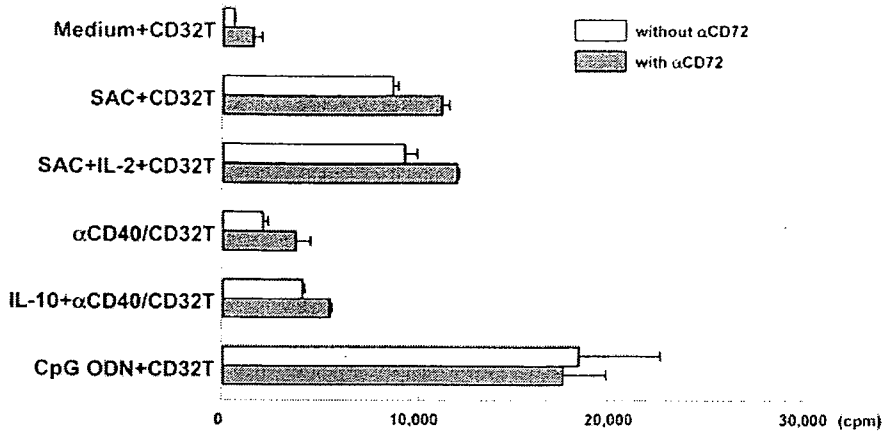

Fig. 3. CD72 stimulation enhances B cell proliferation. Purified human B cells were cultured with or without anti-CD72 (2.5 μg/ml)/CD32T in the presence of SAC (0.01%), SAC plus IL-2 (50 ng/ml), anti-CD40 (1 μg/ml)/CD32T, IL-10 (50 ng/ml) plus anti-CD40/CD32T or CpG ODN (2 μg/ml) for 72 h as described in Fig. 2A. Proliferation assays were performed in triplicate, and standard deviations are shown. The results depicted are representative of three independent experiments.

CD72 signaling inhibits Ig production

To identify further functions of CD72 in B cells, we investigated the impact of CD72 signaling on Ig synthesis by human peripheral B cells. Following stimulation with anti-CD72/CD32T, synthesis of IgG, IgA and IgM by purified human B cells was diminished in the presence of SAC plus IL-2 (Fig. 4A), whereas the Ig levels were barely reduced in the presence of anti-CD40/CD32T or CpG ODN (Fig. 4A). A significant decline in Ig production in the presence of SAC plus IL-2 was found with anti-CD72 treatment in a dose-dependent manner (Fig. 4B). In contrast, marginal reduction of Ig production was observed with anti-CD72 in the presence of CpG ODN (Fig. 4B). CD72 stimulation did not increase apoptosis in activated B cells in the presence of SAC plus IL-2 (data not shown). These findings indicate that CD72 signaling inhibits B cell Ig synthesis preferentially via B cell receptor (BCR) signaling.

CD72 signaling suppresses differentiation of B cells into plasma cells

The observation that B cell Ig production is reduced by CD72 stimulation prompted us to ascertain whether CD72 stimulation influences the differentiation of B cells into plasma cells. Accordingly, we investigated the effect of CD72 on circulating human B cell differentiation using morphological and flow cytometric analyses in various B cell activation systems. Flow cytometric analysis indicated that the percentage of plasma cells decreases when anti-CD72/CD32T is added in the presence of SAC plus IL-2 (Fig. 5A, B), while CD72 signaling does not influence the percentage of plasma cells in the presence of anti-CD40/CD32T or CpG ODN (Fig. 5A). May–Giemsa staining also showed that the number of plasma cells is reduced by anti-CD72 treatment in the presence of SAC plus IL-2 (data not shown). These results indicate that CD72 signaling inhibits B cell differentiation into plasma cells via BCR signaling.

CD72 signaling reduces XBP-1 expression at the mRNA and protein levels in activated B cells

Since plasma cell differentiation was inhibited by CD72 signaling, we examined the expression of XBP-1, PRDI-BF1/Blimp-1 and BSAP, which are known to be critical for plasma cell differentiation, so as to solve this inhibition mechanism. The expression of XBP-1 at the mRNA level in B cells was remarkably diminished by the addition of anti-CD72/CD32T in the presence of SAC plus IL-2, while CD72 signaling had marginal effects on XBP-1 expression in the presence of anti-CD40/CD32T (Fig. 6A). Conversely, the expression of BSAP mRNA was increased with CD72 stimulation in the presence of SAC plus IL-2. The expression of PRDI-BF1 mRNA was not affected by the addition of anti-CD72/CD32T (Fig. 6A). In our preliminary experiments, the expression of XBP-1 appeared at a maximum level at day 3 to day 5 of culture (data not shown). The expression of XBP-1 at day 5 was reduced by anti-CD72 treatment in the presence of SAC plus IL-2, while a marginal reduction of XBP-1 expression was observed following CD72 stimulation in the presence of anti-CD40/CD32T. However, as shown by a densitometric analysis, expression of XBP-1 under CD40 signaling was considerably low both with and without CD72 stimulation. In the presence of CpG ODN, the XBP-1 expression levels were faint and unaffected by anti-CD72/CD32T (Fig. 6B). Since

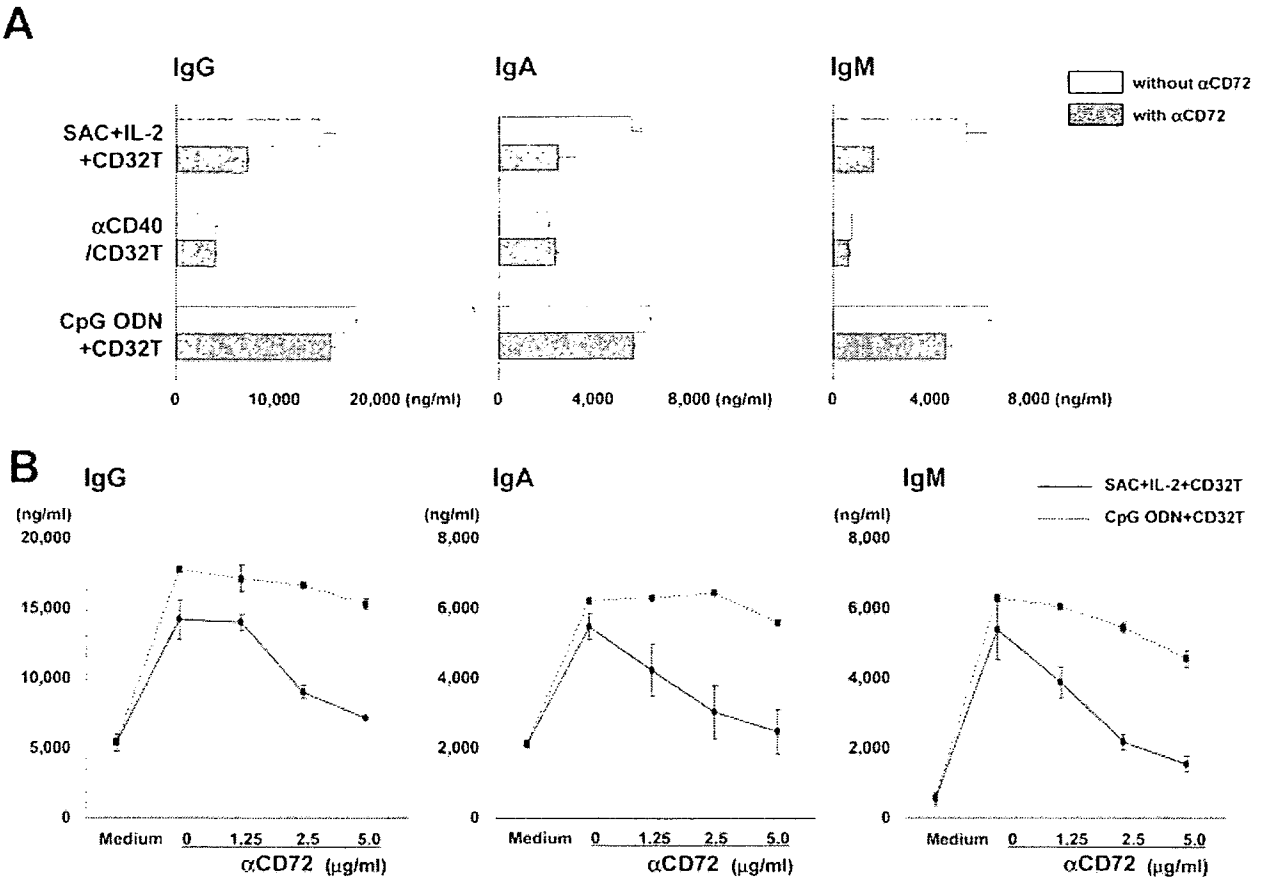

Fig. 4. CD72 stimulation influences Ig production by B cells. (A) Purified human B cells were cultured with or without anti-CD72 (5 μg/ml)/CD32T for 7 days as described in Fig. 2A. Ig concentration in the culture supernatant was measured using ELISA as described in the Materials and methods. All assays were performed in triplicate, and standard deviations are shown. The results depicted are representative of three independent experiments. (B) Purified human B cells were cultured with different concentrations of anti-CD72 cross-linked with CD32T for 7 days as described in Fig. 2A. All assays were performed in triplicate, and standard deviations are shown. The results depicted are representative of two independent experiments.

CD72 is expressed preferentially in naive B cells, we examined whether CD72 signaling differently affects XBP-1 expression on naive and memory B cells. CD72 stimulation only mildly diminished the expression of XBP-1 in memory B cells, while a strong reduction was found in naive B cells (Fig. 6C). These findings indicate that the inhibition of plasma cell differentiation via CD72 signaling is strongly associated with the degradation of XBP-1, especially in naive B cells.

Discussion

In this study, we provide evidence for a unique function of the CD72 molecule in B cells, namely in preventing naive B cells from producing Ig and differentiating into plasma cells by diminishing XBP-1 expression. To our knowledge, this study provides the first description of CD72 expression on the surface of quiescent naive B cells and CD72-mediated suppression of their differentiation. These findings indicate that CD72 affects the humoral immune response by controlling naive B cell Ig synthesis.

