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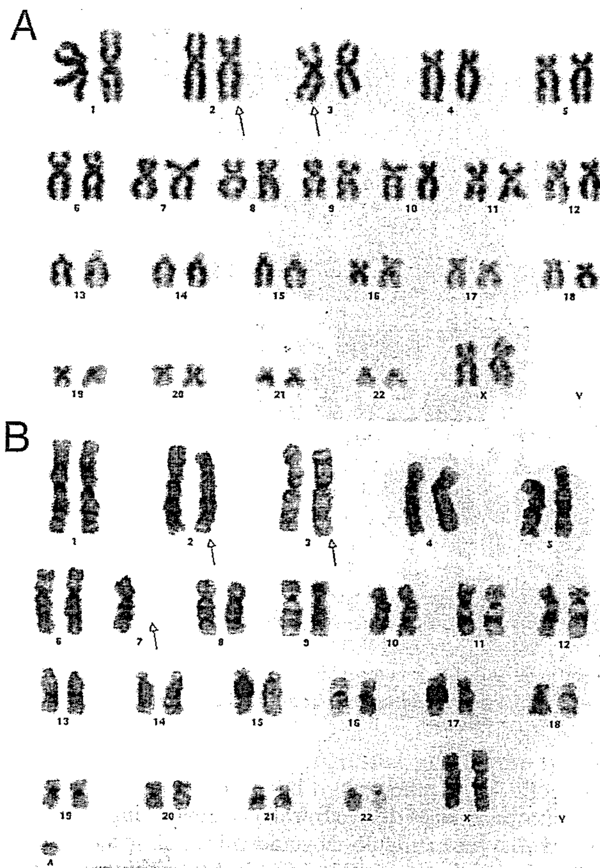

Figure 2. Results of G-band karyotyping of bone marrow cells. A, 46, XX, t(2;3) (p23;q27) at the onset of secondary myelodysplastic syndrome. B, 46, XX, t(2;3) (p23;q27), −7, +mar at progression. Arrows indicate abnormal chromosomes.

position. In the present case, many potential tumorigenic factors, such as immunosuppression and cytokines, were present, suggesting the involvement of multiple pathways in leukemogenesis.

Another possible mechanism for MDS in this patient is hepatitis-associated aplastic anemia followed by secondary MDS. Development of MDS and acute myelogenous leukemia in patients with hepatitis-associated aplastic anemia has been reported to be more common than previously suggested [17]. Most patients with hepatitis-associated aplastic anemia who do not have an HLA-matched donor available for SCT respond well to immunosuppressive therapy [18]. Therefore, the lack of immunosuppressant treatment after LT in this patient might have accelerated the clonal evolution of hematopoietic stem cells into MDS.

In conclusion, periodic cytogenetic monitoring and analysis of hematopoietic chimerism helped to reveal a rare case of early MDS with t(2;3) and monosomy 7 that occurred in donor cells 2 years after allogeneic PBSCT and 1 year after LT. This study suggests that hepatitis-associated aplastic anemia, profound immunosuppression followed by repeated infections, and dysregulated microenvironment of the bone marrow after administrations of G-CSF and EPO may have played critical roles in the development of monosomy 7 in donor cells.

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Phagocytosis of codeveloping megakaryocytic progenitors by dendritic cells in culture with thrombopoietin and tumor necrosis factor-α and its possible role in hemophagocytic syndrome

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Tumor necrosis factor-α (TNF-α) and thrombopoietin (TPO) have been shown to induce the differentiation and proliferation of CD34+ cells toward dendritic cells (DCs) in the presence of multiacting cytokines. We hypothesized that the costimulation of TPO and TNF-α generates megakaryocytic progenitors and DCs together from human CD34+ cells and that the interaction of these cells may indicate a physiologic and/or a pathologic role of DCs in megakaryopoiesis. When highly purified human CD34+ cells were cultured for 7 days with TPO alone, the generated cells expressed megakaryocytic markers, such as CD41, CD42b, and CD61. The addition of TNF-α with TPO remarkably decreased the number of megakaryocytic progenitor cells without affecting the cell yield. Almost half of the cells thus generated expressed CD11c, and most of them were positive for CD4 and CD123. Furthermore, CD11c+ cells were found to capture damaged CD61+ cells and to induce autologous T-cell proliferation, although the cytokine productions were low. We also confirmed an engulfment of CD61+ cells and their fragment by CD11c+ cells in bone marrow cells from patients with hemophagocytic syndrome. These findings suggest that DCs generated under megakaryocytic and inflammatory stimuli are involved in megakaryopoiesis and the subsequent immune responses to self-antigens. (Blood. 2006;107:1366-1374)

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Introduction

One of the earliest mediators of the acute phase response of infection, inflammation, and/or tissue damage is tumor necrosis factor-α (TNF-α).[1] TNF-α, a proinflammatory cytokine, is mainly produced by activated macrophages and lymphocytes,[2,3] and it has a multifunctional effect on various cell types.[4-6] For example, TNF-α acts as a positive and negative regulator on myeloid-cell proliferation and differentiation during hematopoiesis.[7-10] It is also well documented that TNF-α enhances the proliferation of human CD34+ cells while also promoting the development of dendritic cells (DCs) in the presence of stem-cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and/or interleukin-3 (IL-3).[11,12]

Thrombopoietin (TPO) is a primary and sole factor for megakaryopoiesis,[13] because the genetic elimination of *c-mpl* or *tpo* leads to profound thrombocytopenia in mice due to a greatly reduced number of megakaryocytic progenitors and mature megakaryocytes.[14] TPO also acts in synergy with IL-3 and SCF on hematopoietic stem cells to induce cell-cycle progression and to increase the both primitive and committed hematopoietic progenitor cells of all lineages.[15-17] Furthermore, TPO cooperates with FLT3 ligand (FLT3-L) and SCF in the generation of DC precursors from human CD34+ cells,[18-20] and human DCs generated from CD34+ cells following incubation with SCF, GM-CSF, and TNF-α either with or without TPO express the TPO receptor c-mpl.[21] As a result, both TPO and TNF-α enhance the proliferation of CD34+ cells and differentiation toward DCs in the presence of multipotent cytokines such as SCF, FLT3-L, and IL-3.

We recently showed that nonerythroid cells were cogenerated from human CD34+ cells during erythroid differentiation in the presence of IL-3/SCF/erythropoietin with TNF-α and expressed DC phenotypes. The CD11c+ DCs physically and selectively associate with developing damaged and immature self-erythroid cells and then phagocytose them.[22] This phenomenon may not be limited to the erythroid lineage. We hypothesized that TNF-α in the course of the inflammatory response by viral and microbial infection facilitates DC development during hematopoiesis, thus leading to phagocytosis of damaged self-progenitor cells by DCs. To confirm this hypothesis, we examined the effect of TNF-α and TPO on CD34+ cells.

We herein provide evidence that TNF-α inhibits the generation of megakaryocytic progenitors from CD34+ cells in the presence of TPO while inversely increasing the number of CD4+ CD11c+ CD123+ nonmegakaryocytic cells that show a DC phenotype. We also found that DCs physically associate with immature

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megakaryocytic cells during differentiation and then phagocytose damaged cells. Next, DCs acquire an ability to induce autologous T-cell proliferation in vitro However, they produce cytokines, which are more tolerogenic than immunogenic, and the T cells activated by them also do not produce a significant amount of immune cytokines. Interestingly, DCs with an immature phenotype in bone marrow from hemophagocytic syndromes are also found to capture $CD61^+$ cells. These findings may suggest the possibility that the phagocytosis of damaged cells by DCs under daily life conditions with either a weak or strong inflammatory response could thus play a pivotal role in regulating the immune responses against hematopoietic progenitor cells.

Materials and methods

Reagents

Bovine serum albumin (BSA), Iscove modified Dulbecco medium (IMDM), and propidium iodide (PI) were purchased from Sigma (St Louis, MO). Fetal calf serum (FCS), penicillin, and streptomycin were obtained from Flow Laboratories (McLean, VA). Insulin (porcine sodium, activity 26.3 U/mg; United States Pharmacopoeia) was obtained from Calbiochem of Behring Diagnostics (La Jolla, CA). Fluorescein isothiocyanate (FITC)–labeled monoclonal antibodies (mAbs) specific for CD4 (SK7), CD8 (SK1), CD19 (4G7), CD20 (L27), and CD34 (8G12) and phycoerythrin (PE)–labeled antibody for CD34 (8G12) were purchased from Becton Dickinson (Mountain View, CA). The PE-CD120a (TNFR-I: 16803.1) and PE-CD120b (TNFR-II: 22235) were obtained from R&D Systems (Minneapolis, MN), and PE-CD11c (B-ly6) and PE-CD83 (HB15a) were from Immunotech (Marseilles, France). PE-CD14 (TUK4), FITC-CD14 (TUK4), PE-CD41 (5B12), PE-CD42b (AN51), mouse anti–human glycophorin A (GPA: JC159), normal mouse serum, goat anti–rabbit IgG conjugated to peroxidase-labeled dextran polymer, diaminobenzidine (DAB)–substrate chromogen system, and fuchsin-substrate chromogen system were from DAKO Japan (Kyoto, Japan). Cyanin-labeled CD4, FITC-CD86 (FUN-1), PE-conjugated CD61 (VI-PL2), CD80 (L307.4), CDw123 (IL-3Rα; 9F5), c-mpl (BAH-1), major histocompatibility complex (MHC) class I (-A, -B, -C) (B9.12.1) and MHC class II (-DR) (B8.12.2), and unconjugated antimannose receptor were purchased from PharMingen (San Diego, CA). Anti–single-stranded DNA (anti-ssDNA) antibody was donated by Dr T. Sugiyama (Akita University, Japan).[23] Anti-CD3 (OKT3) was purified from ascites.

TNF-α was purchased from R&D Systems (Minneapolis, MN). TPO, IL-3, and SCF were kind gifts from the Kirin Brewery (Tokyo, Japan) and granulocyte colony-stimulating factor (G-CSF) from Chugai Pharmaceutical (Tokyo, Japan). Vitamin B_{12} and folic acid were purchased from Sankyo Pharmaceutical (Tokyo, Japan). ^{3}H-thymidine was purchased from Perkin Elmer (Yokohama, Japan).

