recombinant human EPO (rhEPO). rhEPO-related PRCA reached a peak incidence mainly in Europe in 2001 to 2002, largely related to a change of formulation, and of uncoated rubber stoppers (leachates present), in a particular rhEPO product, Eprex, and subcutaneous administration (Casadevall *et al*, 2002; Rossert *et al*, 2004).

The two most important initial steps in the management of anti-EPO antibody-mediated PRCA are transfusions for symptomatic anaemia and stopping the administration of rhEPO (Rossert *et al*, 2004). Since PRCA in this setting is immune-mediated, and since spontaneous remissions after cessation of rhEPO therapy are rare, immunosuppressive therapy should be provided in most cases (Verhelst *et al*, 2004; Rossert *et al*, 2005). Anti-rhEPO antibodies cross-react not only with the endogenous hormone, but also with all rhEPO molecules, including darbepoetin alfa (Casadevall *et al*, 2002). Rechallenge with rhEPO preparations may cause an anamnestic antibody response, making it less possible for the antibody to either spontaneously disappear or return to clinically unimportant levels, and may induce the formation of allergic skin and systemic reactions (Weber *et al*, 2002).

Recently, a novel peptide-based EPO receptor agonist called Hematide, which does not cross-react with anti-EPO antibodies, has been developed (Stead *et al*, 2006). Hematide is a synthetic, dimeric peptidic erythropoiesis-stimulating agent covalently linked to polyethylene glycol and is being developed for the treatment of anaemia associated with chronic renal failure and cancer. Because its primary amino acid sequence is unrelated to that of rhEPO, Hematide is unlikely to induce a cross-reactive immune response against endogenous EPO and is reported to correct anaemia induced by anti-rhEPO antibodies in a rat PRCA model (Woodburn *et al*, 2007).

Table I. Treatment of pure red cell aplasia (PRCA).

Agent	Response rate (CR + PR)*	Mean time to response	Need for maintenance therapy	Feasibility of long-term maintenance§
Corticosteroids; CS (methyl-prednisolone/ prednisone/prednisolone)	30–62%	2·5 weeks† 9 weeks in patients with primary idiopathic PRCA‡ (33% of patients achieved remission within 2 weeks)	Required† (Most patients relapsed during the taper of CS) Required in patients with primary idiopathic PRCA‡	Unacceptable for the dose to maintain remission
Cyclosporine A; CsA	65–87%	12 weeks in patients with primary idiopathic PRCA‡ (65% of responders achieved remission within 2 weeks)	Required in patients with primary idiopathic PRCA‡ (86% relapsed after discontinuation of CsA while 11% relapsed during the maintenance of CsA)	May be durable but needs careful monitoring
Cyclophosphamide; CY (CY + CS)	7–20% (40–60%)	11 weeks†	Unknown: required in some patients	Unacceptable

Agent	Relapse free survival (RFS)	Median overall survival (OS)
Corticosteroids; CS (methyl-prednisolone/ prednisone/prednisolone)	80% of patients relapse within 24 months after remission during dose-reduction† Median RFS: 33 months in patients with primary idiopathic PRCA‡ (88% of patients relapsed during CS maintenance)	14-year OS in patients with primary idiopathic PRCA treated with CS or with various combinations except for CsA†
Cyclosporine A; CsA	Median RFS: 103 months in patients with primary idiopathic PRCA‡ (Including patients who relapsed after the discontinuation of CsA)	12-year OS in patients with primary idiopathic PRCA responding to remission induction therapy with CsA‡ 10-year OS was 95% and the median OS has not yet been reached in all patients‡
Cyclophosphamide; CY (CY + CS)	Unknown: the duration of remission induced by CY seems to be prolonged as compared to patients induced by CS*	Unknown

Patients with primary acquired and secondary PRCA are included if not otherwise indicated.
*References are indicated in the text.
†Referenced by Clark *et al* (1984).
‡Referenced by Sawada *et al* (2007).
§Referenced by Clark *et al* (1984), Sawada *et al* (2007), Radis *et al* (1995) and Reinhold-Keller *et al* (2000).

Thus, a recent animal study suggested that a possible alternative strategy might be to administer Hematide to patients with PRCA due to anti-rhEPO antibodies, which should enable ongoing stimulation of erythropoiesis.

Future prospects: proposal for a first-line therapy in primary acquired PRCA

There are several options for inducing remission of PRCA, but many patients with acquired PRCA require immunosuppressive therapy to maintain remissions. As summarized in Table I, CS, CsA and CY plus CS are almost equally effective for inducing remissions of PRCA, but the most important difference between these agents is the feasibility of long-term maintenance. Although the relapsed patients can be re-treated with the same agents, such as CS or CS plus CY, the cumulative side effects and toxicity become unacceptable. Considering the recurrent nature of acquired PRCA, we suggest CsA as first-line therapy for these patients at a dose of 2·5–3 mg/kg twice daily to achieve trough CsA levels of 150–250 ng/ml for a maximum of three to four months. This trough CsA level has been empirically determined according to a consensus from a multicentre randomized study in Japan for aplastic anaemia (Teramura *et al*, 2007). Maintenance therapy with CsA is a requisite for most patients to prevent relapse. Since nephrotoxicity constitutes the major limiting side effect of CsA, careful and progressive decrease of the dosage to the minimum required for maintenance of remission is appropriate. The mean maintenance dose of CsA in Japanese patients who were continuing their first remission for more than 24 months was 2·2 ± 0·8 mg/kg per day with a range of 1·1–3·8 mg/kg per day, 40% of the initial dose (Sawada *et al*, 2007), which suggests difficulty in reducing CsA under this dosage to maintain remissions. Adequate prevention and treatment of infections secondary to immunosuppression are also necessary for successful management of these patients.

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Cut-off value of red-blood-cell-bound IgG for the diagnosis of Coombs-negative autoimmune hemolytic anemia

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Direct antiglobulin test (DAT)-negative autoimmune hemolytic anemia (Coombs-negative AIHA) is characterized by laboratory evidence of in vivo hemolysis, together with a negative DAT performed by conventional tube technique (CTT) in clinically suspected AIHA patients. The immunoradiometric assay (IRMA) for red-blood-cell-bound immunoglobulin G (RBC-IgG) can be used to diagnose patients in whom CTT does not detect low levels of red cell autoantibodies. We investigated the diagnostic cutoff value of the IRMA for RBC-IgG in Coombs-negative AIHA and calculated its sensitivity and specificity. Of the 140 patients with negative DAT by CTT referred to our laboratory with undiagnosed hemolytic anemia, AIHA was clinically diagnosed in 64 patients (Coombs-negative AIHA). The numbers of Coombs-negative AIHA and non-AIHA patients changed with age and gender. The cutoff values were determined from receiver operating characteristic (ROC) curve according to age and gender. The IRMA for RBC-IgG proved to be sensitive (71.4%) and specific (87.8%) when using these cutoffs. Using these cutoffs for 41 patients with negative DAT referred to our laboratory in 2006, all the pseudonegative cases were treated with steroids before the test. The 31 untreated cases could be grouped using one cutoff value of 78.5 and showed 100% sensitivity and 94% specificity, independent of gender and age. Results indicate that RBC-IgG could become a standard approach for the diagnosis of Coombs-negative AIHA, when measured before treatment. Am. J. Hematol. 84:98–101, 2009. © **2008 Wiley-Liss, Inc.**

Introduction

The detection of red-blood-cell-bound immunoglobulin G (RBC-IgG) and complement by direct antiglobulin test (DAT) remains the main serological assay in the diagnosis of autoimmune hemolytic anemia (AIHA) [1]. Several methodologies have been investigated for detection and evaluation of these autoantibodies. DAT by conventional tube technique (CTT) is the method most commonly used in the blood centers and is still considered a gold standard [2]. A positive DAT is almost always seen in association with AIHA [3] and forms the characteristic of the serological diagnosis of AIHA [4,5]. However, it has also been shown that a negative DAT does not exclude the diagnosis of AIHA [5,6] and 1–10% of patients with AIHA have been reported to show a negative DAT [7–9]. These patients, designated "Coombs-negative AIHA" patients, may carry lower numbers of IgG molecules per RBC, yielding a negative tube DAT and in vivo hemolysis [10]. Also, they may have only RBC-IgA autoantibodies or monomeric IgM molecules that induce the clinical and hematological features typical of AIHA [11]. The immunoradiometric assay (IRMA) [12], the complement fixation antibody consumption test [13] and the enzyme-linked antiglobulin test (ELAT) for RBC-IgG, as well as the enzyme-linked immunosorbent assay (ELISA) for IgG eluted from RBCs [14,15] are representative methods to quantitatively detect RBC-IgG. Flow cytometry [16] and the gel test [17,18] are semiquantitative methods. Despite the availability of these sensitive methods, there are no established standard tests for the diagnosis of Coombs-negative AIHA, which often makes it difficult for clinicians to diagnose AIHA in patients with DAT-negative hemolytic anemia. Therefore, the aim of the study was to assess the clinical utility of RBC-IgG levels in the diagnosis of Coombs-negative AIHA patients and to calculate the cutoff values, sensitivity, and specificity after a 1-year follow-up period.

Results

A total of 192 surveys were returned for analysis; a response rate of 78%. The mean age of participants was 51.0 ± 22.9 years (range 0.9–85) and 49% of participants were female. There were no significant differences in age and gender (49.7 ± 27.7 years old and 63% female) between responders and nonresponders to the survey. Of the responders, 144 were DAT-negative. Forty-seven percent of the DAT-negative hemolytic anemia patients (n = 68) were classified as AIHA; 64 had warm-type AIHA and four had cold-type AIHA. Significant differences were found in %Retic (P = 0.03), MCV (P = 0.01), LDH (P = 0.03), IDBIL (P = 0.03), and RBC-IgG (P < 0.0001) levels between the Coombs-negative AIHA and non-AIHA groups. There were no significant differences in age, gender, Hb, and Hp between the two patient groups.

