Rituximab Inactivates Signal Transducer and Activation of Transcription 3 (STAT3) Activity in B-Non-Hodgkin’s Lymphoma through Inhibition of the Interleukin 10 Autocrine/Paracrine Loop and Results in Down-Regulation of Bcl-2 and Sensitization to Cytotoxic Drugs

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ABSTRACT

Development of the chimeric mouse antihuman CD20 antibody, Rituximab, presented a notable advance in the treatment of patients with non-Hodgkin’s lymphoma (NHL). Its use allowed the specific targeting of tumor B cells without the systemic toxicity of traditional therapies. The mechanisms by which Rituximab induces its antitumor activity are not fully understood. We have shown previously that Rituximab down-regulates Bcl-2 expression in some B-NHL cell lymphoma lines through an interleukin 10 (IL-10)-dependent autocrine loop, an effect that renders the resistant cells susceptible to chemotherapeutic drugs. The objective of this study was to delineate the signaling pathway by which Bcl-2 is controlled by Rituximab and IL-10. We hypothesized that the down-regulation of IL-10 by Rituximab decreases activation of the signal transducer and activator of transcription 3 (STAT3) protein, which in turn, is responsible for decreased levels of Bcl-2. We demonstrate by phosphoprotein immunoblotting and gel shift analyses that endogenous IL-10 induces activation of STAT3 in the 2F7 cell line. Furthermore, we show that Rituximab and anti-IL-10 antibody treatment decreases the ability of STAT3 to bind to its DNA binding site. The decrease in STAT3 activation by these treatments correlates with a decrease in Bcl-2 expression. Additionally, piceatannol, an inhibitor of STAT3 activation, down-regulates the expression of Bcl-2. Altogether, these results demonstrate that Bcl-2 expression is under the regulation of the STAT3 signaling pathway, which is regulated by endogenously secreted IL-10. Hence, Rituximab-induced down-regulation of IL-10 expression is responsible for the down-regulation of Bcl-2 and sensitization of NHL cells by therapeutic drugs. Furthermore, these findings support the notion that circulating IL-10 in vivo may control the resistance of NHL to drug-mediated cytotoxicity.

INTRODUCTION

Treatment of NHL has undergone a significant change since the introduction of monoclonal antibodies. Traditionally, patients have been treated with standard chemotherapeutic regimens that have offered partial responses and led to eventual relapse. Subsequent administration of higher doses of chemotherapy proved to be more cytotoxic and did not necessarily result in longer rates of remission.

Monoclonal antibodies have provided an alternative approach to malignant disease. Their use allows for targeting of cell surface markers, making them more specific than traditional chemotherapeutic approaches. They are also less systemically toxic and less myelosuppressive than therapeutic drugs. Their antitumor activity has been shown to include antibody-dependent cellular cytotoxicity (1), complement-dependent cytotoxicity (1), inhibition of cell proliferation (2), and induction of apoptosis (3–5). Our laboratory has extended the use of monoclonal antibody usage, specifically the chimeric anti-CD20 monoclonal, Rituximab, to include its potential as a sensitizer of tumor cells to chemotherapeutic drug-mediated cytotoxicity (3, 6, 7).

Rituximab is a monoclonal antibody that specifically targets the CD20 antigen on the surface of normal and malignant human B cells (8). Its primary use has been for the treatment of follicular and low-grade NHL but is now in wide use for an assortment of B-cell cancers and proliferative disorders (9). Although CD20 is also expressed on normal B cells, Rituximab does not evoke an immunosuppressed profile in patients (8). CD20 is a M, 33,000–37,000 cell surface phosphoprotein that is expressed specifically within the B-cell lineage from pre-B cells to mature B cells (10). The function of CD20 is not yet fully understood. Its role has been implicated in the differentiation and proliferation in B-lymphocyte development (11). CD20 is also not internalized when bound by Rituximab antibody (12), making it an ideal target for immune therapy.

Our studies have shown that Rituximab can reverse the drug-resistant phenotype of NHL tumor cells to a drug-sensitive phenotype (3, 6, 7). By delineating the mechanism(s) by which this sensitization is achieved, we have observed that, specifically in ARLs, Rituximab down-regulates both the secretion of tumor-derived IL-10 and the expression of Bcl-2 (6). The down-regulation of Bcl-2 is believed to be the major reason for the sensitization of these tumor cells to therapeutic drugs, although the possibility of other factors and pathways being involved are not excluded.