CD72 signaling activates and induces proliferation of B cells as previously reported [7, 8], which occurs in the presence of BCR stimulation. In this study, we found that CD72 signaling has a marginal effect on CD40 and little effect on CpG signaling, which is mediated through toll-like receptor 9 (TLR9), in the activation and proliferation of B cells. It has been shown that anti-CD72 treatment induces tyrosine phosphorylation of phospholipase Cγ and CD19 and activates Lyn, Blk and Btk but not Syk [23, 24]. These observations suggest that ligation of CD72 may transmit positive signals for B cell activation. Tyrosine phosphorylation of some cytoplasmic proteins following the stimulation with anti-CD72 was also recognized using our system. However, evidence has suggested a potential role of CD72 as a

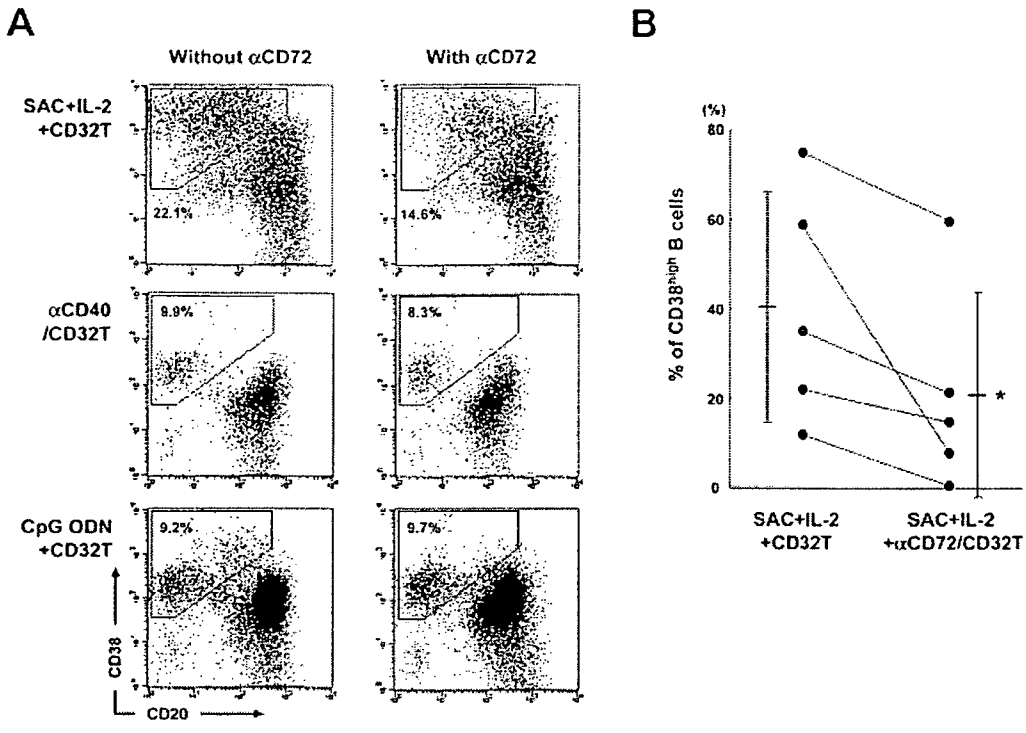

Fig. 5. Plasma cell differentiation from B cells after CD72 ligation. Purified human B cells were cultured with or without anti-CD72 (5 μg/ml)/CD32T for 7 days as described in Fig. 2A. Then cultured cells were stained with anti-CD20-FITC and anti-CD38-PE and then evaluated by flow cytometry. (A) The fraction of $CD38^{high}$ cells, indicated by the polygonal gate on the charts, is considered to be a plasma cell population. The results depicted are representative of five independent experiments. (B) The difference in the percentage of plasma cell populations following culture with and without anti-CD72 stimulation is shown for five independent experiments. Mean ± standard deviations of the percentages are shown at the sides (without and with anti-CD72, 40.4±25.9 and 20.6±23.0, respectively; *$p<0.05$). The value was statistically analyzed by unpaired t-test.

negative regulator of B cell responses [1, 25, 26]. The cytoplasmic domain of CD72 contains two ITIM. It has been shown that cross-linking of the BCR enhances tyrosine phosphorylation of CD72 and its association with the protein tyrosine phosphatase SHP-1, suggesting that CD72 might negatively regulate B cell activation [1]. Kumanogoh et al. reported that in mice, CD72 is the natural receptor for CD100 and that its stimulation induces tyrosine dephosphorylation of CD72 and dissociation of SHP-1 from CD72, implying that CD100 turns off negative B cell signaling [9]. Similar findings were recently reported in a study using COS7 cells transfected with human CD72 [27].

Given the prevailing view that CD72 signaling activates B cells, we were surprised to find that following a BCR-mediated stimulus, Ig production and plasma cell differentiation were diminished upon CD72-mediated activation. Using cytometric analyses and morphological studies, we demonstrated changes in the percentages of plasma cell populations following a CD72 stimulus. In our model, sorted $CD38^{high}$ cells exhibit the typical morphology of plasma cells as previously demonstrated [14, 16, 28]. In contrast, CD72 signaling did not contribute to Ig production or plasma cell differentiation via CD40 or the TLR9 pathway. These data demonstrate that the inhibitory effects of CD72 signaling are selective for the BCR pathway and have no impact on the response mediated by CD40 or the TLR9 pathway. Our studies strongly suggest that CD72 stimulation contributes to the pathway upstream of BCR, which is mediated by the engagement of antigen.

The mechanisms for the inhibition of the differentiation of B cells into plasma cells by CD72 signaling are unclear and remain speculative. Evidence has shown that XBP-1 is important for the differentiation of B cells into plasma cells [18, 29–33]. Little is known, however, about the role of XBP-1 in plasma cell differentiation. In this study, we found that CD72 signaling strongly reduces the expression of XBP-1 but not PRDI-BF1/Blimp-1. Blimp-1 is known for its ability to induce B cell differentiation by repressing the proto-oncogene c-*myc* promoter activity in a binding site-dependent manner [20]. The reason for the discrepancies in XBP-1 and PRDI-BF1/Blimp-1 expression is possibly explained as follows. The expression levels of Blimp-1 in activated B cells are not affected by the absence of XBP-1, and thus Blimp-1 does not compensate for the absence of XBP-1 [18]. Furthermore, XBP-1 does not directly repress

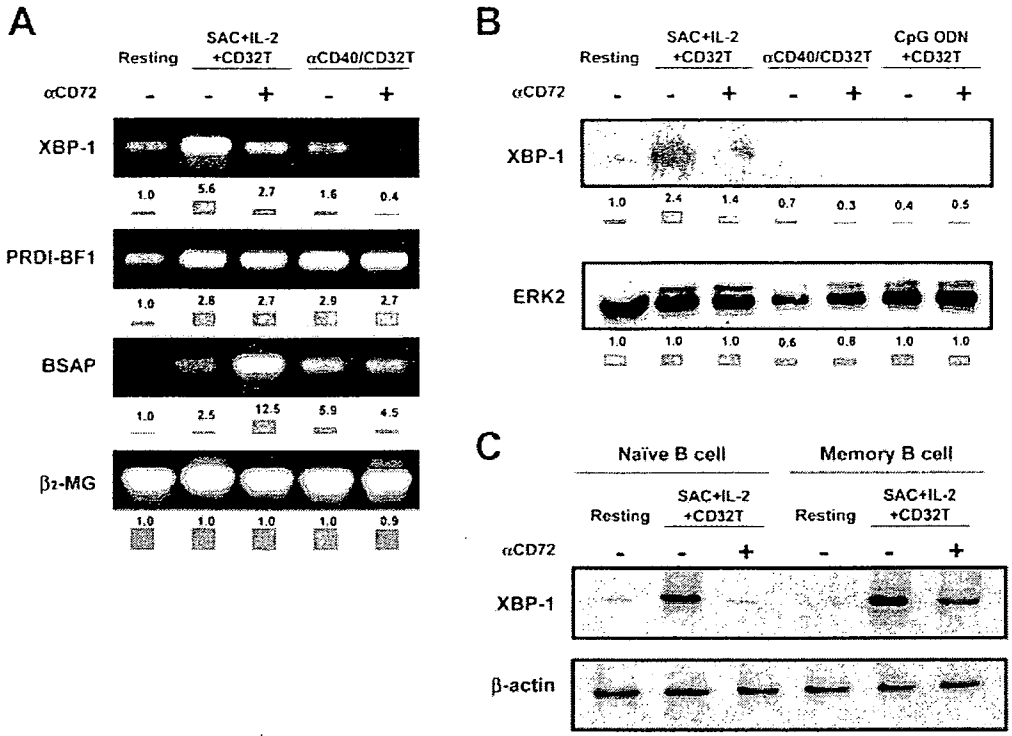

Fig. 6. Effects of CD72 signaling on XBP-1 expression at the mRNA and protein levels. Purified human B cells were cultured with or without anti-CD72 (5 μg/ml)/CD32T for 5 days as described in Fig. 2A. (A) After extraction of total RNA from cultured B cells (1×10^6/lane), RT-PCR was performed with β_2-MG mRNA as a control. The results depicted are representative of three independent experiments. The fold induction is shown directly below each band. (B) After making lysates from cultured B cells (1×10^6/lane), they were analyzed by immunoblotting with Ab specific for XBP-1 and ERK2. The results depicted are representative of three independent experiments. The fold induction is shown directly below each band. (C) Purified human B cells (2×10^6/lane) were cultured with or without anti-CD72 (10 μg/ml)/CD32T for 4 days as described in Fig. 2A. Cell lysates were analyzed by immunoblotting with Abs to XBP-1 and β-actin. A similar result was obtained in another experiment.

activation of the c-*myc* promoter [18]. It has been suggested that XBP-1 acts downstream of Blimp-1 or through a separate pathway in its regulation of B cell differentiation [18].

Our observations that CD72 signaling enhances BSAP expression may be relevant to the depression of plasma cell differentiation through CD72. Transient expression studies have shown that the promoter activity of XBP-1 is strongly down-regulated by the transcription factor BSAP, which may account for the high expression of XBP-1 seen in some plasma cell lines at a stage of development when BSAP is no longer present [33]. BSAP-binding sites have been identified in genes coding promoter regions of the surrogate light chain complex, Blk, mb-1 and CD72, suggesting that BSAP is a transactivator for these genes [34]. BSAP, which is increased by CD72 signaling, may inhibit XBP-1 expression, and CD72 signaling may be enhanced by the increased expression of CD72 through BSAP binding.

It is also interesting that CD72 stimulation reduces the expression of CD27 molecules on B cells. CD27 affects plasma cell differentiation via PRDI-BF1/Blimp-1 in the presence of SAC plus IL-2 [14]. We believe that the influence of the CD27/CD70 interaction on PRDI-BF1/Blimp-1 expression was minimal because of the absence of T cells and low levels of CD70 expression in activated B cells in our system. CD72 may inhibit plasma cell differentiation via two distinct pathways, albeit primary through XBP-1.

The research using the $CD72^{-/-}$ mouse revealed that CD72 signaling is essential for B cell maturation, as reported by Pan et al. [2]. Anti-CD72 and CD72 ligand (CD100) remove inhibitory signaling by CD72 [9], and it is possible that CD72 is a positive regulator of plasma cell differentiation on the basis of our data that anti-CD72 inhibits plasma cell differentiation. Therefore, our data can be reconciled with those from Pan et al. regarding CD72 as positive regulator in B cell differentiation, both in early B cell and mature B cell development.

We analyzed whether anti-CD72 affects the target molecule of CD72, XBP-1, in memory B cells. As expected, a mild reduction of XBP-1 expression was recognized in memory B cells, while strong reduction

was found in naive B cells (Fig. 6C). Therefore, though CD72 itself acts as an agonist for B cell differentiation, we believe that memory B cells have a great advantage in plasma cell differentiation under the least impact of CD72 stimulation due to low expression of CD72. This advantage in memory B cells confirms the ability to elicit more rapid and vigorous responses to subsequent antigenic encounters. While naive B cells can differentiate into antibody-producing plasma cells, they do not undergo somatic hypermutation, thus producing only low-affinity antibodies *in vitro* [28]. Therefore, it is important immunologically that CD72 signaling suppresses plasma cell differentiation from naive B cells.

We have concluded that signaling by anti-CD72, which is probably induced by CD72 ligand *in vivo*, is an antagonist for plasma cell differentiation. However, it is difficult to determine whether CD72-mediated suppressive effects on plasma cell differentiation are caused by direct signaling via CD72 or by removal of inhibitory signals downstream of the BCR. CD72 stimulation may have a different pathway that is not involved in BCR signaling, because it has been reported that CD72 signaling activated B cells in association with CD19, and CD72 ligation can deliver positive signals that are independent of BCR [23, 35].

In conclusion, we have identified a novel inhibitor of differentiation of peripheral naive B cells into plasma cells. Molecules on T cells regulate Ig synthesis by direct T and B cell interaction. Activated T cells express CD40 ligand (CD154), CD70 and CD100 on their surface. The interaction of CD70 and CD27 promotes plasma cell differentiation from memory B cells through BCR signaling by increasing PRDI-BF1/Blimp-1 expression [14]. The interaction of CD100 and CD72 reduces the differentiation of naive B cells into plasma cells specifically through BCR signaling by diminishing XBP-1 expression, inhibiting the production of low-affinity antibodies. Further studies will be required to elucidate the molecular mechanisms by which CD72 reduces plasma cell differentiation. Clarification of these mechanisms will contribute to a better understanding of the development of plasma cells and will provide strategies to promote protective immunity.

