Inhibition of the Raf–MEK1/2–ERK1/2 Signaling Pathway, Bcl-\textsubscript{xl} Down-Regulation, and Chemosensitization of Non-Hodgkin’s Lymphoma B Cells by Rituximab

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ABSTRACT

Rituximab (Rituxan, IDEC-C2B8) has been shown to sensitize non-Hodgkin’s lymphoma (NHL) cell lines to chemotherapeutic drug-induced apoptosis. Rituximab treatment of Bcl-2–deficient Ramos cells and Bcl-2–expressing Daudi cells selectively decreases Bcl-\textsubscript{xl} expression and sensitizes the cells to paclitaxel-induced apoptosis. This study delineates the signaling pathway involved in rituximab-mediated Bcl-\textsubscript{xl} down-regulation in Ramos and Daudi NHL B cells. We hypothesized that rituximab may interfere with the extracellular signal-regulated kinase (ERK) 1/2 pathway, leading to decreased Bcl-\textsubscript{xl} expression. Rituximab (20 \textmu g/mL) inhibited the kinase activity of mitogen-activated protein kinase kinase (MEK) 1/2 and reduced the phosphorylation of the components of the ERK1/2 pathway (Raf-1, MEK1/2, and ERK1/2) and decreased activator protein-1 DNA binding activity and Bcl-\textsubscript{xl} gene expression. These events occurred with similar kinetics and were observed 3 to 6 hours after rituximab treatment. Rituximab-mediated effects were corroborated by using specific inhibitors of the ERK1/2 pathway, which also reduced Bcl-\textsubscript{xl} levels and sensitized the NHL B cells to paclitaxel-induced apoptosis. Previous findings implicated a negative regulatory role of the Raf-1 kinase inhibitor protein (RKIP) on the ERK1/2 pathway. Rituximab treatment of NHL B cells significantly up-regulated RKIP expression, thus interrupting the ERK1/2 signaling pathway through the physical association between Raf-1 and RKIP, which was concomitant with Bcl-\textsubscript{xl} down-regulation. These novel findings reveal a signaling pathway triggered by rituximab, whereby rituximab-mediated up-regulation of RKIP adversely regulates the activity of the ERK1/2 pathway, Bcl-\textsubscript{xl} expression, and subsequent chemosensitization of drug-refractory NHL B cells. The significance of these findings is discussed.

INTRODUCTION

The B-cell lineage restricted marker CD20 is expressed on mature B cells with minimal expression on early pre-B cells and normal plasma cells. Approximately 80% to 85% of non-Hodgkin’s lymphomas (NHLs) are B-cell malignancies in origin, and >95% of these express surface CD20. CD20 is neither shed from the cell surface (1) nor modulated or internalized on antibody (Ab) binding (2), which makes it a suitable target for immunotherapy. The chimeric mouse antihuman CD20 monoclonal Ab (mAb) rituximab [Rituxan, IDEC-C2B8 (3)] has significant antitumor activity and, alone or in combination with chemotherapy, has been successfully used in the treatment of patients with follicular or low-grade lymphoma (4) and aggressive diffuse large B-cell lymphoma in elderly patients (5). Treatment of CD20\textsuperscript{+} NHL B cells with rituximab triggers multiple cell-damaging mechanisms. Possible antitumor mechanisms involve Ab-dependent cellular cytotoxicity, complement-dependent cytotoxicity, and induction of apoptosis (6).

The in vivo effectiveness of the combination of rituximab and drugs in the treatment of drug-resistant tumors suggests that rituximab can modify the drug-resistant phenotype by interfering with signaling pathways. In fact, we have reported that rituximab interferes with the intracellular signal transduction pathways and sensitizes NHL B-cell lines to drugs via selective down-regulation of transcription of the antiapoptotic gene products Bcl-2 [in AIDS-related NHL (ARL); ref. 7] or Bcl-\textsubscript{xl} (in non-ARL; ref. 8). These proteins exert their protective effects mainly in the membrane of mitochondria, where they prevent loss of membrane potential, cytochrome c efflux on apoptotic stimuli, and the initiation of apoptosis (9). Bcl-2 and the long alternatively spliced variant of the Bcl-x gene, Bcl-\textsubscript{xl}, are predominantly expressed in lymphomas (10) and protect the cells from apoptosis induced by drugs, thus conferring a multidrug-resistant phenotype (9, 11, 12).

Activation of the extracellular signal-regulated kinase (ERK) 1/2 pathway by fibroblast growth factor-2 rescues small-cell lung carcinoma cells from apoptosis induced by etoposide, via up-regulation of Bcl-2 and Bcl-\textsubscript{xl} (13). Thus, we hypothesized that rituximab may inhibit the constitutive activity of the ERK1/2 pathway, leading to inhibition of Bcl-\textsubscript{xl} transcription. A negative regulatory role for the Raf-1 kinase inhibitor protein (RKIP) on the ERK1/2 pathway is described (14, 15). RKIP exerts its suppressive effects mainly in association with Raf-1, thus rendering it incapable of relaying the signal to downstream molecules. Overexpression of RKIP significantly reduces the transformation efficiency of Raf-1 kinase domain BXB and decreases activator protein (AP)–1–dependent transcription (14, 15). Thus, we further hypothesized that rituximab may up-regulate RKIP expression, resulting in inhibition of the ERK1/2 pathway, diminished Bcl-\textsubscript{xl} expression, and chemosensitization of NHL B cells.

