for other strategies for preventing HHV-6 infection, because ganciclovir is associated with BM suppression. This study showed that the earlier the reactivation occurred, the more the increase in the HHV-6 viral load; the viral load increased within 48 h after the negative test to a level greater than 5×10^2/mL. Thus, it may be important to ensure that the test result for HHV-6 DNA measurement can be returned on the day of blood sampling, or at least by the following morning, to make the preemptive approach successful. However, most hospitals are unable to get results in this short time. It is possible that prophylactic PFA administration before the time of leukocyte recovery would be a more reasonable approach rather than preemptive PFA administration after the identification of HHV-6 DNA.

To prevent limbic encephalitis, what needs to be treated with PFA is HHV-6 DNAemia that occurs before the rise in the leukocyte count. Therefore, prophylactic administration of PFA from day 7 or earlier to day 20 may be a more reasonable approach than preemptive PFA administration guided by HHV-6 DNA detection to prevent limbic encephalitis in UCBT recipients. The efficacy of such prophylactic administration of PFA after UCBT is now being examined in a prospective study.

Conflict of interest

The authors declare no conflict of interest.

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BIOLOGY

Mycophenolic Acid Inhibits Natural Killer Cell Proliferation and Cytotoxic Function: A Possible Disadvantage of Including Mycophenolate Mofetil in the Graft-Versus-Host Disease Prophylaxis Regimen

Kinya Ohata, J. Luis Espinoza, Xuzhang Lu, Yukio Kondo, Shinji Nakao

To determine how immunosuppressant agents used for graft-versus-host disease (GVHD) prophylaxis affect natural killer (NK) cells, we examined the effects of cyclosporine (CSP), tacrolimus (TAC), mycophenolic acid (MPA, an active form of mycophenolate mofetil), and methotrexate (MTX) on the proliferation and cytotoxicity of NK cells. The proliferation of NK cells from healthy individuals in the presence of interleukin (IL)-2 and IL-15 was suppressed to 51% ± 16% of that of the controls with CSP, to 31% ± 19% with TAC, to 14% ± 6% with MPA, and to 87% ± 18% with MTX. Both CSP and TAC increased the proportion of $CD16^{-}CD56^{bright}$ cells, a NK cell subset capable of secreting high amount of cytokines, and also enhanced NKp30 expression, whereas MPA markedly decreased the proportion of $CD16^{-}CD56^{bright}$ cells and reduced the expression of all activating NK cell receptors, including NKG2D, NKp30, NKp44, and NKp46. MPA also reduced the cytotoxicity against K562 cells from 61% ± 15% to 17% ± 7% and that against Daudi cells from 44% ± 4% to 4% ± 4%, whereas the other 3 drugs did not diminish these cytotoxicities. The inhibition of NK cell proliferation and cytotoxicity against leukemic cell lines by MPA was partially abolished by the inclusion of guanosine in the culture. Similar to the effect of MPA on T cells, MPA inhibited the down-regulation of p27 on NK cells induced by the incubation of NK cells in the presence of IL-2. These results suggest that MPA is a potent inhibitor of NK cells, and that its inclusion in the GVHD prophylaxis regimen might diminish the graft-versus-leukemia effect of NK cells.

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KEY WORDS: Allogeneic stem cell transplantation, Graft-versus-leukemia effect, Cyclosporine, Tacrolimus, Methotrexate, p27

INTRODUCTION

Graft-versus-host disease (GVHD) and disease relapse are major causes of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (allo-HSCT) for hematologic disease. Both murine and human studies show that natural killer (NK) cells mediate a number of potentially beneficial effects after allo-HSCT, including the elimination of residual malignant cells and virally infected cells [1,2]. The Perugia group demonstrated that NK cells play a critical role in the development of the graft-versus-leukemia (GVL) effect [2,3]. Patients with acute myelogenous leukemia (AML) who received a haploidentical transplant from a killer immune globulin–like receptor (KIR) ligand–mismatched donor in the GVHD direction showed a marked reduction in the relapse rate compared with an otherwise similar group of AML patients without this mismatch (3% vs 47%) [4].

In contrast to several subsequent reports supporting that original report, a large retrospective study of T cell–replete transplantation from the National Marrow Donor Program showed no decrease in the relapse rate of AML following KIR-mismatched transplantation [5]. Similarly negative results have been reported by other groups as well [6,7]. Those studies differed in various respects, including donor source (related vs unrelated), HLA parity of donors (haploidentical vs matched unrelated), T cell content in the graft (strictly depleted vs T cell replete), and the use of posttransplantation immunosuppression (absent vs present). The GVHD prophylaxis regimen may particularly affect the potency of the GVL effect by

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NK cells. Wang et al. [8] recently reported that cyclosporine (CSP) augments NK cell cytotoxicity against leukemia cell lines.

In addition to CSP and methotrexate (MTX), new immunosuppressants, including tacrolimus (TAC) and the inosine monophosphate dehydrogenase (IMPDH) inhibitor mycophenolate mofetil (MMF), are being successfully used for GVHD prophylaxis. Although sparing NK cells from the inhibitory effect of immunosuppressants is potentially important to retain the GVL effect after allo-HSCT, precisely how these new drugs influence NK cells remains to be clarified. In the present study, we compared the effects of 4 different immunosuppressants on NK cells and found a potent inhibitory effect of mycophenolic acid (MPA), an active form of MMF, on NK cells.

MATERIALS AND METHODS

$CD3^-CD56^+$ NK Cell Enrichment

Peripheral blood mononuclear cells (PBMCs) were isolated from 7 healthy donors using lymphocyte separation media. After washing with phosphate-buffered saline, PBMCs were subjected to NK cell enrichment using the negative selection with immunomagnetic beads (Dynal NK cell isolation kit; Invitrogen Dynal AS, Smestad, Norway) according to the manufacturer's recommendations. Enriched NK cells contained more than 85%-90% $CD56^+CD3^-$ (data not shown).

NK Cell Culture

Enriched NK cell populations were cultured in Dulbecco's Modified Eagle's Medium/Hams F12 (2:1) with 10% human AB^- sera, 2-β mercaptoethanol (24 μM), L-ascorbic acid (24 mg/L), sodium selenite (50 μg/L), ethanolamine (50 μM), and penicillin-streptomycin (100 U/mL of each). At the start of each culture, interleukin (IL)-2 (100 U/mL) and IL-15 (10 ng/mL) were added to the culture medium [8]. Then 4 different immunosuppressive drugs were added to the culture at the following concentrations: CSP, 1000 ng/mL; TAC, 20 ng/mL; MPA, 10 μg/mL; and MTX, 100 ng/mL. Each drug was diluted with ethanol and included in the culture at the indicated concentration. Vehicle control cultures were set up in parallel using ethanol (EtOH) at a final concentration of 0.1%. In some experiments, guanosine was included in the culture at a concentration of 100 μM with MPA.

Reagents and Cell Lines

The immunosuppressants were kindly provided by the producers: CSP, Novartis Pharma, Tokyo, Japan; TAC, Astellas Pharma, Tokyo, Japan; MTX, Wyeth, Tokyo, Japan; and MPA, Roche Bioscience, Tokyo, Japan. Guanosine was purchased from Sigma-Aldrich (St Louis, MO). K562 and Daudi were purchased from the Health Science Research Resources Bank (Osaka, Japan) and RIKEN BRC (Ibaraki, Japan), respectively.

Phenotypic Analysis of NK Cells

The NK cell surface phenotype was determined by 3-color flow cytometry. Fresh or cultured NK cells were stained with various monoclonal antibodies specific to cell surface proteins, including CD3, CD56, and CD16 (BD Pharmingen, Franklin Lakes, NJ) and NKG2D, NKp30, NKp44, and NKp46 (Beckman Coulter, Marseille, France), and then analyzed with a FACScan flow cytometer (BD Bioscience, Mountain View, CA).

Carboxy Fluoroscein Succinimidyl Ester Staining

NK cells were stained with carboxy fluoroscein succinimidyl ester (CFSE) using the Cell Trace CFSE Cell Proliferation Kit (Invitrogen, Carlsbad, CA). After a 10-minute incubation with CFSE, NK cells were cultured in the presence or absence of each drug. The CFSE content of the NK cells was measured using the FACScan flow cytometer at various time points during the culture.

