Anti-Moesin Antibodies in the Serum of Patients with Aplastic Anemia Stimulate Peripheral Blood Mononuclear Cells to Secrete TNF-α and IFN-γ[1]

Hiroyuki Takamatsu,[2*†] J. Luis Espinoza,[2*] Xuzhang Lu,[*] Zhirong Qi,[*] Katsuya Okawa,[‡] and Shinji Nakao[3*]

Moesin is an intracellular protein that links the cell membrane and cytoskeleton, while also mediating the formation of microtubules and cell adhesion sites as well as ruffling of the cell membrane. To determine the roles of anti-moesin Abs derived from the serum of patients with aplastic anemia (AA) in the pathophysiology of bone marrow failure, we studied the expression of moesin on various blood cells and the effects of anti-moesin Abs on the moesin-expressing cells. The proteins recognized by anti-moesin mAbs were detectable on the surface of T cells, NK cells, and monocytes from healthy individuals as well as on THP-1 cells. The peptide mass fingerprinting of the THP-1 cell surface protein and the knock-down experiments using short hairpin RNA proved that the protein is moesin itself. Both the anti-moesin mAbs and the anti-moesin polyclonal Abs purified from the AA patients' sera stimulated THP-1 cells and the PBMCs of healthy individuals and AA patients to secrete 60–80% as much TNF-α as did LPS 100 ng/ml. Although the polyclonal Abs induced IFN-γ secretion from the PBMCs of healthy individuals only when the PBMCs were prestimulated by anti-CD3 mAbs, the anti-moesin Abs were capable of inducing IFN-γ secretion from the PBMCs of AA patients by themselves. Anti-moesin Abs may therefore indirectly contribute to the suppression of hematopoiesis in AA patients by inducing myelosuppressive cytokines from immunocompetent cells. ***The Journal of Immunology*****, 2009, 182: 703–710.**

Acquired aplastic anemia (AA)[4] is a syndrome characterized by pancytopenia and bone marrow (BM) hypoplasia (1). The T cell-mediated suppression of hematopoiesis is considered to be the most important mechanism responsible for the development of this syndrome because approximately 70% of AA patients respond to immunosuppressive therapy, such as antithymocyte globulin and cyclosporine (2, 3). In addition to a large body of evidence for T cell involvement in the pathogenesis of AA (4–7), recent studies have revealed the presence of Abs specific to self-Ags in the serum of AA patients (8–11). Although some of these Abs are directed toward Ags that are abundant in hematopoietic cells (e.g., kinectin (Ref. 8) and DRS-1 (Ref. 9)), their roles in the pathophysiology of AA are unclear.

Of the various autoAbs detected in the autoimmune diseases, some are known to exhibit stimulatory effects on the target cells rather than inhibitory effects, such as anti-thyroglobulin Abs in Basedow's disease (12) and anti-desmoglein Abs in pemphigus vulgaris (12, 13). The autoAbs specific to platelet-derived growth factor receptors in patients with scleroderma and those with extensive chronic graft-vs-host diseases trigger an intracellular loop, involving Ha-Ras-ERK 1 and 2 (ERK 1/2)-reactive oxygen species (Ha-Ras-ERK 1/2-ROS), and augment collagen gene expression as well as myofibroblast phenotype conversion of normal human primary fibroblasts (14, 15). The anti-proteinase 3 Abs detected in Wegener's granuloma stimulate monocytes through the binding of cell surface proteinase 3 to secrete IL-8 (16). The autoAbs detected in AA patients may also be involved in the pathophysiology of BM failure by way of other mechanisms than the direct toxicity against the hematopoietic cells, though there has been no evidence for such functional autoAbs in AA patients.

We previously demonstrated that Abs specific to moesin, a membrane-cytoskeleton linker protein in the cytoplasm, were detectable in approximately 40% of AA patients (11). Moesin is an intracellular protein that links the cell membrane and cytoskeleton, and mediates the formation of microtubules and cell adhesion sites as well as ruffling of the cell membrane (17). On the other hand, some reports have identified molecules that were recognized by anti-moesin mAbs on the surface of blood cells such as T cells and macrophages (18, 19). Because these immune cells are an important source of myelosuppressive cytokines such as TNF-α and IFN-γ, it is conceivable that anti-moesin Abs in AA patients may bind such moesin-like molecules on these immune cells and affect the cytokine secretion from these cells.

To test these hypotheses, we studied the expression of moesin on blood cells and the effects of anti-moesin Abs on the moesin-expressing

*Cellular Transplantation Biology, Kanazawa University Graduate School of Medical Science, and †Internal Medicine, NTT WEST Kanazawa Hospital, Kanazawa, Ishikawa, and ‡Biomolecular Characterization Unit, Frontier Technology Center, Kyoto University Graduate School of Medicine, Kyoto, Japan

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[2] H.T. and J.L.E. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Shinji Nakao, Cellular Transplantation Biology, Kanazawa University Graduate School of Medical Science, Kanazawa, Ishikawa 920-8641, Japan. E-mail address: snakao@med3.m.kanazawa-u.ac.jp

[4] Abbreviations used in this paper: AA, aplastic anemia; BM, bone marrow; pAb, polyclonal Ab; PB, peripheral blood.

cells. The present study revealed a novel function of autoAbs, which may contribute to the pathophysiology of BM failure.

Materials and Methods

Study subjects (patients)

Sera were obtained from 19 patients with AA and 4 healthy individuals. BM plasma was obtained from five patients with AA and three healthy individuals. All AA patients had severe AA and were positive for anti-moesin Abs. The samples were cryopreserved at −80°C until use. Peripheral blood (PB) was obtained from 7 patients with AA and 10 healthy individuals and BM was aspirated from 3 healthy individuals. The PBMCs were isolated using lymphoprep (Nycomed). All patients and healthy volunteers provided an informed consent before sampling according to the Declaration of Helsinki. This study was approved by the human research committee of Kanazawa University Graduate School of Medical Science.

Cell lines

Molt-4, THP-1, U937, K562, Daudi, and Jurkat cell lines were purchased from the Health Science Research Resources Bank. A megakaryoblastic leukemia cell line UT-7, a myeloid leukemia cell lines OUN-1, and a myelodysplastic syndrome cell line TF-1 were provided by Dr. N. Komatsu of Jichi Medical School, Dr. M.Yasukawa of Ehime University, and Dr. S. Ogawa of the University of Tokyo, respectively.

Purification of anti-moesin Abs in the sera of patients with AA

The anti-moesin polyclonal Abs (pAbs) were purified from the patients' serum with affinity chromatography using a protein G column (mAb Trap kit, no. 17-1128-0; GE Healthcare) and recombinant moesin protein (11) fixed on an agarose-gel column (1 ml, HiTrap NHS-activated HP, no. 17-0716-01; GE Healthcare) according to the manufacturer's instruction. In brief, 20 ml of serum from AA patients was applied to the Protein G column. After washing with the binding buffer, the whole IgG was eluted with the elution buffer and neutralized with the neutralizing buffer. The purified IgG was then applied to the recombinant moesin-fixed affinity chromatography column. After washing with the binding buffer (75 mM sodium phosphate (pH 8.0)), anti-moesin pAbs were eluted with the elution buffer (100 mM glycine-HCl and 500 mM NaCl (pH 2.7)). The purified anti-moesin pAbs were dialyzed in PBS at 4°C overnight using a spectra/Por Float-A-Lyzer column (Spectrum Laboratories; no. 235118). The purity of the isolated anti-moesin pAbs was confirmed by PAGE followed by Coomassie Brilliant Blue staining.

Flow cytometry

Mouse anti-moesin mAb (clone 38/87; Neomarkers) which was labeled with FITC (Immuno-Biological Laboratories) was used in combination with mAbs specific to CD3-PE (BD Pharmingen; no. 555333), CD19-PE (BD Pharmingen; No. 555413), CD4-PE (BD Pharmingen; No. 347327), CD-8-PE (BD Pharmingen; no. 555367), CD14-PE (BD Pharmingen; No. 555398), CD11b-PE (BD Pharmingen; No. 555388), CD34-PE (BD Pharmingen; no. 348057), and CD3-Cy-Chrome (BD Pharmingen; no. 555334). Isotype-matched control mAbs (BD Pharmingen) were used as negative controls. For the detection of moesin-like molecules on leukocytes and leukemia cell lines, 1 μl of anti-moesin mAbs and 2 μl of PE-labeled mAbs were added to 50 μl of cell suspension containing 1×10^6 cells and the suspension was incubated on ice for 30 min. For the detection of moesin-like molecules by pAbs, 1×10^6 THP-1 cells were washed twice with PBS containing 1% BSA (Sigma-Aldrich; no. A8022) and resuspended in 200 μl of a PBS containing 2% FCS, 2% goat serum, and 2% BSA. The cell suspension was incubated for 30 min at 4°C. Then, 5 μg/ml anti-moesin pAbs or isotype control human IgG pAbs isolated from healthy individuals were added to the cell suspension and incubated for 1 h at 4°C. The cells were washed twice with PBS containing 1% BSA followed by incubation with a secondary Ab (goat anti-human IgG FITC-labeled Ab; Sigma-Aldrich; no. F5512) diluted 1/100 in PBS containing 2% goat serum and were incubated at 4°C for 30 min. Finally, the cells were washed twice with PBS containing 1% BSA and subjected to flow cytometry.

Stimulation of THP-1 cells with PMA/LPS

THP-1 cells (10^6) were suspended in 2 ml of RPMI 1640 containing 10% FCS and 20 ng/ml PMA (Wako Chemicals; no. 545-00261) and incubated for 24 h at 37°C in a CO_2 incubator. A total of 10 ng/ml LPS (Sigma-Aldrich; no. L2880) was added to the cell suspension and further incubated for 20 h. The cultured cells were analyzed for the expression of moesin-like molecules by flow cytometry using anti-moesin mAbs (clone 38/87; Neomarkers).

Isolation of monocytes and T cells

Monocytes were isolated by plastic adherence as previously described (20). In brief, 5×10^6 PBMCs/well were distributed into 12-well plates (Corning) and allowed to adhere in a 5% CO_2 incubator at 37° for 2 h in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS, 100 U/ml penicillin - 0.1 mg/ml streptomycin (Invitrogen; No.15140-148) and 10 μg/ml polymyxin B (Aerosporin) (Sigma-Aldrich; no. 194538). Nonadherent cells were removed and the remaining adherent cells on the plates were used as monocytes. T cells were purified by negative selection using the Human T Cell Enrichment Columns (R&D Systems; no. HTCC-500) following the manufacturer instructions. The purity of enriched T cells and monocytes was approximately 90% as determined by flow cytometry using anti-CD3-PE and anti-CD-14-PE mAbs, respectively.

