Phase I Study of Rituximab-CHOP Regimen in Combination with Granulocyte Colony-Stimulating Factor in Patients with Follicular Lymphoma

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ABSTRACT

Purpose: Rituximab is an anti-CD20 monoclonal antibody, and it is used to treat B-cell lymphomas. Antibody-dependent cellular cytotoxicity (ADCC) is considered one of the mechanisms through which rituximab exerts its effects. Granulocyte colony-stimulating factor (G-CSF) enhances the cytotoxicity of neutrophils through ADCC, and it can be speculated that a combination of rituximab and G-CSF may augment the treatment efficacy of rituximab.

Experimental Design: We administered rituximab with CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) treatment with G-CSF to 15 patients with follicular lymphoma, and we investigated the safety and efficacy of this regimen. We investigated ADCC activity in neutrophils and the expression of cell surface antigens including Fcγ receptor type I (FcγRI (CD64)) on neutrophils to determine the optimal dose of G-CSF.

Results: Adverse reactions occurred in 14 of 15 patients and consisted mainly of grade 3/4 hematological toxicity. The response rate was 100%, with complete remission in 12 patients (80%) and partial remission in 3 patients (20%). At 14 months, the median length of the observation period, 2 of 12 patients had relapsed. G-CSF administration increased ADCC activity between the 2 μg/kg G-CSF and 5 μg/kg G-CSF groups, indicating that the optimal dose of G-CSF was 2 μg/kg.

Conclusions: We conclude that the combination of rituximab-CHOP and G-CSF is well tolerated. We plan to carry out a randomized trial to compare efficacy between rituximab-CHOP treatment and treatment with a combination of rituximab-CHOP and G-CSF.

INTRODUCTION

Follicular lymphoma progresses slowly, and the short-term prognosis is relatively good. It has been reported that the 5-year survival rate of patients with stage III or IV follicular lymphoma is 55–60%, and the 10-year survival rate is approximately 35% (1). However, because a standard treatment has not been established, most patients continue to suffer from the disease and die due to histological progression. Recently, monoclonal antibody (mAb) therapy has been shown to be effective against indolent B-cell lymphoma. Rituximab is an anti-CD20 mAb, and it achieved a response rate of approximately 50% among patients with indolent B-cell lymphoma who had relapsed after chemotherapy (2). Rituximab has also been used as initial therapy in patients with follicular lymphoma and a low tumor burden, with an overall response rate of 73% and complete remission (CR) in 20% (3). Czuczman et al. (4) reported that treatment with rituximab and CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) [R-CHOP] achieved CR in 22 of 40 patients (55%) and an overall response in 38 patients (95%) with low-grade B-cell lymphoma, and CR was maintained for 46.8–86.3 months in 21 patients.

Rituximab is a chimeric IgG1κ mAb with a mouse variable region and human constant regions, and it recognizes the CD20 antigen on B lymphocytes. It has been shown that rituximab exerts cytotoxic effects through complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC; Ref. 5) and that it directly induces apoptosis (6). Clynes et al. (7) demonstrated that ADCC through Fcγ receptor (FcγR) substantially contributes to the cytotoxic action of rituximab. More recently, an association between the FcγR3A-158V genotype and a better response to rituximab treatment was found, suggesting that FcγRIIIa is involved in the antitumor mechanism of rituximab (8, 9). Taken together, these studies support that FcγR-dependent mechanisms play a role in the effects of rituximab treatment. In vitro studies have confirmed that granulocyte colony-stimulating factor (G-CSF) up-regulates the expression of FcγRI (CD64) on polymorphonuclear (PMN) cells, which play an important role in exerting ADCC activities (10, 11), and it has been considered that G-CSF augments the effects of rituximab. In addition, experiments using a bispecific antibody (FccRI × CD20) have confirmed that G-CSF enhances cytotoxic activities regulated by FccRI by increasing the number of effector cells, although it does not up-regulate FccRI (CD89) expression (12). Meanwhile, rituximab inhibits interleukin (IL)-10 expression and down-regulates Bcl-2 expression, induc-
ing apoptosis of tumor cells (13). However, G-CSF increases IL-4 and IL-10 levels in the peripheral blood, thereby predominantly activating Th-2 cells (14). Therefore, it is important to investigate whether the addition of G-CSF to rituximab in chemotherapy regimens can enhance the antitumor effects. In this study, we investigated the optimal dose of G-CSF when used in combination with R-CHOP, we investigated the expression of cell surface antigens including FcγRI (CD64) and ADCC activity to assess the enhanced antitumor effects of G-CSF in combination therapy with R-CHOP for untreated follicular lymphoma, and we report the safety of this combination regimen.

