Acquired Resistance to Rituximab Is Associated with Chemotherapy Resistance Resulting from Decreased Bax and Bak Expression

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Abstract

**Purpose:** Targeting malignant B cells using rituximab (anti-CD20) has improved the efficacy of chemotherapy regimens used to treat patients with non-Hodgkin’s lymphoma. Despite the promising clinical results obtained using rituximab, many patients relapse with therapy-resistant disease following rituximab-based treatments. We have created a cell line model of rituximab resistance using three B-cell non-Hodgkin’s lymphoma–derived cell lines (Raji, RL, and SUDHL-4). In an attempt to define strategies to overcome rituximab resistance, we sought to determine the chemotherapy sensitivity of our rituximab-resistant cell lines (RRCL).

**Experimental Design:** Parental, rituximab-sensitive cell lines (RSCL) Raji, RL, and SUDHL-4, along with RRCLs derived from them, were exposed to several chemotherapeutic agents with different mechanisms of action and the ability of these agents to induce apoptotic cell death was measured. Expression of multidomain Bcl-2 family proteins was studied as potential mediators of chemotherapy/rituximab resistance.

**Results:** We found that RRCLs are resistant to multiple chemotherapeutic agents and have significantly decreased expression of the Bcl-2 family proteins Bax, Bak, and Bcl-2. RRCLs do not undergo rituximab- or chemotherapy-induced apoptosis but die in a caspase-dependent manner when either wild-type Bax or Bak is exogenously expressed. Furthermore, forced expression of Bak sensitized RRCL to chemotherapy-induced apoptosis.

**Conclusions:** Whereas a single or limited exposure of lymphoma cells to rituximab may lead to a favorable ratio of proapoptotic to antiapoptotic Bcl-2 family proteins, repeated exposure to rituximab is associated with a therapy-resistant phenotype via modulation of Bax and Bak expression.

A hallmark of cancer is evasion of cell death, which is often achieved by aberrant expression of Bcl-2 family proteins in a manner that favors cell survival (1). Follicular non-Hodgkin’s lymphoma (NHL) was the first cancer in which disruption of the Bcl-2 family of proteins was found to play a significant role in the pathogenesis of malignant B cells (2). The ratio of antiapoptotic to multidomain proapoptotic Bcl-2 family proteins seems to be supremely important in determining cell fate and is prognostic in follicular B-cell NHL (3) and chronic lymphocytic leukemia (4). Additionally, the expression of Bax was found to be inversely correlated with overall prognosis and chemotherapy responsiveness when comparing subtypes of NHL (5). These data suggest that expression of Bcl-2 family proteins affects the overall survival of lymphoma patients by determining the sensitivity of lymphoma cells to therapy. Therefore, it seems probable that shifting the balance of antiapoptotic to proapoptotic Bcl-2 family members to favor survival would lead to the therapy-resistant phenotype commonly found in relapsed or refractory lymphoma.

NHLs encompass a heterogeneous group of malignancies with different pathophysiology, clinical behavior, and response to therapeutic intervention. Treatment strategies for NHL have evolved from initial use of external beam radiation therapy and alkylating agents into a combined approach using non–cross-resistant combination chemotherapy regimens, monoclonal antibodies, immunoconjugates, and novel small molecules targeting pathways involved in lymphomagenesis. Rituximab, a chimeric monoclonal antibody directed against surface CD20, has changed the paradigm for treatment of patients with B-cell lymphoma. As a single agent, rituximab has consistently provided clinical responses in ~50% of previously treated patients (6–10). In addition to being directly cytotoxic, rituximab can sensitize malignant B cells to chemotherapy. Phase II and III studies showed that addition of rituximab to systemic chemotherapy is associated with improvement in response rates, time to progression, and even overall survival when compared with chemotherapy alone (11–13). Additionally, several in vitro studies have shown that rituximab can sensitize cells to the action of chemotherapeutic agents by affecting cellular signaling in a manner that down-regulates Bcl-2 or Bcl-xL (14, 15).
Despite these promising data, re-treatment with single agent rituximab is associated with resistance in ~60% of patients who responded to their first rituximab therapy (8). Similarly, we found that repeated exposure of three distinct B-cell lymphoma cell lines (Raji, RL, and SUDDL-4) to rituximab resulted in significant resistance to rituximab-induced cell death (16). Here we report that three rituximab-resistant cell lines (RRCL) derived from two distinct rituximab-sensitive cell lines (RSCL; Raji and RL, are also markedly resistant to a panel of chemotherapeutic agents and to direct killing by rituximab due to a block in the initiation of apoptosis. This apoptotic block seems to be the result of decreased expression of Bax and Bak in each RRCL. Reduced Bax and Bak expression correlated with resistance to chemotherapy and direct apoptosis induced by rituximab but not with rituximab-associated complement-dependent cytolysis or antibody-dependent cellular cytotoxicity. Restoration of Bax or Bak expression resulted in rapid cell death of most transfected RRCLs. Additionally, RRCLs that survived forced expression of Bak were resistant to chemotherapy-induced apoptosis. These data indicate that repeated exposure of malignant B cells to rituximab resulted in reduced expression of Bax and Bak, which contributes to their rituximab/chemotherapy-resistant phenotype by blocking initiation of apoptosis.