The ROC curves for RBC-IgG levels, using clinical diagnosis as an indicator of AIHA, are shown in Fig. 1. Table I summarizes the AUCs, confidence intervals for AUCs, and likelihood ratios (LRs) for laboratory variables, as well as sensitivities and specificities, which were calculated at the

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optimal cutoff values (maximum point of efficiency curve). RBC-IgG showed the highest AUC values; the other three indices showed low AUC values. These data suggested that only RBC-IgG can effectively distinguish between the Coombs-negative AIHA and non-AIHA patients (see Fig. 2). Use of the cutoff point value (83) showed the sensitivity (70%), specificity (84%), and LR (4.8).

To discover the clinically significant cutoff values, the numbers of Coombs-negative AIHA patients were investigated according to age and gender. In females, there were two groups in the distribution of patients: one in the patients aged less than and one in those aged more than 45 years. In males, there was one peak in the patients aged more than 60 years. Together with the ROC curve for each group, the optimal cutoff values (maximum points of efficiency curve) were calculated for RBC-IgG levels. In females aged less than 45 years and those aged more than 45 years, the optimal cutoff values were 96 and 128, respectively. In males aged less than 60 years and those aged more than 60 years, the optimal cutoff values were 60 and 102, respectively.

Use of these cutoff points for 140 DAT-negative hemolytic anemia patients showed the slightly good sensitivity (71%), specificity (88%), and LR (5.9). Using these cutoff points, 41 cases of DAT-negative hemolytic anemia, which were referred to our laboratory in 2006, were categorized and showed slightly better sensitivity (78%), specificity (94%), and LR (14.1). Interestingly, all the pseudonegative cases had been treated with steroids (see Fig. 3). The 31 untreated cases could be grouped using one cutoff value as 78.5 (Fig. 3b) and showed high sensitivity (100%), specificity (94.1%) and LR [16]. Two patients with non-AIHA hemolytic diseases (drug-induced hemolytic anemia and myelodysplastic anemia) showed positive RBC-IgG, which might suggest the involvement of immunological hemolytic mechanisms [19]. In our laboratory, some patients with myelodysplastic anemia tended to show positive RBC-IgG (data not shown) mechanisms of which are analyzed by our collaborators.

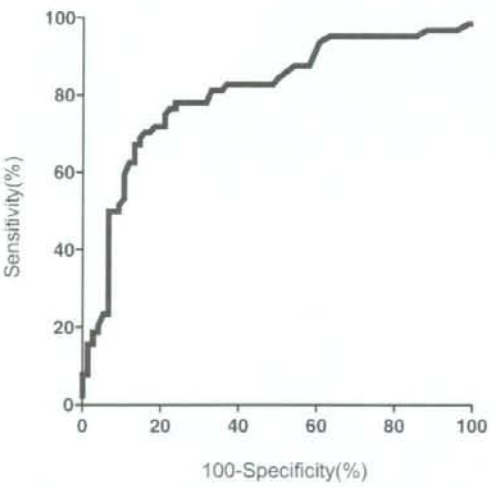

Figure 1. Receiver operating characteristic (ROC) curves for red-blood-cell-bound immunoglobulin G (RBC-IgG) (area under the ROC curves (AUC) 0.81), when the clinical diagnosis of AIHA by the attending doctors after 1-year follow-up was the gold standard.

Discussion

In the management of DAT-negative hemolytic anemia, it is important to distinguish Coombs-negative AIHA patients from other hemolytic anemia, because steroid treatment has major effects on AIHA [6], but steroids have also been associated with several serious side effects [20], which makes clinicians hesitate to use steroids to treat DAT-negative hemolytic anemia patients without diagnosis of AIHA.

In our laboratory, the immunoradiometric assay (IRMA) [12] is used to detect RBC-IgG quantitatively rather than semiquantitative methods such as flow cytometry [16] and gel column [17] for two reasons. First, in our laboratory IRMA has been used since 20 years ago as a central laboratory in Japan and its cost is supported by a grant for research on intractable diseases from the Ministry of Health, Labor and Welfare of Japan. Second, gel column method showed occasionally pseudopositive in some cases, the reason of which has remained unclear and flow cytometry requires normal RBCs as negative control in each measurement. Although the simple methods are desired, quantitative measurements should be used to guarantee their ability to measure subthreshold IgG.

Previously, we reported the value of RBC-IgG (33 ± 13) in 100 healthy Japanese adults and the RBC-IgG required to be DAT-positive (335 ± 72) [21]. Previous studies have reported very similar values [12,22]. The usefulness of RBC-IgG in diagnosis for Coombs-negative AIHA had been reported [23,24]. In previous studies, patients with Coombs-negative AIHA were reported to have abnormal levels of IgG, ranging from 70 to 434 [25] or from 76 to 350 [6]. There are, however, no reports referring to the IgG cutoff value, sensitivity, specificity, and LR. Practically, in our laboratory some non-AIHA samples from patients with hemolysis showed higher values than normal healthy individuals (Figs. 2 and 3), which might suggest the involvement of immunological hemolytic mechanisms [19]. In addition, some Coombs-negative AIHA patients had values very close to the normal range (see Fig. 2). So, in the practical diagnostic procedure, cutoff values must be calculated from RBC-IgG levels of DAT-negative patients with hemolysis rather than from normal RBC-IgG levels of healthy individuals. We have adopted the clinical diagnosis as the gold standard for the diagnosis of Coombs-negative AIHA because there are no established standards and many clinicians have previously clinically diagnosed Coombs-negative AIHA by the presence of hemolysis, denial of other

TABLE I. Area Under the ROC (Receiver Operating Characteristic) Curve (AUC), Confidence Intervals (95%) of AUCs, and Likelihood Ratio for Diagnosing AIHA in Patients With DAT-Negative Hemolytic Anemia (n = 140)

Parameter	AUC ± SE	Confidence interval (95%)	Cutoff point	Sensitivity	Specificity	Likelihood ratio
%Retic (%)	0.61 ± 0.05	0.51–0.71	6.8	54.9	82.8	1.9
MCV (fL)	0.65 ± 0.05	0.54–0.75	94.7	72.6	51.8	1.5
LDH (U/l)	0.62 ± 0.05	0.51–0.73	386	64.7	62.5	1.7
IDBIL (mg/dl)	0.62 ± 0.05	0.52–0.72	1.2	66.7	59.1	1.6
RBC-IgG	0.81 ± 0.04	0.73–0.88	83.0	70.3	84.2	4.8

Cutoff points, sensitivities, and specificities for each test are indicated in maximum points of the ROC curves. Calculated likelihood ratios are based on the cutoff points.

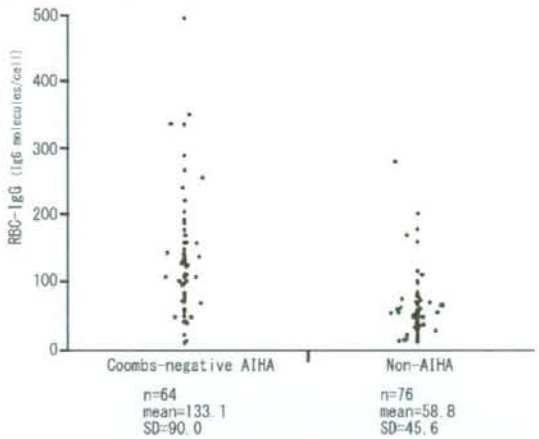

Figure 2. Red-blood-cell-bound IgG (RBC-IgG) of Coombs-negative AIHA and non-AIHA patients between 2003 and 2006. A significant difference was found in RBC-IgG levels ($P < 0.0001$) between the Coombs-negative AIHA and non-AIHA groups.

hemolytic diseases, and responsiveness to steroid treatments with respect of RBC-IgG value [26].

RBC-IgG levels of non-AIHA patients tended to increase with advanced age, especially in females. RBC-IgG levels of Coombs-negative AIHA in females also tended to increase with age and showed a gap at about 45 years of age, which gave two peaks at the ages of 25 and 65 years. In males, there was a smaller increase in RBC-IgG levels with advanced age, but Coombs-negative AIHA was most common in patients aged more than 60 years. These distribution trends were also reported in a previous report in Japanese patients [27]. The report suggested that these tendencies might be attributed to characteristics of AIHA, regardless of DAT-positive or -negative characteristics, and not to the population composition in Japan. In light of these trends, more effective cutoff values could be calculated using age- and gender-stratified analyses. Moreover, exclusion of the treated samples could increase the sensitivity and specificity of RBC-IgG and so it can be considered a standard approach for the diagnosis of Coombs-negative AIHA. Therefore, we propose that the RBC-IgG level should be measured for the diagnosis of Coombs-negative AIHA and the cutoff value used should be 78.5 if RBC-IgG is measured before treatment, and that after treatment the RBC-IgG level might range from the Coombs-negative value to as low as that seen in normal healthy individuals (see Fig. 3).

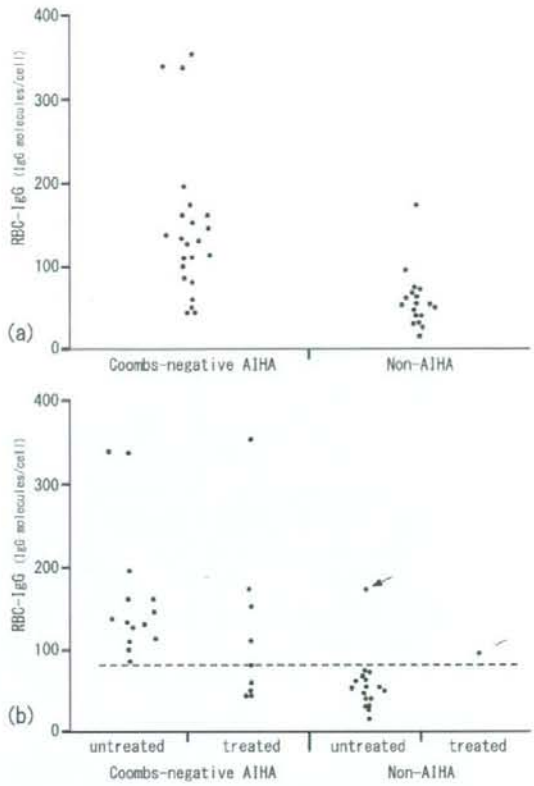

Figure 3. Red-blood-cell-bound IgG (RBC-IgG) of Coombs-negative AIHA and non-AIHA groups in 2006 (a). Each group consists of two subgroups treated or untreated with steroid drugs (b). A dotted line indicates 78.5 IgG molecules per cell. There were two non-AIHA patients with negative DAT, whose RBC-IgG levels were higher than 78.5. The black arrow represents a patient with drug-induced hemolytic anemia and the white arrow indicates a patient with hypoplastic myelodysplastic syndrome.

