Upregulated Expression of BCL-2 in Multiple Myeloma Cells Induced by Exposure to Doxorubicin, Etoposide, and Hydrogen Peroxide

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Enhanced expression of the antiapoptotic gene BCL-2 may participate in chemoresistance. To ascertain if multiple myeloma cells surviving exposure to chemotherapy after their BCL-2 expression, we treated the myeloma cell lines 8226, IM-9, and U266 as well as a primary myeloma cell culture with various injurious agents. Doxorubicin, etoposide, and hydrogen peroxide consistently induced a concentration- and time-dependent upregulation of BCL-2 expression in all myeloma target cell types assayed by flow cytometry and Western blot analysis. In contrast, serum starvation, dexamethasone, and anti-fas antibodies had no effect on expression. Enhanced expression of BCL-2 was relatively selective as treatments had no effect on expression of Ig light chains, BCL-X, or actin. An reverse transcriptase-polymerase chain reaction assay showed increased levels of BCL-2 RNA in 8226 cells as early as 4 hours after treatment with doxorubicin at a time when cell recoveries were not decreased. Thus, doxorubicin stimulates BCL-2 expression in individual 8226 cells rather than simply allowing a selected survival of high BCL-2-expressing cells in culture. Doxorubicin-treated 8226 cells with upregulated BCL-2 expression were relatively resistant to a second exposure of doxorubicin. In addition, BCL-2-transfected IM-9 cells, with enhanced expression of BCL-2 which was comparable to that achieved by initial exposure to doxorubicin, were resistant to doxorubicin and etoposide cytotoxicity. These data suggest that exposure to chemotherapeutic agents may enhance BCL-2 expression in surviving myeloma cells and contribute to acquired chemo-resistance.

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MATERIALS AND METHODS

Cell lines and culture media. The myeloma cell lines 8226, IM-9, and U266 were kind gifts from J. Epstein (Little Rock, AR) and K. Anderson (Dana-Farber Cancer Institute, Boston, MA). These lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL-complete media). A fresh myeloma culture was initiated with Ficoll-Hypaque (Sigma, St Louis, MO) purified peripheral blood mononuclear cells from a patient with plasma cell leukemia. These malignant cells proliferated spontaneously in vitro and, within 1 week of seeding, were completely (100%) comprised of mature appearing plasma cells. These cells were studied within the first 3 months of their in vitro life span.

Drugs. Doxorubicin was obtained from Astra Pharmaceutical Products, Inc (Westborough, MA). Etoposide was obtained from Bristol Laboratories (Evanston, IN). Dexamethasone was obtained from Sigma Laboratories. Anti-fas antibody was purchased from Kamiya, Inc (Thousand Oaks, CA). All other reagents were purchased from Sigma unless described otherwise.

Flow cytometry for BCL-2 expression- FACs analysis for BCL-2 expression was performed as previously described. Briefly, (phosphate-buffered saline (PBS)-washed cells were treated with 0.6% paraformaldehyde for 1 hour at 4°C and then resuspended in Tween solution for 15 minutes at 37°C. After washing x2, cells were stained with antimouse BCL-2 antibody (DAKO) as previously described. They were then exposed to the secondary antibody fluorescein isothiocyanate (FITC)-conjugated F(ab')2 fragment of rabbit antimouse IgG (Dako). Cells were then analyzed by flow cytometry with a FACScan apparatus (Becton Dickinson). The corresponding control consisted of identical staining except for the use of an irrelevant mouse IgG of identical isotype instead of the anti-BCL-2 antibody. Intensity of fluorescence was first expressed

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on a log scale and converted to linear scale and is expressed as mean channel fluorescence above control. Expression was assessed on the FACSscan flow cytometer with at least 1,000 events evaluated for each group. The FACSscan is standardized for fluorescent intensity each morning such that a given amount of photon output appears in the exact same channel. In figures, expression is presented relative to untreated cells whose expression was arbitrarily chosen to be 1. The initial BCL-2 mean channel fluorescence before culture was never significantly different from levels in cells cultured for 24 to 96 hours in the absence of doxorubicin, etoposide, or hydrogen peroxide. In some experiments, similarly treated 8226 cells were also stained for \( \lambda \)-light chain expression (8226 are \( \lambda \)-light chain secretors). Mouse antihuman \( \lambda \)-light chain IgG2a antibodies (Sigma) were used directly conjugated to FITC. Control staining consisted of irrelevant mouse IgG2a conjugated with FITC. In some experiments, relative expression of BCL-2 was compared among different groups by reference to calibrated beads, (Quantum Series; Flow Cytometry Standards Corp, Research Triangle Park, NC) which were run in parallel.

