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Differential Regulation of Doxorubicin-induced Mitochondrial Dysfunction and Apoptosis by Bcl-2 in Mammary Adenocarcinoma (MTLn3) Cells*

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Various anticancer drugs cause mitochondrial perturbations in association with apoptosis. Here we investigated the involvement of caspase- and Bcl-2-dependent pathways in doxorubicin-induced mitochondrial perturbations and apoptosis. For this purpose, we set up a novel three-color flow cytometric assay using rhodamine 123, annexin V-allophycocyanin, and propidium iodide to assess the involvement of the mitochondria in apoptosis caused by doxorubicin in the breast cancer cell line MTLn3. Doxorubicin-induced apoptosis was preceded by up-regulation of CD95 and CD95L and a collapse of mitochondrial membrane potential ($\Delta\psi$) occurring prior to phosphatidylserine externalization. This drop in $\Delta\psi$ was independent of caspase activity, since benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone did not inhibit it. Benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone also blocked activation of caspase-8, thus excluding an involvement of the death receptor pathway in $\Delta\psi$ dissipation. Furthermore, although overexpression of Bcl-2 in MTLn3 cells inhibited apoptosis, dissipation of $\Delta\psi$ was still observed. No decrease in $\Delta\psi$ was observed in cells undergoing etoposide-induced apoptosis. Immunofluorescent analysis of $\Delta\psi$ and cytochrome *c* localization on a cell-to-cell basis indicates that the collapse of $\Delta\psi$ and cytochrome *c* release are mutually independent in both normal and Bcl-2-overexpressing cells. Together, these data indicate that doxorubicin-induced dissipation of the mitochondrial membrane potential precedes phosphatidylserine externalization and is independent of a caspase- or Bcl-2-controlled checkpoint.

Upon anticancer drug treatment, a number of cellular stress response pathways are activated. Some of these pathways are linked to mitochondrial perturbations that are often associated with apoptosis. Thus, the release of proapoptotic factors from the intermembrane space into the cytosol, including cytochrome *c*, apoptosis-inducing factor, and Smac/DIABLO, occurs after cytostatic treatment (1–4). The mechanisms regulating the release of cytochrome *c* include specific pore formation in the outer mitochondrial membrane and opening of the permeability transition pore (reviewed in Ref. 5). As a consequence of both the loss of the electrochemical gradient caused by pore opening and rupture of the outer mitochondrial membrane, the mitochondrial membrane potential ($\Delta\psi$)[1] generally collapses. There is general agreement that cytosolic cytochrome *c* interacts with Apaf-1, ATP, and procaspase-9, resulting in the activation of the latter, followed by caspase-3 activation and initiation of a proteolytic cascade (6). However, the exact sequence of events resulting in disruption of mitochondrial function and release of cytochrome *c* from the mitochondria in apoptosis caused by anticancer drugs is not yet clear.

There are two prominent pathways that may cause mitochondrial dysfunction during apoptosis. First, death receptor activation through CD95/CD95L and caspase-8 activation during apoptosis is under several circumstances upstream from mitochondrial perturbations. Thus, active caspase-8 may cause cleavage of the proapoptotic Bcl-2 member Bid and, as a consequence, mitochondrial dysfunction (7–9). Several anticancer agents, including doxorubicin and etoposide, can up-regulate CD95 and CD95L (10–12). However, there is controversy on the relative importance of this pathway in anticancer drug-induced mitochondrial perturbations and apoptosis. Thus, in some cell types, inhibition of this pathway using either Fas-linked interleukin-β-converting enzyme inhibitory protein, CrmA, or dominant negative Fas-associated death domain abrogates apoptosis, whereas in other cell types little effect was observed (reviewed in Ref. 12). Although up-regulation of death receptor pathway components was found in some solid tumor cell lines, the extent of subsequent involvement of the mitochondrial pathway remains unclear (11, 13). Up-regulation and/or translocation of Bax and/or other proapoptotic Bcl-2 family members to the mitochondria is a second major pathway for mitochondrial perturbation preceding the onset of apoptosis. At the mitochondria, Bax invokes cytochrome *c* release and loss of $\Delta\psi$ (14), possibly via direct pore formation (15, 16) or by association with the voltage-dependent anion channel (17, 18). Importantly, the antiapoptotic Bcl-2 family members Bcl-2 and Bcl-x_L generally inhibit these mitochondrial perturbations (19, 20). Various tumor cells have increased expression of proteins that inhibit either the death receptor pathways (*e.g.* Fas-linked interleukin-β-converting enzyme inhibitory protein) or apoptosis caused by Bax (*e.g.* Bcl-2 or Bcl-x_L) (21–25). These tumor

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[1] The abbreviations used are: $\Delta\psi$, mitochondrial membrane potential; AMC, 7-amino-4-methylcoumarin; APC, allophycocyanin; AV, annexin V; CLSM, confocal laser-scanning microscopy; CMXRos, Mitotracker™ Red CMXRos; $DiOC_6$, 3,3′-dihexyloxacarbocyanine; Neo cells, neomycin-resistant cells; PI, propidium iodide; PS, phosphatidylserine; Rho123, rhodamine 123; zVAD, benzyloxycarbonyl-Val-Ala-DL-Asp; fmk, fluoromethylketone; α-MEM, α-minimal essential medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid.

cells are generally more resistant to anticancer drug-induced apoptosis. It is unclear whether such resistance includes mitochondrial protection. Therefore, we have investigated the relative roles of caspase activation and Bcl-2-dependent pathways in mitochondrial perturbations and apoptosis caused by the anticancer drug doxorubicin.

Doxorubicin is often used in the treatment of solid tumors, including breast, liver, and bone tumors (26). It causes DNA damage and formation of reactive oxygen species, eventually resulting in apoptosis (27). Although doxorubicin causes mitochondrial injury in cardiac muscle cells (28) and some other cell types, these effects were studied primarily in lymphoid cells (29, 30). The molecular mechanism of mitochondrial injury and its role in the induction of apoptosis in adenocarcinoma cells remains, however, largely unclear. As discussed above, the dissipation of $\Delta\psi$ is one of the markers for mitochondrial involvement in apoptosis. So far, dissipation of $\Delta\psi$ caused by doxorubicin has been determined either in the total cell population (30–32) or in "viable" cells based on scatter properties (33). These methods do not allow proper distinction of the exact cell population in which the changes in $\Delta\psi$ occurred: genuinely viable, apoptotic or (secondary) necrotic cells. As a consequence, the identification of the exact sequence of events in doxorubicin-induced apoptosis was precluded. In the present study, we set up three-color flow cytometry with rhodamine 123, annexin V-allophycocyanin (APC), and propidium iodide to assess the involvement of the mitochondria in doxorubicin-induced apoptosis. For this purpose, we used the rat mammary adenocarcinoma cell line MTLn3, which is often used as a model to study molecular mechanisms of metastasis formation (34, 35) and responses to drug therapy both *in vitro* and *in vivo* (36–38). We have previously characterized in detail the induction of apoptosis by anticancer drugs in these cells (38).

In the present study, we show that the doxorubicin-mediated collapse of $\Delta\psi$ is a primary event preceding PS externalization. Moreover, despite the fact that doxorubicin causes up-regulation of CD95 and CD95L, prevention of caspase-8 activation does not prevent loss of $\Delta\psi$. Furthermore, although Bcl-2 inhibits apoptosis, dissipation of $\Delta\psi$ is still observed. Analysis of $\Delta\psi$ and cytochrome *c* localization on a cell-to-cell basis indicates that the collapse of $\Delta\psi$ and cytochrome *c* release are mutually independent in both normal and Bcl-2-overexpressing cells. Together, these data indicate that doxorubicin-induced dissipation of the mitochondrial membrane potential precedes PS externalization and is independent of a caspase- or Bcl-2-controlled checkpoint.

EXPERIMENTAL PROCEDURES

Chemicals—α-Modified minimal essential medium with ribonucleosides and deoxyribonucleosides (α-MEM), fetal bovine serum (FBS), penicillin/streptomycin, LipofectAMINE Plus, and Geneticin (G418 sulfate) were from Invitrogen. Collagen (type I, rat tail) was from Upstate Biotechnology, Inc. (Lake Placid, NY). Doxorubicin, propidium iodide (PI), 7-amino-4-methylcoumarin (AMC), $DiOC_6$, rhodamine 123, and RNase A were from Sigma. Benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk) and acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin were from Bachem (Bubendorf, Switzerland). Annexin V was from Roche Molecular Biochemicals. Hoechst 33258, the Alexa™ 488 protein labeling kit, and Mitotracker™ Red CMXRos were from Molecular Probes (Leiden, The Netherlands). APC was from Prozyme (San Leandro, CA). All other chemicals were of analytical grade.