Stimulation of THP-1 cells, PBMCs, monocytes, and T cells with anti-moesin Abs

THP-1 cells, PBMCs, monocytes, or T cells were suspended in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS. Polymyxin B (Aerosporin) (Sigma-Aldrich; no. 194538) was added at 10 μg/ml to eliminate any contaminating endotoxin. The endotoxin concentration in the pAbs and the reagents used for culture was <10 pg/ml as demonstrated by chromogenic *Limulus* amebocyte lysate assay (Seikagaku). The cells (5×10^5) were incubated for 48 h in the presence of 5 μg/ml of anti-moesin mouse mAbs (clone 38/87, IgG1; Neomarkers) or 5–10 μg/ml of pAbs isolated from the serum of AA patients as described above. Mouse mAbs (Coulter Clone; IgG1, no. 6602872) and control human IgG pAbs isolated from healthy individuals were used as negative controls. This Ab concentration was selected based on an estimated concentration of anti-moesin pAbs in the serum of an AA patient. For prestimulation of PBMCs to induce IFN-γ secretion, the cells were incubated for 1 h in the presence of 100 ng/ml of anti-CD3 mAbs (Clone OKT3) (eBioscience; functional grade no. 16-0037). For co-stimulation of isolated T cells to induce IFN-γ secretion, the cells were cultured for 48 h on a 48-well tissue culture plate that was coated with 100 ng/ml of anti-CD3 mAbs (clone OKT3) (eBioscience; functional grade no. 16-0037) overnight at 4°C and washed. Then, 100 ng/ml LPS or 10 μg/ml PHA (Sigma-Aldrich; no. L4144) was included instead of anti-moesin Abs as the positive controls for the induction of TNF-α or IFN-γ secretion, respectively, and control human IgG pAbs isolated from healthy individuals were added as a negative control.

Western blotting

Western blotting was performed using THP-1 cell lysates. The specific bands were visualized by anti-moesin mAbs, pAbs from AA patients, or control human IgG pAbs from healthy individuals as described in a previous report (11). The expression levels of α-tubulin were determined as an internal control using Western blotting with anti-α-tubulin mAbs (Sigma-Aldrich; no. T 5168).

Isolation of proteins on the surface of THP-1 cells

The THP-1 cells were treated with sulfo-NHS-SS-biotin, and the cell surface proteins were isolated with avidin-fixed columns according to the manufacturer's instructions (Pierce). Thereafter, 1×10^7 cells were washed twice with 8 ml of ice-cold PBS. The cells were suspended in 10 ml PBS containing 2.5 mg sulfo-NHS-SS-Biotin and incubated for 30 min at 4°C. Then, 500 μl of quenching solution was added to the cell suspension and the cells were washed with 10 ml TBS twice. The cell pellet was lysed in 500 μl of lysis buffer containing 60 μl of preotease inhibitor cocktail (Sigma-Aldrich; no. P-8340) and then disrupted by sonication. The biotin-labeled membrane proteins were isolated by an immobilized NeutrAvidin Gel column. The isolated membrane proteins were subjected to Western blotting and peptide mass fingerprinting.

Peptide mass fingerprinting

Mass spectrometric identification of 80- and 75-kDa proteins on the surface of the THP-1 cells was performed as previously described (21). In brief, the proteins fractionated by SDS-PAGE were visualized by Coomassie Brilliant Blue staining and the 80- and 75-kDa bands were excised from gels, followed by in-gel digestions with trypsin (Promega) in a buffer containing 50 mM ammonium bicarbonate (pH 8.0) and 2% acetonitrile overnight at 37°C. Molecular mass analyses of the tryptic

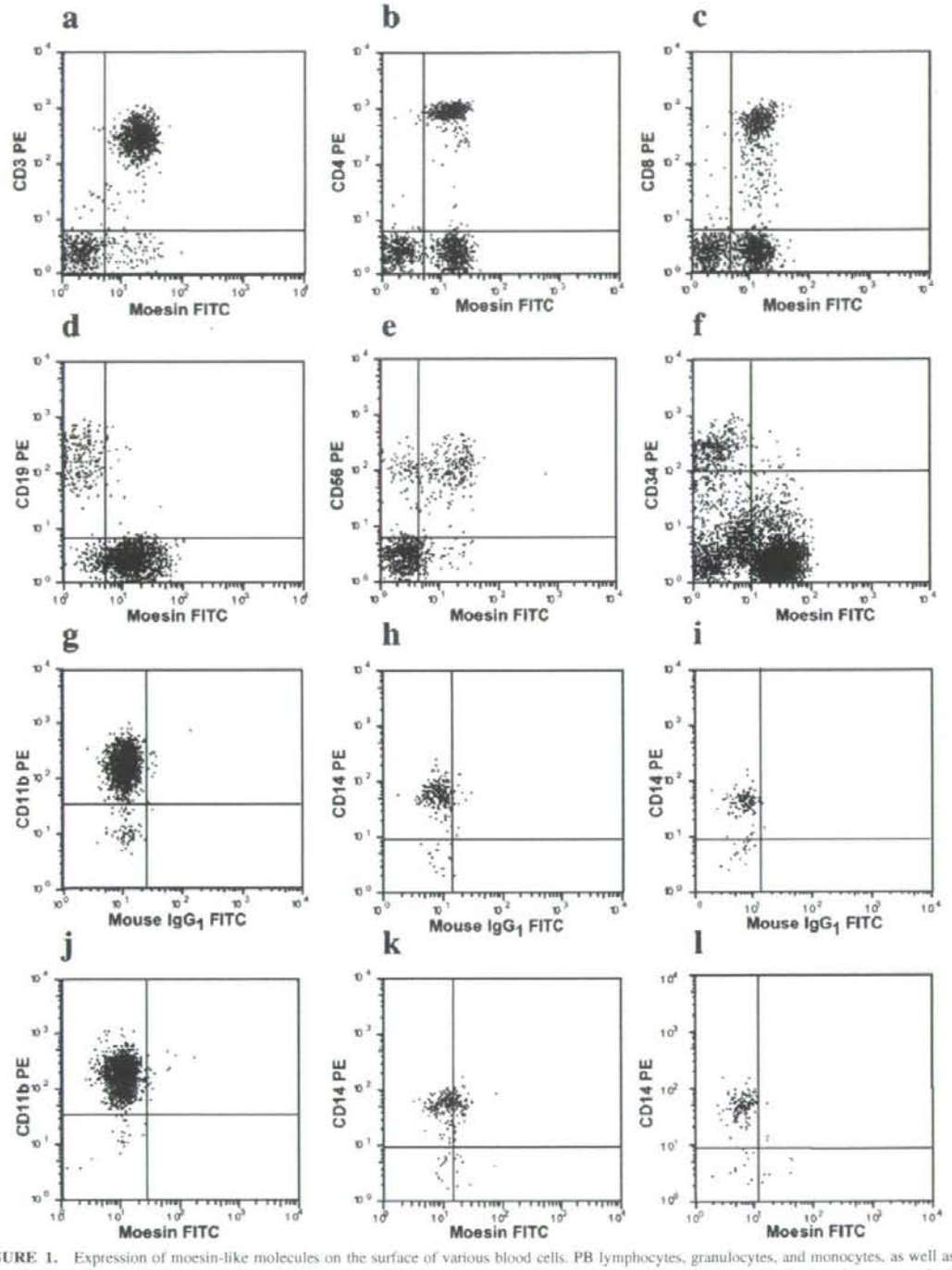

FIGURE 1. Expression of moesin-like molecules on the surface of various blood cells. PB lymphocytes, granulocytes, and monocytes, as well as BM mononuclear cells, of a healthy individual and a patient with AA were analyzed by flow cytometry. The gate was set up for lymphocytes (*a–d*), $CD3^-$ lymphocytes (*e*), granulocytes (*g* and *j*), and monocytes (*h* and *k*) derived from a healthy individual and monocytes (*i* and *l*) derived from an AA patient. BM mononuclear cells (*f*) of a healthy individual were included in the analysis. One representative result of three experiments is shown.

peptides were performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using an ultraflex TOF/TOF (Bruker Daltonics). The proteins were identified by comparisons between the molecular weights determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and the theoretical peptide masses of proteins registered in NCBInr.

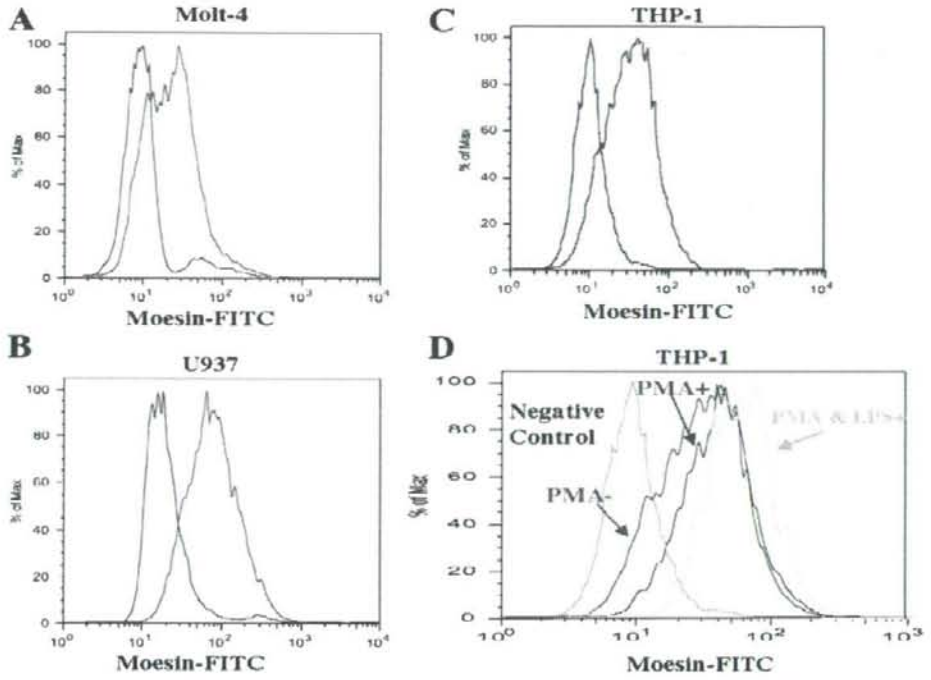

FIGURE 2. Expression of moesin-like molecules on the surface of T cell and monocytic leukemia cell lines. *A–C*, Three leukemia cell lines were examined for the cell surface expression of moesin-like molecules. Left lines, mouse IgG used as negative control; right lines, FITC-labeled anti-moesin mAbs. *D*, THP-1 cells were cultured in the presence or absence of 20 ng/ml PMA for 24 h and then the PMA-stimulated cells were further cultured in the presence of 10 ng/ml LPS for 20 h. The cultured cells were analyzed for the expression of moesin-like molecules by flow cytometry. One representative result is shown.

Transfection of moesin short hairpin (shRNA)

Moesin shRNA plasmid (pENTR/moesin-shRNA-264) (22) was kindly provided by Dr. G. M. Kelly of the University of Western Ontario (Ontario, Canada). THP-1 cells were transfected by electroporation using a Gene Pulser II Electroporation System (Bio-Rad). In brief, 3–5 μg of moesin shRNA plasmid or control shRNA (pENTR/U6-GW/lacZ^{shRNA}) was mixed with 800 μl of Opti-Mem I medium (Invitrogen) containing 1×10^6 THP-1 cells and incubated on ice for 10 min. The cells were electroporated in a 4-mm cuvette (Bio-Rad) at the setting of 300 V of voltage pulse and 960 μF of capacitance. Immediately after electroporation, the transfected THP-1 cells were left on ice for 10 min and then 3 ml of RPMI 1640 containing 10% FCS was added to the cell suspension followed by overnight incubation at 37°C. The cells were rinsed and cultured in 3 ml of fresh RPMI 1640 containing 10% FCS for 72 h at 37°C in a CO_2 incubator and were analyzed for the expression of moesin-like molecules by flow cytometry using FITC-labeled anti-moesin mAb (clone 38/87; Neomarkers).