IL-10 is a pleiotropic cytokine that plays a modulatory role in the lymphoid and myeloid response (13). It signals through the IL-10 receptor using primarily the JAK/STAT pathway (14). It induces the activation and proliferation of B lymphocytes as well as confers survival advantages to certain B-cell tumors (15–17). We have demonstrated in recent studies that IL-10 induces drug resistance in ARL tumor B cells (6, 17). More specifically, ARL tumor cells secrete IL-10 to create autocrine/paracrine loops that induce or up-regulate protective factors in those cells. In our ARL tumor model, neutralization of endogenously secreted IL-10 sensitized the tumor cells to the cytotoxic effects of chemotherapeutic drugs (6). These findings supported the notion that IL-10 is a protective factor in ARL and that Rituximab-mediated down-regulation of IL-10 increases tumor cell sensitivity to chemotherapeutic drugs.

Bcl-2 is a member of the Bcl-2 family of proteins that acts as a regulator of programmed cell death (18). The family is comprised of antiapoptotic and proapoptotic proteins. Bcl-2 is perhaps the best...
characterized member of this family, having been shown to protect cells from apoptotic stimuli, including drug cytotoxicity (19). The overexpression of Bcl-2 in NHL cells has been implicated in their resistance to chemotherapy, and its regulation plays a pivotal role in the ability of Rituximab to drug-sensitize ARL to cytotoxic drugs (6).

The objective of this study was to delineate the signaling pathway by which Rituximab-mediated inhibition of IL-10 secretion inhibited Bcl-2 protein expression. This study examined: (a) the role of endogenously secreted IL-10 in activating the transcription factor STAT3; (b) the role of Rituximab in regulating STAT3 activity; and (c) the relationship between STAT3 activity and Bcl-2 expression. Knowing the details of such regulation will allow for the modification and development of more effective treatments in ARL and perhaps NHL in general.

MATERIALS AND METHODS

Cell Lines and Reagents. 2F7 is a CD20-positive Burkitt’s lymphoma cell line established from a male with AIDS (20) and was generously provided by Dr. Otoniel Martinez-Maza (Jonsson Comprehensive Cancer Center, Los Angeles, CA). Cell culture was performed using RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with 10% heat-inactivated fetal bovine serum (Gemini, Calabasas, CA). Fetal bovine serum-supplemented medium is hereafter referred to as complete medium. All cells were cultured in 5% atmospheric CO2 at 37°C.

Antibodies used for IL-10 capture in ELISAs and for IL-10 neutralization in cell culture were purchased from PharMingen (San Diego, CA). IL-10 polyclonal antibodies for detection in ELISAs were produced from rabbits in our laboratory and partially purified by ammonium sulfate. Recombinant IL-10 was acquired from PeproTech (Rocky Hills, NJ). Bcl-2 antibody for immunoblotting was purchased from Dako (Carpinteria, CA). Antibodies against STAT3 and phosphorylated STAT3 were obtained from New England BioLabs (Beverly, MA). The inhibitor of STAT3 activation, piceatannol, was purchased from Sigma Chemical Co. (St. Louis, MO).

Monoclonal Antihuman CD20 Antibody, Rituximab (IDEC-2C8). The monoclonal antibody, Rituximab, is specific for human CD20 and has been used primarily in the treatment of low-grade and follicular NHL. Rituximab was generated by fusing the human IgG1 constant regions to the murine antihuman CD20 antibody, IDEC-2B8, variable regions (8) and was kindly provided by Dr. Christos Emmanouilides (Department of Medicine, University of California Los Angeles School of Medicine, Los Angeles, CA).

Cytotoxicity/Growth Inhibitory Assay. Cytotoxicity/growth inhibition was performed using the XTT assay (Refs. 21 and 22; Boehringer Mannheim, Indianapolis, IN) and carried out as described previously (7). Cells were treated with Rituximab (20 μg/ml; Ref. 23), CDPD (1 μg/ml; Ref. 24), fludarabine (20 μM; Ref. 25), vinblastine (0.1 μg/ml; Ref. 26), and Adriamycin (1 μg/ml; Ref. 27) or combinations of Rituximab with drugs. Control cells, i.e., grown in complete medium alone, served as the assay baseline and negative control. Wells with 200 μl of complete medium only served as the plate blank. All samples were done in triplicate. After 24 h of incubation, the XTT assay was used to quantitate cell inhibition in each sample. The inhibitory effect of each treatment was calculated as the percentage of inhibition as compared with the untreated controls.