Cell Culture—MTLn3 rat mammary adenocarcinoma cells were originally developed by Dr. D. R. Welch (Jake Gittlen Cancer Research Institute, The Pennsylvania State University College of Medicine, Hershey, PA) and used between passages 46 and 56. They were cultured in α-MEM supplemented with 5% (v/v) FBS (complete medium). For experiments, cells were plated at a density of 4×10^3 cells/cm^2 in Corning plates (Acton, MA) and grown for 3 days in complete medium supplemented with 50 units of penicillin/liter and 50 mg of streptomycin/liter (penicillin/streptomycin). Cells were exposed to doxorubicin for 1 h in Hanks' balanced salt solution (137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM $Na_2HPO_4 \cdot 2H_2O$, 0.4 mM KH_2PO_4, 1.3 mM $CaCl_2$, 4 mM $NaHCO_3$, 25 mM HEPES, 5 mM D-glucose, pH 7.4). After removal of doxorubicin, cells were recovered in α-MEM containing 2.5% (v/v) FBS and penicillin/streptomycin for the indicated periods. In some experiments, cells were recovered in α-MEM containing 2.5% (v/v) FBS, penicillin/streptomycin and 100 μM zVAD-fmk.

Construction of Bcl-2-overexpressing Cells—Subconfluent MTLn3 cells were transfected with pcDNA3 (Neo) or pcDNA3 containing human Bcl-2 (gift from Dr. James L. Stevens) using LipofectAMINE Plus reagent, and after reaching confluence they were selected for neomycin resistance (G418; 100 μg/ml). For both vectors, three clones were selected and used for up to six passages, during which they stably expressed Bcl-2 in over 95% of cells based on immunofluorescence.

In some experiments, we used porcine renal proximal tubular cell line LLC-PK1 expressing either Bcl-2 (pkBCL-2 clone 6) or the empty vector (pkNEO clone 1) that have been described previously (39). LLC-PK1 cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) FBS and penicillin/streptomycin. For experiments, cells were plated overnight in Dulbecco's modified Eagle's medium plus penicillin/streptomycin without FBS on collagen-coated 6-cm culture dishes to form a subconfluent monolayer as described previously (40). Thereafter, cells were treated with varying concentrations of doxorubicin in Dulbecco's modified Eagle's medium/penicillin/streptomycin for 24 h.

Determination of Cell Death—For annexin V/propidium iodide (AV/PI) staining, cells were washed twice in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 2H_2O$, 1.4 mM KH_2PO_4, pH 7.4) containing 1 mM EDTA (PBS-EDTA) and subsequently trypsinized with 0.13 g/liter trypsin in PBS-EDTA. Medium, washes, and cells were combined and centrifuged (5 min, 200 × *g*, 4 °C), and the pellet was washed once with PBS-EDTA. Cells were allowed to recover from trypsinization in complete medium (30 min, 37 °C). Externalized phosphatidylserine (PS) was labeled (15 min, 0 °C) with Alexa™ 488-conjugated annexin V in AV buffer (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1.0 mM $MgCl_2 \cdot 6H_2O$, 1.8 mM $CaCl_2 \cdot 2H_2O$, pH 7.4). Propidium iodide (2 μM) in AV buffer was added 1 min prior to analysis on a FACScalibur flow cytometer (BD PharMingen).

For cell cycle analysis, trypsinized and floating cells were pooled, washed with PBS-EDTA, and fixated in 70% (v/v) ethanol (30 min, −20 °C). After two washes with PBS-EDTA, cells were incubated with PBS-EDTA containing 50 μg/ml RNase A and 7.5 μM PI (45 min, room temperature) and subsequently analyzed by flow cytometry.

Caspase-3-like activity was determined as described previously (38). Briefly, cells were trypsinized as described for AV/PI, washed once in PBS-EDTA, and resuspended in lysis buffer (10 mM HEPES, 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, pH 7.0). Cells were lysed by four cycles of freezing and thawing followed by centrifugation (30 min, 13,000 × *g*, 4 °C). To 10 μg of cell lysate protein, 80 μl assay buffer was added (100 mM HEPES, 10% (w/v) sucrose, 0.1% (v/v) Nonidet P40, 10 mM dithiothreitol, 25 μM acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin, pH 7.25), and the release of AMC was monitored (45 min, 37 °C) in a fluorescence plate reader (HTS 7000 bioassay reader; PerkinElmer Life Sciences). Free AMC was used as a standard, and caspase activity was expressed as pmol of AMC/min/mg of protein.

Determination of Mitochondrial Membrane Potential—Mitochondrial membrane potential was essentially performed as described before (41, 42) with some modifications. Briefly, cells were harvested as described for AV/PI staining. Following recovery in complete medium, cells were incubated with 1 μM rhodamine 123 (Rho123) and APC-conjugated annexin V (43) in AV buffer (30 min, 37 °C). Cells were centrifuged (30 s, 400 × *g*, room temperature), and the pellet was resuspended in AV buffer containing 2 μM propidium iodide 1 min prior to analysis by confocal laser-scanning microscopy (CLSM; Bio-Rad) or flow cytometry. As an alternative method to determine the mitochondrial membrane potential, we used $DiOC_6$ (0.1 μM) instead of Rho123 (30, 44, 45). Selective localization of $DiOC_6$ at the mitochondria was confirmed by CLSM.

Soft Agar Colony Assay—MTLn3 Neo and Bcl-2 cells were treated with varying concentrations of doxorubicin as described above. After 24 h, cells were trypsinized, and viable cells (trypan blue exclusion) were counted. Next, 12,500 cells were plated in 1 ml of top agar (0.33% (w/v) agarose in complete medium in the presence of amphotericin B (250 ng/ml)) on top of 2.5 ml of bottom agar (0.66% (w/v) agarose in complete medium in the presence of amphotericin B (250 ng/ml)) in duplicate in six-well plates, as described by Kiley *et al.* (35). After 1 week, a top layer of 2.5 ml of bottom agar was added. After 14 days, 150

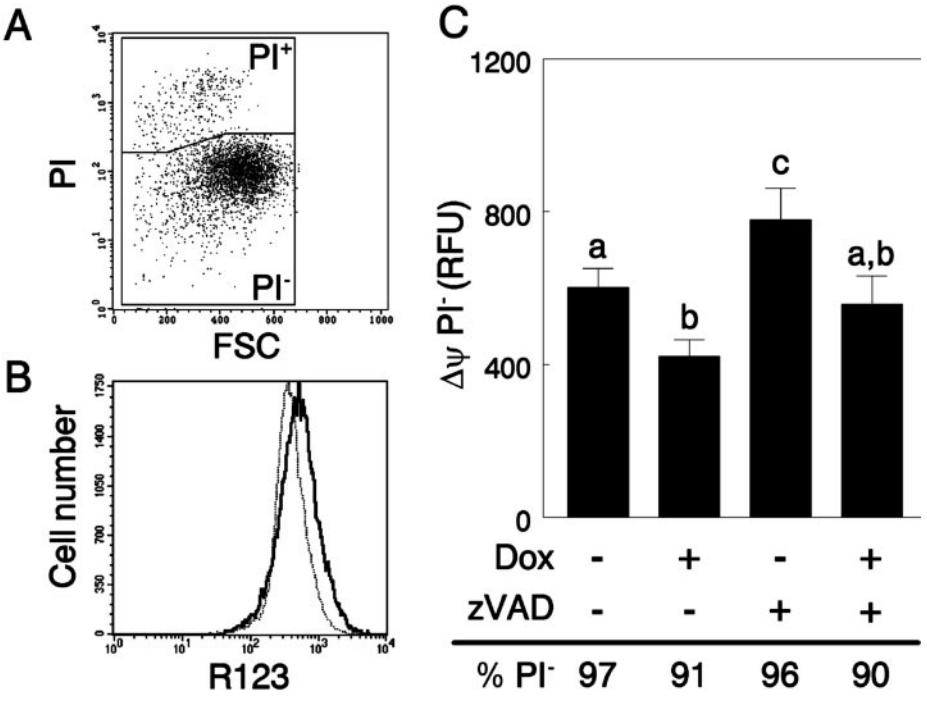

FIG. 1. **Doxorubicin-induced loss of Δψ in nonnecrotic cells.** MTLn3 cells were exposed to 17 μM doxorubicin (*Dox*) or vehicle for 1 h and subsequently allowed to recover for 24 h in the absence or presence of 100 μM zVAD-fmk. Δψ was determined by flow cytometry in nonnecrotic, PI^- cells (defined as shown in *A*) using rhodamine 123 (R123; *B* and *C*). In *B*, the *thick line* indicates control cells, and the *dashed line* indicates doxorubicin-treated cells. *RFU*, relative rhodamine 123 fluorescence units; *FSC*, forward scatter of cells. Data shown are the mean of three independent experiments ± S.E. *Lowercase letters* indicate statistical significance as described under "Experimental Procedures."

μl of a 5 μg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution in medium was added to the wells, and after overnight incubation (37 °C), digital images of the wells were taken with a Nikon CCD camera. Colonies were counted using a particle-counting option in Image Pro (Media Cybernetics, Silver Spring, MD).