**Materials and Methods**

**Cell lines.** All cell lines are maintained in RPMI 1640 (Sigma Chemical) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals), 5 mmol/L HEPES, 100 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen). RSCLs Raji and RL were purchased from American Type Culture Collection. RRCLs were created from each cell line by repeated 24-h exposure to increasing concentrations of rituximab (0.1-128 μg/mL) in the absence (2R lines) or presence (4RH lines) of human serum as a source of complement (1:1.000-1:1.875). RSCLs and RRCLs were further cloned by limiting dilution.

**Antibodies and reagents.** Antibodies to Bax (2D2), Bak (N-20), Beclin-1 (S-18), and Mcl-1 (S-19) were obtained from Santa Cruz Biotechnology; poly(ADP-ribose) polymerase (PARP)-1 from Axxora; and Moesin (Ab-1) from Lab Vision. Propidium iodide (PI), 3,3′-dihexyloxy-acarbocyanine (DiOC6), and poly(ADP-ribose) polymerase (PARP)-1 from Invitrogen. **Cell death and apoptosis.** Cells were incubated at a concentration of 0.5 × 10⁶/ml in complete media containing cisplatin, etoposide, vincristine, Adriamycin, paclitaxel, gemcitabine, or thapsigargin (see figure legends for specific doses). At 24-, 48-, and 72-h time points, aliquots were removed and stained with FITC-conjugated Annexin V (Invitrogen) and 5 μg/mL PI- or allophycocyanin-conjugated Annexin V (Invitrogen) and 10 nmol/L SYTOX Green (when using Adriamycin) in Annexin binding buffer (10 mmol/L HEPES, 150 mmol/L potassium chloride, 1 mmol/L magnesium chloride, 1.3 mmol/L calcium chloride, 1 mg/mL glucose, 0.5% bovine serum albumin, pH 7.4). Following staining, 10,000 events were collected on a FACScan or FACScalibur (Becton Dickinson). Data were analyzed using WinList software (Verity Software House) and percentages compared using paired t tests in SPSS 14.0 software (SPSS, Inc.).

**Complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity measurement by ⁴¹Ca-release assay.** Standard ⁴¹Ca-release assays were done as previously described (16) to determine the ability of rituximab to induce complement-dependent cytotoxicity and antibody-mediated cellular cytotoxicity of Raji, Raji 4RH, and clones derived from each.
eGFP-negative cells were sorted using a FACS Aria cytometer (Becton Dickinson) and treated overnight with 100 μmol/L cisplatin. Caspase activity in treated and untreated cells was then assessed using the Caspase-Glo 3/7 Assay (Promega). Relative luminometric units were measured using an Ascent Fluoroskan FL. Relative luminometric unit values were normalized to vector-transfected cells and fold cisplatin-induced caspase induction was calculated from normalized values.