ELISA for IL-10 Detection. Briefly, supernatants from tumor cells treated with Rituximab (20 μg/ml) or medium alone were collected at 6, 12, 18, and 24 h. IL-10 detection and quantification was accomplished by ELISA as described previously (28). The absorbance values for all samples were determined on an Enmax microplate reader (Molecular Devices Corp., Sunnyvale, CA) at 405 nm.

RT-PCR for IL-10 Transcriptional Regulation. We used RT-PCR to detect transcriptional regulation of IL-10 in 2F7 tumor cells treated with or without Rituximab (29, 30). 2F7 cells (2 ×10^5) were treated in 12-well plates (Costar, Cambridge, MA) with Rituximab (20 μg/ml) or medium alone for 6, 12, 18, and 24 h at 37°C. Total RNA was extracted from 2F7 cells at the specified time points using the single-step guanidinium thiocyanate-chloroform method with STAT 60 reagent (Tel-Test “B,” Inc., Friendswood, TX). Total RNA (1 μg) was reverse transcribed to first-stranded cDNA for 1 h at 42°C using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Grand Island, NY). Reactions for RT-PCR consisted of 20 μM random hexamer primers, 125 μM of each deoxynucleotide triphosphate, 10 μM DTT, and 4 μl of 5X first strand buffer. Amplification IL-10 and GAPDH transcript expression was performed on 2F7 cDNA using the hot-start technique (31, 32). Primers used in this study included human IL-10 upstream (5’-CCA ACA GAA GCT TCT ATT CC-3’) and downstream (5’-CCG TCG AAC AAT AAA TAT TG-3’) and GAPDH upstream (5’-GAA CAT CCC TCG CTC TAC TG-3’) and downstream (5’-GTT CCT GCT GTA GGC AAA TTC GTT G-3’). All amplifications were performed with a DNA Thermo Cycler 480 (Perkin-Elmer, Norwalk, CT) and analyzed on 1% agarose (Sigma Chemical Co.) gels in TBE (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA, pH 8.0).

Immunoblotting Analysis of Protein Expression. To analyze regulation of protein expression and phosphorylation, cells were treated in six-well plates (Costar) at 10^5/ml with 20 μg/ml Rituximab, 1 μg/ml neutralizing IL-10 antibody, 100 μM piceatannol, DMSO (equal volume of piceatannol), or complete medium medium alone. At specified time points (6, 12, 18, 24 h) of treatment, cells were transferred to 5 ml of polypropylene tubes (Fisher Scientific, Pittsburgh, PA) and pelleted for 5 min at 1400 rpm × g in an Omnipaque RT (American Scientific Products) or Marathon 3200RT (Fisher Scientific) microcentrifuge. Pellets were transferred to microcentrifuge tubes (Fisher Scientific) and were lysed on ice with cold RIPA buffer (1% NP40, 0.1% SDS, 0.5% deoxycholic acid, and 1% PBS) supplemented with protease inhibitor cocktail tablets (Boehringer Mannheim, Indianapolis, IN). Cells were further lysed by sheering using 1-mm insulin syringes (Becton Dickinson, Franklin Lakes, NJ) and subsequently centrifuged at 14,000 rpm × g at 4°C for 20 min. Lysates were quantified for protein concentration using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). An equal volume of sample buffer (6.2 mM Tris (pH 6.8), 2.3% SDS, 5% mercaptoethanol, 10% glycerol, and 0.02% bromphenol blue) was then added to the lysates, boiled for 10 min, and stored at −80°C. Primary antibody for Bcl-2 was diluted 1:500; antibodies for STAT3 and phosphorylated STAT3 were diluted 1:1000.

Electrophoretic Mobility Shift Assays. The electrophoretic mobility gel shift assay was used for analysis of STAT3 binding (33). Cells were pelleted after treatments and washed twice with ice-cold PBS. After washing, cells were lysed in 1 ml of NP40 lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl2, and 0.5% NP40) on ice for 5 min. Samples were centrifuged at 2000 rpm × g at 4°C for 5 min in a microcentrifuge to pellet the nuclei, and supernatants were subsequently removed. Nuclei were washed once in NP40 buffer and twice in cold PBS. Nuclei were then lysed in nuclear extraction buffer (20 mM HEPES (pH 7.9), 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) and sonicated at 4°C for 30 min.

Nuclear lysates were incubated with 32P-labeled, double-stranded SIE probe (BrdU forward, 5’-GAT CCA TTT CCC GTA AAT CA-3’; reverse, 5’-TGA TTT ACG GGA AAT GGA TC-3’). All amplifications were performed with a DNA Thermo Cycler 480 (Perkin-Elmer, Norwalk, CT) and analyzed on 1% agarose (Sigma Chemical Co.) gels in TBE (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA, pH 8.0).