PATIENTS AND METHODS

Study Design. This was an open-label, single-arm Phase I study of the R-CHOP regimen in combination with G-CSF for the treatment of follicular lymphoma grade 1/2 with a gradual increase to five cohort patients in each of three groups. The five patients in group 1 initially received R-CHOP with 0 μg/kg G-CSF; the five patients in group 2 initially received R-CHOP with 2 μg/kg G-CSF, and the five patients in group 3 received R-CHOP with 5 μg/kg G-CSF. In patients in groups 1 and 2 who showed no safety problems, the dosage of G-CSF was increased to the next level. The optimal dose of G-CSF, when given in combination with the R-CHOP regimen to patients with follicular lymphoma grade 1/2, was determined. The efficacy of each treatment regimen was assessed by the level of cell surface expression of FcγRI (CD64) and ADCC activity. In addition, the safety of these regimens was investigated. Patients with follicular lymphoma grade 1/2 who were being treated at Kitasato University School of Medicine or Fujita Health University School of Medicine were enrolled. This study was approved by the ethics committee at each center and performed in accordance with the guidelines of the Declaration of Helsinki.

Eligibility Criteria. Eligible patients had histologically documented follicular lymphoma of grade 1 or grade 2 as defined by the WHO lymphoma classification (15). It was confirmed that CD20 antigen was expressed on the surface of lymphoma cells by either immunohistochemical analysis or flow cytometry using B1 or L26 anti-CD20 mAb. All patients were being treated for follicular lymphoma for the first time. Patients who were between the ages of 20 and 80 years at the time of the study with an expected survival of >4 months and a performance status of 0–2 using the Eastern Cooperative Oncology Group scale were included. Patients with stage III or IV disease, as assessed by the Ann Arbor classification, were included (16). Pretreatment laboratory examination was performed within 1 month of study entry, and patients with the following range of laboratory results were included: absolute neutrophil count > 1.5 × 109/liter; platelets > 75 × 109/liter; creatinine < 1.5 × the upper limit of normal; bilirubin < 2.0 × the upper limit of normal; and aspartate transaminase < 5 × the upper limit of normal. Patients with uncontrolled infection, concomitant malignancy (excluding carcinoma in situ of the cervix and skin basal cell carcinoma), unstable angina pectoris, symptomatic cardiac arrhythmia, clinical heart failure, or symptomatic pleural effusions were excluded. Pregnant or lactating women as well as patients who had clinically apparent central nervous system lymphoma were also excluded from the study. All patients gave informed consent for both treatment and sample collection in accordance with institutional policy.