This study tested both of the above hypotheses using the Bcl-2\textsuperscript{−}/Bcl-\textsubscript{xl}\textsuperscript{−} Ramos and the Bcl-2\textsuperscript{−}/Bcl-\textsubscript{xl}\textsuperscript{−} Daudi NHL B-cell lines, and the following questions were investigated: (a) whether rituximab inhibits the ERK1/2 pathway; (b) whether rituximab decreases AP-1 DNA binding activity; (c) whether pharmacological interruption of the ERK1/2 pathway mimics rituximab-mediated effects such as inhibition of Bcl-\textsubscript{xl} expression, chemosensitization, and inhibition of proliferation; and (d) whether rituximab up-regulates RKIP expression and potentiates its association with Raf-1, thus interfering with the activity of the ERK 1/2 signaling pathway.

MATERIALS AND METHODS

Tumor Cell Lines

The CD20\textsuperscript{+} human B-cell lines Daudi and Ramos (American Type Culture Collection, Manassas, VA) were maintained in sterile 75-cm\textsuperscript{2} tissue culture flasks in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (to ensure the absence of complement) as described previously (7, 8). The pEBB-puro-Bcl-x-HA construct was generated by polymerase chain reaction (PCR) cloning of human Bcl-x, which was then inserted into the BamHI and NotI sites of pEBB-puro-HA in-frame with the 3′ influenza

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hemagglutinin (HA) tag (16). The cells were then pulsed using electroporation.

Fig. 1. Rituximab-mediated effects, alone and in combination with paclitaxel, on the NHL B-cell lines. A, Ramos and Daudi cells were either left untreated (control) or pretreated with rituximab (20 \( \mu \)g/mL, 24 hours). Thereafter, the cells were washed, fresh medium was added, and the cells were incubated with various concentrations of paclitaxel (0.1, 1, and 10 \( \mu \)mol/L, 18 hours; ref. 8). The cells were then stained with PI solution, and cell cycle analysis was assessed by flow cytometry. The percentage of apoptosis is represented as the percentage of tumor cells with hypodiploid DNA accumulating at the sub-G\(_0\) phase of the cell cycle. An aliquot of the same samples was stained with anti-active caspase-3 mAb to validate the PI procedure (24). B, Synergy in apoptosis is achieved by the combination of rituximab and paclitaxel as determined by isobolographic analysis (26). C, examination of a panel of pro- and antiapoptotic proteins after exposure to rituximab. Tumor cells were either left untreated (control) or treated with rituximab (20 \( \mu \)g/mL, 24 hours), and total cell lysates (40 \( \mu \)g) were subjected to immunoblot analysis. D, Tumor cells were left untreated or treated with rituximab (20 \( \mu \)g/mL, 24 hours) or 2MAM-A3 (15 and 20 \( \mu \)g/mL, 7118
at 250 V/975 μF and then selected and maintained in 2.5 μg/mL puromycin. This cell line was provided by Dr. G. Cheng (University of California at Los Angeles, Los Angeles, CA). Tumor cell cultures were incubated in a controlled atmosphere incubator at 37°C with saturated humidity and an atmosphere of 95% air and 5% CO₂ at a density of 0.5 × 10⁶ cells/mL.

**Reagents**

A stock solution of paclitaxel [6 mg/mL in dimethyl sulfoxide; Bristol Myers Squibb (New York, NY)] was kept at room temperature. For each experiment, paclitaxel was diluted with medium to obtain the indicated concentrations. The dimethyl sulfoxide concentration did not exceed 0.1% in any experiment. Rituximab (stock, 10 mg/mL) was obtained commercially.

Mouse anti-Bcl-xL, anti-Mcl-1, anti-phospho-p-38 (Ty(147), anti-c-Jun, and anti-c-Fos mAbs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-Bcl-2 mAb was purchased from DAKO (Carpinteria, CA). Rabbit anti-Bad, anti-Bid, and anti-Bax polyclonal Abs were purchased from Cell Signaling (Beverly, MA). Rabbit anti-survivin, anti-c-IAP-1, anti-c-IAP-2, and anti-XIAP polyclonal Abs were purchased from Proscience (Pittsburgh, CA) and Trevegen (Gaithersburg, MD), respectively. Mouse anti-actin mAb was purchased from Chemicon (Temecula, CA). Rabbit anti-RKIP (NH₂-terminal) polyclonal Ab was purchased from Zymed (San Francisco, CA). Rabbit anti-phospho-mitogen-activated protein kinase MEK (MEK) 1/2 (Ser217), anti-MEK1/2, anti-c-Raf-1, anti-phospho-c-Raf-1 (Ty(338/339)), anti-ERK1/2, anti-phospho-ERK1/2 (Thr(185/187)), anti-phospho-c-Jun NH₂-terminal kinase (JNK) 1/2 (Thr(183/185)) polyclonal Abs; the mitogen-activated protein kinase (MAPK) substrate 4 (significant); and subsequently incubated with 30 μL of Immuno-Pure Plus Immobilized Protein A beads (ref. 22; Pierce, Rockford, IL) for 4 hours at 4°C on ice-cold radioimmunoprecipitation assay buffer. The supernatants were incubated overnight at 4°C at 4°C for 5 minutes to pellet the nuclei, and supernatants were subsequently removed. Nuclei were washed once in Nonidet P-40 buffer and twice in ice-cold PBS. Nuclei were then lyzed in nuclear extraction buffer [20 mmol/L HEPE (pH 7.9), 25% glycerol, 0.42 mol/L NaCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 0.5 mmol/L dithiothreitol] and sonicated at 4°C for 30 seconds. A detergent-compatible protein assay kit was used to determine the protein concentration of the nuclear extracts. Nuclear proteins were mixed for 30 minutes at room temperature with biotin-labeled oligonucleotide AP-1 probe (5’-CCCTTGTAGCTCAGCCCG-GAA-3’; ref. 25) using the electrophoretic mobility shift assay (EMSA) kit purchased from Panomics Inc. (Redwood City, CA) according to the manufacturer’s instructions, as described previously (24). For the supershift assays, 1 μL of the appropriate Abs was added to the nuclear extracts for 20 minutes on ice before the addition of the labeled probe.

