Biochemical and Biophysical Research Communications 390 (2009) 48–53

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Escape mechanisms from antibody therapy to lymphoma cells: Downregulation of *CD20* mRNA by recruitment of the HDAC complex and not by DNA methylation

Takumi Sugimoto [a], Akihiro Tomita [a,*], Junji Hiraga [a,b], Kazuyuki Shimada [a], Hitoshi Kiyoi [c], Tomohiro Kinoshita [a], Tomoki Naoe [a]

[a] Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, Nagoya, Japan
[b] Department of Hematology, Toyota Memorial Hospital, Toyota, Japan
[c] Department of Infectious Diseases, Nagoya University School of Medicine, Nagoya, Japan

ARTICLE INFO

Article history:
Received 15 September 2009
Available online 19 September 2009

Keywords:
CD20
Rituximab
Epigenetics
Histone deacetylases
DNA methytransferases

ABSTRACT

Although rituximab is a critical monoclonal antibody therapy for CD20-positive B-cell lymphomas, rituximab resistance showing a CD20-negative phenotypic change has been a considerable clinical problem. Here we demonstrate that *CD20* mRNA and protein expression is repressed by recruitment of a histone deacetylase protein complex to the *MS4A1* (*CD20*) gene promoter in CD20-negative transformed cells after treatment with rituximab. *CD20* mRNA and protein expression were stimulated by decitabine (5-Aza-dC) in CD20-negative transformed cells, and was enhanced by trichostation A (TSA). Immunoblotting indicated that DNMT1 expression was first downregulated 1 day after treatment with 5-Aza-dC, but IRF4 and Pu.1, the transcriptional regulators of *MS4A1*, were still expressed with or without 5-Aza-dC. Interestingly, CpG methylation of the *MS4A1* promoter was not observed in CD20-negative transformed cells without 5-Aza-dC. A chromatin immunoprecipitation (ChIP) assay indicated that the Sin3A–HDAC1 co-repressor complex was recruited to the promoter and dissociated from the promoter with 5-Aza-dC and TSA, resulting in histone acetylation. Under these conditions, IRF4 and Pu.1 were continually recruited to the promoter with or without 5-Aza-dC and TSA. These results suggest that recruitment of the Sin3A–HDAC1 complex is related to downregulation of CD20 expression in CD20-negative B-cells after treatment with rituximab.

Introduction

Rituximab is the first therapeutic monoclonal antibody targeting human malignant tumors, and is now an indispensable molecular-targeting drug for CD20-positive B-cell lymphomas [1–3]. Although the effectiveness is significant, resistance to rituximab has also become a considerable problem [4].

Several mechanisms of the resistance have been suggested, including loss of CD20 protein expression after rituximab use [5–12] and CD20 gene mutations [13]. Furthermore, other mechanisms have also been suggested [4] such as internalization of CD20 protein [14], interference with accessibility of rituximab to CD20 protein by inhibitory factors, rapid metabolism of the antibody, abnormalities in B-cell signaling in tumor cells [15], abnormalities of apoptosis [16], antibody-dependent cell-mediated cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC) [17].

Very recently, we reported observation of downregulation of CD20 protein expression in CD20-positive B-cell lymphoma patients after treatment with rituximab-containing combination chemotherapies [6,7]. In those cases, it was strongly suggested that aberrant downregulation of *MS4A1* expression was closely related to the loss of CD20 protein expression, and that expression of CD20 and rituximab sensitivity were partially restored by some molecular-targeting drugs [6,7]. Although these findings suggest that epigenetic mechanisms, in part, contribute to the downregulation of CD20 expression, the molecular mechanisms are still not clear. Furthermore, a recent report indicated that reduced CD20 protein expression in *de novo* diffuse large B-cell lymphoma is associated with a poor survival rate [18]. Thus, understanding the mechanisms of downmodulation of CD20 protein expression is likely to be very important from both basic research and clinical viewpoints.

In this report, we show that the recruitment of a histone deacetylase (HDAC) co-repressor complex to the *MS4A1* promoter

* Corresponding author. Address: Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, Tsurumai-Cho 65, Showa-ku, Nagoya 466-8560, Japan. Fax: +81 52 744 2161.
E-mail address: atomita@med.nagoya-u.ac.jp (A. Tomita).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.09.059

region, but not DNA methylation [19], is involved in CD20-negative phenotypic changes in B-cell lymphoma cells after treatment with rituximab. We show that the complex dissociated from the promoter in the presence of a DNA methyltransferase (DNMT) inhibitor and a HDAC inhibitor [20], resulting in partial restoration of CD20 expression.

Materials and methods

Cell culture conditions and treatment with epigenetic drugs. RRBL1 [6], Raji, and NALM6 cells were cultured in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO, USA) with 10% fetal calf serum. Five-Aza-dC (5-aza-2′-deoxycytidine; Sigma, St. Louis, MO) and TSA (Sigma) at final concentrations of 100 μM and 100 nM, respectively, were added directly to the culture medium.