ELISA

The TNF-α and IFN-γ concentration in the culture supernatant, as well as in PB serum and BM plasma was measured using ELISA kits (Mabtech;

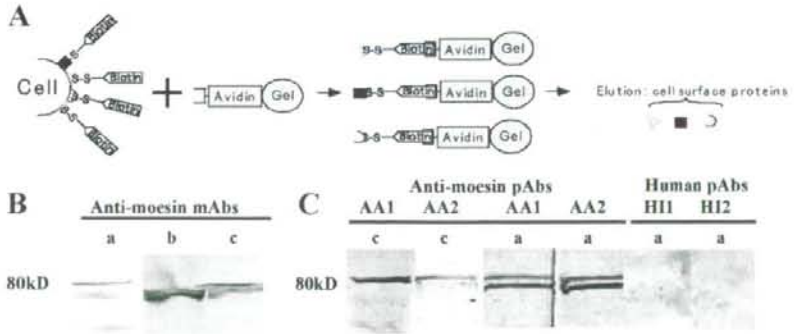

FIGURE 3. Isolation and identification of proteins on THP-1 cells recognized by anti-moesin Abs. *A*, THP-1 cells were treated with sulfo-NHS-SS-biotin and the cell surface proteins were isolated with avidin-fixed columns. *B*, Three different protein lysates (*a*, whole cells; *b*, cytoplasmic proteins; and *c*, surface proteins) were subjected to Western blotting with anti-moesin mAbs. *C*, THP-1 cell lysates (*a*) and surface proteins (*c*) isolated from THP-1 cells were subjected to Western blotting using anti-moesin pAbs purified from two AA patients' sera (AA1 and AA2) or non-specific control human IgG pAbs purified from two healthy individuals' sera (HI1 and HI2).

AB, No. 3510-1H-20, and Mabtech; AB, No. 3420-1H-6) according to the manufacturer's instructions. The OD absorbance at 450 nm was determined using a SLTEAR 340 ATELISA reader (SLT-Labinstruments). For determination of cytokine levels in the PB serum and BM plasma, the following additional procedures were performed. Samples were centrifuged at 10,000 rpm for 10 min. ELISA plates were covered with 200 μl/well of TNF-α assay diluent (eBioscience; No. 00-4202-AD) or IFN-γ assay diluent (Mabtech; No. 3652-D) for 1 h at room temperature before adding samples to block nonspecific reactions. TNF-α assay diluent (eBioscience; No. 00-4202-AD) and IFN-γ assay diluent (Mabtech; No. 3652-D) were used to dilute biotinylated mAb TNF-α-II solution and biotinylated mAb 7-B6-1, respectively.

Statistics

The results are given as the mean ± SD. Comparisons were made using the paired *t* test.

Results

Expression of moesin-like molecules on the surface of various blood cells

To confirm the expression of moesin-like molecules on the PB and BM cells, various leukocyte subsets were examined using flow cytometry with anti-moesin mAbs. Fig. 1 shows the representative results of flow cytometry on one healthy individual. Moesin-like molecules were detectable on T cells, NK cells, and monocytes on their surface but not on B cells, neutrophils, and BM $CD34^+$ cells as shown in Fig. 1. All three healthy individuals and the three AA patients showed similar results except that moesin-like molecules were not detectable on monocytes derived from the three AA patients. The mean fluorescence intensity values of the monocytes from healthy individuals and AA patients were 11.5 ± 2.2 and 6.6 ± 2.1, respectively, and the difference was significant (mean fluorescence intensity ± SD, $p < 0.05$, unpaired *t* test). In addition to the leukocyte subsets from the healthy individuals, moesin-like molecules were detectable on a T cell leukemia cell line Molt-4 as well as on monocytic leukemia cell lines U937 and THP-1 (Fig. 2), while they were undetectable on myeloid leukemia cell lines such as K562, UT-7, OUN-1, and TF-1. They were either undetectable on the Burkitt lymphoma cell line, Daudi, or T cell lymphoma cell line, Jurkat (data not shown). The treatment of THP-1 with 20 ng/ml PMA for 24 h and/or 10 ng/ml LPS for 20 h augmented the expression of moesin-like molecules (Fig. 2*D*), thus, indicating an up-regulation of the moesin-like molecules associated with the differentiation of THP-1 cells into macrophages.

Identification of moesin on the surface of THP-1 cells

To identify the proteins on THP-1 cells recognized by anti-moesin Abs, the THP-1 cells were treated with sulfo-NHS-SS-biotin and the cell surface proteins were isolated with avidin-fixed columns (Fig. 3*A*). Western blotting of the isolated proteins with anti-moesin mAbs showed two clear bands of which the sizes were 75 and 80 kDa (Fig. 3*B*). Mass fingerprinting of the eluted protein revealed the 80 kDa protein to be moesin. The 75 kD band proved to be nucleolin and eukaryotic translation elongation factor 2. To confirm that anti-moesin pAbs in the serum of AA patients can bind to this cell surface moesin, anti-moesin pAbs were purified from the AA patients' sera (AA1 and AA2) with recombinant moesin proteins using affinity chromatography and then were used for Western blotting. As shown in Fig. 3*C*, the serum-derived anti-moesin pAbs bound to moesin derived from the surface proteins of THP-1.

Effect of moesin-specific shRNA on the expression of moesin on THP-1 cells

To further confirm the expression of moesin on the surface of THP-1 cells, the cells were transfected with moesin shRNA using electroporation. Flow cytometry showed a decrease in the moesin expression level on the surface of the THP-1 cells transfected with moesin shRNA in comparison to the THP-1 cells transfected with negative control shRNA (Fig. 4*A*). When the THP-1 cells transfected with different dosages of moesin-specific shRNA were examined by Western blotting, the moesin expression by the THP-1 cells was decreased in a dose-dependent manner. The control shRNA specific to LacZ had no effect on moesin expression.

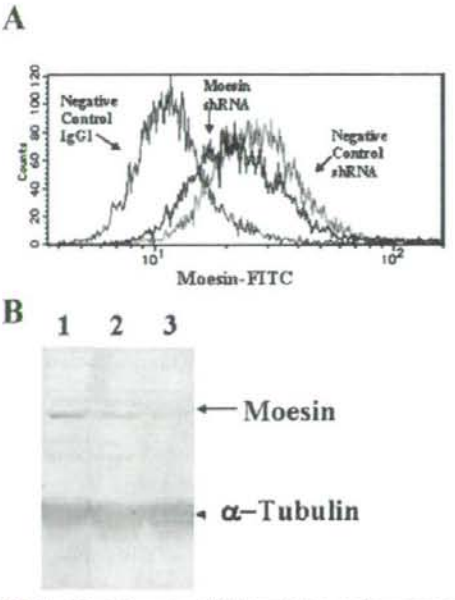

FIGURE 4. Effect of moesin shRNA transfection on the expression of moesin by THP-1 cells. *A*, THP-1 cells transfected with 5 μg of moesin shRNA or control shRNA were examined for the expression of moesin with flow cytometry. The blue line, non-transfected THP-1 cells stained with control mouse IgG1 mAbs; the green line, moesin shRNA transfected cells stained with anti-moesin IgG1 mAbs; the red line, negative control shRNA transfected cells stained with anti-moesin IgG1 mAbs. *B*, Negative control shRNA or moesin-specific shRNA transfected THP-1 cell lysates were examined by Western blotting. 1, 5 μg control shRNA; 2, 3 μg moesin shRNA; 3, 5 μg moesin shRNA.

Effect of anti-moesin Abs on THP-1 cells

To determine whether anti-moesin Abs have some effects on THP-1 cells, the THP-1 cells were cultured in the presence of anti-moesin Abs or control IgG for 48 h and the TNF-α concentration of the culture supernatant was measured using ELISA. Both the anti-moesin mAbs and pAbs induced a significantly greater amount of TNF-α from the THP-1 cells than did the control IgG (Fig. 5*A*). The amount of TNF-α induced by anti-moesin pAbs (5 μg/ml) was almost comparable to that induced by LPS (100 ng/ml) (Fig. 5*A*). The anti-moesin pAbs' binding to moesin on the THP-1 cells was ascertained by flow cytometry (Fig. 5*B*).

Effect of anti-moesin pAbs on PBMCs, monocytes, and T cells from healthy individuals and AA patients

The expression of moesin on the T cells and monocytes as well as the TNF-α secretion from the THP-1 cells induced by anti-moesin pAbs suggested that anti-moesin pAbs in the AA patients' sera might also stimulate these immune cells from healthy individuals and AA patients to secrete cytokines. When the PBMCs from healthy individuals were incubated for 48 h in the presence of 5

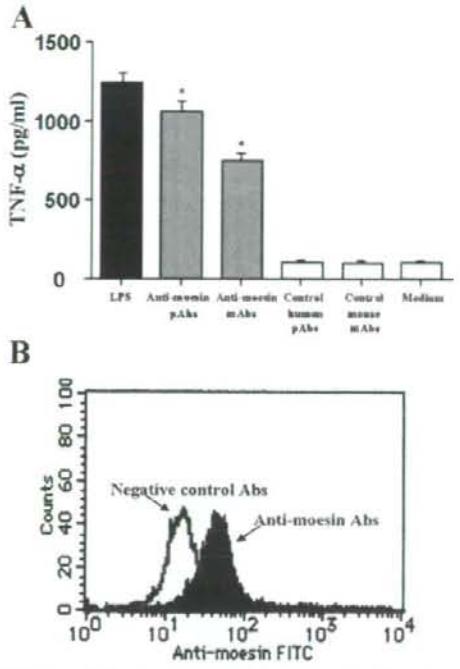

FIGURE 5. TNF-α release from THP-1 cells stimulated by anti-moesin Abs. *A*, THP-1 cells were cultured for 48 h with 5 μg/ml of anti-moesin Abs or control Abs. Anti-moesin pAbs, anti-moesin polyclonal IgG isolated from the serum of AA patients; control human pAbs, control human IgG pAbs isolated from healthy individuals; anti-moesin mAbs, anti-moesin mouse IgG1 mAbs (clone 38/87); control mouse mAbs, control mouse IgG1 mAbs. Then, 100 ng/ml LPS was used as a positive control. The data represent the mean TNF-α concentration ± SD of three experiments. *, $p < 0.01$ vs control Abs. *B*, The detection of moesin on THP-1 cells by anti-moesin pAbs purified from the serum of an AA patient.