Results

**RRCLs are resistant to chemotherapy-induced apoptosis.** We have previously shown that repeated exposure of B-cell lymphoma cell lines Raji, RL, and SUDHL-4 to rituximab induced a rituximab-resistant phenotype (Table 1; ref. 16). Parental SUDHL-4 cells were markedly resistant to chemotherapy when compared with Raji or RL (data not shown), making fair comparison of chemotherapy sensitivity between RRCLs derived from SUDHL-4 and Raji or RL impossible. Surprisingly, RRCLs derived from the parental cell lines Raji or RL displayed significant resistance to multiple chemotherapeutic agents with distinct mechanisms of action (Fig. 1). PI or SYTOX Green staining of chemotherapy-treated cells revealed that 73% to 97% (P < 0.05) of RRCL remained viable at doses of multiple chemotherapeutic agents that induced ~50% cell death in Raji or RL (Fig. 1A and B). RSCls Raji and RL were found to die in a time- and dose-dependent manner, whereas RRCLs were resistant to up to several log fold increases of chemotherapy and were not found to undergo a significant decrease in viability over 72 h of treatment with chemotherapy (Supplementary Fig. S1).

To determine at what point in the chemotherapy-induced apoptotic pathway RRCLs are defective, we examined several defining events of apoptosis. We found that Annexin V bound to the surface of viable (PI−) chemotherapy-treated RSCl but not RRCL, 24 h after incubation (Fig. 1C), consistent with induction of early apoptosis in RSCl. Concurrently, "executor" caspases (i.e., caspase-3 and/or caspase-7) were activated in chemotherapy-treated RSCl, but not RRCL (Fig. 1D). Chemotherapy-induced caspase-3/7 activity along with cell death of RSCl was blocked by coincubation with the pan-caspase inhibitor zVAD-fmk, suggesting that chemotherapy induced apoptotic cell death of RSCl (Fig. 1D). During apoptosis, many proteins, including PARP and Mcl-1, are cleaved and/or degraded in a caspase-dependent or caspase-independent manner. PARP is cleaved by caspase-3/7 (19) whereas Mcl-1 is degraded by the proteasome in a caspase-independent manner (20). Following chemotherapy treatment, RSCl, but not RRCL, exhibited PARP cleavage and Mcl-1 degradation (Fig. 1E). Despite marked differences in chemotherapy-induced apoptosis, RSCl and RRCL exhibited nearly complete chemotherapy-induced growth arrest at doses used (Supplementary Fig. S2) and expressed similar levels of functional multidrug resistance protein pumps (data not shown). Taken together, these data suggest that resistance to chemotherapy-induced cell death in RRCL is the result of a defect early in the apoptotic cascade.

**RRCLs maintain their mitochondrial membrane potential (ΔΨm) following chemotherapy treatment.** On induction of apoptosis by chemotherapeutic agents, the proapoptotic Bcl-2 family proteins Bax and/or Bak oligomerize on the outer mitochondrial membrane, causing loss of ΔΨm with subsequent collapse of electron transport and release of mitochondrial proteins into the cytoplasm (21). At low concentrations, the cell-permeable dye DiOC6 accumulates in mitochondria with intact ΔΨm and fluoresces bright green. Mitochondria with compromised ΔΨm cannot efficiently take up DiOC6, leading to a reduction in green fluorescence (DiOC6(Dim)). Following chemotherapy treatment, we found significant loss of DiOC6 fluorescence (ΔΨm) in RSCl but not RRCL (Fig. 2A). In accord with these results, we found that reduction of Alamar Blue, a substrate of several reducing enzymes involved in mitochondrial electron transport, was maintained to a greater degree in RRCL than in RSCl following chemotherapy exposure (Fig. 2B). These data suggest that mitochondria in RRCL do not respond normally to apoptotic stimuli and implicate alterations in mitochondria or mitochondria-associated proteins in the resistance of RRCL to chemotherapy-induced apoptosis.