Cell preparations

Human $CD34^+$ cells and $CD34^-$ cells were purified from healthy volunteers, who had signed informed consent forms approved by the Akita University School of Medicine Committee for the Protection of Human Subjects, and stored in liquid nitrogen until use as previously described.[24] The thawed $CD34^+$ cells were suspended in IMDM containing 30% FCS and 100 U/mL DNAse and then were washed twice with IMDM containing 20% FCS. Next, the cells were seeded in 50 mL polystyrene flasks (Corning Costar, Cambridge, MA) at 2×10^5 to 5×10^5 cells per milliliter in IMDM containing 5% heat-inactivated pooled human AB plasma, 1% BSA, 10 μg/mL insulin, 10 μg/mL vitamin B_{12}, 15 μg/mL folic acid, 50 nM β-mercaptoethanol, 50 U/mL penicillin, and 50 U/mL streptomycin in the presence or absence of 100 ng/mL TPO and/or at the indicated doses of TNF-α for the various periods at 37°C in a 5% CO_2/5% O_2 incubator.

Bone marrow aspirate was obtained from 3 patients (patients 1 to 3; Table 1) with hemophagocytic syndrome who had signed informed consent forms approved by the Akita University School of Medicine Committee for the Protection of Human Subjects. The diagnosis was made according to the diagnostic criteria for hemophagocytic lymphohistiocytosis.[25] Smear preparations of bone marrow were air dried and stored at −80°C.

Table 1. Characteristics of each of 3 patients with hemophagocytic syndrome*

	Patient no.		
	1	2	3
Sex/age, y	M/70	M/58	F/67
Underlying diseases	DIC‡	HIV/AIDS§	DLBCL‖
Clinical findings			
Nonremitting high fever	+	+	+
Hepatosplenomegaly	+	+	+
Peripheral blood			
White cells (neutrophils), × 10^9/L	2.7 (1.6)	0.8 (0.5)	1.0 (0.6)
Hemoglobin, g/L	85	65	61
Platelet, × 10^9/L	96	31	6
Biochemistry¶			
Triglycerides, mg/dL	ND	120	106
Fibrinogen, mg/dL	97	78	481
Ferritin, μg/L	1746	ND	2861
sIL-2R, U/mL	3870	ND	6280
FDP, μg/mL	53.2	38.6	8
Hemophagocytosis in bone marrow†	+	+	+
Outcome	Remission	Rapidly fatal	Remission

sIL-2R indicates soluble interleukin-2 receptor; FDP, fibrin degeneration products; DIC, disseminated intravascular coagulation syndrome; HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; DLBCL, diffuse large B-cell lymphoma; ND, not determined.

*According to the diagnostic guidelines developed by the Histiocyte Society, the diagnosis of hemophagocytic lymphohistiocytosis (HLH) requires the presence of all 5 major criteria: *(1)* fever, *(2)* splenomegaly, *(3)* cytopenia involving 2 or more cell lines, *(4)* hypertriglyceridemia or hypofibrinogenemia, *(5)* hemophagocytosis. Either criterion *(a)* low or absent natural killer cell activity or a combination of criteria, *(b)* ferritin above 500 mg/L, and *(c)* sIL-2R above 2400 U/mL may substitute for 1 of the major criteria.[25]

†Details are shown in Table 3.

‡Chronic DIC after surgery for aortic abdominal aneurysm.

§Diagnosed as AIDS at acute onset of *Pneumocystis carinii* pneumonia. Cytomegalovirus antigenemia assay was also positive.

‖DLBCL with bone marrow invasion. Some cases of DLBCL have a prominent background of reactive T cells and histiocytes.

¶Reference ranges for normal values are 30 to 150 mg/dL for triglycerides, 160 to 360 mg/dL for fibrinogen, below 234 μg/L for ferritin, 135 to 483 U/mL for sIL-2R, and less than 10 μg/mL for FDP.

Flow cytometry

The cells collected from the cultures were washed twice with phosphate-buffered saline (PBS) containing 3% FCS, 2 mM EDTA, and 0.05% NaN_3 (staining medium) and stained with FITC- and PE-labeled mAbs, and they then were analyzed using a FACS Calibur (Becton Dickinson). To determine the TNF receptor (TNFR) expression, the cells were incubated at 37°C for 2 hours without TNF-α before staining with specific mAbs, because TNFRs were down-regulated by TNF-α in the culture medium (data not shown).

Enzymatic immunohistochemistry

The cells were spun onto slides using a Cytospin 3 (Shandon Lipshaw, Pittsburgh, PA), and smear specimens of bone marrow were fixed in 100% methanol, dried, and examined by enzymatic immunohistochemistry as described.[26] After blocking endogenous peroxidase activity with 3% hydrogen peroxide, the preparations were incubated with mouse mAbs, followed by goat anti–mouse IgG conjugated to peroxidase-labeled dextran polymer, and then were visualized with a DAB-substrate chromogen system. For double staining, the preparations were further incubated with rabbit antihuman antibody, followed by goat anti–rabbit IgG conjugated to alkaline phosphatase-labeled dextran polymer using a fuchsin-substrate

chromogen system. ssDNA-specific rabbit polyclonal IgG antibodies were used to recognize hexadeoxynucleotides with various base sequences in apoptotic cells,[23] and the stained specimens were incubated with goat anti–rabbit IgG conjugated to peroxidase-labeled dextran polymer. Specimens that were incubated with normal serum with secondary antibody served as negative controls.

Confocal microscopy

For confocal microscopy, cells generated after 5 days of culture with TPO and TNF-α were fixed using PermaFluor Aqueous Mounting Medium (Thermo Shandon, Pittsburgh, PA), permeabilized with BD FACS Permeabilizing Solution (Becton Dickinson), and then stained with PE-CD11c and FITC-CD61 as described previously.[22] Cells engulfing $CD61^+$ cells were observed using a CLSM 510 confocal laser scanning microscope (Carl Zeiss Microscope Systems, Oberkochen, Germany) equipped with a 40 ×/65 μm (FITC) or 40 ×/72 μm (PE) oil-immersion objective lens at zoom 5. Fluorochromes were excited using an argon laser at 488 nm for FITC and helium neon at 543 nm for PE. Detector slits were configured to minimize cross talk between channels. Z-sliced optical sections were collected with an optimal interval of 0.45 mm and processed using Carl Zeiss software and Adobe Photoshop 6.0 software (Adobe Systems, San Jose, CA).

T-cell proliferation assays

Autologous T lymphocytes were prepared as a negative fraction from a nylon-fiber syringe[27] of thawed $CD34^-$ cells. Cells generated in the culture with TPO plus TNF-α or TNF-α alone for 7 days were used as a stimulator after γ-irradiation at 30 Gy (3000 rad) and then were cocultured with autologous responder T cells (1×10^3 cells per well) in RPMI 1640 medium containing 10% heat-inactivated human AB serum in round-bottom 96-well microculture plates. Anti-CD3 mAb was added into some cultures as positive controls. ^{3}H-thymidine (1 μCi per well [37 κBq/well]) incorporations for the last 18 hours of 5 days of culture were measured by a liquid scintillation counter (TopCount NXT; PerkinElmer LAS, Boston, MA), and the results were expressed as the mean ± SD of triplicate cultures.

Cytokine measurement

Culture supernatants were obtained from autologous mixed lymphocyte reactions (MLRs) and culture of $CD34^+$ cells at 4×10^5 in 2 mL complete medium with TPO (100 ng/mL) and TNF-α (100 ng/mL) for 7 days in 24-well flat-bottomed Falcon tissue culture plates (Becton Dickinson, Franklin Lakes, NJ). Cytokine activities were assessed with cytometric bead array system (Pharmingen) according to the manufacturer's protocol.

Results

TNF-α inhibits the generation of megakaryocytic progenitors while increasing the nonmegakaryocytic cells from $CD34^+$ cells supported by TPO

Human $CD34^+$ cells (98.5% ± 1.0% purity; mean ± SD) were cultured with TPO in the presence of various concentrations of TNF-α ranging from 0 to 250 ng/mL. Seven days later, the total number of recovered cells was counted, and CD41, CD42b, and CD61 expressions, specific markers for the megakaryocytic lineage, were examined using a cytofluorometer. The total number of cells increased 1.4-fold during the 7 days of culture with TPO alone, and 89%, 43%, and 87% of the generated cells expressed CD41, CD42b, and CD61, respectively, thus indicating that most of them consisted of megakaryocytic progenitors. The addition of TNF-α significantly decreased the number of $CD41^+$, $CD42b^+$, and $CD61^+$ cells in a dose-dependent manner while conversely increasing $CD41^-$, $CD42b^-$, and $CD61^-$ cells (Figure 1A). A low dose of TNF-α (0.25 ng/mL) tended to increase the cell yield but reduced the proportion of $CD41^+$, $CD42b^+$, and $CD61^+$ cells. Then

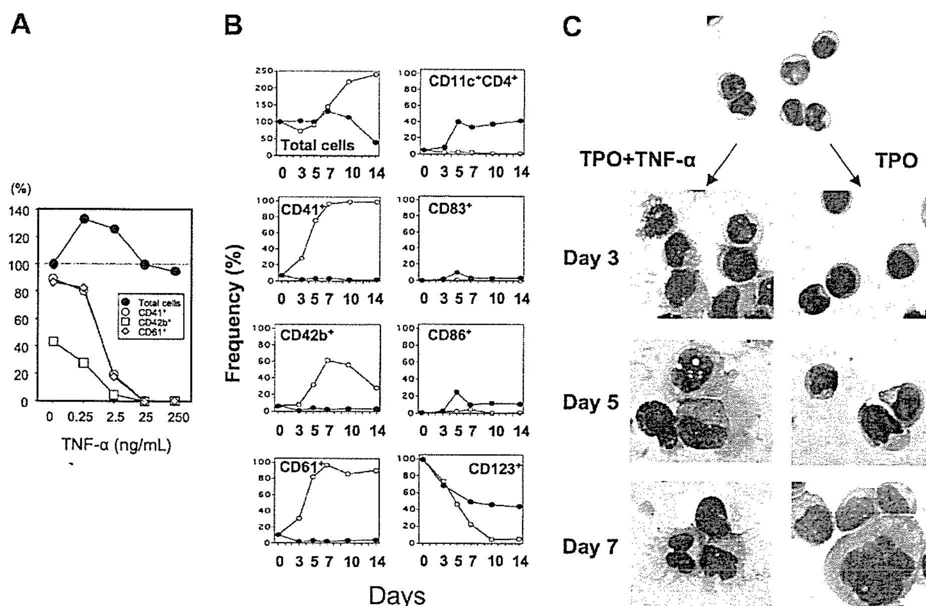

Figure 1. TNF-α inhibits the generation of megakaryocytic progenitors but increases the number of nonmegakaryocytic cells from $CD34^+$ cells supported by TPO. (A) Human $CD34^+$ cells at 7.5×10^5/mL were cultured with 100 ng/mL TPO in the presence of various concentrations of TNF-α ranging from 0 to 250 ng/mL. Seven days later, the total numbers of recovered cells (●) were counted, and CD41 (○), CD42b (□), and CD61 (◇) expressions were examined by a cytofluorometer. The cell yields were represented as the percent relative to the total number of cells without TNF-α. Marker-positive cells were also shown as the percent of the total number of cells in an individual group. The result shown is representative of 3 independent experiments. (B) $CD34^+$ cells were cultured as in panel A in the presence (●) or absence (○) of 100 ng/mL TNF-α. Cell counts and phenotype analyses were performed at various time points. The results shown are representative of 3 independent experiments. (C) Morphologic differences in the cells differentiated by TPO with and without TNF-α as in panel B. Cytospin specimens were stained with May-Grünwald-Giemsa solution at the indicated time points (× 1000).