Treatment. Patients were treated with R-CHOP and G-CSF (lenograstim). The CHOP regimen consisted of 750 mg/m2 cyclophosphamide, 50 mg/m2 doxorubicin, and 1.4 mg/m2 vincristine, up to a maximal dose of 2 mg, i.v. on day 1, with 100 mg of prednisone orally per day for 5 days. Patients were given six cycles of CHOP, once every 3 weeks. Rituximab, at a dose of 375 mg/m2, was given on day −2 of cycles 1, 2, 4, and 6. Between 30 and 60 min before the start of rituximab infusion, the patient was given oral acetaminophen (650 mg) and diphenhydramine hydrochloride (50 mg). Corticosteroids were never given as premedication. In the first infusion of rituximab, the rituximab solution was administered i.v. at an initial rate of 50 mg/h, and the rate was escalated in 50 mg/h increments every 30 min, to a maximum rate of 400 mg/h. If the patient developed hypersensitivity or an infusion-related event, the infusion was temporarily slowed or stopped and then resumed at one-half the previous rate on improvement of the patient’s symptoms. Subsequent rituximab infusions were administered at an initial rate of 100 mg/h, and the rate was increased in 100 mg/h increments at 30-min intervals to a maximum rate of 400 mg/h as tolerated. G-CSF was administered on days −4, −3, and −2 in cycles 1, 2, 4, and 6, and it was administered 3–6 h before rituximab infusion on day −2. G-CSF was administered s.c. The patients in group 1 initially received 0 μg/kg G-CSF with the R-CHOP regimen, the patients in group 2 initially received 2 μg/kg G-CSF with the R-CHOP regimen, and the patients in group 3 received 5 μg/kg G-CSF with the R-CHOP regimen. If there were no safety problems in a group 1 or group 2 patient, the dosage of G-CSF was increased to the next level.

Response Criteria. The tumor response was assessed after the six cycles of treatment or at the end of treatment. Disease assessment included the following evaluations: physical examination and assessment of performance status and B symptoms (baseline; weeks 6, 12, and 18; and every 3 months through 2 years); chest X-ray (baseline); bone marrow aspiration and biopsy (baseline, and to confirm a CR, if they were positive at baseline); and computed tomography or magnetic resonance imaging (baseline; weeks 6, 12, and 18; and every 3 months through 2 years). The tumor responses were classified as CR, partial remission, stable disease, or progressive disease according to the international workshop for non-Hodgkin’s lymphoma response criteria (17). These classifications were defined as follows: CR, the disappearance of all lesions and of radiological or biological abnormalities observed at diagnosis and the absence of new lesions; partial remission, regression of all measurable lesions by >50%, the disappearance of nonmeasurable lesions, and the absence of new lesions; stable disease, regression of measurable lesions by ≤50% or no change in the nonmeasurable lesions, and no growth of existing lesions or no appearance of new lesions; progressive disease, the appearance of new lesions, growth of the initial lesions by >25%, or growth of measurable lesions that had regressed during treatment by >50% of their smallest dimensions.

ADCC Assay. The ADCC assay was performed as described previously (12). In brief, target cells were labeled with 51Cr (100 μCi/106 cells) at 37°C for 1 h. Cells were washed
three times and resuspended in culture medium at a concentration of 5 × 10^6 cells/ml. Cells were then sensitized with antibodies (final concentration, 5 μg/ml). The neutrophils of a patient and target cells were added to 96-well, flat-bottomed microtiter plates at an E:T ratio of 10:1 and adjusted to a final volume of 200 μl. The plates were centrifuged at 200 × g for 1 min and incubated at 37°C under 5% CO2 for 20 h. 51Cr release was measured in triplicate and expressed as cpm. The percentage of specific lysis was calculated using the following formula: percentage of specific lysis = (experimental cpm – spontaneous cpm)/(maximal cpm – spontaneous cpm) × 100. The maximal 51Cr release was determined by adding saponin (5% (v/v), 100 μl) to target cells; spontaneous 51Cr release was determined by measuring 51Cr release from unsensitized target cells in the absence of effector cells. ADCC experiments were performed in duplicate.

**Immunophenotyping.** After cells were washed once in PBS and resuspended in PBS containing 1% BSA (w/v), immunophenotyping was performed as described previously (18), using phycoerythrin R-conjugated mAbs (Beckman-Coulter, Hialeah, FL) directed against CD3, CD19, CD20, CD64 (FcRI), or CD89 (FcRII). An irrelevant isotype-matched control mAb was used in all experiments. Flow cytometry was carried out using a FACScan (Becton Dickinson, San Jose, CA). The mean fluorescence intensity was measured by flow cytometry, and data were always corrected for the mean fluorescence intensity in the presence of the irrelevant control antibody.