Cytotoxicity Assay of NK Cells against Leukemia Cell Lines

NK cell cytotoxicity against K562 and Daudi cells was assessed using a standard chromium-release assay [9]. The percentage of specific lysis was calculated using the following formula: 100 × (count per minute [cpm] released from test sample − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release).

Western Blot Analysis

The preparations of activated T cells, as well as their MPA and IL-2 treatments, were as described by Quemeneur et al. [10]. Freshly isolated NK cells and activated T cells were cultured for the indicated times in the presence or absence of IL-2 with and without MPA. Lysates of NK cells and T cells were subjected to Western blot analysis as described previously [11], with minor modifications. In brief, the cultured cells were sonicated using a Sonifier B-12 cell disrupter (Branson, Danbury, CT). Equal amounts of the lysate proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes (Millipore, Schwalbach, Germany). The membranes were incubated with the primary antibodies (rabbit anti-human p27 pAb; Ana Spec, San Jose, CA) and mouse anti-human α-tubulin antibody (Sigma-Aldrich), and then incubated with the appropriate horseradish

peroxide–labeled secondary antibody. Specific bands were visualized using a peroxidase chemiluminescent substrate (Pierce, Rockford, IL) and analyzed using an LAS-4000 luminescent image analyzer (Fujifilm, Tokyo, Japan).

Statistical Analyses

The significance of differences in cell number, percentage of $CD16^+CD56^-$ cells, and cytotoxicity among NK cells cultured in the presence of different immunosuppressants was assessed by the Student *t* test. The significance of differences in the expression levels of NKG2D, NKp30, NKp44, and NKp46 among NK cells cultured with different immunousuppressants was assessed by the Mann-Whitney *U* test. A *P* value <.05 was considered significant.

RESULTS

Effects of Immunosuppressants on NK Cell Proliferation

NK cells isolated from 7 healthy donors were cultured in the presence of interleukin (IL)-2 (100 U/mL) and IL-15 (10 ng/mL) with CSP 1000 ng/mL [12], TAC 20 ng/mL [13], MTX 100 ng/mL [14], MPA 10 μg/mL [15], or vehicle control (EtOH 0.1%), and the number of NK cells in each culture was measured after 7 days (Figure 1A). The concentration of each immunosuppressant in the culture medium was similar to the peak level measured in the blood after the administration of standard doses. CSP, TAC, and MPA significantly inhibited the proliferation of NK cells, with MPA having the strongest inhibitory effect, whereas MTX did not influence NK cell proliferation.

Next, freshly isolated NK cells were labeled with the membrane dye CFSE and cultured under the same conditions as described above. The CFSE levels in NK cells harvested at days 1, 5, and 9 of culture were determined by flow cytometry. Although all 4 immunosuppressants inhibited the decline of CFSE levels associated with NK cell proliferation, the inhibitory effect of MPA was most prominent (Figure 1B). MPA blocked the CFSE decline almost completely, even in cells harvested after 9 days of culture. The inhibitory effect of MPA on NK cell proliferation was dose-dependent, as shown in Figure 1C. MPA inhibited NK cell proliferation at a much lower concentration (1 μg/mL) than that achieved after the administration of standard-dose MMF. These results indicate that MPA inhibits NK cell proliferation more potently than the other immunosuppressants at a concentration

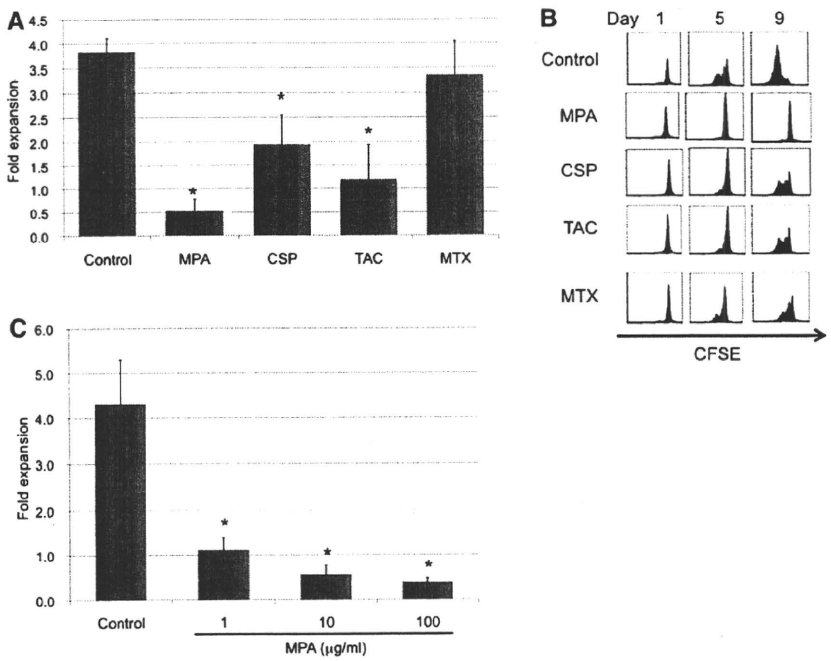

Figure 1. Effect of immunosuppressants on the proliferation of NK cells induced by IL-2 and IL-15. (A) NK cells isolated from healthy individuals were cultured in the presence of IL-2 (100 U/mL) and IL-15 (10 ng/mL) with a vehicle control (EtOH 0.1%, MPA 10 μg/mL, CSP 1000 ng/mL, TAC 20 ng/mL, or MTX 100 ng/mL) for 7 days. The vertical axis represents the fold expansion (mean ± SD; n = 6) of NK cells calculated by dividing the number of cells after culture by that before culture. An asterisk indicates values showing significantly less degree of proliferation ($P < .05$) than the vehicle controls. (B) CFSE contents in $CD3^-CD56^+$ cells were determined using flow cytometry at days 1, 5, and 9 of culture. The figure shows a representative set of histograms from a healthy individual. The results from 5 other individuals showed similar results. (C) NK cells were cultured in the presence of IL-2 (100 U/mL) and IL-15 (10 ng/mL) with MPA (1-100 μg/mL) for 7 days. The vertical axis represents the fold expansion (mean ± SD; n = 4) of NK cells calculated by dividing the number of cells after culture by that before culture. An asterisk indicates values showing significantly less proliferation ($P < .05$) compared with the vehicle controls.

similar to that measured in blood after administration of standard doses.

Changes in NK Cell Phenotype Associated with Immunosuppressants

NK cells can be separated into 2 subsets on the basis of CD56 density and CD16 expression [16], namely $CD56^{dim}CD16^{+}$ cells, characterized by potent cytotoxicity, and $CD56^{bright}$ $CD16^{-}$ cells, featuring high cytokine secretion activity. The 1-week culture of NK cells with EtOH alone resulted in an increase in $CD56^{bright}CD16^{-}$ cells, with a reciprocal decrease in $CD56^{dim}CD16^{+}$ cells. The addition of MPA markedly reduced the proportion of $CD56^{bright}CD16^{-}$ cells and left $CD56^{dim}CD16^{+}$ cells unchanged. CSP and TAC reduced the proportion of $CD56^{dim}CD16^{+}$ cells and reciprocally increased that of $CD56^{bright}CD16^{-}$ cells, whereas MTX did not affect the proportion of either subset (Figure 2A). Figure 2B shows the proportion of the $CD56^{bright}CD16^{-}$ NK cell subpopulation after 7 days of culture with immunosuppressants or EtOH alone. The median proportion of this NK cell subset was significantly lower in the culture with MPA (4.0%) than in the vehicle control (15.5%). In contrast, the proportion was significantly higher in the culture with CSP (36.5%) and TAC (33.0%) than in the control, whereas the proportion of the culture with MTX (17.5%) was comparable to that in the control.

Effect of Immunosuppressants on NK Cell Receptor Expression

Activating NK cell receptor expression after a 1-week culture was compared among the NK cells treated with the different immunosuppressants using the relative geometric mean fluorescent intensity (MFI) of NKG2D, NKp30, NKp44, and NKp46 to that of the control culture. NKG2D expression was decreased significantly after the treatment with TAC and MPA. In contrast, NKp30 expression was augmented significantly by CSP and TAC (Figure 3; $P < .05$). Of note, the expression of all NK-activating receptors decreased significantly after MPA treatment (Figure 3; $P < .05$), suggesting poor cytotoxic function of MPA-treated NK cells.