Immunoblotting—Attached cells were scraped in ice-cold TSE+ (10 mM Tris-HCl, 250 mM sucrose, 1 mM EGTA, pH 7.4, containing 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium vanadate, 50 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride). Floating cells in the medium and in one wash of PBS were pelleted (5 min, 200 × *g*, 4 °C) and pooled with scraped cells in TSE+. The protein concentration in the supernatant was determined using the Bio-Rad protein assay using IgG as a standard. Fifteen μg of total cellular protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore Corp., Etten Leur, The Netherlands). Blots were blocked with 5% (w/v) nonfat dry milk in TBS-T (0.5 M NaCl, 20 mM Tris-HCl, 0.05% (v/v) Tween 20, pH 7.4) and probed for Bcl-2 (C-2; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), p53 (polyclonal antibody 240; Santa Cruz Biotechnology), CD95 (FL335; Santa Cruz Biotechnology), CD95L (clone 33; Transduction Laboratories), caspase-8 (kindly provided by Prof. J. Borst (46)), active caspase-3 (CM-1; kindly provided by Dr. A. Srinivasan (47)), or protein kinase Cδ (δ14K; kindly provided by Dr. S. Jaken (48)), followed by incubation with secondary antibody containing horseradish peroxidase and visualization with ECL reagent (Amersham Biosciences AB, Uppsala, Sweden).

Cytosolic Fractions for Cytochrome c Immunoblotting—Cytosolic fractions were prepared as described by Boesen-de Cock (46). Briefly, floating cells in the medium and one wash of mitobuffer (50 mM PIPES-KOH (pH 7.4), 220 mM mannitol, 68 mM sucrose, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) were pelleted (5 min, 200 × g, 4 °C) and pooled with attached cells scraped in 100 μl of ice-cold mitobuffer. Cells were pelleted (1 min, 400 × *g*, room temperature), resuspended in 100 μl of mitobuffer, and allowed to swell on ice for 30 min. Cells were homogenized by passing the suspension through a 25-gauge needle (10 strokes). Homogenates were centrifuged (15 min, 13,000 × *g*, 4 °C), and supernatants were collected. Thirty μg of cytosolic protein was separated on a 15% SDS-PAGE gel and transferred to polyvinylidene difluoride membrane. Cytochrome *c* was detected with anti-cytochrome *c* monoclonal antibody (7H8.2C12; BD PharMingen) using the Western Star kit (Tropix, Bedford, MA).

Immunocytochemistry—Cells were cultured on 12-mm collagen-coated glass coverslips and fixated in fresh 4% (w/v) paraformaldehyde in PBS. Coverslips were blocked in TBP (0.5% (w/v) bovine serum albumin and 0.05% (v/v) Tween 20 in PBS, pH 7.4) (1 h, room temperature) and subsequently incubated with primary antibody in TBP (1 h, room temperature). Coverslips were washed twice in PBS containing 0.05% Tween 20 and incubated with Alexa™ 488-, Cy3- or Cy5-conjugated secondary antibodies in TBP (45 min, room temperature). After washing, coverslips were incubated with 2 μg/ml Hoechst 33258 in PBS (15 min, room temperature), washed in PBS, and mounted in Aqua PolyMount (Polysciences, Warrington, PA). In some experiments, cells were incubated with 200 nM MitoTracker Red CMXRos in complete medium (15 min, 37 °C) prior to fixation. Primary antibodies used were Bcl-2 (C-2; Santa Cruz Biotechnology) and cytochrome *c* (6H2B4; BD PharMingen). Imaging occurred by confocal laser-scanning microscopy (Bio-Rad).

Statistical Analysis—Student's *t* test was used to determine whether there was a significant difference between two means ($p < 0.05$). When multiple means were compared, significance was determined by one-way analysis of variance ($p < 0.05$). For analysis of variance analysis, letter designations are used to indicate statistically significant differences. Means with a common letter designation within one *figure* are not different; those with a different letter designation are significantly different from all other means with different letter designations. For example, a mean designated as *a* is significantly different from a mean designated *b*, but neither is different from a mean designated *a*,*b*.

RESULTS

Doxorubicin Decreases the Δψ Prior to the Onset of PS Externalization—The mitochondria are key organelles in the control of apoptosis. Therefore, we investigated the involvement of mitochondrial dysfunction in doxorubicin-induced apoptosis. The Δψ is a sensitive measure for mitochondrial functioning (reviewed by Kroemer and Reed (49)). Previously, we reported on the apoptotic effects of doxorubicin on MTLn3 cells (38). Doxorubicin induced apoptosis in a time-dependent manner, as determined by annexin V-staining, analysis of DNA content, and caspase activity. The onset of apoptosis occurred between 8 and 16 h after exposure, with maximal caspase-3 activity at 24 h.

In the present study, we analyzed Δψ after doxorubicin treatment by flow cytometry using Rho123. Initially, Rho123 fluorescence was only quantified in the PI (*i.e.* viable) population, gated as indicated in Fig. 1*A*. Doxorubicin caused a decrease in Δψ in PI^- cells (Fig. 1*B*). PI^- cells, however, include two cell populations: genuinely viable cells as well as early apoptotic cells. These cells can be distinguished by the absence or presence of externalized PS (viable and apoptotic cells, respectively)

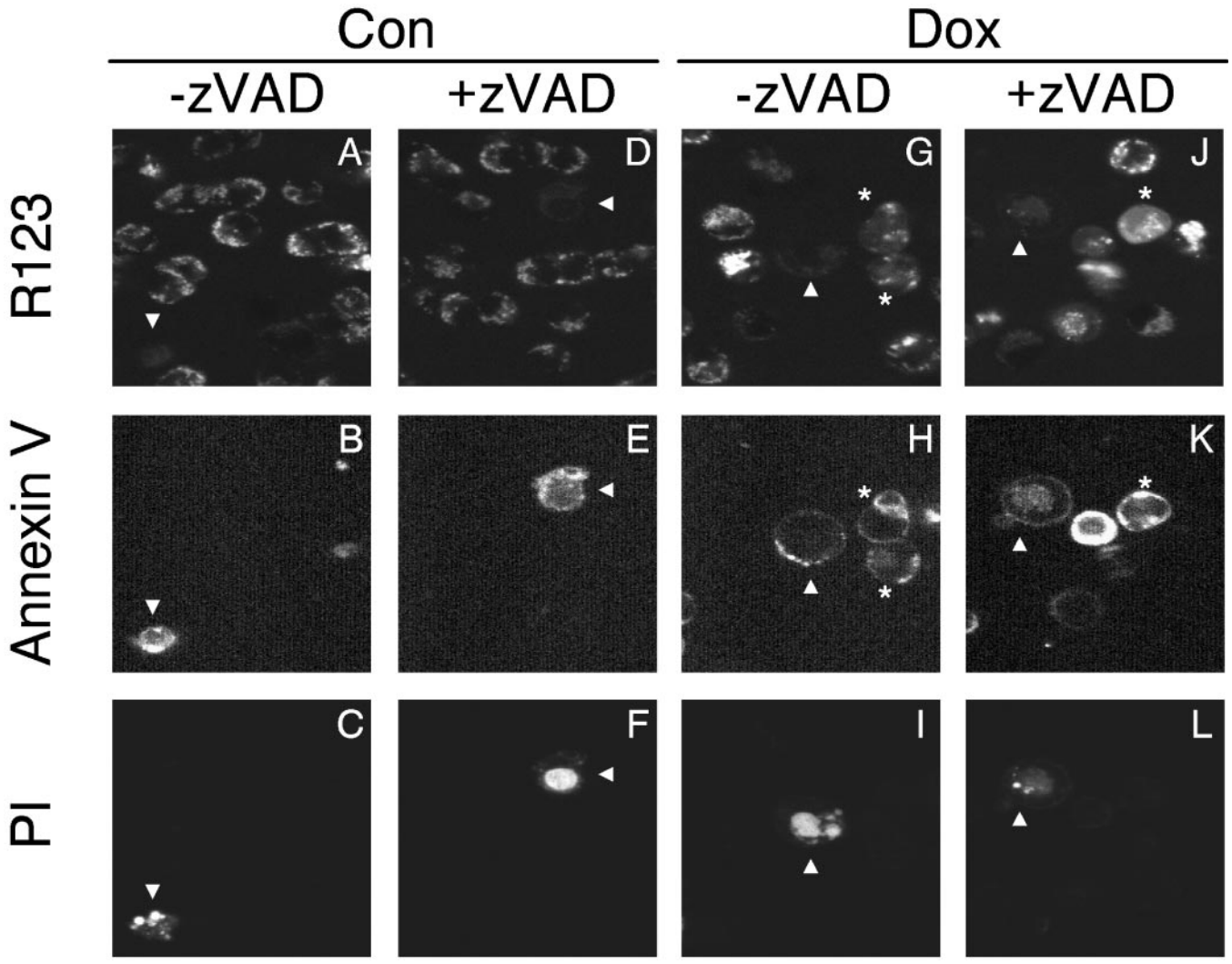

FIG. 2. **Rhodamine 123 staining in the mitochondria decreases in AV^-/PI^- cells upon exposure to doxorubicin (*Dox*).** MTLn3 cells were treated as described in the legend to Fig. 1, harvested as for flow cytometry, and subsequently stained with rhodamine 123 (*R123*; *A*, *D*, *G*, and *J*), annexin V-APC (*B*, *E*, *H*, and *K*), and PI (*C*, *F*, *I*, and *L*). Samples were analyzed using CLSM. The *asterisks* indicate cells that are AV^+/PI^- (*i.e.* apoptotic) and that display diffuse R123 staining. The *arrowheads* indicate cells that are AV^+/PI^+ (*i.e.* "secondary" necrotic). Data shown are representative for three independent experiments. *Con*, control.