**Reverse Transcription-Polymerase Chain Reaction**

The reverse transcription-PCR analysis was performed as described previously (21, 24). After reverse transcription, 2.5 μL of cDNA were amplified using the following Bcl-xL gene-specific primers: forward, 5’-ACCATGTCTC- CAGGCAACCGGGAGACT-3’; and reverse, 5’-TCAATTTCGCAGTGA- GAGTGGACC-3’. Internal control for equal cDNA loading in each reaction was assessed using the following gene-specific glyceraldehyde-3-phosphate dehydrogenase primers: forward, 5’-GAACATCATCCCTGCCTCTACTG-3’; and reverse, 5’-TGTCGTTGAGCCAAATTCGTGTT-3’.

**Luciferase Bcl-x Promoter Reporter Assay**

A 650-bp region of Bcl-x promoter spanning −640 to +9 relative to the transcription start site was inserted between the XhoI and HindIII sites of the pGL2-Basic luciferase reporter vector to generate the Bcl-x wild-type (WT) promoter as described previously (16). Cells were then transfected by electroporation using pulse at 270 V/975 μF with 10 μg of Bcl-x WT promoter or empty plasmid. After transfection, the cells were cultured in 12-well plates and allowed to recover for 36 hours. The cells were then either left untreated or treated with P0D98059 (20 μg/mL) or rituximab (20 μg/mL) for 18 hours. Cells were then harvested in 1× lysis buffer, and luciferase activity was measured according to the manufacturer’s instructions (Promega, Pittsburgh, PA).

**XTT Proliferation Assay**

Inhibition of proliferation was assessed using the standard 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanide inner salt (XTT) assay kit (Roche, Indianapolis, IN), which measures the metabolic activity of viable cells (27). The percentage of proliferation was calculated using the background-corrected reading as follows: Proliferation (%) = [(absorbance of sample wells/absorbance of untreated cells)] × 100.

**Isobolographic Analysis for Determination of Synergy**

Determination of the synergistic versus additive versus antagonistic cytotoxic effects of the combination treatment of the tumor cells by rituximab and paclitaxel was assessed by isobolographic analysis as described previously (26).

**Statistical Analysis**

Assays were set up in triplicates, and the results were expressed as the mean ± SD. Statistical analysis and P value determinations were done by

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7 hours followed by paclitaxel treatment (10 mmol/L, 18 hours) and analyzed for the percentage of apoptosis. E. Bcl-xL-overexpressing cells were treated under the conditions described in A–D and analyzed by Western blot and flow cytometry. Results represent the mean ± SD of duplicate samples from three independent experiments. *, P <0.05 (significant); NS, not significant, compared with paclitaxel treatment alone.
two-tailed paired t test with a confidence interval of 95% for determination of
the significance of differences between the treatment groups. P < 0.05 was
considered to be significant. Analysis of the variance was used to test the
significance among the groups. InStat 2.01 software was used for analysis.

RESULTS

Rituximab Sensitizes the Ramos and Daudi NHL B Cells to
Paclitaxel-Induced Apoptosis by Down-Regulation of
Bcl-xL Expression

Optimal concentration of rituximab (20 μg/mL) (28) sensitizes the
CD20+ (>95%) Ramos and Daudi cells to apoptosis induced by
clinically achievable subtoxic concentrations of paclitaxel (8, 29). A
close correlation between PI+ cells and those possessing active
caspase-3 was established (ref. 24; Fig. 1A). Rituximab alone did not
induce significant apoptosis beyond the background levels in both cell
lines. All three concentrations of paclitaxel induced significant apo-
pptosis in rituximab-pretreated Ramos and Daudi cells compared with
the untreated control cells (Fig. 1A). The observed augmentation of
apoptosis by the combination treatment of rituximab and paclitaxel
was synergistic as determined by isobolographic analysis (Fig. 1B).

Western blot analysis of the total cell lysates (40 μg) of tumor cells
[± rituximab (20 μg/mL), 24 hours] showed that in Bcl-2−deficient
Ramos (30) and Bcl-2−expressing Daudi cells, rituximab selectively
decreased the expression of Bcl-xL, but had no effect on the expression
of several other apoptosis-associated proteins tested (Fig. 1C). To
confirm the protective role of Bcl-xL, in paclitaxel-induced apoptosis,
2MAM-A3, which specifically impairs the function of Bcl-xL (19),
was used. The cells were grown either in complete medium (control)
or in complete medium supplemented with various concentrations of
2MAM-A3 (15 and 20 μg/mL, 7 hours) followed by treatment pacli-
taxel (10 mmol/L, 18 hours). As shown, 2MAM-A3 by itself was inefficient in killing the tumor cells, but it was capable of significantly
augmenting paclitaxel-induced apoptosis in both cell lines, albeit
to varying degrees (Fig. 1D).