Materials and methods

Antibodies and reagents

Anti-CD27 mAb (8H5, IgG1) was provided by Dr. T. Morimoto (Dana-Farber Cancer Institute, Boston, MA) [36, 37]. FITC-conjugated anti-CD20 mAb (anti-CD20-FITC), anti-CD27-FITC, anti-IgD-FITC, PE-conjugated anti-CD20 mAb (anti-CD20-PE) and anti-CD38-PE were purchased from DAKO Japan (Tokyo, Japan). Anti-CD69-PE, anti-CD80-PE, anti-CD86-PE, anti-HLA-DR-PE and purified anti-CD40 mAb (MAB89, IgG1) were purchased from Immunotech (Westbrook, MA). Anti-CD72-FITC, Peridinin Chlorophyll Protein (PerCP)-conjugated anti-CD20 mAb, PE-conjugated streptavidin, PerCP-conjugated streptavidin and purified anti-CD72 mAb (J4–117, IgG2b) were purchased from BD Biosciences (San Jose, CA). Conjugation of biotin to anti-CD27 mAb or anti-CD72 mAb was performed by the standard technique using *N*-hydroxysuccinimido-biotin (Sigma Chemical, St. Louis, MO). *Staphylococcus aureus* Cowan strain (SAC) was obtained from Sigma Chemical, and human recombinant IL-2 and IL-10 were obtained from Genzyme (Cambridge, MA). The CpG ODN (CpG ODN 2006) with the sequence 5′ TCG TCG TTT TGT CGT TTT GTC GTT 3′ was used.

Cell preparation

Highly purified human B cells were obtained from the peripheral blood of healthy adult volunteers using the human B cell enrichment cocktail RosetteSep (IgG1; StemCell Technologies, Vancouver, Canada), which uses antibodies that are bound as bispecific antibody complexes directed against common cell surface antigens on human hematopoietic cells (CD2, CD3, CD16, CD36, CD56) and glycophorin A on red blood cells. Purifications were performed according to the manufacturer's instructions (StemCell Technologies). The resulting B cells did not show any signs of proliferation or activation, and their purity exceeded 90% in most experiments. The residual cells contained neutrophils with <1% $CD3^+$ T cells and <1% $CD56^+$ NK cells.

Human memory and naive B cells were obtained from purified B cells. The separation into the two populations was performed by an immunomagnetic method (MACS system) using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) coated with the anti-CD27 mAb (Miltenyi Biotec) according to the protocol of the supplier.

Preparation and fixation of CD32 transfectants

CD32T were prepared as described elsewhere [28, 38, 39]. CD32T were then incubated with 1% paraformaldehyde in PBS at room temperature for 5 min. After washing with PBS three times, the cells were cultured in RPMI 1640 with 10% fetal calf serum for 30 min and then used for B cell cultures to reinforce the stimulation levels of anti-CD40 or anti-CD72.

Flow cytometric analysis

Double- and triple-color analyses of B cell surface molecules were performed using a FACScan cytometer (Becton Dickinson, San Jose, CA). The antibody-coated cells were gated on living cells by cell size and granularity and were then counted by means of flow cytometric analyses.

B cell proliferation assay

Purified human B cells were cultured in 96-well round-bottom plates (Nunc, Roskide, Denmark) for 72 h at 37°C in a humidified atmosphere with 5% CO_2. The cultures were then pulsed with 0.5 μCi [^{3}H]-thymidine. After 15 h incubation, the cells were harvested using an automatic cell harvester

(Packard, Meriden, CT), and [^{3}H]-thymidine incorporation was measured on a liquid scintillation analyzer (Packard).

Ig assay by ELISA

Purified human B cells were cultured for 7 days as described for the proliferation assay. The supernatants were harvested and added to 96-well flat-bottom plates coated with goat anti-human Ig (Southern Biotechnology, Birmingham, AL) for the detection of IgG, IgA and IgM. Standard human IgG, IgA or IgM (Sigma) was also added to the plates. After overnight incubation at 4°C, the supernatants were discarded, and the wells were washed with 0.05% Tween 20 in PBS. Alkaline phosphatase-conjugated goat anti-human IgG, IgA or IgM (Sigma) at a dilution of 1:2500 was added for the detection of IgG, IgA and IgM, respectively. After 2 h incubation at room temperature, color detection was performed with 3-[cyclohexylamino]-1-propanesulfonic acid buffer containing p-Nitrophenyl phosphate (Sigma). Calibration was performed with PBS at standard zero levels. No cross-reaction among IgG, IgA and IgM occurred in this ELISA system.

RT-PCR

Cultured cells were dissolved in 1 mL TRIzol reagent (Life Technologies, Grand Island, NY). Total RNA was extracted by the acid-guanidine-thiocyanate-phenol-chloroform method and was then reverse transcribed into cDNA using oligo (dT) primer (Life Technologies) and Superscript II reverse transcriptase (Life Technologies) in a total volume of 20 μl. The following oligonucleotide primers were used: for XBP-1, 5′ GGT GCG TAG TCT GGA GCT AT 3′ and 5′ GCA AAA GTG TCC TCC CAA GAA T 3′; for PRDI-BF1, 5′ AGC TGA CAA TGA TGA ATC TCA 3′ and 5′ CTT GGG GTA GTG AGC GTT GTA 3′; and for BSAP, 5′ CAA CCA ACC AGT CCC AGC TTC 3′ and 5′ TCA CAA TGG GGT AGG ACT GCG 3′ (sense and antisense, respectively). The β_2-microglobulin (β_2-MG) sense primer 5′ GCT ATG TGT CTG GGT TTC AT 3′ and antisense primer 5′ ATC TTC AAA CCT CCA TGA TG 3′ were used as controls. A total of 2 μl cDNA was amplified in using each primer and Taq DNA polymerase (Life Technologies) by 35 cycles of the following steps: denaturation (94°C, 1 min), annealing (55°C, 1 min) and elongation (72°C, 2 min). The final polymerization step was extended for 5 min more. The amplified products were analyzed on a 1.2% agarose gel, and the relative integrated OD of mRNA bands was estimated with the NIH Image program (Scion, Frederick, MD).

Immunoprecipitation

Cells were lysed in buffer containing 1% Nonidet P-40, 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10% Glycerol and protease inhibitors and were immunoprecipitated with rabbit anti-Blk polyclonal Ab (C-20; Santa Cruz Biotech, Santa Cruz, CA) with protein G-Sepharose (Amersham Biosciences AB, Uppsala, Sweden).

Western blotting

Cultured cells were lysed in buffer similar to that used for immunoprecipitation. Total cell lysates or immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Bedford, MA). Membranes were incubated with recombinant horseradish peroxidase-conjugated anti-phosphotyrosine Ab (Amersham Biosciences, Buckinghamshire, UK) or rabbit anti-XBP-1 polyclonal Ab (M-186; Santa Cruz Biotech), rabbit anti-Blk polyclonal Ab, rabbit anti-extracellular signal regulatory kinase (ERK) 2 polyclonal Ab (C-14; Santa Cruz Biotech) or anti-β-actin (H-196; Santa Cruz Biotech) followed by reaction with peroxidase-conjugated anti-rabbit Ab (Amersham Biosciences). Proteins were then visualized by an ECL system (Amersham Biosciences). The relative integrated OD of protein bands was estimated with the NIH Image program (Scion).

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A comparison of the defective granulopoiesis in childhood cyclic neutropenia and in severe congenital neutropenia

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Background and Objectives. Cyclic neutropenia (CyN) in childhood and severe congenital neutropenia (SCN) are congenital disorders that cause chronic neutropenia. Mutations in the neutrophil elastase gene, *ELA2*, have been reported in patients with CyN and in those with SCN. We examined granulopoietic defects in CyN patients with those in SCN patients.

Design and Methods. Three patients with CyN and four with SCN were enrolled in this study. Bone marrow cells were enriched based on the expression of CD34, Kit, and granulocyte colony-stimulating factor receptor (G-CSFR). The purified cells were assayed for colony formation, proliferation, and mRNA expression of granular enzymes.

Results. All patients showed heterozygous mutations of *ELA2*. Flow cytometric analysis demonstrated no differences in the frequency of CD34, Kit, and G-CSFR expression between CyN patients and normal subjects. Significant differences in granulocyte/macrophage (GM)-colony formation of $CD34^+/Kit^+$ cells were observed among CyN patients, SCN patients, and normal subjects in response to hematopoietic factors. Impaired granulopoiesis was found in both $CD34^+/Kit^+/G\text{-}CSFR^+$ and $CD34^+/Kit^+/G\text{-}CSFR^-$ cells in patients with CyN, whereas this impairment was observed only in $CD34^+/Kit^+/G\text{-}CSFR^+$ cells in SCN patients, as previously reported. The mRNA expression of granular enzymes in myeloid precursors and the transcription levels during myeloid cell differentiation in CyN patients were comparable to those in normal subjects, in contrast to the abnormal transcription of granular enzymes in SCN patients.

Interpretation and Conclusions. These results suggest that the underlying granulopoietic abnormalities differ between CyN and SCN, and emphasize the presence of additional genetic pathophysiology specific to each disease.

Keywords: cyclic neutropenia, granulopoiesis, neutrophil elastase, severe congenital neutropenia.

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Human cyclic neutropenia (CyN) in children is a rare blood disorder characterized by regular oscillations in the numbers of circulating blood neutrophils, monocytes, eosinophils, lymphocytes, platelets, and reticulocytes.[1-4] A profound decrease in circulating neutrophils is observed at regular 3-week intervals. During phases of neutropenia, patients frequently suffer from symptoms of fever, malaise, aphthous stomatitis, lymphadenopathy and, occasionally, very severe infections. With increasing neutrophil counts, the infections and accompanying symptoms normally disappear. Bone marrow examinations have indicated that the cycling of peripheral blood cells is preceded by oscillations of granulopoietic cells. Severe congenital neutropenia (SCN), also known as Kostmann-type neutropenia, is characterized by an early onset in childhood, recurrent life-threatening infections, and profound neutropenia with an absolute neutrophil count of <200/μL in the peripheral blood.[5-9] The bone marrow usually shows a paucity of mature myeloid cells with an arrest of maturation of neutrophil precursors at the promyelocyte-myelocyte stage of differentiation.

Recently, mutations of the gene encoding neutrophil elastase (NE), *ELA2*, have been identified in both patients with CyN and those with SCN.[10-14] Missense or deletion mutations in the NE gene detected in patients with CyN or SCN are assumed to be involved in the pathogenesis of these disorders. We previously demonstrated that primitive myeloid progenitor cells in patients with SCN show abnormal transcriptional regulation of primary granule

enzymes, including NE, during myeloid proliferation and differentiation.[15] However, neither the mutation of *ELA2* nor the transcriptional abnormality can account for the functional defects in the SCN and CyN phenotypes.[13,14] Although these two disorders characterized by chronic neutropenia have similar genetic molecular abnormalities, the significance of the mutations of *ELA2* in the defective myelopoiesis of patients with CyN or SCN remains unclear.