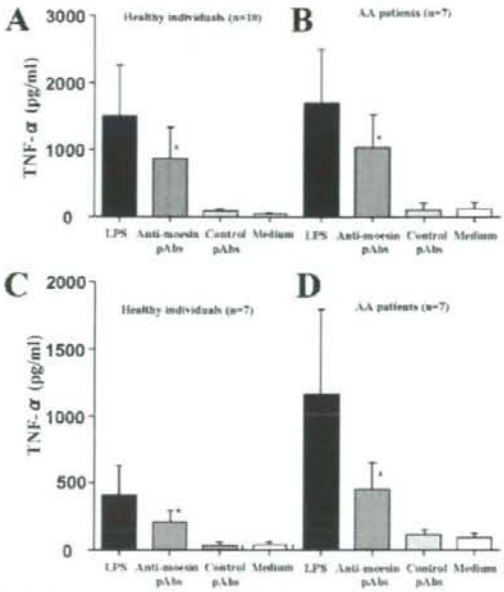

FIGURE 6. TNF-α release from PBMCs or monocytes stimulated by anti-moesin pAbs. The PBMCs or isolated monocytes were cultured for 48 h in the presence of 5 μg/ml of anti-moesin IgG pAbs or human control IgG pAbs isolated from the serum of AA patients or healthy individuals. Then, 100 ng/ml of LPS was used as a positive control. PBMCs isolated from 10 healthy individuals (*A*) and 7 AA patients (*B*), and monocytes separated from the PBMCs of 7 healthy individuals (*C*) and 7 AA patients (*D*), were used as targets. The data represent the mean TNF-α concentration ± SD. *, $p < 0.005$ vs control Abs.

μg/ml of anti-moesin pAbs, the amount of TNF-α in the culture medium was approximately 10 times more than those of control cultures and was more than half of that of the culture stimulated by 100 ng/ml of LPS (Fig. 6*A*). The same concentration of anti-moesin pAbs induced a similar amount of TNF-α from the PBMCs from AA patients (Fig. 6*B*). On the other hand, when monocytes isolated from the PBMC of healthy individuals or AA patients were used as a target, anti-moesin pAbs induced less than half the amount of TNF-α of that induced from whole PBMCs (Fig. 6, *C* and *D*).

The unexpectedly high inducibility of TNF-α secretion from the PBMCs by the anti-moesin pAbs prompted studies on the inducibility of IFN-γ secretion from the PBMCs by the Abs. Fig. 7, *A* and *B*, shows the effect of anti-moesin Abs on the IFN-γ secretion from PBMCs. Although anti-moesin pAbs alone could not induce IFN-γ secretion from the PBMCs derived from healthy individuals, the Abs stimulated PBMCs that were prestimulated with anti-CD3 mAbs to secrete nearly as much IFN-γ as that PHA did. In contrast, the PBMCs from the AA patients could secrete IFN-γ in response to anti-moesin pAbs without the prestimulation by anti-CD3 mAbs, and the amount of IFN-γ was approximately 40% as much as that of the culture stimulated by 10 μg/ml of PHA. In contrast, T cells isolated from the PBMC of healthy individuals or AA patients could not secrete a significantly larger amount of IFN-γ in response to anti-moesin pAbs compared with that in response to control IgG pAbs (Fig. 7, *C* and *D*), and the amount of IFN-γ secreted by T cells was one-tenth as much as that by PBMCs.

When the sera of the 16 AA patients comprising 7 anti-moesin Ab-positive and 9 anti-moesin Ab-negative patients were examined using ELISA, no significant differences in TNF-α and IFN-γ concentrations were observed between the 2 groups (TNF-α: 88.0 ± 106.3 pg/ml in anti-moesin Abs-positive patients, 90.1 ± 161.3 in anti-moesin Abs-negative patients; IFN-γ: 44.6 ± 33.8 pg/ml in anti-moesin Abs-positive patients, 47.5 ± 44.9 pg/ml in anti-moesin Abs-negative patients). None of the sera derived from four healthy donors showed detectable levels of TNF-α (>5 pg/ml) and IFN-γ (>5 pg/ml). On the other hand, when the BM plasma from five patients with AA was examined using ELISA, three anti-moesin Abs-positive patients showed higher levels of TNF-α (129, 338, and 349 pg/ml) compared with those of TNF-α (13 and 128 pg/ml) in two anti-moesin Abs-negative patients. IFN-γ concentrations of three anti-moesin Abs-positive patients were 29, 123, and 133 pg/ml, while those of two anti-moesin Abs-negative patients were 13 and 80 pg/ml. None of the BM plasma derived from three healthy donors showed detectable levels of TNF-α (>5 pg/ml) and IFN-γ (>5 pg/ml).

Discussion

The present study revealed that the proteins recognized by the anti-moesin Abs are detectable on the surface of various leukocytes subsets including T cells, NK cells, and monocytes as well as on T lymphocytic and monocytic leukemia cell lines. Moesin is an intracellular protein that links the cell membrane and cytoskeleton, and mediates the formation of microtubules and cell adhesion sites

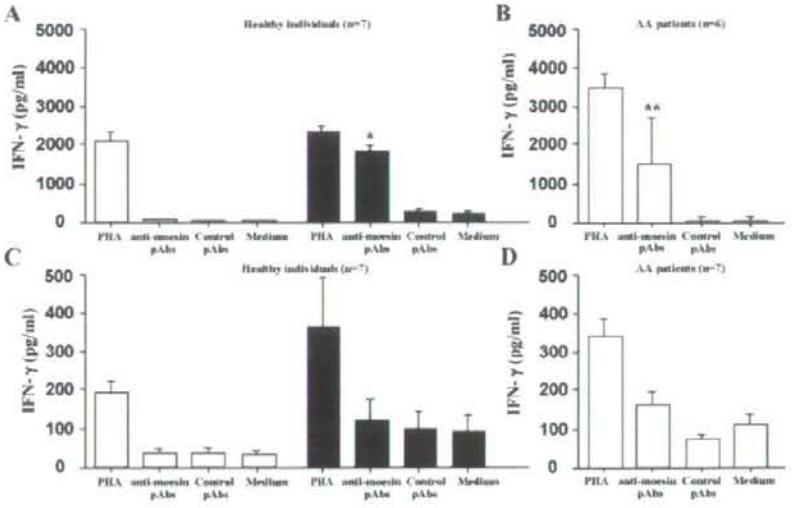

FIGURE 7. IFN-γ release from PBMCs or T cells stimulated by anti-moesin Abs. The PBMCs or isolated T cells were cultured for 48 h in the presence of 5 μg/ml of anti-moesin IgG pAbs or human control IgG pAbs isolated from the serum of AA patients or healthy individuals. Then, 10 μg/ml PHA was used as a positive control. Unprimed PBMCs (□) or CD3-primed PBMCs (■) were used for the culture. PBMCs were isolated from seven healthy individuals (*A*) and six AA patients (*B*). Uncostimulated T cells (□) or CD3-costimulated T cells (■) were used for the culture. T cells were isolated from the PBMCs of seven healthy individuals (*C*) and seven AA patients (*D*). The data represent the mean IFN-γ concentration ± SD. *, $p < 0.0001$ vs control Abs; **, $p = 0.04$ vs control Abs.

as well as ruffling of the cell membrane (17). This membrane-linking protein is expressed by various blood cells including megakaryocytes and granulocytes (23), but its expression was thought to be localized inside the cell membrane and not on the cell surface. Some studies revealed that anti-moesin Abs could bind to the surface of T cells (18) and macrophages (19) in keeping with our observation. However, none of the previous studies characterized the cell surface protein recognized by the anti-moesin Abs. Using biotin-labeled membrane proteins coupled with an avidin gel column and peptide massfinger printing, the present study identified the cell surface 80 kDa protein to be moesin. The decrease in the cell surface moesin induced by moesin shRNA has substantiated the presence of moesin on the cell surface of THP-1 cells.

Little is known about the function of anti-moesin Abs in vitro and in vivo. In contrast to our results, Amar et al. (24, 25) found that anti-moesin mAbs (clone 38) suppressed LPS-induced TNF-α secretion from monocytes through binding of moesin-like molecules on the cell surface. They used a different anti-moesin mAbs (clone 38) from the mAbs (clone 38/87) used in the present study. When we examined the effect of clone 38 mAbs on TNF-α secretion from THP-1 cells induced by LPS using the same condition as the one described by Amar et al. (24), a dose-dependent inhibition of TNF-α secretion was observed (data not shown). In contract to clone 38/87 mAb and pAbs from AA patients' sera, the clone 38 mAbs alone did not induce TNF-α secretion from THP-1 cells. Because the clone 38 preparation contains 1.5 mM sodium azide as a preservative, it is most likely that the dose-dependent inhibition of TNF-α secretion by clone 38 mAbs was due to toxic effect of sodium azide. Alternatively, clone 38 mAb which recognizes the C-terminal portion (554–564 amino acid residues) of moesin may exert a different effect on THP-1 cells from the effect of mAb clone 38/87 which recognizes the middle portion (317–398 amino acid residues) of moesin and from the effect of pAbs purified from AA patients' sera.

The present study revealed that both mAbs and pAbs specific to moesin stimulated the THP-1 cells to secrete TNF-α at an Ab concentration compatible to that in the serum of the AA patients. Moreover, anti-moesin pAbs were as potent as LPS in inducing TNF-α secretion from the PBMCs derived from healthy individuals and the AA patients. Our preliminary analyses on the activation of signaling pathways leading to TNF-α secretion from THP-1 cells showed the phosphorylation of ERK1/2 kinase induced by anti-moesin Abs (49th American Society of Hematology annual meeting abstract #1690, 2007 and submitted). In two patients from whom anti-moesin pAbs were purified, the Abs induced TNF-α release from autologous PBMCs. High concentrations of TNF-α were indeed present in the BM sera of two patients with high anti-moesin Ab titer. Although no difference in the serum TNF-α level was observed between anti-moesin Ab-positive and -negative patients, these findings suggest that anti-moesin Abs may induce a subtle amount of TNF-α from the monocytes or macrophages in the BM, thereby contributing to the pathogenesis of AA.

In contrast to TNF-α, IFN-γ was not induced by the anti-moesin pAbs alone from the PBMCs from healthy individuals, though anti-moesin pAbs augmented IFN-γ secretion from the PBMCs prestimulated with anti-CD3 mAbs. On the other hand, anti-moesin pAbs stimulated the PBMCs from the AA patients to secrete as much IFN-γ as did PHA. It has been shown that T cells from AA patients are in an activated state and are prone to produce IFN-γ in response to suboptimal stimuli (26). The amount of secreted TNF-α from isolated monocytes as well as the amount of secreted IFN-γ from isolated T cells was greatly reduced compared with those from unfractionated PBMCs. The inability to secrete a sufficient amount TNF-α and IFN-γ of isolated monocytes and T cells suggests that the interaction between monocytes and T cells may be required to efficiently respond to extrinsic stimuli as described by previous reports (27, 28). When the anti-moesin Abs titers in the serum were longitudinally measured in three patients, the Abs titer decreased in two patients in association with the response to immunosuppressive therapy, while the Abs titer increased in one patient who became dependent on transfusions due to the relapse of AA in comparison to the titer detected in remission (data not shown). The high titer TNF-α levels in BM plasma of patients showing high anti-moesin Abs titers and the decrease in the Ab titers in parallel with disease amelioration support the hypothesis that anti-moesin Abs are involved in the pathogenesis of AA by way of myelosuppressive cytokine induction from immunocompetent cells. One may wonder why high titer anti-moesin Abs in some AA patients do not induce hypercytokinemia. However, inability of T cell-stimulating Abs such as anti-CD3 Abs to induce IFN-γ secretion in vivo has been shown by previous reports (29, 30). There may be some regulatory mechanisms that mitigate T cell activation by stimulating Abs in vivo.