To ascertain the protective role of Bcl-xL, against drug-induced
apoptosis, Ramos cells were stably transfected with a HA-tagged
Bcl-xL−expressing construct (16). These cells expressed higher levels of
Bcl-xL compared with the parental cell line (Fig. 1E, a) and exhibited higher resistance to paclitaxel [and cisplatin, etoposide, and
Adriamycin (data not shown)] compared with the parental cell line.
Rituximab only reduced the levels of endogenous Bcl-xL in these cells
but not the ectopic Bcl-xL, driven by the overexpressing vector (Fig.
1E, a), and rituximab was not as efficient in sensitizing these cells to
paclitaxel compared with the parental cells (Fig. 1E, b). However,
higher concentrations of 2MAM-A3 (35 μg/mL) than those used for
the parental cells (15 and 20 μg/mL; Fig. 1D) sensitized the HA-
Bcl-xL cells to paclitaxel (Fig. 1E, c).

These results demonstrate that Bcl-xL regulates the resistant phe-
notype and that rituximab, through down-regulation of Bcl-xL, sensi-
tizes the Ramos and Daudi cells to paclitaxel-induced apoptosis in a
synergistic manner.

Rituximab Diminishes the Constitutive Activity of the
ERK1/2 MAPK Signaling Pathway in Ramos and Daudi
NHL B-Cell Lines

The ERK1/2 MAPK signaling pathway regulates the transcriptional
expression of Bcl-xL (31, 32). Total cell lysates of Ramos and Daudi
cells treated with rituximab (1–24 hours) were subjected to immuno-
blootting using phospho-specific and non–phospho-specific Abs for
proteins in the ERK1/2 pathway. A slight increase in the phospho-
dependent state of Raf-1 and MEK1/2 was observed 1 hour after
rituximab treatment. After this transient period, however, rituximab
decreased the phospho-dependent state of Raf-1, MEK1/2, and
ERK1/2 in a time-dependent manner beginning 3–6 hours after treat-
ment, which was maintained up to 24 hours. The basal level (phospho-
dependent state) of these proteins remained unaltered during the
entire (24-hour) experiment (Fig. 2A). The temporal expression of
Bcl-xL, which is regulated in part by the ERK1/2 pathway (33, 34),
was also examined. Rituximab decreased the expression of Bcl-xL at
the protein level as a function of time starting between 3 and 6 hours
after treatment; the decrease was more pronounced at later time points
(Fig. 2A).

In contrast to our findings with monomeric rituximab, previous
findings demonstrated that cross-linking rituximab induces apoptosis
and activates the MAPK pathways in NHL and B-cell chronic lympho-
phytic leukemia cells (33, 34). To validate these observations,
cross-linked rituximab was generated by using the secondary antihu-
man immunoglobulin (50 μg/mL) in combination with rituximab.
Cross-linking rituximab, but not monomeric rituximab, resulted in
robust and sustained activation of ERK1/2 and p38 (and to a lesser
extent, JNK1/2; Fig. 2B) but had no effect on the basal levels of these
proteins (data not shown). Activation of the MAPK pathways was
accompanied by a substantial induction of apoptosis in both cell lines
at later time points (18–24 hours; data not shown).

To ascertain whether the observed dephosphorylation also resulted
in decreased kinase activity of the ERK1/2 pathway, an in vitro (in
gel) kinase assay was performed. The MEK1/2 kinase activity (using
ERK1/2 peptide containing residues 172–192 as substrate) of tumor
cells (±20 μg/mL rituximab) was assessed. Rituximab decreased
MEK1/2 kinase activity as shown by the inability of rituximab-treated
cells to phosphorylate the ERK1/2 peptide (Fig. 2C), which was
reversed on treatment with cross-linked rituximab (data not shown).

Collectively, these results demonstrate the ability of rituximab to
negatively regulate the activity of the ERK1/2 signaling pathway and
concomitantly decrease the protein level of Bcl-xL. These results also
show that the molecular signaling pathways triggered by monomeric
versus cross-linked rituximab are different.

Pharmacological Inhibition of the ERK1/2 MAPK Signaling
Pathway Mimics the Effects of Rituximab

Sensitization to Paclitaxel-Induced Apoptosis. The involvement of
the ERK1/2 signaling pathway in the resistance of Ramos and
Daudi cells to paclitaxel and inhibition of this pathway by rituximab
suggest that specific inhibition of this pathway should mimic ritux-
imab. PD098059 exerts its effects by binding to the inactive form of
MEK1/2 and prevents MEK1/2 activation by Raf-1, thus inhibiting
the activation of ERK1/2 (17). U0126 blocks the phosphorylation and
activation of ERK1/2 (18), and GW5074 is a specific inhibitor of
Raf-1 (20). Optimal concentrations of the inhibitors were determined
by pilot studies and are in accordance with previous reports (17, 18,
20). The cells were pretreated with the inhibitors (GW5074: 10
μmol/L, 45 minutes; PD098059: 20 μg/mL, 45 minutes; U0126: 10
μmol/L, 45 minutes) followed by paclitaxel (10 mmol/L, 18 hours)
treatment. Paclitaxel (10 μmol/L) induced modest apoptosis, whereas
the inhibitors were nontoxic to the cells. However, the inhibitors
sensitized the cells to paclitaxel (Fig. 3A).