**Diminished expression of Bax and Bak in RRCL.** The Bcl-2 family of proteins is known to play an essential role in the control of apoptosis following chemotherapy exposure. For example, murine embryonic fibroblasts deficient in both Bax and Bak are resistant to apoptotic death induced by chemotherapy, growth factor withdrawal or tBid (22). Given the resistant phenotype and intact mitochondrial potential observed in chemotherapy-treated RRCL, we compared the expression of a panel of Bcl-2 family proteins between RSCl and the RRCL derived from them. Expression of the proapoptotic proteins Bax and Bak and the antiapoptotic protein Bcl-2 were markedly reduced in RRCL compared with parental RSCl, whereas expression of Bcl-xl and Mcl-1 was increased (Fig. 3A). Quantification of Western blots showed that Bax expression was reduced to an undetectable level whereas Bak expression was reduced 9.7-, 7.6-, and 8.2-fold in Raji 2R, Raji 4RH, and RL 4RH cell lines, respectively (Fig. 3B). Additionally, physiologically relevant ratios of proapoptotic to antiapoptotic Bcl-2 family proteins were significantly decreased in RRCL compared with RSCl (Fig. 3C), mainly due to decreased expression of Bax and Bak. Based on these observations, we hypothesize that decreased Bax and Bak expression in RRCL led to an apoptosis-resistant, and therefore chemotherapy-resistant, phenotype.
Interestingly, quantitative real-time PCR revealed no significant differences in mRNA coding for Bax and Bak when comparing parental Raji or RL cells with RRCLs derived from them (Fig. 3D). Sequencing of cDNAs derived from RRCL revealed that the coding region of Bak remained wild type in all RRCL whereas each RRCL contained a deletion of one deoxyguanosine residue within the (G)₈ tract of Bax (data not shown). These frameshift mutations within Bax led to insertion of a premature stop codon and abrogation of a mature Bax protein product in RRCL. The mechanism underlying decreased Bak expression in RRCL remains unclear yet must occur at a posttranscriptional level because relative BAK1 mRNA expression is similar in RRCL and RSCL.

**Decreased Bax and Bak in RRCL is associated with apoptosis resistance.** Whereas it seemed likely that decreased expression of Bax and Bak proteins contributed to chemotherapy resistance in RRCL, the contribution of these proteins to rituximab resistance remained unclear. Clones derived from Raji RSCL and Raji 4RH RRCL displayed Bcl-2 family expression profiles similar to those of the cell lines from which they were isolated (Fig. 4A). Additionally, clones derived from Raji were sensitive to chemotherapy whereas those derived from Raji 4RH were

![Graphs](image-url)

**Fig. 1.** RRCLs Raji 2R, Raji 4RH, and RL 4RH are resistant to chemotherapy-induced apoptosis. Parental Raji (A) and RL (B) B-cell NHL cell lines were found to contain a significantly (*, *P < 0.05*) higher percentage of dead cells (PI + or SYTOX Green +) than RRCLs derived from them following 72-h exposure to 100 μmol/L cisplatin, 16 μmol/L Adriamycin, 100 μmol/L etoposide, 50 μmol/L gemcitabine, 1 μmol/L paclitaxel, 50 μmol/L vincristine, or 1 μmol/L thapsigargin (approximate LD₅₀ doses for parental cells). Columns, average of at least three independent experiments; bars, SD. C. RRCLs (Raji 2R or 4RH) do not initiate apoptosis as measured by surface phosphatidylserine exposure (Annexin V +) following 24-h incubation with cisplatin, vincristine, or any other agent used in these studies (data not shown). Data from one representative experiment (n = 3). D. Raji 2R or 4RH RRCLs fail to activate caspase-3 and/or caspase-7 following cisplatin exposure (24 h; *, *P < 0.01). The percentage of dead cells (PI +) at the conclusion of the experiment (72 h) is provided below the appropriate data groups. Note that treatment with cisplatin (100 μmol/L) in the presence of zVAD-fmk (40 μmol/L) did not induce caspase activity or appreciable cell death in Raji cells, indicating that the majority of cisplatin-induced cell death is caspase dependent. Data from one representative experiment (n = 3) done in triplicate. E. PARP cleavage and Mcl-1 degradation were readily observed in Raji cells but not in RRCL treated with vincristine (50 μmol/L) over a 48-h time course.
resistant to chemotherapy (Fig. 4B). Although all cells tested (RSCL, RRCL, and clones) were CD20 positive, rituximab was capable of inducing relatively little (<25%) direct cell death in any cell line or clone tested. Consistent with the hypothesis that RRCLs are resistant to apoptosis, direct killing by rituximab was consistently greater in Raji and Raji clones when compared with Raji 4RH and Raji 4RH clones (Fig. 4C). These data suggest that direct apoptotic pathways induced by chemotherapy or rituximab are blocked in RRCL, likely due to decreased expression of Bax and Bak.