Cytotoxicity of NK Cells Treated with Immunosuppressants

Figure 4A shows specific cytotoxicities against K562 and Daudi cells at an effector:target ratio of 1:1. MPA-treated NK cells showed significantly lower cytotoxicity against K562 cells (61.3% ± 14.5% vs 16.7% ± 6.7%; $P < .01$) and Daudi cells (44.0% ± 3.6% vs 4.3% ± 4.0%; $P < .01$) compared with the control. There was a trend toward a higher cytotoxicity against Daudi cells in the CSP- or TAC-treated NK cells, but this enhancing effect was not observed against K562 cells. MTX did not affect NK cell cytotoxicity against either cell line. The reduced cytotoxicity against K562 and Daudi cells by NK cells treated with MPA was dependent on the MPA dose (Figure 4B).

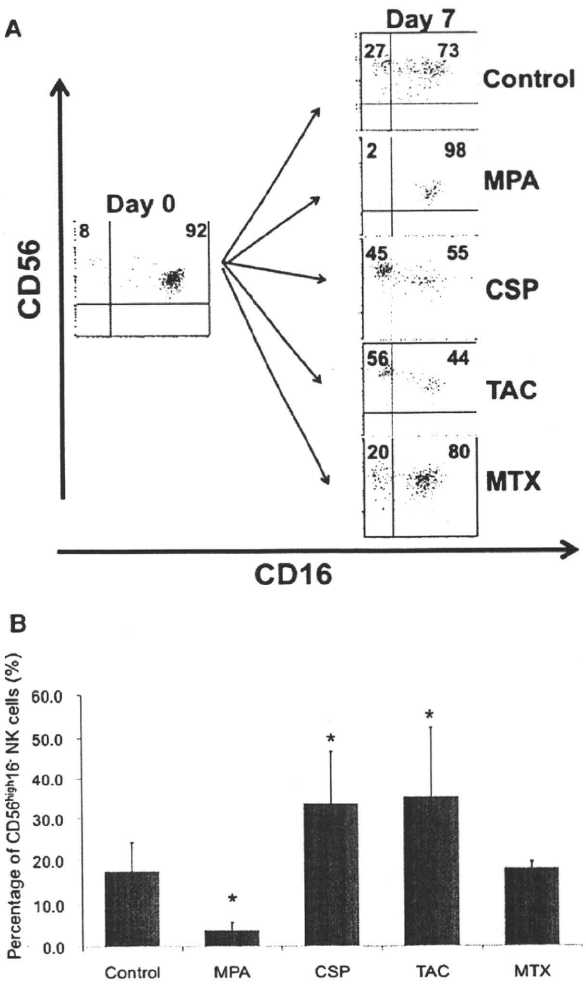

Figure 2. Changes in the NK cell phenotype after a 1-week culture with different immunosuppressants. NK cells were cultured in the presence of IL-2 (100 U/mL) and IL-15 (10 ng/mL) with different immunosuppressants or a vehicle for 7 days, and the percentages of $CD56^{dim}CD16^{+}$ and $CD56^{hig}CD16^{-}$ cells before and after culture were determined. (A) Scattergrams of a representative donor from 4 different ones. The analyses of other individuals produced similar results. (B) The percentages of $CD56^{high}CD16^{-}$ NK cells in the total $CD3^{-}CD56^{+}$ cells after culture in the presence of various immunosuppressants. The y-axis represents the mean percentage ± SD of $CD56^{high}CD16^{-}$ NK cells calculated from 4 different experiments. An asterisk indicates a value showing a significant difference in the $CD56^{high}CD16^{-}$ NK cell percentage compared with the vehicle control.

Effect of Guanosine on the Inhibition of NK Cell Proliferation and Cytotoxicity by MPA

MPA has been shown to inhibit T cell proliferation by depleting pools of guanosine triphosphate (GTP) in activated T cells through IMPDH inhibition, whereas guanosine has been shown to be able to restore the proliferation of MPA-treated T cells by increasing the GTP concentration in T cells [17,18]. To

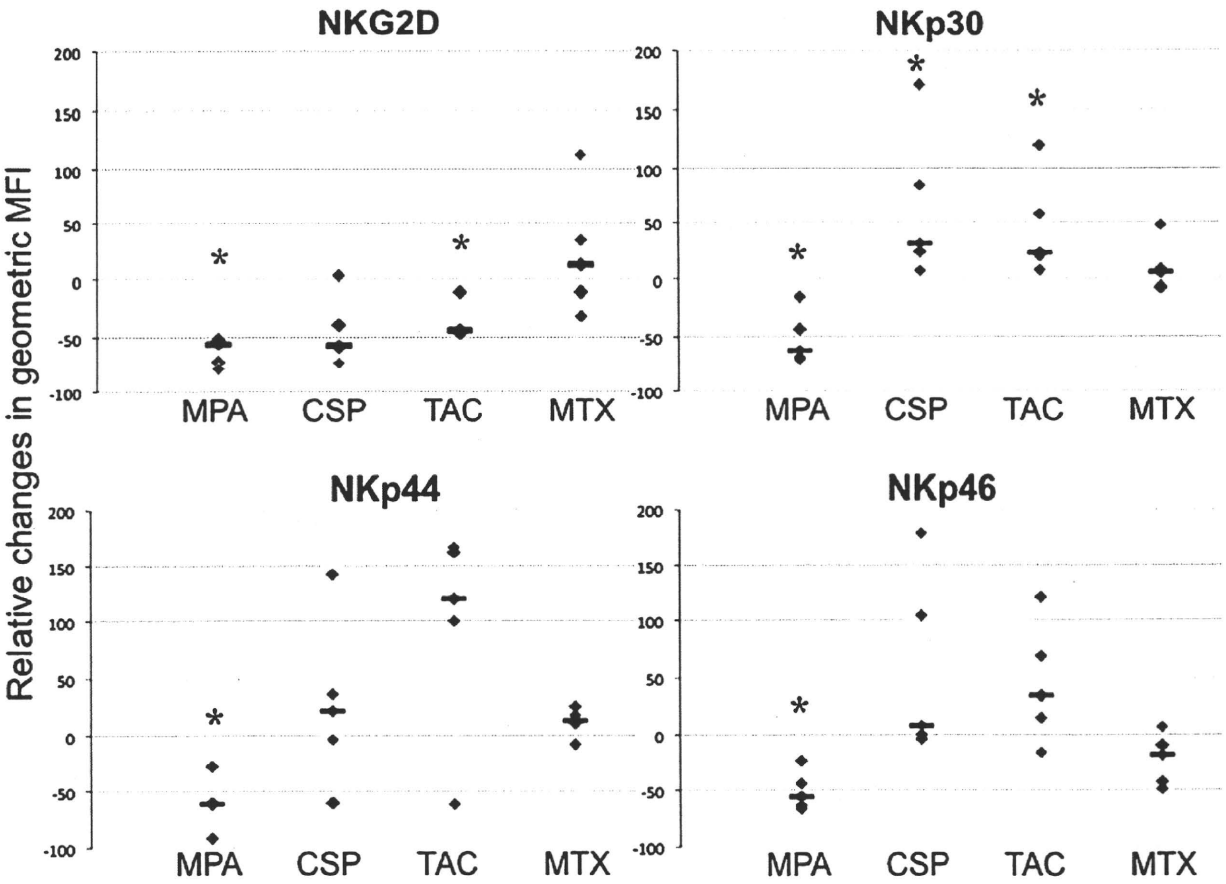

Figure 3. Changes in the expression of NK cell–activating receptors after culture with immunosuppressants. The changes in the expression levels of NKp30, NKp44, NKp46, and NKG2D in NK cells from 5 different individuals after a 1-week culture with immunosuppressants are shown. The vertical axis represents relative changes in the geometric MFI (gMFI) that is calculated by (gMFI of the receptor in each immunosuppressant -gMFI of the receptor in control)/gMFI of the receptor in control) × 100. An asterisk indicates a significant difference compared with the vehicle control. "–" denotes the mean of the relative change.

determine whether the same mechanism is involved in the NK cell inhibition by MPA, we examined the effects of guanosine on NK cell proliferation and cytotoxicity. As shown in Figure 5A, the addition of guanosine to the culture partially restored the NK cell proliferation that had been markedly inhibited by MPA; NK cell expansion increased from 0.7 ± 0.2-fold to 2.6 ± 0.9-fold by guanosine. Guanosine significantly increased the cytotoxicity against K562 by MPA-treated NK cells (from 16.0% ± 7.0% to 36.7% ± 3.2%) (Figure 5B), indicating that IMPDH inhibition is a major mechanism of NK cell inhibition by MPA.