To determine the specificity of the inhibitors, tumor cells were
treated with inhibitors under the conditions described above. Total cell
lysates were analyzed by immunoblotting using specific Abs for
pERK1/2, p-p38, and pJNK1/2 MAPKs. The inhibitors specifically
blocked ERK1/2 phosphorylation but had no effect on p38 and
JNK1/2 phosphorylation. 2MAM-A3 had no modulatory effect on
pERK1/2, p-p38, or pJNK1/2 (Fig. 3B). The phospho-independent

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state of these signaling molecules was unaffected by the inhibitors (data not shown). Together, these results show that GW5074, PD098059, and U0126 specifically inhibit the ERK1/2 signaling pathway by decreasing the phosphorylation of ERK1/2. These results also show that pharmacological disruption of the ERK1/2 pathway using specific inhibitors, such as rituximab, sensitizes the cells to paclitaxel.

Inhibition of Cell Proliferation. Rituximab inhibits cellular proliferation of Ramos, Daudi, and other NHL B-cell lines (7, 8). We examined whether the ERK1/2 pathway affected by rituximab treatment was involved in the proliferation of the Ramos and Daudi cells. An aliquot of the samples from Fig. 3A (10^4 cells per sample) was used in a 24-hour XTT assay to analyze the antiproliferative effects of rituximab and the inhibitors. The results presented in Fig. 3C demonstrate that specific pharmacological inhibitors of the ERK1/2 pathway mimic rituximab in decreasing the proliferation rate of the Ramos and Daudi cells.

Regulation of Bcl-xL Expression by the ERK1/2 Pathway

It has been reported that activation of the ERK1/2 pathway leads to transcriptional activation of AP-1 and AP-1–dependent Bcl-xL gene expression (31, 32). Rituximab decreased the activation of the

Fig. 2. Rituximab inhibits the ERK1/2 signaling pathway. A, dephosphorylation of the components of the ERK1/2 pathway by monomeric rituximab. B, activation of the MAPK pathways by cross-linked rituximab. C, inhibition of MEK1/2 kinase activity by monomeric rituximab. Ramos and Daudi cells were grown in complete medium in the absence (control) or presence of rituximab (20 μg/mL) for various time points (1, 3, 6, 12, and 24 hours) or cross-linked rituximab (20 μg/mL, rituximab +50 μg/mL, antihuman immunoglobulin). Total cell lysates (40 μg) were subjected to Western blot analysis using phospho-specific and non–phospho-specific Abs for various components of the ERK1/2, p38, and JNK1/2 MAPK pathways (A and B) or kinase assay (C). The results are representative of three independent experiments.

Fig. 3. Pharmacological inhibition of the ERK1/2 signaling pathway. A, sensitization of the Ramos and Daudi cells to paclitaxel-induced apoptosis. Tumor cells were either left untreated (control) or treated with GW5074 (10 μmol/L, 45 minutes), U0126 (10 μmol/L, 45 minutes), or PD098059 (20 μg/mL, 45 minutes). Thereafter, the cells were incubated with paclitaxel (10 nmol/L, 18 hours) and subjected to PI staining. B, specificity of the inhibitors for the ERK1/2 pathway. Tumor cells were treated with inhibitors under the conditions mentioned above [also treated with 2MAM-A3 (20 μg/mL, 7 hours)]. Total cell lysates (40 μg) were used in immunoblotting experiments with Abs specific for pERK1/2, p-p38, and pJNK1/2 MAPKs. C, inhibition of cellular proliferation by rituximab and ERK1/2 inhibitors. An aliquot of the samples from A (10^4 cells per sample; also treated with 2MAM-A3 (20 μg/mL, 7 hours)) was used in a 24-hour XTT proliferation assay to measure the proliferation rate of Ramos and Daudi cells on treatment with rituximab and the inhibitors. Results represent the mean ± SD of triplicate samples from two independent experiments. *, P <0.05 (significant).
ERK1/2 MAPK pathway; thus, alteration in the DNA binding activity of AP-1 on rituximab treatment was examined. The results demonstrate that AP-1 DNA binding activity was significantly reduced in the presence of rituximab as early as 3 to 6 hours after treatment, and that it remained decreased in the presence of rituximab during the entire experiment (24 hours). Rituximab-mediated decrease in AP-1 DNA binding activity was corroborated by the use of PD908059 (20 μg/mL, 45 minutes; Fig. 4A; refs. 29 and 30). Furthermore, the specificity of the EMSA assay was demonstrated by including crucial controls (e.g., positive control, no nuclear extract, unlabeled free probe, and unrelated probe). Because the AP-1 complex is composed of Jun and Fos family members, supershift experiments using c-Jun and c-Fos Abs were performed. The postulated AP-1 bands showed significant shift on the addition of the Abs to the nuclear extracts, confirming the involvement of AP-1 (Fig. 4A).

Additional evidence for the direct role of rituximab and the ERK1/2 pathway in the regulation of Bcl-xL expression was provided by luciferase reporter assays. To this end, a 650-bp DNA fragment spanning the Bcl-x S' promoter region (Bcl-x WT) was inserted into pGL2-Basic luciferase plasmid (16). Using electroporation, the cells were transfected with this plasmid, and the cells were allowed to recover for 36 hours. The cells were then treated with either rituximab (20 μg/mL) or PD908059 (20 μg/mL) for an additional 18 hours. Then, the cells were harvested using 1× lysis buffer, and luciferase activity was measured with an analytical luminescence counter. Transfection with the WT promoter resulted in robust luciferase activity, albeit to a varying degree. However, treatment of the cells with rituximab or PD908059 substantially diminished the luciferase activity (Fig. 4B). These results corroborate the above-mentioned findings demonstrating the regulation of Bcl-xL by rituximab and the ERK1/2 pathway.