Immunoblotting. Cells ($\sim 5 \times 10^5$) were lysed in 100 μl of lysis buffer (50 mM Tris–HCl, pH 8.0, 1.5 mM $MgCl_2$, 1 mM EGTA, 5 mM KCl, 10% glycerol, 0.5% NP-40, 300 mM NaCl, 0.2 mM PMSF, 1 mM DTT, and a complete mini protease inhibitor tablet (Roche)). After centrifugation at 10,000 *g* for 10 min, the supernatants were placed in new tubes, and 100 μl of 2× SDS sample buffer was added. After boiling for 5 min, samples were separated with SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Immunoblotting was carried out as described previously [21,22] using anti-CD20, -IRF4, -Pu.1, -GAPDH antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-DNMT1 antibody (Abcam, Cambridge, MA, USA).

RNA preparation and reverse transcriptase-polymerase chain reaction (RT-PCR). RNA from cell lines (1×10^5 cells) was obtained using Trizol (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was prepared as reported previously [7,22].

For RT-PCR, the following primers were designed: CD20-U; 5′-ATGAAAGGCCCTATTGCTATG-3′, CD20-L; 5′-GCTGGTTCACAGTTGTATATG-3′, β-actin-U; 5′-TCACTCATGAAGATCCTCA-3′, and β-actin-L; 5′-TTCGTGGATGCCACAGGAC-3′. Semi-quantitative RT-PCR with AmpliTaq Gold was performed as described previously [6].

Methylation status of the MS4A1 promoter. To examine the methylation status, bisulfite sequencing was performed. Genomic DNA was prepared with a QIAamp DNA Blood Mini kit (Qiagen, Valencia, CA, USA). Bisulfite treatment was performed using EpiTect Bisulfite kits (Qiagen). After bisulfite treatment, PCR of the *MS4A1* promoter was performed using the specific primers as follows, MS4A1-pro-MSPU; 5′-GGTAGTATGAGTATGTTAGGTAGTT-3′, MS4A1-pro-MSPL; 5′-TTTTCCTTACCTAAATCTCCAAAA-3′. PCR fragments were cloned into a pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced.

Flow cytometry (FCM) analysis. Cell surface antigens of RRBL1 with or without 5-Aza-dC and TSA treatment were analyzed using a BD FACSCalibur Flow Cytometer (BD Bioscience, Franklin Lakes, NJ, USA) with anti-CD20 antibody (Leu-16 PE, BD) and mouse IgG1 κ isotype control (PE-Cy7, BD).

Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was performed as described previously [22,23]. For immunoprecipitation (IP), the following antibodies were used; anti-Pu.1, -IRF4 (Santa Cruz Biotechnology), -acetylated H4 (Millipore, Billerica, MA, USA), -Sin3A, and anti-HDAC1 (Abcam) antibodies. Immunoprecipitated DNA was used for semi-quantitative PCR using LA-Taq polymerase (TAKARA, Ohtsu, Japan). The following primers for the *MS4A1* promoter and 3′-intron sequence (negative control) were used; CD20pro-U; 5′-CTAAAAGTGAAGCCAGAAGG-3′, CD20pro-L; 5′-GGAGGGTGAGTGGTGTAGT-3′, CD20-3′U; 5′-GCTGACCTCACATAACTCCT-3′, CD20-3′L; 5′-GAAATCCCTCAGACTCAGAC-3′.

Immunoprecipitation (IP) assay. The IP assay was carried out as described previously [22]. Whole cell lysate was obtained from RRBL1 cells (1×10^7) using 800 μl of lysis buffer. After adding 800 μl of lysis buffer without NP-40 and NaCl, the lysate was divided into four tubes (400 μl each) and IP using anti-IRF4, -Sin3A, and -HDAC1 antibodies was performed. The precipitated samples were applied to SDS–PAGE followed by immunoblotting. For the pre-IP samples, 5% of the whole cell lysate was used.

Results

CD20 protein and mRNA expression were stimulated by treatment with 5-Aza-dC in CD20-negative transformed cells

As we reported previously [6,7], the downregulation of CD20 protein and mRNA expression has been observed in some CD20-positive B-cell lymphoma patients after treatment with rituximab-containing chemotherapies. We also reported that the downregulation of CD20 expression was partially stimulated by treatment with the epigenetic drugs 5-Aza-dC and TSA. RRBL1 cells were established from a patient with B-cell lymphoma who showed a CD20-negative phenotypic change after treatment with rituximab [6]. To examine the mechanisms of stimulation of CD20 expression by 5-Aza-dC, we examined the protein expression pattern that may affect CD20 gene transcription in RRBL1 cells. RRBL1 cells were treated with 5-Aza-dC for 24 h, and were then washed and incubated for up to 7 days (Fig. 1A). During this procedure, the cells were harvested several times as indicated and analyzed using semi-quantitative RT-PCR and immunoblotting (IB) (Fig. 1B). *CD20* mRNA and protein expression were stimulated by 5-Aza-dC, and the peak of expression was observed around day 3 after treatment with 5-Aza-dC (lane 6). After day 5, CD20 protein expression had gradually decreased. DNMT1 depletion was confirmed at 24 h after treatment with 5-Aza-dC (lane 4) as reported previously [24]. IRF4 and Pu.1 are transcription factors that interact with the *MS4A1* promoter and regulate CD20 expression [25]. IRF4/Pu.1 was almost constantly expressed throughout the 5-Aza-dC treatment duration (lanes 3–8), but only a modest upregulation was observed after treatment with 5-Aza-dC around day 2 (lane 5). These results suggested that DNMT1 depletion by 5-Aza-dC may be related to stimulation of *MS4A1* expression.