Western blot analysis. Following washing in cold PBS, cells were lysed in 50 \( \mu \)L of lysis buffer (1% Triton-X 100 [Sigma], 0.5% NP40, 10 \( \mu \)L Tris, pH 7.4, 150 \( \mu \)L NaCl, 1 \( \mu \)L EDTA, 1 \( \mu \)L EGTA, 0.2 \( \mu \)L Na3VO4, 0.2 \( \mu \)L NaF, 0.2 \( \mu \)L phenylethlylsulfonfluoride). Lysates were cleared at 14,000 rpm for 15 minutes at 4°C. Twenty-five micrograms of protein from each sample was boiled for 5 minutes in 1 × sodium dodecyl sulfate (SDS) gel-loading buffer (125 \( \mu \)L Tris, pH 6.8, 5% glycerol, 2% SDS, 1% mercaptoethanol and 0.006% bromophenol blue). Proteins were separated by 12.5% SDS-polyacrylamide gel electrophoresis and transferred onto PUDF membranes. The membranes were blocked for 1 hour at room temperature in 3% bovine serum albumin, 5% nonfat dried milk, 10 \( \mu \)L Tris, pH 7.5, 100 \( \mu \)L NaCl and 0.1% Tween 20. After 4 washes, the membranes were incubated with 0.5 \( \mu \)g/mL of rabbit antihuman BCL-2 antibody (gift of Dr John Reed, La Jolla, CA) for 1 hour. After 4 more washes, the membranes were overlaid with 1 \( \mu \)g/mL of HRP-labeled goat antirabbit IgG antibody (Amersham, Arlington Heights, IL) and the BCL-2 protein band was detected with an ECL system.

Reverse transcriptase-polymerase chain reaction (RT-PCR). The RT-PCR assay was performed as previously described.14 Briefly, total RNA was extracted from 107 cells by the guanidium-thiocyanate acid phenol method, treated with DNase (30 minutes at 37°C), extracted with phenol/chloroform and precipitated with ethanol. For cDNA synthesis, 5 \( \mu \)g of RNA was incubated with 100 \( \mu \)L random hexameteroildes in 46.5 \( \mu \)L for 10 minutes at 70°C, chilled and brought up to buffer conditions including 200 U Moloney murine leukemia virus RT as recommended by the supplier. Samples were incubated for 60 minutes at 37°C and 5 minutes at 95°C to denature RT. For the PCR, 10 \( \mu \)L of cDNA was used as template for PCR. A reaction mixture of 100 \( \mu \)L contained the template in 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, 1.5 mmol/L MgCl2, 0.001% gelatin, each primer at 0.5 umol/L, each dNTP at 200 umol/L and 2.5 U of Taq DNA polymerase. The BCL-2 sense primer = 5′CGACGACCTTCTCCCGCCGTACCCG3′ and the antisense primer = 5′CGGCAATGCTGGGGCGTACAGGCC3′ giving an expected PCR product of 318 bp. The B-actin sense primer = 5′GTGGGCGGCCCAAGGACCAC3′ and the antisense primer = 5′CGGCAATGCTGGGGCGTACAGGCC3′ giving an expected product of 548 bp. The samples were overlaid with 50 \( \mu \)L of mineral oil, denatured for 7 minutes at 94°C and subjected to 17 to 25 cycles of amplification in an automated thermal cycler. The cycling conditions were 1 minute at 94°C and 1 minute at 72°C. Fifteen microliters of amplified product was electrophoresed on 2% agarose gels and the gels were stained with ethidium bromide.

Transfection of IM-9 cells. The full length BCL-2 cDNA was a gift from Dr Dale Bredesen (University of California Los Angeles). It was subcloned into the pCI expression vector at EcoRI sites. Plasmids for transfection were purified with maxiprep kits. Transfection was performed by lipofection. Briefly, plasmid and lipofectin (Boehringer, Indianapolis, IN) were diluted to 0.05 and 0.1 \( \mu \)g/mL, respectively, with 200 \( \mu \)L HEPES buffer, pH 7.4. Diluted plasmid DNA, and lipofectin were mixed at a ratio of 1:2 and kept at room temperature for 15 minutes. One hundred and fifty microliters of the above mixed solution was added to IM-9 cells cultured in 6 well plates (500,000 cells/ 5 mLs). After 10 hours of exposure, transfection media was removed and cells were refed with fresh media for another 24 hours. At that point, transfected cells were selected in 800 \( \mu \)g/mL of G418 (GIBCO-BRL, Grand Island, NY). After transfected cells grew out in G418 (2 weeks), they were cloned by limiting dilution.

