which annexin II acts on the tunica media tissue directly or via a system other than MMP. Investigation of annexin II expression in the normal aorta without atherosclerosis is needed, but we could not get the normal aortic wall in this study.

In summary, the present study suggests that annexin II plays an important role in the development of atherosclerosis and degradation of media in AAAs. To clarify such specific processes, further detailed studies are necessary.

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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

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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

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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/m², melphalan 80 mg/m², 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 $3.2–4.5 \times 10^9$/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.

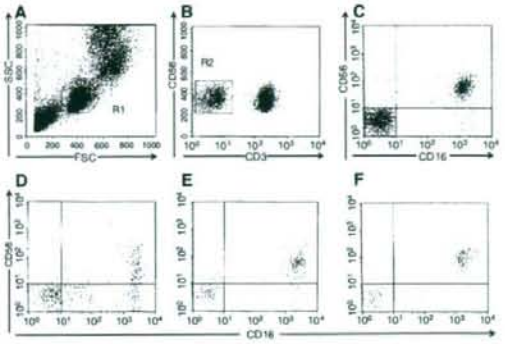

Figure 1 Phenotype of the $CD16^+$ NK cells in the peripheral blood. Representative results of flow cytometry on $CD3^-$ lymphocytes from SCT recipients and healthy individuals are shown. Gates were set up to exclude any $CD3^+$ lymphocytes as shown in (A) and (B); (C) a healthy individual; (D) a CBT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

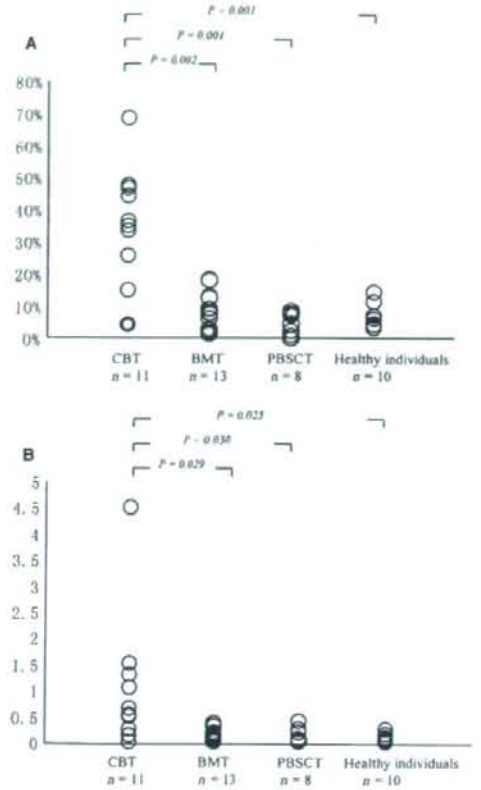

Figure 2 The proportion (A) and the absolute count (B) of $CD3^-CD16^+CD56^-$ in the PB of SCT recipients and healthy individuals. An increase in the proportion of $CD3^-CD16^+CD56^-$ NK cells (20% or more) in the PB $CD16^+$ NK cells and an increase in the absolute count of the same NK cell subset (>0.5 × 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.

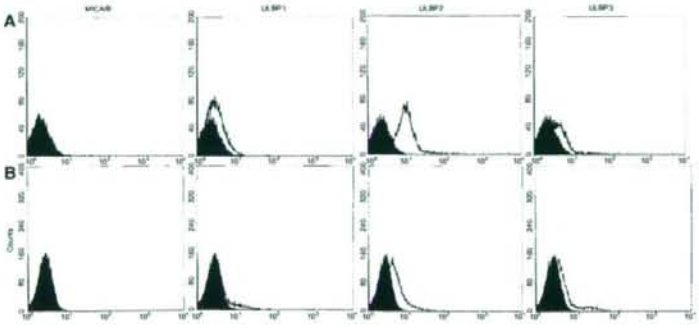

Figure 3 Expression of NKG2D ligands on leukemic cells from Patient 1. (A) leukemic cells obtained before CBT; (B) leukemic cells obtained after relapse. The proportion of ULBP2 expressing leukemic cells decreased from 59% to 9%.

