluorometer (Coulter, Miami, FL). HE (2 μM; 15 min at 37°C; Molecular Probes) was used to measure superoxide anion generation as described (18, 20, 30). $DiOC_6(3)$ fluorescence was recorded in FL1; HE fluorescence was recorded in FL3. In control experiments, cells were labeled in the presence of the uncoupling agent carbonyl cyanide *m*-chlorophenylhydrazone (50 μM; Sigma) or the ROS-generating agent menadione (1 mM) as described (18, 20). The frequency of cells having lost part of their chromosomal DNA (subdiploid cells) was determined by propidium iodide staining of ethanol-permeabilized cells as described (31). Background values of apoptosis of uncultured peripheral blood leukocytes or cell lines cultured in the absence of any apoptosis inducer (<15% of $DiOC_6(3)^{low}$, <5% HE^+, and <5% hypoploid cells) were subtracted from the experimental values.

[4] S. Geley, unpublished data.

RESULTS AND DISCUSSION

Chemotherapeutic Agents Cause a Disruption of the $\Delta\Psi_m$ Preceding Nuclear Apoptosis *in Vitro*. As pointed out in the "Introduction," an early $\Delta\Psi_m$ disruption has been observed in a number of different physiological (receptor-induced) models of apoptosis. As shown previously (20), exposure of U937 myelomonocytic lymphoma cells to an apoptosis-inducing combination of TNF and CHX first causes a $\Delta\Psi_m$ collapse and then causes an increase in the percentage of cells exhibiting chromatin loss as a sign of end-stage apoptosis. This $\Delta\Psi_m$ collapse can be conveniently measured using the potential-sensitive dye $DiOC_6(3)$, which emits a green fluorescence. The $\Delta\Psi_m$ collapse first affects cells with a low capacity of oxidizing HE (which is nonfluorescent) into Eth (which emits a red fluorescence). Thus, as described (20), cells first disrupt their $\Delta\Psi_m$ and then hyperproduce ROS responsible for the HE→Eth conversion and undergo nuclear apoptosis. This was also observed when apoptosis was induced by a number of agents used in chemotherapy (etoposide, doxorubicin, and ara-C; Figs. 1 and 2*A*). When U937 cells are treated with the topoisomerase type 2 inhibitor etoposide during a limited period (2 h), most cells possessing a low $\Delta\Psi_m$ and a still-normal capacity of HE→Eth conversion still have a normal (≥ diploid) DNA content (Fig. 2*B*). If etoposide-treated purified $\Delta\Psi_m^{low}$ or $\Delta\Psi_m^{high}$ cells are subjected to further culture for 60 min at 37°C, only a minority of $\Delta\Psi_m^{high}$ cells undergoes chromatinolysis, whereas a major portion of purified $\Delta\Psi_m^{low}$ cells with a still HE→Eth^{low} phenotype acquires a subdiploid phenotype (Fig. 2, *A* and *B*). These data confirm previous observations on glucocorticoid-induced apoptosis (16, 18), indicating that $\Delta\Psi_m^{low}$ but not $\Delta\Psi_m^{high}$ cells are primed for rapid DNA fragmentation.

Tumor Cells Dying from Apoptosis *ex Vivo* Manifest an Early $\Delta\Psi_m$ Disruption. In an attempt to confirm the data obtained in an *in vitro* system (Figs. 1 and 2) *in vivo*, we recovered peripheral blood leukocytes from patients with leukemias with >80% tumor cells among leukocytes. Cells were obtained before and after initiation of standard chemotherapy, frozen, and then cultured simultaneously *in vitro*. As demonstrated in Fig. 3, tumor cells undergo apoptosis spontaneously. Prior exposure to chemotherapeutic agents *in vivo* accelerates the apoptotic process *in vitro* in two cases of acute myeloid leukemia as well as a case of mantle cell lymphoma. This effect is minor (2–4-h acceleration) but highly significant ($P < 0.001$, paired Student *t* test). As observed for cell lines (Fig. 1 and see

