Fibroblast Growth Factor Receptor 3 (FGFR3) Associated with the CD20 Antigen Regulates the Rituximab-induced Proliferation Inhibition in B-cell Lymphoma Cells

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Background: The mechanism of cytotoxicity by rituximab treatment remains to be elucidated.

Results: FGFR3 was physically associated with CD20 under stimulation with rituximab, and it affected the cytotoxicity.

Conclusion: FGFR3 participates in the rituximab-induced proliferation inhibition in B-cell lymphoma cells.

Significance: Identification of the signal molecules associated with the therapeutic antibody complex is useful for finding the target molecules for concomitant therapy.

Rituximab is reported to inhibit the proliferation of lymphoma cells through an unknown CD20-mediated signal transduction pathway. Herein, we investigated cell surface molecules involved in the CD20-mediated signal transduction pathway by using a recently developed technique named enzyme-mediated activation of radical sources (EMARS)3 reaction. Using this method, we found that under stimulation with rituximab and another anti-CD20 antibody B-Ly1, CD20 was physically associated with fibroblast growth factor receptor 3 (FGFR3) as well as some other receptor tyrosine kinases in Raji cells. However, under stimulation with a noncytotoxic anti-CD20 antibody 2H7, CD20 was not associated with FGFR3 but with the PDGFR receptor β. When the tyrosine kinase activity of FGFR3 was inhibited by the chemical inhibitor PD173074 or an siRNA knockdown strategy, the proliferation inhibition by rituximab was attenuated, indicating that FGFR3 participates in the rituximab-dependent signal transduction pathway leading to proliferation inhibition. These observations raise the possibility that concomitant targeted therapy toward FGFR3 might improve the efficacy and safety of the rituximab therapy.

Targeted therapy has been developed for the last 2 decades and has made remarkable progress. It is categorized into two types, one being small molecules and the other being monoclonal antibodies. Now we have several successful therapeutic antibodies, such as alemtuzumab (Campath-1H), trastuzumab (Herceptin®), and rituximab (Rituxan®) (1, 2). These therapeutic antibodies are supposed to work mainly by mediating two immune reactions, namely the antibody-dependent cellular cytotoxicity (ADCC) and the complement-dependent cytotoxicity (CDC). However, alternative mechanisms are also involved in the action of the therapeutic antibodies. For instance, antibodies bind to their antigen receptors as a ligand and induce pharmacological effects such as cytotoxicity and cytokine secretion. These antibodies are called a “signaling antibody” and are applied to clinical therapy (3). However, their action mechanisms remain to be elucidated in many signaling antibodies. Identifying the signal molecules associated with their antigen receptors may be useful for solving this problem.

We focused on rituximab in this study because it is one of the most successful therapeutic antibodies for the treatment of B-cell lymphoma (4–6). The target of rituximab is CD20 expressed on the cell surface of B-cell lymphoma cells. Rituximab is reported to inhibit proliferation of lymphoma cells through the CD20-mediated signal transduction pathway (7), although the pharmacological effect of rituximab is considered to be mainly mediated by antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity (7). This finding indicates that rituximab is a signaling antibody. However, little is known about which signaling molecules participate in the rituximab-stimulated CD20 pathway. CD20 is a member of the membrane-spanning 4A family. Its genuine molecular function is unclear, although it is implicated as a Ca2+ transporter. Because membrane-spanning proteins are robustly associated with the plasma membrane, their cross-linking with antibody may affect the organization of the membrane structure. Actually, rituximab treatment brings about the translocation of CD20 into the lipid raft fraction (7, 8). This reorganization of the membrane structure results in the formation of a transmembrane signal cluster, including CD20, which may initiate a signal transduction pathway leading to cytotoxicity (supplemental Fig. 1). A similar situation is observed in the immunoreceptors, which form a functional transmembrane cluster under stimulation and subsequently generate immune reaction signals (9).