Materials and Methods

The study was performed over a period of 4 years from 2003 to 2006 at the laboratory of the Center for Community Medicine, Jichi Medical University, Tochigi, Japan, after approval by the Institutional Ethics Panel Committee.

Patients. During a 4-year period, 261 samples from 245 patients were referred to our laboratory for quantitation of RBC-IgG. Of these, 54 (22%) were DAT-positive and 191 (78%) were DAT-negative, as shown by analysis of polyspecific DAT by CTT (Ortho Diagnostics, USA) and monospecific DAT by CTT using anti-IgG and anti-C3d antibodies (Ortho Diagnostics, USA) following manufacturer's instructions.

Sample preparation. Heparinized whole blood (10 ml) samples were collected. The RBC layer was prepared by centrifuging the whole blood at 1,000 rpm for 20 min. The supernatant plasma and buffy coat were discarded. One milliliter samples of packed RBCs were diluted in 10 ml of phosphate-buffered saline (PBS), pH 7.0, 0.15 M. The diluted RBCs were passed through a cotton-wool column to exclude neutrophils and monocytes, according to the method of Jeje et al. [12]. The RBCs were washed four times with PBS, and the resulting RBCs (0.3 ml) were suspended in 0.4 ml of PBS.

Immunoradiometric assay (IRMA) for RBC-IgG. IRMA for RBC-IgG was performed according to the method of Jeje et al. [12] with some modifications. Samples of ^{125}I-labeled anti-human IgG antisera derived from goat (Du Pont, Wilmington, DE) were diluted in PBS containing 3% bovine serum albumin with a specific activity of ~10,000 cpm/200 μl (Wakojunyaku, Osaka, Japan). A volume of 400 μl of the washed RBCs was incubated for 1 h at 37°C with 200 μl of the diluted anti-human IgG. IgG beads were prepared using the methods described by Jeje et al. [12]. Human IgG and beads (SephasorbTM HP) were purchased from Sigma Chemical (St. Louis, MO) and Pharmacia Fine Chemicals (Uppsala, Sweden), respectively. Two hundred microliter samples of IgG beads (2×10^6) were added to the mixture of RBCs and ^{125}I-labeled anti-human IgG and incubated at 37°C for 30 min. The RBCs were lysed by the addition of 80 μl of 20% Triton X-100 (Sigma). The beads were washed four times with 20% Triton X-100-containing PBS, and the radioactivity was measured using a gamma counter (Aroka, Tokyo, Japan). A standard curve was generated using human IgG standards (10–10,000 ng IgG/ml; Sigma). The percent inhibition of binding was plotted against each concentration of IgG. Using the standard curve, RBC-IgG levels were calculated after counting the number of RBCs. Each attending doctor was informed of the RBC-IgG level within 3–10 days of ordering.

Clinical diagnosis questionnaire. At 1 year after referral to our laboratory, follow-up investigations were performed; the attending doctor used a questionnaire to assess the patient's clinical diagnosis. The bases of

clinical diagnosis of Coombs-negative AIHA were in vivo hemolysis (low hemoglobin (Hb) concentration, high percentage of reticulocyte (%Retic), high indirect serum bilirubin (IDBIL) level, high lactate dehydrogenase (LDH) level, low haptoglobin (Hp) level and/or high erythropoiesis level in bone marrow) and exclusion of other anemic icteric diseases without hemolysis (such as megaloblastic anemia, myelodysplastic syndrome, erythroid leukemia, congenital dyserythropoietic anemia, hepatobiliary diseases, and constitutional jaundice). AIHA was diagnosed by measuring the RBC-IgG level, steroid-reactivity and exclusion of alloimmune hemolytic anemia and drug-induced hemolytic anemia.

Statistical analysis. Patients who had negative DAT were divided into AIHA and non-AIHA groups on the basis of clinical diagnosis. The normality of the laboratory variables was analyzed using the Kolmogorov-Smirnov test with Lilliefors significance correction. As most variables were not normally distributed, a Mann-Whitney *U*-test was used to determine the differences between Coombs-negative AIHA and non-AIHA patients. The median range and interquartile range were also calculated for all variables.

The accuracy of the tests for diagnosis of AIHA in DAT-negative hemolytic anemia patients was evaluated using receiver operating characteristic (ROC) curves [28]. By this method, a test that is perfect has 100% sensitivity and no false-positives (1-specificity = 0) and will have an area under the curve (AUC) of 1.0, whereas a test that has no diagnostic value would have an AUC of 0.5. The 95% confidence intervals and LRs were also calculated.

JMP 7.0.1 for Macintosh (SAS Institute, Cary, NC) and GraphPad Prism 4.0c for Macintosh (GraphPad Software, San Diego, CA) were the statistical software used.

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Review Article

Roles for deregulated receptor tyrosine kinases and their downstream signaling molecules in hematologic malignancies

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Growth, survival and differentiation of hematopoietic cells are regulated by the interactions between hematopoietic growth factors and their receptors. The defect in these interactions results in a failure of hematopoiesis, while aberrantly elevated and/or sustained activation of these signals cause hematologic malignancies. Among them, constitutively activating mutations of the receptor tyrosine kinases (RTKs), such as c-Kit, platelet-derived growth factor receptor (PDGFR) and FLT3, are often involved in the pathogenesis of various types of hematologic malignancies. Constitutive activation of RTKs is provoked by several mechanisms including chromosomal translocations and various mutations involving their regulatory regions. Chromosomal translocations commonly generate chimeric proteins consisting of the cytoplasmic domain of RTKs and the dimerization or multimerization motif of the fusion partner, resulting in the constitutive dimerization of RTKs. On the other hand, missense, insertion or deletion mutations in the regulatory regions, such as juxtamembrane domain, activation loop, and extracellular domain, also cause constitutive activation of RTKs mainly by preventing the auto-inhibitory regulation. Oncogenic RTKs activate downstream signaling molecules such as Ras/MAPK, PI3-K/Akt/mTOR, and STATs as well as ligand-activated wild type RTKs. However, their signals are quantitatively and qualitatively different from wild type RTKs. Based on these findings, several agents that target oncogenic RTKs or their downstream molecules have been developed: imatinib and FLT3 inhibitors for RTKs themselves, farnesyltransferase inhibitors, mTOR inhibitors and MEK inhibitors for the downstream signaling molecules. As promising results have been obtained in several clinical trials using these agents, the establishment of these molecular targeted agents is expected. (*Cancer Sci* 2008; 99: 479–485)

Growth, differentiation, and survival of hematopoietic cells are regulated by a number of soluble factors such as cytokines and hormones as well as intercellular interactions through the cell surface antigens. Cytokines bind to their cognate cell-surface receptors, which immediately activate intracellular cascades either through their intrinsic enzymatic activity or their association with other catalytic proteins. Then, the activated intracellular downstream signaling cascades control the transcription of their effector genes directly or indirectly. The loss of function of this signaling leads to the failure of hematopoiesis. Conversely, aberrantly elevated and/or sustained activation of these signals can be a causative event of hematologic malignancies.[1–3] Among them, considerable attention has been paid to oncogenic receptor tyrosine kinases (RTKs). These include receptors for macrophage colony-stimulating factor (c-FMS or CSF-1R), FLT3, stem cell factor (c-KIT), and platelet-derived growth factor (PDGF), which are also involved in normal hematopoiesis. Here, we will briefly review the recent findings on the mechanisms of aberrant activation of RTKs and their roles in the development of hematological malignancies. Also, we will focus on several new agents that target these oncogenic RTKs and their downstream molecules for the treatment of hematologic malignancies.

Structures and classification of RTKs

Receptor tyrosine kinases are membrane-bound enzymes composed of a characteristic extracellular ligand-binding domain, a transmembrane domain, a highly conserved intracellular kinase domain, and a C-terminal tail. RTKs include 20 subfamilies: class I including epidermal growth factor receptor; class II including insulin-like growth factor-1 (IGF1) receptor; class III including PDGF receptor (PDGFR), c-FMS, c-KIT, and FLT3R; and class IV including fibroblast growth factor receptor (FGFR). Among them, type III RTKs, which are characterized by five immunoglobulin-like extracellular domains and two intracellular kinase domains separated by the kinase insert, are frequently mutated and involved in the pathogenesis of hematopoietic malignancies (Fig. 1).[2,3]

Mechanisms of activation of RTKs

Generation of fusion genes by chromosomal translocations. Reciprocal chromosomal translocations involving RTKs are observed in several types of hematologic malignancies. Among RTKs, PDGFR and FGFR1 are the frequent targets of this type of activation (Table 1). For example, TEL(ETV6)-PDGFRβ is formed in t(5;12)(q31;p12), Huntington interacting protein-1 (HIP1)-PDGFRβ in t(5;7)(q33;q11.2), H4/D10S170-PDGFRβ in t(5;10)(q33;q11.2), Rabaptin-5-PDGFRβ in t(5;17)(q33;p13), and CEV14-PDGFRβ in t(5;14)(q33;q32).[2,3] The fusion partners are completely unrelated, but commonly possess the motifs, which are assumed to be utilized for the ligand-independent homodimerization of each RTK (Fig. 2). Most patients with the PDGFRβ rearrangement reveal common clinical features resembling chronic myelogenous leukemia (CML) or chronic myelomonocytic leukemia (CMML), which are characterized by leukocytosis accompanied by marked eosinophilia, a variable degree of monocytosis, and splenomegaly. Transformation to acute leukemia occurs in only a minority of these patients with a highly variable latent period from 9 months to 12 years. Exceptionally, CEV14-PDGFRβ caused by t(5;14)(q33;q32) has been reported to be associated with acute myeloid leukemia (AML) relapse.[4] In addition to PDGFRβ,

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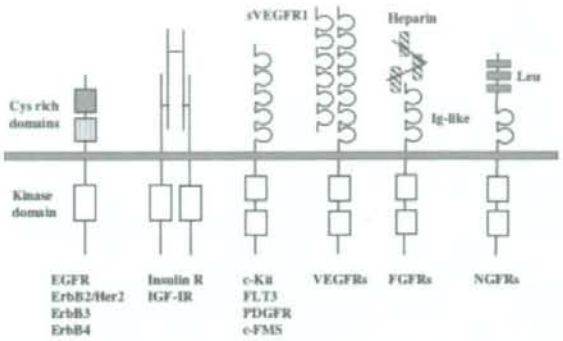

Fig. 1. Structures and classification of receptor tyrosine kinases.