RESULTS

Exposure to doxorubicin or etoposide upregulates BCL-2 expression in 8226 myeloma cells. To assess effects on BCL-2 expression, 8,226 cells were exposed to increasing concentrations of doxorubicin for 2 hours and washed, and recultured for increasing durations, after which viable cells were analyzed for BCL-2 content by flow cytometry. As shown in Fig 1, doxorubicin consistently upregulated BCL-2 expression and this was related to the concentration used. BCL-2 expression increased as the doxorubicin concentration increased until becoming optimal at 10^{-6} mol/L. Viability of cells remained over 75% at all these concentrations of doxorubicin. However, further increases in doxorubicin concentration to 5 \( \times \) 10^{-6} mol/L resulted in decreased BCL-2 expression (Fig 1) and this was associated with worsening viability of cells. Upregulated expression was first detected after 24 hours and continued to increase up to 72 hours of culture. When DNA was extracted from the doxorubicin-treated groups and electrophoresed, we found that only the cell groups that showed viability below 70% also showed apoptotic DNA ladder-like cleavage (that treated with 5 \( \times \) 10^{-5} mol/L, not shown). This concentration- and time-depen-
BCL-2 AND MULTIPLE MYELOMA

Fig 2. Doxorubicin increases BCL-2 expression but has no effect on light chain expression. Myeloma 8226 cells were cultured without (A and C) or with 10⁻⁶ mol/L doxorubicin (B and D) for 2 hours, washed and recultured for 48 hours. Cells were then stained for BCL-2 (A and B) or λ-light chain expression (C and D). Shown is FACs analysis profile of cells stained with control antibody (darkened profile) or with specific anti-BCL-2 or anti-λ antibody (open profile).

dent doxorubicin-induced increase in BCL-2 expression has been consistently detected in 10 experiments. The mean x 10⁻⁶ mol/L doxorubicin) was 2.3 ± 0.5 (mean ± SD) for 24 hours, 4.4 ± 0.6 at 48 hours and 5.1 ± 0.4 at 72 hours. These values are all significantly different from the arbitrarily chosen control of 1 (P < .05). The actual data from a typical experiment where media-control or doxorubicin-treated (10⁻⁶ mol/L) cells were analyzed for BCL-2 or λ-light chain expression (used as a control intracellular antigen) is shown in Fig 2. As shown, BCL-2 expression was markedly upregulated while expression of λ-light chains was unaffected, indicating some selectivity of the doxorubicin effect on BCL-2 expression.

In separate experiments, relative BCL-2 expression was assessed by comparison to calibrated beads expressing different levels of FITC fluorescence. This allowed us to convert flow data into the number of molecules of equivalent soluble fluorochrome per cell (MESF). The fluorescence intensity obtained with the isotype matched unreactive antibody was subtracted from that obtained with anti-BCL-2 to calculate net MESF values. The baseline expression of BCL-2 was significant (MESF3328 ± 650), especially when compared to the DOHH2 lymphoma cell line (MESF3865 ± 575), which contains the 14:18 translocation. Following 48 hours exposure to 2 × 10⁻⁷ mol/L doxorubicin, the MESF increased to 7950 ± 475 and, following exposure to 10⁻⁶ mol/L doxorubicin, the MESF increased to 21,550 ± 850 (as compared to nonexposed targets whose MESF remained at 3500 ± 585).

Exposure to a different topoisomerase inhibitor, etoposide (VP-16), also resulted in upregulated expression of BCL-2 (Fig 3). As with doxorubicin treatment, enhanced expression due to VP-16 was also concentration- and time-dependent. In addition, once again, optimal expression was detected with concentrations of drug that did not decrease cell viability or degrade DNA into endonucleosomal fragments. The cells cultured with 5 × 10⁻⁶ mol/L VP-16 showed 75%, 52%, and 34% viability at 24, 48, and 72 hours, respectively. In addition, at each time point, ladder-like DNA fragmentation was detected on gels from these targets. In contrast, targets cultured at all other concentrations showed viability above 75% and intact DNA. This experiment was repeated twice with similar results. The mean increase in BCL-2 expression from the three experiments induced by 10⁻⁶ mol/L VP-16 was 3.3 ± 0.4 at 48 and 3.7 ± 0.5 at 72 hours. These values are significantly different from the control value of 1 (P < .05).