DNA methylation status of the MS4A1 promoter

To explain the activation of *CD20* mRNA and protein expression after treatment with 5-Aza-dC in RRBL1 cells, we next examined the CpG methylation status of the *MS4A1* promoter (Fig. 1C). Interestingly, CpG islands were not observed on the promoter region located ~5 kb upstream from the transcription start site, and only four CG sites were found on the promoter from the −1000 to +100 region. Bisulfite sequencing was carried out to confirm methylated CpG. As shown in Fig. 1C, no CpG methylation was observed on the three CpG sites around the transcription start site in RRBL1 cells. In NALM6 cells, a CD20-negative lymphoblastic leukemia cell line, several methylated CpGs were observed. Furthermore, the same analysis was performed using primary tumor cells from a patient suffering from CD20-negative transformed B-cell lymphoma after treatment with rituximab-containing combination chemotherapies. (Detailed information about this patient is described in our previous paper as UPN3 [7]). The three CpG sites were not methylated, as observed in RRBL1 cells (Fig. 1C, UPN3). These results suggest that transcriptional activation of *MS4A1* by 5-Aza-dC may not be regulated by its promoter CpG demethylation in RRBL1 cells.

Histone deacetylase inhibitor TSA enhances CD20 expression by 5-Aza-dC in CD20-negative transformed cells

Next, we analyzed the effect of a HDAC inhibitor in addition to 5-Aza-dC on *MS4A1* expression in RRBL1 cells. CD20 protein expression in RRBL1 cells was confirmed using immunoblotting

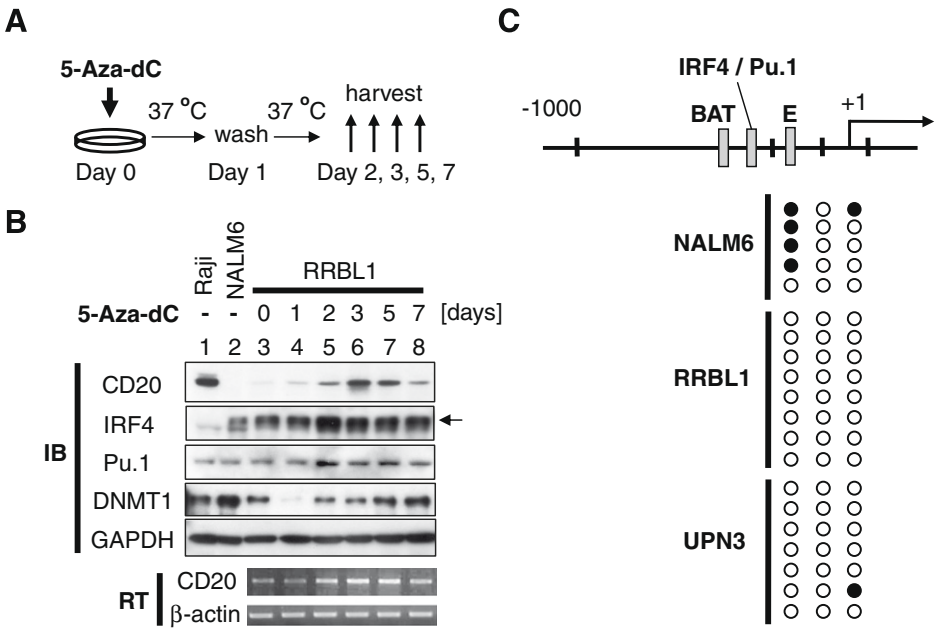

Fig. 1. CD20 protein and mRNA expression were transiently stimulated by treatment with a DNMT inhibitor. (A) Schematic representation of 5-Aza-dC treatment of the CD20-negative transformed B-lymphoma cells. RRBL1 cells were incubated at 37 °C for 24 h, and then washed twice with RPMI medium with 10% FCS without 5-Aza-dC. Cells were further incubated for up to 7 days, and were harvested at days 1, 2, 3, 5, and 7. (B) Protein expression was examined using immunoblotting (IB) with the indicated antibodies. The mRNA expression level was determined using semi-quantitative RT-PCR (RT). The black arrow indicates the band for IRF4. Raji and NALM6 cells were used as positive and negative controls, respectively. GAPDH and β-actin were measured as internal controls. (C) The structure of the *MS4A1* promoter near the transcription start site (from −1000 to +100) is depicted. The BAT-box, IRF4/Pu.1 binding sites, and E-box are shown as shaded boxes. Only four CpG sites, which are putative methylation sites, were found and are shown as black vertical bars. The methylation status of the three CpG sites around the transcription start site in NALM6, RRBL1, and primary B-lymphoma cells that show CD20-negative transformation was analyzed with bisulfite sequencing. Five to eight clones were analyzed from each sample. Black and open circles indicate methylated and non-methylated CpGs, respectively.