Bone marrow cells from patients with CyN and those with SCN frequently display abnormal responses to hematopoietic factors in *in vitro* culture.[16-25] A defective response to haematopoietic factors, including granulocyte colony-stimulating factor (G-CSF), may be an important pathophysiologic mechanism underlying both CyN and SCN. We previously reported the presence of qualitative and quantitative abnormalities in the proliferation of primitive myeloid progenitor cells expressing G-CSF receptor (G-CSFR) in patients with SCN in response to hematopoietic factors, including G-CSF.[24,25] In patients with CyN, abnormally increased concentrations of G-CSF and granulocyte/macrophage colony-stimulating factor were required to stimulate maximal granulocyte colony formation of bone marrow cells.[16-18] The requirement of hematopoietic factor for maximal growth could be demonstrated at the $CD34^+$ progenitor cell level.[18] Thus, hematopoietic factors and their receptors may play a pivotal role in furthering our understanding of abnormal granulopoiesis in patients with CyN or SCN. In this study we examined abnormalities in primitive myeloid cells expressing CD34, Kit, and G-CSFR from patients with CyN or SCN. A comparison of CyN and SCN samples revealed a number of differences between the two disorders in terms of the abnormalities of affected myeloid cells.

Design and Methods

Patients

Three patients with CyN and four patients with SCN (characteristics presented in Table 1) were enrolled in this study. The diagnosis of CyN was made based on oscillations in the number of circulating blood neutrophils at regular 3-week intervals. The diagnosis of SCN, also known as Kostmann's syndrome, was made based on the established criteria, including an absolute neutrophil count below 200/μL in the peripheral blood, and maturation arrest at the promyelocyte or myelocyte stage in the bone marrow. No circulating antineutrophil antibodies were detected by granulocyte indirect immunofluorescence tests in either the CyN or SCN patients. The onset of recurrent infections was observed before the age of 24 months. All patients with CyN had received G-CSF in cases of severe infection during the neutropenic phase. All patients with SCN had a history of recurrent life-threatening infections and were receiving G-CSF with monitoring for hematologic problems, including myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML). None of the patients developed MDS and/or AML during the administration of G-CSF. We have previously reported some of the features of two of the four patients with SCN (SCN 2 and 3).[15,25] The bone marrow cells used in this study were collected during a period in which the patients were not receiving G-CSF, with the exception of one patient with SCN whose bone marrow cells were obtained during G-CSF treatment.

Cytokines

Recombinant human G-CSF, recombinant human interleukin-3 (IL-3), and recombinant human stem cell

Table 1. Characteristics of patients.

	Age (years)/ Sex	WBC (/μL)	Neutrophils (%)	Eosinophils (%)	Monocytes (%)	Lymphocytes (%)	Site of ELA2 mutation[1]	Treatment
Patients with CyN								
CyN 1	4/M	3,300	0	10	5	84	Exon 5, G4943A (Arg191Gln)	G-CSF[2]
CyN 2	5/F	3,400	0	5	6	87	Intron 4, G4716A	G-CSF[2]
CyN 3	12/M	4,400	0	11	14	74	Exon 4, C4534T (Pro110Leu)	G-CSF[2]
Patients with SCN								
SCN 1	0.9/M	4,100	0	9	10	77	Exon 5, C4953A (Cys194stop)	BMT[3]
SCN 2	0.3/F	4,900	2	3	21	69	Exon 4, C4495T (Ser97Leu)	BMT[3]
SCN 3	0.4/M	5,500	0	16	15	68	Exon 5, G4924A (Gly185Arg)	G-CSF[4]
SCN 4	0.2/M	4,000	1	7	10	80	Exon 5, G4981C (Ala204Pro)	BMT[3]

The patients' data reflect the findings at diagnosis. The white blood cell count (WBC) and differentials of patients with CyN represent the nadir in periodic oscillations. One patient with SCN (SCN 3) received G-CSF continuously without developing MDS or AML. [1]Nucleotide position corresponds to GenBank entry AC Y00477. Amino acid number one is the first after the presignal peptide. [2]G-CSF was intermittently administered when severe infections occurred during a neutropenic phase. [3]Allogeneic bone marrow transplantation (BMT) from an HLA-matched donor. [4]Administration of G-CSF four days per week was maintained for nine years without the development of either MDS or AML.

factor (SCF) were supplied by the Kirin Brewery Co. Ltd. (Tokyo, Japan). The recombinant human ligand for flk2/flt3 (FL) was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). Unless otherwise specified, the concentrations of factors used were as follows: G-CSF, 100 ng/mL; SCF, 100 ng/mL; FL, 20 ng/mL; and IL-3, 100 U/mL.

Bone marrow cell separation

Bone marrow samples were obtained with the informed consent of patients and/or their guardians. Normal bone marrow cells for this study were taken from healthy adult volunteers after obtaining informed consent. The study protocol was approved by the Institutional Review Board for Human Research, Hiroshima University Graduate School of Biomedical Sciences. The light density bone marrow cells obtained by centrifugation over Lymphoprep (1.077 g/mL; Nycomed Pharma AS, Oslo, Norway) were washed three times with phosphate-buffered saline (PBS) containing 2% human AB serum (Sigma Chemical Co., St. Louis, MO, USA) and 0.1 mg/mL of DNase I (type II-S; Sigma Chemical Co.), and resuspended in an α-modification of Eagle's medium (αMEM; ICN Biomedicals, Inc., Aurora, OH, USA) containing 10% FBS (ICN Biomedicals, Inc.). Cells were incubated in plastic culture flasks (Becton Dickinson Labware, Lincoln Park, NJ, USA) at 37°C for 1 hour to remove adherent cells. Non-adherent cells were either used in the described purification or were cryopreserved by a standard procedure using 10% DMSO, and then stored in liquid nitrogen until use. Cells, fresh or thawed, were washed and resuspended in PBS-human serum-DNase solution containing 0.1% sodium azide for subsequent immunofluorescence staining. No differences in the results of the experiments were observed between fresh and cryopreserved cells when the cells were purified according to the following procedure.

Purification of bone marrow cells

Cell purification was performed according to previously reported methods with modification.[26,27] In brief, cells (2×10^7/mL) were incubated with fluoroscein isothiocyanate (FITC)-labeled monoclonal anti-CD34 antibody (clone 581, Beckman Coulter, Inc., Fullerton, CA, USA) for 30 minutes at 4°C. FITC-conjugated mouse IgG1a was used as an isotype control. After the addition of propidium iodide (PI, Sigma Chemical Co.) at a concentration of 1 μg/mL, the cells were washed twice and resuspended in PBS-human serum-DNase-sodium azide solution. The initial enrichment of $CD34^+$ cells was carried out by setting a FACS Vantage (Becton Dickinson Immunocytometry Systems, San José, CA, USA), equipped with a 4-W argon laser, to recognize both FITC-positive and negative PI fluorescence, as well as low to medium forward scatter and low side scatter. The enriched $CD34^+$ cells were further stained with a phycoerythrin (PE)-conjugated anti-Kit (clone 95C3, Beckman Coulter, Inc.) for 30 to 40 minutes at 4 °C. After the addition of PI at a concentration of 1 μg/mL, cells were washed twice, then $CD34^+/Kit^+$ cells were sorted by FACS Vantage. When G-$CSFR^{+/-}$ cells on $CD34^+/Kit^+$ cells were purified, enriched $CD34^+$ cells were further stained with Kit-PE, and with biotin-conjugated anti-G-CSFR (clone LMM741, PharMingen, San Diego, CA, USA) for 30 to 40 minutes at 4°C. The cells were then washed twice and stained with streptavidin labeled with allophycocyanin (APC, Molecular Probes, Inc., Eugene, OR) for 15 minutes at 4°C. After the addition of PI at a concentration of 1 μg/mL, cells were sorted by FACS Vantage. The appropriate isotype controls, FITC-, PE- and biotin-conjugated mouse IgG1a, were used to identify background staining.

Clonal cultures

The clonal cell culture was performed in 35-mm Falcon suspension culture dishes (Becton Dickinson Labware). In the serum-deprived culture, 1 mL of the culture mixture contained purified cells, 1% deionized crystallized BSA, 300 μg/mL fully iron-saturated human transferrin (98% pure), 10 μg/mL soybean lecithin, 6 μg/mL cholesterol, 1×10^{-7} M sodium selenite, 10 μg/mL insulin, 4.5 mM L-glutamine, 1.5 mM glycine (all from Sigma Chemical Co.), as well as 1.2% 1,500-centipoise methylcellulose (Shinetsu Chemical, Tokyo, Japan), and designated cytokines.[26,27] The cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2/95% air. On day 14 of incubation, granulocyte-macrophage colonies were scored on an inverted microscope using the criteria described previously.[28] Each granulocyte-macrophage colony contained pure granulocyte colonies consisting of primarily neutrophils and their precursors, and mixed granulocyte-macrophage colonies consisting of mainly neutrophils, macrophages/monocytes, and their precursors. The number of colonies reported represents the means of values recorded in triplicate cultures.

Liquid suspension cultures

Ten thousand purified cells were cultured in 24-well microtrays (Corning Coaster Inc., Corning, NY, USA), in serum-deprived liquid suspension media consisting of the same supplements used in the clonal culture described above. Incubation was carried out at 37°C in a humidified atmosphere with 5% CO_2/95% air. The number of cells in each well was scored serially. Aliquots were centrifuged onto slides using Shandon's Cytospin 2 Centrifuge (Shandon Inc., Pittsburgh, PA,

Table 2. Primers for sequencing and real-time PCR.

	Sequence	Nucleotides
Primers for sequencing of the NE gene		
1 Forward primer	CGGAGGGGCAGAGACCCCGGA	1252-1272
Reverse primer	AGACCGGGACGCGGGGTCCGA	1972-1992
Sequencing primer		
Forward primer	CACCCGGTGTGTCCCCAGGCA	1768-1788
2 Forward primer	CTCGAGCACCTTCGCCCTCAG	2065-2085
Reverse primer	TCAACGGCCCATGGCGGGTAT	2497-2517
3 Forward primer	CCTGCCCTGCAGGATCCCAGA	4363-4383
Reverse primer	GGAGAGTGTGGGTGTGGGCAG	5113-5133
Sequencing primer		
Forward primer	AGGAACCGTGGGATCGCCAGC	4599-4619
Reverse primer	TGCAGACGTTGCTGCGACGGC	4660-4680
Primers and probes for real-time PCR		
Neutrophil elastase		
Forward primer	GCTAATCCACGGAATTGCCTC	668-688
Reverse primer	CTCGGAGCGTTGGATGATAGA	768-788
Probe	TTTGCCCCGGTGGCACAGTTTGTA	732-755
Myeloblastin		
Forward primer	TTCTTCTGCCGGCCACATA	586-604
Reverse primer	ATCCCCAGATCACGAAGGAGT	698-718
Probe	CATTTGCACTTTCGTCCCTCGC	606-627
Myeloperoxidase		
Forward primer	TCTTCACCAATGCCTTCC	1641-1658
Reverse primer	GCAAAAAAGACCCTGCTGAGG	1741-1761
Probe	TCATCCAACCCTTCATGTTCCGCC	1674-1697
Lactoferrin		
Forward primer	CCGAGGCCACAAAATGCTT	395-413
Reverse primer	ATGGCCTGGATACACTGGATG	480-500
Probe	TGGCCCTCCTGTCAGCTGCATAAA	444-467
β-actin		
Forward primer	CAGGTCATCACCATTGGCAAT	777-797
Reverse primer	TCTTTGCGGATGTCCACGT	898-916
Probe	TTGAAGGTAGTTTCGTGGATGCCACAGG	853-880

Nucleotide positions correspond to GenBank entries NM_001972 (neutrophil elastase), X00351 (β-actin), NM_002774 (myeloblastin), X04876 (myeloperoxidase), and NM_002343 (lactoferrin).

USA) for morphological examination of Wright-Giemsa staining.

