Cancer Therapy: Preclinical

Regulation of CD20 in Rituximab-Resistant Cell Lines and B-cell Non-Hodgkin Lymphoma

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Abstract

Purpose: The aim of this research was to further investigate the contribution of CD20 antigen expression to rituximab activity and define the mechanisms responsible for CD20 downregulation in rituximab-resistant cell lines (RRCL).

Experimental Design: Rituximab-sensitive cell lines, RRCL, and primary neoplastic B cells were evaluated by chromium-51 release assays, ImageStream image analysis, immunohistochemical staining, flow cytometric analysis, CD20 knockdown, promoter activity, chromatin immunoprecipitation (ChIP) analysis of CD20 promoter, and CD20 plasmid transfection experiments to identify mechanisms associated with CD20 regulation in RRCL.

Results: RRCL exhibited a gradual loss of CD20 surface expression with repeated exposure to rituximab. We identified a CD20 antigen surface threshold level required for effective rituximab-associated complement-mediated cytotoxicity (CMC). However, a direct correlation between CD20 surface expression and rituximab-CMC was observed only in rituximab-sensitive cell lines. CD20 promoter activity was decreased in RRCL. Detailed analysis of various CD20 promoter fragments suggested a lack of positive regulatory factors in RRCL. ChIP analysis showed reduced binding of several key positive regulatory proteins on CD20 promoter in RRCL. Interleukin-4 (IL-4) induced higher CD20 promoter activity and CD20 expression but modestly improved rituximab activity in RRCL and in primary B-cell lymphoma cells. Forced CD20 expression restored cytoplasmic but not surface CD20, suggesting the existence of a defect in CD20 protein transport in RRCL.

Conclusions: We identified several mechanisms that alter CD20 expression in RRCL and showed that, whereas CD20 expression is important for rituximab activity, additional factors likely contribute to rituximab sensitivity in B-cell lymphoma. Clin Cancer Res; 18(4); 1039–50. ©2012 AACR.

Introduction

The incorporation of rituximab in the management of non-Hodgkin lymphoma (NHL) has improved the response rate, progression-free survival (PFS), and overall survival (OS). In aggressive lymphoma, patients who do not respond to rituximab-combined chemotherapy in the first-line setting represent a group of patients with a very poor clinical outcome (1, 2).

The primary mechanisms of action of rituximab include antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CMC), and induction of apoptosis and antiproliferation (3). The acquisition of phenotypic changes in cancer cells or host immune cells over time may affect rituximab responsiveness and underscores the complexity of the potential mechanisms of resistance to anti-CD20 monoclonal antibodies (mAb).

Both Martin and colleagues (4) and Gisselbrecht and colleagues (2) have individually reported that the use of rituximab as first-line therapy in patients with diffuse large B-cell lymphoma results in selection for cells more resistant to subsequent salvage regimens, indicating that prior rituximab is changing the biology of relapsed/refractory B-cell NHL (B-NHL). Downregulation of CD20 has been observed in a number of case reports of patients with relapsed/refractory B-cell lymphoma who became unresponsive to rituximab-based therapies and has been postulated to be one of the most important etiologies contributing to rituximab resistance (5–8). Because this has not been extensively studied in a large number of paired patient samples, it is unknown whether CD20 is the most important, if not limiting factor, in the context of rituximab resistance.
Translational Relevance

Rituximab resistance is a clinical problem that has arisen as a result of the incorporation of rituximab in the management of B-cell lymphoma. The incidence of rituximab resistance may significantly increase in the near future following the adoption of prolonged rituximab maintenance schedules by a significant percentage of practicing oncologists. CD20 downregulation has been reported to be associated with a decrease in rituximab sensitivity in B-cell non-Hodgkin lymphoma. However, many other alterations, such as altered proapoptotic versus antiapoptotic molecules, have been reported to contribute to rituximab resistance. It is currently unknown whether CD20 expression is the major factor responsible for the decrease in the biologic activity of rituximab. Our current work investigated by several approaches, the contribution of CD20 to rituximab sensitivity in a well-defined rituximab-resistant cell line model. We have identified reduced CD20 promoter activity and a defect in CD20 transport as two novel mechanisms responsible for CD20 downregulation in rituximab-resistant cell line.