Upregulated BCL-2 expression was also detected by Western blot analysis (Fig 4). As shown, BCL-2 expression was increased 4.2 x fold by doxorubicin (10⁻⁶ mol/L, lane 6) as determined by scanning densitometry and 3.7 x fold by etoposide (10⁻⁶ mol/L, lane 8). Also shown in Fig 4 is the unaltered expression of BCL-X-L protein (lanes 1 through 3), another member of the BCL family of proteins. These results further support the relative specificity of doxorubicin's effect on BCL-2 expression.

Doxorubicin-induced upregulation of BCL-2 was not a peculiarity of the 8226 myeloma cell line. We also detected the phenomenon in IM-9 and U266 myeloma cell lines. Following a 2 hour exposure and 48 hours of reculturing, 5 × 10⁻⁷ mol/L doxorubicin and 10⁻⁶ mol/L VP-16 increased BCL-2 expression 5.3 x fold and 4.1 x fold versus control untreated cells in IM-9 targets (mean of 3 separate FACs experiments) and 3.9 x fold and 3.7 x fold versus control cells in U266 targets. In addition, a freshly explanted myeloma cell culture initiated from a patient with plasma cell leukemia showed a similar enhanced expression. As shown in Fig 5, exposure of these cells to doxorubicin or VP-16 resulted in a 2 to 3 x fold increase in expression.
**Effect of other inducers of apoptosis on BCL-2 expression.**
Doxorubicin is an effective inducer of apoptosis in myeloma target cells. Thus, the ability of doxorubicin to increase expression of BCL-2, a protein that inhibits apoptotic death, suggested the upregulation of BCL-2 served as a protective mechanism of the target cell. To test whether triggering of apoptotic pathways in general would lead to upregulation of BCL-2 as a protective mechanism, we also exposed 8226 targets to dexamethasone, anti-fas antibody and serum deprivation. In preliminary experiments (and others not shown), we confirmed that each of these conditions induced apoptosis of 8226 cells (confirmed by morphologic analyses and detection of DNA ladder-like fragmentation) and delineated the effective concentration range, which induced apoptosis. Targets were then exposed to a wide range of drug concentrations with the highest concentration being capable of inducing at least 50% apoptosis. As shown in Table 1, doxorubicin and etoposide again increased BCL-2 expression. However, serum deprivation and equitoxic concentrations of anti-fas antibody and dexamethasone were unable to enhance BCL-2 expression. Table 1 also shows the significant upregulation of BCL-2 induced by doxorubicin and etoposide, which was associated with unaltered target cell viability although cell recoveries were significantly reduced. Note that the concentrations of dexamethasone and anti-fas antibodies, which were ineffective in altering BCL-2 expression, did induce similar cell viabilities and recoveries. This experiment was repeated twice with identical results and similar experiments using concentrations of anti-fas antibody between 0.05 to 1 \( \mu \text{g/mL} \) and dexamethasone concentrations of \( 1 \times 10^{-8} \) to \( 5 \times 10^{-6} \) \( \text{mol/L} \) never showed an upregulation of BCL-2 expression. However, hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) consistently increased BCL-2 expression. The concentration-dependence was similar to that of doxorubicin and VP-16 vis-à-vis associated cell viability and profile of DNA electrophoresis. Optimal BCL-2 expression was detected between 24 and 48 hours following exposure to 0.5 \( \text{mol/L} \) \( \text{H}_2\text{O}_2 \) (Fig 6), a concentration that

![Western analysis of BCL-2 expression. Myeloma 8226 cells were cultured in media alone (lanes 1, 4, and 7), in 2 \( \times 10^{-3} \) \( \text{mol/L} \) doxorubicin (lanes 2 and 6), 10 \( \times 10^{-4} \) \( \text{mol/L} \) doxorubicin (lanes 3 and 6) or 10 \( \times 10^{-5} \) \( \text{mol/L} \) VP-16 (lane 8) for 2 hours, recultured for 48 hours, and protein then extracted for Western blotting with anti-BCL-2 or anti-BCL-X antibodies. Bands were semi-quantified by densitometry analysis.](image)