Real-time quantitative polymerase chain reaction

Total cellular RNA extracted from fresh and cultured cells using the guanidinium thiocyanate extraction method was converted into cDNA by reverse transcriptase. The primers and probes of NE, myeloblastin, myeloperoxidase, and lactoferrin, and the β-actin for PCR used in this study are listed in Table 2. The commercial reagents (TaqMan PCR Reagent Kit, Applied Biosystems, Foster City, CA, USA) used in this study, as well as the PCR conditions, were those recommended by the manufacturer.[15] Thus, 10 μL of cDNA and 5 μL of oligonucleotides together with a final concentration of 200 nmol/L of primers and 100 nmol/L of TaqMan hybridization probe were added to a 25 μL reaction mixture. The amplification conditions for quantification were an initial 2 minutes of incubation at 50°C, 10 minutes at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute. The reactions were performed with the ABI PRISM 7700 sequence detection system equipped with a 96-well thermal cycler (Applied Biosystems). The data were collected and subsequently analyzed using Sequence Detector v1.6 software (Applied Biosystems). NE, myeloblastin, myeloperoxidase and lactoferrin values were corrected for the values obtained for β-actin from the same cDNA. In brief, the mean of experimentally obtained NE, myeloblastin, myeloperoxidase, or lactoferrin copy numbers obtained from a sample run in triplicate was divided by the expected value based on the amount of cDNA added to a reaction to obtain a β-actin normalizing value. In preliminary experiments we confirmed that the β-actin copy number was linearly dependent on the amount of cDNA that had been extracted, varying relative to the number of bone marrow cells.

Sequencing of PCR products

Mutational analysis was performed by sequencing PCR-amplified genomic DNA extracted from peripheral blood leukocytes or light density bone marrow cells with Applied Biosystems PRISM BigDye terminator chemistry on an ABI PRISM 310 Analyzer (Applied Biosystems). Each exon of neutrophil NE was sequenced from both directions in each individual. The primers used for the sequencing are listed in Table 2. The presence of mutation was confirmed using restriction endonuclease digestion of the relevant PCR fragment according to a method described by Ancliff *et al.*[12]

Statistical analysis

The statistical significance of the data was determined by ANOVA and unpaired two-group t-test using StatView software (version 5.0, SAS Institute, Inc., Cary, NC, USA).

Results

Mutations of ELA2

Several types of mutations in the gene encoding NE have been identified in CyN and SCN patients.[10-14] As shown in Table 1, heterozygous mutations were identified in all patients enrolled in this study. The site of mutation in three patients with CyN was consistent with that reported in the previous study.

The mutation found in one CyN patient (CyN 3) was reported to correspond to that in several patients with SCN. There have been no reports on the loci of mutations in patients with SCN (SCN 1 and SCN 4).

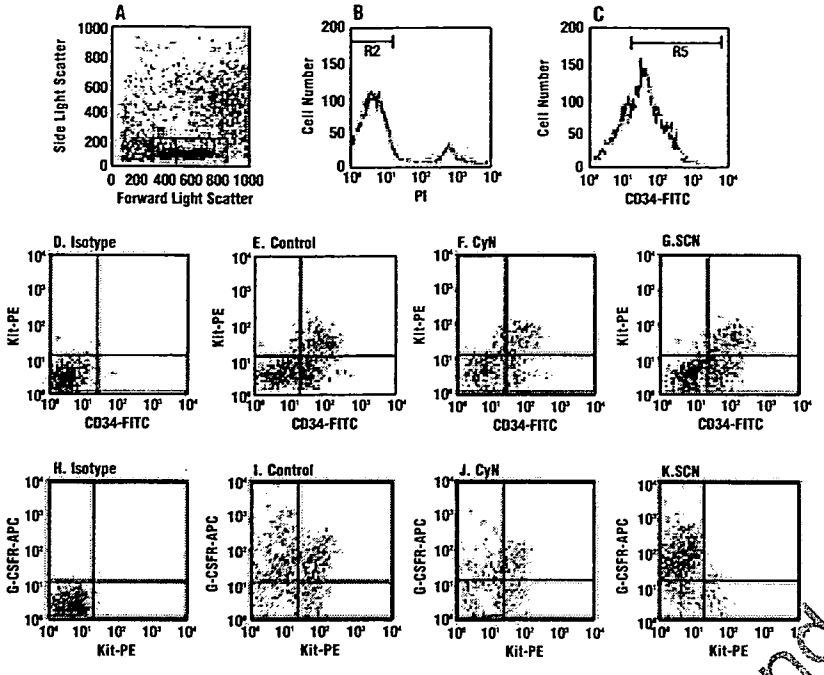

Figure 1. Flow cytometric analysis of bone marrow cells based on the expression of CD34, Kit, and G-CSFR. Low to medium forward scatter, and low side scatter (R1), and negative for PI fluorescence gates (R2) were used in all analyses (A, B). Mouse IgG1-FITC, IgG1-PE, and IgG1-biotin were used as isotype controls. The expression of CD34 and Kit (CD117) within the gated cells described above (R1 and R2) is shown for an isotype control (D), a representative normal subject (E), for a patient with CyN (F), and for a patient with SCN (G). The expression of Kit and G-CSFR (CD114) within R1, R2, and R3 (positive for CD34 in panel C) gates is shown for an isotype control (H), a representative normal subject (I), for a patient with CyN (J), and for a patient with SCN (K).

Flow cytometric analysis of bone marrow cells

We previously reported a remarkably reduced frequency of CD34⁺/Kit⁺/G-CSFR⁺ cells in patients with SCN.[25] The representative expression of CD34, c-Kit and G-CSFR on bone marrow cells in a patient with CyN, a patient with SCN, and a normal subject is shown in Figure 1. The expression of both CD34 and c-Kit on bone marrow cells was comparable among CyN patients, SCN patients, and normal subjects. Table 3 summarizes the quadrant percentage of Kit⁺/G-CSFR⁺, Kit⁺/G-CSFR⁻, Kit⁻/G-CSFR⁺, and Kit⁻/G-CSFR⁻ cells in CD34⁺ cells in three patients with CyN, patients with SCN, and normal subjects. A statistically significant difference in the frequency of Kit⁺/G-CSFR⁺ expression on CD34 cells was observed among CyN patients, SCN patients, and normal subjects as determined by ANOVA ($p< 0.001$). Post-hoc analysis showed that the frequency of Kit⁺/G-CSFR⁺ expression on CD34 cells in patients with SCN was clearly lower than that in CyN patients or normal subjects ($p<0.001$). There was no difference between patients with CyN and normal subjects in terms of the expression of CD34, Kit, and G-CSFR.

GM-colony formation of CD34⁺/Kit⁺ cells in response to various hematopoietic factors

Light density bone marrow cells were enriched for primitive myeloid progenitors using CD34 antibody and anti-Kit receptor antibody, because CFU-GM was enriched in the CD34⁺/Kit⁺ fraction. CD34⁺/Kit⁺ cells were cultured under serum-deprived conditions that included various hematopoietic factors. As shown in Table 4, significant differences in the number of GM colonies formed in response to G-CSF alone and to combinations of SCF, FL, and IL-3 with or without G-CSF were found among CyN patients, SCN patients, and normal subjects on analysis by ANOVA ($p<0.001$). Post-hoc analysis (Tukey's method) revealed that CD34⁺/Kit⁺ cells in normal subjects yielded a greater number of GM colonies than did those in patients with CyN or SCN ($p<0.01$), and CD34⁺/Kit⁺ cells in CyN patients yielded a greater number of GM colonies than did those in SCN patients ($p<0.05$). These results imply that patients with SCN have a more severe granulopoietic defect than do patients with CyN. In one patient with CyN (CyN 1), bone

Table 3. Frequency of the expression of Kit and G-CSFR on CD34 cells.

	Quadrant percentages of cells					
Cells	CyN 1	CyN 2	CyN 3	CyN (Mean ± SD, n=3)	SCN (Mean ± SD, n=4)	Normal Subjects (Mean±SD, n=12)
Kit⁺/G-CSFR⁺	35.3	38.2	29.0	34.2±3.8[a]	5.9±3.5[a]	30.4±9.8[a]
Kit⁺/G-CSFR⁻	12.5	15.3	23.5	17.1±4.7	15.4±8.9	18.1±5.2
Kit⁻/G-CSFR⁺	25.3	21.1	28.1	24.8±2.9	45.2±21.0	27.4±8.5
Kit⁻/G-CSFR⁻	27.0	25.3	19.4	23.9±3.3	33.5±13.6	24.2±13.6

Data demonstrating the quadrant percentage of each fraction in CD34⁺ cells illustrated in Figure 1-I, J, and K. [a]ANOVA revealed statistical significance ($p< 0.001$). [b]$p< 0.001$, Normal subjects or CyN vs. SCN by post-hoc analysis (Tukey's method).

Table 4. Formation of GM colonies of CD34⁺/c-Kit⁺ cells supported by various factors.

Factor(s)	CyN 1	CyN 2	CyN 3	Number of GM colonies[a] CyN (Mean±SD, n=3)	SCN (Mean±SD, n=4)	SCN (Range, n=4)	Normal Subjects (Mean±SD, n=10)	Normal Subjects (Range, n=10)
G-CSF	8	9	13	10±3[b]	4±2[b]	2-5	19±5[b]	14-35
SCF, FL, IL-3	31	28	38	32±5[b]	19±5[b]	16-24	53±12[b]	41-68
SCF, FL, IL-3, G-CSF	62	65	70	66±4[b,c]	31±8[b,c]	25-37	95±20[b]	76-128

Cultures were performed under serum-deprived conditions including 500 CD34⁺/c-Kit⁺ cells and the designated factors. CyN patient data represent the mean values in triplicate cultures. SCN and control data represent 4 and 10 subjects, respectively. [a]ANOVA revealed statistical significance (p< 0.001). [b]p< 0.01, Normal subjects vs. CyN or SCN by post-hoc analysis (Tukey's procedure). [c]p< 0.05, CyN vs. SCN by post-hoc analysis (Tukey's procedure).

Table 5. Formation of GM colonies of purified cells supported by various factors.

Factor(s)	CyN 1	CyN 2	CyN 3	Number of GM colonies[a] CN (Mean±SD, n=3)	SCN (Mean±SD, n=4)	SCN (Range, n=4)	Normal Subjects (Mean±SD, n=10)	Normal Subjects (Range, n=10)
CD34⁺/Kit⁺/G-CSFR⁺ Cells								
G-CSF	8	15	16	13±4[b,c]	3±1[b,c]	1-4	28±8[b]	21-35
SCF, FL, IL-3	21	30	25	25±3[b,c]	15±5[b,c]	9-18	41±8[b]	28-47
SCF, FL, IL-3, G-CSF	39	49	55	48±8[b,c]	29±8[b,c]	21-35	70±5	56-76
CD34⁺/Kit⁺/G-CSFR⁻ Cells								
G-CSF	0	1	1	1±1	3±1	1-4	2±1	0-3
SCF, FL, IL-3	9	20	13	14±6[d]	23±6	16-30	21±6[d]	22-35
SCF, FL, IL-3, G-CSF	25	32	26	28±4[d]	39±9	28-46	38±7[d]	32-51

Cultures were performed under serum-deprived conditions including 250 CD34⁺/Kit⁺/G-CSFR⁺ or CD34⁺/Kit⁺/G-CSFR⁻ cells and the designated factors. CyN patient data represent the mean values in triplicate cultures. SCN and control data represent 4 and 10 subjects, respectively. [a]ANOVA revealed statistical significance (p< 0.001); [b]p< 0.01, Normal subjects vs. CyN or SCN by post-hoc analysis (Tukey's procedure). [c]p< 0.05, CyN vs. SCN by post-hoc analysis (Tukey procedure). [d]p< 0.01, Normal subjects vs. CyN by post-hoc analysis (Tukey's procedure).

marrow aspiration was performed at three different time points over 21 days. No significant differences in the GM-colony formation of CD34⁺/Kit⁺ cells were observed relative to the point of oscillation (data not shown). These findings suggest the possibility that the primitive myeloid progenitor cells of patients with CyN and those with SCN are defective in their response not only to G-CSF, but also to other hematopoietic factors involved in granulopoiesis.[29-31]

GM-colony formation of CD34⁺/Kit⁺ cells expressing G-CSFR

The CD34⁺/Kit⁺ cells were further purified to obtain CD34⁺/Kit⁺/G-CSFR⁺ and CD34⁺/Kit⁺/G-CSFR⁻ cells. No difference in quadrant percentages of Kit⁺/G-CSFR⁺ and Kit⁺/G-CSFR⁻ cells on CD34⁺ cells was noted between patients with CyN and normal subjects (Figures 1-I and J). This result is completely different from that previously reported for SCN patients.[25] Similar to the results seen in CD34⁺/Kit⁺ cells, the number of GM colonies formed was significantly different (ANOVA, $p<0.001$) in CD34⁺/Kit⁺/G-CSFR⁺ cells among CyN patients, SCN patients, and normal subjects (Table 5). The ranking established according to the number of GM colonies formed was similar to that in CD34⁺/Kit⁺ cells. In contrast to the case of CD34⁺/Kit⁺/G-CSFR⁺ cells, no difference in GM colony formation was observed between normal subjects and patients with SCN in the case of CD34⁺/Kit⁺/G-CSFR cells. However, GM-colony formation of CD34⁺/Kit⁺/G-CSFR cells in patients with CyN was significantly reduced compared with that in normal subjects and in patients with SCN in response to hematopoietic factors ($p< 0.01$). These findings suggest that the affected cell population differs between patients with CyN and those with SCN.