To further define the mechanisms of resistance to rituximab, our group of investigators generated and fully characterized several rituximab-resistant cell lines (RRCL; refs. 9, 10). We observed several changes in RRCL, notably downregulation of surface CD20 antigen and CD20 mRNA and upregulation of complement-inhibitory proteins (CIP), CD55 and CD59. Concurrent deregulation of proapoptotic (Bax/Bak) and antiapoptotic (Mcl-1, Bcl-XL) proteins was observed and associated with concomitant chemotherapy resistance (9, 10).

Forced expression of CD20 in a CEM-T cell line model revealed a direct correlation between increasing surface CD20 expression and augmented rituximab-CMC, but not ADCC (11). Similar results were observed when primary chronic lymphocytic leukemia (CLL) and lymphoma samples were studied (11).

Several investigators have studied potential mechanisms that may play a role in CD20 expression changes observed in previously rituximab-treated relapsed/refractory B-cell lymphoma. For instance, Terui and colleagues found CD20 gene mutations leading to C-terminus truncated forms of CD20 in 22% of patients with rituximab-relapsed/refractory lymphomas (12). Beers and colleagues (13) and Beum and colleagues (14) showed CD20 internalization into lysosomes or “shaving” following rituximab exposure, respectively. In this report, we identify 2 novel mechanisms that regulate CD20 expression in rituximab-refractory lymphoma cells, study the chronologic changes of surface CD20 in the context of acquired resistance to rituximab, and further evaluate the role of surface CD20 density in the biologic activity of rituximab.

Our data suggest that, whereas CD20 levels are important in rituximab sensitivity at an early stage, as lymphoma cells are repeatedly exposed to rituximab, additional gains (i.e., upregulation of Mcl-1, Bcl-XL, CIPs) or losses (downregulation of Bax and Bak) of other proteins further impair the antitumor activity of rituximab alone or in combination with chemotherapeutic agents.

Materials and Methods

Cell lines

The generation of RRCLs from rituximab-sensitive cell lines (RSCL) and the preresistant passages was done as previously described (9, 10) and summarized in Supplementary Table S1. Final resistant cell lines (RRCL, tenth passage) and prior passages were used for the experiments described later. Single-cell clones from RSCL and RRCL were further obtained by limiting dilution.

Primary B-cell isolation from patient specimens

Malignant B cells were isolated by magnetic-activated cell sorting (MACS; negative selection) from biopsy tissue obtained from patients with B-NHL under Roswell Park Cancer Institute (Buffalo, NY) nontherapeutic protocols I-42804 and I-42904 (Supplementary Table S2). B-cell purity was assessed by flow cytometry using antibodies to CD19 and CD20 (Becton Dickinson). Greater than 95% pure CD19-positive cells were obtained from each biopsy processed. Collected cells were resuspended in media and used in corresponding experiments.

Chromium-51-release assay

Rituximab-CMC was determined as previously described. (9, 10) Briefly, viable lymphoma cells labeled with chromium-51 at 37°C, 5% CO2 for 2 hours were plated in 96-well plates at a cell concentration of 1 × 10^5 cells per well. Cells were then exposed to rituximab or isotype and human serum (1:4 dilution) for 6 hours at 37°C and 5% CO2. 51Cr-release was measured from the supernatant by standard gamma counter. The percentage of lysis was calculated as follows: [test cpm – background cpm]/[maximum cpm – background cpm]. Pooled human serum was obtained from healthy donors (Institutional Review Board-approved protocol CIC-016).

ImageStream analysis

Surface CD20 expression density was analyzed by the ImageStream Technology (Amnis Inc.). In brief, 2 × 10^6 cells were stained with anti-human CD20-FITC or isotype control and then fixed by 2% paraformaldehyde. Cells were illuminated in the ImageStream system by a bright-field lamp and a 488-nm excitation laser for fluorescein isothiocyanate (FITC). Data were analyzed by ImageStream IDEAS image analysis software. Cells were gated for single, focused populations, which were then analyzed for the mean cell size (by mean surface area) and mean surface CD20 expression (by mean CD20-FITC intensity). Mean surface CD20 density was calculated as...
follows: mean surface CD20-FITC intensity/mean surface area (CD20-FITC per μm²)

**CD20 knockdown**

Forty-eight hours after transfection, using CD20 siRNA duplexes (On-TARGET plus SMARTpool siRNA; Dharmacon/Thermo Scientific) or siRNA control (Qiagen), cells were harvested and subjected to a second transfection using similar experimental conditions. Forty-eight hours after the second transfection, cells were harvested for CD20 expression analysis by immunoblotting, ImageStream, and rituximab-CMC by standard \(^{51}\)Cr-release assay.