A previous study demonstrated the presence of anti-moesin Abs in 14–34% of patients with rheumatoid arthritis (11, 31), and a

case-control study on AA conducted by the International Agranulocytosis and Aplastic Anemia Study Group revealed that a past history of rheumatoid arthritis is significantly associated with the later development of AA (32). The anti-moesin pAbs derived from patients with rheumatoid arthritis also enhanced TNF-α secretion from THP-1 cells (data not shown). It is, therefore, possible that AA and rheumatoid arthritis may share pathogenetic mechanisms leading to a breakdown of immunologic tolerance to moesin. Anti-TNF-α therapy has been successfully used for patients with rheumatoid arthritis (33–35) as well as for some patients with myelodysplastic syndrome (36, 37). Recent reports have shown the efficacy of anti-CD20 Abs in restoring hematopoietic functions of AA (38, 39). Therefore, autoAbs capable of inducing cytokine secretion like anti-moesin Abs may be a new target of therapy for AA.

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Disclosures

The authors have no financial conflict of interest.

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ORIGINAL ARTICLE

CD16$^+$ CD56$^-$ NK cells in the peripheral blood of cord blood transplant recipients: a unique subset of NK cells possibly associated with graft-versus-leukemia effect

Xuzhang Lu[1], Yukio Kondo[1], Hiroyuki Takamatsu[1], Kinya Ohata[1], Hirohito Yamazaki[2], Akiyoshi Takami[3], Yoshiki Akatsuka[4], Shinji Nakao[1]

[1]Cellular Transplantation Biology, Kanazawa University Graduate School of Medical Science, Kanazawa, Ishikawa, Japan; [2]The Protected Environmental Unit, Kanazawa University Hospital, Kanazawa, Ishikawa, Japan; [3]Division of Transfusion Medicine, Kanazawa University Hospital, Kanazawa, Ishikawa, Japan; [4]Division of Immunology, Aichi Cancer Research Institute, Nagoya, Aichi, Japan

Abstract

A marked increase in CD16$^+$ CD56$^-$ NK cells in the peripheral blood (PB) was observed in a cord blood transplant (CBT) recipient with refractory acute myeloid leukaemia (AML) in association with attaining molecular remission. CD16$^+$ CD56$^-$ NK cells isolated from the patient became CD16$^+$CD56$^+$NKG2D$^+$ when they were cultured in the presence of IL-2. Although cultured CD16$^+$CD56$^-$ NK cells retained the killer-cell immunoglobulin receptor (KIR)-ligand (KIR-L) specificity and the patient's leukemic cells expressed corresponding KIR ligands, they killed patient's leukemic cells expressing ULBP2. The cytotoxicity by cultured CD16$^+$CD56$^-$ NK cells was abrogated by anti-ULBP2 antibodies. When leukemic cells obtained at relapse after CBT were examined, both the ULBP2 expression and susceptibility to the cultured NK cells decreased in comparison to leukemic cells obtained before CBT. An increase in the CD16$^+$CD56$^-$ NK cell count (0.5×10^9/L or more) in PB was observed in seven of 11 (64%) CBT recipients but in none of 13 bone marrow (BM) and eight peripheral blood stem cell (PBSC) transplant recipients examined during the similar period after transplantation. These findings suggest an increase in CD16$^+$CD56$^-$ NK cells to be a phenomenon unique to CBT recipients and that mature NK cells derived from this NK cell subset may contribute to the killing of leukemic cells expressing NKG2D ligands *in vivo*.

Key words CD56$^+$CD16$^-$ NK cell; NKG2D; graft-versus-leukemia; cord blood transplantation

Correspondence Shinji Nakao, Cellular Transplantation Biology, Kanazawa University Graduate School of Medical Science, Kanazawa University Hospital, 13-1 Takara-machi Kanazawa, Ishikawa 920-8640, Japan. Tel: +81-76-265-2274; Fax: +81-76-234-4252; e-mail: snakao@med3.m.kanazawa-u.ac.jp

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Cord blood transplantation (CBT) is being increasingly used for treatment of hematologic malignancies because its efficacy in the treatment of adult patients has been proven based on the findings of recent studies (1–4). One possible drawback of CBT is the less potent graft-versus-leukemia (GVL) effect than that of bone marrow transplantation (BMT) or peripheral blood stem cell transplantation (PBSCT) due to the immaturity of T cells contained in the cord blood (CB) graft (5). However, a recent study has shown the relapse rate after CBT to be comparable to that after BMT or PBSCT from human leukocyte antigen (HLA) matched sibling donors (1). Moreover, an analysis on the outcome of CBT for adult patients with acute myeloid leukaemia (AML) in Japan revealed that the rate of leukemic relapse after HLA-mismatched CBT was lower than that after HLA-matched CBT despite the fact that the incidence of graft-versus-host disease (GVHD) was similar between the two groups (Cord Blood Bank Network of Japan; unpublished observation). These clinical findings suggest that immunocompetent cells other than T cells may mediate the GVL effect after CBT.

Natural killer (NK) cells play a major role in the development of GVL effect after an HLA-mismatched stem cell transplantation (SCT) (6, 7). The GVL effect by NK cells depends on the presence of

HLA-mismatches and T cell recovery after SCT (8). Because CBT is often carried out from HLA-mismatched donors and is also associated with delayed T cell recovery (9–11), NK cells may be more likely to contribute to the development of GVL effect after CBT than after BMT or PBSCT. Few studies, however, have previously focused on the GVL effect by NK cells after CBT.

CB has a unique subset of NK cells characterized by a phenotype CD16$^+$CD56$^-$ (12–14). This NK cell subset is thought to be immature NK cells capable of differentiating into CD16$^+$CD56$^+$ NK cells (15). We recently observed an apparent increase in this NK cell subset in a patient who underwent reduced-intensity CBT for the treatment of relapsed AML after PBSCT from an HLA-compatible sibling donor. The patient achieved a molecular remission of AML in association with the NK cell increase. This observation prompted the characterization of CD16$^+$CD56$^-$ NK cells of this patient and other patients after allogeneic SCT. The present study revealed that CD16$^+$CD56$^-$ NK cells may potentially play a role in the development of the GVL effect in patients whose leukemic cells express NKG2D ligands.

Materials and methods

Patients

Peripheral blood (PB) was obtained from 11 CBT, 13 BMT (10 from related and three from unrelated donors), and eight PBSCT patients 2–135 months after transplantation. None of the patients had active graft-versus-host disease requiring corticosteroids at time of sampling or signs of infection. The original diseases of the CBT recipients included AML in four, non-Hodgkin's lymphoma (NHL) in four, myelodysplastic syndromes (MDS) in two and renal cell carcinoma in one. In the BMT recipients, those were AML in four, acute lymphoblastic leukemia (ALL) in four, MDS in three, chronic myeloid leukaemia (CML) in one, and aplastic anaemia (AA) in one while in the PBSCT recipients, those were AML in four, ALL in one, biphenotypic leukemia in two and NHL in one. All CBT recipients received an HLA-mismatched graft; the number of HLA mismatches between donor and recipient were two in seven, three in three and four in one. No HLA mismatch was observed between each donor and the BMT or PBSCT recipient except for six PBSCT recipients whose mismatches with their donors was one in two, two in one and three in one. This study was approved by our institutional review board and all patients gave their informed consent for the phenotypic and functional analyses of their peripheral blood mononuclear cells (PBMCs).

Phenotype analysis of PBMC after SCT and leukemia cells

The cell surface phenotype was determined by three-color flow cytometry. The cells were stained with various monoclonal antibodies (mAbs) specific to cell surface proteins including CD3, CD56, CD16, CD158a, CD158b (Becton Dickinson Pharmingen), NKG2A, NKG2D, NKp30, NKp44 and NKp46 (Beckman Coulter, Marseille, France). The expression of NKG2D ligands on leukemic cells from a CBT recipient was determined using mAbs specific to MICA/B (Becton Dickinson Pharmingen), ULBP1, ULBP2 and ULBP3 (R&D Systems, Minneapolis, MN).

Cell separation

PBMCs were isolated using density gradient centrifugation. NK cells were enriched by negative selection using immunomagnetic beads (Dynal NK cell isolation kit; Dynal Biotech, Lake success, NY) according to the manufacturer's recommendation (16). NK cell purity was confirmed by flow cytometry. CD16$^+$CD56$^+$ and CD16$^+$CD56$^-$ NK cells were separated from the enriched NK cells with anti-CD56-coated microBeads (MACS) by passing them through two sequential large-scale columns (Milteny Biotec, Gladbach, Germany) according to the manufacturer's instructions. CD158b$^+$ and CD158b$^-$ NK cells were separated with anti-CD158b-FITC Abs and anti-FITC microbeads.

NK cell culture

Isolated 2×10^6 CD16$^+$CD56$^+$ and CD16$^+$CD56$^-$ subsets were cultured with or without 2×10^5 irradiated (45 Gy) K562 cells transfected with the membrane-bound form of IL-15 and human 4-1BBL (K562-mb15-41BBL) kindly provided by Dr. Dario Campana of University of Tennessee College of Medicine (17) in RPMI1640 containing 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/mL streptomycin and 100 IU/mL IL-2 for 14 d. The cultured NK cells were washed with RPMI1640 and then were used for the cytotoxicity assay.

Transfection of 721-221 cells with retroviral vector

An HLA class I-negative B cell line 721–221 was transfected with retrovirus vectors containing HLA-C*0301 (.221-Cw3) or HLA-C*0401 (.221-Cw4) as described previously (18). Transfectants were selected in the presence of 0.1 mg/mL neomycin and 0.1 mg/mL puromycin. The surface expression of HLA-C molecules was confirmed by flow cytometry using a mAb HLA-ABC (Immunotech, Marseille, France). A clone exhibiting the highest

level of HLA-C expression was used as a target in the cytotoxicity assay.

Cytotoxicity assay

NK cell cytotoxicity was assessed using the standard chromium release assay, as described previously (19). In blocking experiments, anti-ULBP Abs were added at 10 μg/mL to the ^{51}Cr labeled target cells and target cells were incubated at 37°C for 30 min before the addition of NK cells. The percentage of specific lysis was calculated using the formula: 100 × (count per minute [cpm] released from test sample-cpm spontaneous release)/(cpm maximum release − cpm spontaneous release).

Statistical analysis

The significance of difference in the PB $CD16^+CD56^-$ cell count between CBT recipients and recipients of BM, PBSCT, or healthy individual was assessed by Student's *t*-test. The significance of difference in the time of sampling after SCT between CBT, BMT and PBSCT was assessed by Mann-Whitney test. *P*-values <0.05 were considered to be significant.