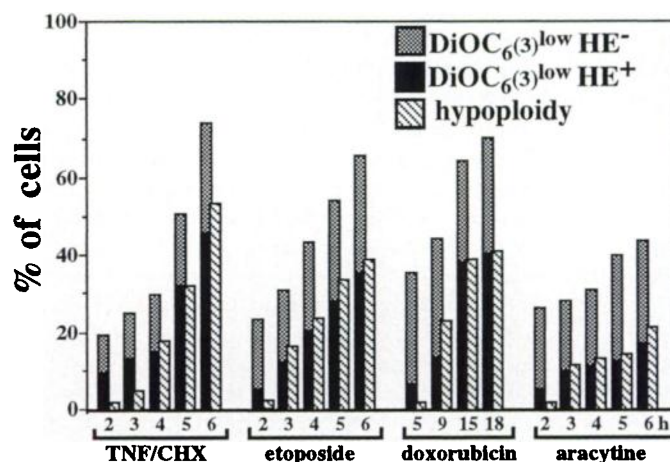

Fig. 1. Chronology of TNF or chemotherapy-induced apoptosis in myelomonocytic U937 cells. U937 cells were cultured during the indicated interval with either TNF + CHX or one of the three indicated apoptosis-inducing compounds, followed by determination of the frequency of cells with a low ΔΨ [$DiOC_6(3)^{low}$ cells] with either a normal production of ROS (HE^-) or enhanced ROS generation (HE^+) as described in Ref. 20.

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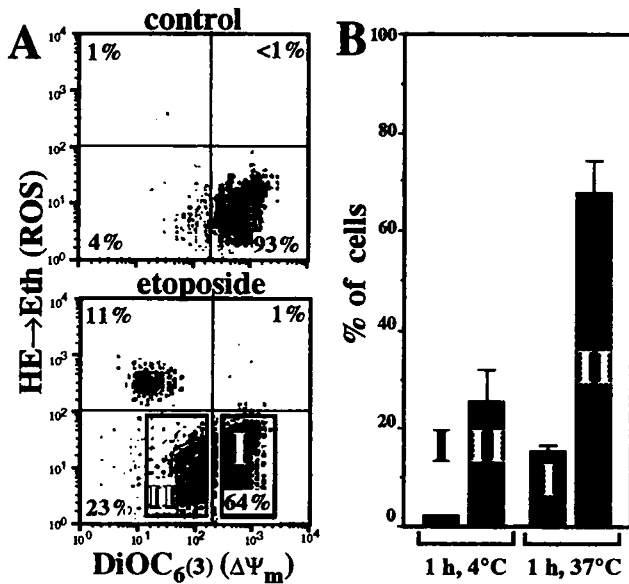

Fig. 2. Cells with a disrupted $\Delta\Psi$ are programmed to undergo nuclear apoptosis. *A*, purification of $\Delta\Psi_m{}^{low}$ and $\Delta\Psi_m{}^{high}$ cells. U937 cells were cultured for 2 h in the presence of etoposide as in Fig. 1, followed by the staining of cells with a combination of $DiOC_6(3)$ and HE. Cells with a normal $DiOC_6(3)^{high}$ phenotype (fraction *I*) or cells showing a reduced $DiOC_6(3)$ incorporation and a still-normal HE→Eth conversion (fraction *II*) were purified cytofluorometrically and reanalyzed to confirm that they differ in the $\Delta\Psi_m$. *B*, nuclear apoptosis of purified $\Delta\Psi_m{}^{low}$ and $\Delta\Psi_m{}^{high}$ cells. Cells were cultured for 60 min at 37°C or 4°C, followed by ethanol fixation and analysis of DNA content using propidium iodine. The percentages of subdiploid cells ($X \pm$ SE of triplicates) are represented as histograms.

below), full-blown nuclear apoptosis of tumor cells is preceded by a disruption of the $\Delta\Psi_m$, and both the $\Delta\Psi_m$ and the loss of nuclear DNA are accelerated by *in vivo* chemotherapy (Fig. 3).