RESULTS

Rituximab Down-Regulates the Production of IL-10. Recent studies in our laboratory have demonstrated that Rituximab can sensitize the B-NHL cell lines, DHL-4 (3), 10C9, and 2F7 (Table 1; Refs. 6 and 7) to chemotherapeutic drugs. The 2F7 cell line constitutively secretes IL-10 when cultured in vitro, and Rituximab down-regulates the expression and secretion of IL-10 in these cells (6). The decrease in endogenous IL-10 was shown to result in the decrease of Bcl-2 expression in these tumor cells, rendering them sensitive to chemotherapeutic drugs (6). To delineate the kinetics of IL-10 down-regulation mediated by Rituximab treatment, 2F7 tumor cells were grown in complete medium in the presence or absence of Rituximab (20 μg/ml; Fig. 1A). IL-10 secretion was adjusted for cell number by decreasing the cell number in control samples to that of treated samples and applying that ratio to the IL-10 (pg/ml) secreted in the...
INHIBITION OF STAT3 ACTIVATION BY RITUXIMAB

Table 1 Sensitization of chemotherapeutic drugs by Rituximab

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Complete medium</th>
<th>Rituximab (μg/ml)</th>
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<tbody>
<tr>
<td>Complete medium</td>
<td>0 ± 3.1</td>
<td>9.82 ± 1.7</td>
</tr>
<tr>
<td>CDDP (1 μg/ml)</td>
<td>9.1 ± 2.0</td>
<td>32.3 ± 3.9</td>
</tr>
<tr>
<td>Fludarabine (20 μM)</td>
<td>0 ± 4.5</td>
<td>36.6 ± 6.2</td>
</tr>
<tr>
<td>Vinblastine (0.1 μg/ml)</td>
<td>12.4 ± 3.6</td>
<td>37.5 ± 3.1</td>
</tr>
<tr>
<td>Adriamycin (1 μg/ml)</td>
<td>6.5 ± 1.1</td>
<td>28.6 ± 1.4</td>
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* All combination treatments are significantly more cytotoxic than single agents alone, P < 0.05.
* Cytotoxicity/growth inhibition assays were performed using the XTT assay. Tumor cells were seeded at a concentration of 10,000 per well in 96-well culture plates. Cells were treated with Rituximab (20 μg/ml), chemotherapeutic drugs, or a combination of both for 24 h. Drugs and concentrations were as follows: CDDP (1 μg/ml), fludarabine (20 μM), vinblastine (0.1 μg/ml), and Adriamycin (1 μg/ml). CDDP, Adriamycin, and vinblastine were added to 2F7 cells simultaneously with Rituximab when treated in combination. Fludarabine was added 6 h after addition of Rituximab. Cells grown in complete medium alone served as the baseline. After 24 h of treatment, the XTT assay was used to quantify cytotoxicity/growth inhibition in each sample.
* Values shown are means of triplicates ± SD from XTT assays after 24 h incubation.

Fig. 1. Rituximab down-regulates secretion of IL-10 in 2F7 lymphoma cells. The 2F7 cells were seeded at 10^5 per ml and left untreated or treated with Rituximab (20 μg/ml). The plates were then incubated at 37°C in CO2 incubator, and the supernatants or total RNA was collected. A, cells from one untreated and one treated well were counted at 6, 12, 18, or 24 h. IL-10 secretion was determined by ELISA, and the amount of secretion was quantified using an ELISA kit. B, IL-10 samples compared with the untreated control (P < 0.05). C, RT-PCR amplification of IL-10 transcripts taken from 2F7 cDNA at 6, 12, 18, and 24 h revealed that Rituximab inhibits the transcription of the IL-10 gene, as compared with the untreated cells, which showed IL-10 increase over time.
IL-10 (min.)

Control 0 5 10 15

STAT3P

STAT3

Actin

Fig. 2. IL-10 induces activation of STAT3. 2F7 tumor cells were cultured in six-well plates (10^5/ml) and serum starved overnight (15 h) in RPMI containing 0.1% FCS. The cells were collected, pelleted, and resuspended in IL-10 (1 ng/ml) for 5, 10, or 15 min. Control 2F7 cells were left untreated. The cells were lysed and immunoblotted for phosphorylated STAT3, unphosphorylated STAT3, and actin. Treatment with IL-10 is shown to induce phosphorylation of STAT3 protein as early as 5 min. However, no change in STAT3 protein expression was observed. IL-10 was shown to induce activation of STAT3 phosphorylation as soon as the 5-min mark. Densitometric analysis of the Western blots was performed on a Power Mac computer using the public domain NIH Image program and illustrates the induction of phosphorylation and subsequent decrease to just above the control baseline.