Based on the above-mentioned observations, we next examined whether rituximab and/or ERK1/2-specific inhibitors modulate Bcl-xL transcription. Tumor cells were either left untreated (control) or treated with rituximab (20 μg/mL, 1–24 hours), 2MAM-A3 (20 μg/mL), GW5074 (10 μM), PD908059 (20 μg/mL), or U0126 (10 μM), and total RNA was extracted and reverse transcribed to first-strand cDNA. Complementary DNA primers specific for Bcl-xL mRNA were used in a PCR. As shown, 2MAM-A3 had no effect on Bcl-xL gene expression, whereas rituximab and the inhibitors decreased the transcription of Bcl-xL (Fig. 4C, a). Rituximab inhibited Bcl-xL transcription in a time-dependent manner, beginning as early as 1 hour after treatment, and the inhibition was more pronounced at later time points (Fig. 4C, b). Together, these results denote the ability of rituximab to inhibit the ERK1/2 pathway, decrease AP-1 DNA binding activity, and down-regulate Bcl-xL transcription.

**Rituximab-Mediated Up-Regulation of RKIP Parallels Bcl-xL Down-Regulation**

The above-mentioned data demonstrate that rituximab (and the inhibitors) inhibits the ERK1/2 pathway. Thus, we investigated the possible mechanism by which rituximab exerts this inhibitory effect. Recently, RKIP has been identified as a negative regulator of the ERK1/2 signaling pathway (14, 15). Therefore, we examined whether RKIP induction was associated with rituximab-mediated inhibition of the ERK1/2 pathway and chemosensitization of the Ramos and Daudi cells. We observed a time-dependent induction of RKIP in rituximab (20 μg/mL)-treated Ramos and Daudi cells. Intriguingly, the induction of RKIP coincided with Bcl-xL down-regulation (also observed as early as 3–6 hours; Figs. 5A and B).

It has been shown that RKIP interferes with the ERK1/2 pathway via physical interaction with Raf-1 (14, 15), thus we examined whether rituximab potentiates the association between Raf-1 and RKIP. Tumor cells were grown in the presence or absence of rituximab (20 μg/mL, 24 hours), and total cell lysates were used in an immunoprecipitation assay. Using anti-Raf-1 Ab, Raf-1 was precipitated, and the membranes were subsequently immunoblotted with anti-RKIP polyclonal Ab. As depicted (Fig. 5C), the association of RKIP with Raf-1 was significantly enhanced by rituximab. In addition, the lysates contained similar levels of Raf-1, and, consistent with findings in Fig. 5A and B, the rituximab-treated samples exhibited a higher level of RKIP (Fig. 5C). These results suggest that rituximab up-regulates RKIP and enhances the association of RKIP with Raf-1, events that can account for the inhibition of the ERK1/2 pathway.

**DISCUSSION**

We have reported previously that rituximab interferes with the intracellular signaling pathways and sensitizes various NHL cells to drug-induced apoptosis. The observed chemosensitization was due to the selective inhibition of antiapoptotic gene products Bcl-2 (in 2F7 ARL; ref. 7) and Bcl-xL (in non-ARL cells) by rituximab (8). The current study delineates the signaling pathway used by rituximab for selective inhibition of Bcl-xL in Ramos and Daudi cells. Rituximab decreases the phosphorylation-dependent state of the components of the ERK1/2 signaling pathway concomitant with the up-regulation of RKIP expression. Induction of RKIP enhances the physical association of RKIP with Raf-1, resulting in decreased activity of the ERK1/2 pathway, diminished AP-1 DNA binding activity, down-regulation of Bcl-xL expression, and subsequent chemosensitization of the cells. These events occurred with similar kinetics and were observed 3 to 6 hours after rituximab treatment. Using specific inhibitors corroborated rituximab-mediated inhibition of the ERK1/2 pathway and Bcl-xL expression. These findings reveal for the first time the interruption of the ERK1/2 pathway by rituximab.

The ERK1/2 pathway is constitutively activated in Ramos and Daudi cells (Fig. 2). Our findings demonstrate that monomeric rituximab is incapable of inducing apoptosis and inhibits the ERK1/2 pathway in Ramos and Daudi cells. However, previous reports have demonstrated activation of the ERK1/2, p38, and JNK1/2 MAPK pathways and induction of apoptosis by cross-linking rituximab (33, 34). In agreement with these reports, cross-linking rituximab (20 μg/mL + 50 μg/mL antihuman immunoglobulin) induced robust and sustained phosphorylation of ERK1/2 and p38 MAPK (Fig. 2B) and induced significant apoptosis in Ramos and Daudi cells (data not shown). Li et al. (35) identified novel mechanisms that may explain the opposing effects of p38 in apoptosis induction, whereby activation of p38 by mitogen-activated protein kinase kinase (MKK)3/6 was followed by rapid dephosphorylation of MEK1/2 and subsequent apoptosis. It is possible that cross-linking rituximab inhibits this pathway, whereas a different pathway is modulated by monomeric rituximab. These findings suggest that the pathways used by monomeric and cross-linked rituximab are different.