In this study, we propose a strategy to explore cell surface signal molecules to modulate the action of rituximab, using the enzyme-mediated activation of radical sources (EMARS)3 reaction (10, 11) developed by us to label proximal molecules within

6 This article contains supplemental Experimental Procedures and Figs. 1–4.
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9 The abbreviations used are: EMARS, enzyme-mediated activation of radical source; RTK, receptor tyrosine kinase; FGFR3, FGF receptor β.
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a limited distance (200–300 nm) from the probed molecule in living cells. This reaction is useful for the identification of molecules coming close to the target molecule under stimulation. By using this reaction, we have revealed the spatiotemporal association between β1 integrin and ErbB4 that is involved in the fibronectin-dependent cell migration (12). To find CD20-related signal molecules in this study, physically associated molecules with CD20 under stimulation with rituximab and other anti-CD20 antibodies, B-Ly1 and 2H7 were compared by using the EMARS reaction. B-Ly1 suppresses proliferation of B-cell lymphomas as well as rituximab (13), although 2H7 has no effect on cell growth (14). In this study, we found that the fibroblast growth factor receptor 3 (FGFR3) was associated with CD20 under stimulation with rituximab and B-Ly1 but not under stimulation with 2H7. We further found that FGFR3 plays a critical role in the rituximab-dependent proliferation inhibition in B-cell lymphoma cells.

EXPERIMENTAL PROCEDURES

Cell Culture—in the Burkitt lymphoma cell line, Raji and BJAB cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum at 37 °C under humidified air containing 5% CO2. HEK293 cells were cultured in DMEM supplemented with 5% fetal bovine serum at 37 °C under humidified CO2. BJAB cells were cultured in RPMI 1640 medium supplemented with 5% CO2.

Preparation of HRP- or Fluorophore-conjugated Antibodies—Rituximab, which was a generous gift from Zenyaku-Kogyo Co. (Tokyo, Japan), mouse monoclonal anti-CD20 antibody 2H7 (Gene Tex), and mouse monoclonal anti-CD20 antibody B-Ly1 (Abcam) were conjugated to HRP using a peroxidase labeling kit NH2 (Dojindo) following the manufacturer’s instruction. Rituximab and 2H7 antibodies were labeled with FITC (Sigma) and Alexa 647-carboxylic acid, succinimidyl ester (Invitrogen), respectively, and subsequently purified using a G-50-Sepharose spin column (Amersham Biosciences) to remove excess labeling reagent. In the case of rabbit anti-FGFR3 antibody (Santa Cruz Biotechnology) and anti-caspase-3 antibody (Cell Signaling) for Western blot, the antibody was labeled with Zenon NHS biotin (Pierce) in PBS at 37 °C for 15 min. After washing with Tris-buffered saline to quench the reaction, the cells were lysed with a detergent-containing buffer for lipid raft extraction (25 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1% Triton X-100, and protease inhibitor mixture (Nacalai)) followed by incubation on ice for 20 min. The mixtures were subsequently homogenized using a glass homogenizer and 10 strokes. The homogenized samples were mixed with 80% sucrose solution resulting in the final concentration of 40% sucrose. The solution was transferred to a centrifugation tube and the discontinuous sucrose density gradient was prepared by layering successively two decreasing sucrose density solutions (30 and 5% sucrose solution) onto this sample solution. The gradient solution was centrifuged at 160,000 × g for 18 h at 4 °C by using Beckman TL-100 ultracentrifugal unit equipped with TLS-55 swing rotor. After centrifugation, the 3rd to 6th fractions from the top (total of 12 fractions; 200 µl/fraction) were collected. 50 µl of each fraction was mixed with 500 µl of the lipid raft lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1% Triton X-100, and 1% glycerol) and then incubated at 37 °C for 15 min to lyse the lipid raft. The mixtures were applied to Proteome Profiler™ human phospho-RTK array, respectively.