STAT3 and STAT5 signaling pathways of the IFN-α/β receptors (49). The IL-10 receptor is a member of the IFN receptor family and signals through the same JAK/STAT pathway (50). We examined whether piceatannol, like Rituximab, also inhibited activation of STAT3 in our IL-10-dependent, STAT3-activated 2F7 tumor model. 2F7 tumor cells cultured in piceatannol (50 μM) for 24 h showed an inhibition of STAT3 phosphorylation (Fig. 3). Pulsing with IL-10 (1 ng/ml) after 24 h of piceatannol treatment induced very little STAT3 phosphorylation (Fig. 3). Pulsing with IL-10 (1 ng/ml) after 24 h of piceatannol treatment induced very little STAT3 phosphorylation (Fig. 3), further abrogated STAT3 DNA-binding activity. Saturating levels of exogenous IL-10 maintained STAT3 activated in anti-IL-10 samples (Fig. 4, Lane 5). IL-10 could

Rituximab Down-Regulates STAT3 Binding Activity in 2F7 Tumor Cells. As shown previously, Rituximab down-regulates the secretion of IL-10 in 2F7 tumor cells (Fig. 1). Considering that IL-10 is responsible for the phosphorylation state of STAT3, we sought whether Rituximab altered the ability of STAT3 to bind its DNA sequence. STAT3 activation was detected by electrophoretic mobility shift assays using 2F7 nuclear extracts and the SIE, which binds activated STAT3 (42). Treatments with Rituximab (20 μg/ml), neutralizing IL-10 antibody (1:10,000), piceatannol (50 μM), DMSO (equal volume as piceatannol), and exogenous IL-10 (1 ng/ml) were carried out for 24 h. DNA binding proved to be inhibited upon Rituximab treatment (Fig. 4, Lane 2) and could be reestablished with IL-10 (Fig. 4, Lane 3). Neutralizing IL-10 antibody (Fig. 4, Lane 4) and piceatannol (Fig. 4, Lane 6), which have been shown to inhibit STAT3 phosphorylation (Fig. 3), further abrogated STAT3 DNA-binding activity. Saturating levels of exogenous IL-10 maintained STAT3 activated in anti-IL-10 samples (Fig. 4, Lane 5). IL-10 could

Fig. 3. Constitutively secreted IL-10 initiates signaling of STAT3 and is inhibited by anti-IL-10 antibody and piceatannol. 2F7 tumor cells showed a constitutive activation of STAT3 after 24 h of culture in complete medium (Control), as analyzed by Western blotting. Cells serum starved (SS) for 24 h showed no STAT3 activation. Cells serum starved for 24 h and then pulsed with IL-10 (1 ng/ml) for 10 min induced STAT3 phosphorylation but not when pulsed with complete medium (C.M.). Cells cultured in complete medium and supplemented with IL-10 neutralizing antibody displayed no activation of STAT3 after 24 h. Piceatannol (Pic.) abrogated the constitutive activation of STAT3 over 24 h and also inhibited its activation when pulsed with IL-10 (1 ng/ml). The piceatannol solvent, DMSO, had no effect on constitutive or pulsed IL-10-mediated STAT3 phosphorylation.

Fig. 4. STAT3 DNA binding is inhibited by Rituximab, IL-10 neutralization, and piceatannol treatment. To analyze STAT3 binding inhibition, electrophoretic mobility shift assays were performed with P32-labeled SIE probe and nuclear lysates from 2F7 cells treated with Rituximab (20 μg/ml), anti-IL-10 antibody (1:10,000), piceatannol (50 μM), DMSO (same volume as piceatannol), or IL-10 (1 ng/ml) for 24 h as described in “Materials and Methods.” Constitutive DNA binding of activated STAT3 was down-regulated by Rituximab and abrogated by both neutralizing IL-10 antibody and piceatannol. In the presence of IL-10 coculturing for 24 h, STAT3 activation was restored in Rituximab and anti-IL-10 treated cells. Piceatannol continued to inhibit STAT3 activation, even in the presence of exogenous IL-10. DMSO had no effect on constitutive or exogenous IL-10-mediated STAT3 binding. The final lane shows cold probe, at 10X the concentration of labeled probe, was able to out compete STAT3 binding in control lysates.