**CD20 promoter activity analysis by luciferase assay**

The PCR fragments of CD20 promoter were amplified by PCR from both RSCL and RRCL genomic DNA by several pairs of specific primers (Supplementary Table S3), ligated individually into the polyclinker of pGL3 basic vector (Promega), before being cotransfected with a pEF-RL vector to monitor transfection efficiency (Nucleofector kit V, Lonza). The empty pGL3 basic vector was used to determine the baseline activity. Transfected cells were cultured in complete medium for 48 hours before conducting luciferase assay (Promega). In addition, differences in expression of CD20-positive transcription regulatory factors were evaluated by immunoblotting.

**Chromatin immunoprecipitation assay**

Cells from RSCL and RRCL were cross-linked and lysed before chromatin was sheared to 200 to 500 bp using Fisher Sonic Dismembrator (Model 300). Immunoprecipitation was conducted with Magna chromatin using Fisher Sonic Dismembrator (Model 300). Immuno-lysed before chromatin was sheared to 200 to 500 bp. Immunoprecipitation assay was performed by Sigma-Aldrich; anti-Bak (610982) was from BD Biosciences; and IRF-4 (F-4) x sc-48338X and normal mouse immunoglobulin G (IgG):sc-2025 were from Santa Cruz Biotechnology, Inc.; anti-Bax (B5897) was from Sigma-Aldrich; anti-Bax (B5897) was from Sigma-Aldrich; anti-Bak (610982) was from BD Biosciences; and IRF-4 (F-4) x sc-48338X and normal mouse immunoglobulin G (IgG):sc-2025 were from Santa Cruz Biotechnology. Normal rabbit IgG was from Cell Signaling. CD20-FTC, CD55-FTC, CD59-FTC, and FITC mouse IgG1 isotype control were from BD Biosciences. Rituximab (anti-CD20) and trastuzumab (anti-Her-2/neu, as isotype control) were from Genentech Inc.

**Statistics**

All the in vitro experiments were repeated in triplicate, and the results were reported as the mean with SE determined by SPSS. Significant differences were calculated by Student t test. P values < 0.05 were considered statically significant.

**Results**

Repeated exposure to rituximab led to a gradual reduction of CD20 expression during the development of RRCL

RRCL were generated by exposing sensitive cells to escalating doses of rituximab for 24-hour time periods in the presence or absence of human serum. The process required RSCL to be exposed 10 times to rituximab with or without human serum, at the end, 3 cell lines were isolated: RL4RH, Raji2R, and Raji4RH with a rituximab-resistant phenotype. CD20 surface levels that were further characterized (9, 10). To determine the timing and cumulative dose of rituximab necessary to negatively affect CD20 expression, changes in CD20 surface expression were studied by Western blotting and ImageStream analysis in Raji, Raji4RH, and various pre-Raji4RH passages. At the same time, we correlated rituximab-CMC versus surface CD20 expression in RSCL and RRCL.

Overall, there was 70% reduction in total CD20 expression in RRCLs even at very early stages in the process of acquiring resistance to rituximab. Significant CD20 downregulation was observed as early as in pre-Raji4RH passage 4 (Fig. 1A). Moreover, there was a gradual...
reduction in surface CD20 density, as determined by the unit of CD20-FITC per \( \mu \text{m}^2 \) (Fig. 1A). Raji had surface CD20 density of approximately 300 CD20-FITC per \( \mu \text{m}^2 \). As Raji cells were exposed to 8 increasing doses of rituximab (pre-Raji4RH passage 8), the surface CD20 density decreased by 50% (150 CD20-FITC per \( \mu \text{m}^2 \)). Raji4RH (tenth and final cell passage) was found to have 67% decrease in surface CD20 density (100 CD20-FITC per \( \mu \text{m}^2 \); Fig. 1B). Parallel to the changes in CD20 surface expression, we found a gradual decrease in rituximab-CMC. Rituximab activity decreased by approximately 33% after 5 cycles of rituximab exposure (pre-Raji4RH passage 5), by 60% in pre-Raji4RH cell passage 8, and by 90% in Raji4RH cells (Fig. 1C). There was a direct correlation between the degree of CD20 downregulation and rituximab-CMC (Fig. 1D). Similar results of CD20 reduction and a corresponding loss of rituximab-CMC were seen in Raji cells exposed to rituximab without human serum (Raji 2R; data not shown).