as identified by annexin V staining. Therefore, the possibility existed that the drop in $\Delta\psi$ was mainly present in cells that were already apoptotic (*i.e.* have externalized PS). Alternatively, apoptotic cells may have a disturbed intracellular distribution of Rho123 that does not reflect the mitochondrial membrane potential. To investigate these possibilities in more detail, MTLn3 cells were stained with Rho123 and propidium iodide as well as APC-labeled AV. This enabled us to determine the relationship between loss of $\Delta\psi$ and onset of apoptosis in PI^- cells. Using CLSM (Fig. 2), we observed that in control cells, the Rho123 staining is intense and strictly located at the mitochondria; little variability is observed between cells (*A–C*). In contrast, in doxorubicin-treated cells, there is considerable variability in Rho123 staining (*G–I*). Thus, whereas in all AV^-/PI^- cells Rho123 is located at mitochondria, some of these cells have a markedly decreased $\Delta\psi$. In contrast, in AV^+/PI^- cells the Rho123 staining is more diffuse (*asterisks*).

Because of the variability in Rho123 staining in AV^-/PI^- cells and the more diffuse localization of Rho123 in AV^+/PI^- cells, we reevaluated the Rho123 fluorescence intensity measurements by flow cytometry. Doxorubicin caused a clear increase in the percentage of AV^+/PI^- cells as determined by three-color flow cytometry with Rho123, PI and APC-annexin V (Fig. 3, *A* and *B*). Moreover, in accordance with the CLSM observations, the decrease of $\Delta\psi$ caused by doxorubicin was already present in AV^-/PI^- cells (*i.e.* prior to PS externalization) (Fig. 3, *C* and *D*). Similar observations were made with another structurally unrelated fluorescent dye, $DiOC_6$ (Fig. 4), that is also often used to determine the mitochondrial membrane potential in living cells (30, 44, 45). We also investigated whether the collapse of $\Delta\psi$ in MTLn3 cells was restricted to doxorubicin. The anticancer agent etoposide did not induce dissipation of $\Delta\psi$ under conditions that resulted in comparable levels of apoptosis (Fig. 3, *E* and *F*).

Doxorubicin-induced Dissipation of $\Delta\psi$ Is Independent of Caspase Activation—Next we wanted to determine the mechanism for the early mitochondrial perturbations in doxorubicin-induced apoptosis. Up-regulation of CD95/CD95L, which may occur in a p53-dependent manner, and subsequent activation of caspase-8 have been implicated in doxorubicin-induced apoptosis (12, 50, 51). Therefore, we examined the kinetics of expression and/or cleavage of these proteins. Treatment of MTLn3 cells with doxorubicin rapidly induced up-regulation of p53, CD95, and CD95L (Fig. 5*A*). Caspase-8 cleavage was first observed at 12 h after exposure and may therefore be responsible for the collapse of the mitochondrial membrane

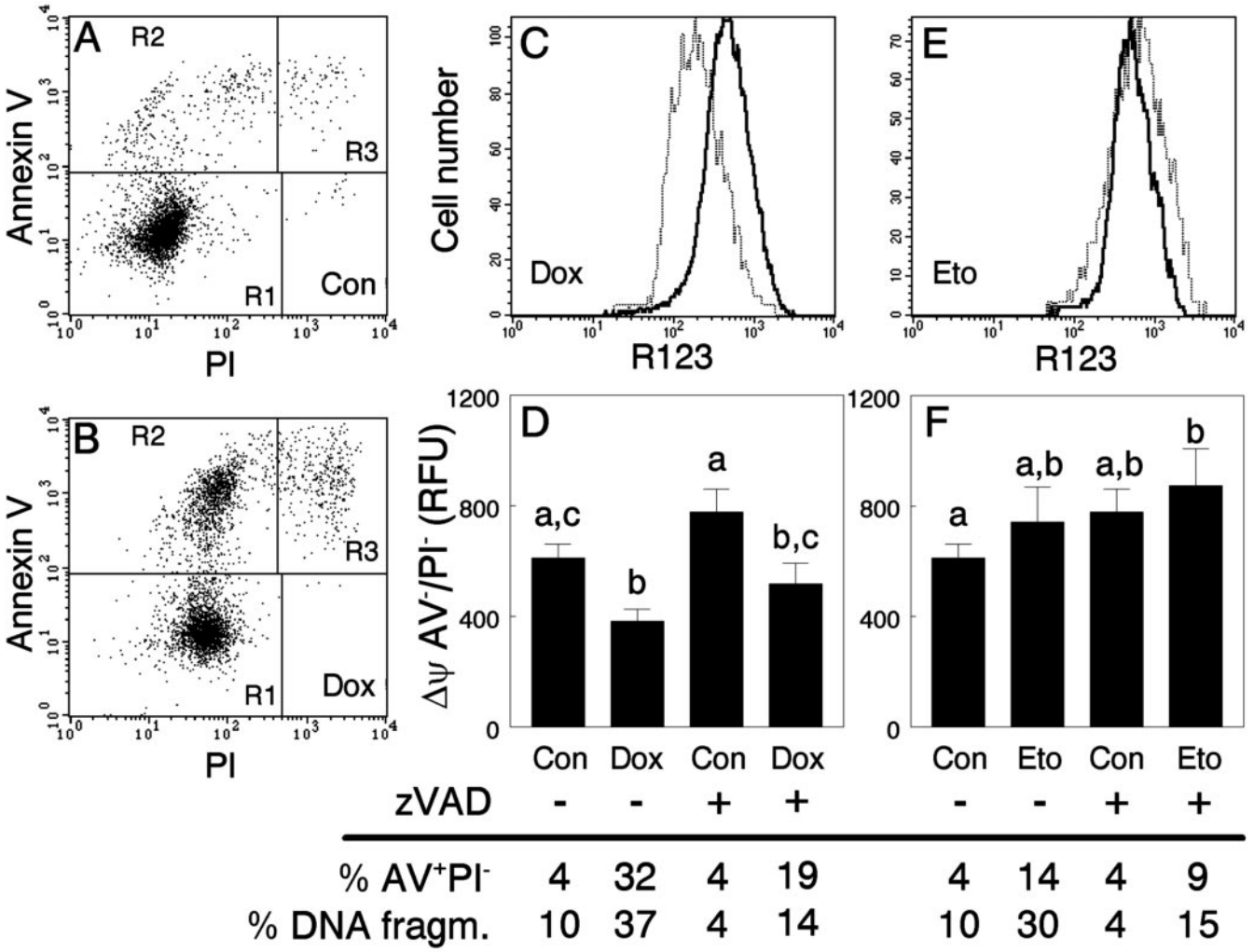

FIG. 3. **Three-color flow cytometry shows that collapse of Δψ by doxorubicin (*Dox*) occurs prior to PS exposure.** MTLn3 cells were treated as described in the legend to Fig. 1 or with 50 μM etoposide under the same conditions and stained for rhodamine 123 (*R123*), annexin V, and PI prior to analysis by flow cytometry. The plots show clear separation of viable (AV^-/PI^-; *R1*), apoptotic (AV^+/PI^-; *R2*), and necrotic (AV^+/PI^+; *R3*) cells in control (*A*) and doxorubicin-treated cells (*B*). The decrease in Δψ is shown as the mean fluorescence intensity of R123 in doxorubicin-treated (*C* and *D*), or etoposide-treated AV^-/PI^- cells (*E* and *F*). Control samples (*Con*) are indicated with a *thick line* (*C* and *E*), and doxorubicin (*C*) and etoposide (*Eto*; *E*) are indicated with a *dashed line*. The percentage of DNA fragmentation in *D* and *E* indicates the percentage of cells with sub-G_1/G_0 DNA content as determined by flow cytometric cell cycle analysis. Data shown are the mean of three independent experiments ± S.E. *Lowercase letters* indicate statistical significance as described under "Experimental Procedures."

potential. To investigate the involvement of caspases in the dissipation of Δψ, we treated MTLn3 cells with the pancaspase inhibitor zVAD-fmk. zVAD-fmk protected against doxorubicin-induced apoptosis (Fig. 3*D*) and, importantly, abrogated the cleavage of caspase-8 as well as caspase-3 (Fig. 5*B*). In contrast, in the presence of zVAD-fmk, the dissipation of Δψ caused by doxorubicin in viable cells was also about 35% (Fig. 3*D*). Also, evaluation of the intracellular Rho123 localization by CLSM revealed that the Rho123 distribution within either AV^-/PI^- or AV^+/PI^- cells was similar in the absence or presence of zVAD-fmk (Fig. 2, *G* and *J*, *asterisks*). These combined data indicate that the decrease in Δψ caused by doxorubicin in viable cells occurs independent of CD95/CD95L up-regulation and caspase-8 activation.