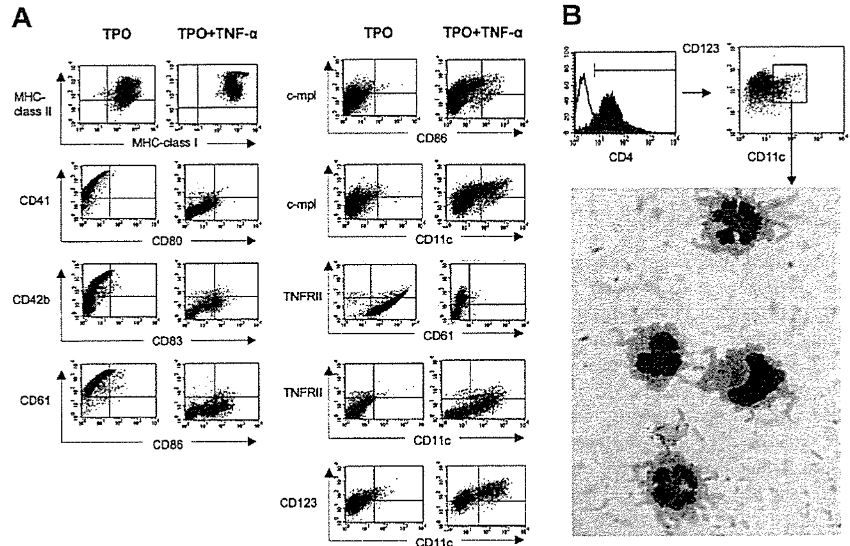

Figure 2. Phenotypic analyses of nonmegakaryocytic cells. (A) Cells generated after 7 days cultured as described for Figure 1 were assessed for their phenotype with cytofluorometer using various combinations of mAbs. (B) The purification of CD11c$^+$ CD123$^+$ cells and their morphology. Cytospin specimens of sorted CD11c$^+$ cells were stained with May-Grünwald-Giemsa solution (arrow). Original magnification, × 1000.

half-maximal dose of TNF-α to inhibit the generation of megakaryocytic cells ranged from 0.25 to 2.5 ng/mL.

Characterization of nonmegakaryocytic cells

To understand the kinetics of nonmegakaryocytic-cell development in our system, purified CD34$^+$ cells were cultured for 14 days with 100 ng/mL TPO in the presence or absence of 100 ng/mL TNF-α, and the surface phenotypes were monitored at the indicated time points (Figure 1B). The number of total cells recovered from the cultures with and without TNF-α were comparable to each other for 7 days. During this period, the proportions of CD41$^+$, CD42b$^+$, and CD61$^+$ cells dramatically increased only when TNF-α was not added. The cell yield substantially increased in the culture with TPO alone but decreased with TPO plus TNF-α thereafter. Very few cells expressed CD41, CD42b, and CD61 in the presence of TNF-α throughout the culture period, thus suggesting that TNF-α affects CD34$^+$ cell development by inhibiting the generation of megakaryocytic progenitors. Interestingly, CD11c$^+$ cells were inversely generated as early as at day 5, and some of them expressed CD86 and CD83. Then morphologic changes of cells also showed the development of cells with DC features, such as dendrites and eccentric nuclei (Figure 1C).

To further characterize the DC-like cells in detail, the surface phenotypes were monitored on day 7 (Figure 2A). All cells became positive for MHC class II in the presence of TNF-α. A significant number of CD11c$^+$ cells and CD86$^+$ cells coexpressed c-mpl and TNFRII. In addition, CD61$^+$ cells expressed not only c-mpl but also TNFRII. None of the TNFRI$^+$, CD3$^+$, CD8$^+$, CD19$^+$, CD20$^+$, and CD56$^+$ cells was present in either of the culture conditions (data not shown).

CD123 (IL-3 receptor-α), a marker of plasmacytoid DCs or interferon-producing cells (IPCs),[28] was detected in 70% ± 19% of the total cells and 97% of CD11c$^+$ cells (Figure 2A). Chen et al[20] demonstrated that TPO and FLT3-L allow human CD34$^+$ cells to differentiate into CD11c$^-$ CD123^{high} IPCs, CD11c$^+$ immature DCs, and CD14$^+$ monocytes. Therefore, we examined the CD123 expression on CD11c$^+$ DCs using purified cells gated on CD4, which is expressed on DCs.[28] CD4$^+$ cells consisted of 92% of bulk cells at day 7, and all of them were positive for CD123 (Figure 2B). The sorted CD11c$^+$ CD123$^+$ cells showed a uniform picture characterized by abundant dendrites and eccentric nuclei.

Megakaryocytic cells and DCs are closely associated together during their development in the presence of TNF-α

DCs are known to develop from proliferating precursor cells in aggregates. We stained cells with megakaryocyte-specific and DC-associated markers to visualize the localization of megakaryocytic progenitors and DCs (Figure 3). The CD34$^+$ cells proliferated

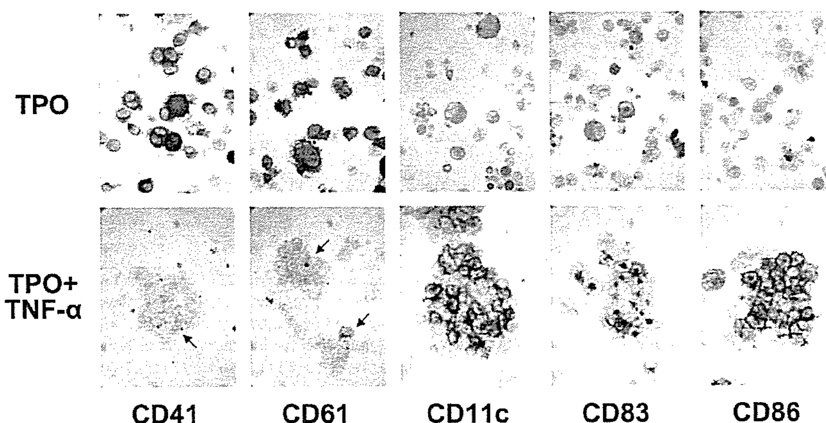

Figure 3. DCs develop in aggregates along with megakaryocytic cells in the presence of TPO and TNF-α. The aggregates formed were collected by gentle pipetting after 5 days of culture with TPO in the presence or absence of TNF-α as in Figure 2. The cytospin specimens were stained with megakaryocyte-specific and DC-associated markers. Original magnification, × 40.

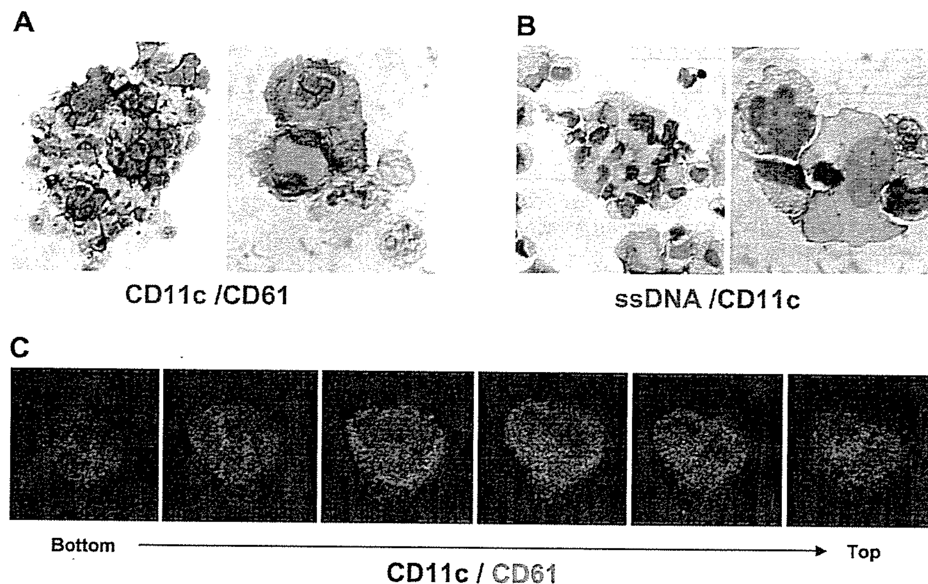

Figure 4. Physical association and phagocytosis of CD61$^+$ cells by CD11c$^+$ cells. Five days after culture with 100 ng/mL TPO and 2.5 ng/mL TNF-α, the cells were cytospun and stained with anti-CD11c (brown) and anti-CD61 (red) (A) or with anti-CD11c (red) and anti-ssDNA (brown) (B); original magnification, × 1000. (C) Cells placed on coverslips were fix-permeabilized, stained with FITC-CD61 (green) and PE-CD11c (red), and then were observed by laser scanning confocal microscopy. A serial 3-dimensional reconstruction of the cells demonstrates the phagocytosis of CD61$^+$ cells (green) by CD11c$^+$ cells (red).

as single cells without forming aggregates in the absence of TNF-α, and they expressed CD41 and CD61. In the presence of TNF-α, the aggregates were found to consist of CD11c$^+$, CD83$^+$, and CD86$^+$ cells, and they contained fragments that were positive for CD41 or CD61 (Figure 3, arrows).

Immature DCs are known to be capable of capturing apoptotic and necrotic cells,[29,30] thus leading to antigen presentation, which thus induces immunity and tolerance.[31,32] Therefore, it is possible that developing immature DCs in aggregates engulf damaged megakaryocytic progenitor cells. To address this question, CD34$^+$ cells were cultured for 5 days with 100 ng/mL TPO and 2.5 ng/mL TNF-α, half-maximal dose of inhibition, to maintain the shape of megakaryocytic progenitor cells. Cytospin specimens of the aggregated cells were stained with CD11c in combination with anti-CD61 (Figure 4A) or anti-ssDNA (Figure 4B). CD11c$^+$ cells were often shown to be associated with CD61$^+$ megakaryocytic progenitor cells in aggregates, and many ssDNA$^+$ cells were present in the aggregates. When the aggregates were dispersed with gentle pipetting, large parts of CD11c$^+$ cells associate with (63% ± 5%) or engulfed (15% ± 4%) CD61$^+$ cells. In addition, ssDNA was also found in the cytoplasm of CD11c$^+$ cells, which associated with ssDNA$^+$ cells. These observations indicate that DCs capture dying megakaryocytic progenitors. This was also confirmed with confocal microscopy, which showed that the CD11c$^+$ cells phagocytosed CD61$^+$ cells (Figure 4C).