Inhibition of FGFR3 Phosphorylation by PD173074 Inhibitor Treatment—Raji cells were cultured with or without PD173074, 1-tert-butyl-3-[6-(3,5-dimethoxyphenyl)-2-(4-diethylamino-butylamino)pyrido[2,3-d]pyrimidin-7-yl]-urea (10 or 30 μM; Sigma), in culture medium at 37 °C for 2 h. After treatment, the cells were lysed with the lysis buffer and immunoprecipitated with 1 µg of rabbit anti-FGFR3 antibody (Santa Cruz Biotechnology) and Ab-capture protein A-Sepharose (Protenova) at 4 °C for overnight. The immunoprecipitated samples were sub-
jected to SDS-PAGE (6% gel, under a reducing condition) followed by blotting onto PVDF membrane. The membrane was first treated with HRP-conjugated 4G10 platinum antibody (Millipore). After washing, the membranes were developed with an Immobilon Western chemiluminescent HRP substrate (Millipore). After stripping the antibody with Western blot stripping buffer (Thermo), the same membrane was treated with HRP-labeled anti-FGFR3 antibody as described above. After washing, the membranes were developed. Each Western signal was quantified using ImageJ software (National Institutes of Health).

**Proliferation Assay of Anti-CD20 Antibody-treated Cells**—Raji and BJAB cells (3 × 10⁴ cells/ml) were treated with or without 10 or 30 nM PD173074 in culture medium at 37 °C for 2 h. After treatment, rituximab, 2H7, or B-Ly1 (1 g/ml) were separately added. The plate was cultured at 37 °C under humidified air containing 5% CO₂. After 3 days, the cells were harvested and suspended in 0.5% trypan blue/PBS solution, and living cell numbers were counted using the cell counting chamber under microscopy. In addition, these cells were also observed with IMT-2 phase contrast microscopy equipped with a DP-12 digital camera unit (Olympus).

**Knockdown of FGFR3 Using siRNA Strategy**—The knockdown sequences of FGFR3, CGACAAGGAGCTAGAGGTT (Takara), and the negative control sequence, TCTTAATCGCGTATAAGGC (Takara), with the hairpin sequence were inserted into the pSINsi-hU6 retrovirus vector (Takara) at BamHI and Clal ligation sites, respectively, following the manufacturer’s instructions. The plasmid was transfected into HEK293 cells with Lipofectamine 2000 (Invitrogen), and the transfected cells were cultured in DMEM supplemented with 10% fetal bovine serum for 48 h. The supernatant was collected and treated with Raji cells (1.5 × 10⁶ cells in 6-cm culture dish) for 48 h. After treatment, the cells were diluted with RPMI 1640 medium supplemented with 5% fetal bovine serum and G418 (800 μg/ml), and cultured in a 24-well culture plate. The colonies grown on the plate were collected and subjected to Western blot analysis using anti-FGFR3 antibody to select appropriate FGFR3 knockdown cells.

**Analysis of Rituximab-CD20 Complex**—Raji and BJAB cells (5 × 10⁶ cells) were treated with or without 30 nM PD173074 in culture medium at 37 °C for 2–2.5 h. After treatment, the cells were collected and treated with or without rituximab (5 g). The cells were subsequently incubated at 37 °C for 5, 10, 15, 20, and 30 min. Each cell sample was lysed with lysis buffer, and then the rituximab-CD20 complex was isolated with Dynabeads-protein A (Invitrogen). The purified samples were subjected to SDS-PAGE (4–20% gradient gel, under a reducing condition). The gel was stained with flamingo gel stain solution (Bio-Rad) to see the total protein. The purified samples were also subjected to SDS-PAGE (4–20% gradient gel, under a reducing condition) followed by blotting onto PVDF membrane. The membrane was treated with HRP-conjugated 4G10 platinum antibody (Millipore). After washing, the membranes were developed with an Immobilon Western chemiluminescent HRP substrate (Millipore). After stripping the antibody with Western blot
stripping buffer, the same PVDF membrane was re-stained with rabbit anti-CD20 antibody (1:1000; Thermo) and anti-rabbit IgG-HRP (1:5000; Promega).