![Fig 4. Western analysis of BCL-2 expression. Myeloma 8226 cells were cultured in media alone (lanes 1, 4, and 7), in 2 \( \times 10^{-3} \) \( \text{mol/L} \) doxorubicin (lanes 2 and 6), 10 \( \times 10^{-4} \) \( \text{mol/L} \) doxorubicin (lanes 3 and 6) or 10 \( \times 10^{-5} \) \( \text{mol/L} \) VP-16 (lane 8) for 2 hours, recultured for 48 hours, and protein then extracted for Western blotting with anti-BCL-2 or anti-BCL-X antibodies. Bands were semi-quantified by densitometry analysis.](image)

![Table 1. Equitoxic Concentrations of Dexamethasone or Anti-fas Antibody or Serum Starvation Do Not Increase BCL-2 Expression](table)

<table>
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<tr>
<th>Conditions</th>
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<th>% Recovery</th>
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<td>Etoposide</td>
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8226 cells cultured for designated intervals in described conditions. Exposure to dexamethasone (10 \( \times 10^{-4} \) \( \text{mol/L} \)) and etoposide (10 \( \times 10^{-4} \) \( \text{mol/L} \)) was for 2 hours followed by washing and reculturing for 24 or 48 hours. Exposure to anti-fas (0.1 \( \mu \text{g/mL} \)) was for the entire 8 or 24 hours. Percent viability assessed by trypan staining; percent recovery assessed relative to media control (\( =100\% \)); BCL-2 expression as mean channel fluorescence above control.

![Fig 5. Doxorubicin and VP-16 increase BCL-2 expression of freshly cultured myeloma cells. Myeloma cells from a patient with plasma cell leukemia were cultured for 2 hours in media, doxorubicin (\( \square = 10^{-5} \text{mol/L} \); \( \square = 10^{-4} \text{mol/L} \)) or VP-16 (\( \square = 10^{-5} \text{mol/L} \); \( \square = 10^{-4} \text{mol/L} \)), washed and then recultured for 24 or 48 hours after which BCL-2 expression was assayed by FACs analysis and compared to control cells (arbitrarily shown to be \( =1 \)). This experiment was repeated \( \times 1 \) with similar results.](image)
allowed unaltered viability (consistently over 70%) at all time points and no ladder-like DNA fragmentation. Higher concentrations (1 mol/L) resulted in reduced viability (30% to 65%) at 24 and 48 hours, ladder-like DNA fragmentation and an inability to enhance BCL-2 expression (Fig 6).

**Upregulated BCL-2 expression is not due to selective survival in culture of a high-expressing subpopulation of cells.** There are two alternative explanations for the upregulated BCL-2 expression detected in cultures following doxorubicin treatment. The drug might directly or indirectly increase the expression of BCL-2 in individual cells of the culture. Alternatively, a small subpopulation, with already enhanced expression of the BCL-2 survival factor, may selectively survive in culture following doxorubicin exposure. Although viabilities of cells with doxorubicin-induced heightened expression of BCL-2 were never significantly below control cell cultures, cell recoveries (relative to control cultures) were consistently decreased to the 45% to 70% range. Thus, it was theoretically possible that a survival advantage of high-expressing cells in culture could have accounted for the detected increase in expression. To differentiate between these possibilities, we exposed 8226 cells to media or doxorubicin for 2 hours, washed the cells, recultured for 4 hours and then analyzed expression of BCL-2 RNA by an RT-PCR assay. At this early time point, viabilities as well as cell recoveries in all cultures were basically identical (over 95% viability and recoveries of 94% to 107% relative to media-treated control cells). As shown in Fig 7, as early as 4 hours, cultures exposed to 10^{-6} mol/L doxorubicin showed an increased level of BCL-2 RNA (2.7 \times 10^{1} fold over control by densitometric analysis). As shown, there was no alteration in the level of control actin transcripts in the cultures. Exposure to the lower concentration of doxorubicin (2 \times 10^{-7} mol/L) did not show an increased level of BCL-2 RNA. This experiment was repeated with identical results (3.1 \times 10^{1} fold increase in BCL-2 RNA resulting from exposure to 10^{-6} mol/L doxorubicin and no significant increase from 2 \times 10^{-7} mol/L concentrations).