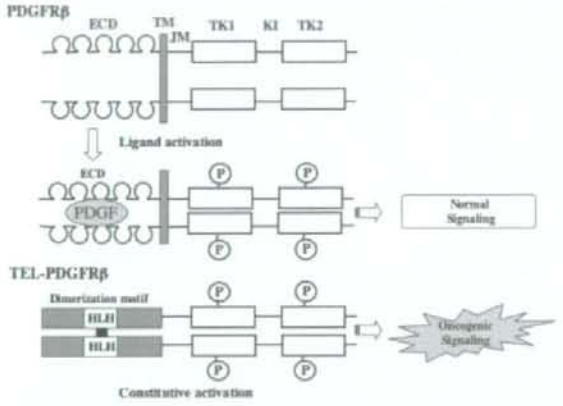

Fig. 2. Structures of wild type and chimeric platelet-derived growth factor receptors (PDGFRs). The representative fusion product of ETV6-PDGFRβ is shown. Helix-loop-helix domain (HLH) in the TEL mediates the dimerization of fused PDGFRβ. ECD, extracellular domain; TM, transmembrane domain; JM, juxtamembrane domain; TK1, tyrosine kinase 1 domain; TK2, tyrosine kinase 2 domain; KI, kinase insert.

PDGFRα is also involved in chromosomal translocation. FIP1L1-PDGFRα generated by the interstitial deletion at 4q12 was reported to act as a causative gene for hypereosinophilic syndrome (HES)/chronic eosinophilic leukemia (CEL).[5] Moreover, a recent paper identified novel fusion genes, KIF5B-PDGFRα from t(4;10)(q12;p11), STRN-PDGFRα from t(2;4)(p24;q12), and ETV6-PDGFRα from t(4;12)(q2?3;p1?2) in patients with CEL.[6,7] Meanwhile, a rare variant translocation t(4;22)(q12;q11) yielding BCR-PDGFRα was detected in a patient with CML-like myeloproliferative disorder (MPD).[8] On the other hand, the rearrangements of FGFR1 generated by chromosomal translocations involving 8p11 cause MPDs with common clinical features characterized by splenomegaly and marked eosinophilia.[3,9] So, these MPDs are now recognized as a distinct disease entity referred to as '8p11 myeloproliferative syndrome (EMS)' or 'stem cell leukemia–lymphoma syndrome (SCLL)'.[9] The most frequent type of chromosomal translocation is t(8;13)(p11;q12), which yields the ZNF198-FGFR1 fusion product. Other fusion genes are FOP-FGFR1 caused by t(6;8)(q27;p11), CEP110-FGFR1 by t(8;9)(p11;q33), and BCR-FGFR1 by t(8;22)(p11;q22). Among EMS, patients with BCR-FGFR1 translocation have clinical and morphological characteristics very similar to typical, BCR-ABL-positive CML. In addition, t(4;14)(p16.3;q32.3) leading to the overexpression of FGFR3 was identified in approximately 15% of multiple myeloma patients and cell lines.[10] In some cases, the translocated FGFR3 gene contains an activating mutation K650E. FGFR3 is also involved in the t(4;12)(p16;p13)-associated peripheral T cell lymphoma (PTCL) that progresses into AML, which generates the TEL-FGFR3 fusion protein with constitutive tyrosine kinase activity.[11]

Juxtamembrane domain mutation. The juxtamembrane (JM) domain of RTKs interacts with N- and C-terminal kinase lobes and prevents the activation loop from adopting active configuration, thereby serving as a negative regulatory domain. The mutations of this region including a missense point mutation, a deletion, and an insertion have been found in c-Kit, Flt3 and PDGFRβ genes (Fig. 3). The JM mutations of c-Kit were first identified in mast cell lines: a point mutation (Val560Gly) in a human mast cell leukemia line, HMC-1; the deletion of seven amino acids (ΔThr573–His579) in a murine mastocytoma cell line (FMA3).[12,13] In addition, JM c-Kit mutations were detected in canine mast cell tumors, one of the most popular and aggressive neoplasms in dog.[14] Subsequently, similar JM mutations of c-Kit were found to be present in 50–80% of cases of gastrointestinal stromal tumors (GISTs).[15,16] Furthermore, JM domain mutations of PDGFRα including the missense point mutation, insertion, and deletion, were identified in the GISTs without c-Kit mutations.[17]

The mutations of the FLT3 genes are in-frame internal tandem duplications (ITDs) involving the JM and adjacent N-terminal kinase domain, which result in the duplication of a stretch of several amino acids.[18] ITD is detectable in about 20% of AML cases covering all subtypes in FAB classification and in 10% of myelodysplastic syndrome (MDS) cases, while it is quite rare in

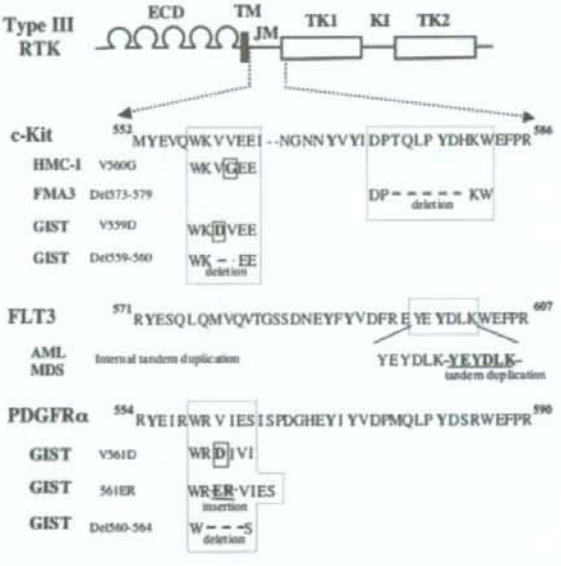

Fig. 3. Juxtamembrane domain (JM) mutations of type III receptor tyrosine kinases (RTKs). Representative mutations of type III RTKs, c-Kit, Flt3 and platelet-derived growth factor receptor β (PDGFRβ) are shown. Conserved amino acid sequence around the mutated regions are depicted. Conserved tyrosine residues are written in bold. Internal tandem duplications of FLT3 are tandem insertion of various length of amino acid sequences. The mutations of PDGFRα are found in GISTs without c-Kit mutations. HMC-1, a human mast cell leukemia cell line; FMA3, a murine mastocytoma cell line; GIST, gastrointestinal stromal tumor; AML, acute myeloid leukemia.

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acute lymphoblastic leukemia (ALL). In addition, FLT3-ITD has been shown to be a poor prognostic factor for AML cases in several studies.[3,19] To clarify the mechanism of aberrant activation of RTKs caused by the JM domain mutations, Ma and Irusta made alanine-scanning mutations in the JM domain of c-Kit and PDGFRβ, respectively.[20,21] Consequently, they found that the alanine mutation at the structurally corresponding residues in c-Kit and PDGFRβ induced the constitutive activation. Based on the structural analysis made by Irusta, the JM domain was supposed to form a short α-helix followed by a three-stranded β-sheet very similar to known structures of WW domains. Furthermore, they found that activation mutations were clustered in the central portions of the first and second β strands and along one face of the β-sheet. These results suggest that various mutations such as point mutations, deletion, and insertion would invalidate the auto-inhibitory effect of the JM domain through the disruption of the WW structure. Previous papers including ours showed that both c-Kit and FLT3 carrying the JM domain mutations constitutively form a homodimer via the extracellular and juxtamembrane domains.[22,23] Together, it was speculated that JM mutations would induce the conformational change facilitating the receptor dimerization, which results in the constitutive activation of RTKs.