In addition to direct apoptosis, rituximab is capable of inducing complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity of CD20-positive cells. Using our Raji RSCL clones and Raji 4RH RRCL clones, we found that expression of Bax and Bak did not correlate with the ability of rituximab to induce complement-dependent cytotoxicity or antibody-dependent cellular cytotoxicity (Fig. 4). Clone 11 derived from Raji contained relatively high levels of Bax and Bak but was resistant to complement-dependent cytotoxicity, whereas clone 7 derived from Raji 4RH contained relatively low levels of Bax and Bak and was sensitive to complement-dependent cytotoxicity (Fig. 4D). The ability of rituximab to induce antibody-dependent cellular cytotoxicity was similar when comparing Raji and Raji clones containing relatively high levels of Bax and Bak with Raji 4RH and Raji 4RH clones containing relatively low levels of Bax and Bak (Fig. 4E). These data suggest that Bax and Bak do not play a role in resistance to rituximab-associated complement-dependent cytotoxicity or...
antibody-dependent cellular cytotoxicity, likely because these two mechanisms of cell death do not rely on mitochondrial perturbation and downstream activation of the apoptotic cascade.

**Transfection of Bax or Bak into RRCL promotes apoptosis.** To determine if the reduced level of Bax and/or Bak expression contributed to the apoptosis-resistant phenotype of RRCL, constructs containing the coding region of BAXx or BAK1 followed by an internal ribosomal entry site (IRES) and a gene cassette encoding enhanced green fluorescence protein (eGFP) were transfected into RRCL. Twenty-four hours following transfection, cells were stained with PI and analyzed by flow cytometry. Transfection efficiency was between 40% and 60% in all experiments (data not shown). A significant increase in PI+ dead cells was observed in RRCL transfected with either BAXx or BAK1 compared with empty vector in the absence of any added apoptotic stimuli (e.g., chemotherapy; [Fig. 5A]). Curiously, the PI+ dead cells were predominantly eGFP negative and Bax expression was nearly undetectable by Western blot in BAXx-transfected cells despite the observation that these cells displayed significant PARP cleavage and Mcl-1 degradation (Fig. 5B). This suggested several possibilities. First, the forced expression of Bax or Bak promoted rapid onset of cell death accompanied by shutdown of protein synthesis, loss of plasma membrane integrity, and associated leakage of soluble eGFP from transfected cells. Second, the transfection process itself was lethal to the cells, resulting in a high percentage of cell death in the absence of protein expression. To distinguish