and flow cytometry (FCM) (Fig. 2A and B) following treatment with 5-Aza-dC and/or TSA. When RRBL1 cells were treated with 5-Aza-dC or TSA alone, minimal activation of CD20 protein expression was observed using immunoblotting (Fig. 2A, lanes 4 and 5) and FCM (Fig. 2B, 5-Aza-dC). In the presence of 5-Aza-dC and TSA, CD20 protein expression was significantly increased (Fig. 2A, lane 6, and B, 5-Aza-dC + TSA). These results suggested that *MS4A1* expression is, in part, regulated by epigenetic mechanisms such as histone modification including lysine acetylation, rather than DNA CpG methylation of the *MS4A1* promoter.

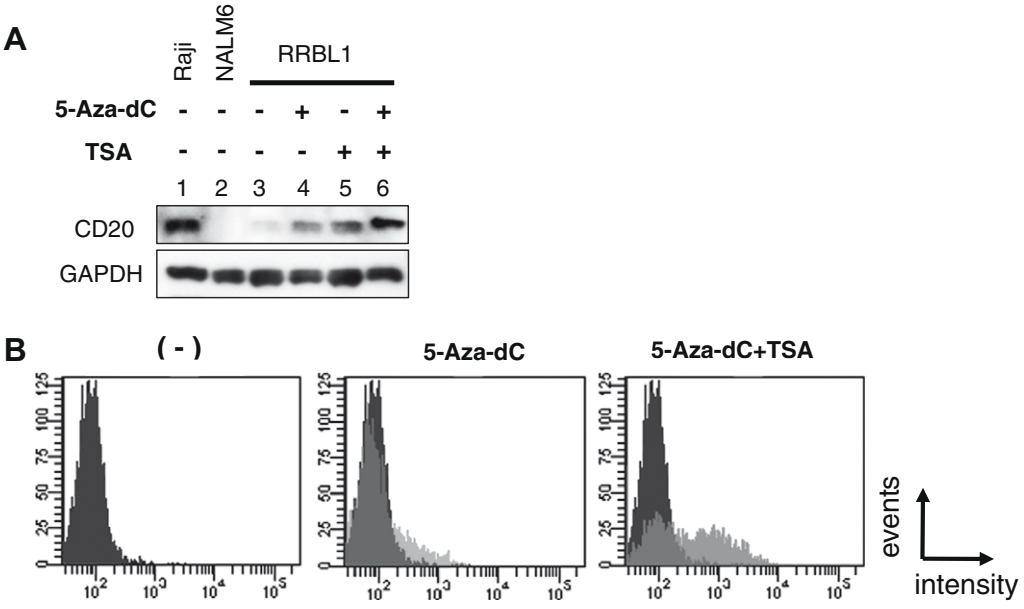

Fig. 2. CD20 protein expression by 5-Aza-dC was enhanced by TSA. CD20 protein expression was shown with IB (A) and FCM (B) with or without epigenetic drugs. For 5-Aza-dC treatment, RRBL1 cells were incubated with 5-Aza-dC for 24 h followed by washing and two additional days of incubation. TSA was added at the start of day 3, and cells were incubated for 24 h. If cells were not treated with 5-Aza-dC, washing was also carried out at day 1 to adjust the incubation conditions. All the cells were harvested at day 4 and utilized for IB and FCM analyses. The untreated and treated cells with the epigenetic drugs were depicted as black and gray areas, respectively (B).

Recruitment of co-repressor proteins and histone deacetylation on the MS4A1 promoter in the absence of epigenetic drugs

To study the molecular mechanisms of transcriptional repression of *MS4A1*, a ChIP assay was performed. RRBL1 cells were incubated with or without 5-Aza-dC and TSA, and a ChIP assay was carried out using anti-Pu.1, -IRF4, -Sin3A, -HDAC1, and -acetylated-histone H4 antibodies. After IP, precipitated genomic DNA was utilized in semi-quantitative PCR using primers for the *MS4A1* promoter (Fig. 3A) and the 3′-intron sequences as a negative control. IRF4 and Pu.1 interactions were consistently observed on the promoter region, but not on the 3′-intron region (Fig. 3B, lanes 5–8). Sin3A and HDAC1, which form a transcription repressor protein complex [26], interacted with the promoter region only in the absence of 5-Aza-dC and TSA (Fig. 3B, lanes 11 and 13). Acetylated-histone H4 was observed at the promoter region with 5-Aza-dC and TSA, but the acetylation was decreased in the absence of the two drugs (lane 9). In the 3′-intron region, histone acetylation was consistently observed with or without 5-Aza-dC and TSA. These results strongly suggest that the Sin3A–HDAC1 co-repressor complex may be recruited to the *MS4A1* promoter through some transcription factors in the absence of epigenetic drugs, resulting in histone deacetylation and transcriptional repression. In addition, the recruitment may be dissociated from the promoter by adding 5-Aza-dC and TSA, resulting in histone acetylation and transcription activation.