RESULTS
CD20-associated Cell Surface Molecules under Stimulation with Distinct Anti-CD20 Antibodies Revealed by the EMARS Method—Because distinct anti-CD20 antibodies exhibit diverse functionality in terms of proliferation of B-cell lymphoma cells (15), it was assumed that CD20 forms different transmembrane signal cluster complexes to send distinct signals under stimulation with these antibodies. To investigate the cell surface molecules associated with CD20 when rituximab and 2H7 were treated, the EMARS method using HRP-conjugated antibodies was performed. The principle of the EMARS method is shown in supplemental Fig. 2. We separately treated two lines of B-cell lymphoma cells, Raji and BJAB, with HRP-conjugated rituximab or 2H7 antibody at 37 °C for 15 min and then reacted with azide biotin under a living condition as described under “Experimental Procedures.” Following the EMARS reaction, the membranes were solubilized, and the total protein extract was subjected to Western blot to detect biotinylated molecules. As shown in Fig. 1A, similar biotinylated bands were observed between the rituximab- and 2H7-treated cells in both Raji (upper panel) and BJAB (lower panel) cells, although the intensity of the band at 37 kDa was stronger in the rituximab-treated cells than the 2H7-treated cells.

Next, we applied both the rituximab- and 2H7-treated EMARS products to two kinds of antibody array systems for the identification of biotinylated immunoreceptors and RTKs. The analysis using the RTK array revealed that FGFR3, ROR1, and Dtk were predominantly biotinylated in the rituximab-treated Raji cells (rituximab-HRP in Fig. 1B), whereas only PDGF receptor β was weakly labeled in the 2H7-treated Raji cells (2H7-HRP in Fig. 1B). We additionally performed the EMARS method using another anti-CD20 antibody B-Ly1. The RTK antibody array analysis showed a slightly different pattern of labeled molecules, including ErbB4, FGFR3, and Dtk, from that using rituximab (B-Ly1-HRP in Fig. 1B). When analyzed in BJAB cells, the biotinylated RTKs were quite similar to that in Raji cells (Fig. 1C). In the analysis using the immunoreceptor antibody array, CD22, CD23, FcγRIIA, FcRH5, and TREM-2 were predominantly biotinylated in the rituximab-treated Raji
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FIGURE 3. Role of FGFR3 in the proliferation inhibition of rituximab- and B-Ly1-treated cells. A, effect of PD173074 on the tyrosine phosphorylation of FGFR3 in Raji cells. Raji cells were treated with the indicated concentration of PD173074 for 2 h and then homogenized with the lysis buffer. The immunoprecipitate (IP) with an anti-FGFR3 antibody was subjected to Western blot (WB) using the anti-phosphotyrosine antibody 4G10 and HRP-conjugated anti-FGFR3 antibody. Intensity of signals was quantified using ImageJ software (National Institutes of Health), and the phosphorylation rate was calculated as the intensity of bands stained with 4G10 divided by the intensity of corresponding bands stained with anti-FGFR3 antibody (indicated in the lower area of the column). B, effect of the FGFR3 kinase inhibitor PD173074 on cell proliferation and viability. Raji cells were pretreated with none (open bar), 10 nM (gray bar), or 30 nM (closed bar) of PD173074 and subsequently treated without (mock) or with rituximab or 2H7 antibody as described under “Experimental Procedures.” The number of living cells was counted after 3 days. Seven independent experiments were performed. One-way analysis of variance was used to test differences among more than two means for significance (*, p = 0.0016; rituximab). C, morphological observation of rituximab-treated Raji cells in the presence or absence of PD173074 with phase contrast microscopy. D, effect of the PD173074 in B-Ly1-treated Raji cells. Raji cells were pretreated with none (open bar) or 30 nM (closed bar) of PD173074 and subsequently treated without (mock) or with B-Ly1 as described under “Experimental Procedures.” The number of living cells was counted after 3 days. Four independent experiments were performed. The statistical analysis for the calculation of two-tailed probability value was performed using Student’s t tests (*, p = 0.00049; B-Ly1). E, effect of the PD173074 in rituximab- and B-Ly1-treated BJAB cells. Four independent experiments were performed. The statistical analysis for the calculation of two-tailed probability value was performed using Student’s t tests (*, p = 0.0014; rituximab; #, p = 0.042; B-Ly1).