Autologous T-cell activation by DCs generated in the presence of TPO and TNF-α

We hypothesized that DCs phagocytosing self-megakaryocytic cells might thus modify self-proteins and, as a result, they can present antigen to autologous T cells. We therefore assessed autologous T-cell proliferation using cells generated during a 7-day culture with TPO plus TNF-α or TNF-α alone (Figure 5). CD3-dependent T-cell proliferation induced by these DCs served as positive controls. TNF-α alone was able to induce DC development from CD34$^+$ cells, although the cell yield was much lower than the culture with TPO and TNF-α (data not shown). DCs generated in the culture with TPO plus TNF-α, but not TNF-α alone, were found to potently activate autologous T cells, although T-cell proliferation was much lower than that induced by anti-CD3 mAb.

When T cells were cocultured with DCs at a 2:1 ratio, low but significant amounts of IL-2 (6.5 ± 1.7 pg/mL) and IL-6 (12.5 ± 2.4 pg/mL) were detected. However, no IFN-γ, IL-4, and IL-10 were detected. In contrast, large amounts of IFN-γ (825 ± 70 pg/mL), TNF-α (233 ± 21 pg/mL), and IL-10 (416 ± 68 pg/mL) as well as IL-6 (193 ± 35 pg/mL) were produced when T cells were stimulated via CD3 with DCs, although no IL-2 activity was detected at this time point.

Cytokine secretion by developing cells during the culture

Cytokines secreted during the culture are believed to have an important autocrine and paracrine effect on developing cells both in

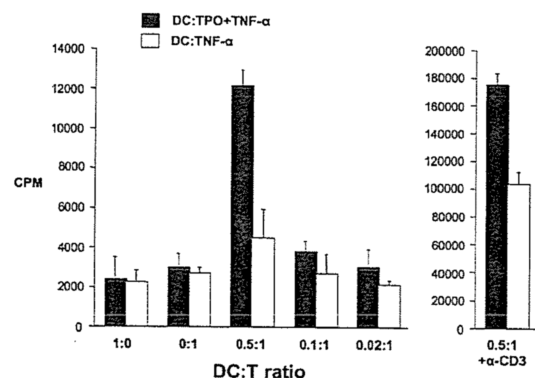

Figure 5. Autologous T-cell proliferation by DCs generated in the presence of TPO and TNF-α. The cells prepared by culture for 7 days with 100 ng/mL TNF-α in the presence (■) or absence (□) of 100 ng/mL TPO were used at graded doses as stimulator cells after γ-irradiation. In some MLRs, anti-CD3 mAb was added. Proliferation was assessed by adding ^{3}H-thymidine (1 μCi per well [37 κBq/well]) during 72 to 90 hours of culture. Results represent the mean ± SD of triplicate cultures.

Table 2. Cytokine secretion profile of the generated cells

Cytokine combination (s)	Concentration, pg/mL				
	INF-γ	IL-6	IL-8	IL-10	IL-12p70
TPO	< 5	< 5	302 ± 18	< 5	< 5
TPO + TNF-α	< 5	5.5 ± 0.8	> 5000	< 5	< 5
TNF-α	< 5	7.0 ± 0.8	3152 ± 74	< 5	< 5

Purified human CD34+ cells were cultured for 7 days with or without TPO and TNF-α, and the supernatants were collected. The cytokines secreted during culture were measured using a cytometric beads array system.

vivo and in vitro. As a result, we assessed the cytokine activities in the media of $CD34^+$ cell culture in the presence of TPO and/or TNF-α (Table 2).

TPO alone induced a small amount of IL-8, whereas TNF-α induced the secretion of IL-6 and IL-8 but not that of IL-1β, IL-10, or IL-12p70. The combination of TPO and TNF-α further enhanced IL-8, but not IL-6, secretion. These results suggest that TNF-α is a primary factor to induce the other proinflammatory cytokines and that TPO synergizes with TNF-α for only IL-8 secretion.

Hemophagocytosis by $CD11c^+$ cells in vivo is confirmed

Our observation clearly shows that developing DCs under hematopoietic and inflammatory conditions phagocytose codifferentiated progenitor cells with damage by inflammatory cytokines. We therefore attempted to corroborate that our observation in vitro also takes place in situ. To do so, we stained specimens of bone marrow smears from patients with hemophagocytic syndrome with DC markers, such as CD11c, CD83, CD86, and mannose receptor, and the megakaryocytic marker CD61 or the erythroid marker GPA. As shown in Figure 6, hemophagocytic cells (Figure 6A) were weakly positive for CD83 (Figure 6B), while they were strongly positive for CD86 (Figure 6C), mannose receptor (Figure 6D), and CD11c (Figure 6E-F). Double staining with anti-CD11c and anti-CD61 showed that $CD11c^+$ cells phagocytose platelets (Figure 6E-F,H) and $CD61^+$ substrates (Figure 6G) in vivo. Our previous report demonstrated that nonerythroid cells generated from $CD34^+$ cells treated with erythropoietin, SCF, IL-3, and TNF-α expressed DC phenotype and that $CD11c^+$ DCs phagocytosed immature erythroid cells.[22] We therefore stained bone marrow cells from the patients with $CD11c^+$ and erythroid marker, GPA (Figure 7A-C). As a result, $CD11c^+$ cells in the patients' bone marrow were consistently shown to capture erythroid as well as megakaryocytic cells. In addition, staining with α-naphthyl butyrate (α-NB) of hemophagocytic cells showed a similar perinuclear distribution of granules in immature DCs generated from purified normal human $CD34^+$ cells (Figure 7D-E). Such a distribution pattern of α-NB–positive granules was different from pancellular staining in macrophages.

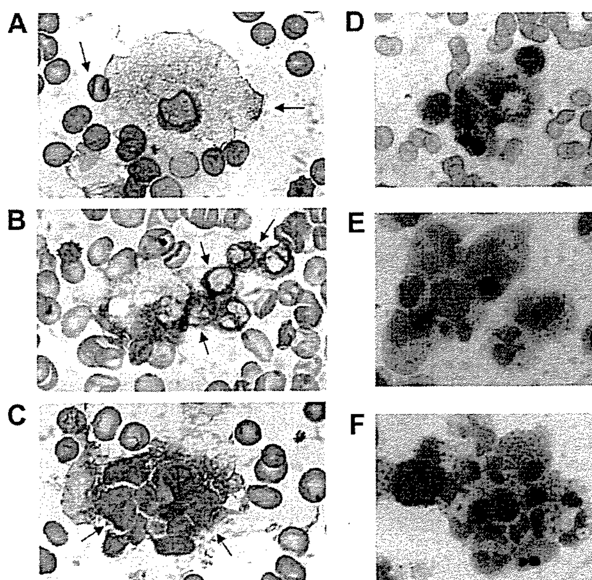

Figure 7. Capture of erythroid progenitor cells expressing GPA by $CD11c^+$ cells from bone marrow of hemophagocytic syndrome. Bone marrow smear preparations from patients with hemophagocytic syndromes were stained with CD11c (red)/GPA (brown) for patient 1 (A) or CD11c (brown)/GPA (red) for patient 2 (B) and patient 3 (C). The arrows indicate GPA-positive erythroid cells. Staining with α-NB of hemophagocytic cells is also shown in patient 3 (D) and DCs generated from purified normal human $CD34^+$ cells in the presence of TPO and TNF-α (day 5 [E]; day 7 [F]).

To obtain more detailed information about hemophagocytic cells in these patients, we clarified the frequency of monocytic cells macrophages by May-Grünwald-Giemsa staining and $CD11c^+$ cells associated with/capturing dying hematopoietic progenitor cells by immunohistochemical staining (Table 3). The frequencies of hemophagocytic cells

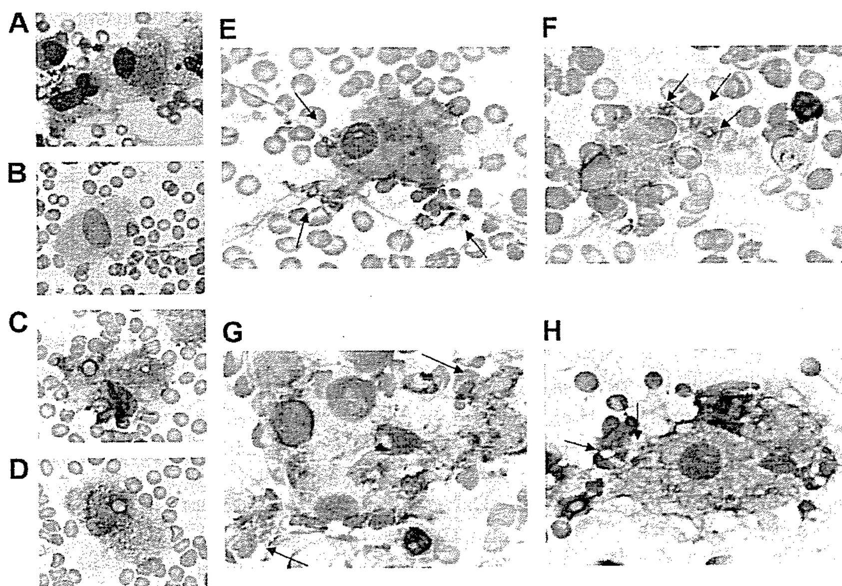

Figure 6. Hemophagocytosis by $CD11c^+$ cells in bone marrow cells from patients with hemophagocytic syndrome. Bone marrow smear preparations from patients with hemophagocytic syndrome were stained with May-Grünwald-Giemsa staining (patient 1) (A), CD83 staining (patient 1) (B), CD86 staining (patient 1) (C), mannose receptor staining (patient 1) (D), or CD11c (brown)/CD61 (red) double staining for patient 1 (E), patient 2 (F-G), and patient 3 (H). The arrows indicate $CD61^-$ platelets or substrates.