Tyrosine kinase domain (TKD) point mutations. TKD point mutations of the c-Kit, FLT3 and PDGFR genes are commonly observed at the Asp residue in the activation loop (Fig. 4). This type of mutation was first identified by us as Asp816Val in a human mast cell leukemia line, HMC-1, which also carried the JM domain mutation.[12] Subsequently, the mutations of c-Kit at the same site (Asp816→Val, Tyr, Phe, His) were found in patients with aggressive mastocytosis, MDS, and MPDs associated with mastocytosis.[24–26] Furthermore, the same mutations have been detected in a subset of AML patients,[27,28] especially in about 10% of AML cases called CBF AML, in which the AML1(Runx1) complex is deregulated by chromosomal translocations such as t(8;21) and inv(16). Although patients with CBF leukemia commonly have a good prognosis, the concurrent TKD mutation of c-Kit makes their prognosis poor.[27,28] FLT3 also has TKD point mutations at Asp835 to Tyr, Val, or various amino acids, which is found in 7% of AML patients and in some patients with ALL or MDS.[29,30] In addition, several novel alterations of FLT3 TKD such as a point mutation at codon 840 or 841, a small deletion yielding Δ835 or Δ836, and a 6-bp of small insertion between the codon 840 and 841 were identified in AML patients.[31,32] Of note, FLT3 TKD mutations are biologically distinct from and have a significantly more favorable prognosis than FLT-ITD in patients with AML.[32] Furthermore, the activation loop mutations of PDGFRα were found in patients with GIST without c-Kit mutations.[17] The activation mechanism of the TKD mutations has been extensively analyzed using the c-Kit mutants. We previously showed that c-Kit is constitutively activated by the substitution of Asp814 not only into Val and Tyr, but also into a wide variety of amino acids,[33] suggesting that Asp814 would play a crucial role in regulating enzymatic activity of c-Kit. Although the JM domain mutations induced constitutive dimerization via the extracellular domain, the TKD mutant does not show a similar reaction. By generating c-Kit mutants lacking the extracellular domain, we demonstrated that TKD mutants can dimerize independently of the extracellular domain.[34] Furthermore, using a series of c-Kit mutants carrying Tyr→Phe conversion of the intracellular 20 tyrosine residues or C-terminal deletion, we found that Tyr719Phe mutation and C-terminal 70 amino acids deletion abolished kinase activity of c-Kit.[35] Although the C-terminal deletion disrupted kinase activity of the wild type c-Kit, Tyr719Phe did not, suggesting that Tyr719 is specifically required for the activation of the c-Kit TKD mutant. Since Tyr719 is the binding site for the p85 subunit of PI3-K, it is postulated that the binding between Tyr719 of c-Kit and the p85 subunit is necessary for the c-Kit TKD mutant to form an active configuration. Recently, the crystal structure analysis of the RTK revealed that the activation loop in the wild-type RTK is oscillating between an active and inactive conformation, which is shifted to an active form when tyrosine residues in the loop are phosphorylated. So, it is speculated that activation loop mutations would evoke a conformational change similar to that caused by the tyrosine phosphorylation in the loop, thereby inducing constitutive activation of RTKs.

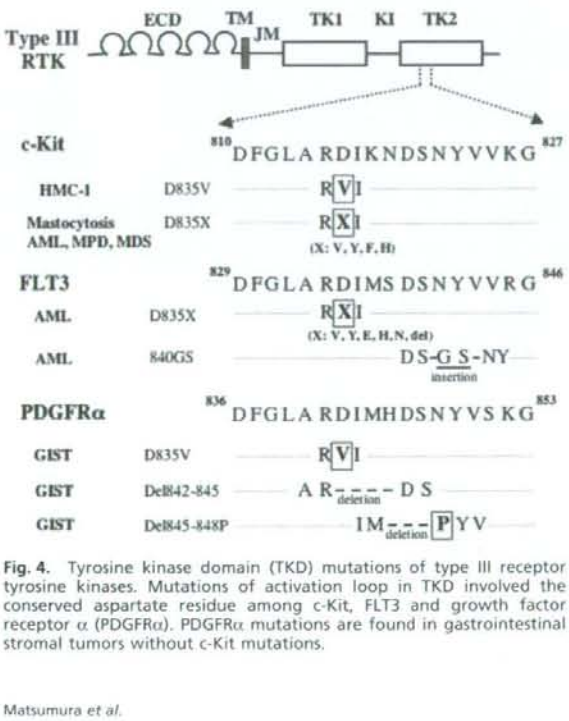

Fig. 4. Tyrosine kinase domain (TKD) mutations of type III receptor tyrosine kinases. Mutations of activation loop in TKD involved the conserved aspartate residue among c-Kit, FLT3 and growth factor receptor α (PDGFRα). PDGFRα mutations are found in gastrointestinal stromal tumors without c-Kit mutations.

Extracellular domain (ED) mutations. ED mutations in c-Kit, c-FMS and FGFR3 have been identified in several hematological malignancies. c-Kit mutations in the ED were located in the exon 8, causing the in-frame deletion plus insertion with consistent loss of Asp419. Gari *et al.* reported that this mutation was associated with AML harboring a mutation in either subset of CBF AML; 7/21 cases (33%) with inv(16) and 1/19 cases (5.2%) with t(8;21).[36] In agreement with their findings, Care *et al.* detected the same mutation in 15/63 cases (23.8%) with inv(16) and 1/22 cases (4.5%) with t(8;21).[37] In addition, Boell *et al.* found the c-Kit mutation in the exon 8 in 10/46 cases (22%) with inv(16) and 6/50 cases (12%) with t(8;21).[38] Similarly, the ED mutations of c-FMS at Leu301 located in the fourth immunoglobulin domain were found in acute myelomonocytic leukemia and MDS.[39,40] Although c-Kit mutants harboring the ED mutations have not been verified to be constitutively active, the ED mutants of c-FMS was proved to be constitutively active and oncogenic.[41] Since the fourth Ig-like domain is implicated in the stabilization of ligand-induced dimerization, these mutations are supposed to induce a conformational shift facilitating dimerization and activation. The ED mutations of FGFR3 involving Arg248 or Tyr373 were identified in a small subset of multiple myeloma. FGFR possesses three Ig-like domains, and the first Ig-like domain interacts with the ligand binding domain in the second and third Ig-like domains.[42] This intramolecular interaction is assumed to prevent the accidental activation of FGFR by the ligand, serving as an autoinhibition mechanism.

So, the ED mutation of FGFR was supposed to disturb this autoinhibitory mechanism, resulting in the constitutive activation.

Signal transduction from RTKs

Upon the ligand-binding to the extracellular domain, RTK dimerizes and induces transphophorylation of tyrosine residues in the cytoplasmic domains, which serve as docking sites for several adaptor molecules harboring SH2 domain or PTB (phosphotyrosine binding domain). These adaptor molecules recruit and activate downstream signaling molecules such as Ras/MAPK, PI3-Kinase, phospholipase C-γ, JNK, STATs, NF-κB pathways through tyrosine- or serine/threonine-phosphorylations (Fig. 5). Among them, Ras/MAPK, PI3-K and STAT pathways act as the major oncogenic signaling pathways.[1–3]

Ras family of proteins belongs to the large superfamily of GTPases that localize to the inner surface of the plasma membrane.[43,44] Ras proteins play a pivotal role in a number of signaling pathways mediated by RTKs. Ligand-activated and autophosphorylated RTKs create phosphotyrosyl-binding sites for adapter proteins such as Shc and Grb2, which in turn recruit guanine nucleotide exchange factors (GEFs), thereby inducing Ras activation. Once induced, Ras activates Raf serine/threonine kinase, which then phosphorylates MAPK kinases (otherwise known as MEKs) (Fig. 5). In addition to Ras, PKC is a potent activator of Raf-1/MAPK cascade, whereas its role as a downstream effector of RTKs has not been clearly demonstrated. Activated MAPKs (or extracellular signal-regulated kinases [ERKs]), move into the nucleus and phosphorylate and activate nuclear transcription factors such as Elk-1. ERKs can also activate other kinases such as RSKs (also known as MAPK-activated protein kinases), which regulate cell-cycle regulation and apoptosis. ERK-activated RSK kinase catalyzes the proapoptotic Bcl-2 family protein Bad, thereby inhibiting Bad-mediated apoptosis. Furthermore, the Ras-Raf-MEK-ERK cascade modulates cellular proliferation by regulating the expression and activity of several proteins, including cell-cycle regulators (e.g. cyclin D1, $p21^{WAF(1)}$ $p27^{KIP(1)}$ and cdc25A) and transcription factors (e.g. c-*fos*, c-*jun*, and c-*myc*). Aberrant MEK and ERK activity has been demonstrated in AML and CML cells.[1,3,43,44] As for the roles of Ras/MAPK in the growth and survival of hematopoietic cells, we and other groups previously showed that dominant-negative Ras and a chemical inhibitor of MAPK blocked proliferation and survival of hematopoietic cells mediated by oncogenic RTKs, respectively, indicating the importance of the Ras/MAPK signaling in the RTK-dependent malignant transformation of hematopoietic cells.[45,46]

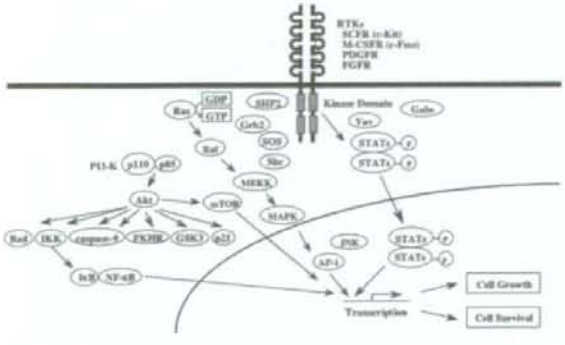

Fig. 5. Signal transduction from receptor tyrosine kinases (RTKs). Both ligand activated RTKs and oncogenic RTKs activate a common set of signaling molecules such as Ras/MAPK, PI3-K/Akt, and STATs. However, their signals are supposed to be quantitatively and/or qualitatively different.