Bcl-2 Hyperexpression Confers Protection against both Mitochondrial and Nuclear Manifestations of Apoptosis. To evaluate the apoptosis-protective role of the proto-oncogene Bcl-2, a B leukemia cell line transfected with the human *Bcl-2* gene (23) or vector-transfected controls was treated by chemotherapy *in vitro*. As shown in Fig. 4*A*, Bcl-2 hyperexpression inhibits the manifestations of apoptosis at both the mitochondrial and the nuclear levels. Bcl-2 hyperexpression also inhibits apoptosis in B leukemia cell lines responding to chemotherapy, γ-irradiation, or cyclosporin A (Fig. 5). In this context, it should be mentioned that the cyclosporin A-induced apoptosis observed in long-term experiments (24 h; Fig. 5; Ref. 32) can be dissociated from its short-term effects on mitochondrial permeability transition (<1 h; Ref. 33). Thus, the cyclosporin A derivative *N*-methyl-val-4-cyclosporin A, which fails to affect the calcineurin pathway (and thus loses its immunosuppressive effect) yet conserves its inhibitory effects on the pre-apoptotic $\Delta\Psi_m$ disruption (34), fails to induce apoptosis in WEHI-231 cells (data not shown). Thus, the apoptosis-inducing effect of cyclosporin A is probably linked to its immunosuppressive effect.

Interestingly enough, Bcl-2 hyperexpression prevents the ceramide-induced $\Delta\Psi_m$ disruption in both intact T-cell hybridomas (Fig. 4*A*) and anucleate cells (cytoplasts; Fig. 4*B*), indicating that the protective effect of Bcl-2 does not depend on its nuclear localization. In conclusion, Bcl-2 confers apoptosis resistance via a cytoplasmic (non-nuclear) action and prevents a major biochemical manifestation of apoptosis ($\Delta\Psi_m$ collapse) upstream of nuclear DNA fragmentation. This observation is compatible with a $\Delta\Psi_m$-stabilizing effect of Bcl-2 on isolated mitochondria (19).

Bcl-X$_L$ Shares the Mitochondrial Effects of Bcl-2. Bcl-2 and Bcl-X$_L$ share several features, namely some aspects of their primary structure, apoptosis-inhibitory effects, and predominant localization to the outer mitochondrial membrane (35–37). As shown in Fig. 5, this parallelism extends to the fact that transfection-enforced hyperexpression of Bcl-X$_L$ inhibits chemotherapy- or γ-irradiation-induced $\Delta\Psi_m$ disruption and DNA hypoploidy (Fig. 3) exactly as does Bcl-2 hyperexpression (Fig. 3 and 4). Thus, as compared to control cells, cell