Because the constitutive activation of the ERK1/2 signaling pathway confers a chemoresistance phenotype on tumor cells and induces their rapid proliferation (36–38), inhibition of this pathway may confer drug sensitivity. Thus, interruption of this pathway is a target for therapeutic intervention for the treatment of leukemia and other tumors (36–38). Herein, we demonstrate that rituximab inhibits the ERK1/2 pathway and sensitizes the cells to drug-induced apoptosis. The phosphorylation-dependent state of Raf-1, MEK1/2, and ERK1/2 is significantly decreased 3 to 6 hours after rituximab treatment in NHL B cells, concomitant with inhibition of MEK1/2 kinase activity (Fig. 2). Moreover, rituximab sensitized these cells to paclitaxel (Fig. 1A), suggesting a novel function for rituximab as a negative regulator.
Fig. 4. Rituximab diminishes constitutive AP-1 DNA binding activity and Bcl-\textsubscript{xL} gene expression. A, After overnight growth in RPMI 1640 + 1% fetal bovine serum, Ramos and Daudi cells were washed and grown in either complete medium (control) or complete medium supplemented with rituximab (20 μg/mL; 1, 3, 6, 12, and 24 hours) or PD098059 (20 μg/mL; 45 minutes). Ten micrograms of nuclear lysates were subjected to EMSA. Specificity of the assay was confirmed by the inclusion of appropriate controls. For the supershifts, 1 μL of the appropriate Abs was added to the nuclear extracts 20 minutes before the addition of the labeled probe. B, A Bcl-x promoter fragment spanning the −640 to +9 region relative to the transcriptional start site was cloned into pGL2-Basic luciferase reporter vector (16). The cells were then transfected with 10 μg of this reporter plasmid or empty vector. Thirty-six hours after transfection, the cells were either left untreated or treated with PD098059 (20 μg/mL) or rituximab (20 μg/mL). The samples were harvested after 18 hours and assessed for luciferase activity. C, Tumor cells were either left untreated or treated with the inhibitors [rituximab (20 μg/mL), GW5074 (10 μmol/L), PD98059 (20 μg/mL), or U0126 (10 μmol/L)], and total RNA was extracted and reverse transcribed to first-strand cDNA. Complementary DNA (2.5 μg) of various sample conditions was used in PCR analysis using Bcl-\textsubscript{xL}-specific primers. The intensity of the bands was normalized to the levels of the corresponding glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH). The results are representative of two independent experiments.
Rituximab inhibits the proliferation rate of NHL B-cell lines through an unknown mechanism (7, 8). The ERK1/2 pathway is implicated in the proliferation of tumor cells (36, 38). Here we demonstrate that rituximab inhibits the constitutive activation of the ERK1/2 pathway in Ramos and Daudi cells and also reduces the rate of proliferation (Fig. 3C). Therefore, these findings suggest a link between rituximab-mediated inhibition of proliferation and inhibition of the ERK1/2 pathway. However, inhibition of proliferation cannot be explained solely by the inhibition of Bcl-xL, because 2MAM-A3 did not reduce the proliferation rate to the levels achieved by rituximab.

We have established several lines of evidence for the involvement of the ERK1/2 pathway in the regulation of Bcl-xL expression. First, robust luciferase reporter activity was observed on transfection of the cells with WT Bcl-xL promoter, which was significantly reduced by rituximab and PD098059 (Fig. 4B). Secondly, specific inhibitors of the ERK1/2 pathway inhibited Bcl-xL transcription (Fig. 4C) and sensitized the cells to drug-induced apoptosis at levels comparable with those achieved by rituximab (Fig. 3A). Expression of Bcl-xL is regulated by several transcription factors including Ets, nuclear factor κB, signal transducers and activators of transcription, and AP-1 (31). Conversely, on activation, the ERK1/2 pathway leads to the activation of various transcription factors including AP-1. Mutational analysis has implicated AP-1 in the regulation of Bcl-xL (42); however, our data do not establish a direct role of AP-1 in the regulation of Bcl-xL expression. Our findings establish a correlation between rituximab-mediated inhibition of AP-1 DNA binding activity and inhibition of the ERK1/2 pathway and Bcl-xL down-regulation. Nonetheless, the likelihood of the contribution of other transcription factors to the regulation of Bcl-xL by rituximab has not been ruled out.

The protective role of Bcl-xL against chemotherapy-triggered apoptosis (11, 12) was confirmed by using 2MAM-A3, which binds to Bcl-xL at the hydrophobic groove formed by the highly conserved BH1, BH2, and BH3 domains, thus impairing the antiapoptotic ability of Bcl-xL (19). Although unable to regulate transcription or translation of Bcl-xL, 2MAM-A3 sensitized the cells to paclitaxel (and other drugs; data not shown), at levels comparable with those achieved by rituximab, via functional impairment of Bcl-xL. These findings support our contention that down-regulation of Bcl-xL expression by rituximab is critical for chemosensitization.