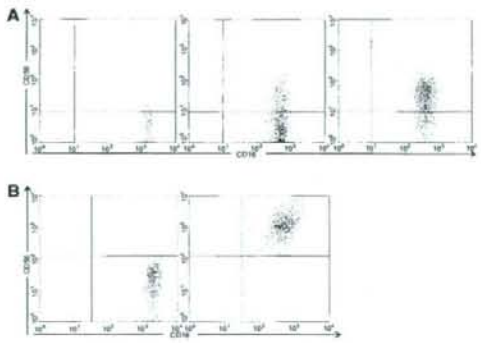

Figure 4 Phenotypic change of CD16⁺CD56⁻ NK cells with time associated with *in vitro* culture. Isolated CD16⁺CD56⁻ cells from Patient 1 were cultured in the presence of 100 IU/L IL-2 without (A) or with K562-mb15-41BBL (B). CD16⁺CD56⁻ NK cells from CBT recipients became CD16⁺CD56⁺ after the *in vitro*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

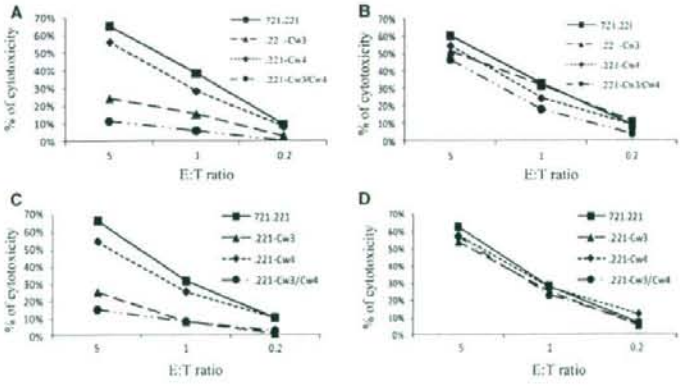

Figure 5 Specificity of NK cells derived from CD16⁺CD56⁻ NK cells. Cultured NK cells derived from CD16⁺CD56⁻ cells of Patient 1 (A and B) and Patient 2 (C and D) were separated into CD158b⁺ (A and C) and CD158b⁻ cells (B and D) and were examined for the cytotoxicity against 721-221 cells and 721-221 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.

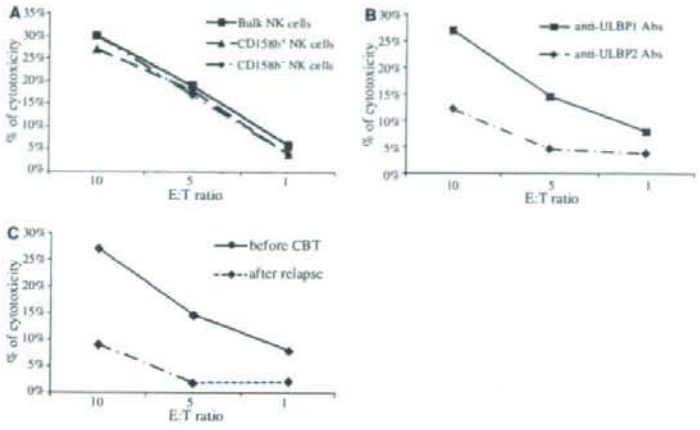

Figure 6 Cytotoxicity of cultured NK cells against leukemic cells. (A) Unseparated and separated NK cells were tested against leukemic cells obtained before CBT; (B) Leukemic cells were incubated in the presence of anti-ULBP1 or ULBP2 Abs before incubation with cultured NK cells; (C) Cytotoxicity of 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.