PI3-K is another important signaling pathway controlling serine/threonine phosphorylation.[1,3] PI3-K consists of two subunits, the p85 regulatory subunit and the p110 catalytic subunit (Fig. 5). The p85 subunit binds to the ligand-activated and autophosphorylated RTKs. As a result, the p110 subunit and their downstream substrate Akt (also called PKB) are recruited to the membrane. PI3-K/Akt pathway activates several downstream targets including p70 RSK, forkhead transcription factors (FOXOs), and NF-κB. The serine/threonine kinase Akt is an important component of the cell survival machinery. PI3-K-activated Akt provokes a number of signaling events. For example, Akt phosphorylates an NF-κB inhibitor, IκB. Upon phosphorylation, IκB is degraded by 26S proteasome and releases NF-κB, which then moves into the nucleus and induces a number of target genes involved in cell survival such as Bcl-XL and IAPs. Akt also phosphorylates the proapoptotic protein Bad, which leads to higher levels of free anti-apoptotic Bcl-XL, thereby inhibiting the cell-death protease, caspase-9. A tumor suppressor, PTEN, is a phosphatase that removes a phosphate from the 3 position of the inositol ring of the PIP3,4,5 phospholipids. PTEN has been shown to act as a negative regulator for Akt through its phosphatase activity. Furthermore, the mammalian target of rapamycin (mTOR) is known to be another important downstream effector of the PI3-K/Akt signaling pathway, which mediates cell survival and proliferation as a serine/threonine kinase. With regard to the biologic roles of PI3-K/Akt in hematologic malignancies, we previously showed that tyrosine 719 in c-Kit, which is utilized for the PI3-K-binding, is essential for the transforming activity of the TKD mutant of c-Kit by exchanging tyrosine residues in the cytoplasmic domain to phenylalanine (as described in the above section).[35,47] However, since $KIT^{WT-Tyr719Phe}$ knock-in mouse did not show an apparent abnormality in hematopoiesis, it was speculated that PI3-K signaling is dispensable for normal hematopoiesis under physiologic conditions.[48,49]

STATs are coded by six known mammalian genes and include 10 different proteins including different isomers of STAT1, 3, 4, and 5.[50,51] Like other transcription factors STATs have a well-defined structure including a DNA-binding domain, a conserved NH2-terminal domain, a COOH-terminal transactivation domain, and SH2 and SH3 domains. Upon tyrosine phosphorylation by upstream TKs, activated STATs dimerize and translocate into the nucleus, where they activate specific target genes (Fig. 5). A number of previous studies have shown that STATs mediate cytokine-dependent cell growth and survival by regulating the expression of cyclins, c-*myc*, and Bcl-XL. Besides the normal signaling from ligand-activated cytokine receptors, the TKD mutant of c-Kit induces constitutive activation of STAT3 and STAT1 in a ligand-independent manner.[52,53] TEL-PDGFRβ can activate STAT1 and STAT5 through JAK-independent pathway in BaF3 cells, while wild-type PDGFRβ can scarcely activate these STATs.[54,55] FLT-ITD also activates STAT3 and STAT5 constitutively, which is far more effective than ligand-activated wild type FLT3.[45,46] Thus, the aberrant activation of STAT is the common characteristic of constitutive active RTKs. Regarding the biologic functions of STATs as downstream signaling molecules of oncogenic RTKs, it was shown that dominant-negative STAT3 suppressed the growth and survival mediated by oncogenic c-Kit.[52] Also, we previously showed that dominant negative STAT5 inhibited the growth of 32D cells transformed by FLT-ITD as efficiently as dominant negative Ras.[46] These results suggest that STATs would play a crucial role in malignant transformation caused by oncogenic RTKs. In order to identify the target genes activated by FLT3-ITD, we previously compared the mRNA expression profile between the cells expressing FLT3-ITD and those expressing wild type FLT3 by a microarray analysis.[56] Consistent with the previous observation that STATs

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Table 1. Chromosomal translocations involving receptor tyrosine kinases

Genetic abnormality	Chromosomal translocation	Disease type
TEL-PDGFRβ	t(5;12)(q31;p12)	Atypical CML/CMML
HIP1-PDGFRβ	t(5;7)(q33; q11.2)	
H4-PDGFRβ	t(5;10)(q33; q11.2)	
Rabaptin-5-PDGFRβ	t(5;17)(q33;p13)	
CEV14-PDGFRβ	t(5;14)(q33;q32)	AML relapse
FIP1L1-PDGFRα	Interstitial deletion at 4q12	CEL
KIF5B-PDGFRα	t(4;10)(q12;p11)	
STRN-PDGFRα	t(2;4)(p24;q12)	
ETV6-PDGFRα	t(4;12)(q2?3; p1?2)	
BCR-PDGFRα	t(4;22)(q12;q11)	CML-like MPD
ZNF198-FGFR1	t(8;13)(p11;q12)	8p11 myeloproliferative syndrome (EMS)/stem cell leukemia–lymphoma syndrome (SCLL)
FOP-FGFR1	t(6;8)(q27;p11)	
CEP110-FGFR1	t(8;9)(p11;q33)	
BCR-FGFR1	t(8;22)(p11;q22)	
FGFR3 overexpression	t(4;14)(p16.3; q32.3)	Multiple myeloma
TEL-FGFR3	t(4;12)(p16;p13)	PTCL?AML

CML, chronic myelogenous leukemia; CMML, chronic myelomonocytic leukemia; AML, acute myeloid leukemia; CEL, chronic eosinophilic leukemia; MPD, myeloproliferative disorder; PTCL, peripheral T cell lymphoma.

are aberrantly activation by FLT3-ITD, several known target genes of STATs were specifically induced by FLT-ITD. Among STAT3/5 target genes, we found that Pim-2 mRNA was induced by FLT3-ITD. In addition, the inhibition of Pim-2 activity by the kinase-dead Pim-2 mutant suppressed the transforming activity of FLT3-ITD. These results suggest that the STAT5/Pim-2 cascade alone may be sufficient to execute the biologic function of FLT3-ITD, whereas other molecules can also contribute to this process. As for other STAT3/5 target genes, we found that SOCS2 and SOCS3 were also specifically induced by FLT3-ITD. Since SOCS family proteins can act as a negative regulator of JAK/STAT pathways, this result may indicate that the negative feedback system still works even in the cells transformed by oncogenic RTKs. However, as we previously found that the SOCS1 gene was silenced by the hypermethylation in its promoter region in about 70% of AML cases, this result may indicate that this SOCS-mediated anti-oncogenic system may be disrupted in more advanced stage or in some type of hematologic malignancies.[57] Other interesting findings from the microarray analysis of FLT3-ITD are the repressed expression of transcription factors, C/EBPα and PU.1, which are critical mediators of myeloid differentiation. Because loss of function mutations of C/EBPα or PU.1 have been found in AML, our results suggest that FLT3-ITD would work as both a differentiation blocker and an augmenter of proliferation and survival.

New drugs targeting oncogenic RTKs and their downstream molecules

As described above, aberrant activation of RTKs and their downstream molecules are involved in the pathogenesis of various hematologic malignancies, suggesting that these molecules are particularly attractive targets for therapy.[1–3]

Imatinib originally synthesized to inhibit activity of BCR-ABL was found to inhibit that of PDGFR and c-Kit more efficiently. In fact, imatinib has been shown to be effective on GIST carrying the mutations of c-Kit or PDGFR, and its clinical use is now admitted. In addition, imatinib has been shows to be effective on CMML patients with t(5;12)(q33;p13) yielding TEL-PDGFRβ and HES/CEL patients with the interstitial deletion at 4q12 yielding FIP1L1-PDGFRα.[5,58]

Also, several FLT3 inhibitors, such as MLN518, PKC412, CEP701, and SU11248, have been shown to be selectively cytotoxic to AML blasts harboring the mutation either FLT-ITD or FLT-TKD *in vitro*.[59,60] However, in several clinical trails, the effects of these agents were observed in a minority of patients and were only transient. So, the development of a more potent new drug is required to control deregulated FLT3 activity in AML patients.

Inhibition of Ras signal transduction has been the subject of intense research. Ras activity can be inhibited by preventing its membrane localization.[43,44] Farnesyltransferase, which induces the COOH-terminal prenylation of Ras, is the key enzyme responsible for membrane localization of Ras as well as geranylgeranyltransferase I that regulates geranylgeranylation. So, several farnesyltransferase inhibitors (FTIs) have already been developed, and clinical trials for cancers including hematologic malignancies are now under way.[43,44] Among them, two FTIs, R115777 and SCH66336, have been reported to be effective against several hematologic malignancies such as AML, high risk MDS, imatinib-resistant CML, acute or blastic phase of CML, and multiple myeloma, alone or in combination with other drugs. However, when considering the pharmacological effects of FTIs, it should be kept in mind that FTIs also exert their anti-oncogenic effects through several Ras-independent mechanisms.[61] Compounds such as geldanamycin and derivatives of radicicol destabilize Raf protein and interfere with Raf signaling.[1,3] Several staurosporine derivatives such as UCN-01, CGP41251, and PKC412 can inhibit PKC and MAPK signaling, and have been examined in preclinical and clinical studies.[62] Also, MEK inhibitors such as PD098059, PD184352, and UO126 are able to modulate cellular proliferation, differentiation, and apoptosis. PD184352 is now subjected to Phase I trials.[63]

Pharmacological inhibitors of PI3-K, wortmannin, and LY294002 have shown to be effective in preclinical studies.[64] In addition, rapamycin (sirolimus), the prototypic mTOR inhibitor, exhibits anti-oncogenic activity on AML cells. Three rapamycin analogs, temsirolimus, everolimus, and AP23573, are in clinical trials for various hematologic malignancies: temsirolimus for mantle cell lymphoma, AP23573 for acute leukemia, and everolimus for lymphoma (Hodgkin and non-Hodgkin) and multiple myeloma.[65] In addition, perifosine, an inhibitor of Akt that exhibited

considerable anti-oncogenic activity on multiple myeloma, is being examined in relapsed multiple myeloma.

Future perspectives

Over the past decade, extensive efforts have been made to elucidate the role of cellular signaling pathways in cellular growth, differentiation, apoptosis, and malignant transformation, which enabled us to establish molecular targeted therapeutics. However, these drugs still have considerable adverse effects on normal tissues and their effects were not satisfactory at all in terms of curing the malignancies. So, we have to further understand the complex pathophysiology of each disease. Also, based on these findings, we have to devise the agents that specifically target the malignant cells without spoiling normal tissues. These attempts would truly realize the concept of molecular-targeted therapeutics and shed light on patients with hematologic malignancies.