Bcl-xL is abundantly expressed in lymphomas (10) and protects the cells from apoptosis induced by DNA-damaging agents and metabolic, microtubule, and topoisomerase inhibitors (11, 12). An inverse correlation between Bcl-xL levels and sensitivity to 122 standard anticancer agents has been established (12). Also, Bcl-xL acts independently of wild-type p53 function or cell type and, through modulation of apoptosis, plays a major role in the determination of cellular response to a wide variety of drugs (9–13). Our results suggest that in Ramos and Daudi cells, Bcl-xL is the main antiapoptotic factor, and the ability of rituximab to negatively modulate the expression of Bcl-xL may explain its effectiveness in combination with chemotherapy in the treatment of some cases of NHL. This contention was further supported by using Bcl-xL-overexpressing cells, which expressed higher resistance against a battery of structurally and functionally diverse antineoplastic agents (cisplatin, Adriamycin, etoposide, and paclitaxel; data not shown). Functional impairment of Bcl-xL (by 2MAM-A3) sensitized these cells to paclitaxel. Rituximab was only able to reduce the endogenous levels of Bcl-xL in these cells and was not able to reduce the ectopically expressed Bcl-xL, driven by the overexpressing plasmid, and it exerted a modest sensitizing attribute, suggesting that the level of Bcl-xL is critical for drug-resistance. Our findings with Bcl-2—expressing Daudi and Bcl-2—deficient Ramos cells reveal that rituximab-mediated chemosensitization is independent of Bcl-2 expression, which is in agreement with recent findings of the ERK1/2 pathway. This was corroborated by pharmacological interruption of the ERK1/2 pathway using specific inhibitors, which also sensitized the cells to paclitaxel at levels comparable with those achieved by rituximab (Fig. 3A). Our results corroborate previous reports in which MEK inhibition synergized with UCN-01 (39) and augmented the apoptotic effects of paclitaxel (40, 41). In our studies, pretreatment with rituximab followed by paclitaxel was optimal for sensitization, and pretreatment with the ERK1/2 inhibitors followed by paclitaxel mimics the effects of rituximab. Thus, inhibition of the ERK1/2 pathway seems to be required before paclitaxel treatment to trigger apoptosis in this model. However, studies by Yu et al. (41) in the U937 leukemia cell line demonstrated that pretreatment with the inhibitors followed by paclitaxel did not sensitize the cells to paclitaxel. The discrepancy may be due to differences in the cell lines used as well as differences in the duration of paclitaxel treatment (6 hours in their studies versus 18 hours in the present study) or the activation status of the proteins in the signaling cascade.
The inhibition of the ERK1/2 pathway might occur via several different mechanisms. It might be through the de-activation of the src family kinase Lyn. Indeed, a decrease in p-Lyn by rituximab was observed (data not shown). Alternatively, it might be due to the modulation of RKIP expression that inhibits the ERK1/2 pathway (14, 15). In fact, our findings reveal that rituximab up-regulates the expression of RKIP and facilitates the association of RKIP with Raf-1 (Fig. 5). Physical association between RKIP and Raf-1 will abrogate the ability of Raf-1 to phosphorylate and activate downstream molecules such as MEK1/2 and ERK1/2. In vitro, RKIP disrupts the interaction between Raf-1 and MEK, thus behaving as a competitive inhibitor for MEK, and inhibits AP-1–dependent gene expression by suppressing the ERK1/2 pathway (14, 15). Our results corroborate these findings and demonstrate that rituximab-mediated RKIP induction diminishes the phosphorylation of the components of the ERK1/2 pathway, reduces AP-1 DNA binding activity, and decreases Bcl-x(L) transcription and translation, all of which occur with similar kinetics. These findings provide a novel mechanism induced by rituximab that regulates cell survival and sensitizes the cells to paclitaxel through induction of RKIP and inhibition of the ERK1/2 pathway. Thus, RKIP expression regulates drug sensitivity. The novel and important role of RKIP as a mediator of drug-induced apoptosis is not limited to the NHL model. Ectopic expression of RKIP sensitizes drug-resistant cells to undergo apoptosis (44). Furthermore, down-regulation of RKIP expression in tumor cells confers drug resistance by releasing its inhibitory constraint of ERK1/2 and nuclear factor κB major survival pathways (14, 15, 45). The mechanism by which rituximab and other chemotherapeutic agents regulate RKIP expression, however, is unknown and under investigation. The role of RKIP in the regulation of cell survival and apoptosis in cancer cells may be clinically important. For instance, a novel antimetastatic function for RKIP in prostate cancer has recently been proposed (46), showing the involvement of the ERK1/2 pathway in tumor progression and metastasis and further confirming the specific interaction of RKIP with the ERK1/2 pathway. Notably, the invasion of tumor cells was abrogated only in the presence of PD98059. Thus, the modulation of RKIP expression in cancer cells may dictate the outcome of tumor progression and response to apoptosis-inducing stimuli.

In conclusion, we have described a novel mechanism by which rituximab affects both proliferative and apoptotic signaling pathways schematically represented in Fig. 6. Accordingly, rituximab up-regulates RKIP expression and interferes with the constitutively active ERK1/2 pathway, resulting in diminished AP-1 DNA binding activity and Bcl-x(L) expression. Decreased levels of Bcl-x(L) will in turn lower the apoptosis threshold, and the cells will be sensitized to drug-induced apoptosis through the type II mitochondrial pathway (8). Pharmacological interruption of the ERK1/2 pathway (e.g., GW5074, PD98059, and U0126) or functional impairment of Bcl-x(L) (e.g., 2MAM-A3) mimics the antiproliferative and chemosensitizing effects of rituximab. Hence, this study identifies several potential targets for therapeutic intervention (namely, the components of the ERK1/2 pathway, Bcl-x(L), and RKIP) and might provide a rational molecular basis for the therapeutic use of rituximab and/or ERK1/2 inhibitors in combination with chemotherapeutic compounds.

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