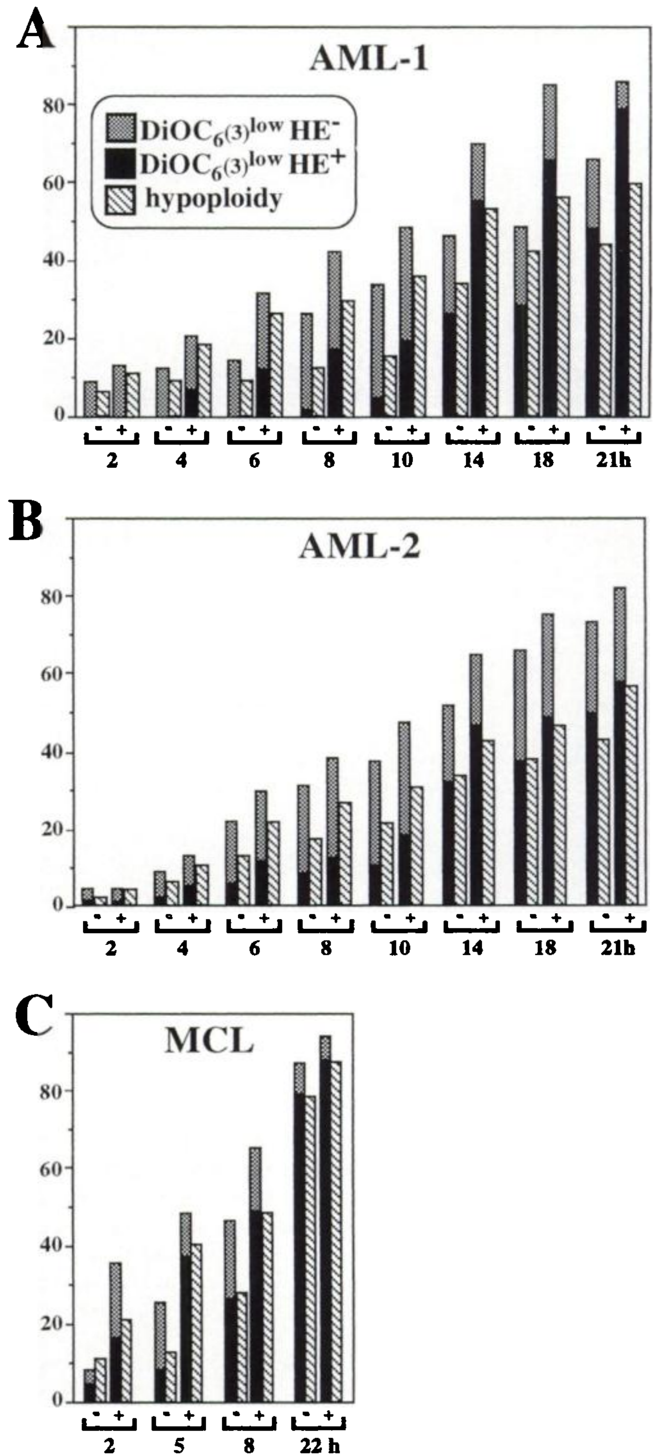

Fig. 3. Spontaneous apoptosis of tumor cells in culture is preceded by $\Delta\Psi$ disruption. Peripheral blood leukocytes from two patients with acute myeloid leukemia (*A* and *B*) or one patient with mantle cell lymphoma (*C*) were cultured during the indicated intervals in complete medium *in vitro*. Cells were recovered either before chemotherapy (−) or 38 h (*A*), 60 h (*B*), or 4 days (*C*) after initiation of standard chemotherapy *in vivo* (+). Aliquots of cryopreserved cells were cultured and analyzed simultaneously. One of two experiments yielding similar results is shown.

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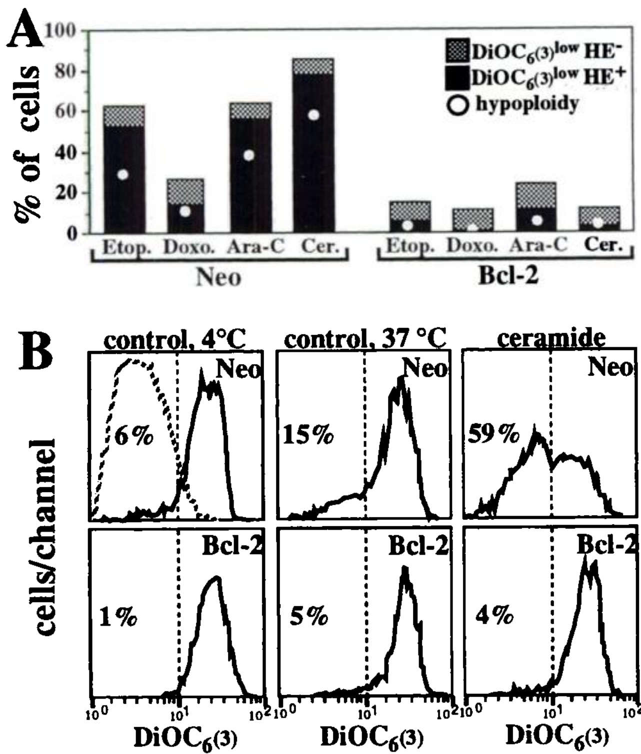