Table 3. Frequencies of CD11c+ hemophagocytic cells in the bone marrow of patients with hemophagocytic syndrome

	Patient no.		
	1	2	3
Cellularity	Hypo	Normo	Hypo
Frequencies, %			
By May-Grünwald-Giemsa staining			
Monocytic cells/macrophages in total bone marrow	35.8	15.0	26.0
Hemophagocytes in total bone marrow	13.6	3.8	5.2
CD11c+ cells by immunohistochemical staining			
In total bone marrow	31.0	11.6	27.8
In hemophagocytes	84.4	62.2	70.3
Associated with/capturing CD61+ cells/substrates	36.1	42.0	9.7
Associated with/capturing GPA+ cells/substrates	94.4	53.6	91.4

GPA indicates glycophorin A; hypo, hypocellular marrow; normo, normocellular marrow.

were in the range of 3.8% ± 13.6% of total bone marrow cells, which consisted of 35.8% ± 15.0% of monocytic cells/macrophages and 31.0% ± 11.6% of CD11c+ cells. In addition, CD11c+ cells were composed of 84.4% ± 62.2% of hemophagocytic cells, and they captured megakaryocytic as well as erythroid cells. These data strongly suggest the possibility that hemophagocytic cells in the bone marrow of patients, at least in part, are DCs.

Discussion

This study demonstrated that TNF-α inhibits the generation of megakaryocytic progenitor cells from human CD34+ cells in the presence of TPO and inversely increases nonmegakaryocytic cells with feature of DCs. These cells expressed the typical surface markers of DCs, such as CD11c, CD4, and CD86. They were closely associated with codeveloping immature megakaryocytic progenitor cells and then captured them. Interestingly, the cells thus generated were capable of inducing autologous MLRs. Like DCs generated by TPO and TNF-α, capture of CD61+ cells by CD11c+ cells in bone marrow was also observed in patients with hemophagocytic syndrome, thus indicating that similar phenomena can take place in vivo. These findings suggest that megakaryocytic and inflammatory costimuli on hematopoietic stem/progenitor cells may induce hemophagocytosis of megakaryocytic cells in a physiologic situation.

We documented that TNF-α inhibits the generation of megakaryocytic progenitor cells from human CD34+ cells in the presence of TPO in a dose-dependent fashion with a half-maximal dose ranging from 0.25 to 2.5 ng/mL. Although considerable advances have been made during the past 2 decades in our understanding of the biology and the clinical role of the TNF superfamily, the role of TNF-α in megakaryopoiesis remains controversial. TNF-α stimulates colony formation by a megakaryoblastic leukemia cell line (CMK) established from a patient with Down syndrome,[33] and it also induces megakaryocytic differentiation of the HIMeg-1 cells,[34] a cell line derived from a patient with chronic myeloid leukemia capable of monocytic and megakaryocytic differentiation. In contrast, TNF-α has been well documented to inhibit the megakaryopoiesis of normal progenitor cells. Lu et al[35] reported that TNF-α suppressed colony-forming-unit megakaryocytes derived from human CD34+ cells in the presence of IL-11, IL-3, SCF, and TPO. TNF-α almost completely abrogated the growth of human CD34+ CD38− progenitor cells in response to TPO alone as well as SCF/FLT3/TPO.[36] It is very likely that the differences between various cell lines and primary cells account for some of the discrepancy between observations made by different researchers. The mechanism by which TNF-α inhibits normal megakaryocytic differentiation remains unclear.

Although TPO has been shown to be the primary regulator of platelet production,[13] many studies have demonstrated that TPO supports the proliferation and long-term expansion of primitive CD34+ cells in synergy with FLT3-L, SCF, and/or IL-3.[15-17] We further demonstrated that TPO and TNF-α permit CD34+ cells to differentiate into CD4+ CD11c+ CD123+ DCs and CD4+ CD11c− CD123+ cells. The finding that TPO alone did not induce the generation of CD11c+ immature DCs suggests that TNF-α thus plays a critical role in synergy with TPO. The coexpression of TNFR-II and c-mpl on CD11c+ cells suggests that TPO and TNF-α act in synergy with the downstream signaling pathways of both receptors.

In suspension cultures, a rapid increase in the proportion of CD11c+, CD83+, or CD86+ cells was observed as early as day 5. Although CD34+ cells can also differentiate into monocytes/macrophages, it takes 10 to 14 days in vitro.[37] Day 5 CD11c+ DCs were capable of capturing codeveloping megakaryocytic progenitors. Most CD11c+ cells coexpressed CD4 and CD123. Therefore, the CD4+ CD11c+ CD123+ cells are most likely DCs in an immature stage. The remaining nonmegakaryocytic cells consisted of CD4+ CD11c− CD123+ cells. However, a dot plot analysis showed a continuous CD11c expression in CD123+ cells on day 5 (Figure 2). The proportion of CD11c+ CD4+ cells reached a plateau by day 5, while that of CD123+ cells gradually decreased by day 7 to the equivalent with CD11c+ CD4+ cells. Therefore, some CD11c− CD123+ cells may not be in a transitional stage to CD11c+ CD123+ cells. Blom et al[28] have shown that CD34+ CD45RA+ CD123+ cells develop into mature DCs in cultures with SCF or FLT3-L through the differentiation pathway of plasmacytoid DC (CD11c− CD123+) precursor cells. More recently, Chen et al[20] showed that TPO cooperates with FLT3-L in the generation of plasmacytoid DC precursors from human CD34+ cells. DCs derived from plasmacytoid precursor cells are known to lack CD11c and CD1a.[38] The possibility that CD11c− CD123+ cells are capable of differentiating plasmacytoid DCs upon transfer to the culture with FLT3-L and TPO remains to be elucidated. However, the CD4+ CD11c+ CD123+ DCs are possibly myeloid lineage DCs, because they do not produce a large amount of IFN-α in response to CpG-ODN (data not shown).

The potent effects of TNF-α on generation of DCs and, in turn, the inhibition of TPO-induced megakaryopoiesis are also of particular interest, because the CD11c+ DCs closely associated with codeveloping megakaryocytic progenitor cells and also phagocytosed them (Figure 4). Moreover, those DCs induced autologous MLRs, whereas CD11c+ cells generated by TNF-α alone did not (Figure 5). As previously reported,[39,40] phagocytosis of necrotic but not apoptotic cells induces DC maturation. Under in vitro culture conditions, developing DCs possibly captured both apoptotic and necrotic cells, thus leading to DC maturation. Indeed, the proportions of CD11c+ cells were comparable with each other, but a slight increase in the expression of costimulatory molecules was noted in DCs induced by TPO plus TNF-α. However, DCs generated with TNF-α alone and TNF-α and TPO showed comparable amounts of IL-6 and IL-8, although the latter produced more IL-8 than the former (Table 2).

An infection with a virus, such as Epstein-Barr virus,[41] mumps virus,[42] dengue virus,[43] and hepatitis A virus,[44] has been suggested to result in fatal hemophagocytic syndrome, which is associated

with an overproduction of IFN-γ, TNF-α, IL-1, and IL-6,[41,45] which are known to be potent activators of macrophages. Well-differentiated macrophages have been observed to phagocytose hematopoietic cells in the bone marrow.[46] We herein also show that CD11c$^+$ CD83$^+$ CD86$^+$ cells in patients with hemophagocytic syndrome contained CD61$^+$ cells and their fragments and GPA-positive cells intracellularly (Figures 6-7; Table 3), thus suggesting the involvement of DCs in hemophagocytosis.

Recent studies indicate that DCs are involved in the counterregulation of potential autoimmune T-cell responses[47] and that CD11c$^+$ DCs that have not been activated by pathogen-related or endogenous inflammatory stimuli can significantly contribute to peripheral tolerance by inducing the inactivation and/or deletion of specific T cells.[48] On the other hand, DCs expressing endogenous self-peptides or pulsed ex vivo with immunogenic self-peptides can induce severe autoimmune disease.[47,49] The data presented in this work demonstrate that TNF-α has strong effects on cytokine secretion by developing cells, most probably immature DCs, including the up-regulation of early proinflammatory cytokines (IL-6 and IL-8). On the other hand, TNF-α–treated DCs have been reported to be semimature and induced regulatory cells upon inoculation in vivo.[50,51] Therefore, limited immune response or induction of regulatory T cells may prevent T cell–mediated autoimmune diseases in a steady state as well as in pathologic situations.

In conclusion, this is the first report showing that in the presence of TNF-α the nonmegakaryocytic cells with typical feature of DCs are cogenerated from human CD34$^+$ cells during megakaryocytic differentiation by TPO. The CD4$^+$ CD11c$^+$ CD123$^+$ DCs are physically associated with and phagocytose either developing or dying immature megakaryocytic cells. A similar phenomenon showing engulfment of CD61$^+$ fragment by CD11c$^+$ cells was also observed in the bone marrow cells from patients with hemophagocytic syndrome. Therefore, it may be conceivable that DCs with phagocytic activity during the development in bone marrow may play a crucial role in the maintenance of tolerance for self-substances derived from hematopoietic progenitor cells.

Acknowledgments

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Case Report

Late-Onset Fatal Epstein-Barr Virus–Associated Hemophagocytic Syndrome following Cord Blood Cell Transplantation for Adult Acute Lymphoblastic Leukemia

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Abstract

A 43-year-old Japanese woman underwent unrelated cord blood transplantation (CBT) during remission for acute lymphoblastic leukemia with t(4;11)(q21;q23). Tacrolimus was given for prophylaxis of graft-versus-host disease. The posttransplantation clinical course was mostly uneventful, and the leukemia remained in remission. Fourteen months after CBT, the patient developed pancytopenia and hepatic dysfunction with persistent high-grade fever. The bone marrow was hypocellular with increased numbers of macrophages and hemophagocytes. The numbers of Epstein-Barr virus (EBV) copies in peripheral blood samples were remarkably high. Although the patient showed complete donor-type hematopoiesis, the titer of viral capsid antigen immunoglobulin G was low, and the results of a test for EBV nuclear antigen were negative. There was no clinical response to the reduction of immunosuppressive therapy or to the administration of high-dose methylprednisolone, human immunoglobulin, or acyclovir. The patient died 466 days after CBT of massive gastrointestinal hemorrhage due to bone marrow and hepatic failures. This case demonstrates that fatal EBV-associated hemophagocytic syndrome (HPS) can occur more than 1 year after CBT. This report is the first of a case of late-onset EBV-associated HPS following CBT.