cells (rituximab-HRP in Fig. 1D), whereas only CD23 and SHP-2 were labeled in the 2H7-treated Raji cells (2H7-HRP in Fig. 1D). These results indicate that in associated molecules with CD20 under stimulation with rituximab, 2H7 and B-Ly1 are distinct from each other, suggesting that the transmembrane signal transduction assemblies formed by the stimulation with anti-CD20 antibodies vary depending on the antibody clones.

To exclude the possibility that stimulation with antibodies affects the expression of FGFR3, we examined expression levels of FGFR3. As the result, we confirmed that there was no effect of mock, rituximab, and 2H7 treatment on the FGFR3 expression level (Fig. 1E).

To characterize the difference in the membrane structures between the rituximab-CD20 and the 2H7-CD20 complexes, their localizations were observed with a confocal laser scan microscopy after staining Raji cells simultaneously with fluorescein-labeled rituximab and Alexa 647-labeled 2H7. Although images of rituximab and 2H7 apparently overlapped on the cell surface, some parts were differently stained as green and red spots (Fig. 2A, merge). This observation suggests that rituximab and 2H7 bind CD20 residing at different molecular clusters. Alternatively, the treatments with rituximab and 2H7 may form different molecular clusters around CD20, respectively.

It was reported that rituximab treatment results in the translocation of CD20 into the lipid raft fraction (15). Because the lipid rafts are well known to function as a platform of signal transduction, this reorganization of the membrane structure triggered by rituximab may initiate a signal transduction leading to the proliferation inhibition. To investigate what molecules are transferred into the lipid rafts in response to the rituximab treatment, the detergent-insoluble membrane microdomain fraction was isolated after treatment of Raji cells with or without rituximab and 2H7. Then whole proteins in the detergent-insoluble membrane microdomain fraction were labeled with sulfo-N-hydroxysuccinimide-biotin and subjected to the RTKs antibody array. As shown in Fig. 2B, no RTK was detected in the lipid raft fraction from the cells with mock treatment. By contrast, FGFR3 and ROR1 were detected in the rituximab-treated lipid rafts (rituximab), and FGFR3, PDGF receptor β, Flt-3, and MCSF receptors were detected in 2H7-treated lipid rafts (2H7). Thus, FGFR3 was recovered in the lipid raft fraction in both rituximab- and 2H7-treated cells, whereas it was associated only with the rituximab-treated CD20 molecule (Fig. 1B). These findings suggest that not 2H7 but rituximab is able to recruit FGFR3 for the CD20-resident cluster, although FGFR3 is transferred into the lipid rafts by stimulation with both antibodies (schematic model is shown in Fig. 2C).
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FGFR3 Participates in the Signaling Pathway Leading to the Proliferation Inhibition of Rituximab- and B-Ly1-treated Raji Cells—Because our EMARS method revealed a physical interaction of FGFR3 and CD20 following the rituximab treatment, we investigated the issue of whether FGFR3 participates in the signal transduction pathway leading to the proliferation inhibition of rituximab-treated B-cell lymphoma cells. To this end, effects of the FGF family kinase inhibitor PD173074 that inhibits FGFR3 in a low concentration (IC_{50} = 5–10 nM) (16, 17) was examined. We first confirmed that only FGFR3 was tyrosine-phosphorylated among the FGF family proteins (FGFR1 to FGFR4) in the rituximab and 2H7-treated Raji cells (data not shown). PD173074 decreased the phosphorylation level of FGFR3 in the range of 10–30 nM (Fig. 3A), indicating that the tyrosine phosphorylation occurred by autophosphorylation. Raji cells pretreated with or without PD173074 were treated with or without rituximab and 2H7 and subsequently cultured in the ordinary growth medium. After 3 days, the number of living cells in each sample was counted. As shown in Fig. 3B, rituximab decreased the number by ~50% as compared with mock treatment, although 2H7 had no effect (open bars). PD173074 had no effect on cell proliferation and viability of mock-treated and 2H7-treated cells, but attenuated the suppression by the rituximab treatment (rituximab). Furthermore, effects of PD173074 on the morphology of rituximab-treated Raji cells were investigated. As shown in Fig. 3C, rituximab-treated cells were aggregated and damaged (rituximab). In contrast, PD173074-cotreated cells were aggregated but vigorous (rituximab +10 or 30 nM PD173074). These results indicate that the tyrosine kinase activity of FGFR3 modulates the proliferation inhibition by rituximab. PD173074 also attenuated the proliferation inhibition by B-Ly1 in Raji cells (Fig. 3D). In contrast to the observation in Raji cells, PD173074 augmented the proliferation inhibition by rituximab and B-Ly1 treatment in BJAB cells (Fig. 3E).