MPA Inhibits Down-Regulation of CDK Inhibitor p27

The down-regulation of the CDK inhibitor $p27^{Kip1}$ is a critical event in the control of G1- to S-phase transition of T cells [19] and is dependent on IL-2 signaling [20], possibly through the $p21^{ras}$/Raf/mitogen-activated or extracellular signal–regulated protein kinase/extracellular signal–regulated kinase pathway [21]. MPA has been shown to inhibit down-regulation of $p27^{Kip1}$ and also to interfere with the cytokine-dependent signals that control the cell cycle, thereby inhibiting T cells from entering the mid-G1 phase of the cell cycle [10,22].

To determine whether a similar cell cycle inhibition is induced in NK cells by MPA, we examined expression of the $p27^{Kip1}$ in cultured NK cells and T cells in the presence or absence of MPA. As shown in Figure 6, the degradation of $p27^{Kip1}$ was induced in both T and NK cells within 24 hours in the culture with IL-2 (100 U/mL). Degradation of $p27^{Kip1}$ did not occur when the T and NK cells were stimulated with IL-2 in the presence of 10 μg/mL of MPA.

DISCUSSION

MMF is being increasingly used in GVHD prophylaxis after allo-HSCT because its use in combination with calcineurin inhibitors (CIs) produces comparable neutrophil recovery to that seen with the MTX + CI regimen [23]. The MMF + CI regimen is commonly

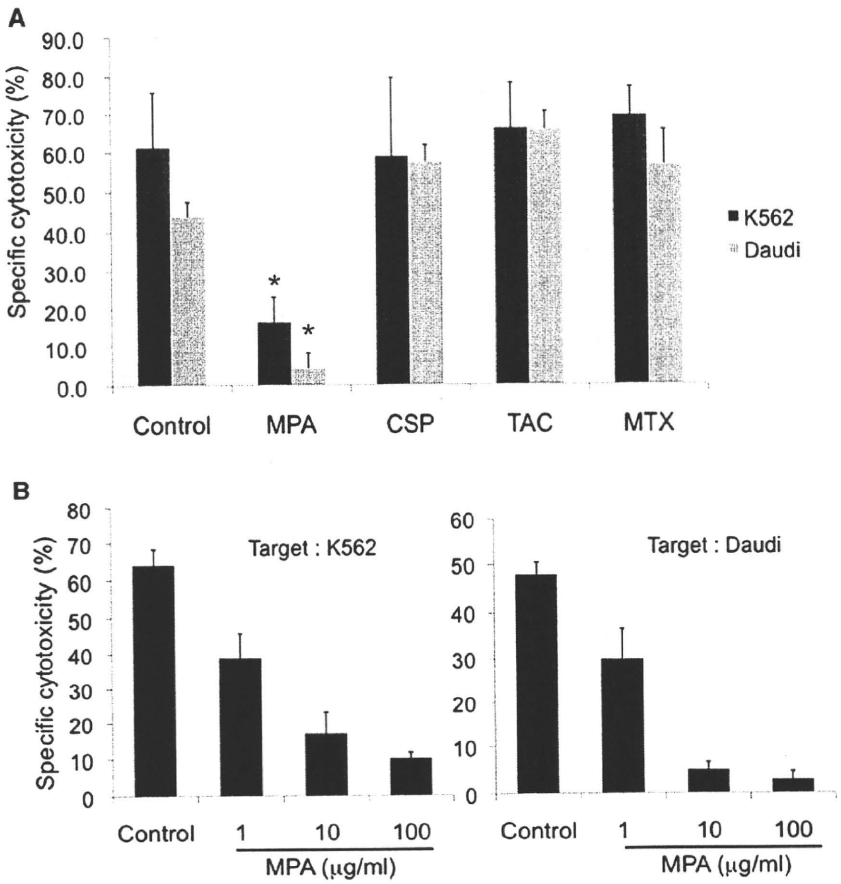

Figure 4. Cytotoxicity of cultured NK cells against leukemia cell lines. (A) Cytotoxicities by NK cells against K562 and Daudi cells at a 1:1 E:T ratio after the 1-week culture in the presence of different immunosuppressants were compared. The mean ± SD cytotoxicity from experiments using 3 different donors is shown. An asterisk indicates a significant difference compared with the control. (B) Cytotoxicities by NK cells against K562 and Daudi cells at a 1:1 E:T ratio after the 1-week culture in the presence of different concentration of MPA were compared. The mean ± SD cytotoxicities of experiments using NK cells from 3 different donors are shown.

used as a GVHD prophylaxis regimen after cord blood transplantation [24], with the anticipation of rapid hematologic recovery. Although the inhibitory effect of MPA on T cells has been studied extensively, its effect on NK cells remains unclear. The present study is the first to document the potent inhibitory effects of MPA on NK cells. MPA's inhibiting effects on NK cell proliferation and cytotoxic activity are apparently more evident than those of other immunosuppressants, including CSP, TAC, and MTX. The NK-activating receptor expression of NK cells is down-regulated by MPA, and the inhibitory effect is partially abrogated by the addition of guanosine to the NK cell culture.

Wang et al. [8] recently reported that CSP inhibits NK cell proliferation in a dose-dependent manner, while increasing the proportion of $CD56^{bright}CD16^{-}$ NK cells as well as enhancing NK cell cytotoxicity against K562 and the human Burkitt's lymphoma cell line Raji. The present study confirms that the culture of NK cells in the presence of CSP results in enhanced cytotoxicity of NK cells against Daudi cells, as well as an increased proportion of $CD56^{bright}CD16^{-}$ NK cells. Another CI, TAC, exhibited similar effects as CSP on NK cells. In accordance with the previous report, both CSP and TAC significantly augmented the surface expression of NKp30. The NKp30 expression level has been reported to correlate with the cytotoxicitic function of NK cells [25]. Thus, the augmented NKp30 expression might account for the increased cytotoxicity by CI-treated NK cells.

IMPDH is a rate-limiting enzyme in the de novo synthesis of guanosine nucleotides. Mitogenic stimulation of T cells results in a marked increase in IMPDH activity and a 5-fold increase in the guanine nucleotide pool [26,27]. MPA inhibits type II IMPDH, which is expressed in activated lymphocytes more abundantly than the type I IMPDH expressed by most leukocytes [28]. The MPA-induced deprivation of guanosine nucleotides from lymphocytes results in decreased glycosylation and expression of some adhesion molecules, thereby decreasing the recruitment of lymphocytes and monocytes into sites of inflammation and graft rejection [29]. The present study shows that the inhibitory effects of MPA on NK cells are also mediated in

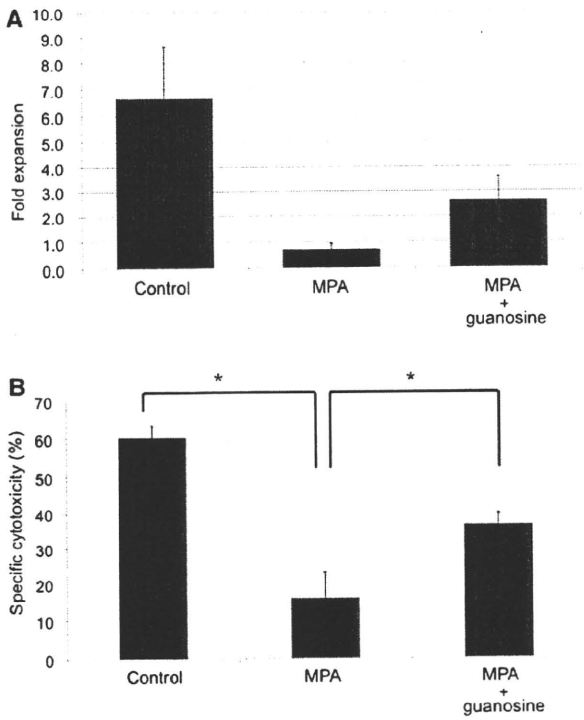

Figure 5. Effect of guanosine on the MPA-induced NK cell inhibition. NK cells were cultured in the presence of 10 μg/mL MPA with or without 100 μM guanosin for 1 week, and the effects of guanosine on MPA-induced NK cell growth inhibition (A) and cytotoxicity inhibition (B) were assessed. The vertical axis in A and B represent the mean ± SD of the fold expansion calculated by dividing the cell number after culture by that before culture and of the cytotoxicity by cultured NK cells against K562 cells at a 1:1 E/T ratio determined from 4 different donors. An asterisk indicates a significant difference ($P < 05$) compared with vehicle controls.

part by the deprivation of guanosine nucleotides. However, given MPA's remarkable inhibitory effect on NK cells, other mechanisms besides inhibition of IMPDH might play a role in this NK cell inhibition.