Overexpression of Bcl-2 in MTLn3 Cells Inhibits Doxorubicin-induced Apoptosis—If caspases are not involved in doxorubicin-induced collapse of Δψ, which pathway(s) may then be responsible? Antiapoptotic Bcl-2 family members, including Bcl-2 and Bcl-x_L, inhibit apoptosis by preventing mitochondrial perturbations during the apoptotic process. Therefore, we tested whether the decrease of Δψ in genuinely viable cells upon doxorubicin treatment also occurred in MTLn3 cells over-expressing Bcl-2. We constructed MTLn3 cells stably overexpressing Bcl-2 and characterized the protective action of Bcl-2 in doxorubicin-induced apoptosis. Three different clones were obtained, which all showed strong overexpression of Bcl-2 (Fig. 6*A*). Immunofluorescent staining for Bcl-2 together with the mitochondrial marker Mitotracker Red (CMXRos), indicated that most of the Bcl-2 was located at the mitochondria (Fig. 6*A*). Very little Bcl-2 staining was detectable in Neo control cells. In Bcl-2-overexpressing (Bcl-2) cells, doxorubicin did not induce statistically significant DNA fragmentation at concentrations up to 25 μM, whereas in Neo control cells 39% apoptosis was observed (Fig. 6*B*). DNA fragmentation is a late, caspase-dependent event in apoptosis. Therefore, we also determined the effect of Bcl-2 overexpression on caspase activation. Doxorubicin caused a clear induction of caspase-3-like activity in Neo cells (156 ± 11 pmol of Asp-Glu-Val-Asp-7-amino-4-methylcoumarin/min/mg of protein (control) *versus* 528 ± 76 pmol of Asp-Glu-Val-Asp-7-amino-4-methylcoumarin/min/mg of protein (doxorubicin)). Although some induction of caspase activity was observed in Bcl-2 cells (25 ± 11 pmol of Asp-Glu-Val-Asp-7-amino-4-methylcoumarin/min/mg of protein (control) *versus* 120 ± 6 pmol of Asp-Glu-Val-Asp-7-amino-4-methylcoumarin/

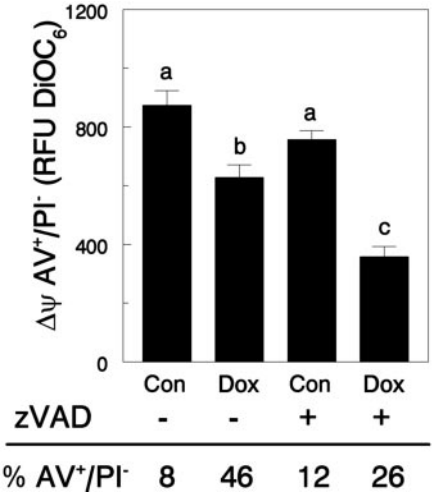

FIG. 4. **Doxorubicin-induced dissipation of $\Delta\psi$ as determined by DiOC$_6$ staining.** MTLn3 cells were treated with doxorubicin as described in the legend to Fig. 1 and stained for DiOC$_6$, annexin V, and PI prior to analysis by flow cytometry as described under "Experimental Procedures." Data shown are the mean of three independent experiments ± S.E. Characters indicate statistical significance as described under "Experimental Procedures."

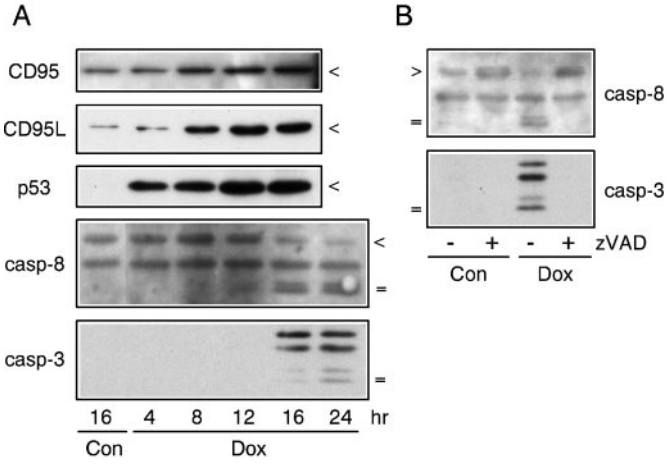

FIG. 5. **Doxorubicin-induced up-regulation of CD95, CD95L, and p53, which was accompanied by zVAD-fmk-inhibitable caspase-8 and -3 cleavage.** MTLn3 cells were treated with vehicle or 17 μM doxorubicin for 1 h and allowed to recover in the absence (*A*) or presence (*B*) of 100 μM zVAD-fmk for the indicated times (*A*) or 24 h (*B*) and immunoblotted for CD95, CD95L, caspase-8 (*casp-8*), p53, and caspase-3 (*casp-3*). The *arrowheads* and *double lines* indicate the full-length proteins and cleavage fragments, respectively. Data shown are representative for three independent experiments.

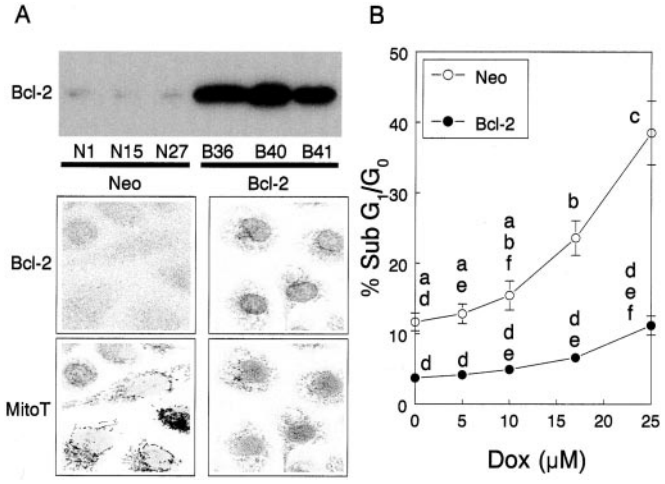

FIG. 6. **Bcl-2 overexpression inhibits doxorubicin-induced apoptosis of MTLn3 cells.** Neomycin-resistant (Neo) cells and Bcl-2 overexpressing (Bcl-2) cells were subjected to Western blotting and immunofluorescent staining with a Bcl-2 antibody and mitochondrial staining using MitoTracker Red (*MitoT*; *A*). Neo and Bcl-2 cells were exposed to the indicated concentrations of doxorubicin for 1 h and subsequently allowed to recover for 24 h. Apoptosis was determined by cell cycle analysis (*B*). Data shown are representative for (*A*) or the mean of (*B*) three independent experiments with three individual clones ± S.E. Characters indicate statistical significance as described under "Experimental Procedures."

min/mg of protein (doxorubicin)), the absolute activity was less than 25% of doxorubicin-treated Neo cells.

In a previous report (38), we demonstrated that inhibition of caspases using zVAD-fmk did not completely block doxorubicin-induced PS externalization in MTLn3 cells. This indicated that part of the PS externalization occurred in a caspase-independent, possibly Bcl-2-sensitive manner. Therefore, we next investigated whether Bcl-2 was able to block doxorubicin-induced PS externalization. Whereas inhibition of caspases reduced DNA fragmentation to control levels in Neo cells (Fig. 7*A*), PS externalization caused by doxorubicin treatment was only marginally affected by zVAD-fmk (Fig. 7*B*). In contrast, Bcl-2 overexpression protected against DNA degradation as well as PS externalization induced by doxorubicin (Fig. 7). zVAD-fmk had only a slight additional protective effect on DNA fragmentation, but it had no effect on PS externalization. Importantly, whereas the resistance of Bcl-2 cells to doxorubicin was not due to inhibition of the induction of p53, CD95, or CD95L expression, caspase-8 and caspase-3 activation were markedly reduced in Bcl-2 cells (Fig. 7*C*). The inhibition of caspase-8 cleavage in Bcl-2 cells further strengthens the notion that caspase-8 cleavage occurs secondary to mitochondrial perturbations. These observations indicate clear differences in the regulation of several apoptotic features in doxorubicin-induced apoptosis by Bcl-2 on the one hand and caspases on the other hand.