Proliferation of CD34⁺/Kit⁺ cells in liquid suspension culture

The proliferation of CD34⁺/Kit⁺/G-CSFR⁺ and CD34⁺/Kit⁺/G-CSFR⁻ cells in response to various hematopoietic factors, including G-CSF, was examined in a serum-deprived liquid suspension culture. The total number of cells in the wells was recorded

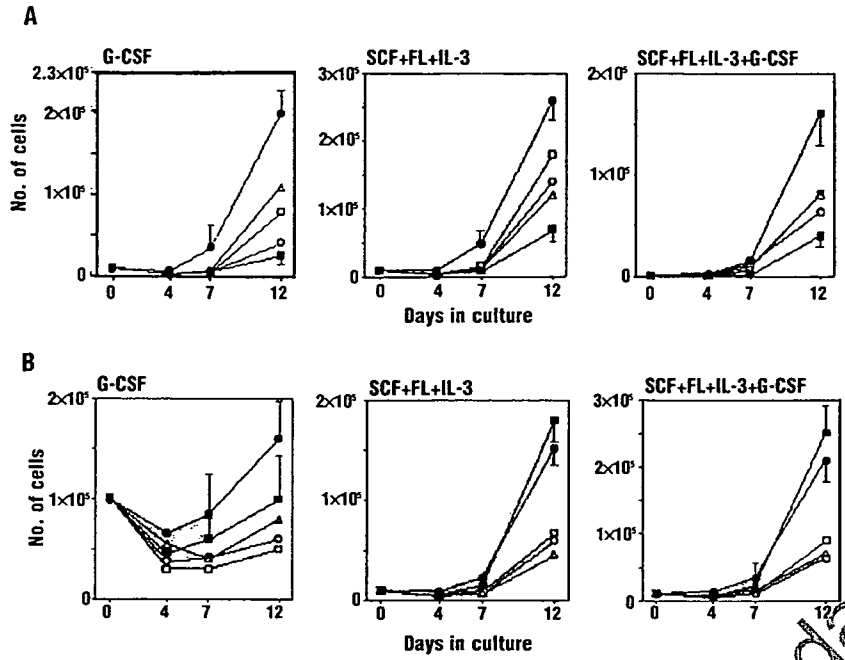

Figure 2. Proliferation of CD34⁺/Kit⁺/G-CSFR⁺ and CD34⁺/Kit⁺/G-CSFR⁻ cells in liquid suspension culture. Ten thousand purified CD34⁺/Kit⁺/G-CSFR⁺ (A) and CD34⁺/Kit⁺/G-CSFR⁻ cells (B) were cultured with the hematopoietic factors indicated under serum-deprived conditions. Data represent the cell number (mean ± SD) in six normal subjects (●), in four patients with SCN (■), and in three patients with CyN (CyN 1, ○; CyN 2, □; CyN 3, △).

serially. As shown in Figure 2, the proliferation of CD34⁺/Kit⁺/G-CSFR⁺ cells in response to G-CSF alone, or to SCF, FL, and IL-3, with and without G-CSF, was less in patients with CyN than in normal subjects. A more substantial decrease in the proliferation of CD34⁺/Kit⁺/G-CSFR⁺ cells in response to hematopoietic factors was found in SCN patients than in CyN patients. Similarly, the CD34⁺/Kit⁺/G-CSFR⁻ cells of patients with CyN proliferated less in response to SCF, FL, and IL-3, with and without G-CSF, than did the corresponding cells from normal subjects or patients with SCN. These results are consistent with the data showing a reduced level of GM colony formation in CD34⁺/Kit⁺/G-CSFR⁺ cells and a comparably reduced level in CD34⁺/Kit⁺/G-CSFR⁻ cells in semisolid culture. Thus, defective granulopoiesis in patients with CyN was primarily manifested in the level of CD34⁺/Kit⁺ cells, irrespectively of the expression of G-CSFR.

Granular protein mRNA expression in myeloid precursor cells

We recently demonstrated that the primitive myeloid progenitor cells in patients with SCN show an abnormal transcriptional regulation of primary granule enzymes, including NE, during myeloid proliferation and differentiation.[15] In the culture of CD34⁺/Kit⁺ cells with G-CSF, granular protein mRNA expression, including that of NE, was examined using real-time quantitative PCR. As shown in Figure 3, the transcription level of primary granule enzymes in CD34⁺/Kit⁺ cells was enhanced to relatively high levels on days 4 and 7, and then decreased on day 12 in normal subjects, as previously reported.[15] The transcription levels of primary granule enzymes in patients with CyN showed a pattern similar to that of normal subjects. The cell composition reflecting proliferation and differentiation in this culture was almost the same between CyN patients and normal subjects,

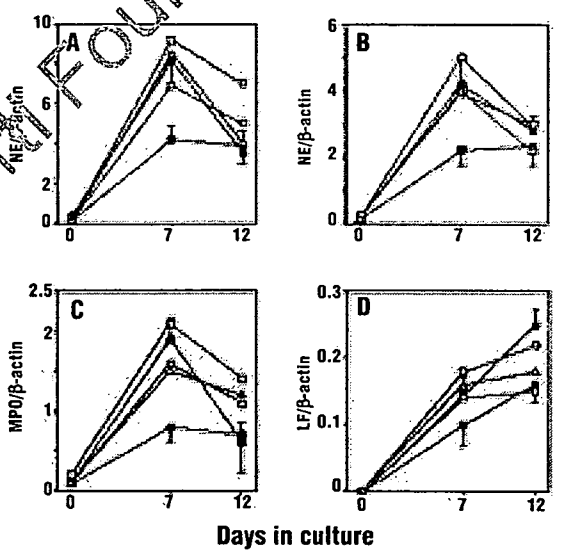

Figure 3. Expression of neutrophil elastase (NE), myeloblastin (MBN), myeloblastin myeloproxidase (MPO) and lactoferrin (LF) genes during the culture of CD34⁺/Kit⁺ cells with G-CSF. Total cellular RNA extracted from cells during culture with G-CSF under serum-deprived conditions was converted into cDNA and submitted to a quantitative real-time PCR analysis. The ratios of the copies of NE (A), MBN (B), MPO (C), and LF (D) to those of β-actin are presented as the mean ± SD of four normal subjects (●), the mean ± SD of four patients with SCN (■), and that of three patients with CyN (CyN 1, ○; CyN 2, □; CyN 3, △).

Table 6. Expression of granular protein genes in myeloid precursor cells of bone marrow.

	CyN 1	CyN 3	SCN (Mean±SD, n = 4)	Normal Subjects (Mean±SD, n = 5)
Neutrophil elastase	12.6	24.4	2.9±1.5	17.1±6.4
Myeloblastin	3.5	7.2	1.1±0.6	4.6±1.8
Myeloperoxidase	3.6	6.5	0.9±0.3	3.9±1.6
Lactoferrin	1.5	2.2	1.7±0.6	3.5±2.9

Fresh bone marrow cells were stained with CD14-FITC, CD16-FITC, and CD13-PE, and then the CD13+/CD14-/CD16- cells were sorted by FACS Vantage. cDNA in CD13+/CD14-/CD16- cells were extracted and then subjected to real-time quantitative PCR analysis. The ratios of the copies of neutrophil elastase, myeloblastin, myeloperoxidase and lactoferrin, respectively, to the copies of β-actin are presented.

as determined by cytospin preparation (*data not shown*). Although abnormal proliferation was observed in CyN patients, the difference in transcription levels between CyN patients and normal subjects was not significant. In contrast, initial up-regulation and subsequent down-regulation of primary granule enzyme transcription were not clearly observed in the SCN patients, as previously reported.[15] In addition, no differences in the levels of lactoferrin expression were observed among normal subjects, CyN patients, and SCN patients.

To confirm the levels of transcription in myeloid cells, bone marrow myeloid precursor cells were isolated from fresh light density bone marrow cells from two patients with CyN. The cells positive for CD13 and negative for both CD14 and CD16 were purified in order to avoid contamination by monocytes and mature neutrophils. Cytospin preparation with Wright-Giemsa staining revealed that more than 90% of cells were myeloblasts, promyelocytes, and myelocytes. The cDNA extracted from these cells was subjected to quantitative real-time PCR analysis. As shown in Table 6, no significant differences in the levels of transcription of primary granule enzymes were noted between patients with CyN and normal subjects. The transcription levels in CyN patients were normal, irrespective of the time point in the course of the CyN cycle (*data not shown*). These observations suggest that the regulation of transcription of primary granule proteins in patients with CyN is normal. Thus, the mutations of the NE gene observed in CyN patients may not be directly involved in the transcription of primary granule proteins.