The Sin3A–HDAC1 co-repressor complex is found in RRBL1 cells with or without epigenetic drugs

To show that loss of Sin3A–HDAC1 interaction with the *MS4A1* promoter was due to protein complex dissociation and not degradation, we confirmed the protein expression in RRBL1 cells with and without epigenetic drugs using IB. As shown in Fig. 4A, HDAC1 and Sin3A protein expression levels did not change in the presence of epigenetic drugs. Next, we performed an IP assay using anti-IRF4, -Sin3A, and -HDAC1 antibody to confirm that Sin3A and HDAC1 exist as a protein complex, and to examine whether the Sin3A–HDAC1 co-repressor complex was recruited by IRF4 in the absence of epigenetic drugs. The Sin3A–HDAC1 interaction was confirmed with an IP assay using anti-Sin3A and -HDAC1 antibodies (Fig. 4B, lanes 4 and 5), but interaction of IRF4 with this complex was not observed (lane 3). These results indicate that the Sin3A–HDAC1 complex exists in RRBL1 cells with or without 5-Aza-dC and TSA, and that the recruitment of the complex to the *MS4A1* promoter may not involve a direct interaction with IRF4.

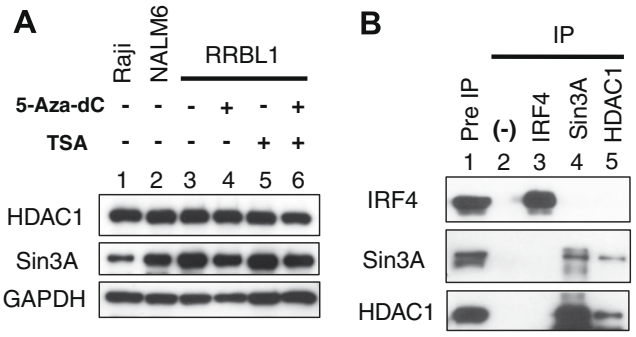

Fig. 4. The Sin3A–HDAC1 co-repressor complex is stably expressed in RRBL1 cells with or without 5-Aza-dC and/or TSA. (A) IB was performed using the RRBL1 lysate after treatment with 5-Aza-dC and/or TSA. Raji and NALM6 cells were used as expression controls. Similar levels of expression of HDAC1 and Sin3A were observed in each sample. (B) Whole cell lysate of RRBL1 cells was obtained using lysis buffer. Lysates were divided into four samples and used for IP using anti-IRF4, -Sin3A, and -HDAC1 antibodies. Five percent of the whole cell lysate was used for the pre-IP samples (lane 1). As a negative control, antibodies for IP were omitted (lane 2). IB indicated that endogenous Sin3A–HDAC1 interacted in RRBL1 cells without epigenetic drugs, but significant interaction with IRF4 was not observed in this assay system.