Bcl-2 Does Not Protect against Doxorubicin-induced Dissipation of $\Delta\psi$—In many cell types, Bcl-2-mediated protection against apoptosis is associated with preservation of mitochondrial homeostasis (19). Since Bcl-2 protected against PS externalization, we next checked whether Bcl-2 could also prevent the decrease in $\Delta\psi$ induced by doxorubicin as observed in AV$^-$/PI$^-$ MTLn3 (see Fig. 3, *C* and *D*). Flow cytometric analysis (Rho123, PI, and annexin V-APC staining) of Neo cells indicated that doxorubicin induced a dissipation of $\Delta\psi$ of ~60% in AV$^-$/PI$^-$ cells. Surprisingly, doxorubicin caused a similar dissipation of $\Delta\psi$ in AV$^-$/PI$^-$ Bcl-2 cells (Fig. 8*A*). The $\Delta\psi$ of apoptotic AV$^+$/PI$^-$ cells remained unchanged under all conditions in both Neo and Bcl-2 cells (Fig. 8*B*). As mentioned above, doxorubicin still caused some activation of caspases in Bcl-2 cells. Therefore, the possibility existed that low caspase activity contributes to the dissipation of $\Delta\psi$. To exclude this possibility, both Neo and Bcl-2 cells were treated with doxorubicin in the presence of zVAD-fmk. Co-incubation with zVAD-fmk did not affect the doxorubicin-induced decrease of $\Delta\psi$ in both Neo and Bcl-2 cells. Next we investigated whether these findings are also applicable to other cells. For this purpose, we used the immortalized porcine renal proximal tubular epithelial cell line LLC-PK1 that is also sensitive to doxorubicin (40). As expected, LLC-PK1 cells stably transfected with Bcl-2 (pkBcl2 clone 6) were resistant to doxorubicin-induced apoptosis compared with empty vector control cells (pkNEO clone 1). In addition, cells that were not yet apoptotic after doxorubicin treatment (*e.g.* AV$^-$/PI$^-$ cells) had a decreased $\Delta\psi$ (Table I). zVAD-fmk did block the doxorubicin-induced apoptosis of pkNEO and pkBcl2 cells; however, no protection was observed against the decrease

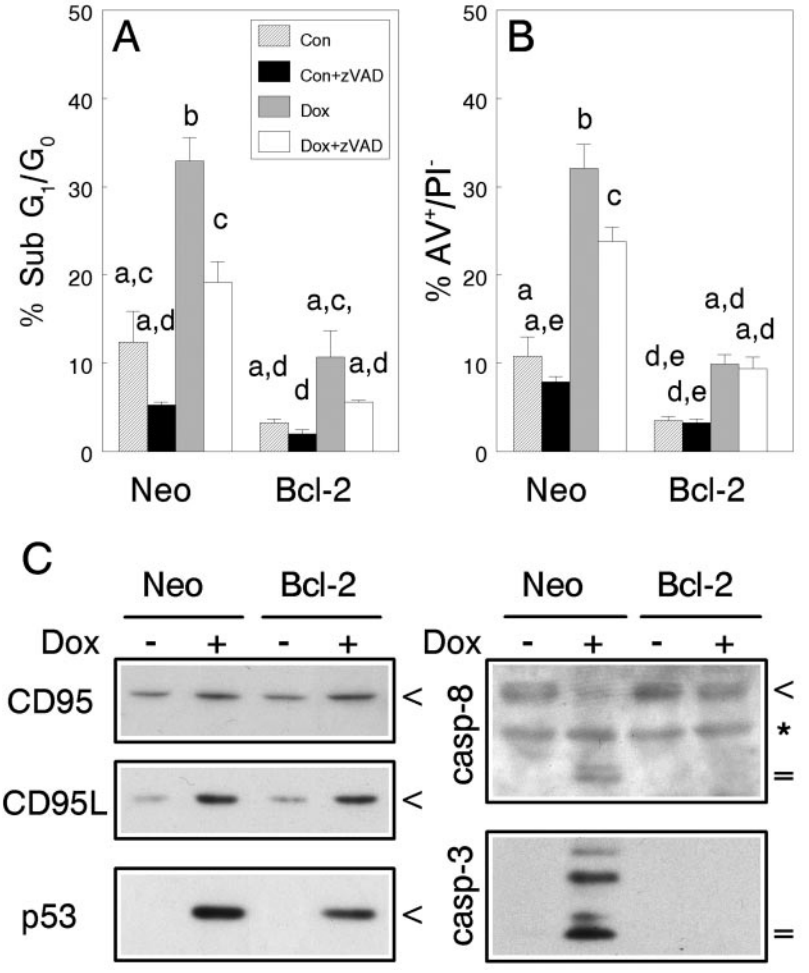

FIG. 7. **Differential regulation of DNA fragmentation and PS externalization by Bcl-2 and caspases.** Neo and Bcl-2 cells were treated as described in the legend to Fig. 1. Apoptosis was determined by cell cycle analysis (*A*) and AV/PI staining (*B*) in split samples. Western blot samples were also taken at 12 h (CD95, CD95L, p53) or 24 h (caspase-8 and -3) after exposure to 17 μM doxorubicin (*C*). Data represent the mean of (*A*, *B*) or are representative for (*C*) three independent experiments with three individual clones ± S.E.

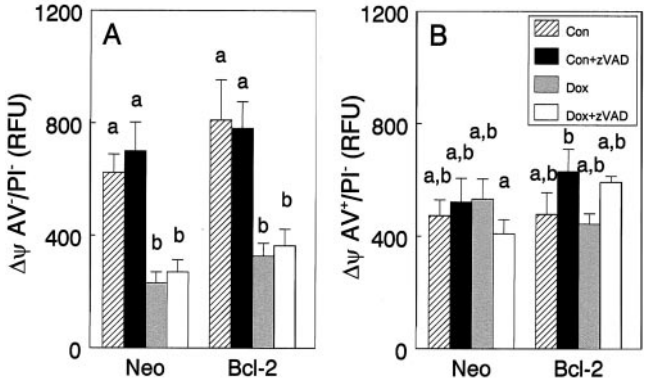

FIG. 8. **Bcl-2 does not inhibit loss of Δψ induced by doxorubicin in MTLn3 cells.** The samples shown in Fig. 7, *A* and *B*, were also used for analysis of Δψ using R123 in viable (AV^-/PI^-; *A*) and apoptotic (AV^+/PI^-; *B*) cells. *RFU*, relative rhodamine 123 fluorescence units. Data represent the mean of three independent experiments with three individual clones ± S.E. *Lowercase letters* indicate statistical significance as described under "Experimental Procedures."

of Δψ (Table I). In conclusion, these data indicate that the dissipation of Δψ caused by doxorubicin is an event that occurs upstream of a checkpoint controlled by either the Bcl-2 family or caspases.

Bcl-2 Does Not Protect against Long Term Survival of MTLn3 Cells after Doxorubicin Treatment—Bcl-2 clearly protected against doxorubicin-induced apoptosis. However, the fact that no protection was observed against the doxorubicin-induced dissipation of the mitochondrial membrane potential suggested that compromised mitochondrial function may affect long term survival. Indeed, in MTLn3 cells overexpressing Bcl-2, doxorubicin still caused a strong reduction in clonogenic survival in a soft agar assay (Fig. 9).

Cytochrome c Release Is Not Strictly Correlated with Loss of Δψ—Cytochrome *c* release from the mitochondria is a critical event in formation of the apoptosome and subsequent activation of caspase-9 and -3 (6). In our cells, the doxorubicin-induced loss of Δψ was not inhibited at all by caspase inhibition. Moreover, Bcl-2 did not protect against the dissipation of Δψ either. It has been reported that in various models of apoptosis, the protective effect of Bcl-2 occurs through inhibition of mitochondrial cytochrome *c* release (52, 53). This suggests that the loss of Δψ, on the one hand, and cytochrome *c* release with subsequent caspase-activation, on the other hand, may be independent events in doxorubicin-induced apoptosis of MTLn3 cells. We first characterized the cytochrome *c* release in Neo cells. To investigate the relationship between the loss of Δψ and cytochrome *c* release, we used the mitochondrial membrane potential-sensitive dye CMXRos (54) in combination with immunofluorescent staining of cytochrome *c*. For this purpose, cells were incubated with CMXRos just prior to fixation of the cells. This procedure allowed us to evaluate Δψ, cytochrome *c* localization, and caspase-3 activation in individual attached cells by CLSM. In Neo control cells, cytochrome *c* was clearly visible as a punctate staining that clearly co-localized with CMXRos, indicating mitochondrial localization (Fig. 10*A*). Exposure to doxorubicin caused a decreased staining of CMXRos; in some cells, no mitochondrial localization of CMXRos was evident (*arrowhead*). Also, the punctate localization of cytochrome *c* staining was lost (Fig. 10*A*, *arrowhead* and *asterisk*), which was evident in ~15% of the cells (Fig. 10*B*). To determine

TABLE I

Effect of Bcl-2 overexpression on doxorubicin-induced apoptosis and loss of Δψ in LLC-PK1 cells

pkNEO and pkBCL-2 cells were treated with varying concentrations of doxorubicin in the absence or presence of z-VAD-fmk (100 μM) in Dulbecco's modified Eagle's medium containing penicillin/streptomycin for 24 h. Thereafter, total percentage of cell death (AV^+/PI^- and AV^+/PI^+) and Δψ were determined as described under "Experimental Procedures." Data shown are the mean ± S.E. of three independent experiments. RFU, relative rhodamine 123 fluorescence units.