**Additional factors influence rituximab-CMC in RRCL**

Previously, we showed that RRCL have other phenotypic changes leading to cross-resistance to other mAbs (upregulation of CD55 and CD59) or chemotherapy agents (downregulation of Bax/Bak; refs. 9, 10). Whereas we found a linear correlation between rituximab-CMC and CD20 downregulation as lymphoma cells acquired resistance to rituximab, other factors may have contributed significantly to the acquisition of rituximab resistance. To this end, we isolated single-cell clones from the heterogeneous populations of RSCL (Raji and RL) and the RRCL (Raji4RH, Raji2R, and RL4RH) by limiting dilution and expansion. Single-cell clones were verified by ImageStream data (data not shown).

Differences were observed between single-cell clones of RSCL and RRCL in terms of surface CD20 expression, cell size, Bel-2 family member levels, and *in vitro* responsiveness to rituximab. Cell clones isolated from Raji2R cells had a larger size, lower surface CD20 expression (Fig. 2A), and lower levels of Bax/Bak than did parental Raji cells (Supplementary Fig. S1). Furthermore, taking into consideration both changes in size and CD20 surface expression, the decrease in mean surface CD20 density in Raji2R was magnified when compared with Raji cells (Fig. 2B). As previously noted, Raji2R cells were essentially resistant to rituximab-CMC.

Using ImageStream studies, we defined a threshold of CD20 antigen density expression required for efficient rituximab-CMC at approximately 150 CD20-FITC/\( \mu \text{m}^2 \). A direct correlation between surface CD20 density and rituximab-CMC was observed in Raji but not in Raji2R cell...
clones, suggesting the existence of additional factors influencing rituximab activity in RRCL. CD20 antigen density ranged from 150 to 300 CD20-FITC/μm² in cell clones isolated from Raji cells, and rituximab-CMC exhibited a linear correlation with CD20 levels. In contrast, most of the cell clones isolated from Raji2R cells had a much lower CD20 antigen density (50 to 150 CD20-FITC/μm²) and minimal rituximab-CMC activity (Fig. 2B).

We further compared the degree of rituximab-CMC between cell clones isolated from Raji (RSCL) and Raji2R (RRCL) with equivalent levels of CD20 antigen. In some subclones, CD20 antigen levels appeared primarily responsible for rituximab-CMC (e.g., Raji clone 6 and Raji2R). On the other hand, several RSCL and RRCL clones exhibited similar responsiveness to rituximab at different CD20 levels (Raji clone 6 vs. Raji2R clones 3, 4, or 10). Moreover, several pairs of Raji subclones with similar CD20 expression levels showed variable responsiveness to rituximab (Raji clone 3 vs. 20 and 16; 21 vs. 5 and 7; and 2 vs. 14). These data suggest the existence of other factors affecting rituximab activity (Fig. 2B).

To evaluate whether the expression of CIPs, CD55 and CD59, could account for the discrepancies between CD20 expression and rituximab-CMC in Raji clones, we determined their surface expression levels by ImageStream. There were differential surface expression levels of both CIPs within Raji single-cell clones (Fig. 2C and D). Raji clones 3 and 7, in particular, expressed higher CD59 than their pair counterparts, clones 16 and 21, respectively. This potentially explained why even though these 2 pairs of clones expressed a similar surface CD20 density, significant differences in rituximab-CMC sensitivity were seen.

While overexpression of CIPs could explain the resistant phenotype observed, we previously showed that RRCL are lysed by mAbs targeting CD52 (alemtuzumab; ref. 17). This observation further supports a cause and effect between CD20 expression and a decrease in rituximab activity observed in our in vitro lymphoma model.

Overall, we observed that rituximab-CMC is dependent on CD20 expression in RSCL and may be further affected by the surface expression of CIPs. In RRCL, it appears that CIPs may play a more dominant role. However, CD20 levels observed were below the threshold level for rituximab activity in RRCL clones.

**Transient CD20 knockdown in RSCL negatively affected rituximab-CMC**

To further investigate the significance of CD20 expression in rituximab activity and to investigate whether additional mechanisms might be involved in the process of acquired resistance to rituximab, we knocked down endogenous CD20 in RSCL (Raji) using the On-TARGETplus SMART-pool siRNA against CD20 (M5A41), which consisted of 4 siRNA duplexes. Cell lysates from untransfected Raji, Raji2R, as well as mock- and CD20 siRNA-transfected Raji were analyzed for endogenous expression of CD20.