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Key words: PTLD; EBV; HPS; CBT; Late onset

1. Introduction

Posttransplantation lymphoproliferative disease (PTLD) is one of the severe complications that may arise after allogeneic hematopoietic stem cell transplantation (HSCT) [1-4]. Previous reports have proposed that PTLD occurring within 1 year of transplantation be defined as early PTLD and that PTLD occurring after 1 year be defined as late PTLD [5-7]. The majority of cases of PTLD after allogeneic HSCT occur within 100 days after transplantation and result from the proliferation of B-lymphocytes of donor origin by Epstein-Barr virus (EBV) reactivation [4,8,9]. EBV can also be the major triggering factor producing hemophagocytic syndrome (HPS). In patients with EBV-associated HPS, the EBV-infected T-cells or natural killer (NK) cells are monoclonally or oligoclonally proliferating, and hypercytokinemia plays a major role, causing hemophagocytosis, cellular damage, and the dysfunction of various organs [10]. EBV-associated HPS is frequently fatal, particularly if not diagnosed at an early stage. However, early diagnosis of EBV-associated HPS is not easy because of extranodal prediction and the absence of specific symptoms. Although there are a few reports of EBV-associated PTLD following cord blood cell transplantation (CBT) [11-15], the actual frequency and outcome of EBV infection and reactivation following CBT have not been fully described. In this report, we describe a case of EBV-associated HPS occurring more than 1 year after allogeneic CBT. To our knowledge, this report is the first of a case of late-onset EBV-associated HPS following CBT.

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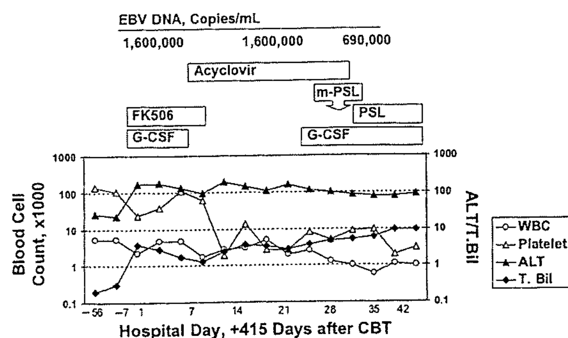

Figure 1. Clinical course of and therapy for the patient. EBV indicates Epstein-Barr virus; FK506, tacrolimus; m-PSL, methylprednisolone; PSL, prednisolone; G-CSF, granulocyte colony-stimulating factor; WBC, white blood cells; ALT, alanine aminotransferase; T. Bil, total bilirubin; CBT, cord blood transplantation.

2. Case Report

Acute lymphoblastic leukemia (ALL) with t(4;11)(q21;q23) involving MLL-AF4 rearrangement was diagnosed in a 43-year-old Japanese woman. At disease onset, the white blood cell count was 148,000/μL, 89% of which were lymphoblastic cells expressing CD19 and HLA-DR. EBV serologic tests revealed the following antibody titers: viral capsid antigen immunoglobulin G (VCA IgG), ×160; VCA IgM, <×10; early antigen for DR components (EADR) IgG, <×10; and EBV nuclear antigen (EBNA), ×20. The patient had no central nervous system involvement. Induction chemotherapy consisting of Adriamycin (doxorubicin), vincristine, prednisolone, and L-asparaginase achieved hematologic and cytogenetic remission, and consolidation and intrathecal chemotherapy followed. The patient did not have any suitable family or unrelated donor for HSCT. Four months after diagnosis, the patient received a conditioning regimen of total body irradiation, cyclophosphamide, and cytosine arabinoside, followed by an infusion of allogeneic cord blood stem cells containing 1.9×10^5 $CD34^+$ cells/kg from an unrelated male donor mismatched at 1 HLA-DR locus and 1 ABO minor locus. Information regarding the EBV serologic status of the graft was not available. Tacrolimus was given for prophylaxis of acute graft-versus-host disease (GVHD). The neutrophil count rose to 500/μL on day 24, and the platelet count increased to 2.0×10^4/μL on day 49. Acute GVHD was not observed, and tacrolimus therapy was gradually tapered. Examination of a bone marrow aspirate on day 106 showed hematologic and cytogenetic remission. Fluorescence in situ hybridization analysis of peripheral lymphocytes showed complete donor-type chimerism. The patient was discharged on day 113.

Eight months after CBT, the patient's condition was complicated by a herpes zoster infection, which was responsive to acyclovir treatment. Because of chronic GVHD with a skin rash, oral mucositis, and test results showing an abnormal liver function, treatment with low-dose tacrolimus was continued. Complete blood cell counts were almost within the normal range. On day 415 after CBT, the patient was admitted to our hospital with a 1-month history of persistent high-grade fever. A physical examination showed palpable hepatomegaly 5 cm below the costal margin, but the spleen was not palpable. There were no enlarged lymph nodes on palpation. A blood examination demonstrated a white blood cell count of 2700/μL (61% lymphocytes), a hemoglobin level of 9.5 g/dL, and a platelet count of 2.1×10^4/μL. Blood chemistry evaluations revealed the following: aspartate aminotransferase, 249 IU/L; alanine aminotransferase, 177 IU/L; alkaline phosphatase, 2608 IU/L; lactate dehydrogenase, 352 IU/L; total bilirubin, 3.9 mg/dL; direct bilirubin, 3.4 mg/dL; ferritin, 1400.2 ng/mL; total cholesterol, 98 mg/dL; triglycerides, 290 mg/dL; and C-reactive protein, 7.4 mg/dL. Levels of fibrin degradation products, D dimer, and fibrinogen were 5.94 μg/mL, 6.79 μg/mL, and 200.5 mg/dL, respectively. A bone marrow examination on day 416 after CBT showed hypocellular marrow with hemophagocytosis. There was no obvious proliferation of leukemic cells, but the numbers of $CD8^+$ lymphocytes were increased in both the peripheral blood and the bone marrow. The results of a flow cytometric analysis of lymphocyte subsets in the blood and bone marrow were as follows: 91.1% $CD3^+$, 13.1% $CD4^+$, 84.7% $CD8^+$, 8.8% $CD56^+$, and 7.1% $CD20^+$ cells in the blood, and 88.3% $CD3^+$, 9.0% $CD4^+$, 80.5% $CD8^+$, 10.5% $CD56^+$, and 1.7% $CD20^+$ cells in the bone marrow. A cytogenetic study of bone marrow cells showed a normal karyotype with a Y chromosome. The serologic status of EBV was as follows: VCA IgG, ×10; VCA IgM, <×10; EADR IgG, <×10; and EBNA, <×10. EBV genomes were detectable in peripheral blood plasma by the polymerase chain reaction (PCR). A computed tomography scan of the abdomen demonstrated hepatosplenomegaly without apparent lymph node swelling. The ferritin level increased to 18,268.8 ng/mL. From these clinical findings, a diagnosis of posttransplantation EBV-associated HPS was established. Despite the reduction and interruption of tacrolimus therapy, the pancytopenia and hepatic failure rapidly progressed (Figure 1). A repeat bone marrow examination on day 445 after CBT demonstrated severe hypocellular marrow with increased numbers of macrophages, some of which were actively phagocytosing red cells and neutrophils (Figure 2). A real-time quantitative PCR assay for the EBV genome in the peripheral blood on day 452 showed marked elevation to 160×10^4 copies/mL (reference range, <150 copies/mL). The administration of high-dose methylprednisolone and cyclosporin A in addition to blood transfusion, antibiotics, human immunoglobulin, acyclovir, and granulocyte colony-stimulating factor were not effective, and the patient died 466 days after CBT of massive gastrointestinal hemorrhage due to bone marrow and hepatic failures.

3. Discussion

PTLD following HSCT represents a heterogeneous group of disorders that range from benign polyclonal proliferation to malignant monoclonal lymphoma [16,17]. In recipients following HSCT, EBV infection can be acquired from donor B-cells in the graft or be caused by the reactivation of EBV already present in the host [8,18]. It is

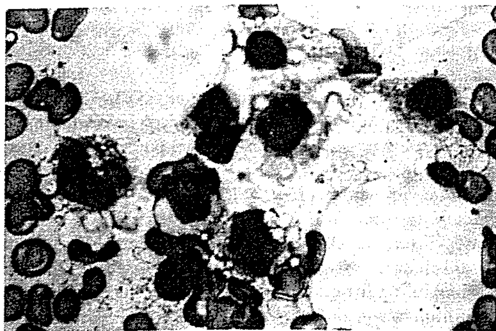

Figure 2. A bone marrow smear demonstrated severe hypoplasia (nucleated cell count, 24,500/μL) with highly elevated numbers of macrophages (7.8% of bone marrow nucleated cells), some of which were actively phagocytosing red cells and neutrophils (May-Giemsa, original magnification ×400).

generally believed that early PTLD usually arises from donor B-cells and that late-onset PTLD originates from residual host B-cells [8,9]. CBT may have a low risk of EBV-associated PTLD, because cord blood appears to carry few herpes viruses. In fact, there are only a few reports of PTLD following CBT [11-15]. Barker et al reported EBV-associated PTLD in 5 of 272 recipients of unrelated-donor umbilical CBTs [14]. EBV-associated PTLD was diagnosed in these patients 4 to 14 months (median, 6 months) after transplantation. The patients had apparent lymph node enlargement, but none of them showed EBV-associated HPS. To our knowledge, this report is the first of a case of late-onset EBV-associated HPS following CBT. In CBT for adult patients, reactivation of EBV frequently occurs, because of the high percentage of seropositive recipients. Although the majority of PTLD cases are of B-cell origin, PTLD of NK/T-cell origin has rarely been described. Late-onset EBV-negative lymphoid tumors are reported more frequently in this population [6], but EBV-associated HPS is rare. Although we do not have direct evidence in the present case, T-cells or NK cells might have been infected with EBV, similar to the EBV-associated HPS cases that have been observed in other clinical settings.

The origin of the CD8$^+$ cells that proliferated in the blood and bone marrow appears to have been donor-derived T-lymphocytes, because the patient achieved complete donor chimerism in blood lymphocytes and bone marrow cells more than 100 days after transplantation and the majority of the CD8$^+$ cells expressed CD3. The clonality of these proliferating CD8$^+$ cells was not determined. Progression of EBV infection may result in lytic, latent, or immortalizing infection, and these infections can be distinguished on the basis of virus production and the expression of different viral proteins and antigens. Although there is no information regarding the presence or absence of EBV in the graft, reactivation of latent virus is likely to be behind the mechanism of EBV infection in the present case.

The prognosis of EBV-associated PTLD after HSCT is poor, particularly for the late-onset type, and the disease has a high mortality rate [8,18]. Reduction of immunosuppressive therapy should be attempted initially in almost all patients and has been reported to result in the regression of PTLD lesions, with remission rates of 50% in PTLD following organ transplantation [19]. Porcu et al reported that reduction of immunosuppression and antiviral therapy for PTLD after renal transplantation are a highly successful therapeutic combination [20], but this therapy was not effective in our case. The inconsistent response to the reduction of immunosuppressive therapy likely reflects heterogeneity in the disease type, the intensity of immunosuppressive therapy, the EBV status of the PTLD, and the presence of monoclonal disease. Safety profiles and the efficacy of cytotoxic chemotherapy containing etoposide [21] remain unknown in patients after HSCT. Although donor lymphocyte infusion may be effective for EBV-associated PTLD [22,23], this treatment modality is not feasible after CBT.