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not activate STAT3 in the presence of piceatannol (Fig. 4, Lane 7), because the mechanism of inhibition of piceatannol has been shown to involve blocking of JAK1 association with the IL-10 receptor downstream (49). DMSO had no effect on STAT3 activation beyond control levels (Fig. 4, Lanes 8 and 9). The final lane shows that cold probe at 10× the concentration of labeled probe was able to out compete STAT3 binding in control lysates. These findings demonstrate that Rituximab treatment of 2F7 cells inhibits STAT3 binding to DNA and inhibits transcription of corresponding gene products.

Bcl-2 Overexpression Is Inhibited by Hindering STAT3 Phosphorylation.

It has been reported that Bcl-2 expression is under the control of several transcription factors, including STAT3 (39, 53–57). Several putative STAT3 DNA binding sites are present in the Bcl-2 promoter. Thus, we hypothesized that Bcl-2 expression in 2F7 cells may be attributable to the control of STAT3. To correlate the level of Bcl-2 expression with STAT3 activation, protein levels were determined by Western blotting over a 24-h time period at 6-h intervals in the presence of varying treatments, i.e., complete medium alone (control), Rituximab (20 μg/ml), anti-IL-10 (1:10,000), piceatannol (50 μM), or DMSO (same volume as piceatannol).

As expected, STAT3 phosphorylation was inhibited by treatment with Rituximab, anti-IL-10, and piceatannol (Fig. 5). STAT3 phosphorylation in untreated (medium) cells was observed at 12 h and seen to increase at 18 and 24 h. The Rituximab-treated cells also exhibited STAT3 phosphorylation at 12 h with subsequent increases at 18 and 24 h but were not as highly phosphorylated as control cells. The levels of STAT3 were not affected. The levels of Bcl-2 increased as a function of time in medium-treated cells but did so to a lesser extent when treated with Rituximab. The anti-IL-10 and piceatannol-treated cells inhibited Bcl-2 overexpression as well, seen most notably at 12, 18, and 24 h. This demonstrates that Bcl-2 overexpression is dependent on IL-10 secretion by 2F7 cells. Because IL-10 secretion augments with time of culture (Fig. 1A), Bcl-2 expression does well. When comparing the kinetics and levels of Bcl-2 to STAT3 phosphorylation (Fig. 5), a good correlation was noted between their levels of expression under controls and treatments. Control samples demonstrated that overexpression of Bcl-2 is observed by 12 h, which is also when STAT3 has been shown to be phosphorylated. Thus, Bcl-2 levels were decreased, as was inhibition of STAT3 phosphorylation by Rituximab and anti-IL-10 treatments. Complete inhibition of STAT3 phosphorylation by piceatannol resulted in significant inhibition of Bcl-2, albeit not totally, suggesting the existence of another mechanism of Bcl-2 regulation independent of STAT3 phosphorylation. Overall, the findings suggest that STAT3 activation by IL-10 results in the regulation of Bcl-2 via STAT3 binding sites.

**DISCUSSION**

Rituximab therapy has been used in NHL patients with much success (58, 59). However, 50% of patients still do not respond (60). We have shown that Rituximab and chemotherapeutic drugs can work in a synergistic manner to kill tumor cells (3, 6, 7). Knowing at least one pathway by which Rituximab can sensitize these tumor cells can lead to ways in which to improve its efficacy. Our laboratory has reported recently on the down-regulation of both IL-10 and Bcl-2 by Rituximab treatment in ARLs, leading to their sensitization to chemotherapeutic drugs (6). We present findings here that delineate a signaling pathway in which Rituximab decreases the transcription and secretion of IL-10 in 2F7 tumor cells, thus interrupting the IL-10 autocrine/paracrine loops that signal through the STAT3 signaling pathway. The reduction of endogenous IL-10 decreases the constitutive STAT3 activation seen in these cells and subsequently decreases the overexpression of Bcl-2.

STAT3 has been found constitutively activated in many types of tumor cells (35–37). This led to the belief that it is somehow involved in the persistence of tumor growth or onset of oncogenesis. Studies investigating such theories have revealed that STAT3 can be responsible for the up-regulation of antiapoptotic factors such as Bcl-2 and Bcl-xL (39, 56, 57, 61), demonstrating that by one or more mechanisms, functional STAT3 serves as an antiapoptotic factor.