Furthermore, ablation of FGFR3 in Raji cells by a retroviral siRNA knockdown system (Fig. 4A) attenuated the proliferation inhibition by rituximab and B-Ly1 treatment (Fig. 4B) in accordance with the results using PD173074 (Fig. 3, B and D). These results indicated that FGFR3 regulates the rituximab- and B-Ly1-dependent proliferation inhibition in a cell line-dependent manner.

Rituximab-dependent Proliferation Inhibition Is Not Caused by the Caspase-3-dependent Apoptosis but Is Dependent on the Cell Cycle Phase—To elucidate the mechanism of the rituximab-dependent proliferation inhibition, we first examined whether apoptosis is responsible for it. Raji cells were treated with rituximab and then incubated for 8, 16, and 24 h. Each sample was applied to Western blot analysis using an anti-caspase-3 and an anti-poly(ADP-ribose) polymerase antibody as described under “Experimental Procedures.” The cleaved forms of caspase-3 and poly(ADP-ribose) polymerase were not observed at all the incubation times (supplemental Fig. 3, A and B), suggesting that the rituximab-dependent proliferation inhibition is not caused by the classical caspase-3-dependent apoptosis. It was also revealed using an apoptosis antibody array that there is no difference in the levels of apoptotic signal molecules between mock and the rituximab-treated cells (supplemental Fig. 3C).

Next, we investigated the effect of the cell cycle phase on rituximab treatment. When Raji and BJAB cells were treated with 1.5% DMSO (see supplemental “Experimental Procedures”), the cell population at the G0 phase was significantly increased in both cell lines (supplemental Fig. 4A). These G0-arrested cells had more resistance against the rituximab treatment (80% survival in Raji cells in supplemental Fig. 4B) than cells not arrested (50% survival in Fig. 3B), indicating that rituximab-induced proliferation inhibition is dependent on the cell cycle phase.

FGFR3 Regulates the Assembly of the Rituximab-stimulated CD20 Complex—To elucidate the role of FGFR3 in the assembly of the rituximab-stimulated CD20 signal complex, effects of the FGFR3 kinase inhibitor PD173074 on the complex formation were time-dependently investigated after addition of rituximab. The rituximab-CD20 complex was isolated from the rituximab-treated Raji cells by immunoprecipitation with protein A-immobilized Dynabeads and subjected to SDS-PAGE followed by staining for proteins with a fluorescence reagent. As shown in Fig. 5A, more proteins were detected in the rituximab-CD20 complex in the presence of PD173074. This molecular clustering reached a peak at 15 min after rituximab treatment. Simultaneously, tyrosine-phosphorylated molecules in the complex were examined with the anti-phosphotyrosine antibody 4G10 (Fig. 5B). Phosphorylation signals were more robust in the absence of PD173074 and their intensity reached a peak at 15 min after rituximab treatment. Considering the coin-(Disease Name)
cidence of timing between the phosphorylation and the molecular assembly, it was suggested that tyrosine phosphorylation of the components in the CD20 complex by FGFR3 prevents the molecular assembly.