Fig. 4. Bcl-2-mediated stabilization of $\Delta\Psi_m$ in cells undergoing apoptosis and in cytoplasts. *A*, Bcl-2-mediated inhibition of apoptosis in T-cell hybridoma cells transfected with the human *Bcl-2* gene or a vector conferring resistance to neomycin. Cells were cultured for 14 h in the presence of etoposide, doxorubicin, ara-C, or ceramide, followed by determination of the mitochondrial and nuclear features of apoptosis. *B*, anucleate cells (cytoplasts) undergo a Bcl-2-inhibitable $\Delta\Psi_m$ disruption in response to ceramide. Cytoplasts prepared from *bcl-2*-transfected cells or from vector-only transfected controls (*Neo*) were left at 4°C or cultured for 3 h at 37°C in the presence or absence of ceramide, followed by cytofluorometric assessment of the $\Delta\Psi_m$ using the potential-sensitive dye $DiOC_6(3)$. *Numbers*, the percentage of cells exhibiting low $DiOC_6(3)$ incorporation. *Dotted line*, control cytoplasts marked in the presence of the $\Delta\Psi_m$-dissipating protonophore carbonyl cyanide *m*-chlorophenylhydrazone.

lines transfected with Bcl-X_L exhibit a delay in both mitochondrial and nuclear features of apoptosis induced by etoposide, doxorubicin, ara-C, cyclosporin A, and ionizing radiation (Fig. 5). In conclusion, Bcl-X_L and Bcl-2 have similar protective effects on mitochondrial features of apoptosis.

CrmA Overexpression Prevents the $\Delta\Psi_m$ Disruption and Apoptosis Induced by Fas but not by Chemotherapy. The cowpox protein CrmA functions as an inhibitor of cysteine proteases involved in the apoptotic cascade and possesses a relative specificity for the pro-apoptotic ICE and possibly apopain/CPP32/Yama (38, 39). In accordance with published studies (40–44), transfection of human lymphoma cells with crmA confers resistance to apoptosis induction by cross-linking of the Fas/APO-1/CD95 surface antigen, given that Fas/APO-1/CD95-triggered apoptosis is dependent on ICE activation (40, 41). Transfection with a sense construct of *crmA* protects against the Fas-induced $\Delta\Psi_m$ disruption as well as DNA hypoploidy (Fig. 6). In sharp contrast, crmA fails to inhibit the $\Delta\Psi_m$ disruption and subsequent apoptotic death of lymphoma cells induced by etoposide, doxorubicin, or ara-C. Thus, CrmA-inhibitable proteases (ICE and CPP32/Yama) are not rate-limiting in chemotherapy-induced apoptosis. This set of data underlines the postulate that Bcl-2 and CrmA-inhibitable apoptotic pathways can be distinguished (43, 44). Moreover, the fact that CrmA overexpression prevents Fas-induced apoptosis but not chemotherapy-induced apoptosis is incompatible with a postulated Fas mediation of chemotherapy-induced apoptosis (45). Finally, these results indicate that in no instance can the mitochondrial and nuclear manifestations of apoptosis be dissociated, a finding that supports the intimate linkage between both phenomena.