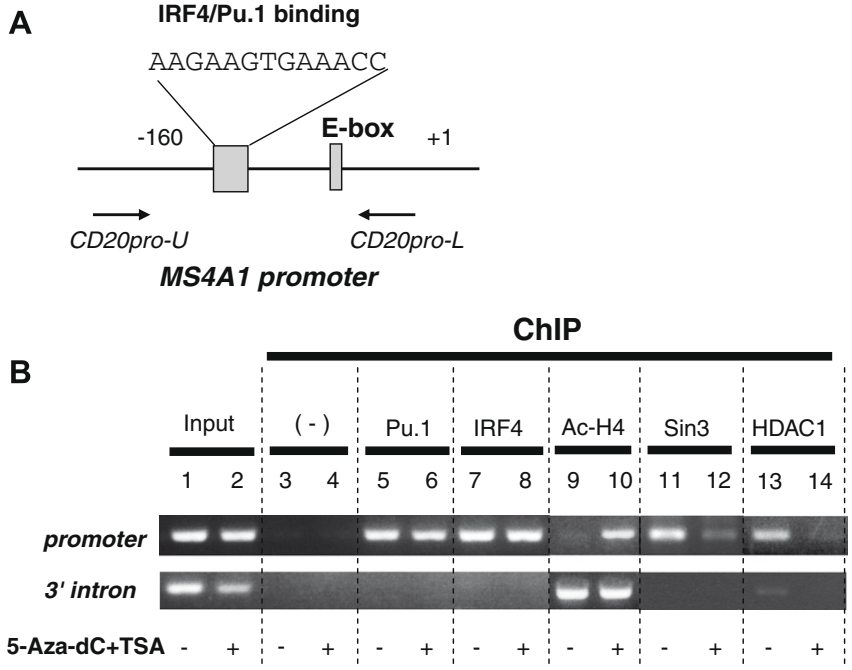

Fig. 3. ChIP assay of the *MS4A1* promoter. The primer set used for amplification of the *MS4A1* promoter (−160 to +1) is shown in (A). The positions of the upper and lower primers are indicated as black arrows. (B) A ChIP assay using anti-Pu.1, -IRF4, -Sin3A, -HDAC1, and -acetylated-histone H4 was performed using the cell lysate from cells treated with or without 5-Aza-dC and TSA. Semi-quantitative PCR was performed, and the amplified DNA fragments were visualized by 1.5% agarose gel electrophoresis. As a positive control, lysate without the IP step was used (input). A ChIP sample without antibodies was used as a negative control (−). PCR using the primers for the 3′-intron region of *MS4A1* was also used as a control. Sin3A and HDAC1 recruitment to the promoter was observed in lanes 11 and 13, and accumulation of histone deacetylation was seen in lane 9.

Discussion

In clinical practice, CD20 expression abnormalities have been reported. Johnson et al. [18] reported that 43 out of 272 (16%) patients with diffuse large B-cell lymphoma (DLBCL) showed reduced CD20 expression using FCM analysis at the time of initial diagnosis, and that the survival rate of this phenotype was significantly lower than that of patients with CD20-positive phenotype. Furthermore, we previously reported that a CD20-negative phenotypic change after using rituximab resulted in resistance to salvage chemotherapies with or without rituximab [6,7]. We observed that all of these patients died of disease progression within 1 year after the diagnosis of CD20-negative transformation, suggesting that the CD20-negative phenotype may be related to the poor prognosis. From these findings, we realized the importance of investigating the mechanisms of downmodulation of CD20 expression to explore overcoming strategies including salvage combination chemotherapies with anti-CD20 antibodies.

In this study, we firstly investigated the effect of 5-Aza-dC on RRBL1 cells. DNMT1 protein reduction was observed 1 day after adding 5-Aza-dC, followed by temporal upregulation of CD20 protein expression (Fig. 1B). This phenomenon suggested that CpG demethylation of the *MS4A1* promoter region was a result of DNMT1 depletion. But interestingly, significant CpG islands were not located at the promoter, suggesting that *MS4A1* activation by 5-Aza-dC was not regulated directly by *MS4A1* promoter methylation.

The next hypothesis we investigated was that expression of transcription factors, which is critical for *MS4A1* expression, was regulated by the methylation status of the promoter DNA. We analyzed the protein expression level of IRF4/Pu.1, and only a modest upregulation was observed. Furthermore, the ChIP assay showed that IRF4/Pu.1 recruitment to the *MS4A1* promoter was fairly stable in the presence or absence of 5-Aza-dC and TSA (Fig. 3B). On the other hand, Sin3A–HDAC1 recruitment and histone deacetylation was observed in the absence of epigenetic drugs. Because previous reports have indicated that HDACs form large protein complexes, such as Sin3 [26], NuRD/Mi-2 [27], and N-CoR/SMRT co-repressor complexes [26,28], and are recruited to the specific promoter by transcription factors, we analyzed whether the Sin3A–HDAC1 complex interacts with IRF4 in RRBL1 cells. Using an IP assay, we observed that HDAC1 interacts with Sin3A but not with IRF4 (Fig. 4B). We also analyzed the recruitment of the proteins N-CoR, HDAC3, and TBLR1 (transducin β-like protein 1 relating protein), which are all expressed in the same co-repressor complex *in vivo* [21–23,26,28], to the promoter region using the ChIP assay. Significant recruitment of these proteins was not seen in this assay (data not shown).

Thus, these findings suggest that, (1) *MS4A1* repression is not directly regulated by methylation of its promoter and (2) transcription factors other than IRF4 recruit the Sin3A–HDAC1 co-repressor complex to the *MS4A1* promoter to repress transcription through histone deacetylation. Our previous report [6] showed that treatment with TSA without 5-Aza-dC upregulates CD20 expression in RRBL1 cells within 1 day, suggesting that the activity of HDAC may be more critical for *MS4A1* expression than the activity of DNMTs. One explanation for why 5-Aza-dC can stimulate *MS4A1* expression is that the expression of some transcription factors, whose expression is critical for CD20 expression, may be regulated by CpG methylation of the gene promoters. The maximal effect of 5-Aza-dC on CD20 protein expression was seen at 3 days after treatment with 5-Aza-dC, which is consistent with this hypothesis. The knockdown of endogenous DNMT1 using the siRNA technique may help explain the importance of DNMT1 for *MS4A1* repression. On the other hand, the possibility that CpG islands in *MS4A1* that affect its expression are in a location that is relatively remote (~5 kb) from the transcription start site cannot be excluded. Further investigation is needed.

In our study, the efficiency of stimulating CD20 protein expression in CD20-negative transformed cells using epigenetic drugs is not complete (Fig. 2B). As we showed previously [7], this efficiency may not be sufficient to overcome resistance to rituximab. Using the newer generation humanized-anti-CD20 monoclonal antibodies, such as ofatumumab [29], GA-101 [30], and others, which have higher antibody binding capacity with CD20 and/or a higher CDC/ADCC activity, may help overcome the resistance. We also anticipate the use of those therapies in combination with epigenetic drugs such as HDAC and/or DNMT inhibitors. Further investigation is still needed.