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**Fig. 3.** Altered expression of multiple Bcl-2 family proteins in RRCL. Western blot analysis of total cell lysates from Raji or RL cells versus Raji-derived or RL-derived RRCL revealed dramatic decreases in Bax, Bak, and Bcl-2 expression and slight increases in Bcl-xL and Mcl-1 expression (A). Quantification of fluorescent signal produced by chemiluminescent detection of Western blots from three independent experiments showed the degree of down-regulation of Bax, Bak, and Bcl-2 in RRCL when compared with RSCL (B; P<0.05). Physiologically relevant proapoptotic to antiapoptotic Bcl-2 family ratios were determined and represented graphically with each point representing data from one experiment and lines representing the average ratio for RSCL or RRCL (C). In all cases, RRCL were found to have significantly lower proapoptotic to antiapoptotic Bcl-2 family protein ratios than RSCL. Quantitative real-time PCR analysis of BAX or BAK1 expression revealed that no significant difference (12- or 0.5-fold compared with Raji) between all cell lines tested (D). Fold expression was calculated from quadruplicate samples using the \(\Delta \Delta C_t\) method with expression in Raji cells used as a baseline. Subsequent analysis of the coding region of BAX revealed that all RRCLs contained frameshift mutations in their (G)₈ tract (data not shown).
between these possibilities, we incubated transfected cells in media containing the pan-caspase inhibitor zVAD-fmk or transfected cells with inactive mutant forms (mBH3) of BAX (17) or BAK1 (18). Inhibition of apoptosis by zVAD-fmk or transfection with the BAX mBH3 or BAK1 mBH3 constructs led to an obvious increase in eGFP expression in the absence of any apparent cell death as assessed by PI staining (Fig. 5C). Bax or Bak expression was readily detected in transfected RRCL incubated in the presence of zVAD-fmk or RRCL transfected with BAX mBH3 or BAK1 mBH3 (Fig. 5D). Additionally, PARP cleavage and Mcl-1 degradation were not observed in mutant transfectants or wild-type transfectants incubated with zVAD-fmk or transfection with the BAX mBH3 or BAK1 mBH3 constructs. Direct killing by rituximab was relatively low in all cell lines but consistently higher in Raji and RRCL clones compared with Raji 4RH and Raji 4RH clones (C). Columns, average percent of dead cells following 72-h incubation with 10 mg/mL isotype (Herceptin) or rituximab (n = 3); bars, SD. **, P < 0.05, compared with isotype-treated cells. The ability of rituximab to induce complement-dependent cytotoxicity (D) or antibody-dependent cellular cytotoxicity (E) did not correlate with Bax or Bak expression in clones. Columns, average specific lysis measured by 51Cr release following 6-h incubation with 10 mg/mL isotype (Herceptin) or rituximab in the presence of 25% human serum or human peripheral blood mononuclear cells (PBMC) at 40:1 (n = 3); bars, SD. **, P < 0.05, compared with isotype-treated cells.

examined the ability of cisplatin to induce apoptosis in eGFP+, Bak-transfected Raji 2R RRCL. We were able to take advantage of a small population of eGFP+/PI- Raji 2R cells detectable 24 h following transfection with vector, BAK1, or BAK1 mBH3. A small number of cells (20,000-100,000), sufficient for measurement of caspase activity, were sorted by flow cytometry into Bakhigh/eGFP+ (+) and Baklow/eGFP- (-) populations (Fig. 6A). Western blot analysis confirmed that BAK1-transfected eGFP+ cells contained more Bak protein than eGFP- cells (Fig. 6B), although addition of zVAD-fmk was necessary to obtain enough viable eGFP+ cells to collect cell lysates. Importantly, when Bakhigh/eGFP+ (+) and Baklow/eGFP- (-) RRCL populations were exposed overnight to cisplatin only, Bakhigh/eGFP+ (+) cells transfected with wild-type Bak displayed marked caspase activity (Fig. 6C). It therefore seems that wild-type Bak (and likely Bax) expression in RRCL controls sensitivity to chemotherapy-induced apoptosis and that the significant reduction of Bax and Bak in RRCLs led to their resistance to apoptotic stimuli including chemotherapy and rituximab.
Discussion

Development of therapy resistance continues to be a major obstacle in the treatment of NHL. The majority of NHL patients who respond to standard chemotherapy or rituximab-based treatment regimens eventually relapse, usually with subsequent therapy-resistant disease. Using a cell line model, we show here that resistance to chemotherapy and direct killing by rituximab can result from decreased expression of Bax and Bak following repeated exposure of cells to rituximab. Clinically, expression of Bax and/or Bak protein has not been convincingly correlated with survival in aggressive diffuse large cell lymphoma (23, 24). In indolent lymphoma, however, lower levels of Bax expression seem to be inversely correlated to inherent chemosensitivity when comparing across several lymphoma subtypes (5). More comprehensive studies examining several potential prognostic factors in follicular lymphoma and chronic lymphocytic leukemia have shown positive correlations between survival and the ratio of proapoptotic to antiapoptotic proteins. Specifically, a low ratio of Bak (or Bax) to Bcl-2 was associated with increased survival of several years in patients with follicular lymphoma (3). Additionally, a low ratio of Bax to Mcl-1 was associated with resistance to rituximab therapy in chronic lymphocytic leukemia patients (3, 4). These data highlight the potential contribution of the ratio of proapoptotic to antiapoptotic Bcl-2 family proteins to the fate of lymphoma cells, especially in the context of ongoing therapeutic intervention.