In this paper, we show that chemotherapy-induced apoptosis obeys the same rule as physiological (receptor-mediated) apoptosis induced by TNF, glucocorticoids, or ligation of Fas/APO-1/CD95 in the sense that nuclear apoptosis is preceded by the disruption of the $\Delta\Psi_m$. This holds true for chemotherapy with different agents, including the topoisomerase inhibitor etoposide, the anthracycline antibiotic doxorubicin, and the antimetabolite ara-C, both *in vitro* in cell lines and *in vivo* in tumor cells. Although the inhibitor of ICE-like protease crmA

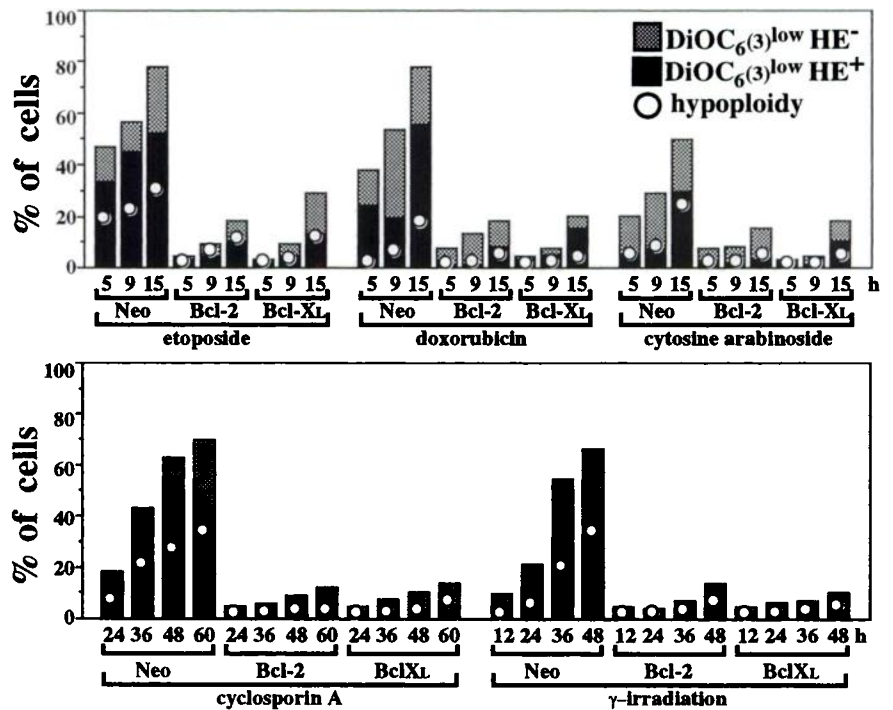

Fig. 5. Effect of transfection-enforced hyperexpression of Bcl-2 or Bcl-X on the induction of mitochondrial and nuclear signs of apoptosis by different treatments in WEHI-231 leukemia cells. Cells transfected with a neomycin resistance vector only (*Neo*) served as controls.

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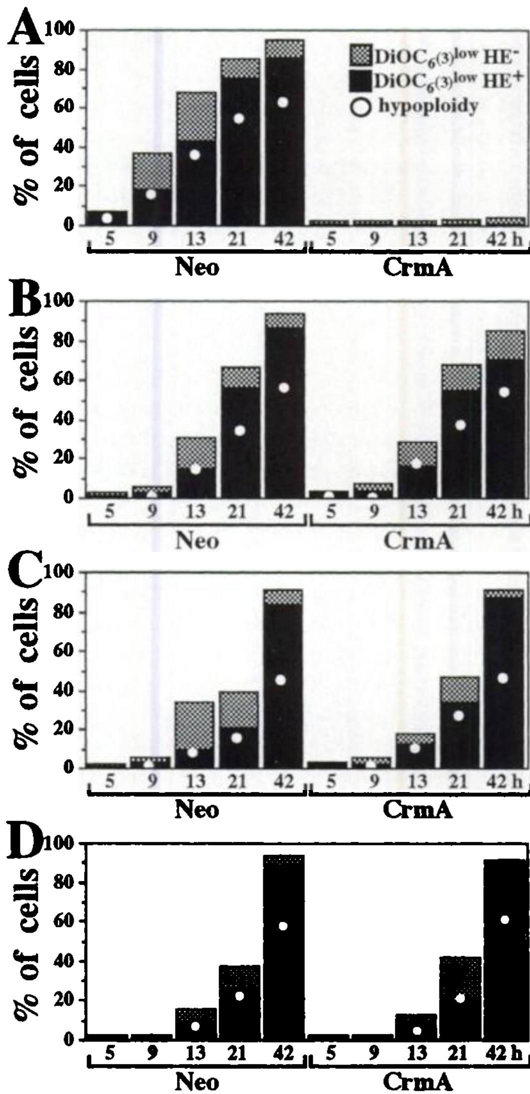