Because adoptive immunotherapy with donor lymphocyte infusion is not available for EBV-associated PTLD following CBT, early diagnosis is important. Some recent reports have demonstrated that the EBV burden in the peripheral blood following HSCT is predictive of the development of EBV-associated PTLD [24-26]. Sequential monitoring of EBV DNA may be useful for identifying EBV reactivation and the early diagnosis of patients showing only extranodal prediction [24-26]. Hoshino et al proposed diagnostic criteria and a therapeutic strategy based on quantitative analysis of EBV DNA to classify the severity of EBV infection in HSCT [24]. According to these criteria, we classified our case as the most severe type of EBV-associated PTLD.

The efficacy of using a chimeric anti-CD20 monoclonal antibody for PTLD after HSCT has recently been reported, with response rates of 50% to 60% [27,28]. This therapy appears to be effective for patients following CBT as well [14]. However, this treatment may not be effective against EBV-associated HPS in patients with proliferating EBV-infected T-cells or NK cells. Hypercytokinemia plays a major role in EBV-associated HPS and causes hemophagocytosis, cellular damage, and the dysfunction of various organs [10]. Therefore, special therapeutic strategies are needed to control the cytokine storm generated by EBV and to suppress proliferating EBV genome–containing cells during the early stage of HPS [10]. In this respect, plasma exchange may be a therapeutic option for posttransplantation EBV-associated HPS during its early course, because exchange transfusion, plasma exchange, plasmapheresis, or hemodialysis has been used during induction immunochemotherapy for EBV-associated hemophagocytic lymphohistiocytosis in children [21,29,30].

In conclusion, EBV-associated PTLD may be encountered with increasing frequency because of the rising number of CBT procedures being performed for adult recipients. Although both innate and stimulated immunity to EBV have been demonstrated in cord blood lymphocytes [31-33], the true capacity of native neonatal T-cells to regulate reactivation of EBV already present in the host is unknown. We should be aware of not only early-onset PTLD but also late-onset EBV-associated HPS after CBT in adult recipients.

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Tohoku J. Exp. Med., 2006, **209**, 61-67

Case Report

Successful Autologous Peripheral Blood Stem Cell Transplantation Using Thiotepa in a Patient with Systemic Sclerosis and Cardiac Involvement

Atsushi Komatsuda, Yoshinari Kawabata, Takahito Horiuchi, Mutsuhito Motegi, Masatoyo Ozawa, Naohito Fujishima, Masaaki Kume, Makoto Hirokawa, Hideki Wakui, Akihiko Yamaguchi[1] and Ken-ichi Sawada

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Komatsuda, A., Kawabata, Y., Horiuchi, T., Motegi, M., Ozawa, M, Fujishima, N., Kume, M., Hirokawa, M., Wakui, H., Sawada, K. and Yamaguchi, A. *Successful Autologous Peripheral Blood Stem Cell Transplantation Using Thiotepa in a Patient with Systemic Sclerosis and Cardiac Involvement.* Tohoku J. Exp. Med., 2006, **209** (1), 61-67 —— A 19-year-old man with systemic sclerosis (SSc) was hospitalized for autologous peripheral blood stem cell transplantation (auto-PBSCT) due to progressive scleroderma and cardiac involvement despite conventional treatment. During the administration of cyclophosphamide (60 mg/kg/day for 2 days) for mobilization and collection of CD34$^+$ selected peripheral blood stem cells, he developed congestive heart failure. Echocardiogram showed hypokinetic asynergy from the septum to posterior wall, which might indicate underlying cardiac damage. We were also concerned about the risk of high-dose cyclophosphamide-induced cardiotoxicity. Since the dose-limiting toxicity of thiotepa, an alkylating agent, is myelosuppression, and cardiac toxicity due to thiotepa is less common, we used a conditioning regimen consisting of thiotepa (10 mg/kg/day, day –5) and low-dose cyclophosphamide (50 mg/kg/day, days –3 and –2), instead of the conventional high-dose cyclophosphamide (50 mg/kg/day × 4 days/course). The post-transplant course was uneventful, and the modified Rodnan skin thickness score improved from 32 to 15. The present case report demonstrates that thiotepa can be employed as a conditioning regimen for auto-PBSCT in SSc patients with cardiac involvement in order to reduce cyclophosphamide-induced cardiotoxicity. ——— autologous peripheral blood stem cell transplantation (PBSCT); cardiotoxicity; cyclophosphamide; systemic sclerosis; thiotepa
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Systemic sclerosis (SSc) is a generalized disease of the small arteries, microvessels, and diffuse connective tissue, characterized by extensive fibrosis and vascular obliteration in the skin and visceral organs including the gastrointestinal tract, lungs, heart, and kidneys. Although the eti-

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ology of SSc remains unknown, this disease is characterized by predominant T-cell activation, production of autoantibodies to Scleroderma-70 (Scl-70) (anti-topoisomerase I) and cytokine release (Sakkas and Platsoucas 2004).

Recent studies in a large series of patients with SSc demonstrated that the prognosis is associated with the extent of skin involvement and/or affected visceral organs (Ferri et al. 2002; Scussel-Lonzetti et al. 2002). Significantly worse prognosis was observed in the diffuse cutaneous subset, in male gender, and in patients with lung, heart, and renal involvement (Ferri et al. 2002). Standardized mortality ratios were 2.71, 2.76, and 6.17 in the limited, intermediate, and diffuse subsets, respectively (Scussel-Lonzetti et al. 2002). Although cyclophosphamide has documented but limited efficacy in patients with SSc and alveolitis (White et al. 2000), effective treatment has not been found to prevent disease progression in SSc.

Autologous transplantation of $CD34^+$ selected cells after high-dose chemotherapy has recently emerged for patients with a variety of diseases, including hematological diseases, solid tumors, and immunological diseases resistant to conventional therapy (Kawabata et al. 2003). $CD34^+$ selected autologous peripheral blood stem cell transplantation (PBSCT) has been initiated as a promising new therapy for patients with refractory diffuse SSc with early visceral organ involvement in the United States and Europe since the 1990's (McSweeney et al. 2002; Farge et al. 2002, 2004). Improvement of the disease was observed in the majority of patients after PBSCT, although there were some deaths related to either the PBSCT procedure or disease progression; the projected 2-year and 5-year survival rates were 79% and 72%, respectively (McSweeney et al. 2002; Farge et al. 2004). In most SSc patients or patients with other diseases, high-dose cyclophosphamide was introduced in mobilization and conditioning regimens for PBSCT (Itoh et al. 2004; Sacchardi et al. 2004; Tang et al. 2004). It is well known that high-dose cyclophosphamide can cause an acute form of cardiotoxicity within 10 days of administration (Gharib and Burnett 2002). Furthermore, cardiovascular manifestations including pericardial disease, myocardial disease, and arrhythmias frequently occur in patients with SSc (Janosik et al. 1989). In general, eligibility criteria for PBSCT require preserved cardiac function, defined as left ventricular ejection fraction (LVEF) more than 50% (Nishio et al. 2001; Itoh et al. 2004; Tang et al. 2004). However, patients with SSc or other diseases complicated by cardiac dysfunction will have significantly increased treatment-related mortality. Therefore, when such patients receive autologous PBSCT (auto-PBSCT) using high-dose cyclophosphamide, we need to pay close attention to its cardiotoxicity (Saccardi et al. 2004).

Here we report a patient with progressive and refractory SSc, who received successful $CD34^+$ cell-selected auto-PBSCT using conditioning regimens incorporating thiotepa to reduce high-dose cyclophosphamide-induced cardiotoxicity.

Case Report

A 19-year-old man was admitted to Akita University Hospital on November 17, 2003 to receive auto-PBSCT. He had a history of bilateral hand edema and elbow joint pain from December 2000, and Raynaud's phenomenon from February 2001. He was diagnosed with SSc at Senboku General Hospital, based on skin manifestations (Raynaud's phenomenon, bilateral sclerodactyly, proximal scleroderma, and bilateral hand edema) (Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee 1980) and the presence of anti-nuclear antibodies and anti-Scl-70 antibodies. He was treated with prednisolone (PSL) (10 mg/day) and cilostazol (200 mg/day) from June 2001. From August 2001, he developed polyarthralgia and showed progression of scleroderma including the face, neck and trunk. From October 2001, he was treated with 30 mg/day PSL for weeks. Treatment was partially effective for polyarthralgia, but skin symptoms became progressive with a tapering course. He demonstrated bilateral fingertip ulcers and limitation of both fist closure and finger extension.

On admission, height was 170 cm, weight

49.9 kg, body temperature 36.5°C, pulse rate 100 beats/min, respiratory rate 16 breaths/min, and blood pressure 108/54 mmHg. Physical examination demonstrated bilateral sclerodactyly and proximal scleroderma, face, neck, and trunk. Modified Rodnan skin thickness score (mRSS) (Clements et al. 1993) was 32. The lungs and heart were normal. The liver and spleen were not palpable. Bowel sounds were normal.

The erythrocyte count was 513 × $10^4/\mu l$, hemoglobin 14.2 g/dl, hematocrit 43.2%, leukocyte count 7,100/μl (neutrophils 74%, monocytes 6%, and lymphocytes 20%), and platelet count 296 × $10^3/\mu l$. Urinalysis gave normal results. The serum total protein was 7.3 g/dl, albumin 3.8 g/dl, blood urea nitrogen 8.0 mg/dl, and creatinine 0.6 mg/dl. Human atrial natriuretic peptide was 18.0 pg/ml (normal range: less than 40.0 pg/ml) and brain natriuretic peptide (BNP) was 50.5 pg/ml (normal range: less than 18.4 pg/ml). Thyroid function tests were normal. C-reactive protein was 1.8 mg/dl. Serological study indicated the presence of anti-nuclear antibodies with a speckled pattern (2,560-fold), anti-Scl-70 antibodies (169.4 IU/ml), anti-double-stranded (ds) DNA antibodies (63.6 IU/ml), and anti-ribonucleoprotein (RNP) antibodies (52.5 IU/ml). Serum CH50 was 23 U/ml, C3 42 mg/dl, and C4 8 mg/dl. Circulating immune complexes (IC) measured by the C1q binding assay were 7.4 μg/ml (less than 3.0 μg/ml). Although high titers of anti-ds DNA antibodies, anti-RNP antibodies and IC, and hypocomplementemia were observed, he had no clinical features associated with systemic lupus erythematosus or vasculitis.