STAT3 is also known to be the primary initiator of transcription in the IL-10 signal transduction pathway. Because Rituximab treatment down-regulates IL-10 and Bcl-2 but not other proteins, such as IL-6, tumor necrosis factor-α, Bcl-xL, Bad, Bax, and p53 (6), we proceeded to investigate whether the down-regulation of Bcl-2 by Rituximab treatment was attributable to a consequence of the down-regulation of IL-10 and inhibition of STAT3 activation. Evidence is presented that IL-10 triggers the activation of STAT3 and that the inhibition of tumor-derived IL-10 by Rituximab correlates with the inhibition of STAT3 activation, as observed by gel shift analysis (Fig. 4). Activation of STAT3 is clearly inhibited by anti-IL-10 antibody and piceatannol as well. These findings demonstrate the importance of tumor-derived IL-10 in the constitutive signaling of the STAT3 transcription factor in the ARL microenvironment. To further illustrate the role of endogenous IL-10, the inhibition of its signaling by either Rituximab, anti-IL-10, or piceatannol not only correlated with decreased STAT3 phosphorylation but also with the demise in Bcl-2 expression. The Bcl-2 protein level was not completely abrogated because STAT3 is not believed to be the only factor involved in Bcl-2 expression. It is, however, shown to be the main reason Bcl-2 is constitutively up-regulated in these cells.
The Bcl-2 gene promoter is poorly characterized for STAT3 binding sites and activity. Using luciferase assays, Stephanou et al. (57) demonstrated that STAT3 and CT-1, a STAT3 activator, both increased the Bcl-2 promoter activity in a pre-B-cell line. This would indicate that STAT3-responsive elements do indeed exist within the promoter region. No promoter mapping was done in their study, however. Analysis of the Bcl-2 promoter sequence published by Seto et al. (62) revealed eight putative binding sites that matched the STAT3 consensus binding sequence of TTNNNNNAA. Which of these binding sites actively binds STAT3 and how many are important for transcription are currently unknown. Nevertheless, our findings demonstrate that STAT3 inactivation by Rituximab and activation by IL-10 results in the regulation of STAT3 binding to corresponding DNA sequences and, therefore, infers that STAT3 binding will also take place at binding sites on the Bcl-2 promoter and regulate Bcl-2 transcription.

With respect to Bcl-2 regulation in general, there is little information on the interaction between IL-10-mediated signaling and known Bcl-2 regulators, such as c-Myb, WT1, AML1/ETO, BCR-ABL, CREB, Ras, and Pax proteins (53–55, 63–66). Although STAT3 is shown to be the major contributor to Bcl-2 overexpression in 2F7 tumor cells, it does not exclude the involvement of other factors acting as intermediates. In 2F7 tumor cells, the up-regulation of Bcl-2 by IL-10 autocrine/paracrine loops has been shown to be specific for Bcl-2 because no other Bcl-2 family members tested, i.e., Bcl-xL, Bax, Bad, and Bid, were regulated (6). STAT3 has been shown to up-regulate c-myc levels (67); however, we have shown previously that Rituximab does not alter the expression of c-myc mRNA in these tumor cells (6). Because Rituximab inhibits STAT3 activation, it seems safe to assume that the effect of STAT3 on 2F7 tumor cells is independent of c-myc activity.

Work in our laboratory elucidated a novel mechanism involved in the sensitization of drug-resistant ARL tumor cells by the anti-CD20 antibody, Rituximab (6). The findings indicated that IL-10 autocrine/paracrine loops might be responsible for the up-regulation of Bcl-2, and that Rituximab disrupted this pathway by interfering with the transcription of tumor-derived IL-10 in these ARL cells. The loss of high Bcl-2 expression correlated with the reversal of drug resistance in the tumor cells.

The involvement of STAT3 in Bcl-2 up-regulation and subsequent resistance to drug-induced apoptosis makes the STAT3 pathway an ideal target for anticancer therapy. Inhibitors to this pathway can be used against tumor cells dependent on growth factors or protective factors regulated by STAT3. It has been shown that disruption of the JAK/STAT3 pathway is not detrimental to normal cells in vitro or in animal models (68, 69). Moreover, STAT3 inhibition in some tumor cell systems induces cell death (38). This may be attributable to the fact that disruption of STAT3 affects the signal transduction networks that serve to provide tumors with advantages for survival. Normal tissue may survive because of the redundancy of STAT signaling in normal processes, where advantages for outgrowth are not an issue. Further studies are necessary to investigate the systemic effects of STAT3 inhibitors, such as piceatannol, on patients with resistant or refractory lymphoma. Such inhibitors may serve a role such as drug adjuvants.