Laliberte et al. [22] attributed MPA's antiproliferative effect on T cells to the inhibition of cyclin D/cyclin-dependent kinase 6 induction and down-regulation of the CDK inhibitor $p27^{Kip1}$ following phytohemaglutinin stimulation of peripheral blood lymphocytes. $p27^{Kip1}$ is a member of a family of CDK inhibitors that includes $p21^{Cip/Waf1}$ and $p57^{Kip2}$ [20]. It binds to both CDK2 and CDK6 and controls the activity of cyclin D/CDK6 and cyclin E/CDK2 complexes. As a result, guanine nucleotide depletion by MPA significantly retards the degradation of $p27^{Kip1}$ after T cell activation, and $p27^{Kip1}$ gene expression by T cells, which decreases over time after incubation with IL-2, is blocked by MPA [30]. Our findings indicate that MPA also inhibits the IL-2–induced down-regulation of $p27^{Kip1}$ in NK cells, although the expression level of $p27^{Kip1}$ in NK cells was much lower than that in T cells. It is plausible that the diminished degradation of $p27^{Kip1}$ induced by MPA during NK cell activation leads to an increase in the amount of $p27^{Kip1}$ available for inhibiting cyclin E/CDK2 activity. Rapamycin, another potent immunosuppressant, is known to prevent the activation of cyclin E/CDK2 kinase activity and the degradation of $p27^{Kip1}$ associated with T cell activation [31]. Chen et al. [32] recently demonstrated the potent inhibitory effect of rapamycin (sirolimus) on NK cells. Thus, the inhibition of the $p27^{Kip1}$ degradation might be a common mechanism underlying NK cell inhibition by immunosuppressive agents.

NK cells play an important role in the development of the GVL effect after allo-HSCT, particularly in transplants from HLA-haploidentical donors who have KIR-L mismatches in the GVHD direction. Recently, Dunbar et al. [33] reported an association between high NK cell reconstitution and reduced rates of relapse and death, with no increase in the incidence of GVHD after reduced-intensity conditioning allo-HSCT. Thus, the use of a GVHD prophylaxis regimen that does not impair the reconstitution and function of NK cells early after HSCT is critical. Willemze et al. [34] recently reported a significantly lower relapse rate of AML after cord blood transplantation (CBT) from KIR-L–mismatched donors than after CBT

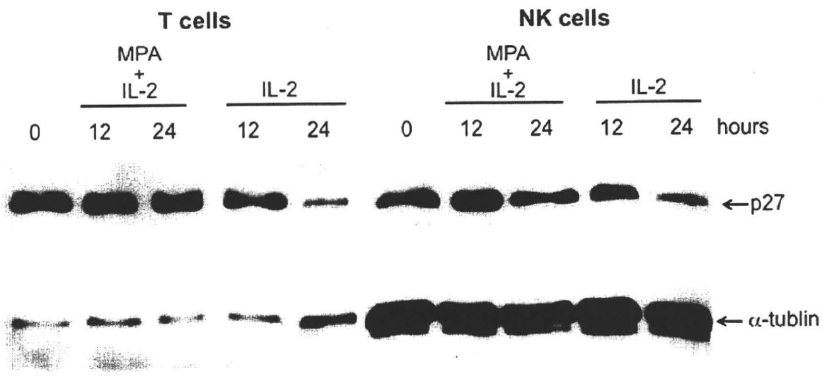

Figure 6. Effect of MPA on $p27^{Kip1}$ expression by T cells and NK cells. T cells and NK cells were harvested at the indicated time points after culture in the presence of IL-2 (100 U/mL) with or without MPA (10 μg/mL), and $p27^{Kip1}$ protein in the T cell and NK cell lysate was detected by Western blot analysis with anti-$p27^{Kip1}$–specific monoclonal antibody. The figure shows representative results from 5 experiments.

from non–KIR-L–mismatched donors. In contrast, another study failed to demonstrate the beneficial effect of KIR-L mismatches in CBT recipients [35]. Only 16% of the 218 patients analyzed in the former study received a GVHD prophylaxis regimen that included MMF [34], whereas 78% of the 257 patients treated in the latter study received MMF [34]. Although a number of factors are involved in the different outcomes between these 2 studies, the use of MMF and the resultant NK cell impairment might be one reason for the difference in the KIR-L–mismatch effect between the 2 studies. Thus, the possible negative effect of MMF on the GVL effect after SCT from KIR-L–mismatched donors merits examination in a prospective randomized study.

In conclusion, our in vitro study shows that MPA has a more potent inhibitory effect on NK cells than other immunosuppresants commonly used for GVHD prophylaxis. Thus, the combination of MTX + CI might be preferred over MMF + CI in terms of the retention of the NK cell–mediated GVL effect as a GVHD prophylaxis regimen.

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LETTER TO THE EDITOR

Reduced-intensity cord blood transplantation without prior remission induction therapy induces durable remission in adult patients with relapsed acute leukemia after the first allogeneic transplantation

To the Editor:

Leukemia relapse following allogeneic stem cell transplantation (allo-SCT) remains a major cause of treatment failure (1, 2). Among treatment options, second allo-SCT appears to increase disease-free survival compared with other interventions (3). The most important factors influencing outcomes of second allo-SCT may be the length of remission after the first transplantation and age (2). While disease status may also be an important factor, the probability of remission by re-induction therapy is generally low for relapsed acute leukemia after allo-SCT (4, 5). Limited data exist on other factors, such as conditioning regimens or use of different donors (2). Reduced-intensity conditioning (RIC) regimens are now widely used in umbilical cord blood transplantation (CBT) (6). However, few reports exist on the use of RIC regimens in second allo-SCT with CB to treat relapsed patients after first allo-SCT (7).

We report the outcomes of RIC CBT without prior re-induction chemotherapy for 11 consecutive adult patients from 2007 to 2009 at our institution (Table 1). Seven had acute myeloid leukemia (AML) and four had acute lymphoblastic leukemia (ALL). Median duration between first SCT and relapse was 6 months (3–24). All but one underwent conditioning with fludarabine (125 mg/m^2), L-PAM (80 mg/m^2), and total-body irradiation (TBI; 4 Gy). To achieve the maximum graft-versus-leukemia effect, a relatively low concentration of continuous tacrolimus was used as graft-versus-host disease (GVHD) prophylaxis (mean concentrations for each 10-d period spanning days 1 to 40 were 8.9, 8.9, 10.2, and 9.8 ng/mL, respectively). Six patients received minimal cytoreductive chemotherapy prior to or during conditioning to control the rapid increase in leukemic cells in peripheral blood. Median duration between relapse to second SCT was 37 d (22–60). Four were in complete remission (CR) at the last follow-up (15–37 months). Except for three patients who died early after the second SCT, eight achieved longer CR after the second SCT than after the first.

According to the definitions for pre-engraftment immune reaction (PIR) (8) and hemophagocytic syndrome (HPS) following SCT (9), PIR was observed in all cases and HPS in seven. In 10 cases, prednisolone (median dose, 1 mg/kg, ranging 0.2–6.9) was introduced for a short period immediately following the diagnosis of PIR to avoid HPS, which may correlate with graft failure of CB. Graft failure was observed in three patients, two of whom underwent further CBTs as salvage treatment.