CD20 siRNA-transfected Raji cells exhibited 60% decrease in endogenous CD20 when compared with untransfected...
and mock-transfected Raji cells (Fig. 3A–C). Despite a significant reduction of surface CD20 antigen (i.e., comparable with levels observed in Raji2R cells), CD20 siRNA-transfected Raji cells had only a 15% decrease in rituximab-CMC when compared with rituximab-CMC in Raji control cells (Fig. 3C). Together these data suggest that CD20 antigen expression contributes partially to rituximab-CMC and that additional factors such as CIPs likely affect the biologic activity of rituximab.

Mechanisms that regulate CD20 expression in RRCL

Subsequently, we investigated potential mechanisms responsible for the global downregulation of CD20 in RRCL. Previously, we showed that the repeated exposure of lymphoma cells to rituximab led to a decrease in CD20 mRNA (9). In contrast with what has been previously reported (12), we did not find mutations in the CD20 gene to explain changes observed at the mRNA and protein level (data not shown).

mRNA half-life is similar between RSCL and RRCL

CD20 mRNA is a balance between transcription and decay rates. However, using actinomycin-D to stop endogenous gene transcription, we did not observe differences in the mRNA half-life of CD20 between Raji and Raji2R or Raji4RH cells (data not shown).

RRCL exhibit a decrease in the CD20 promoter activity when compared with RSCL

We next tested the possibility that reduced CD20 promoter activity was responsible for lower CD20 mRNA in the RRCL. No mutations were found in the gene sequence of the cloned CD20 promoter (−968/+53) from each RRCL (data not shown), indicating that lower CD20 mRNA levels in RRCL were not due to the mutation of the CD20 promoter. On the other hand, a relatively lower CD20 promoter activity in RRCL was observed (Fig. 4B), suggesting that a lower CD20 promoter activity in RRCL might be a contributing factor to reduced CD20 expression in rituximab-resistant lymphomas.

Three major positive regulatory binding sites for CD20 promoter have been identified for positive regulatory factors such as USF (which binds to E-box), PU.1/IRF-4, and Oct-2 (18–21); on the other hand, negative regulators remain unknown. To further dissect the underlying cause for lower CD20 promoter activity in RRCL, we investigated whether a particular region of the CD20 promoter was responsible for the overall decrease in CD20 promoter activity observed in RRCL. Different CD20 promoter fragments encompassing various lengths of the CD20 promoter were cloned into pGL3 vector and cotransfected with pEF-RL (Fig. 4A). Differences in promoter activity between the constructs transfected into RSCL and RRCL showed that all the 3 positive regulatory sites on CD20 promoter (E-box, PU.1/pip site, and BAT box) were required for its maximum activity. CD20 promoter activity significantly decreased as the CD20 promoter constructs increased from 300 to 400 pairs, suggesting the existence of a potential negative regulatory site present within this region of the CD20 promoter (Fig. 4C).

When CD20 promoter activity of each construct was compared between RSCL and RRCL, we observed decreased CD20 promoter activity in RRCL regardless of the CD20 promoter fragment analyzed (Fig. 4C). However, the analysis of changes in expression of USF, PU.1, IRF-4, and Oct-2 between RSCL and RRCL revealed no significant changes as determined by Western blotting (data not shown). As we further compared the association status of these transcription factors with CD20 promoter in RSCL and RRCL by ChIP assay, we observed an approximately 50% reduction in binding of PU.1 (but not IRF-4), Oct-2, USF-1, and USF-2.
to CD20 promoter in both Raji2R and Raji4RH, as compared with Raji (Fig. 4D). Our findings suggest alternative mechanisms, such as the methylation status of the promoter affecting the binding of positive transcription factors, and acetylation of known transcription factors that regulated CD20 promoter activity.

**CD20 promoter activity and surface CD20 expression can be enhanced by IL-4 in RRCL and in primary tumor cells derived from patients with B-NHL lymphoma**

The use of IL-4 has been shown to modulate the level of CD20 expression in malignant cells isolated from patients with CLL (22). Subsequently, we investigated whether CD20 expression level in RRCL could be modulated by IL-4. Of interest, total and surface CD20 expression increased in RRCL following IL-4 exposure in vitro (Fig. 5A).