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Mechanism of Decrease of Oral Bioavailability of Cyclosporin A During Immunotherapy upon Coadministration of Amphotericin B

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ABSTRACT: The trough level of blood concentration of cyclosporin A (CyA) in a patient receiving immunotherapy was observed to decrease following coadministration of amphotericin B (AMB). This clinical observation was confirmed experimentally in Wistar rats intravenously given AMB (1.5 or 3.0 mg/kg) or saline (control) for 4 days, followed by CyA (10 mg/kg). The blood concentration of CyA after i.v. or p.o. administration in both AMB groups was significantly decreased compared with the control. The oral bioavailability of CyA after 1.5 or 3.0 mg/kg AMB treatment was decreased to 67% or 46%, respectively, of that of the control group. AMB treatment increased the expression levels of mdr1a and mdr1b mRNAs in the duodenum to about three times the control, and expression of CYP3A2 mRNA in the liver was increased to about twice the control. The P-gp and CYP3A2 proteins were increased significantly. These findings suggest that the oral bioavailability of CyA is reduced as a result of both increased efflux transport via P-glycoprotein in the duodenum and an increased first-pass effect of CYP3A2-mediated hepatic metabolic activity, induced by AMB. It is suggested that careful monitoring of CyA levels is necessary in the event of AMB administration to patients receiving immunotherapy with CyA. Copyright © 2008 John Wiley & Sons, Ltd.

Key words: cyclosporin A; amphotericin B; P-glycoprotein; CYP3A; drug interaction; oral bioavailability

Introduction

There have been many reports of drug interactions involving cyclosporin A (CyA), and clinically, a patient was encountered who was receiving immunotherapy with CyA in whom the blood concentration of CyA was decreased upon coadministration of amphotericin B (AMB) (Figure 1).

It is well known that CyA is a substrate of both the efflux transporter P-glycoprotein (P-gp) and the metabolic enzyme cytochrome P450 (CYP3A) [1–3]. P-gp and/or CYP3A limit the oral bioavailability of digoxin [4], rifampin [4], vinblastine [5], dextromethorphan [6] and CyA [7,8]. It has already been shown that the blood concentration

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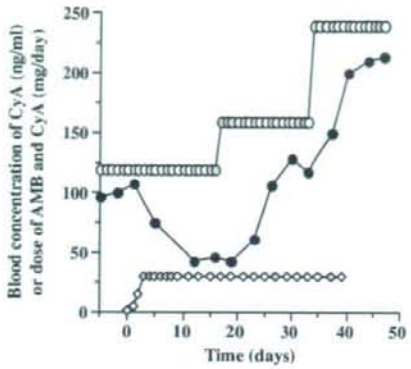

Figure 1. Change of blood concentration–time courses of CyA in a patient (23-year-old man, 56 kg) receiving immunotherapy with CyA, upon coadministration of AMB. He received daily oral administration of CyA, and then was intravenously infused over 6 h with AMB (daily for the first 10 days, every other day for the next 15 days). ● CyA blood concentration; ○ CyA dose; ◇, AMB dose

of CyA is reduced by pretreatment with dexamethasone [9], cyclophosphamide [10] or levothyroxine [11] in rats, owing to induction of P-gp and CYP3A2 in the liver and intestine. In those reports, it was demonstrated that the oral bioavailability of CyA is primarily controlled by the level of CYP3A in the upper small intestine under physiological conditions, whereas after treatment with inducers, P-gp in the upper intestine also plays a significant role as an absorption barrier to CyA. Thus, it was speculated that the reason for the decrease of blood concentration of CyA in the patient mentioned above might have been induction of P-gp and CYP. However, no report is currently available on the drug interaction between CyA and AMB.

Therefore, this study examined the mechanism of the decrease of blood concentration of CyA by using rats treated with AMB.

Materials and Methods

Chemicals

Fungizone® injection (amphotericin B, AMB) and Sandimmun® injection (cyclosporin A, CyA) were purchased from Bristol-Myers Squibb Co. Ltd (Tokyo, Japan) and Novartis Pharma Co. Ltd (Tokyo, Japan), respectively. Oligonucleotide primers were custom-synthesized by Amersham Pharmacia Biotech (UK). Other reagents were purchased from Sigma Co. (MO, USA).

Animal experiments

All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Kanazawa.