Complications included severe infections (bacterial, n = 4; viral, n = 5). Other toxicities greater than grade 2 included mucositis (n = 3), sinusoidal obstruction syndrome (n = 2), and generalized skin pain (n = 2).

While high grade acute GVHD (III or IV) was not observed in the four patients maintaining CR, all had chronic GVHD.

To the best of our knowledge, the largest studies of second allo-SCTs using CB included seven relapsed adult leukemia patients (10) and 22 pediatric (11) leukemia patients; none of the adults and 10 of the pediatric patients received RIC regimens. Compared with these two studies, the interval from first SCT to relapse in the surviving four patients maintaining CR was extremely short in our cohort; in three of four patients, it was 5 months.

Compared with the first SCT, an extremely high incidence of PIR was observed with the second SCT (one case vs. all cases), whereas no difference was seen in the incidence of acute GVHD (grade II–IV) and chronic GVHD. Although the biological mechanisms are unclear, PIR is assumed to reflect alloimmune reaction and is observed frequently in CBTs. (8). While the use of CB and a relatively low tacrolimus concentration may have caused the high incidence of PIR and HPS, PIR may have contributed to the suppression of leukemic cells that remained in the early phase after the RIC regimen.

Graft failure was a devastating complication that has recently been reported to be closely correlated with HPS following CBT (9). As we previously reported, two of three patients who suffered from graft failure and sepsis underwent successful salvage therapy with a one-day regimen using CB (12). However, further studies are needed to clarify how PIR and HPS should be controlled.

Table 1 Characteristics of first and second allogeneic stem cell transplantation

	Case										
	1	2	3	4	5	6	7	8	9	10	11
SCT1											
Diagnosis	AML	AML	AML	AML	AML	ALL	ALL	AML	ALL	ALL	AML
Disease status	CR1	CR1	Rel1	PIF	PIF	CR1	CR2	Rel2	CR1	CR1	CR2
Age, Sex	52F	42M	39F	36M	26F	18M	21M	50M	54F	54F	21F
Donor type	MUD	CB	CB	MUD	SIB	SIB	MUD	MUD	SIB	SIB	Related
No. HLA disparities	None	1	1	None	2	None	None	None	None	None	None
Conditioning regimen	TBI 12 Gy-CY	TBI 12 Gy-CY	BU-CY-TBI 2 Gy	TBI 12 Gy-CY	Flu-Mel-TBI 4 Gy	TBI 12 Gy-CY	TBI 12 Gy-CY	TBI 12 Gy-CY	TBI 12 Gy-CY	TBI 12 Gy-CY	TBI 12 Gy-CY
GVHD prophylaxis	FK-MTX	CSP-MTX	FK	FK-MTX	FK	CSP-MTX	FK-MTX	FK-MTX	CSP-MTX	CSP-MTX	FK-MTX
PIR	–	+	–	–	–	–	–	–	–	–	–
Acute GVHD	II	I	0	II	I	I	II	I	II	II	0
Chronic GVHD	Limited	Extensive	0	Extensive	Extensive	0	Limited	Extensive	Limited	Limited	Limited
Remission duration after SCT1 (months)	12	5	6	20	24	5	7	5	5	3	8
SCT2											
Disease Status at SCT2											
Bone marrow blast cells, %	31.8	49.4	29	64.8	6.4	50	73.8	9	57.8	51.2	9.2
Peripheral blood blast cells, %	3	6	81	80	1	49	1	2	4	16	0
Time from relapse to SCT2, d	42	37	32	28	60	35	22	39	41	31	42
NCC (×107/kg)	2.7	2.4	3.8	2.5	2.4	2.2	3.5	3.7	3.9	2.1	2.3
CD34 (×105/kg)	0.5	0.41	2.81	0.61	1.1	0.9	0.87	1.27	0.81	0.99	0.76
No. of HLA disparities (GVH/HVG)	2/2	2/0	2/2	2/2	1/1	1/1	1/1	2/1	1/1	2/2	1/1
Conditioning regimen	Flu125-Mel80-TBI 4 Gy	Flu150-BU4	Flu125-Mel80-TBI 4 Gy	Flu125-Mel80-TBI 4 Gy	Flu125-Mel80-TBI 4 Gy	Flu125-Mel80-TBI 4 Gy	Flu125-Mel80-TBI 4 Gy	Flu125-Mel80-TBI 4 Gy	Flu125-Mel80-TBI 4 Gy	Flu125-Mel80-TBI 4 Gy	Flu125-Mel80-TBI 4 Gy
Cytoreduction before conditioning	None	AraC	DNR	DNR, MIT, AraC	None	ADR, MIT	DNR	None	None	ADR, AraC	None
GVHD prophylaxis	FK	FK	FK	FK	FK	FK	FK	FK	FK	FK	FK
Neutrophils ≧ 500/μL (d)	34	24*	34	–	38	26*	23	26	36	31	28
PIR	+	+	+	+	+	+	+	+	+	+	+

Table 1 (*Continued*)

	Case										
	1	2	3	4	5	6	7	8	9	10	11
HPS	+	+	+	+	–	+	–	–	–	+	+
Acute GVHD	IV	0*	III	NA	II	II*	II	I	I	I	IV
Chronic GVHD	NA	Extensive*	Extensive	NA	Extensive	Extensive*	0	Extensive	Limited	0	NA
Remission duration after SCT2 (months)	3	37+	9	1	29+	6	7	15+	21+	3	3
OS after SCT2 (months)	3	37+	15	1	29+	15	9	15+	21+	10	3
Cause of death	Viral infection		Relapse	Graft failure		Relapse	Relapse			Relapse	Sepsis

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; Rel, relapse; PIF, primary induction failure; MUD, matched unrelated donor; SIB, sibling; TBI, total-body irradiation; CY, cyclophosphamide; BU; busulfan; Flu, fludarabine; Mel, melphalan; FK, tacrolimus; CSP, cyclosporine; MTX, methotrexate; NCC, nuclear cell count; HPS, hemophagocytic syndrome; GVH, graft-versus-host direction; HVG, host-versus-graft direction; BU, busulfan; AraC, cytosine arabinoside; DNR, daunorubicin; MIT, mitoxantrone; ADR, doxorubicin; NA, not assessed; OS, overall survival; GVHD, graft-versus-host disease; PIR, pre-engraftment immune reaction.

*Results of the 3rd transplantation as a salvage of graft failure.

While haploidentical family donors are candidates for urgent second transplant, CBT could present a useful option by eliciting PIR, which may induce a strong anti-leukemic effect and, if controlled properly, not cause severe acute GVHD.

In conclusion, our study indicates that early RIC CBT using a relatively low concentration of tacrolimus as GVHD prophylaxis may be a useful therapeutic option for patients with acute leukemia who relapse shortly after first transplantation.

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LETTER TO THE EDITOR

Unexpectedly high AUC levels in a child who received intravenous busulfan before stem cell transplantation

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A 6-year-old boy underwent a craniotomy with subtotal resection of the cerebellar vermian tumor. Microscopic examination showed medulloblastoma. Brain magnetic resonance imaging after resection did not show residual disease. He subsequently received four cycles of ICE (ifosfamide, cisplatin, and etoposide) chemotherapy followed by whole craniospinal (24 Gy) and local (30 Gy) radiation therapy. He eventually received tandem high-dose chemotherapy. Conditioning regimens for the first and the second were carboplatin (1200 mg/m^2) + thiotepa (750 mg/m^2) and busulfan 1.1 mg/kg/dose intravenous every 6 h for total 16 doses (17.6 mg/kg) + melphalan 70 mg/m^2 once daily intravenous for 2 days (140 mg/m^2), respectively. Autologous PBSC were infused after high-dose chemotherapy. The numbers of CD-34-positive cells were 5.2×10^6/kg in the first and 7.0×10^6/kg in the second, respectively. Engraftment after the second autologous PBSCT was achieved on day 11. Brain magnetic resonance imaging on day 25 of the second autologous PBSCT showed no evidence of recurrence.