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Regulation of human B lymphopoiesis by the transforming growth factor-β superfamily in a newly established coculture system using human mesenchymal stem cells as a supportive microenvironment

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Objectives. **To characterize and evaluate the validity of a novel coculture system for studying human B-lymphocyte developmental biology.**

Materials and Methods. **We developed a long-term culture system to produce B lymphocytes from human $CD34^+$ cells purified from umbilical cord blood using human mesenchymal stem cells (hMSC) as stroma. We evaluated the effects of several low molecular weight inhibitors, recombinant proteins, and neutralizing antibodies (Abs) as potential regulators of B-lymphocyte development.**

Results. **Our cocultures of 2000 $CD34^+$ cells in the presence of stem cell factor and Flt3-ligand produced 1–5 × 10^5 $CD10^+$ cells after 4 weeks of culture. Surface IgM^+ immature B cells began to appear after 4 weeks. We evaluated the negative-regulatory effects of the transforming growth factor (TGF)-β superfamily on human B lymphopoiesis, and found that adding an anti–activin A antibody enhanced generation of $CD10^+$ cells two- to three-fold. As well, the proportion of $CD10^+$ cells in the generated cells increased markedly, indicating that activin A downregulated B lymphopoiesis more efficiently than myelopoiesis. Addition of TGF-β1 suppressed B-lymphocyte production by 20% to 30%, while addition of an anti–bone morphogenetic protein (BMP)-4 antibody or recombinant BMP-4 had no effect. Therefore, the strength of ability to suppress human B lymphopoiesis seemed to be activin A > TGF-β1 > BMP-4. None of these three factors influenced the emergence of IgM^+ cells.**

Conclusions. **hMSC coculture supported human B lymphopoiesis. Activin A selectively suppressed B lymphocyte production.** © **2008 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

B lymphocytes develop from hematopoietic stem cells within bone marrow (BM), and play an essential role in immune system function. B-lymphocyte production is regulated by an elaborate scheme involving many different soluble or adhesion molecules; failure to control production adequately can promote the development of diseases with quantitative and/or qualitative B-lymphocyte abnormalities [1]. A variety of murine assay systems are used to evaluate the mechanisms of B-lymphocyte regulation, such as Whitlock-Witte–type long-term BM cultures, cocultures of murine BM cells on stromal cell lines, and colony assays. Studies utilizing these culture systems have elucidated many regulatory mechanisms of B-lymphocyte development in mice. For example, CD44 [2], vascular cell adhesion molecule-1, and very-late activation antigen-4 [3] are adhesion molecules essential for B lymphopoiesis. We have also reported that a novel interferon-ζ/limitin suppressed colony formation of B-lymphocyte progenitors [4,5] and that an adipocyte-specific protein, adiponectin,

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inhibited stroma-dependent B-lymphocyte growth through induction of prostaglandins (PGs) [6]. In addition, members of the TGF superfamily [7], the Wnt family [8], and the Notch family [9] are known to regulate proliferation, differentiation, and survival, depending on the developmental stage of the B lymphocytes. Although early B-lymphocyte development was believed to require direct interactions with supportive stromal layers, in previous work we generated $CD45RA^+CD19^+$ B lymphocytes from murine $Lin^-c\text{-}kit^{high}$ and $Lin^-c\text{-}kit^{low}$ cells in the presence of stem cell factor (SCF), Flt3-ligand (FL), and interleukin (IL)-7, in the absence of stromal cells [10,11].

In contrast to mice, systems to evaluate human B-lymphocyte developmental biology are not fully established because of a lack of appropriate human stromal materials. Several culture systems for human hematopoietic cells have utilized murine stromal cell lines as supportive microenvironment [12–15]. Although murine stromal cells produce cytokines that could potentially affect human B-lymphocyte development, some of these molecules have no interspecies cross-reactivity [16]. As well, there are some mechanistic differences between murine and human B-lymphocyte development. For example, signaling through the IL-7 receptor (IL-7R) is critical for adult murine B-lymphocyte development [17,18]. However, in humans, disrupting IL-7R does not induce arrest of B lymphopoiesis [19,20]. In addition, human B-lymphocyte progenitor cells cannot expand without stroma [12], whereas murine progenitor cells can [10,11]. Therefore, assay systems more relevant to human biology are needed. A previous study reported the establishment of a serum-free human BM stromal cell culture; however, the investigators isolated cellular components from fetal BM, which is currently unavailable for research purposes [21,22].

In this study, we show that human mesenchymal stem cells (hMSC) can support the commitment and differentiation of human $CD34^+$ cells into B lymphocytes. Our cocultures of 2000 human $CD34^+$ cells on hMSC in the presence of SCF and FL produced 1–5 $\times$ 10^5 $CD10^+$ B lymphocytes after 4 weeks. Using this coculture system, we determined that members of the transforming growth factor-β (TGF-β) superfamily, activin A and TGF-β1, were negative regulators for early onset of human B lymphopoiesis. The TGF-β superfamily has more than 20 members, including three TGF-βs, two inhibins, three activins, seven bone morphogenetic proteins (BMPs), and nodal [23]. There are two types of receptors for the TGF-β superfamily, type I (activin receptor-like kinase [ALK]-1-7) and type II receptors [24]. Their specific ligand–receptor interactions induce critical effects on a wide range of physiological and pathological processes, such as immune responses, angiogenesis, tumor development, and wound healing [25]. In addition, TGF-βs, activin A, and BMPs have been reported to influence lymphohematopoiesis [7,26,27]. Here, we explore the similarities and/or differences of functions of the TGF-β superfamily members between humans and mice by comparing our results obtained from the human B-lymphocyte coculture with data from several previous reports.

Materials and methods

Origin and isolation of cells

Cord blood (CB) cells were collected from healthy, full-term neonates immediately after delivery by Cesarean section. All participants provided prior informed consent. Mononuclear cells were separated by Ficoll-Paque PLUS (GE Healthcare Bio-Science AB, Uppsala, Sweden) centrifugation. CB $CD34^+$ cells were purified using the Direct CD34 Progenitor Cell Isolation Kit (human; Miltenyi Biotec, Auburn, CA, USA). BM-derived hMSC were purchased from Cambrex Bio Science Walkersville (Walkersville, MD, USA) and maintained in MSC Growth Medium (Cambrex Bio Science Walkersville). Human umbilical vein endothelial cells (HUVEC) were purchased from Cascade Biologics (Portland, OR, USA), and maintained in Humedia-EG2 (Kurabo, Osaka, Japan). The murine stromal cell line MS-5, kindly provided by Dr. Mori (Niigata University), was maintained in α-minimum essential medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS).

Recombinant proteins and reagents

Recombinant human SCF, FL, IL-7, TGF-β1, activin A, and BMP-4 proteins were purchased from R&D Systems (Minneapolis, NY, USA). Granulocyte-colony stimulating factor (G-CSF) was gifted from Kirin Brewery (Tokyo, Japan). N-acetylcysteine was purchased from Sigma (St. Louis, MO, USA), DUP697 from Cayman Chemicals (Ann Arbor, MI, USA), BIO from Calbiochem (Darmstadt, Germany), and SB431542 from TOCRIS Bioscience (Ellisville, MO, USA). Neutralizing antibodies (Abs) against human TGF-β1, activin A, and BMP-4 were purchased from R&D Systems. Follistatin was purchased from Calbiochem.

Cocultures for human B lymphocytes

hMSC were seeded in 12-well tissue culture plates (Iwaki, Tokyo, Japan) 1 or 2 days before coculture. Isolated CB $CD34^+$ cells (2000 cells/well) were plated on subconfluent hMSC layers in MSC growth medium in the presence of 10 ng/mL SCF and 5 ng/mL FL. Half of culture medium was replaced with fresh medium containing the same cytokines twice per week. When appropriate, the cultured cells on hMSC were collected and their phenotypes were analyzed with flow cytometry. In some experiments, cultures were performed in medium containing low molecular weight inhibitors or neutralizing Abs, as indicated. In other experiments, HUVEC or MS-5 cells were used as stroma. In cocultures containing HUVEC or MS-5 cells, the culture media were Iscove's modified Eagle's medium (Gibco) supplemented with 20% FCS and 2 mM glutamine or α-minimum essential medium supplemented with 10% FCS, respectively.

Flow cytometry and cell sorting

Flow cytometry analysis was performed with a FACSCalibur (BD Biosciences Immunocytometry Systems, San Jose, CA, USA) using standard multicolor immunofluorescent staining protocols [28]. Murine monoclonal Abs against the following human cell surface molecules were purchased: phycoerythrin (PE)-CD3,

PE-CD10, allophycocyanin (APC)-CD10, PE-CD19, PE-CD20, fluorescein isothiocyanate (FITC)-CD33, APC-CD33, PE-CD34, APC-CD34, FITC-CD38, FITC-CD45, and PE-glycophorin A from BD Biosciences/BD Pharmingen; PC5-CD19 from Beckman Coulter (Marseilles, France), FITC-IgM from Southern Biotechnology Associates (Birmingham, AL, USA). Cultured cells were categorized as myeloid lineage cells ($CD33^+$ and $CD10^-$), B lymphoid lineage cells ($CD33^-$ and $CD10^+/CD19^+$), or immature B cell ($CD33^-$, $CD10^+$, $CD19^+$, and IgM^+). In some experiments, $CD34^+CD38^-$, $CD34^+CD38^+CD10^-$, and $CD34^+CD38^+CD10^+$ cells were sorted using a FACS Aria (BD Biosciences Immunocytometry Systems).

Limiting dilution assays

Limiting dilution assays were performed in 96-well plates (Iwaki) preseeded with hMSC. CB $CD34^+$ cells were plated at various concentrations from 1 to 100 cells/well. Each well contained 200 μL MSC Growth Medium with 10 ng/mL SCF and 5 ng/mL FL, with or without 10 μM SB431542. Half of culture medium was replaced with fresh medium containing the same cytokines twice per week. After 28 days of coculture, wells with cell expansion were scored. Individual expanded cells were analyzed by flow cytometry, and the number of culture wells containing $CD10^+$ cells was determined.

Reverse transcription polymerase chain reaction

Total RNA was extracted from CB cells and hMSCs using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. RNAs were reverse transcribed and target cDNAs were amplified by polymerase chain reaction using 0.5 U Taq DNA polymerase (Applied Biosystems, Branchburg, NJ, USA; primers sequences are available upon request) [29].