**Cytokine Production Assay.** The levels of IL-4 and IL-10 in serum samples were analyzed by ELISA. ELISA kits for human IL-4 and IL-10 were purchased from BD PharMingen (San Diego, CA). The lower limit of detection was 2.0 pg/ml for human IL-4 and IL-10 were purchased from BD PharMingen.

The mean fluorescence intensity in the presence of the irrelevant control antibody.

### RESULTS

**Patient Characteristics.** The clinical characteristics of the subjects are shown in Table 1. There were 10 males and 5 females with a median age of 56 years (range, 43–63 years). The histological subtype was follicular lymphoma grade 1 in 5 patients and follicular lymphoma grade 2 in 10 patients. Eight patients had stage III disease, and seven patients had stage IV disease.

**Toxicity.** Grade 3–4 hematological toxicities were observed in 10 patients (66.7%). Two patients (13.3%) developed grade 4 neutropenia. One of the two patients was treated with antibiotics for uncomplicated fever and recovered after treatment with G-CSF; the other patient recovered without supportive care. Two patients developed grade 3 thrombocytopenia, and they required platelet transfusions. None of the patients required RBC transfusions. Five of the 10 patients who developed grade 3–4 hematological toxicities had lymphoma invasion in the bone marrow and/or peripheral blood. Toxicity during rituximab treatment was experienced by three patients and consisted mainly of infusion-related adverse events. The four most frequently observed adverse events were fever, chills/rigor, headache, and rash. Infusion-related adverse events typically occurred 1–2 h after the start of infusion. Most of the infusion-related adverse events were classified as mild to moderate (toxicity grades 1 or 2) and occurred during the first infusion. They were effectively managed with prophylactic or supportive antihistamines and antipyretics, and resolved within 24 h. Stomatitis of grade 3–4, which is a nonhematological toxicity, was found in one patient. The five patients in group 1 showed no safety problems, and the G-CSF dosage was increased to 2 μg/kg. The five patients in group 2 showed no safety problems, and the G-CSF dosage was increased to 5 μg/kg.

**Analysis of Cell Surface Antigen Expression on Neutrophils on G-CSF Administration.** The rationale for adding G-CSF to the treatment regimen containing rituximab was to induce expression of FcγRI on the circulating neutrophils and, at the same time, increase the number of neutrophils. As shown in Fig. 1, the number of neutrophils did not change at the time of rituximab infusion in patients who did not receive G-CSF, whereas the number of neutrophils increased remarkably just before the first rituximab infusion in patients who received 2 or 5 μg/kg G-CSF [2 μg/kg G-CSF, number of neutrophils increased from 4.4 ± 0.7 to 15 ± 2.4 (× 10^9/liter), P < 0.01; 5 μg/kg G-CSF, number of neutrophils increased from 4.8 ± 0.6 to 13.4 ± 1.3 (× 10^9/liter), P < 0.01]. Moreover, the level of FcγRI (CD64) expression on neutrophils increased from 62.4 ± 4.1 (mean fluorescence intensity) at baseline to 331.7 ± 23.2 (P = 0.0005) after 3 days of G-CSF (2 μg/kg) administration. Similarly, in group 3 patients who received 5 μg/kg G-CSF, the level of FcγRI (CD64) expression on neutrophils increased from 64.0 ± 5.8 at baseline to 320.4 ± 15.5 three days after the start of G-CSF treatment (P = 0.0005). At 3 days after the start of G-CSF treatment, the mean expression level of FcγRI (CD64) on circulating neutrophils was still elevated. The increase in neutrophil count and induction of FcγRI expression by G-CSF showed similar patterns during the second course and during the sixth course of treatment (data not shown). The expression of FcγRI (CD89) on neutrophils was examined in a similar fashion. There was no remarkable change in the level of FcγRI expression before and after G-CSF administration, irrespective of the dose of G-CSF. The level of ADCC activity increased significantly from 27.7 ± 5.1% at baseline to 68.4 ± 3.8% 3