A 150 µl aliquot of AMB (1.5 or 3.0 mg/kg/day) was intravenously administered to male 8-week-old Wistar rats (Japan slc Co., Hamamatsu, Japan) via a tail vein daily for 4 days. The vehicle control rats received distilled water alone for 4 days. A 100 µl aliquot of CyA (10 mg/kg) was injected via the femoral vein at 24 h after the last treatment with AMB. Alternatively, a 500 µl aliquot of CyA (10 mg/kg) was administered orally at 24 h after the last treatment with AMB. Blood samples (200 µl each) were collected at designated time intervals from the jugular vein of untreated rats and AMB-treated rats under light ether anaesthesia.

Measurement of blood concentration of CyA

The blood concentration of CyA was measured with a TDx analyser using a commercial kit according to the manufacturer's instructions (Dainabot Co. Ltd, Tokyo, Japan). The TDx assay is a fluorescence polarization immunoassay (FPIA) reagent system for the measurement of CyA [12]. The measurement range of blood concentration was 25–1500 ng/ml. The cross-reactivities with the metabolites of CyA were 19.4% for M1 and less than 5% for other metabolites.

Determination of laboratory data

Blood samples were collected from the jugular vein under light ether anesthesia at 24 h after the last treatment with AMB, and the plasma was separated by centrifugation and stored at −80°C. The measurements of laboratory data were entrusted to SRL Co. Ltd (Tokyo, Japan).

Reverse transcriptase-polymerase chain reaction (RT-PCR) assay

Total RNA was isolated from the liver and intestine using an Isogen kit (Wako, Osaka). Synthesis of cDNA from the isolated total RNA was carried out using RNase H-reverse transcriptase (Gibco BRL, Rockville, MD). Reverse transcription (RT) reactions were carried out in 40 mM KCl, 50 mM Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM each of dATP, dCTP, dGTP and dTTP, 10 units of RNase inhibitor (Promega, Madison, WI), 100 pmol of random hexamer, total RNA and 200 units of the Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Berlin, Germany) in a final volume of 50 µl at 37°C for 60 min. A polymerase chain reaction (PCR) was carried out in a final volume of 20 µl, containing 1 µl of RT reaction mixture, 50 mM KCl, 20 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 10 µM each of the mixed oligonucleotide primers, and 1 unit of Taq DNA polymerase (Gibco-BRL). Reported primers were used for rat mdr1a (511 bp) [13], for rat mdr1b (451 bp) [13], for rat CYP3A2 (252 bp) [14] and for rat ß-actin (456 bp) [15]. Each cycle consisted of 30 s at 94°C, 60 s at 62°C and 75 s at 72°C for mdr1a, mdr1b and CYP3A2, and 30 s at 94°C, 60 s at 58°C, and 75 s at 72°C for ß-actin. The PCR reaction was run for 26 cycles for liver, for 34 cycles for intestine and for 22 cycles for ß-actin.

Preparation of microsomes and plasma membrane fraction

For preparation of microsomes, the liver was homogenized in three volumes of 100 mM Tris-HCl buffer (100 mM KCl, 1 mM EDTA, pH 7.4). Microsomes were prepared as reported previously [16] and stored at −80°C until use. The intestine was quickly removed and washed with buffer containing 2 mM HEPES, 0.9% NaCl and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The mucosa was scraped off with a slide glass on ice and homogenized in a buffer containing 300 mM mannitol, 5 mM EDTA, 5 mM HEPES and 1 mM PMSF (pH 7.1). The homogenate was centrifuged at $10\,000 \times g$ for 20 min, and the supernatant was centrifuged at $105\,000 \times g$ for 60 min at 4°C. The pellet was added to the buffer containing 500 mM KCl, 1 mM EDTA, 2 mM DTT and 50 mM KPB (pH 7.4) and again centrifuged at $105\,000 \times g$ for 60 min at 4°C. The resulting pellet was added to the buffer containing 1 mM EDTA, 2 mM DTT and 50 mM KPB (pH 7.4), and stored at −80°C until use.