Chest radiograph did not show either cardiomegaly or reticular shadow in the lung fields. Electrocardiogram demonstrated sinus tachycardia with low QRS voltage. Myocardial scintigraphy was not performed. Pulmonary function test demonstrated normal results of forced expiratory volume in one second (100.4%) and % diffusing capacity for carbon monoxide (94.5%), but a slight reduction of % vital capacity (71.2%). Examination of the upper esophago-gastrointestinal tract demonstrated normal motility.

Based on rapidly progressive skin involvement and mild cardiac involvement despite continuing treatment, we considered his prognosis very poor. We also judged this patient eligible for auto-PBSCT (Burt et al. 2003; Farge et al. 2002; McSweeney et al. 2002). The protocol for auto-PBSCT in this patient was approved by the ethics committee of Akita University Hospital. Written

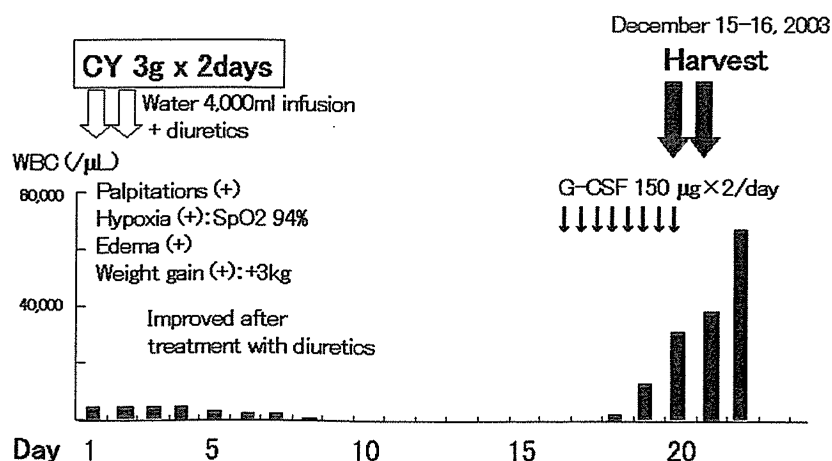

Fig. 1. Clinical course of the patient (I). During mobilization, the patient received high-dose cyclophosphamide (60 mg/kg/day for 2 days). Thereafter, he developed congestive heart failure, but his condition improved after treatment with furosemide. CY, cyclophosphamide; G-CSF, granulocyte-colony stimulating factor; WBC, white blood cell count.

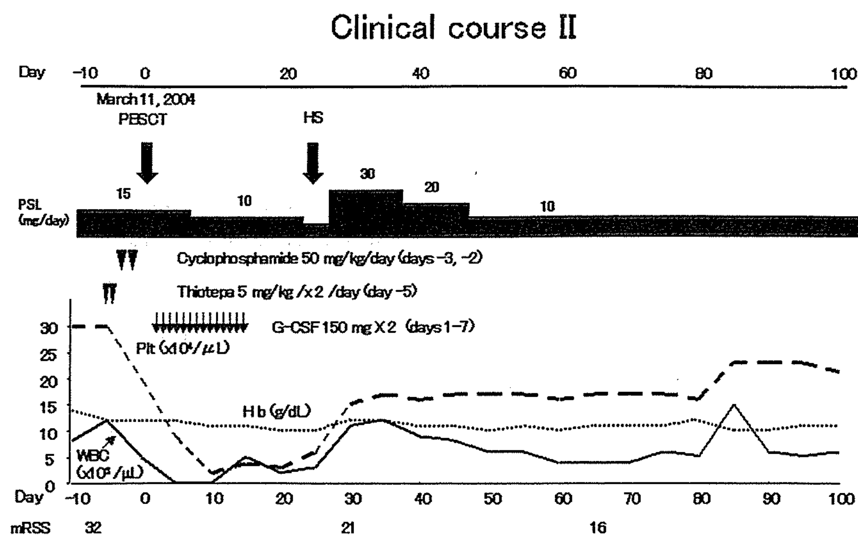

Fig. 2. Clinical course of the patient (II). During conditioning, the patient did not develop congestive heart failure, and he was successfully treated with autologous PBSCT. G-CSF, granulocyte-colony stimulating factor; Hb, hemoglobin; HS, hemophagocytic syndrome; mRSS, modified Rodnan Skin Score; PBSCT, peripheral blood stem cell transplantation; Plt, platelet count; PSL, prednisolone WBC; white blood cell count.

informed consent was obtained. The clinical course is shown in Figs. 1 and 2. Mobilization and collection of peripheral blood stem cells (PBSCs) were achieved with a conventional priming regimen in combination with granulocyte-colony stimulating factor (G-CSF) (Farge et al. 2004). The patient received high-dose cyclophosphamide (60 mg/kg/day for 2 days). Thereafter, he developed palpitations, mild hypoxemia (oxygen saturation [SpO_2]: 94%), edema, and body weight gain (3 kg). At that time, the total volume of intake was about 4,000 ml, and output about 1,600 ml. Electrocardiogram demonstrated sinus tachycardia (120 beats/min) with low QRS voltage. Echocardiogram showed focal hypokinetic asynergy from the septum to posterior wall, mild pericardial effusion, and a normal LVEF (75%). He was treated with furosemide intravenously, and his condition improved. After administration of G-CSF (300 μg/day for 5 days), 23 × 10^6 $CD34^+$ cells/kg were harvested over two consecutive days, and 8.1 × 10^6 $CD34^+$ cells/kg with a purity of 96% were positively selected using CliniMacs (Miltenyi Biotec GmbH, Bergisch, Gladbach, Germany).

We considered that the patient developed congestive heart failure (CHF) due to cardiac involvement of SSc. We were also concerned about the risk of high-dose cyclophosphamide-induced cardiotoxicity. As the conditioning regimen for this patient, we administered thiotepa (two doses of 5 mg/kg on day -5) and low-dose cyclophosphamide (50 mg/kg/day on days -3 and -2) (Aversa et al. 1999). During conditioning, the patient did not develop CHF recurrence. Purified $CD34^+$ selected PBSCs were infused on day 0 (March 11, 2004). On day 13, the erythrocyte count was 418 × $10^4/\mu$l, hemoglobin 10.9 g/dl, hematocrit 33.4%, leukocyte count 7,600/μl (neutrophils 46%, monocytes 13%, lymphocytes 4%, myelocytes 14%, and metamyelocytes 23%), and platelet count 42 × $10^3/\mu$l. On day 22, he was suspected of virus-associated hemophagocytic syndrome, although pathogenic virus was not detected. He was treated with PSL (30 mg/day) and intravenous IgG (5 g/day) and his condition improved thereafter. Two months after PBSCT, rapid improvement in scleroderma was observed; the mRSS decreased from 32 to 16. In December 2005, the mRSS further decreased 15. A serologi-

cal study showed decreased serum titers of anti-Scl-70 antibodies (40 IU/ml), anti-ds DNA antibodies (less than 2 IU/ml), and anti-RNP antibodies (16 IU/ml), and normal serum levels of CH50 (43 U/ml), C3 (115 mg/dl), and C4 (22.6 mg/dl). A pulmonary function test also showed improvement in % vital capacity (82.3%). BNP normalized to 6.4 pg/ml.

Discussion

Primary cardiovascular manifestations of SSc include pericardial disease, myocardial disease, conduction abnormalities, and cardiac arrhythmias (Janosik et al. 1989). In our patient, pre-transplant chest radiograph did not show any abnormal findings, but electrocardiogram demonstrated sinus tachycardia and low QRS voltage, and the BNP level was high. These findings indicated that the patient might have developed cardiac damage due to SSc.

Since adverse prognostic factors have been reported to include male gender (Ferri et al. 2002), diffuse cutaneous SSc, visceral involvement such as heart (Ferri et al. 2002; Ioannidis et al. 2002; Scussel-Lonzetti et al. 2002), and the presence of anti-Scl-70 antibodies (Ioannidis et al. 2002), we considered that our patient would have a very poor prognosis, and was eligible for auto-PBSCT.

We used cyclophosphamide (two daily successive injections of 3,000 mg [60 mg/kg/day]) as employed in the priming regimen in the European Group for Blood and Marrow Transplantation/ European League Against Rheumatism (EBMT/ EULAR) registry (Farge et al. 2004). Although the regimen has been successfully used in many patients with more severe myocardial dysfunction than that in our patient, he developed CHF. Since urine volume at mobilization was less than the total volume of intake resulting in 3 kg weight gain, and CHF improved promptly after intravenous injection of diuretics, CHF might have been caused by the overhydration.

In autologous or allogeneic PBSCT, high-dose cyclophosphamide is widely used for both mobilization and conditioning regimens (Fujimaki et al. 2001; Nishio et al. 2001; Itoh et al. 2004; Sacchardi et al. 2004; Tang et al. 2004). However, high-dose cyclophosphamide can cause an acute form of cardiotoxicity within 10 days of administration, and that a total dose of more than 170-180 mg/kg per course (over 4-7 days) is the risk factor (Gharib and Burnett 2002). Therefore, we are concerned about that patients with cardiac impairment are eligible for PBSCT. An LVEF less than 50% has generally been considered a contraindication for stem cell transplantation (SCT) (Itoh et al. 2004; Tang et al. 2004). However, there are no reports demonstrating the absolute level of cardiac dysfunction that rules out SCT (Itoh et al. 2004). Tang et al. (2004) described that patients with malignant lymphoma complicated by an LVEF less than 50% could safety receive auto-PBSCT, although all patients received a conditioning regimen (cyclophosphamide 120 mg/kg, busulfan 14 mg/kg, etoposide 60 mg/kg). However, Fujimaki et al. (2001) retrospectively analyzed the development of cardiac complications in patients who underwent allogeneic bone marrow transplantation (BMT) or PBSCT with the same regimen of cyclophosphamide (2,250 mg/m^2/day for consecutive days), thiotepa (200 mg/m^2/day for consecutive days), and total body irradiation, and found a significant correlation between LVEF before hematopoietic stem cell transplantation (HCT) and the development of cardiac complication. They recommended that patients with an LVEF less than 55% should received reduced-doses of the conditioning regimen to avoid cardiac toxicity.

Furthermore, high-dose cyclophosphamide (200 mg/kg divided into 50 mg/kg per day on days from -5 to -2 before the transplant) is usually used as the conditioning regimen for autoimmune diseases (Tyndall and Gatwohl 1997; Burt et al. 2003). Saccardi et al. (2004) reported that a direct causal association with cyclophosphamide was suspected in two of five cardiac deaths among 77 SSc patients who received auto-PBSCT in the EBMT database. Careful pre-transplant cardiac assessment is recommended in all SSc patients being considered for auto-PBSCT (Saccardi et al. 2004).

We considered that it was difficult to use conventional high-dose cyclophosphamide as the