On day 64 of the second autologous PBSCT, he developed sudden dyspnea with 90% oxygen saturation in room air. A chest X-ray film showed a ground-glass appearance in both lungs. Chest computed tomography scan also showed bilateral areas of ground-glass opacities, and no centrilobular micronodule. Aspergillus, candida, and cryptococcal antigen for the serum were negative. The β-D-glucan level was significantly elevated (234.4 pg/ml). Intravenous cotrimoxazole therapy was initiated, because pneumocystis pneumonia was thought to have caused pneumonia based on the clinical course. He was placed on mechanical ventilation and methylprednisolone pulse therapy (30 mg/kg × 3 days) was started on day 72. His respiratory condition improved quickly with these treatments and mechanical ventilation was discontinued on day 77. Although polymerase chain reaction for *Pneumocystis jirovecii* in bronchoalveolar lavage fluid was negative, we diagnosed the cause of his pulmonary disorder had been pneumocystis pneumonia. His respiratory condition deteriorated again on day 82. Despite the second course of methylprednisolone pulse and continued cotrimoxazole therapy, he was placed on mechanical ventilation again on day 85 (Figure 1a). No pathogenic bacterial, fungal, or viral agents, including cytomegalovirus were identified by microbiological cultures and serological studies. The β-D-glucan level was also decreased to 33.4 pg/ml. Although various antibiotics, ganciclovir, antifungal drugs, and cotrimoxazole therapy were continued, his pulmonary oxygenation became worse day-by-day. He died on day 133 of the second autologous PBSCT because of respiratory failure (Figure 1b).

An autopsy was performed with the consent of his parents. Microscopically, organizing diffuse alveolar damage was seen, and nuclear enlargement, hyperchromasia, and pleomorphism were seen along alveolar and bronchial epithelium. This cytologic atypia was seen not only in the lung, but also in the urothelium of the renal pelvis. There was no evidence of bacterial, fungal, or viral infection. These pathological findings were consistent with busulfan-induced lung disease. Organizing diffuse alveolar damage is the most common manifestation of busulfan lung toxicity and is associated with bronchiolar and alveolar epithelium atypia.[1] This cytologic atypia is often seen extrapulmonary sites, including urinary bladder, breast, and uterine cervix.[1] The incidence of pulmonary toxicity after high-dose oral busulfan therapy before stem cell transplantation has been reported to be 3.6%.[2] Corticosteroids are effective for treating this disease to various degrees. Although some patients improve, others progress and die.[1]

It has become evident posthumously that busulfan areas under the drug plasma concentration–time curve (AUC) levels at the 1st and 9th doses were significantly elevated (2353 μM × min, 2347 μM × min) (target AUC; 900–1500 μM × min). Busulfan concentrations for the 1st and 9th doses and the accumulation of intravenous busulfan in plasma were assayed using a high-performance liquid chromatography system (Figure 2).[3] Plasma concentrations were analyzed by the non-compartmental method using WinNonlin (version 5.2.1; Pharsight Corp., Mountain View, CA, USA). The AUC from time 0 to infinity (AUC_{inf}) for the 1st dose and at steady state (AUC_{ss}) for the 9th dose was calculated using linear trapezoidal rule. The relationship between high busulfan AUC levels and the occurrence of busulfan-induced lung disease has not been established,[4] although high busulfan AUC levels are commonly associated with hepatic veno-occlusive disease.[5]

The cause of high busulfan AUC levels in our patient is still unclear. He did not have the distinct liver or renal disorder at the time of busulfan administration. Busulfan is metabolized in the liver through conjugation with glutathione by glutathione *S*-transferase (GST) enzymes.[6] GSTA1 is the predominant isoform of GST, which catalyzes the conjugation of busulfan with glutathione. Polymorphisms in GSTA1 are thought to be associated with alterations in the pharmacokinetics of busulfan.[7]

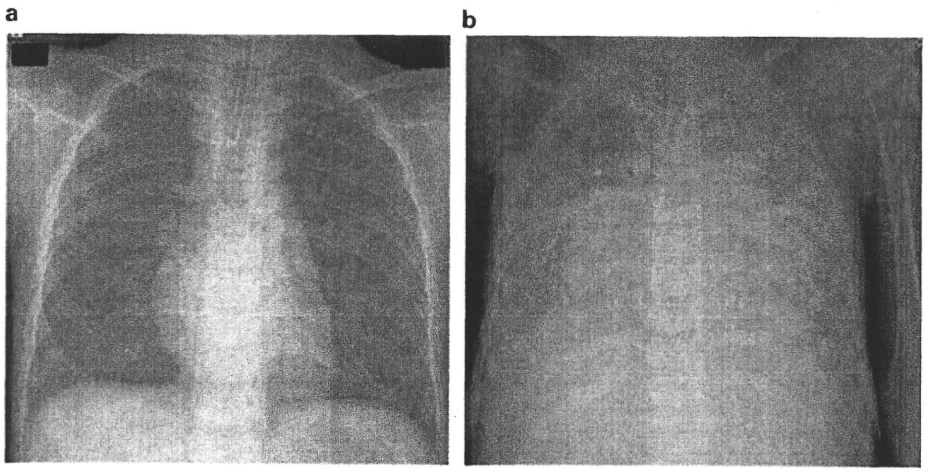

Figure 1 Chest X-ray films. (**a**) (On day 85) bilateral lung fields showed a ground-glass appearance. (**b**) (On day 133) bilateral lung fields showed marked radiopacity and air bronchograms.

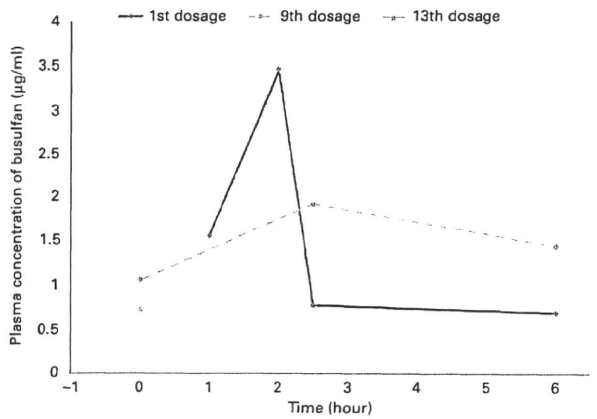

Figure 2 Plasma concentration of busulfan. Peak level of plasma concentration of busulfan is very high (1st dose). Busulfan clearance is poor (before 13th dose).

Johnson *et al.*[7] reported that the GSTA1*B variant had a 2.6-fold higher busulfan AUC level than other variants after intravenous busulfan exposure in the pediatric population. Our patient's genotype of the promoter region of GST A1 by DNA sequencing was GSTA1*A diplotype (-567T, -69C, -52G) which is thought to be more active than the GSTA1 *A/*B. Nevertheless, busulfan AUC levels were significantly elevated. This may indicate that polymorphisms other than GSTA1 polymorphisms may affect busulfan metabolism.

Intravenous busulfan should have a much more predictable pharmacokinetic profile than oral busulfan. Treatment with a fixed dose of 0.80 mg/kg intravenous busulfan achieved the target AUC level (900–1500 μM × min) in 80% of adult patients.[8] The remaining 20% were very close to achieving the target level.[8] A recent European study showed that 91% of children achieved target AUC levels by weight-based dosing.[9] They concluded that this weight-based dosing in children is sufficient without therapeutic drug monitoring and dose adjustment. Our patient received intravenous busulfan according to his body weight, as in the European study. However, his busulfan AUC levels were higher than has been reported elsewhere in the literature.[9,10] To avoid unexpectedly high busulfan AUC levels, therapeutic drug monitoring and dose adjustment should be recommended for all patients who are treated with high-dose busulfan. When therapeutic drug monitoring is not applicable, test dosing of intravenous busulfan before high-dose therapy would be preferable.

Conflict of interest

The authors declare no conflict of interest.

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Successful bone marrow transplantation for children with aplastic anemia based on a best-available evidence strategy

Okamoto Y, Kodama Y, Nishikawa T, Yamaki Y, Mougi H, Masamoto I, Tanabe T, Shinkoda Y, Kawano Y. Successful bone marrow transplantation for children with aplastic anemia based on a best-available evidence strategy.
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Abstract: A best-available evidence strategy, i.e., the best-available donors, conditioning regimens and GVHD prophylaxis were chosen at the time of BMT for AA, was analyzed retrospectively. The outcomes for 18 children with AA who underwent allogeneic BMT were analyzed. The median age was 11 yr (range 4–16), and nine were men. As conditioning regimens, seven had low-dose irradiation + CY, six had ATG + CY + Flu, and five had ATG + CY. Donors were HLA-matched siblings in 10, HLA-mismatched family in one, HLA-matched unrelated in three, and HLA-mismatched unrelated in four. As GVHD prophylaxis, three received CsA alone, nine received CsA + MTX, and six received tacrolimus + MTX. All 18 patients showed engraftment. The median number of days until the neutrophil count exceeded 500/μL was 16 (range 11–21) post-transplant. Five developed more than grade 2 acute GVHD, and three developed extensive cGVHD. One patient died because of interstitial pneumonia complicated with cGVHD. Five-yr OS was 94% (95% CI: 83–105). These results suggest that a strategy of treating patients based on the best-available evidence is acceptable.