For preparation of the plasma membrane, the liver was homogenized in 10 mM Tris-HCl buffer (pH 7.5) containing 2 mM $CaCl_2$ at 4°C. The homogenate was centrifuged at $3000 \times g$ for 10 min, and the supernatant was then centrifuged at $15\,000 \times g$ for 30 min. The pellet was washed, resuspended in 50 mM Tris-HCl buffer (pH 7.2), and twice centrifuged at $10\,000 \times g$ for 5 min, then stored at −80°C until use. The intestine was quickly removed and washed with ice-cold isotonic saline containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The mucosa was scraped off with a slide glass on ice and homogenized in a buffer containing 250 mM sucrose, 50 mM Tris-HCl (pH 7.4) and 1 mM PMSF. The homogenate was centrifuged at $3000 \times g$ for 10 min, and the supernatant was again centrifuged at $15000 \times g$ for 30 min. The resulting pellet was resuspended in 0.5 ml of a buffer containing 50 mM mannitol, 50 mM Tris-HCl (pH 7.4) and 1 mM PMSF, and stored at −80°C until use. Protein contents were measured according to the method of Lowry *et al.* [17].

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting with peroxidase/antiperoxidase staining of the plasma membrane for P-gp and of the microsomes for CYP3A were carried out essentially as described by Laemmli [18] and Guengerich *et al.* [19]. The amounts of sample protein of liver and intestine were 10 and 25 µg for CYP3A or 40 and 80 µg for P-gp, respectively. The sample protein was electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto PVDF membrane filters (Millipore Co., Billerica, MA). After having been blocked with 5% skim milk, the filters were incubated overnight at 4°C with primary antibody, mouse anti-P-gp C219 (Abcam, Cambridge, UK) and rabbit anti-rat CYP3A2 antibody (Daiichi Pure Chemicals Co. Ltd, Tokyo, Japan), and for 1 h with secondary antibody, anti-mouse IgG HRP-linked antibody

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(Cell Signaling, Beverly, MA) and mouse anti-rabbit IgG-HRP (Cell Signaling, Beverly, MA). Thereafter, the sample was extensively washed with phosphate-buffered saline. The immunopositive band was detected by means of a light-emitting nonradioactive detection system (Amersham International plc, Little Chalfont, Buckinghamshire, UK) with Kodak X-Omat R film (Eastman Kodak Co., Rochester, NY).

Data analysis

The pharmacokinetic parameters were estimated according to model-independent moment analysis as described by Yamaoka *et al.* [20]. The data were analysed using Student's *t*-test to compare the unpaired mean values of two sets of data. The number of determinations is noted in each table and figure. A value of $p<0.05$ or 0.01 was taken to indicate a significant difference between sets of data. The electrophoresis results were analysed by using NIH Image software.

Case Report

The patient was a 23-year-old man (56 kg) who had received bone marrow transplantation for acute myelogenous leukaemia 3 months before. He had been receiving immunotherapy with oral administration of Neoral® capsule (Novartis Pharma Co. Ltd.; CyA, 120 mg/day, 2 times a day). The trough blood concentrations of CyA were well maintained at about 100 ng/ml. However, he developed *Aspergillus* pneumonia, which was treated with intravenous infusion with Fungizone® injection (AMB, 1–30 mg/day, over 6 h). Figure 1 shows the relationship between the blood concentration of CyA and the dose of AMB or CyA. The patient was also receiving Saxizon® injection (Kowa Pharmaceutical Co. Ltd; hydrocortisone sodium succinate, 50–100 mg/day) and had been given a preparation of mixed amino acids and multiple vitamins since the transplantation.

The blood concentration of CyA in the patient gradually decreased to about one-third over the 12 days following the start of coadministration of AMB. Although the CyA dose was increased to 160 mg at day 17 after the start of AMB treatment, the blood concentration of CyA did not increase immediately. Subsequently the blood concentration of CyA slowly recovered, reaching about 210 ng/ml with 240 mg/day of CyA after 34 days.

The levels of γ-glutamyltranspeptidase (γ-GTP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), parameters of hepatic function, were initially 52, 45 and 65 IU/l, respectively, and subsequently remained stable within normal ranges. The value of serum creatinine as a parameter of renal function was initially 0.8 mg/dl, but increased slightly to 1.35 mg/dl at day 9 after the start of AMB treatment. Therefore, the AMB administration, which was initially 30 mg/day daily for the first 9 days, was reduced to the same dose every second day until day 40. Following the decrease of AMB dosage frequency, the serum creatinine level rapidly decreased to 0.8 mg/dl. These results suggest that the initial AMB treatment caused slight renal impairment.