<table>
<thead>
<tr>
<th>Table 1 Patient characteristics</th>
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<tr>
<td><strong>Total</strong></td>
<td>15</td>
</tr>
<tr>
<td>Age (yrs); median (range)</td>
<td>56 (43–63)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>10/5</td>
</tr>
<tr>
<td>Performance status (0/1)</td>
<td>14/1</td>
</tr>
<tr>
<td>Stage (III/IV)</td>
<td>8/7</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
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<tr>
<td>Follicular lymphoma grade 1/grade 2</td>
<td>5/10</td>
</tr>
<tr>
<td>LDH* (normal/ elevated)</td>
<td>10/5</td>
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<tr>
<td>Bone marrow involvement</td>
<td>5</td>
</tr>
<tr>
<td>Bulky disease</td>
<td>4</td>
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</table>

* LDH, lactate dehydrogenase.
days after the start of administration of 2 μg/kg G-CSF (P = 0.0004). The level of ADCC activity in the 5 μg/kg G-CSF group also increased significantly from 22.9 ± 5.1% to 70.2 ± 4.5% (P = 0.0004). On G-CSF treatment, the level of ADCC activity changed in a manner similar to the level of FcγRI (CD64) expression on neutrophils, and both the level of ADCC activity and FcγRI (CD64) expression were elevated on the third day of G-CSF treatment. On comparison of the 2 and 5 μg/kg G-CSF groups, there were no significant differences in the number of PMN cells, the level of FcγRI (CD64) expression, or the level of ADCC activity 3 days after the start of G-CSF treatment.

**Serum Levels of IL-4 and IL-10.** Rituximab downregulates IL-10 expression, which mediates the down-regulation of BCL-2 expression and sensitization of lymphoma cells by therapeutic drugs (13). It has been reported that IL-4 enhances CD20 antigen expression (20). However, G-CSF itself induces the release of Th-2 cytokines IL-4 and IL-10 (14). Therefore, there is a possibility that the induction of IL-10 by G-CSF attenuates the effect of rituximab, whereas IL-4 enhances it. We measured the serum levels of IL-4 and IL-10 before and after G-CSF administration, irrespective of the dose of G-CSF (Fig. 1).

**Response to the R-CHOP and G-CSF Regimen.** As shown in Table 2, the response rate to the R-CHOP regimen with or without G-CSF was 100% (CR, 12 patients, 80%; partial remission, 3 patients, 20%). CR was achieved in three of the five patients (60%) who received 0 μg/kg G-CSF, four of the five patients (80%) who received 2 μg/kg G-CSF, and all five patients (100%) who received 5 μg/kg G-CSF. The median length of the observation period was 14 months, and 2 of the 12 patients relapsed. The 2-year survival rate was 90.9%.

**DISCUSSION**

Rituximab, when combined with CHOP chemotherapy, has been shown to have higher response rates and a longer duration of response in B-cell lymphoma, including follicular lymphoma, compared with rituximab alone. In an attempt to further increase the efficacy of this combination, we have added agents that may have other biological effects in immunotherapy such as augmentation of the effect of rituximab by increasing the expression level of CD20 antigen on tumor cells as well as cell-mediated

**Table 2** Results of treatment (n = 15)

<table>
<thead>
<tr>
<th>Response</th>
<th>Response rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR* (including CCR)</td>
<td>12 (80%)</td>
</tr>
<tr>
<td>G-CSF</td>
<td></td>
</tr>
<tr>
<td>0 μg/kg</td>
<td>3/5</td>
</tr>
<tr>
<td>2 μg/kg</td>
<td>4/5</td>
</tr>
<tr>
<td>5 μg/kg</td>
<td>5/5</td>
</tr>
<tr>
<td>PR</td>
<td>3 (20%)</td>
</tr>
<tr>
<td>Relapse (median follow-up, 14 mo)</td>
<td>2/12 (16.6%)</td>
</tr>
<tr>
<td>Survival (median follow-up, 17 mo)</td>
<td></td>
</tr>
<tr>
<td>Overall survival (2 yr)</td>
<td>90.9%</td>
</tr>
<tr>
<td>Disease-free survival (1 yr)</td>
<td>80.0%</td>
</tr>
<tr>
<td>Progression-free survival (2 yr)</td>
<td>68.1%</td>
</tr>
</tbody>
</table>