	Cell death		Δψ AV^-/PI^- (Rho123)	
	pkNEO	pkBCL-2	pkNEO	pkBCL-2
	%	%		
Control	15 ± 1	13 ± 1	189 ± 25 (100)[a]	177 ± 22 (100)
Doxorubicin, 2.5 μM	42 ± 4	24 ± 1	129 ± 29 (68)	100 ± 12 (57)
Doxorubicin, 5 μM	49 ± 2	33 ± 4	128 ± 27 (76)	85 ± 12 (48)
zVAD	14 ± 1	12 ± 1	192 ± 23 (101)	176 ± 21 (100)
zVAD/doxorubicin, 5 μM	12 ± 1	13 ± 1	86 ± 12 (45)	45 ± 12 (25)

[a] Numbers in parentheses are the percentage Δψ of control.

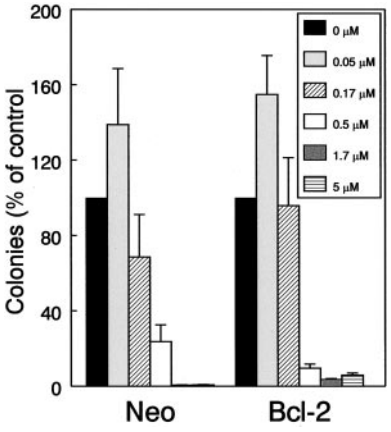

FIG. 9. **Bcl-2 is unable to prevent the inhibition of colony formation of MTLn3 cells by doxorubicin.** Both Neo- and Bcl-2-MTLn3 cells were treated with doxorubicin and plated in soft agar as described under "Experimental Procedures." Colony formation is expressed as the percentage of each vehicle-treated cell line. Untreated Bcl-2 cells grew slightly slower than Neo cells (747 ± 61 and 862 ± 82 colonies/well, respectively). Data shown are the mean of three independent experiments with three Neo and Bcl-2 clones ± S.E.

whether this cytochrome *c* translocation correlated with dissipation of Δψ, we further discriminated between cells with high or low Δψ. This revealed that cytochrome *c* release was not strictly correlated with loss of Δψ, since also cells with still high Δψ showed cytochrome *c* release, although to a lesser extent than cells with low Δψ (Table II). In addition, there were also cells that had almost completely lost Δψ (little or no CMXRos staining; +) but still showed mitochondrial cytochrome *c* staining. The release of cytochrome *c* as well as the loss of Δψ as visualized with CMXRos always preceded the onset of caspase activity; no active caspase-3 staining could be observed in any of the attached cells after doxorubicin treatment (data not shown). In contrast, staurosporin (50 nM, 6 h) was capable of induction of caspase-3 activation prior to cell detachment, probably due to the rapid induction of apoptosis (data not shown).

Bcl-2 Only Partially Inhibits the Release of Cytochrome c—Next we investigated the protective role of Bcl-2 against doxorubicin-induced release of cytochrome *c*. Despite the fact that Bcl-2 protected against apoptosis, doxorubicin induced cytochrome *c* release in cells that were still attached to a similar extent in Bcl-2 cells as in Neo cells (Fig. 10, *A* and *B*). Also, in Bcl-2 cells there was no direct correlation between loss of Δψ and cytochrome *c* release as in Neo cells (Fig. 10*A* and Table II). MTLn3 cells that become apoptotic detach from the substratum. Bcl-2 overexpression decreased the sensitivity to doxorubicin; therefore, fewer detached apoptotic cells are found in the medium. Hence, the percentage of cells with cytochrome *c* release as judged from the immunofluorescent staining of attached cells most likely underestimates the total extent of cytochrome *c* release. This is predominantly the case for Neo cells. For this reason, we also evaluated the total mitochondrial cytochrome *c* release in the pooled floating and attached cells using Western blotting (Fig. 10*C*). No cytochrome *c* release was observed in untreated cells. However, doxorubicin clearly increased the extent of cytochrome *c* release in Neo cells, which was already evident after 16 h. Although in some Bcl-2 cells, cytochrome *c* release was observed after doxorubicin treatment at both 16 and 24 h, the levels were considerably lower than for the Neo cells.

In conclusion, these combined data indicate that loss of Δψ is not necessarily preceded by cytochrome *c* release and that Bcl-2 is unable to completely prevent cytochrome *c* release and loss of Δψ in viable cells. However, Bcl-2 decreases the extent of apoptosis although it cannot completely prevent cytochrome *c* release. This suggests that commitment to apoptosis, at least in part, occurs downstream of cytochrome *c* release.

DISCUSSION

Our investigations on the sequence of several critical mitochondria-related events in doxorubicin-induced apoptosis in mammary adenocarcinoma cells allow several conclusions. First, we found that the dissipation of the Δψ in doxorubicin-induced apoptosis precedes PS externalization in these cells. Importantly, this loss of Δψ is caspase-independent. Therefore, mitochondrial changes resulting in collapse of Δψ appear to be a primary event preceding caspase activation. Second, although overexpression of Bcl-2 protected against caspase activation, PS externalization, and DNA fragmentation, Bcl-2 did not prevent the loss of Δψ caused by doxorubicin. Nevertheless, Bcl-2 inhibited the release of cytochrome *c* from the mitochondria to a large extent. Third, detailed analysis of the relationship between the dissipation of Δψ and cytochrome *c* release indicated that these events are mutually independent in both normal cells and cells that overexpress Bcl-2. Altogether, these data indicate that doxorubicin causes a dissipation of the mitochondrial membrane potential, which is independent of Bcl-2 or caspase-controlled pathways.

Our data indicate that loss of Δψ occurs early in doxorubicin-induced apoptosis in MTLn3 cells as well as in LLC-PK1 cells. This occurs well before cell death (*i.e.* in PI^- cells) and PS externalization, an early event generally associated with the onset of apoptosis (55, 56). In other studies, this could not be established, because the Δψ in doxorubicin-treated cells was determined in a heterogeneous population of cells, containing, besides genuinely viable cells, apoptotic or even necrotic cells, which have no functional mitochondria (30–33). Thus, in another study, only the time course of either loss of Δψ or annexin V binding was studied independently in doxorubicin-induced apoptosis (29). As a consequence, these studies did not make proper distinctions between the population of cells: genuinely viable cells, early apoptotic cells (*i.e.* with externalized PS), or

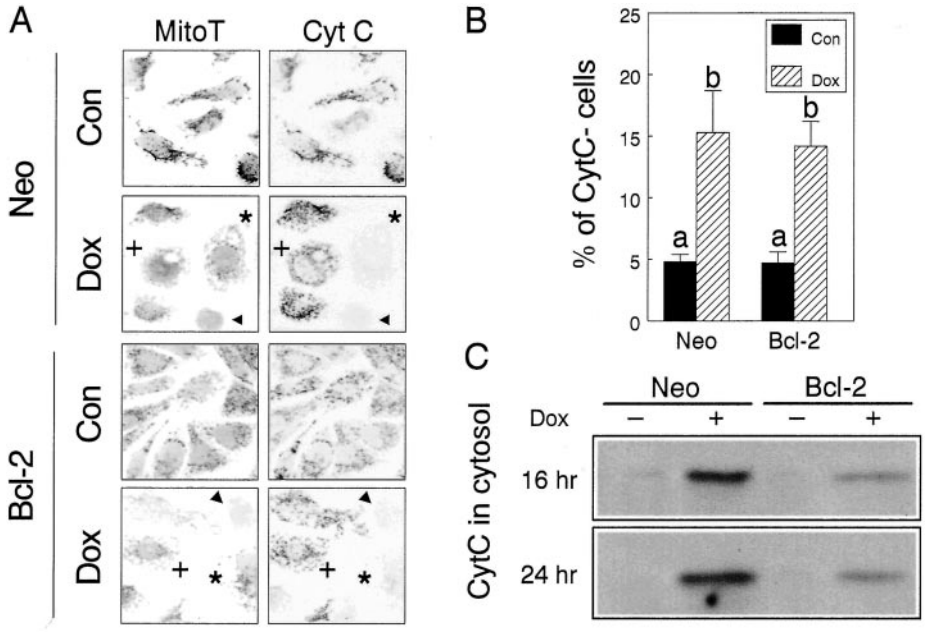

FIG. 10. **Cytochrome *c* release and loss of $\Delta\psi$ are independent events in Neo and in Bcl-2 MTLn3 cells.** Neo and Bcl-2 cells were grown on glass coverslips and treated with 17 μM doxorubicin or vehicle and recovered for 24 h (or 16 h; *C*). Cells were labeled with Mitotracker prior to fixation, stained for cytochrome *c*, and analyzed by confocal microscopy (*A*). These cells were also counted (>100 cells/experiment) for cytochrome *c* staining, and the percentage of cytochrome *c*-negative cells is expressed in *B*. Alternatively, cytosolic fractions were analyzed by Western blotting for cytochrome *c* as described under "Experimental Procedures" (*C*). Data shown are representative for (*A* and *C*) or the mean of (*B*) three independent experiments with three individual clones ± S.E. *Lowercase letters* indicate statistical significance as described under "Experimental Procedures."