Experimental Results

Pharmacokinetics of CyA in rats with AMB treatment

Figure 2 shows the blood concentration–time courses of CyA after i.v. administration of CyA (10 mg/kg) in control rats and in rats pretreated with AMB (1.5 or 3.0 mg/kg/day, i.v.) for 4 days. The blood concentration of CyA in the low-AMB group showed a significant, time-dependent decrease compared with the control, and that in the high-AMB group showed a similar decrease.

Figure 3 show the blood concentration–time courses of CyA after p.o. administration of CyA (10 mg/kg) in control rats and in rats treated with AMB. After p.o. administration, the concentration of CyA reached a maximum within 2–4 h in all three groups. The maximum blood concentrations (C_{max}) of CyA in the control, low-AMB and high-AMB groups were 1.16, 0.37 and 0.26 μg/ml, respectively. The blood concentration of CyA was significantly and dose-dependently decreased by the AMB treatment.

The pharmacokinetic parameters of CyA in the three groups are listed in Table 1. After the i.v.

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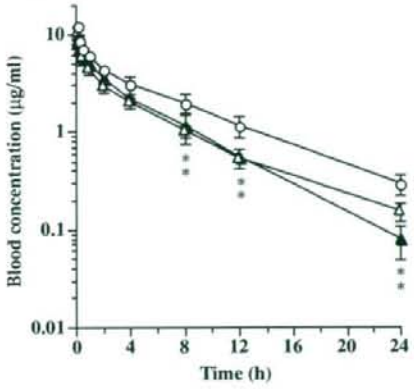

Figure 2. Blood concentration–time courses of CyA after an i.v. administration of CyA (10 mg/kg) in untreated rats (○) and rats treated with AMB at 1.5 mg/kg (△) or 3.0 mg/kg (▲) for 4 days. Rats were given CyA at 24 h after the last AMB treatment. Each point and bar represents the mean ± SD of four rats. **Significant difference between the control group and both AMB groups at $p<0.01$

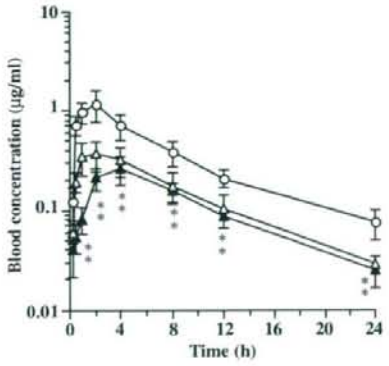

Figure 3. Blood concentration–time courses of CyA after a p.o. administration of CyA (10 mg/kg) in untreated rats (○) and rats treated with AMB at 1.5 mg/kg (△) or 3.0 mg/kg (▲) for 4 days. Rats were given CyA at 24 h after the last AMB treatment. Each point and bar represents the mean ± SD of four rats. **Significant difference between the control group and both AMB groups at $p<0.01$

administration, the values of the area under the blood concentration–time curve from 0 to 24 h ($AUC_{0-24\,h}$) in the two AMB groups were significantly decreased compared with the control,

Table 1. Pharmacokinetic parameters of CyA with or without AMB (1.5 or 3.0 mg/kg) in rats

Parameters	Non treatment	AMB treatment	
		1.5 mg/kg	3.0 mg/kg
i.v. administration			
$AUC_{0-24\,h}$ (μg h/ml)[a]	45.0 ± 2.7	28.4 ± 1.2**	30.5 ± 1.8**
MRT (h)[b]	5.96 ± 0.82	5.01 ± 0.51	4.67 ± 0.53*
CL_{tot} (ml/min)[c]	3.70 ± 0.22	5.87 ± 0.19**	5.47 ± 0.31**
Vd_{ss} (l/kg)[d]	1.32 ± 0.31	1.76 ± 0.35	1.53 ± 0.23
$t_{1/2}$ (h)[e]	5.87 ± 0.78	6.03 ± 0.68	4.18 ± 0.83*
p.o. administration			
$AUC_{0-24\,h}$ (μg h/ml)	8.53 ± 0.78	3.61 ± 0.41**	2.66 ± 0.23**
$t_{1/2}$ (h)	6.32 ± 0.95	5.86 ± 0.82	5.97 ± 0.93
Bioavailability (%)	19.0	12.7	8.7