Results

An increase in the number of $CD16^+CD56^-$ NK cells in a CBT recipient

A 56-yr-old male (Patient 1) who relapsed with AML M0 after PBSCT from a sibling donor underwent CBT following preconditioning with fludarabin 125 mg/m^2, melphalan 80 mg/m^2, and 4 Gy TBI. The patient's leukemia was refractory to chemotherapy and there were 18% leukemic blasts in the PB at the time of preconditioning. He achieved complete chimerism in PB on day 22 after CBT. The WT1 copy number in BM RNA decreased from 13 000 copies/μg RNA before the start of preconditioning to 140 copies/μg RNA on day 60 (20). However, it rose to 1500 copies/μg RNA on day 80 after CBT. Although a molecular relapse was suspected, the WT1 copy number spontaneously decreased to 230 on day 172. Surface phenotype analysis of PB leukocytes on day 84 showed an increase in the count of $CD3^-CD16^+CD56^-$ NK cells (Fig. 1). The $CD16^+CD56^-$ NK cell count remained as high as 3.2–4.5 × 10^9/L for the following 11 months during which he remained in remission. The patient eventually relapsed with AML and died 16 months after CBT. The unexpected long term remission after reduced-intensity CBT associated with an increase in the $CD16^+CD56^-$ NK cell count prompted the characterization of the $CD16^+CD56^-$ NK cells of this patient and other patients who underwent allogeneic SCT.

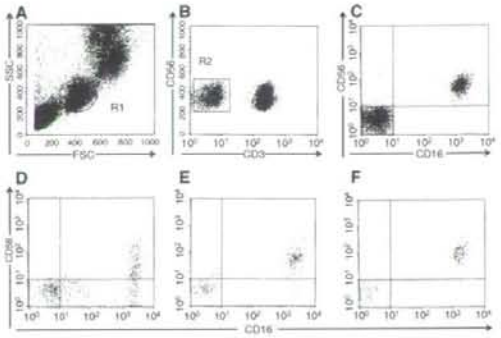

Figure 1 Phenotype of the $CD16^+$ NK cells in the peripheral blood. Representative results of flow cytometry on $CD3^-$ lymphocytes from SCT recipients and healthy individuals are shown. Gates were set up to exclude any $CD3^+$ lymphocytes as shown in (A) and (B); (C) a healthy individual; (D) a CBT recipient (Patient 1); (E) a BMT recipient; (F) a PBSCT recipient.

$CD16^+CD56^-$ NK cells in PB of allogeneic SCT recipients

Because the presence of $CD16^+CD56^-$ NK cells has been reported to be characteristics of CB, the proportion of PB $CD16^+CD56^-$ NK cells as well as their absolute count was determined for other recipients of CB and the other stem cell grafts. An increase in the $CD16^+CD56^-$ NK cell count greater than 0.5 × 10^9/L was seen in seven of 11 CBT recipients but in none of 13 BMT and eight PBSCT recipients (Figs 1 and 2). There was no significant difference in the time of sampling after SCT between CBT recipients and BMT recipients ($P > 0.772$) or CBT recipients and PBSCT recipients ($P > 0.265$). Both the $CD16^+CD56^-$ NK cell proportion and the absolute count were significantly higher in CBT recipients than in other SCT recipients or in healthy individuals. In contrast, there were no significant differences in the count of other NK cell subsets including $CD56^{dim}CD16^+$ and $CD56^{bright}CD16^-$ cells among these three SCT recipient groups (data not shown). A $CD16^+CD56^-$ NK cell increase greater than 1.5 × 10^9/L was restricted to Patient 1 and another CBT recipient with NHL (Patient 2). The $CD16^+CD56^-$ NK cell counts of Patient 2, 5 months and 15 months after CBT were 1.5 × 10^9/L and 1.8 × 10^9/L, respectively.

Surface phenotype of $CD16^+CD56^-$ NK cells and leukemic cells

To characterize this unusual NK cell subset, the surface phenotype was compared between $CD16^+CD56^-$ and $CD16^+CD56^+$ NK cells from Patient 1 and Patient 2

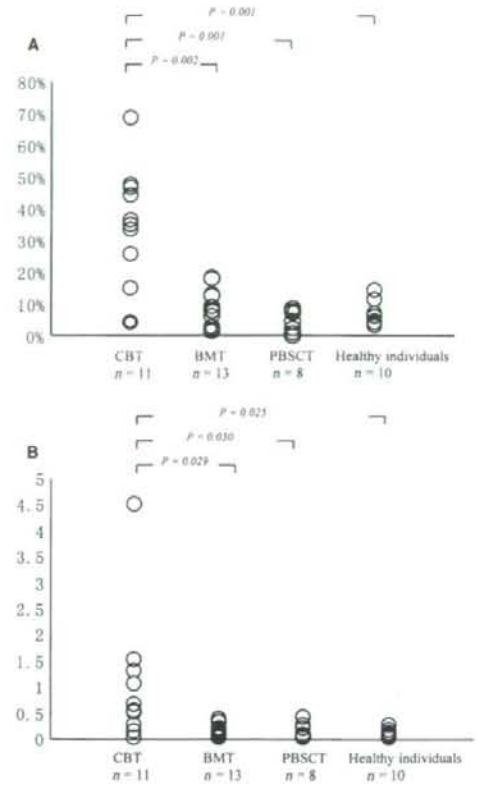

Figure 2 The proportion (A) and the absolute count (B) of $CD3^-CD16^+CD56^-$ in the PB of SCT recipients and healthy individuals. An increase in the proportion of $CD3^-CD16^+CD56^-$ NK cells (20% or more) in the PB $CD16^+$ NK cells and an increase in the absolute count of the same NK cell subset ($>0.5 \times 10^9$/L) were observed in seven of 11 CBT recipients, but in none of allogeneic 13 BM and eight PBSC transplant recipients. The $CD3^-CD16^+CD56^-$ cell count was calculated by multiplying the WBC count with the proportion (%) of this subset among the total cell event.

(Table 1). All $CD16^+CD56^-$ cells, similarly to $CD16^+CD56^+$ cells, expressed CD11a, CD18, but did not express a B-cell marker CD19, or the myeloid marker CD33 (data not shown). There were no differences in the expression levels of two major inhibitory NK receptors CD158a and CD158b between the two NK cell subsets (data not shown). On the other hand, the proportions of cells expressing activating NK receptors including NKG2D in $CD16^+CD56^-$ NK cells tended to be lower than those of $CD16^+CD56^+$ NK cells.

The leukemic cells obtained from Patient 1 before CBT exhibited an NKG2D ligand ULBP2 (Fig. 3). When the leukemic cells obtained after relapse was examined, the ULBP2 expression was observed to have decreased to levels comparable to ULBP1 and ULBP3.

Phenotypic change of $CD16^+CD56^-$ NK cells after *in vitro* culture

$CD16^+CD56^-$ NK cells derived from CB are reported to undergo differentiation *in vitro* in the presence of IL-2 (15, 21) and are therefore thought to be precursors of $CD16^+CD56^+$ NK cells (15). $CD16^+CD56^-$ NK cells were enriched from PBMCs of Patient 1 and Patient 2 and cultured in the presence of 100 IU/ml of IL-2 with or without irradiated K562-mb15-41BBL. In accordance with the results of previous studies, $CD16^+CD56^-$ NK cells from Patient 1 became $CD16^+CD56^+$ after *in vitro* culture (Fig. 4). Cultured $CD16^+CD56^-$ NK showed a tendency toward an increased expression of activating receptors including NKp30, NKp44, NKp46 and NKG2D, but did not show any changes in the expression of inhibitory receptors including CD158a, CD158b and NKG2A (Table 1).

Specificity of cultured $CD16^+CD56^-$ NK cells

Although attaining molecular remission in association with an increase in the $CD16^+CD56^-$ NK cells suggests the involvement of these NK cells in the GVL effect,

Table 1 Phenotype of the NK cell subsets from two CBT recipients

			NKp30		NKp44		NKp46		NKG2D	
			%	MFI	%	MFI	%	MFI	%	MFI
Patient 1	$CD56^+CD16^+$	Fresh	3.7	11.5	0	7.51	56.7	37.9	61.0	35.6
		Cultured	43.1	33.2	71.2	88.9	61.3	48.1	100.0	156.0
	$CD56^+CD16^-$	Fresh	0.0	8.37	0.0	7.57	17.6	12.6	46.7	12.6
		Cultured	14.2	10.4	51.4	31.0	54.2	26.8	99.9	26.8
Patient 2	$CD56^+CD16^+$	Fresh	3.6	6.71	0.0	7.72	42.9	44.3	72.3	44.3
		Cultured	14.2	39.4	51.4	49.4	54.2	54.4	99.5	54.4
	$CD56^+CD16^-$	Fresh	0.0	8.65	0.0	8.31	21.5	16.9	69.0	16.9
		Cultured	58.1	47.6	66.3	51.4	75.2	64.8	98.5	64.8

$CD16^+CD56^-$ and $CD16^+CD56^-$ NK cells were isolated from two CBT recipients and cultured with irradiated K562-mb15-41BBL in the presence of IL-2 for 14 d. Cultured NK showed increased expression of activating NK receptors including NKp30, NKp44, NKp46 and NKG2D.

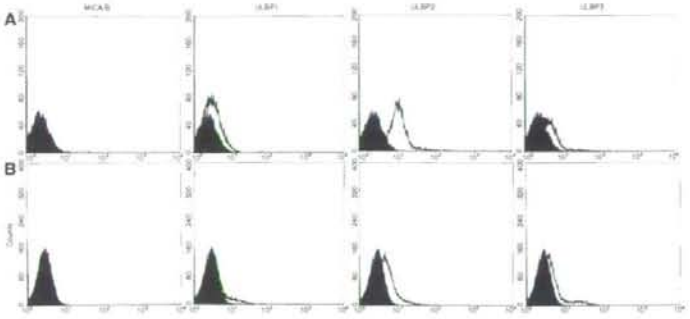

Figure 3 Expression of NKG2D ligands on leukemic cells from Patient 1. (A) leukemic cells obtained before CBT; (B) leukemic cells obtained after relapse. The proportion of ULBP2 expressing leukemic cells decreased from 59% to 9%.

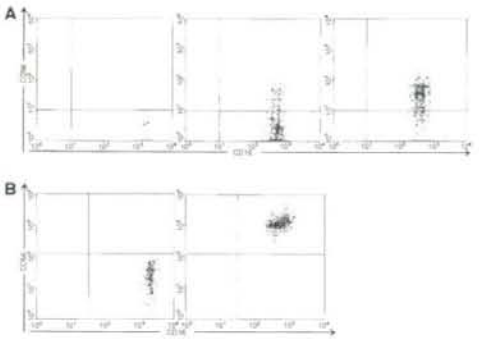

Figure 4 Phenotypic change of CD16⁺CD56⁻ NK cells with time associated with *in vitro* culture. Isolated CD16⁺CD56⁻ cells from Patient 1 were cultured in the presence of 100 IU/L IL-2 without (A) or with K562-mb15-41BBL (B). CD16⁺CD56⁻ NK cells from CBT recipients became CD16⁺CD56⁺ after the *in vitro* culture.

there was no killer-cell immunoglobulin receptor (KIR)-ligand (KIR-L) mismatch between Patient 1 and the CB donor; Patient 1 and the CB donor shared C*0102 and C*0304. To determine whether cultured NK cells derived from CD16⁺CD56⁻ NK cells retain specificity restricted by KIR-L of target cells, cultured NK cells from Patient 1 and Patient 2 who possessed C*0102 and C*1202 were separated into CD158b⁺ and CD158b⁻ NK cells, and were examined for their cytotoxicity against 721–221 cells transfected with different HLA-C alleles (Fig. 5). CD158b⁺ NK cells failed to kill 721–221 cells transfected with HLA-C*0301 (.221-Cw3) while they killed both wild-type 721–221 cells and 721–221 cells transfected with HLA-C*0401 (.221-Cw4). Conversely, CD158b⁻ NK cells not only killed 721–221 cells but they also killed .221-Cw3 and .221-Cw4 cells, thus indicating that the cytotoxicity due to the cultured CD158b⁺ NK cells is inhibited by the KIR-L Cw3 of the target cells.