We further investigated whether IL-4-induced higher CD20 expression in RRCL was associated with effects on CD20 promoter activity. RRCL cotransfected CD20 promoter fragments carrying vector and pEF-RL were incubated with IL-4 or control for 48 hours. "In vitro" exposure of RRCL to IL-4 increased the CD20 promoter activity in RRCL (Fig. 5B).

Moreover, 51Cr-release assay showed a 1.5- to 2-fold increase in rituximab-CMC in RRCLs exposed to IL-4 (Fig. 5C).
Although this result suggests that IL-4 is an attractive strategy to improve CD20 antigen expression and rituximab activity in RRCL, the absolute percentage of rituximab-CMC following IL-4 exposure was only one tenth of rituximab antitumor activity observed in RSCL. As noted before, it is possible that a specific CD20 surface expression threshold is required to fully restore rituximab sensitivity (Fig. 2B).

We further investigated whether IL-4 could modulate CD20 expression and rituximab activity \textit{in vitro} in primary tumor cells isolated from patients with B-cell lymphoma (Fig. 5D). \textit{Ex vivo} IL-4 exposure of primary tumor cells isolated from 6 patients with lymphoma induced CD20 expression that correlated with an improvement in rituximab-CMC. Alternatively, IL-4 did not have an effect on CD20 expression and rituximab-CMC in 7 other specimens of patients with NHL. As patients represented different tumor histologies and disease status (e.g., \textit{de novo} vs. relapsed/refractory), it is difficult to conclude any strong correlation between the observed effects of IL-4 and our preliminary results (Supplementary Table S2).

Reexpression of CD20 expression level in RRCL revealed a potential defect in CD20 protein transport to the cell surface

Finally, we force expressed CD20 in RRCL in an attempt to restore rituximab activity. CD20-carrying BCMGSneo vector was transfected into RRCL. Subsequently, CD20 expression was determined by Western blotting and quantitative flow cytometry and correlated to rituximab activity. Western blot analysis showed that CD20-BCMGSneo-transfected Raji2R cells overexpressed cytoplasmic CD20 (6-fold increase) to similar levels as seen in Raji cells (Fig. 6A). However, \textsuperscript{51}Cr-release assay showed no significant increase in rituximab-CMC (<5% increase in CMC; Fig. 6B). Of interest, while CD20 forced expression was successful in Raji2R as determined by Western blotting, no significant changes in surface CD20 antigen were noted in CD20-BCMGS neo-transfected Raji2R cells. Detailed flow cytometric analysis showed that the majority of the CD20-transfected Raji2R failed to express CD20 on the cell surface despite adequate transfection and CD20 synthesis in the cytoplasmic compartment (Fig. 6C). HeLa and Jurkat T cells, which do not express endogenous CD20, and Raji were used as controls to verify whether this transport defect was specific to RRCL (Supplementary Fig. S2). Unlike Raji2R, which expressed maximum total CD20 by Western blotting 4 days posttransfection, HeLa cells had maximum CD20 expression on day 2, whereas Jurkat T cells failed to express CD20 up to 2 days posttransfection. Detailed analysis of the percentage of cells expressing surface CD20 by flow cytometry showed that HeLa cells had higher percentages of cells expressing surface CD20 than Raji2R (38% vs. 2%).

Figure 5. IL-4 associated increase in intracellular and surface CD20 is associated with enhanced CD20 promoter activity and an increase in rituximab-CMC. Cells were incubated with IL-4 (5 ng/mL) and harvested at indicated time points for total CD20 expression by Western blot analysis and surface CD20 expression by flow cytometry (A). Cells were incubated with RPMI-1640-10 media alone, or with 5 ng/mL IL-4 for 48 hours before cells were lysed for CD20 promoter activity analysis in RRCL by luciferase assay (B) or used to analyze rituximab-CMC by \textsuperscript{51}Cr-release assay (C). B-NHL cells were isolated by negative selection and treated with 5 ng/mL of IL-4 for 48 hours before being subjected to flow cytometric analysis for CD20 expression and \textsuperscript{51}Cr-release assay for rituximab-CMC (D). Ctrl, control; MFI, mean fluorescence intensity.
were unable to further increase CD20 expression in Raji, likely due to an inability to further upregulate CD20 expression regulatory factors, which were operating at maximum levels (Supplementary Fig. S2). This finding suggests the existence of a defect in the transport of CD20 onto the cell surface in RRCLs.