Programmed cell death is essential for the development and maintenance of the mammalian immune system (25). Like many developmentally regulated processes, cancer cells often hijack elements involved in the control of programmed cell death to promote their own survival in a hostile host environment. Bcl-2 was the first protein involved in regulating programmed cell death determined to be an oncogene (2, 26). Several proteins, including Bax and Bak, which share a Bcl-2 homology 3 (BH3) domain, have since been described as both positive and negative regulators of programmed cell death (27). These Bcl-2 family proteins can be divided into multidomain antiapoptotic (Bcl-2, Bcl-xL, Mcl-1, etc.) or...
proapoptotic (Bax and Bak) and “BH3-only” proteins (Bad, Bid, Bim, NOXA, etc.). The multidomain proapoptotic Bcl-2 family proteins Bax and Bak are essential for the execution of programmed cell death (22). On activation of pro-death signals, Bax and Bak undergo structural changes that facilitate their oligomerization on the mitochondrial outer membrane (28, 29). Oligomerization of Bax and/or Bak leads to loss of mitochondrial membrane potential and release of mitochondrial proteins into the cytosol. Released mitochondrial proteins trigger programmed cell death through caspase-dependent or caspase-independent mechanisms (30).

In our cell line model of rituximab resistance, we found significant multiagent chemotherapy resistance that resulted from a block early in apoptosis. This defect in the ability of RRCL to undergo apoptosis resulted from significant reduction in the expression of the multidomain proapoptotic Bcl-2 family proteins Bax and Bak. These cell line data show that the balance between proapoptotic and antiapoptotic Bcl-2 family proteins is critical in determining the response of NHL cells to apoptotic stimuli and, therefore, their sensitivity to multiple therapeutic modalities. Interestingly, creation of a similar RRCL model that shares many similarities to our RRCLs was published during the preparation of this manuscript. The rituximab-resistant clones described by Jazirehi et al. (31) showed chemotherapy resistance associated with alteration of the ratio of proapoptotic to antiapoptotic Bcl-2 family proteins, specifically increased expression of Bcl-2, Bcl-xL, and Mcl-1. Similarly, our RRCLs show increased Bcl-xL and Mcl-1 expression. In contrast, our RRCLs have a dramatic reduction in Bax, Bak, and Bcl-2 protein levels along with a greater degree of chemotherapy resistance. These differences may be due to inherent differences in the parental cell lines used or methods used for creation and maintenance of rituximab-resistant cells used. Regardless of the differences between the rituximab-resistant clones used by Jazirehi et al. (31) and our RRCLs, the induction of chemotherapy resistance through alteration in the expression of Bcl-2 family proteins clearly shows the importance of this family of proteins in acquired resistance to chemotherapy following prolonged exposure of NHL cells to rituximab.

In contrast to the cell line model described by Jazirehi et al. (31), the major defect underlying chemotherapy resistance in our RRCL seems to be decreased expression of Bax and Bak. Wei et al. (22) revealed the requirement for functional redundancy of Bax and Bak in the apoptotic process using a mouse embryonic fibroblast knockout model. Further evidence suggests that the proapoptotic activity of Bax and Bak is kept in check by different mechanisms (32). Whereas the mechanism of regulation remains unclear, it is well established that Bcl-2 can prevent oligomerization of Bax on the mitochondrial membrane (33). Mitochondria-resident Bak, however, interacts with Bcl-xL and Mcl-1 but not Bcl-2 (32). Based on these observations, we determined the ratios of Bax to Bcl-2 and of Bak to Bcl-xL or Mcl-1 and found that these ratios were significantly higher in RSCL than in RRCL (Fig. 3C), suggesting that physiologically relevant Bcl-2 family interactions favor cell survival in RRCL.

Transfection of BAX or BAK1 into RRCL increased the Bax to Bcl-2 or Bak to Bcl-xL/Mcl-1 ratio, respectively (data not shown), and led to spontaneous apoptosis (Fig. 4). However, Bax expression in BAXz-transfected RRCL remained nearly undetectable by Western blot, most likely due to rapid induction of caspase-dependent apoptosis in successfully transfected cells (Fig. 4D). Because RRCLs express relatively low levels of Bcl-2, these data are consistent with a model where the proapoptotic function of Bax is kept in check by Bcl-2.