**Doxorubicin-treated cells with upregulated BCL-2 expression are relatively resistant to a second exposure to doxorubicin.** To test whether doxorubicin-induced upregulation of BCL-2 expression had any potential impact on subsequent survival of cells, we first treated targets with (2 \times 10^{-7} mol/L or 10^{-6} mol/L) or without doxorubicin for 2 hours, washed the cells, cultured for 48 hours, and then challenged with increasing concentrations of doxorubicin in a second 2 hour exposure. Forty-eight hours later cell survival was analyzed. We confirmed in these same doxorubicin-treated groups that BCL-2 was upregulated after the first exposure (3.3 \times fold for 2 \times 10^{-7} mol/L doxorubicin; 4.9 \times fold for 10^{-6} mol/L). As shown in Fig 8, 8226 cells surviving the initial exposure to both concentrations of doxorubicin were relatively resistant to a second exposure.

Enhanced BCL-2 expression in transfected plasma cells induces resistance to doxorubicin. To further support the hypothesis that the previously described upregulation of BCL-2 expression might result in chemoresistance, IM-9 cells were transfected with a BCL-2-containing expression vector or control empty vector. Several transfected clones were isolated with variably enhanced BCL-2 expression. There was a good correlation between resistance to doxorubicin and enhanced expression. Figures 9 and 10 display...
Fig 8. Doxorubicin-treated cells with increased BCL-2 expression are resistant to a second exposure to doxorubicin. Myeloma 9226 cells were exposed to 0 (A), 2 x 10^{-7} mol/L (B), or 10^{-6} mol/L (C) doxorubicin for 2 hours. Cells were then washed, recultured in drug-free media for 48 hours, and then rechallenged with increasing concentrations of doxorubicin 10^{-9} - 5 x 10^{-8} mol/L for 2 hours. Forty-eight hours later survival was analyzed and survival curves were plotted.

Data obtained with one particular clone whose degree of enhanced expression (4.4-4.7 x fold over parent and neo-control transfected cells by densitometry, Fig 9) was comparable to that achieved when IM-9 cells are cultured with doxorubicin. The Western data (Fig 9) also show comparable expression amongst the clones of the other assayed proteins important in cell death, BAX and BCL-X-L. As shown in Fig 10, specific resistance to doxorubicin was present in these BCL-2-transfected cells when assayed at 48 and 72 hours. In addition, these overexpressing targets were relatively resistant after 24 hours of exposure to doxorubicin and after 24 to 72 hours exposure to etoposide (not shown). These data further support the hypothesis that enhanced expression of BCL-2 induced by initial exposure to doxorubicin might specifically result in chemoresistance.

DISCUSSION

These results indicate that doxorubicin, etoposide, and H_2O_2 can upregulate BCL-2 protein levels in multiple myeloma plasma cells. Increased expression was detected in 3 myeloma cell lines as well as a primary myeloma cell culture. Enhanced expression was relatively specific in that expression of several other proteins such as Ig light chains, BCL-X and actin, was unaffected. In addition, heightened expression was not a generalized nonspecific result of injury to these target cells because we have not detected alterations

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Fig 9. Western analysis of transfected IM-9 cells. Protein extracted from parent line (lane 1), neo-control transfected line (lane 2), or BCL-2-transfected clone (lane 3), electrophoresed and BCL-2, BAX, and BCL-X-L expression assessed by Western analysis as described in Materials and Methods.
in expression when the targets were injured by inducers of necrosis (secondary to azide exposure, not shown) or by the apoptosis inducers dexamethasone, serum deprivation, and anti-fas antibody (Table 1).

BCL-2 protects cells against apoptosis induced by many different stimuli including the ones we have used in this study. Maximal enhancement of BCL-2 expression was seen when concentrations of doxorubicin, VP-16 and H2O2 were used that were just below the threshold required for decreased target cell viability and apoptotic DNA cleavage. Further increases in drug concentrations, which induced an apoptotic death may have resulted in sufficiently adverse conditions in remaining viable cells so that they could not achieve BCL-2 upregulation. It is possible that, with the lower concentrations, apoptotic pathways were suboptimally triggered and these aborted pathways, in turn, activated a BCL-2 upregulation as a protective mechanism. Indeed, the survival of the targets following the initial exposure to the injurious agent, as well as the relative resistance of treated targets to a second exposure of doxorubicin is consistent with the protective effects of upregulated BCL-2 expression. In addition, induced enhanced expression of BCL-2 in transfected plasma cells resulted in resistance to doxorubicin and etoposide (Figs 9 and 10). However, we have no experimental evidence yet that the initial cell survival following exposure to doxorubicin or relative resistance to a second doxorubicin exposure is specifically due to the increase in BCL-2 protein levels.