In summary, our data show that surface CD20 antigen expression plays a role in rituximab-CMC. We provide data from several experiments studying the possible mechanisms leading to CD20 downregulation in RRCL and the impact that CD20 expression has on rituximab activity. Whereas reduced CD20 promoter activity appeared to account for the relatively lower CD20 mRNA expression, an altered CD20 protein transport may also contribute to the global downregulation of CD20 in RRCLs. Our data stress the existence of a defect in the transport of CD20 onto the cell surface in RRCLs.

Discussion

The development of rituximab resistance is an emerging clinical problem, and its frequency may increase as a consequence of repeated exposure to rituximab, especially over prolonged periods of time (e.g., maintenance programs) in B-cell lymphoma. A formal study of the frequency of phenotypic changes occurring in rituximab-resistant lymphoma is needed to better understand this clinical concern. The Rituximab Extended Schedule or Retreatment (RESORT) trial is an ongoing Eastern Cooperative Oncology Group clinical study aiming to define the optimal duration of treatment with rituximab as a single agent in patients with indolent CD20– B-cell lymphoma and low tumor burden (23). This study will potentially provide important information on the incidence of rituximab resistance and the phenotypic changes occurring in rituximab-resistant low-grade lymphomas.

Downregulation of CD20 has been observed in patients with relapsed/refractory B-cell lymphoma following prior rituximab therapy (5–7). On the basis of initial studies, it appears that CD20 surface expression is a limiting step for rituximab-CMC, but not ADCC (11). In our model, we observed that the repeated exposure of RRCL to rituximab led to CD20 downregulation and a decrease in the biologic activity of rituximab as determined by standard apoptosis, CMC, and ADCC assays (9). From the laboratory standpoint, CMC assays are easily reproducible with fewer confounding variables than ADCC or apoptosis assays and thus are a valuable tool with which to study the activity of rituximab in vitro. Detailed examination of cell passages showed an incremental loss in CD20 surface expression and corresponding rituximab-CMC activity. Furthermore, we defined a CD20 surface expression threshold level necessary for effective rituximab-CMC.

Given the multiple mechanisms of action of rituximab (i.e., CMC, ADCC, and apoptosis), the overall clinical relevance of variable CD20 surface antigen expression on lymphoma cells in vivo is difficult to ascertain. Whereas CD20 levels appear to play an important role in rituximab-CMC, they appear to be less relevant for rituximab-ADCC. Our group and other investigators have shown that immune effector cells and ADCC play a pivotal role in the antitumor activity of mAbs, including rituximab (24, 25). RRCL exhibited not only resistance to rituximab-CMC but also rituximab-mediated calcium influx, apoptosis, and ADCC (9, 10). It is possible that a gradual decrease in CD20 antigen expression may not affect rituximab-ADCC at early stages, but once CD20 levels drop below a certain threshold (i.e., 150 CD20-FITC/μm²), the capacity of the innate immune cells to kill rituximab-coated lymphoma cells may also be significantly impaired. Our findings are clinically relevant because novel mAbs capable of effectively inducing anti-CD20 CMC or ADCC at even low CD20 levels may be an attractive therapeutic strategy by which to kill rituximab-resistant lymphoma.

Cancer cells develop multiple phenotypic changes in response to selective pressure; therefore, additional alterations besides CD20 downregulation likely contribute to rituximab resistance. Our data not only suggest that CD20 expression plays an important role but also suggest the existence of additional factors affecting rituximab-associated biologic activity (e.g., CIPs). Preresistant cell passages
showed a gradual loss of CD20 expression that directly correlated with a gradual loss of rituximab-CMC, and single-cell clones obtained from RSCL (but not RRCL) exhibited a linear correlation between CD20 expression and rituximab-CMC.

We previously identified additional phenotypic changes in RRCL that can impair rituximab activity. Similar to the findings from the study by Jazirehi and colleagues (26), our study showed that RRCL had a deregulation of the ubiquitin-proteasome system and in Bcl-2 family members led to cross-resistance to chemotherapy agents (9, 10). In addition, we showed differential expressions of CIPs (CD55 and CD59) within the RSCL populations, which together with surface CD20 modulated rituximab-associated activity. This observation could potentially extrapolate to a selective elimination of rituximab-sensitive clones (CD20<sup>hi</sup>, CD55<sup>lo</sup>, CD59<sup>lo</sup>), finally leading to RRCL with altered phenotypes (CD20<sup>lo</sup>, CD55<sup>hi</sup>, CD59<sup>hi</sup>). Additional studies are necessary to define the relative contribution of each of these factors to rituximab resistance in B-cell lymphoma.