More robust expression of Bak was detected in BAK1-transfected RRCL, which contained elevated levels of both Bcl-xL and Mcl-1. Flow cytometric analysis of BAK1 transfectants showed an increased percentage of eGFP+, viable (PI-) cells when compared with BAXz transfectants. The intensity of eGFP expressed by BAK1 transfectants was significantly lower than that by BAXz or BAXz transfectants in which apoptosis was blocked by caspase inhibition or mutation of the Bax or Bak BH3 domain. These data suggest that cells expressing a low
level of the BAK1-ires-EGFP construct remain viable and are consistent with data suggesting that the proapoptotic function of Bak is counterbalanced by expression of Bcl-xL and Mcl-1.

The small population of eGFP+ viable cells obtained 24 h following transfection with BAK1-ires-EGFP allowed us to test whether increased Bak expression could sensitize RRLC to chemotherapy. Using a sensitive luminometric caspase-3/7 activity assay, we were able to determine that Bak\textsubscript{high}/eGFP+ RRLC were in fact more sensitive to cisplatin than Bak\textsubscript{low}/eGFP RRLC (Fig. 5C). Cisplatin-treated, Bak\textsubscript{high}/eGFP+ RRLCs transfected with BH3-mutant Bak were unable to induce caspase activity to the same extent as those transfected with wild-type Bak, suggesting that expression of functional Bak was necessary for cisplatin-induced caspase activity in RRLC. These data show that the level of expression of Bak in RRLC determines their sensitivity to chemotherapy-induced apoptosis and further implicate the decreased expression of proapoptotic Bcl-2 family proteins in the chemotherapy-resistant phenotype found in RRLC.

It remains unclear how repeated exposure of Raji or RL cells to rituximab led to dramatic decreases in expression of both Bax and Bak. The relatively small differences in the amount of BAX or BAK1 mRNA present in RSCCL and RRLC cannot account for the dramatic disparity in Bax and Bak protein expression observed when comparing RSCCL with RRCL (Fig. 3). Frameshift mutations in the tract of eight deoxyguanosines ([G]\textsubscript{8}) within BAX were found in all RRCL derived from Raji and RL RSCCL. Mutations in this region of BAX, known as the (G)\textsubscript{8} tract, are associated with microsatellite instability, resistance to apoptosis, and tumor progression (34, 35). Clinically, mutations in the (G)\textsubscript{8} tract of BAX are commonly seen in colon cancers with the microsatellite mutator phenotype (36). Whereas microsatellite instability and BAX mutations are commonly found in lymphoma and leukemia cell lines (37), BAX mutations were rarely found in primary NHL (38–40). This is likely explained by the low frequency of microsatellite instability in primary B-cell NHL and B-cell NHL following clinical progression (41). Unfortunately, clinical study of the effect of therapy on microsatellite instability in B-cell NHL occurred before Food and Drug Administration approval of rituximab (41). It therefore remains possible that rituximab exposure may be associated with microsatellite instability, which may account for mutations in the (G)\textsubscript{8} tract of BAX observed in RRCL.

In summary, our data suggest that repeated exposure of B-cell NHL cells to rituximab can lead to the development of rituximab and chemotherapy resistance. A mechanism underlying the development of multigagent apoptosis resistance in RRCL was found to be a dramatic alteration in the balance between the proapoptotic and antiapoptotic Bcl-2 family proteins as a result of decreased expression of both Bax and Bak. Our data illustrate the importance of the balance between proapoptotic and antiapoptotic Bcl-2 family proteins in determining the apoptotic, and therefore therapeutic, sensitivity of NHL cells and suggest that there may be additional links between rituximab-induced CD20 signaling and the expression of various Bcl-2 family proteins. Additionally, our RRLCs represent an excellent model for studying regulation of the expression of Bcl-2 family proteins in B-cell lymphoma cells and for identifying novel agents/therapies to resensitize cells to rituximab and/or chemotherapy, such as those targeting the Bcl-2 family of proteins.

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