Because BCL-2 is a survival factor, it was possible that the detected enhanced expression in treated targets could be due to a selective survival in culture of a small subset of cells whose baseline expression was higher before addition of doxorubicin. However, detection of upregulated BCL-2 RNA as early as 4 hours after exposure to 10^{-6} mol/L doxorubicin, at a time when viable cell recoveries were comparable to control groups, suggests that doxorubicin directly or indirectly enhances expression of BCL-2 in individual cells. True confirmation of this would require examination of targets over time at the single cell level rather than through population data like what we have obtained. It is also clear from the RT-PCR results that upregulated protein expression following exposure to 10^{-6} mol/L doxorubicin depends, at least in part, on an upregulation of BCL-2 mRNA levels. It is unclear to us why exposure to 2 \times 10^{-7} mol/L doxorubicin did not result in increased levels of BCL-2 transcripts. Corresponding enhanced protein expression in these latter groups was modest (only approximately 2.5 to 33 \times fold v control values) and the increased level of RNA may have been insufficient to detect. Alternatively, the modest enhancement of protein expression in these groups could have been solely due to posttranscriptional alterations or to the aforementioned selective survival in culture of a BCL-2 higher—expressing subpopulation.

Doxorubicin, etoposide and H2O2 all activate p53.19,20 In contrast, dexamethasone, serum deprivation, and anti-fas antibody, which did not upregulate BCL-2, injure cells through p53-independent mechanisms.21 This suggests the possibility that activated p53 links the pathways of potential injury to the increase in BCL-2 expression in our targets. Although activation of wild type p53, in fact, downregulates BCL-2 levels22,23 rather than enhances expression, the effects of mutated p53 may be variable. Clearly, some mutated forms of p53 can depress BCL-2 levels as shown in transfection studies.24 In addition, in a majority of breast cancer cell lines containing mutated p53, there is an inverse correlation between p53 and BCL-2 levels.24 However, of the few cell lines that overexpressed both p53 and BCL-2, one contained a mutated p53 identical to that of the 8226 cell line we have primarily used in this study (GAG to AAG at codon 285).25 We have not yet sequenced the p53 proteins of the other target cells in this investigation (although the freshly explanted myeloma culture is likely to express a mutated p53 as well since it was obtained from a patients with terminal disease and plasma cell leukemia)26 but it is intriguing to think that certain p53 mutations may facilitate or specifically induce BCL-2 upregulation on activation. In addition, alterations of the BCL-2 gene might influence the effect of p53. In this regard, it is interesting to note that wild type p53 actually upregulates BCL-2/CAT reporter constructs that do not contain a specific p53 negative response element.27

Other potential mediators of the detected upregulated expression of BCL-2 are reactive oxygen intermediates (ROIs). All three successful stimulators of BCL-2 expression (doxorubicin,24 etoposide,27 and H2O2) also induce the generation of ROIs and ROIs may result in gene activation.28 Some support for this hypothesis comes from a recent report by Hu et al29 where H2O2 slightly upregulated BCL-2 levels in acute myelogenous leukemia blasts by itself but even more impressively completely prevented the depression in BCL-2 induced by retinoic acid.

Whether or not the detected in vitro upregulation of BCL-2 induced by doxorubicin and VP-16 will be mirrored by clinical in vivo modulation in patients remains to be seen. To our knowledge, only studies in a small series of neuroblastoma patients to date30 suggests that tumor cells surviving cytotoxic therapy in vivo show enhanced BCL-2 expression. Similar studies in myeloma patients are currently underway. If these studies show enhanced expression of BCL-2 in relapsing patients versus newly diagnosed patients, the data would have potential broad implications for drug resistance in this disease.

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REFERENCES

3. Epstein J, Xiao H, Oba BK: P-glycoprotein expression in
plasma-cell myeloma is associated with resistance to VAD. Blood 74:913, 1999
Upregulated expression of BCL-2 in multiple myeloma cells induced by exposure to doxorubicin, etoposide, and hydrogen peroxide

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