Acknowledgments

This work is supported in part by a Grant-in-Aid for Cancer Research (21-6-3) from the Ministry of Health, Labor and Welfare, a Grant-in-Aid from the National Institute of Biomedical Innovation, and a Grant-in-Aid in Scientific Research (20591116) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. We thank Dr. Shinsuke Iida for providing critical information about IRF4. We also thank Akihide Atsumi, Tomoka Wakamatsu, Yukie Konishi, Mari Otsuka, Eriko Ushida, and Chieko Kataoka for valuable laboratory assistance.

References

[1] B.D. Cheson, Monoclonal antibody therapy for B-cell malignancies, Semin. Oncol. 33 (2006) S2–S14.
[2] B. Coiffier, E. Lepage, J. Briere, R. Herbrecht, H. Tilly, R. Bouabdallah, P. Morel, E. Van Den Neste, G. Salles, P. Gaulard, F. Reyes, P. Lederlin, C. Gisselbrecht, CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma, N. Engl. J. Med. 346 (2002) 235–242.
[3] B.D. Cheson, J.P. Leonard, Monoclonal antibody therapy for B-cell non-Hodgkin's lymphoma, N. Engl. J. Med. 359 (2008) 613–626.
[4] M.R. Smith, Rituximab (monoclonal anti-CD20 antibody): mechanisms of action and resistance, Oncogene 22 (2003) 7359–7368.
[5] M.S. Czuczman, S. Olejniczak, A. Gowda, A. Kotowski, A. Binder, H. Kaur, J. Knight, P. Starostik, J. Deans, F.J. Hernandez-Ilizaliturri, Acquirement of rituximab resistance in lymphoma cell lines is associated with both global CD20 gene and protein down-regulation regulated at the pretranscriptional and posttranscriptional levels, Clin. Cancer Res. 14 (2008) 1561–1570.
[6] A. Tomita, J. Hiraga, H. Kiyoi, M. Ninomiya, T. Sugimoto, M. Ito, T. Kinoshita, T. Naoe, Epigenetic regulation of CD20 protein expression in a novel B-cell lymphoma cell line, RRBL1, established from a patient treated repeatedly with rituximab-containing chemotherapy, Int. J. Hematol. 86 (2007) 49–57.
[7] J. Hiraga, A. Tomita, T. Sugimoto, K. Shimada, M. Ito, S. Nakamura, H. Kiyoi, T. Kinoshita, T. Naoe, Down-regulation of CD20 expression in B-cell lymphoma cells after treatment with rituximab-containing combination chemotherapies: its prevalence and clinical significance, Blood 113 (2009) 4885–4893.
[8] T. Sonoki, Y. Li, S. Miyanishi, H. Nakamine, N. Hanaoka, H. Matsuoka, I. Mori, H. Nakakuma, Establishment of a novel CD20 negative mature B-cell line, WILL2, from a CD20 positive diffuse large B-cell lymphoma patient treated with rituximab, Int. J. Hematol. 89 (2009) 400–402.
[9] I. Jilani, S. O'Brien, T. Manshuri, D.A. Thomas, V.A. Thomazy, M. Imam, S. Naeem, S. Verstovsek, H. Kantarjian, F. Giles, M. Keating, M. Albitar, Transient down-modulation of CD20 by rituximab in patients with chronic lymphocytic leukemia, Blood 102 (2003) 3514–3520.
[10] T. Kinoshita, H. Nagai, T. Murate, H. Saito, CD20-negative relapse in B-cell lymphoma after treatment with rituximab, J. Clin. Oncol. 16 (1998) 3916.
[11] T.A. Davis, D.K. Czerwinski, R. Levy, Therapy of B-cell lymphoma with anti-CD20 antibodies can result in the loss of CD20 antigen expression, Clin. Cancer Res. 5 (1999) 611–615.
[12] A.J. Ferreri, G.P. Dognini, C. Verona, C. Patriarca, C. Doglioni, M. Ponzoni, Re-occurrence of the CD20 molecule expression subsequent to CD20-negative relapse in diffuse large B-cell lymphoma, Haematologica 92 (2007) e1–e2.
[13] Y. Terui, Y. Mishima, N. Sugimura, K. Kojima, T. Sakurai, R. Kuniyoshi, A. Taniyama, M. Yokoyama, S. Sakajiri, K. Takeuchi, C. Watanabe, S. Takahashi, Y. Ito, K. Hatake, Identification of CD20 C-terminal deletion mutations associated with loss of CD20 expression in non-Hodgkin's lymphoma, Clin. Cancer Res. 15 (2009) 2523–2530.
[14] R. Lapalombella, B. Yu, G. Triantafillou, Q. Liu, J.P. Butchar, G. Lozanski, A. Ramanunni, L.L. Smith, W. Blum, L. Andritsos, D.S. Wang, A. Lehman, C.S. Chen, A.J. Johnson, G. Marcucci, R.J. Lee, L.J. Lee, S. Tridandapani, N. Muthusamy, J.C. Byrd, Lenalidomide down-regulates the CD20 antigen and antagonizes direct and antibody-dependent cellular cytotoxicity of rituximab on primary chronic lymphocytic leukemia cells, Blood (2008).