Cytotoxicity of cultured CD16⁺CD56⁻ NK cells against leukemic cells

When leukemic cells obtained from Patient 1 before CBT were used as a target, both CD158b⁺ and CD158b⁻ NK cells showed similar cytotoxicity to that of unfractionated NK cells (Fig. 6). The cytotoxicity was blocked by

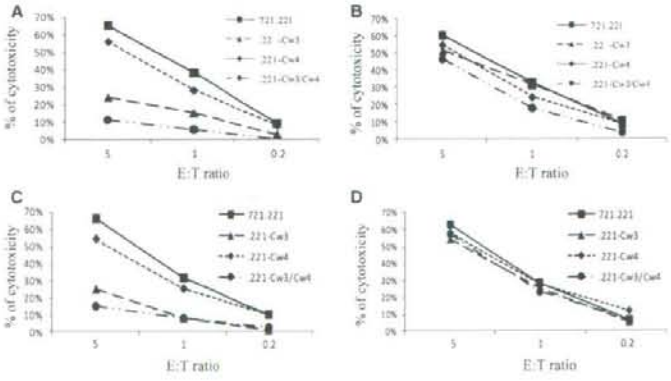

Figure 5 Specificity of NK cells derived from CD16⁺CD56⁻ NK cells. Cultured NK cells derived from CD16⁺CD56⁻ cells of Patient 1 (A and B) and Patient 2 (C and D) were separated into CD158b⁺ (A and C) and CD158b⁻ cells (B and D) and were examined for the cytotoxicity against 721-221 cells and 721-221 transfected with different HLA-C alleles C*0301 (.221-Cw3) and C*0401 (.221-Cw4). The data represent one of two experiments which produced similar results.

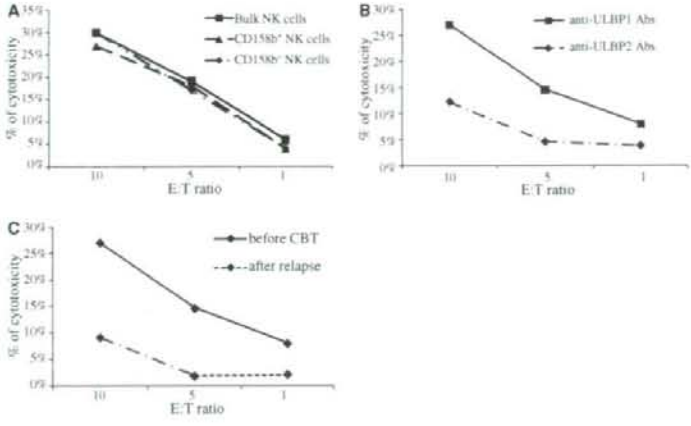

Figure 6 Cytotoxicity of cultured NK cells against leukemic cells. (A) Unseparated and separated NK cells were tested against leukemic cells obtained before CBT; (B) Leukemic cells were incubated in the presence of anti-ULBP1 or ULBP2 Abs before incubation with cultured NK cells; (C) Cytotoxicity of unseparated NK cells were tested against leukemic cells obtained before CBT or after relapse. The data represent one of three experiments which produced similar results.

treatment of leukemia cells with anti-ULPB2 mAbs. Leukemic cells obtained after relapse were relatively resistant to killing by cultured NK cells in comparison to those obtained before CBT.

Discussion

The present study revealed an increase in a unique NK cell subset characterized by CD16$^+$CD56$^-$ in CBT recipients. Although CD3$^-$CD16$^+$CD56$^-$ cells comprise monocytes, an increase in this subset was due to an increase in immature NK cells because they did not express a myeloid marker CD33 and acquired CD56 expression by *in vitro* culture in the presence of IL-2. An increase in NK cells with a similar phenotype has been shown in patients with solid tumors who were treated with IL-2 (21) and in those with HIV infection (22). Our CBT recipients did not receive cytokine therapy nor show any signs of viral infections at sampling. The expression of KIRs including CD158a and CD158b was not depressed in CD16$^+$CD56$^-$ cells of Patient 1 and Patient 2 in contrast to those of HIV patients (22). An *in vitro* culture of CD16$^+$CD56$^-$ NK cells from patients with HIV viremia in the presence IL-2 reportedly failed to induce NKp44 expression while it did induce the NKp44 expression by CD16$^+$CD56$^-$ NK cells from the two CBT recipients. It is therefore unlikely that the increase in the CD16$^+$CD56$^-$ cell count in the CBT recipients was secondary to viral infections.

Gaddy *et al.* demonstrated a novel subset of NK cells characterized by a phenotype CD16$^+$CD56$^-$ to exist in CB (12). They hypothesized that this NK cell subset represents immature NK cells capable of differentiating into CD16$^+$CD56$^+$ NK cells (15). CD16$^+$CD56$^-$ cells of our patients also underwent differentiation into CD16$^+$CD56$^+$ cells when they were cultured in the presence of IL-2. Therefore, CD16$^+$CD56$^-$ cells in PB after CBT may be derived from immature NK cells or NK precursor cells which existed in CB grafts. Previous studies on NK cells from SCT recipients and *ex vivo* engineered CB NK cells did not reveal an increased proportion of CD16$^+$CD56$^-$ cells (23–25). Both Patient 1 and Patient 2 received an HLA-mismatched CB graft although there was no KIR-L mismatch. Notably, Patient 1 had a large leukemic burden at the time of reduced-intensity preconditioning. It is therefore plausible that residual leukemic cells may have stimulated NK cell precursors to recruit CD16$^+$CD56$^-$ NK cells in Patient 1.

Patient 1's leukemic cells obtained before CBT expressed ULBP2. The incubation of CD16$^+$CD56$^-$ NK cells derived from Patient 1 in the presence of IL-2 and the K562 transfectant augmented NKG2D expression and the cultured NK cells showed cytotoxicity against leukemic cells despite that cultured NK cells retained KIR-L specificity and Patient 1's leukemic cells expressed matched KIR-L HLA-C*0304/C*0102. The cytotoxicity by the cultured NK cells decreased against leukemic cells treated with anti-ULBP2 Abs, and also against the leukemic cells obtained from Patient 1 after relapse which were devoid of ULBP2 expression. These findings suggest that mature NK cells derived from CD16$^+$CD56$^-$ NK cells may have exerted GVL effect on Patient 1's leukemic cells by way of interaction of NKG2D and ULBP2. The aberrant expression of NKG2D ligands by leukemic cells has been demonstrated by previous studies (26), but its influence on the outcome of allogeneic SCT has not yet been clarified. The results of the present study

indicate that the susceptibility of leukemic cells to NK cells may depend on both expression of NKG2D ligand on leukemic cells and the expression of NKG2D on effector NK cells. In patients with acute leukemia, leukemic cells are reported to downregulate NKp30 of autologous NK cells, thereby allowing NK cells to escape leukemic cells (27, 28). In the setting of CBT, leukemic cells expressing NKG2D ligands may tend to stimulate NK cell precursors in CB, thus inducing them to undergo differentiation.

The present study demonstrated the expansion of CD16$^+$CD56$^-$ NK cells in the PB of CBT recipients for the first time. These immature NK cells can be expanded *ex vivo* with a help of K562-mb15-41BBL cells as maintaining specificity to KIR-L and cytotoxicity against leukemic cells expressing an NKG2D ligand. Therefore, CB may be a potential source of NK cells which can be utilized for cell therapy. Further studies on a larger number of CBT recipients are needed to determine whether CD16$^+$CD56$^-$ NK cells indeed play a role in the GVL effect.

Acknowledgement

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Expansion of donor-derived hematopoietic stem cells with PIGA mutation associated with late graft failure after allogeneic stem cell transplantation

Kanako Mochizuki, Chiharu Sugimori, Zhirong Qi, Xuzhang Lu, Akiyoshi Takami, Ken Ishiyama, Yukio Kondo, Hirohito Yamazaki, Hirokazu Okumura and Shinji Nakao

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Brief report

Expansion of donor-derived hematopoietic stem cells with *PIGA* mutation associated with late graft failure after allogeneic stem cell transplantation

*Kanako Mochizuki,[1] *Chiharu Sugimori,[1] Zhirong Qi,[1] Xuzhang Lu,[1] Akiyoshi Takami,[1] Ken Ishiyama,[1] Yukio Kondo,[1] Hirohito Yamazaki,[1] Hirokazu Okumura,[1] and Shinji Nakao[1]

[1]Cellular Transplantation Biology, Division of Cancer Medicine, Kanazawa University Graduate School of Medical Science, Ishikawa, Japan

A small population of CD55$^-$CD59$^-$ blood cells was detected in a patient who developed donor-type late graft failure after allogeneic stem cell transplantation (SCT) for treatment of aplastic anemia (AA). Chimerism and *PIGA* gene analyses showed the paroxysmal nocturnal hemoglobinuria (PNH)–type granulocytes to be of a donor-derived stem cell with a thymine insertion in *PIGA* exon 2. A sensitive mutation-specific polymerase chain reaction (PCR)–based analysis detected the mutation exclusively in DNA derived from the donor bone marrow (BM) cells. The patient responded to immunosuppressive therapy and achieved transfusion independence. The small population of PNH-type cells was undetectable in any of the 50 SCT recipients showing stable engraftment. The de novo development of donor cell–derived AA with a small population of PNH-type cells in this patient supports the concept that glycosyl phosphatidylinositol–anchored protein–deficient stem cells have a survival advantage in the setting of immune-mediated BM injury. (Blood. 2008;112:2160-2162)

Introduction

Although small populations of CD55$^-$CD59$^-$ blood cells are often detectable in patients with aplastic anemia (AA), it remains unclear how such paroxysmal nocturnal hemoglobinuria (PNH)-type cells arise.[1] We recently encountered a patient with immune-mediated late graft failure (LGF) following allogeneic stem cell transplantation (SCT) for treatment of AA. Analyses of the patient's peripheral blood (PB) and bone marrow (BM) showed hematopoietic stem cells (HSCs) of donor origin with mutant *PIGA*, supporting the concept that glycosyl phosphatidylinositol–anchored protein (GPI-AP)–deficient stem cells have a survival advantage in the setting of immune mediated BM injury.