Discussion

Several reports have noted that the bone marrow cells of CyN and SCN patients exhibit abnormal *in vitro* growth responses to hematopoietic factors.[16-25] Recently, mutations in the gene encoding NE have been identified in all patients with CyN and in the majority of patients with SCN.[10-14] These findings have provided genetic evidence that mutations in *ELA2* play a key role in the pathogenesis of both CyN and SCN. However, the involvement of *ELA2* mutations in myelopoietic defects in CyN and SCN patients remains unclear. In a comparison of granulopoiesis of myeloid progenitor cells and expression of granular proteins in myeloid precursor cells of patients with CyN and those with SCN, both groups exhibiting heterozygous mutations of the NE gene, different defects were observed. In a previous series we found that affected myeloid progenitor cells in SCN patients were predominantly primitive progenitor cells expressing G-CSFR. In contrast, the cells affected in CyN patients were independent of the presence of G-CSFR, and were found in primitive stage hematopoietic progenitor cells expressing CD34 and Kit (CD117). Human bone marrow cells capable of multilineage differentiation and long-term engraftment are known to be present in the $CD34^+/Kit^+$ population.[32] This effect in CyN patients might be attributable to regulatory defects in multilineage haematopoietic progenitor cells and to characteristics of the periodic decrease in circulating neutrophil levels corresponding with the oscillation of platelets, monocytes, and, occasionally, reticulocytes and lymphocytes.[1-4]

Abnormal responsiveness to hematopoietic factors including G-CSF may be an important pathophysiologic mechanism underlying CyN and SCN. Previous studies have shown that myeloid progenitor cells from CyN patients are 5 to 10 times less responsive to G-CSF and GM-CSF than are normal progenitors.[16-18] A requirement for an increased concentration of hematopoietic factors could be demonstrated at the level of $CD34^+$ cells, especially in response to G-CSF, in both serum-supplemented and serum-deprived cultures.[18] The present study confirmed abnormalities among myeloid cells by using highly purified progenitor cells under serum-deprived conditions. We found a reduction in GM-colony formation and decreased proliferation in response to various hematopoietic factors involved in myelopoiesis. The combination of SCF, FL, IL-3, and G-CSF maximally stimulated GM-colony formation in cells from normal subjects in serum-deprived culture.[24] However, an increased concentration of hematopoietic factors did not induce the formation of the same number of GM colonies as that formed in normal subjects (*data not shown*), implying a decreased number of myeloid progenitor cells in the primitive hematopoietic cell compartment of the $CD34^+/Kit^+$ population. Together, these findings provide evidence that myeloid progenitor cells in CyN patients exhibit qualitative and quantitative abnor-

malities. Avalos and co-workers reported that the lower responsiveness was not the result of a defect in the G-CSF signal transduction pathway at a point distal to G-CSF receptor binding in canine cyclic neutropenia.[19] In humans with CyN, the number of G-CSF binding sites on mature neutrophils has been reported to be normal.[33] The present study also showed no difference in the percentage of cells expressing G-CSFR on $CD34^+/Kit^+$ cells between normal subjects and CyN patients. The difference in G-CSFR expression on $CD34^+/Kit^+$ cells between patients with CyN and those with SCN may suggest a completely different pathophysiologic mechanism underlying these respective disorders. The intensive involvement of G-CSF and G-CSFR in SCN patients may underlie a different prognosis for SCN and CyN patients. A long-term follow-up study of patients included in the Severe Chronic Neutropenia International Registry found that MDS and/or AML developed in some SCN patients, but neither of these disorders developed in CyN patients.[8,34]

The roles played by granular proteins and their expression in the regulation of myelopoiesis are currently unknown. The expression profiles of granule protein mRNA during neutrophilic granulocyte differentiation have already been described.[35-42] Cells positive for NE mRNA transcripts were shown to be present during a very limited period of neutrophil differentiation, and were predominantly found in the promyelocyte and late promyelocyte stages, with a much smaller percentage of positive myelocytes.[37,39,40] We recently demonstrated the dysregulation of transcription in primary granule enzymes during myeloid proliferation and differentiation in SCN patients. In patients with CyN, the expression levels of primary granule constituents were comparable to those in normal subjects during *in vitro* myeloid differentiation. Moreover, no differences were observed between normal subjects and CyN patients in terms of the transcriptional levels of primary granule enzymes in fresh bone marrow cells enriched for myeloblasts, promyelocytes, and myelocytes using CD13, CD14, and CD16 antibodies. These results suggest that the transcription pattern of granular proteins in patients with CyN is normal and, therefore, differs from that in patients with SCN. It appears that mutations of *ELA2* may not be directly involved in the regulation of transcription.

Taken together, the above findings provide no direct evidence of a correlation between the two disorders and the presence of *ELA2* mutations. However, an overlap of mutations of *ELA2* was found in CyN and SCN patients. As demonstrated in this study, faulty myelopoiesis occurred in different subpopulations of myeloid progenitor cells. The expression pattern of granular protein mRNA was also different between these two disorders. Furthermore, in mice the lack of NE as well as the engineered expression of a mutated NE was insufficient to induce an SCN phenotype.[43,44] Further studies are, therefore, necessary to clarify the relationship between *ELA2* mutations and additional underlying molecular defects in the pathophysiology of CyN and SCN.

All authors meet the criteria for being contributing authors. Design of the study, analysis of data, and writing of the manuscript; YS, SK, and MK; cell sorting, TS and MH; ELA2 mutation analysis and real time quantitative PCR data, HK, KN, and OK; cell culture, SO, NI and YS.

All authors were involved in discussing and interpreting the data. The authors declare that they have no potential conflicts of interest.

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Regulation of Aged Humoral Immune Defense against Pneumococcal Bacteria by IgM Memory B Cell

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Elderly persons have a high incidence of lethal infections by encapsulated bacteria. However, mechanisms involved in their poor defense and maintenance of immunological memory have been poorly understood. The present study characterized the population of B cells known as IgM memory B cell compartment and their response by pneumococcal vaccine in elderly people. $CD27^+$ memory B cells, particularly $IgD^+IgM^+CD27^+$ IgM memory B cells, had dramatically declined in the aged. Their Ig syntheses by B cells and the differentiation into plasma cells were diminished in vitro compared with those in adults. A rise of anti-pneumococcal IgM in sera of elderly persons was found with lower levels compared with those in adults after pneumococcal vaccination. Although diminished function itself of aged B cells surely exist, decline of the IgM memory B cell pool is expected to result in a poor humoral immunity against pneumococcal infection in elderly people. *The Journal of Immunology,* **2005, 175: 3262–3267.**

The secondary immune response is crucial for disease prevention and depends entirely on the immunological memory that is carried by memory B cells and memory T cells (1). The mechanisms with which immunological memory is maintained after infections or vaccination are complex and a matter of debate (2). The most dramatic health problem of the aged immune system is the increasing rates of morbidity and mortality from recurrent and invasive infections of the respiratory tract caused by encapsulated bacteria such as *Streptococcus pneumoniae* (2, 3). Related effects include diminished protective immunity after prophylactic vaccines, blunted reactivity to tuberculin skin tests, and re-emergence of latent infections (2). The impaired response of the elderly to most vaccines and the greater susceptibility of the elderly to infections have fostered a view that immune senescence leads to a state of immune deficiency (4).

Human marginal zone B cells in the spleen have been shown to carry somatic hypermutations, and mutated Abs can be raised after immunization with T-independent polysaccharide vaccine (5). Human memory B cells are separated into two populations: $IgM^+IgD^+CD27^+$ so-called IgM memory B cells and IgD^-CD27^+ memory B cells that are composed of IgG^+ and IgA^+CD27^+ so-called switched and IgM-only memory B cells (6). IgM memory B cells do not shift into switched memory B cells by various stimuli in vitro (7). It was suggested recently that IgM memory B cells are generated in the spleen and control *S. pneumoniae* infections (8, 9). *S. pneumoniae* is the chief cause of pneumonia in older adults. The effectiveness of the pneumococcal polysaccharide vaccine for the prevention of bacteremia was demonstrated (10, 11). In addition, functions in the spleen are declined in aged persons (12, 13). These observations led us to suggest that IgM memory B cells are decreased, could represent susceptibility to pneumococcal infections, and could thus be involved in defective immune responses in elderly people.

In this study, we show by phenotypic, gene, and functional analyses that IgM memory B cells indeed dramatically decreased in the aged. We also demonstrate the importance of their pneumococcal polysaccharide vaccination, which was associated with a significant reduction in the risk of pneumococcal bacteremia as reported previously (10), on IgM memory B cells, the origins and characteristics of which were highlighted recently.

Materials and Methods

Study populations

One hundred thirty healthy subjects were enrolled in this study. Adults were 65 healthy blood donors (21–64 years of age, 32 women and 33 men). In the group of elderly persons, 65 elderly persons (65–99 years of age, 37 women and 28 men) living in their homes or in an institution for elderly self-sufficient people were recruited. The volunteers were regarded as eligible if they had no clinically significant diseases and conditions such as diabetes mellitus, cancer, collagen diseases, various kinds of infections, and any treatment with immunosuppressive drugs. Also, none of them suffered from ongoing bacterial infections at the time of analysis.

Blood sampling and cell preparation

Peripheral blood (PB)[2] samples were obtained from healthy volunteers after the informed consent of the study was given. Mononuclear cells (MNCs) were isolated from PB by Ficoll-Hypaque (Pharmacia) density gradient centrifugation. $CD19^+$ B cells were enriched to 95–98% by magnetic cell separation using the MiniMACS system (Miltenyi Biotec).

Flow cytometric analysis

PB MNCs were stained with a combination of anti-IgD-FITC (DAKO Japan) or anti-IgM-FITC (DAKO Japan), anti-CD20-PerCP (Becton Dickinson) or anti-CD19-PerCP (BD Biosciences), and anti-CD27-biotin (8H5; IgG1) (14), followed by streptavidin-PE (Sigma-Aldrich). Conjugation of biotin to anti-CD27 mAb was performed by the standard technique using *N*-hydroxysuccinimido-biotin (Sigma-Aldrich) in our laboratory. Purified B cells after activation were stained with a combination of CD38-FITC (Immunotech) and CD20-PE (DAKO Japan). *Staphylococcus aureus* Cowan strain (SAC) and propidium iodide were obtained from Sigma-Aldrich, and human rIL-2 was obtained from Genzyme. Dead cells were

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[2] Abbreviations used in this paper: PB, peripheral blood; MNC, mononuclear cell; SAC, *Staphylococcus aureus* Cowan strain.

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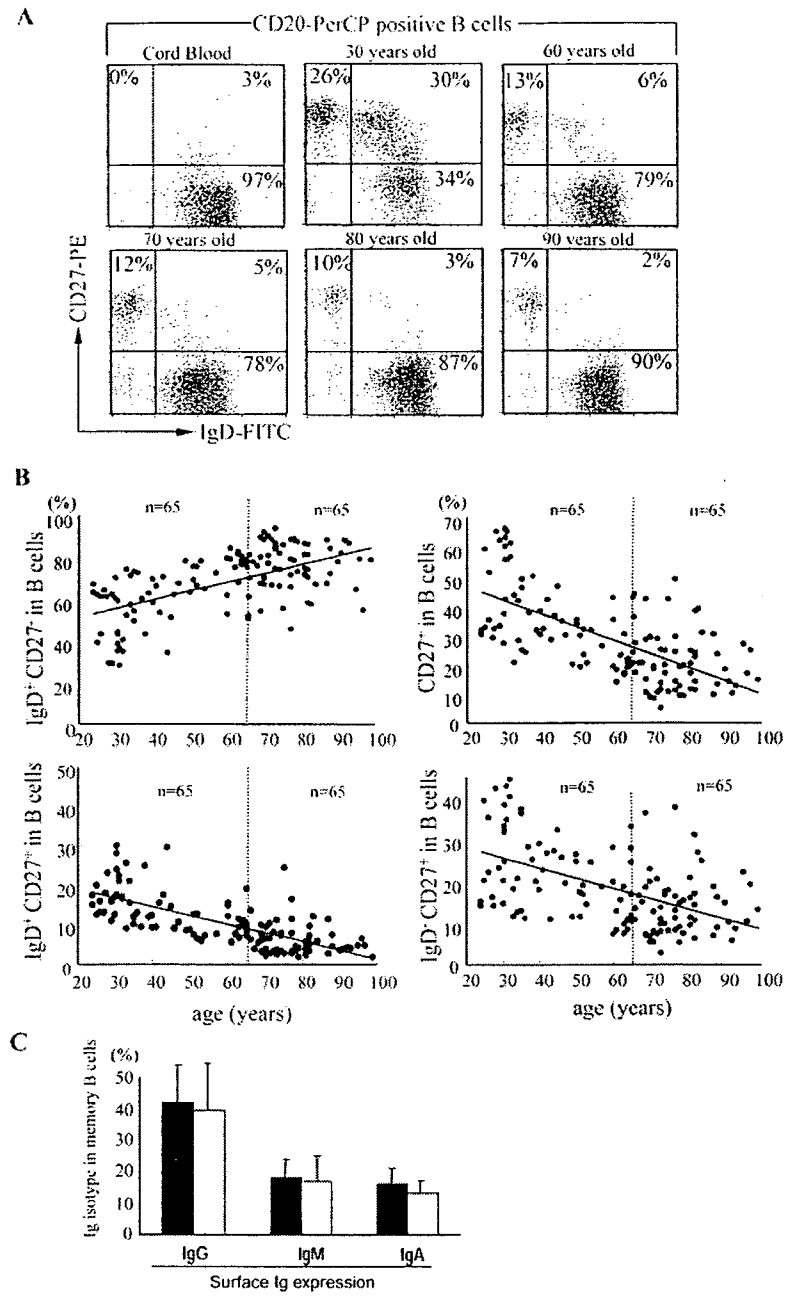