Anti-cytokine treatment appears to be another viable alternative in cancer treatment. Numerous studies have shown that tumor cells develop autocrine/paracrine cytokine loops that provide stimulation for growth, survival, and drug resistance (17, 70, 71). Furthermore, many studies demonstrate that IL-10 is increased in the serum of many NHL patients and that this increase correlates to a lower rate of survival (72–74). Blay et al. (72), for example, reported in their study of 153 patients with a history of NHL that 46% of patients with active NHL had detectable IL-10 serum levels, but that only 6% of patients in remission had detectable IL-10 in their serum. Of their 60 healthy volunteers, no one had detectable serum IL-10. Edelman et al. (74) reported a comparable percentage (44%) of patients with detectable IL-10 in people with ARL, as opposed to none in patients without NHL. These data corroborate our findings that demonstrate that IL-10 is a survival and resistant factor in ARL. Reported exceptions exist. Fluckiger et al. (75) demonstrated that IL-10 induced apoptosis in B-cell chronic lymphocytic leukemia. However, said patients were not reported to have elevated levels of serum IL-10, for which anticytokine therapy could be applied. Fluckiger went on to state that none of the B-NHL or hairy cell leukemia samples apoptosed in the presence of IL-10, thus showing that IL-10-induced cell death was B-cell chronic lymphocytic leukemia specific. If and how IL-10 provides a survival advantage in patients with certain B-cell malignancies is still not clear, but in vitro evidence suggests that some tumor B-cells may become more resistant to chemotherapy in vivo. Immune therapy to neutralize cytokines or cytokine receptors may decrease the ability of these factors to confer survival. Kim et al. (76) showed that IL-15 mutant cytokine/Fcγ2a fusion protein competitively blocked normal IL-15 induced proliferation as well as STAT signaling. Such an approach, in combination with therapeutic drugs, may increase the efficacy of drug treatment. Antisense therapy against cytokines, such as IL-10, or known targets of STAT3 transcription offer yet another alternative, as in the case of Bcl-2 antisense therapy used to sensitize tumors to chemotherapy in melanoma and small cell lung cancer (77–80).

On the basis of our previous and current findings, we propose a model (Fig. 6) whereby, in ARL cells, Rituximab inhibits the transcription and secretion of tumor-derived IL-10. Endogenous IL-10 forms autocrine/paracrine loops that trigger IL-10 receptors to activate STAT3 transcription factors. By inhibiting IL-10 secretion, Rituximab causes the decrease in constitutively activated STAT3. Because STAT3 is shown here to be responsible for the high levels of Bcl-2, the decrease in activated STAT3 results in the suppression of the Bcl-2 overexpression in these cells. Without the overexpression of Bcl-2, the phenotypes of the ARL tumor cells become less resistant to apoptotic stimuli, including the effects of chemotherapeutic drugs.

**Fig. 6.** Proposed mechanism of Bcl-2 down-regulation by Rituximab in AIDS-related lymphoma cells. We diagram a mechanism whereby the targeting of CD20 by Rituximab inhibits IL-10 production by ARL cells. This reduction in IL-10 production disrupts IL-10 autocrine/paracrine loops, which initiate STAT3 activation through the IL-10 receptor. Thus, constitutive levels of activated STAT3 diminish and cease to drive the overexpression of Bcl-2, in effect, making the tumor cells more susceptible to apoptotic stimuli.
In conclusion, our findings demonstrate that Bcl-2 overexpression is attributable in large part to STAT3 activation by IL-10 autocrine/paracrine loops in these ARL tumor cells. Additionally, Rituximab is shown to decrease Bcl-2 protein in a manner that correlates with its ability to inhibit both IL-10 secretion and inhibition of STAT3 phosphorylation. By taking advantage of the several points of signaling between the CD20 stimulation of Rituximab and the downstream regulation of Bcl-2, specific interventions may enhance and augment its ability to sensitize NHLs to drugs.

ACKNOWLEDGMENTS

We extend our gratitude to Drs. Renato Aguilera and Otoniel Martinez-Maza for valuable input and support. Special thanks and appreciation goes to Dr. Hermes Garbán for advice and technical support on the electrophoretic mobility shift assays. We also thank Stephanie Louie for preparation of the manuscript.

REFERENCES


Rituximab Inactivates Signal Transducer and Activation of Transcription 3 (STAT3) Activity in B-Non-Hodgkin's Lymphoma through Inhibition of the Interleukin 10 Autocrine/Paracrine Loop and Results in Down-Regulation of Bcl-2 and Sensitization to Cytotoxic Drugs

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Cancer Res 2001;61:5137-5144.