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Key words: aplastic anemia – bone marrow transplantation – children – best-available evidence

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It is not always easy to design a clinical trial, especially when the number of patients is limited, as in childhood AA. Rapid advances in transplant procedures, including expanding donor availability, improved conditioning regimens, and better GVHD prophylaxis, also make it difficult to perform clinical trials, which can take a long time. Interestingly, a recent report by Lee and coworkers showed that transplant practices worldwide are not uniform (1). In the present analysis, to identify problems that may be associated with a best-available evidence strategy, i.e., the best-available donors, conditioning regimens, and GVHD prophylaxis chosen at the time of BMT, the outcomes of 18 consecutive children with AA who underwent BMT at our hospital were reviewed retrospectively.

Abbreviations: AA, aplastic anemia; ATG, anti-thymocyte globulin; BM, bone marrow; BMT, bone marrow transplantation; cGVHD, chronic graft-versus-host disease; CsA, cyclosporine; CY, cyclophosphamide; DEB, diepoxybutane; FFS, failure-free survival; Flu, fludarabine; G-CSF, granulocyte colony-stimulating factor; GVHD, graft-versus-host disease; Hb, hemoglobin; HLA, human leukocyte antigen; IST, immunosuppressive therapy; IVIG, intravenous immunoglobulin; KPS, Karnofsky Performance Status; MNC, mononuclear cell; MRS, methicillin-resistant staphylococcus; MTX, methotrexate; OS, overall survival; RBC, red blood cells; SCT, stem cell transplantation.

Methods

Patients and methods

Before 2004, IST was given to all AA patients, except in one who was heavily transfusion dependent when he was referred to our hospital. Patients who did not respond to IST underwent BMT. After 2004, BMT was performed if the patient had an HLA-matched/HLA one-locus-mismatched related donor without IST. Patients who did not have a related donor received IST. If the patient did not respond to IST, an unrelated donor was used as an alternative donor. Eighteen consecutive children who underwent BMT at

Kagoshima University were analyzed in this study. Patient, disease, and transplant characteristics are shown in Table 1. Seventeen cases were acquired AA, including one AA post-hepatitis. The remaining patient had congenital AA of unknown origin. Careful physical examinations and a DEB test were performed in all patients to exclude Fanconi anemia and other conditions. The median age was 11 yr (range 4–16), and nine were men. One had very severe, 16 had severe, and one had moderate AA (2, 3). Ten received IST, including ATG + CsA in nine and CsA alone in one congenital AA, prior to BMT, but without success. Eleven were transfusion dependent at the time of BMT. Five had received over 20 units of RBC transfusions prior to BMT. The median time from diagnosis to BMT was 10 months (range 1–52). Only one patient had a poor KPS (4) (< 90) prior to BMT.

In principle, the best donor source, conditioning regimen, GVHD prophylaxis, and supportive care were chosen at the time of transplant.

Conditioning regimen

From 1992 to 2001, seven patients received a low-dose irradiation regimen that consisted of 1.5–2.5 Gy daily for 1–4 days on days −9, −8, −7, and −6 (total 3.0–7.5 Gy) and CY 50 mg/kg once daily i.v. on days −5, −4, −3, and −2 (total dose 200 mg/kg). From 2001 to 2008, five patients received a non-irradiation regimen that consisted of ATG (Zetbulin®; Nihon Zoki, Osaka, Japan, 5 mg/kg or Lymphoglobulin®; Genzyme, Tokyo, Japan 15 mg/kg) once daily i.v. on days −5, −4, −3, and −2 (total dose 20 or 60 mg/kg) and CY 50 mg/kg once daily i.v. on days −5, −4, −3, and −2 (total dose 200 mg/kg) for BMT from a related donor. During the same period, six patients received a Flu-based regimen that consisted of Flu 30 mg/m^2 once daily i.v. on days −5, −4, −3, and −2 (total dose 120 mg/m^2) and CY 50 mg/kg once daily i.v. on days −5, −4, −3, and −2 (total dose 200 mg/kg), and ATG (Zetbulin®; Nihon Zoki, 5 mg/kg or Lymphoglobulin®; Genzyme 15 mg/kg) once daily i.v. on days −5, −4, −3, and −2 (total dose 20 or 60 mg/kg) for BMT from an unrelated donor.

Table 1. Patient-, disease-, and transplant characteristics

Number of patients	18	Range
Median age	11	(4–16)
Patient sex (male/female)	9/9	
Donor sex (male/female)	13/5	
Sex match (recipient/donor)		
Female/female	4	
Male/female	5	
Female/male	1	
Male/male	8	
Pretreatment		
None	8	
IST	10	
Transfusion dependent	13	
Median months from diagnosis to BMT	11	(0.7–52)
Donor		
Related	11	
Unrelated	7	
HLA		
Match	13	
Mismatch	5	
Conditioning		
TBI-based	7	
Non-TBI	11	
Flu-based	6	
GVHD prophylaxis		
CyA based	11	
Tacrolimus based	7	
Median MNC	3.3	(0.52–5.5)
Median CD34-positive cells	2.0	(1.1–3.8)

Donor

The donor source was an HLA-matched sibling in 10, HLA-mismatched family in one (two-locus-mismatch in A/DR alleles by serological typing), HLA-matched unrelated in three, and HLA-mismatched unrelated in four. Seventeen transplants were matched with regard to HLA-A, -B, and -DR by serological typing. Among these 17, seven were also matched and six were not determined by DNA typing. The remaining three were one-locus-mismatched in B or DR and one was two-locus-mismatched in B and DR by DNA typing.

GVHD prophylaxis

GVHD prophylaxis was determined based on the patient's condition, i.e., transfusion dependent or not, HLA disparity, and the source of BM (related/unrelated). Three received CsA alone (5), nine received CsA + MTX (6) and six received tacrolimus + MTX (7) as GVHD prophylaxis. MTX was given at a dose of 15 mg/m^2 on day 1 and at a dose of 10 mg/m^2 on days 3, 6, and 11. In principle, CsA or tacrolimus was continued for six months post-transplant and then was gradually tapered. CsA or tacrolimus was extended for 12 months for patients with unrelated donors.

General supportive care

All patients were transplanted as inpatients. Triggers for blood transfusion depended on symptoms (8). In general, RBC were transfused to keep the Hb level above 7 g/dL. Platelets were given prophylactically to keep the platelet count above 10 000/μL. All patients received G-CSF post-transplant. G-CSF was started on day 5 until the neutrophil count exceeded 5000/μL. All patients received IVIG at a dose of 100–150 mg/kg/dose every week until day 96.

Antimicrobial prophylaxis

Oral polymixin B at a dose of 300×10^4 units/m^2/day, amphotericin B at a dose of 300 mg/m^2/day, vancomycin at a dose of 500 mg/m^2/day, and acyclovir at 10 mg/kg/day were started seven days prior to BMT from 1992 to 2007. Amphotericin B was switched to itoraconazole at a dose of 5 mg/kg/day in 2007. Parenteral antibiotic prophylaxis during the neutropenic period was adopted before 2005 and discontinued thereafter.

Cell dose and RBC or plasma depletion

All patients received BM grafts without T-cell depletion. ABO-compatible marrow and major-, minor- and major + minor-mismatch marrow were transplanted in nine, four, three, and two, respectively. Therefore, RBC depletion, plasma depletion, or both was performed in four, three, and two patients, respectively. The median numbers of MNC and CD34-positive cells transplanted were 3.3×10^8/kg (range 0.52–5.5) and 2.0×10^6/kg (range 1.1–3.8) recipient body weight, respectively.