Methods

Patients

A 59-year-old man underwent allogeneic PBSCT from a human leukocyte antigen (HLA)–matched sibling donor after conditioning with fludarabin (120 mg/m^2), cyclophosphamide (1200 mg/m^2), and antithymocyte globulin (60 mg/kg) for treatment of very severe AA in April 2002 (Table 1) and achieved complete donor chimerism with normal blood cell counts. In January 2006, he developed pancytopenia and was diagnosed as having LGF without residual recipient cells. The patient underwent a second PBSCT from the original donor without preconditioning on February 8, 2006. Pancytopenia resolved completely by day 16 after PBSCT. However, at approximately day 60, the blood counts decreased gradually, and the patient became transfusion-dependent. On day 196 after the second PBSCT, the white blood cell (WBC) count was 5.3×10^9/L with 17% neutrophils, the hemoglobin concentration was 75 g/L, and the platelet count was 22×10^9/L. Treatment with horse antithymocyte globulin (ATG) and cyclosporine was started on day 205 after the second PBSCT. Transfusions were terminated after 88 days of the immunosuppressive therapy. Although the patient presently receives low-dose tacrolimus for treatment of chronic graft-versus-host disease, which developed 1 year after the second PBSCT, his pancytopenia has markedly improved as shown in Table 1. PB and BM of the patient were subjected to analyses of chimerism and flow cytometry to detect CD55$^-$CD59$^-$ cells and *PIGA* gene analysis.

As controls, the PB from 51 SCT recipients (48 with hematologic malignancies and 3 with AA) who achieved a complete recovery of donor-derived hematopoiesis were subjected to flow cytometric analysis for the screening of CD55$^-$CD59$^-$ cells. Of the 51 patients, 4 and 23, respectively, had acute graft-versus-host disease (GVHD) of grade II or higher and chronic GVHD at sampling.

BM aspirates were obtained from the patient's donor and 10 healthy individuals for *PIGA* gene analysis. Informed consent was obtained from all patients and healthy individuals in accordance with the Declaration of Helsinki for blood examination, and the experimental protocol for *PIGA* gene analysis was approved by our participating institutional ethics committee (No.157).

Detection of PNH-type cells

To detect GPI-AP deficient (GPI-AP$^-$), PNH-type cells, we performed high-sensitivity 2-color flow cytometry of granulocytes and red blood cells (RBCs), as described previously.[1] The presence of 0.003% or more CD55$^-$CD59$^-$CD11b$^+$ granulocytes and 0.005% or more CD55$^-$CD59$^-$glycophorin-A$^+$ RBCs was defined as an abnormal increase based on the results in 183 healthy individuals.[2]

Cell sorting and chimerism analysis

CD3$^+$cells were isolated from the PB mononuclear cells of the patient using magnetic-activated cell sorting (MACS) CD3 Microbeads (Miltenyi Biotec, Auburn, CA). The CD55$^-$CD59$^-$CD11b$^+$ granulocytes were separated from the CD55$^+$CD59$^+$CD11b$^+$ granulocytes with a cell sorter (JSAN; Bay Bioscience, Yokohama, Japan). More than 95% of the sorted cells were

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*K.M. and C.S. contributed equally to this work.

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Table 1. Hematologic parameters of donor and recipient

	Donor		Recipient			
Date	Apr 2002	May 2008	Before 1st SCT Apr 2002	Before 2nd SCT Jan 2006	At ATG therapy Aug 2006	After 20 mo of ATG therapy Apr 2008
WBC count, $\times 10^9$/L	7.0	5.1	1.2	1.7	5.3	4.0
Neutrophil proportions, %	77	65	0	0	17	62
RBC count, $\times 10^{12}$/L	4.21	4.43	2.20	2.75	2.07	3.04
Reticulocytes, $\times 10^9$/L	not tested	35	2	3	26	61
Hemoglobin, g/L	146	150	72	89	75	120
Platelet count, $\times 10^9$/L	261	230	19	52	22	54

$CD55^-CD59^-CD11b^+$. The *D1S80* locus was amplified from DNA of different cell populations with an AmpliFLP D1S80 PCR Amplification Kit (Perkin-Elmer Cetus, Norwalk, CT).

PIGA gene analysis

The coding regions of *PIGA* were amplified by seminested PCR or nested PCR from DNA extracted from the sorted PNH-type cells using 12 primer sets[3,4] (Table S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article), and 6 ligation reactions were used to transform competent *Escherichia coli* JM109 cells (Nippon Gene, Tokyo, Japan). Five clones were selected randomly from each group of transfectants and subjected to sequencing with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Amplification refractory mutation system PCR

On the basis of a mutant sequence detected in *PIGA* of the patient, a nested amplification refractory mutation system (ARMS) forward primer with a 3′-terminal nucleotide sequence complementary to the mutant sequence was prepared[5] (Table S1). To enhance the specificity, a mismatch at the penultimate nucleotide position of the mutation site was incorporated in the ARMS forward primer (P1).[6,7] P1 and a reverse primer (P3) were used to amplify a 127 bp fragment containing the mutant sequence from the exon 2 amplified product. PCR was conducted under the following conditions; denaturation for 30 seconds at 94°C, annealing for 60 seconds at 64°C and extension for 90 seconds at 72°C for 20 cycles. Another forward primer (P2), complementary to the wild-type *PIGA* sequence upstream of the mutation site, was used in combination with P3 to amplify an internal control according to the same condition of ARMS-PCR.

Results and discussion

PNH-type cells were not detected in the donor or the patient at the time of development of the first LGF, whereas 0.147% PNH-type granulocytes and 0.019% PNH-type RBCs were detected in the PB

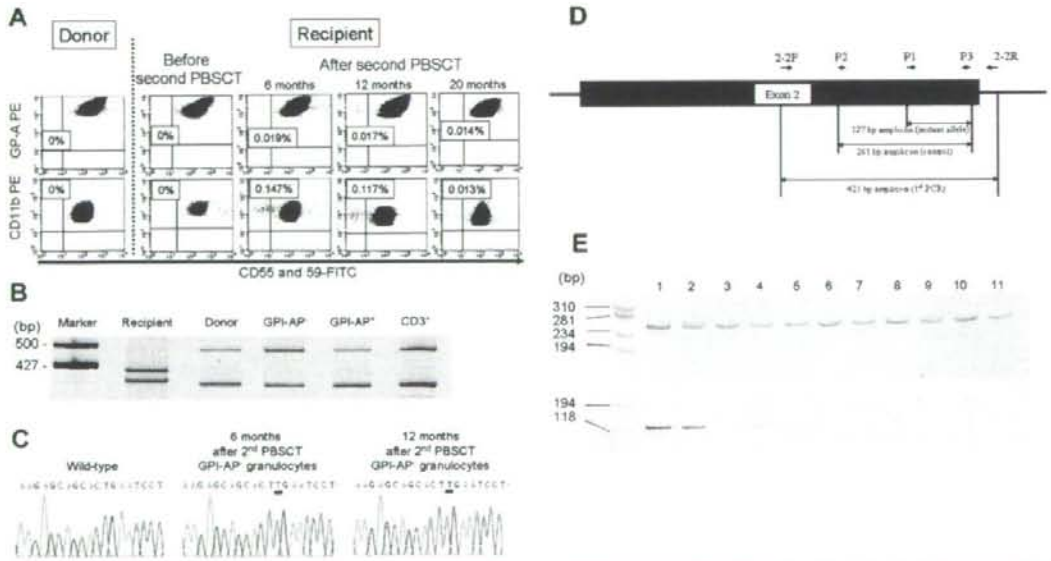

Figure 1. Analysis of PNH-type cells after the second PBSCT. (A) High-sensitivity flow cytometry detected small populations of $CD55^-CD59^-$ cells in both granulocytes and red blood cells at the development of the second LGF as well as in those obtained 6 and 12 months later, but did not detect PNH-type cells in the donor or in the recipient before the second PBSCT. The numbers denote the proportion of PNH-type cells in $CD11b^+$ granulocytes or glycophorin A^+ RBCs. (B) *D1S80* allelic patterns of sorted $GPI\text{-}AP^-$ granulocytes, $GPI\text{-}AP^+$ granulocytes, and $CD3^+$ lymphocytes. The polymerase chain reaction (PCR) products were subjected to 8% polyacrylamide gel electrophoresis and visualized by silver staining. (C) Nucleotide sequences of *PIGA* exon 2 in DNA from PNH-type granulocytes obtained 6 and 12 months after the second PBSCT. (D) A schematic illustration for ARMS-PCR is shown. Primer positions for the first, second are shown by short arrows. A black box and adjacent lines represent exon 2 and introns, respectively. (E) Amplified products of control PCR (the upper gel) and ARMS-PCR (the lower gel) were electrophoresed in 12.5% polyacrylamide gel and visualized by the silver staining. A pMD20-T vector containing the mutated exon 2 fragment was used as a positive control for ARMS-PCR. The template DNA derives from a plasmid containing the mutated exon 2 in lane 1, donor BM in lane 2, donor PB in lane 3, recipient BM in lane 4, recipient PB in lane 5, and BM from healthy individuals in lanes 6 to 11. PCR with a 5′ primer specific to the nucleotide sequence upstream of the mutated sequence amplified a 261 bp fragment from DNA of the donor and all healthy individuals.

obtained at the time of development of the second LGF (Figure 1A). Similar percentages of PNH-type blood cells were detectable in the PB of the patient 6 and 14 months later. When PB from 51 SCT recipients was examined, none of the patients were found to have detectable PNH-type cells (data not shown). PNH-type blood cells were also undetectable in a donor PB sample obtained 21 months later.

The *D1S80* locus allelic pattern of the PNH-type granulocytes in the patient was compatible to that of the donor (Figure 1B). The emergence of donor-derived PNH-type cells and hematologic improvement after immunosuppressive therapy suggest that LGF arises as a result of de novo development of AA which affects the donor-derived hematopoietic stem cells (HSCs).

PIGA gene analysis of the DNA prepared from the sorted PNH-type cells of the patient obtained at the development of LGF and 6 months later showed an insertion of thymine at position 593 (codon 198) in 3 of 5 clones and 5 of 5 clones examined, respectively (Figure 1C). Mutations in other exons were not identified. The presence of a single *PIGA* mutation in PNH-type granulocytes and its persistence over 6 months suggest that these PNH-type cells are derived from a mutant HSC rather than from a committed granulocyte progenitor cell. Moreover, an ARMS-PCR with a 5′ primer specific to the mutated sequence amplified a 127 bp fragment from DNA of the donor BM as well as of the recipient BM and PB while it failed to amplify the same fragment in donor PB and in BM of all 10 healthy individuals (Figure 1D).

These experiments demonstrate that *PIGA*-mutant HSCs were present in the BM of the donor in a dormant state and were transplanted into the recipient and provide, for the first time, in vivo evidence that *PIGA* mutant, GPI-AP–deficient HSCs have a survival advantage in the setting of immune mediated BM injury. Similarly, relative resistance to immune injury likely accounts for the high incidence of PNH observed in association with acquired AA.

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Authorship

Contribution: K.M. and C.S. participated in designing and performing the research. Z.Q. and X.L. performed experiments. K.M., C.S., and S.N. wrote the paper. C.S., A.T., K.I., Y.K., H.Y., and H.O. provided patient care. All authors have approved the final version of the manuscript.

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Correspondence: Shinji Nakao, Cellular Transplantation Biology, Division of Cancer Medicine, Kanazawa University Graduate School of Medical Science, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8641, Japan; e-mail: snakao@med3.m.kanazawa-u.ac.jp.

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