FIGURE 1. Marked decline of IgM memory B cells in elderly persons. *A*, MNCs from cord blood and PB of adults (30 years old) or elderly persons (60, 70, 80, and 90 years old) were stained with anti-IgD-FITC, anti-CD20-PerCP, and anti-CD27-biotin, followed by streptavidin-PE. Three-color analysis was conducted by gating CD20-positive B cells. Data were displayed as density plots with green (FITC) fluorescence, IgD, on the *x*-axis and orange (PE) fluorescence, CD27, on the *y*-axis by the log scale. The percentages of positive cells are indicated. *B*, MNCs from 65 adults (<65 years old) and 65 elderly (≥65 years old) persons are stained as described above. Data are displayed as dot plots with age on the *x*-axis and percentage of positive cells on the *y*-axis. The percentages of IgD^+CD27^- naive B cells and $CD27^+$ memory B cells, including IgD^+CD27^+ (IgM memory) and IgD^-CD27^+ memory B cells, within the $CD20^+$ cell population are shown. The correlation between age and the percentages of IgD^+CD27^- naive B cells, $CD27^+$ memory B cells, IgD^+CD27^+ B cells, and IgD^-CD27^+ B cells within the $CD20^+$ cell population was $r = 0.548$ ($p < 0.001$), $r = -0.651$ ($p < 0.001$), $r = -0.700$ ($p < 0.001$), and $r = -0.521$ ($p < 0.001$), respectively. *C*, MNCs from adults ($n = 7$; ■) or elderly persons ($n = 7$; □) were stained with anti-IgG-FITC, anti-IgM-FITC, or anti-IgA-FITC and with anti-CD20-PerCP and anti-IgD-biotin, followed by streptavidin-PE. Surface IgG, IgM, or IgA expression on IgD^-CD20^+ B cells was evaluated.

removed by staining with propidium iodide (Sigma-Aldrich). Flow cytometric analysis was then performed by FACScan (BD Biosciences).

Ig assay

The amount of serum IgG from adults and elderly persons was measured by a latex agglutination test. For the IgG, IgM, and IgA syntheses, MNCs or purified B cells were cultured with SAC plus IL-2. The cells were cultured for 8 days at 37°C in a humidified atmosphere with 5% CO_2. The final cell density was 2.5–5 × 10^5/ml in a volume of 200 μl/well. The plates were coated with goat anti-human Igs (Southern Biotechnology Associates) for the detection of IgG, IgM, and IgA. The cultured supernatants were harvested and added to 96-well flat ELISA plates (Nunc). The standard human IgG, IgA, or IgM (Sigma-Aldrich) was also added to the plates. After an overnight incubation, supernatants were discarded, and the wells were washed with 0.05% Tween 20 in PBS. Alkaline phosphatase-labeled goat anti-human IgG, IgA, and IgM at a dilution of 1/2500 was added for the detection of IgG, IgA, and IgM, respectively. After 2 h of incubation at room temperature, color detection was performed by 3-[cyclohexylamino]-1-propanesulfonic acid buffer containing *p*-nitrophenyl phosphate (Sigma-Aldrich). Calibration was performed with PBS at standard zero levels. In this ELISA system, no cross-reaction between IgG, IgA, and IgM occurred.

Polysaccharides and sera

Pneumococcal Ags, pneumovax (1 vial of valium is 0.5 ml containing 25 mg of each Ag), which include 23 types of polysaccharides 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F, were obtained from Banyu.

Vaccination

Healthy elderly persons who hoped for pneumococcal vaccination were immunized s.c. in the arm with 1 vial of pneumovax (a 0.5-ml volume containing 25 μg of each of the 23 types of polysaccharides) only one time. Before vaccination and 4 wk after vaccination, sera and PB heparinized samples from the vaccinated persons were obtained and used to measure specific Abs by ELISA and investigate B cell populations by flow cytometric analysis.

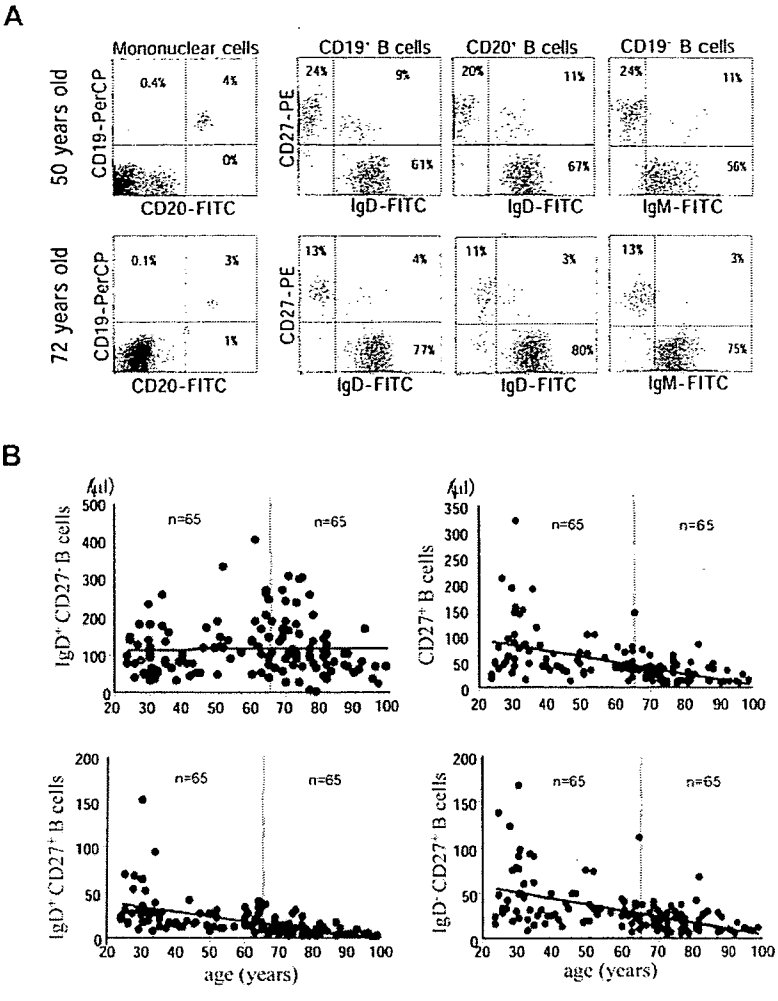

FIGURE 2. B cells evaluated by the expression of CD19 and IgM and absolute numbers of B cell populations. *A*, MNC PB of 50- or 72-year-olds were stained with anti-IgD-FITC or anti-IgM-FITC, anti-CD19-PerCP or anti-CD20-PerCP, and anti-CD27-biotin, followed by streptavidin-PE. Three-color analysis was conducted by gating on CD19- or CD20-positive B cells. Data are displayed as density plots with green (FITC) fluorescence, IgD or IgM, on the *x*-axis and orange (PE) fluorescence, CD27, on the *y*-axis by the log scale. The percentages of positive cells are indicated. *B*, Absolute numbers of IgD^+CD27^- naive B cells and $CD27^+$ memory B cells, including IgD^+CD27^+ (IgM memory) and IgD^-CD27^+ memory B cells within the $CD20^+$ cell population, are shown. The correlation between age and the percentages of IgD^+CD27^- naive B cells, $CD27^+$ memory B cells, IgD^+CD27^+ B cells, and IgD^-CD27^+ B cells within the $CD20^+$ cell population was $r = 0.017$ ($p = 0.851$), $r = -0.513$ ($p < 0.001$), $r = -0.584$ ($p < 0.001$), and $r = -0.530$ ($p < 0.001$), respectively.

IgM Abs against pneumococcal polysaccharides

Specific IgM Abs against pneumococcal polysaccharides were determined by ELISA designed according to the standardized ELISA protocol with few modifications. The 96-well flat ELISA plates (Nunc) were coated with 0.5 μg/ml pneumovax, which is composed of 23 polysaccharides (Banyu) and were blocked by 0.25% BSA in PBS for 4 h. Serum samples were diluted 20 times with PBS including 0.6% BSA. The diluted standard sera ranging from 1/10 to 1/160 and samples were added to each well. After overnight incubation at 4°C, the wells were washed with 0.05% Tween 20 in PBS. Alkaline phosphatase-labeled goat anti-human IgM (Sigma-Aldrich), at a dilution of 1/2500, was added for the detection of specific IgM Ab. After 2 h of incubation at room temperature, color detection was performed by 3-[cyclohexylamino]-1-propanesulfonic acid buffer containing p-nitrophenyl phosphate (Sigma-Aldrich). Absorbance was read at 405 nm.

Statistical analysis

Data from individual experiments were expressed as mean ± SD. Data were analyzed with StatMateIII software (ATMS), and correlation coefficients were obtained using SOKAN in the software. Statistical significance was determined using paired or unpaired Student's *t* test, and $p < 0.05$ was considered to be statistically significant.

Results

Analyses of PB B cell subsets

Circulating PB B cells obtained from 65 healthy adults (<65 years old) and 65 healthy elderly persons (≥65 years old) were examined. As reported previously (15–17), adult B cells could be separated into three subpopulations on the basis of CD27 and IgD expression: IgD^+CD27^- naive B cells, IgD^+CD27^+ (IgM memory) B cells, and IgD^-CD27^+ B cells. In this study, we included IgM-only memory B cells (16 ± 7% in adult IgD^-CD27^+ B cells), which are unclass-switched memory B cells, into IgD^-CD27^+ B cells, a majority of which are composed of switched memory B cells (78 ± 13% in adult IgD^-CD27^+ B cells). In contrast, cord blood B cells consist predominantly of naive B cells (Fig. 1*A*). The percentages of IgM memory B cells and IgD^-CD27^+ memory B cells in PB B cells increased during childhood and adulthood and peaked at ~30 years of age. An especially notable finding in this study revealed that IgM memory B cells markedly reduced with age ($r = -0.700$), and IgD^-CD27^+ memory B cells also reduced to a slight degree ($r = -0.521$), especially in individuals ≥50 years of age (Fig. 1*B*). IgM memory B cells were undetectable to marginal levels in the blood of aged people (>70 years old), and IgD^-CD27^+ memory B cells were apparently present at low frequency. Also, a decrease in the percentage was observed by Student's *t* test in both IgM memory and IgD^-CD27^+ B cells between <65 and ≥65 years of age ($p < 0.001$ and $p < 0.001$, respectively). In contrast, percentages of naive B cells, which are predominant B cells in cord blood, consistently increase with aging (Fig. 1, *A* and *B*). For IgG, IgM, or IgA expression on IgD^-CD27^+ memory B cells, the clear difference was not recognized between young and aged persons (Fig. 1*C*).

We also evaluated B cell populations by the expression of CD19 or IgM. In our experiments, as shown in Fig. 2*A*, when we gated on CD19, the percentages among each B cell population was almost identical to those gated on CD20 (in 10 elderly persons, CD20 or CD19 gated naive B cells, IgM memory B cells, and