[15] A.R. Jazirehi, M.I. Vega, B. Bonavida, Development of rituximab-resistant lymphoma clones with altered cell signaling and cross-resistance to chemotherapy, Cancer Res. 67 (2007) 1270–1281.
[16] B. Bonavida, Rituximab-induced inhibition of antiapoptotic cell survival pathways: implications in chemo/immunoresistance, rituximab unresponsiveness, prognostic and novel therapeutic interventions, Oncogene 26 (2007) 3629–3636.
[17] T. van Meerten, R.S. van Rijn, S. Hol, A. Hagenbeek, S.B. Ebeling, Complement-induced cell death by rituximab depends on CD20 expression level and acts complementary to antibody-dependent cellular cytotoxicity, Clin. Cancer Res. 12 (2006) 4027–4035.
[18] N.A. Johnson, M. Boyle, A. Bashashati, S. Leach, A. Brooks-Wilson, L.H. Sehn, M. Chhanabhai, R.R. Brinkman, J.M. Connors, A.P. Weng, R.D. Gascoyne, Diffuse large B-cell lymphoma: reduced CD20 expression is associated with an inferior survival, Blood 113 (2009) 3773–3780.
[19] G. Egger, G. Liang, A. Aparicio, P.A. Jones, Epigenetics in human disease and prospects for epigenetic therapy, Nature 429 (2004) 457–463.
[20] C.B. Yoo, P.A. Jones, Epigenetic therapy of cancer: past, present and future, Nat. Rev. Drug Discov. 5 (2006) 37–50.
[21] A. Tomita, D.R. Buchholz, Y.B. Shi, Recruitment of N-CoR/SMRT-TBLR1 corepressor complex by unliganded thyroid hormone receptor for gene repression during frog development, Mol. Cell. Biol. 24 (2004) 3337–3346.
[22] A. Atsumi, A. Tomita, H. Kiyoi, T. Naoe, Histone deacetylase 3 (HDAC3) is recruited to target promoters by PML-RARalpha as a component of the N-CoR co-repressor complex to repress transcription in vivo, Biochem. Biophys. Res. Commun. 345 (2006) 1471–1480.
[23] A. Tomita, D.R. Buchholz, K. Obata, Y.B. Shi, Fusion protein of retinoic acid receptor alpha with promyelocytic leukemia protein or promyelocytic leukemia zinc finger protein recruits N-CoR-TBLR1 corepressor complex to repress transcription in vivo, J. Biol. Chem. 278 (2003) 30788–30795.
[24] K. Ghoshal, J. Datta, S. Majumder, S. Bai, H. Kutay, T. Motiwala, S.T. Jacob, 5-Aza-deoxycytidine induces selective degradation of DNA methyltransferase 1 by a proteasomal pathway that requires the KEN box, bromo-adjacent homology domain, and nuclear localization signal, Mol. Cell. Biol. 25 (2005) 4727–4741.
[25] A. Himmelmann, A. Riva, G.L. Wilson, B.P. Lucas, C. Thevenin, J.H. Kehrl, PU.1/Pip and basic helix loop helix zipper transcription factors interact with binding sites in the CD20 promoter to help confer lineage- and stage-specific expression of CD20 in B lymphocytes, Blood 90 (1997) 3984–3995.
[26] M.G. Rosenfeld, V.V. Lunyak, C.K. Glass, Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response, Genes Dev. 20 (2006) 1405–1428.
[27] S.A. Denslow, P.A. Wade, The human Mi-2/NuRD complex and gene regulation, Oncogene 26 (2007) 5433–5438.
[28] J. Li, J. Wang, Z. Nawaz, J.M. Liu, J. Qin, J. Wong, Both corepressor proteins SMRT and N-CoR exist in large protein complexes containing HDAC3, EMBO J. 19 (2000) 4342–4350.
[29] A. Hagenbeek, O. Gadeberg, P. Johnson, L.M. Pedersen, J. Walewski, A. Hellmann, B.K. Link, T. Robak, M. Wojtukiewicz, M. Pfreundschuh, M. Kneba, A. Engert, P. Sonneveld, M. Flensburg, J. Petersen, N. Losic, J. Radford, First clinical use of ofatumumab, a novel fully human anti-CD20 monoclonal antibody in relapsed or refractory follicular lymphoma: results of a phase 1/2 trial, Blood 111 (2008) 5486–5495.
[30] T. Robak, GA-101, a third-generation, humanized and glyco-engineered anti-CD20 mAb for the treatment of B-cell lymphoid malignancies, Curr. Opin. Investig. Drugs 10 (2009) 588–596.