The regulation of CD20 expression in normal and malignant B cells is poorly defined. A limiting factor in understanding CD20 regulation is the absence of immunologic defects in CD20-knockout mouse models (27). Recently, a group of investigators reported the case of a pediatric patient lacking CD20 expression in B cells showing signs and symptoms compatible with immunodeficiency, which has alerted the scientific community to renew studies about the function and regulation of CD20 in normal B-cell differentiation (28).

In contrast with the report from Terui and colleagues, which showed a point mutation in CD20 gene in some patients with rituximab-resistant lymphoma (12), we found no CD20 gene mutations affecting the primary structure of CD20. Moreover, no changes in CD20 mRNA stability were found in RRCL. On the other hand, we identified 2 additional mechanisms that negatively regulate CD20 in RRCLs and lead to a decreased rituximab activity: a lower CD20 promoter activity and/or defect impairing the transport of CD20 to the cell surface.

ChIP analysis showed 50% reduction in association of PL1, USF, and Oct-2 with CD20 promoter in RRCL, potentially explaining reduced CD20 promoter activity in RRCL. Pharmacologic strategies to increase CD20 promoter activity can potentially lead to clinically applicable strategies to restore rituximab sensitivity in rituximab-resistant lymphoma. The use of IL-4 has been shown to modulate CD20 expression in various B-cell lymphoma cell lines (22). In our RRCL, in vitro exposure to IL-4 increased CD20 promoter activity and CD20 antigen expression and enhanced rituximab activity. Similar findings were found in a subset of primary tumor cells isolated from patients with B-cell lymphoma. IL-4-mediated increase in CD20 promoter activity was observed in CD20 promoter fragments containing positive or negative regulatory binding sites, suggesting that IL-4 effects may be mediated by altering the binding status of these regulatory transcription factors. Ongoing studies are seeking to explore differences in the methylation status of the CD20 promoter between RSCL and RRCL that could affect the binding of transcription factors and thereby CD20 expression.

Surface protein transport can be regulated by general or specific factors. These include chaperone proteins that ensure proper protein folding (29–33); the presence of endoplasmic reticulum (ER) retention signals, which need to be cleaved before protein can be transported out of ER (34, 35); posttranslational modification such as glycosylation and phosphorylation, which ensure proper folding and structure stability (36); and/or protein-specific accessory proteins in the ER before surface transport (35, 37, 38). In B cells, the requirement for IgM surface protein expression has been well defined (35, 39, 40). Specifically, the presence of the heterodimers of the coreceptors, Igα and Igβ, has been found to be required for IgM to be transported from ER to the surface transport of normal B cells (35). Similarly, the proper assembly of MHC class I and II molecules requires calnexin and calreticulin accessory protein (38, 41, 42). It is unknown whether any accessory proteins are required for CD20 surface expression. Thus, future research is focused on delineating factors responsible for CD20 transport defects.

In summary, our data suggest that several factors contribute to acquired resistance to rituximab. For the first time, we link an abnormal CD20 promoter activity and/or a defect in the Golgi-to-surface protein transport with a decrease in CD20 expression and resistance to rituximab in B-cell lymphoma. Whereas CD20 antigen expression plays a role in rituximab resistance in B-cell lymphoma, additional factors likely affect anti-CD20 activity (e.g., differences in Bcl-2 family proteins, CIPs, etc.). On the basis of our preclinical studies, it appears that surface CD20 expression may play a role in rituximab sensitivity at early stages of resistance. As lymphoma cells gain additional genetic/phenotypic changes, the role of CD20 expression in rituximab biologic activity may be less relevant at later stages of rituximab resistance. A better understanding of the mechanisms that regulate CD20 antigen expression has the potential to someday contribute to the development of novel therapeutic strategies to overcome rituximab resistance in patients with B-cell lymphoma.

Disclosure of Potential Conflicts of Interest

M.S. Chazquezman has served on advisory boards and has received clinical research support from Genentech, BiogenIdec, and GSK Pharmaceuticals. No potential conflicts of interests were disclosed by other authors.

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