Enzyme-linked immunosorbent assay

TGF-β1 was detected using Immunoassay Kit (Biosource International, Camarillo, CA, USA; sensitivity 15.6 pg/mL). Activin A was detected using DuoSet ELISA Development System (R&D Systems; sensitivity 117.2 pg/mL). Each step of the reactions was performed according to manufacturer's instructions.

Statistical analysis

Student's *t*-test was used to analyze statistically significant differences between data sets. All results are reported as mean values ± standard deviation.

Results

hMSC support B-lymphocyte development from human CB $CD34^+$ cells

The murine stromal cell line MS-5 is used widely to support murine and human lymphohematopoietic cells in culture [12,13]. HUVECs express several adhesion molecules, such as vascular cell adhesion molecule-1 [30], that interact with B lymphocytes. hMSC have the ability to support human hematopoietic stem cells in culture [31], and can enhance engraftment of human hematopoietic stem cell transplantation [32]. Therefore, we compared the ability of these three cell types to support human B lymphopoiesis in coculture. When purified human CB $CD34^+$ cells were cultured on each different monolayer, $CD19^+$ cells were generated within 4 weeks. As shown in Figure 1, cocultures on hMSCs generated many more $CD19^+$ cells than those on MS-5 or HUVEC. In addition, similar supporting activity for human B lymphopoiesis was observed for two different lots of hMSC (data not shown). Finally, HUVEC started to detach from the culture wells within 3 weeks of culture, while hMSC did not.

Therefore, hMSC appear to better support human B-lymphocyte progenitor cell development than HUVEC or MS-5.

SCF and FL enhance human B lymphopoiesis in coculture

In mice, SCF, FL, and IL-7 are critical for early B-lymphocyte development [10,11]. Therefore, coculture of human CB $CD34^+$ cells on hMSC included various combinations of SCF, FL, and IL-7. Although the addition of SCF, FL, or IL-7 individually to the cocultures enhanced production of B lymphocytes slightly (data not shown), many more B lymphocytes were recovered when these factors were added in combination (Fig. 2A). Anti-CD33 Ab recognizes a 67-kD type I transmembrane glycoprotein expressed mainly on monocytes, granulocytes, and myeloid progenitors, but not on lymphocytes and hematopoietic stem cells. Anti-

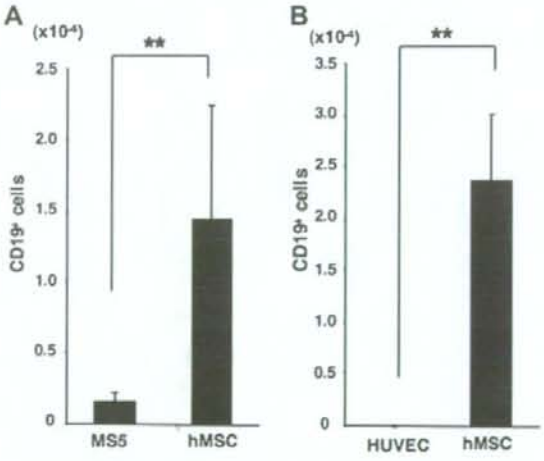

Figure 1. Human mesenchymal stem cells (hMSC) have high supporting activity of human B lymphocytes. Purified cord blood (CB) $CD34^+$ cells (2000 cells/well) were cultured on subconfluent of hMSC (**A**, **B**), MS-5 (**A**), or human umbilical vein endothelial cells (**B**) in the absence of any cytokines for 4 weeks. Total numbers of the generated cells were calculated, and surface phenotypes of the cells were analyzed with flow cytometry. Data are shown as mean ± standard deviation of the generated $CD19^+$ cell numbers in triplicated samples. Statistically differences from control values are shown with two asterisks ($p < 0.01$). Similar results were obtained in three independent experiments.

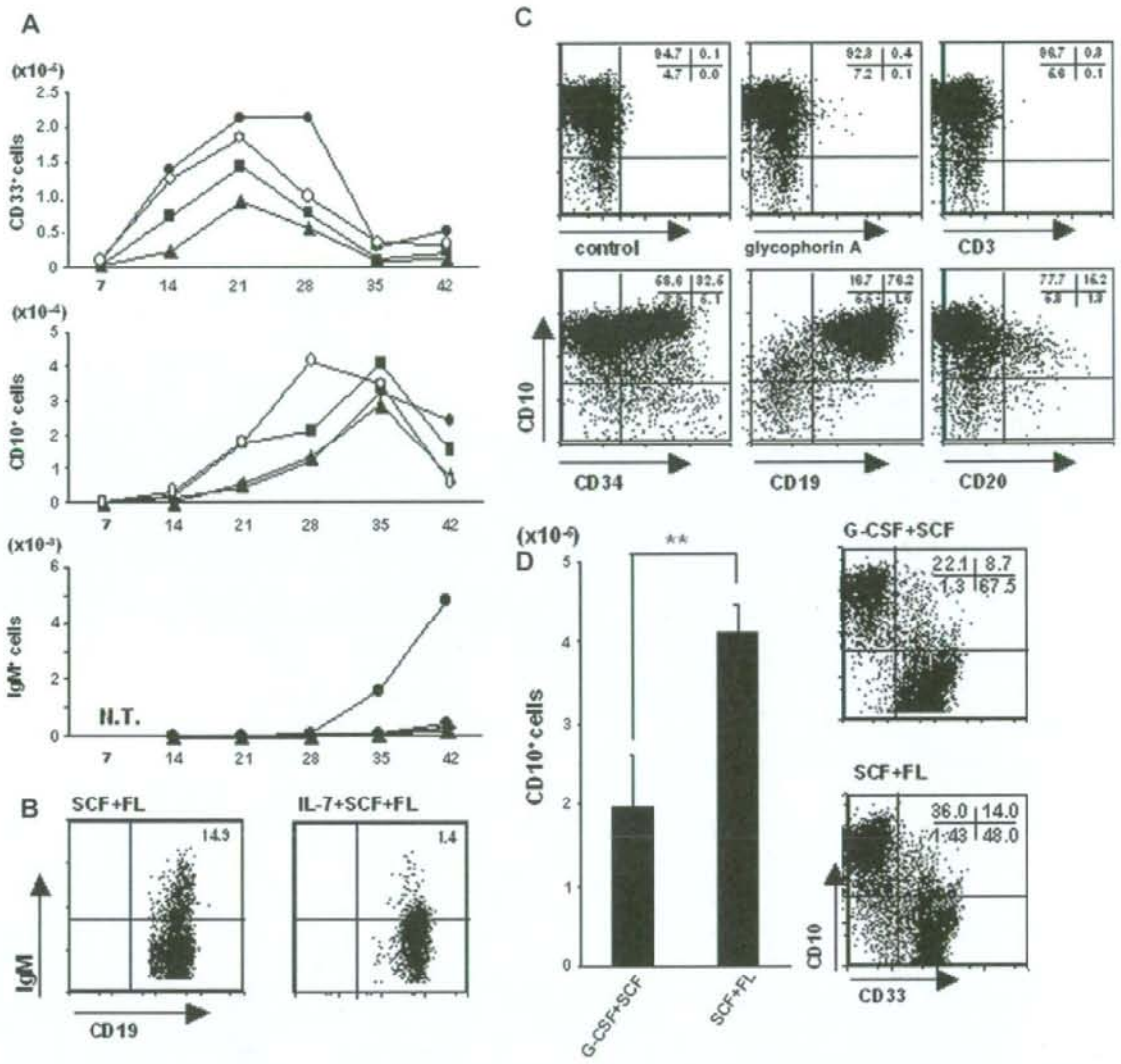

Figure 2. Combination of cytokines enhances human B-lymphocyte production in cocultures of cord blood (CB) $CD34^+$ cells on human mesenchymal stem cells (hMSC). (**A**) Purified CB $CD34^+$ cells (2000 cells/well) were cultured on hMSC in combinations of 10 ng/mL stem cell factor (SCF) + 5 ng/mL, Flt3-ligand (FL) (closed circle), 5 ng/mL FL + 5 ng/mL interleukin (IL)-7 (closed triangle), 5 ng/mL IL-7 + 10 ng/mL SCF (closed square), 10 ng/mL SCF + 5 ng/mL FL + 5 ng/mL IL-7 (open circle) for 6 weeks. The cultured floating cells were collected, and numbers of the generated $CD33^+$ cells (upper panel), $CD10^+$ cells (middle panel), and IgM^+ cells (lower panel) were estimated weekly. Similar results were obtained in three independent experiments. (**B**) Purified CB $CD34^+$ cells (2000 cells/well) were cultured on hMSC in the presence of 10 ng/mL SCF + 5 ng/mL FL or 10 ng/mL SCF + 5 ng/mL FL + 5 ng/mL IL-7, respectively. At day 42, the generated cells were stained with fluorescein isothiocyanate (FITC)-IgM and phycoerythrin (PE)-CD19, and analyzed with flow cytometry. Similar results were obtained in three independent experiments. (**C**) Purified CB $CD34^+$ cells (2000 cells/well) were cultured on hMSC in the presence of 10 ng/mL SCF and 5 ng/mL FL. The generated cells were stained with FITC-CD33 and allophycocyanin (APC)-CD10 as well as the indicated PE-conjugated antibody (Ab), and analyzed with flow cytometry at day 40. Isotype-matched Abs were used as negative controls. Similar results were obtained in three independent experiments. (**D**) Purified CB $CD34^+$ cells (2000 cells/well) were cultured on hMSC in the presence of 10 ng/mL SCF + 5 ng/mL FL or 10 ng/mL SCF + 10 ng/mL G-CSF. Numbers of the generated $CD10^+$ cells were analyzed at 4 weeks of the cocultures. Data are shown as mean ± standard deviation in triplicated samples. Statistically differences from control values are shown with two ($p < 0.01$) asterisks. One representative flow cytometry data for CD10 and CD33 expression was also shown. Similar results were obtained in two independent experiments. NT = not tested.