TABLE II
Cytochrome c release in MTLn3 cells with high or low $\Delta\psi$

Neo and Bcl-2 MTLn3 cells were treated as described in the legend to Fig. 7. The percentage of cytochrome *c*-negative cells in cells with high and low $\Delta\psi$ is expressed. Data shown are mean ± S.E. of three independent experiments (>100 cells/sample).

	Cytochrome *c*-negative cells			
	Neo		Bcl-2	
	Control	Doxorubicin	Control	Doxorubicin
	%	%	%	%
Low $\Delta\psi$	2.5 ± 0.5	10.5 ± 3.0	2.3 ± 0.3	7.5 ± 2.4
High $\Delta\psi$	2.2 ± 0.9	4.8 ± 0.8	2.4 ± 1.0	6.7 ± 1.8

necrotic cells, in which loss of $\Delta\psi$ occurred. Since we have used confocal microscopy and three-color flow cytometry, we could show that mitochondrial changes are a primary event in doxorubicin-induced apoptosis that occurs already in AV^-/PI^- cells. Moreover, this loss of $\Delta\psi$ is not inhibited by the pancaspase inhibitor zVAD-fmk. Fulda *et al.* (31) reported that in SHEP neuroblastoma cells zVAD-fmk blocked doxorubicin-induced collapse of $\Delta\psi$. In this study, however, dissipation of $\Delta\psi$ was determined in the total cell population so that necrosis may have contributed to the observed loss of $\Delta\psi$. The only study to our knowledge that combined annexin V staining with a mitochondrial membrane potential-sensitive dye (but not PI), showed that etoposide-induced loss of $\Delta\psi$ in PC60 cells was preceded by PS externalization (33). In contrast, we showed that etoposide did not affect the mitochondrial membrane potential in viable MTLn3 cells (Fig. 3, *E* and *F*).

Three main pathways for anticancer agent-induced mitochondrial perturbations have been proposed. First, an indirect pathway, which is largely dependent on DNA-damage and/or stress signaling, may result in up-regulation of the death receptor pathway. Activation of caspase-8 is a primary event in CD95 signaling. If this pathway is involved in doxorubicin-induced mitochondrial perturbation, then inhibition of caspases should prevent this (10–12). Second, cellular damage may also result in up-regulation or posttranslational modification of several proapoptotic Bcl-2 family members that directly affect mitochondrial membrane integrity (*e.g.* Bax up-regulation and translocation to the mitochondria or Bad phosphorylation) (57–59). The consensus is that this pathway acts independent of caspase-8 activation and is generally inhibited by Bcl-2 overexpression (20). Third, the effect of doxorubicin in MTLn3 cells may be a direct effect of doxorubicin on the mitochondria, as has been observed in isolated mitochondria from heart and liver (28, 60).

CD95 activation would exert its effect through caspase-8 activation and Bid cleavage, which may engage the mitochondria. In MTLn3 cells, doxorubicin caused up-regulation of CD95 and CD95L. However, since zVAD-fmk prevented caspase-8 activation (Fig. 5*B*) without affecting the mitochondrial perturbations, the CD95/CD95L/caspase-8 pathway plays a minor role in the observed loss of $\Delta\psi$. The fact that zVAD-fmk does not inhibit the dissipation of $\Delta\psi$ also argues against a feedback mechanism in which a moderate release of cytochrome *c* would activate caspase-3, which in turn affects the mitochondria, thereby enhancing loss of $\Delta\psi$ and/or further release of cytochrome *c* (61–63).

Overexpression of Bcl-2 and Bcl-x_L inhibits apoptosis induced by a variety of stimuli, in particular those that utilize the mitochondria-dependent pathway (20, 64). This is often associated with protection against the release of cytochrome *c* as well as collapse of $\Delta\psi$ (52, 53, 65). The Bcl-2-mediated protection of mitochondria is most likely due to the prevention of pore opening caused by Bax- or Bak-dependent mechanisms. We show that in MTLn3 cells that overexpress Bcl-2, mitochondrial changes including loss of $\Delta\psi$ and a moderate cytochrome *c* release still occur upon doxorubicin treatment. This implies that the doxorubicin-induced loss of $\Delta\psi$ is most likely not caused by proapoptotic Bcl-2 family members. Similar effects have been observed in HL60 cells treated with carbonyl cyanide *m*-chlorophenylhydrazone. However, since carbonyl cyanide *m*-chlorophenylhydrazone uncouples mitochondrial respiration, Bcl-2 could not prevent a decrease in $\Delta\psi$ or cytochrome *c* release, despite inhibition of apoptosis (66).

Which possible pathways may then cause dissipation of $\Delta\psi$ by doxorubicin in MTLn3 cells? One possibility is that doxorubicin exerts its effect on the mitochondria through the up-regulation of p53 (67). Doxorubicin clearly increased the levels of p53 in MTLn3 cells. As was to be expected, the Bcl-2-mediated protection against apoptosis did not occur through a reduction in doxorubicin-induced DNA damage, since the up-regulations of p53 and G_2/M arrest were similar in Neo and Bcl-2 cells (Fig. 7*C* and data not shown). Moreover, the expres-

sion levels of CD95 and CD95L were also similar in Neo and Bcl-2 cells. Thus, either p53 or other p53-regulated genes that reside in the mitochondria or translocate there during apoptosis may cause perturbations of the mitochondria, *e.g.* by induction of reactive oxygen species (68–71). Alternatively, we cannot exclude the possibility that doxorubicin has a direct effect on the mitochondria.

Regardless of the mechanism of loss of $\Delta\psi$, the question remains whether collapse of $\Delta\psi$ is causally linked to doxorubicin-induced cytochrome *c* release. Several findings indicate that this may not be the case. First, at the cellular level there are cells, which (i) have a low $\Delta\psi$ and normal mitochondrial staining of cytochrome *c*, (ii) have a high $\Delta\psi$ but no mitochondrial staining of cytochrome *c*, or (iii) have neither $\Delta\psi$ nor mitochondrial staining of cytochrome *c* (Fig. 10*A*). Second, Bcl-2 largely inhibits cytochrome *c* release, although it is unable to prevent doxorubicin-induced collapse of $\Delta\psi$. This is in agreement with previous observations that cytochrome *c* release can occur prior to loss of $\Delta\psi$ (32, 44). Together, these results strongly suggest that the doxorubicin-induced cytochrome *c* release and collapse of $\Delta\psi$ are mutually independent events.

Our data indicate that Bcl-2 was able to inhibit doxorubicin-induced apoptosis despite the fact that some cytochrome *c* release was observed at 16 h after exposure. This appears to be comparable with the observation that Bcl-2 overexpression markedly reduced apoptosis caused by microinjection of cytochrome *c*. Such an effect may be mediated by sequestration of procaspase-9 by Bcl-2, thereby inhibiting formation of the apoptosome and activation of caspase-3 (72–77).

In MTLn3 cells, Bcl-2 overexpression clearly protected against doxorubicin-induced DNA fragmentation as well as PS externalization. Interestingly, zVAD-fmk only partially protected against PS externalization (38). This strongly suggests that PS externalization caused by doxorubicin is largely caused by a caspase-independent pathway that is inhibitable by Bcl-2. The fact that Bcl-2 does not inhibit loss of $\Delta\psi$ further indicates that the PS externalization is not a consequence of dissipation of the mitochondrial electrochemical gradient. Alternatively, Bcl-2-mediated protection against pore formation and/or outer membrane rupture may prevent the release of unknown mitochondrial factors that induce PS externalization.

In conclusion, our data provide the following model for doxorubicin-induced apoptosis. Doxorubicin causes cellular stress, resulting in p53 up-regulation/accumulation and increased levels of CD95 and CD95L. In addition, doxorubicin causes mitochondrial injury, which appears to be independent of the CD95/CD95L/caspase-8 pathway. Although Bcl-2-dependent events and caspase activation are required for the induction of apoptosis, the dissipation of $\Delta\psi$ caused by doxorubicin is independent of a caspase- or Bcl-2-controlled pathway. Further studies are required to elucidate the potential role of p53-dependent pathways in the observed disruption of $\Delta\psi$.

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