Because the effects of PD173074 on the cell proliferation were opposite between Raji and BJAB cells (Fig. 3, B and E), a similar experiment for the CD20 complex formation was done using BJAB cells. In contrast to Raji cells, more proteins were observed in the rituximab-CD20 complex in the absence of PD173074 in BJAB cells (Fig. 5C). This result suggests that tyrosine phosphorylation of the components in the CD20 complex by FGFR3 enhances the molecular assembly. Taken together, FGFR3 regulates the assembly of the rituximab-stimulated CD20 complex (Fig. 6), although the direction of the regulation is variable depending on the cell lines.

DISCUSSION

Therapeutic antibodies serve as a powerful and prospective tool for the therapy against the severe and intractable diseases, such as malignant tumor and autoimmune disease (1, 2). Exploration of a novel therapeutic antibody has been enthusiastically made to gain more effective and safer antibodies. Among therapeutic antibodies, the signaling antibody (3) is especially promising because it gives a specific effect by itself on a particular type of cell expressing its antigen. Moreover, intervention into the signaling pathway stimulated by the signaling antibody is assumed to enhance its therapeutic effect. However, not all signaling antibodies are elucidated with regard to their signaling pathways. Rituximab is one of the most successful therapeutic antibodies for B-cell lymphoma (7). Although rituximab is known to bind to CD20 on lymphoma cells and bring about a cytotoxic effect, the mechanism of this cytotoxic effect is unclear. In contrast to rituximab, some other anti-CD20 monoclonal antibodies have no cytotoxic activity (14, 15). These observations suggest that each clone of anti-CD20 antibody activates a different signaling pathway leading to distinct cellular responses. Hence, we assumed that if we can reveal the differences in associated molecules with CD20 under stimulation with rituximab and other anti-CD20 monoclonal antibodies, we could find a critical cell surface molecule of the signaling pathway in the rituximab-specific CD20-associated molecules. To identify associated molecules with CD20, we applied the EMARS reaction that we previously developed (supplemental Fig. 2) (10, 11).

The feature of this EMARS reaction is that the cross-linking reagent arylazide biotin is activated not by ultraviolet light but by HRP. This reaction can label physically associated molecules...
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The expression level of ROR1 and FGFR3 was demonstrated to increase in hematopoietic dysplasia, such as B-cell lymphoma and multiple myeloma (18–20). In addition, FGFR3 and its mutant form were reported to be involved in the transformation of hematopoietic cells (21). FGFR3 was also shown to mediate cell growth in colorectal (22), bladder (23), and oral (24) cancers. The inhibition of FGFR3 has therefore been regarded as an important strategy for cancer therapy by suppressing cell proliferation (25). These findings prompted us to investigate the involvement of FGFR3 in the signaling pathway stimulated with the rituximab treatment. Instead of the FGFR3 function mentioned above, we found in this study that the specific inhibitor of FGFR3, PD173074, attenuated the rituximab-induced proliferation inhibition in Raji cells, indicating that the FGFR3 kinase participates in the signaling pathway leading to the proliferation inhibition. A similar PD173074 effect was observed in B-Ly1-treated Raji cells (Fig. 3D), and FGFR3 was also associated with the B-Ly1-CD20 complex likely as rituximab (Fig. 1B), suggesting that this association between FGFR3 and antibody-CD20 complex contributes to the proliferation inhibition in Raji cells. Furthermore, the relevance of FGFR3 was supported by the evidence that the FGFR3-knockdown Raji cells canceled the rituximab-stimulated proliferation inhibition (Fig. 4).

Because FGFR3 is a plasma membrane-resident protein and was physically associated with CD20 under stimulation with rituximab, FGFR3 was considered to act at an early phase of the signaling pathway. Therefore, we investigated the effect of PD173074 on the assembly of the rituximab-CD20 complex. Although we had anticipated that inhibition of the tyrosine phosphorylation by PD173074 would abolish the assembly of signaling molecules in the rituximab-CD20 complex, the fact was the opposite. PD173074 enhanced the molecular clustering in the rituximab-CD20 complex. Furthermore, the tyrosine phosphorylation level was found to reach a peak at 15 min after the rituximab treatment in the absence of PD173074 (Fig. 5B), suggesting that tyrosine phosphorylation of the components in the CD20 complex by FGFR3 prevents the molecular assembly that generates the proliferation signal.