*CR, complete remission; CCR, complete clinical remission; G-CSF, granulocyte colony-stimulating factor; PR, partial remission.
cytotoxicity. Several studies have reported that the combination of rituximab and several types of cytokines enhances the cytotoxic effect of rituximab. For example, granulocyte macrophage colony-stimulating factor, IL-4, and tumor necrosis factor α each increase CD20 expression on the surface of tumor cells, enhance the binding of rituximab to tumor cells, and significantly augment apoptosis (20). On the other hand, clinical studies have shown that rituximab in combination with cytokines such as G-CSF (21), IFN-α (22), IL-2 (23), or IL-12 (24) may have higher efficacy than rituximab alone in the treatment of relapsed low-grade lymphoma, and the time to progression with the combined treatment was reported to be a little longer than that with rituximab alone. However, additional studies on whether this leads to improved survival are needed.

The adverse reactions to G-CSF among the patients in the current study were mild and consisted mainly of bone pain and flu-like symptoms, and this is in agreement with a previous study on G-CSF administration (25). Currently, G-CSF is administered to patients who develop neutropenia after R-CHOP therapy, and no enhanced adverse reaction was reported. van der Kolk et al. (21) reported that among patients with relapsed low-grade lymphoma who were treated with rituximab and G-CSF, most of the adverse reactions were of grade 1/2; 29% of patients developed fever, and 19% of patients developed an allergic reaction. The response rate was 42%, which is comparable with that of rituximab monotherapy, but the duration of the response to the combination of G-CSF and rituximab was longer than that to rituximab alone, indicating the usefulness of the combination of G-CSF and rituximab. In the current study, we examined the antitumor effect of G-CSF in combination with the R-CHOP regimen for the treatment of follicular lymphoma. We determined the optimal dose of G-CSF and used the levels of FcγRI (CD64) expression on neutrophils and ADCC activity to assess the antitumor effect. We also investigated the safety of this regimen. We found that the adverse reactions to R-CHOP with G-CSF were comparable with the adverse reactions to R-CHOP, and no new adverse reaction or aggravation of a side effect was observed. A previous study showed that the combination of granulocyte macrophage colony-stimulating factor and R-CHOP yielded an overall response of 100%, but CR was seen in only 21% of the patients, which was lower than the CR rate in the current study, and that the regimen caused heart toxicity in two patients, indicating that G-CSF with R-CHOP is less comparable with the adverse reactions to R-CHOP. Therefore, the optimal dose of G-CSF was considered to be 2 μg/kg. Alas and Bonavida (13) reported that rituximab down-regulated bcl-2 expression in some B-cell lymphoma cell lines through an IL-10-dependent autocrine loop, rendering the resistant cells susceptible to chemotherapeutic drugs, and that the signal transducer and activator of transcription 3 protein was involved. It has also been reported that IL-4 significantly up-regulates CD20 expression on peripheral blood mononuclear cells (20). In addition, because G-CSF stimulates IL-4 and IL-10 release from Th-2 cells, G-CSF-induced IL-10 expression may attenuate the effect of rituximab, whereas G-CSF-induced IL-4 expression may enhance it. Hence, we measured the serum levels of IL-4 and IL-10 in serum samples obtained before and after G-CSF administration. We found that there were no significant differences in the IL-4 and IL-10 levels before and after administration, and it is unlikely that IL-4 and IL-10 modified the effect of rituximab in this study.

In conclusion, R-CHOP with G-CSF was safe for the treatment of untreated follicular lymphoma. We are planning to carry out a randomized controlled trial comparing this regimen and R-CHOP therapy.

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Clinical Cancer Research

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