Fig. 6. Effect of transfection with CrmA on the apoptosis susceptibility of human CEM-7H2 lymphoma cells. Cells stably transfected with the cowpox protease inhibitor CrmA or a vector conferring neomycin resistance only (*Neo*) were cultured in the presence of a cross-linking anti-Fas antibody (*A*), etoposide (*B*), doxorubicin (*C*), or ara-C (*D*) during the indicated interval, followed by the determination of mitochondrial and nuclear signs of apoptosis. Results are representative for three different CrmA-hyperexpressing cell lines.

fails to interfere with chemotherapy-induced apoptosis, our data indicate that overexpression of two apoptosis-inhibitory and structurally related oncoproteins, Bcl-2 and Bcl-X_L, prevents the induction of both the mitochondrial and the nuclear manifestations of apoptosis, suggesting that they act at the level of the mitochondrion (or upstream thereof). We have recently shown that Bcl-2 overexpressed in isolated mitochondria can inhibit the opening of so-called permeability transition pores (19), which are thought to account for the $\Delta\Psi_m$ collapse observed in pre-apoptotic cells (15, 18, 34). In accord with these findings, in several different systems, Bcl-2 is a more efficient inhibitor of apoptosis in cells when it is targeted to cell membranes and in particular to the outer mitochondrial membrane (46–53), suggesting that it does act on mitochondria to prevent apoptosis. Recent crystallographic data (54) indicate that the Bcl-X_L dimer, which also localizes preferentially into the outer mitochondrial membrane (36), possesses a pore-like structure (54), underlining that members of the Bcl-2 family may directly participate in the regulation of ion fluxes. Future efforts will elucidate via which exact molecular mechanisms Bcl-2 and its homologues can influence mitochondrial function(s) to inhibit apoptosis.

A problem that remains elusive concerns the relationship between mitochondrial and other cytoplasmic features of apoptosis, for instance protease activation. Whereas crmA-inhibitable proteases clearly function upstream of apoptosis (at least in the crmA-sensitive Fas-triggered pathway; Fig. 6), it seems that some proteases can act downstream of mitochondria. Thus, two mitochondrial intermembrane proteins (a protease released from the intermembrane space and the CPP32 activator cytochrome *c*) are released from mitochondria undergoing permeability transition and thus may be expected to act downstream of mitochondria (55, 56). Thus, speculatively, mitochondrial permeability transition and activation of cytoplasmic proteases could be intertwined during the early (pre-nuclear) phase of apoptosis.

Irrespective of these theoretical considerations, the present data suggest that the assessment of mitochondrial rather than nuclear features of apoptosis might provide a chronological advantage for the detection of early apoptotic events both *in vitro* and *in vivo*. Moreover, these data may provide a renaissance to earlier studies on mitochondria in tumor cells, especially carcinoma cells, which have been reported to conserve a higher $\Delta\Psi_m$ than normal cells (reviewed in Ref. 57). The notion that a high or normal $\Delta\Psi_m$ is a sign of apoptosis resistance should allow for an operative reinterpretation of these earlier reports.

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