In contrast to Raji cells, the proliferation inhibition effects by rituximab and B-Ly1 were weaker in BJAB cells (Fig. 3E). More surprisingly, PD173074 enhanced this proliferation inhibition in BJAB cells. In accord with this observation, the molecular assembly of the rituximab-CD20 complex was abolished in the presence of PD173074 in BJAB cells (Fig. 5C). These observations suggest that tyrosine phosphorylation of the components in the CD20 complex by FGFR3 enhances the molecular assembly that generates the proliferation signal in BJAB cells.

The downstream signal of FGFR3 leading to the proliferation inhibition remains to be solved. Our additional experiment indicated that the cytotoxicity of rituximab is not caused by the classical caspase-3-dependent apoptosis but was dependent on

with a given molecule on which HRP is set in living cells. In this study, HRP-conjugated anti-CD20 antibodies were used. The HRP conjugation did not affect the binding activity of the original antibodies (data not shown). Although biotinylated molecules as associated molecules were observed in both rituximab-HRP and 2H7-HRP treated cells, the extent of them was stronger in rituximab than 2H7 (Fig. 1A). To identify signaling molecules in these associated molecules, we used the RTKs and immunoreceptors antibody array systems, because antibody array is of high sensitivity and specificity. As the result, associated molecules were quite different between rituximab-HRP- and 2H7-HRP-treated cells. ROR1 and FGFR3 were detected in the rituximab-HRP-treated cell but not in the 2H7-HRP-treated cells. Interestingly, the lipid raft analysis revealed that both rituximab and 2H7 treatment have an ability to promote FGFR3 translocation to lipid raft clusters (Fig. 2B). Considering these findings, it is assumed that the rituximab-CD20 complex is transferred into FGFR3-resident lipid raft clusters, whereas the 2H7-CD20 complex is into distinct lipid raft clusters (Fig. 2C). This difference in the molecular clustering around CD20 may affect the cytotoxic effect of various anti-CD20 antibodies. The underlying mechanism of the translocation of FGFR3 to distinct lipid raft by 2H7 treatment remains to be elucidated. We speculate that the dynamic changes of lipid raft environment including cytoskeletal reorganization by stimulation with 2H7 antibody might promote the lipid raft translocation of FGFR3 without any direct interaction between FGFR3 and the 2H7-CD20 complex.

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The underlying mechanism of the translocation of FGFR3 to
the cell cycle phase (supplemental Figs. 3 and 4). FGFR3 may act on cell cycle-dependent signal molecules. Further investigations are needed to identify the substrates of the FGFR3 kinase.

In this study, we discovered FGFR3 as a physically associated molecule with CD20 under the rituximab treatment by using the EMARS method, and we subsequently demonstrated a critical role of FGFR3 in the proliferation inhibition effect of rituximab. The concomitant therapy with therapeutic antibodies and other medicines has recently been developed (26–29). In rituximab therapy, the R-CHOP chemoimmunotherapy for non-Hodgkin lymphoma is a representative example (30). This strategy seems to be reasonable and promising for effective treatment against intractable diseases, but they usually employ drugs whose mechanisms are irrespective of that of the therapeutic antibody. Our findings raise the possibility that concomitant targeted therapy toward FGFR3 might improve the efficacy of the rituximab therapy from the viewpoint of custom-made medicine for individual patients. This approach is applicable to other therapeutic antibodies to find molecules regulating their actions. Therapeutic antibodies have a risk of adverse responses caused by unforeseen interaction with other molecules than the target molecule (31). Our unbiased approach is also useful for the identification of unforeseen molecular interactions with the target molecule of therapeutic antibodies.

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