Rats were intravenously or orally administered with CyA (10 mg/kg) at 24 h after the last AMB treatment in untreated rats and rats treated with AMB at 1.5 or 3.0 mg/kg for 4 days. Pharmacokinetic parameters were estimated according to model-independent moment analysis. Each value represents the mean ± SD of four rats.

*,**Significant difference between the control group and both AMB groups at $p<0.05$ and 0.01, respectively.

[a] Area under blood concentration–time curve from 0 to 24 h.

[b] Mean residence time from 0 to 24 h.

[c] Blood total clearance.

[d] Distribution volume at the steady-state.

[e] Elimination half-life.

and the values of total clearance (CL_{tot}) were significantly increased. The elimination half-life ($t_{1/2}$) in the high-AMB group was significantly faster than that in the control group. After the p.o. administration, the $AUC_{0-24\,h}$ values of the low- and high-AMB groups were significantly decreased to about 42% and 31% of the control, respectively. However, the $t_{1/2}$ values in the three groups showed no significant difference. The values of oral bioavailability of CyA of the low- and high-AMB groups were decreased to about 67% and 46% of the control, respectively.

Laboratory data in rats after AMB treatment

Table 2 shows the laboratory data for rats treated or not treated with AMB (1.5 or 3.0 mg/kg). There was no clear difference in the body weight among the three groups. The laboratory data reflecting hepatic function were unaffected by the AMB treatment, except for the A/G ratio. The values of blood urea nitrogen (BUN) and serum creatinine were significantly increased by the high-AMB treatment.

RT-PCR analysis of mdr1a, mdr1b and CYP3A2 mRNAs in intestine and liver

Figure 4 shows the effect of AMB treatment (1.5 or 3.0 mg/kg/day, i.v., for 4 days) on the expression of mdr1a, mdr1b and CYP3A2 mRNAs in the duodenum, ileum and liver at 24 h after the last treatment. The expression levels of mdr1a and mdr1b mRNAs were significantly increased in the duodenum, but little changed in the ileum and liver by the AMB treatment. On the other hand, the expression of CYP3A2 was significantly induced, in the liver only, by both AMB treatments.

Table 2. Physical and biochemical data in rats treated with AMB

	Non treatment	AMB treatment	
		1.5 mg/kg	3.0 mg/kg
Body weight (g)	220 ± 7	227 ± 12	216 ± 11
AST (IU/l)	84 ± 5	82 ± 8	88 ± 14
ALT (IU/l)	40 ± 7	35 ± 2	38 ± 13
Albumin (g/dl)	4.07 ± 0.11	3.93 ± 0.15	3.67 ± 0.31
A/G ratio	2.23 ± 0.15	1.97 ± 0.25	1.70 ± 0.31*
Total bilirubin (mg/dl)	0.14 ± 0.04	0.15 ± 0.08	0.14 ± 0.06
BUN (mg/dl)	15.1 ± 1.8	26.1 ± 5.9*	46.8 ± 9.3**
serum creatinine (mg/dl)	0.26 ± 0.03	0.28 ± 0.03	0.37 ± 0.05**

Data were measured at 24 h after the last administration of AMB (1.5 or 3.0 mg/kg i.v.) for 4 days in rats. Each value represents the mean ± SD of four rats.
*,**Significantly different from the control at $p<0.05$ and 0.01, respectively.

Expression of P-gp and CYP3A2 in intestine and liver

Figure 5 shows the effect of the AMB treatment (3.0 mg/kg/day, i.v., for 4 days) on the expression levels of P-gp and CYP3A2 proteins detected by western blot analysis. The protein levels of P-gp in the duodenum and liver were significantly elevated compared with the untreated control level, but the level in the ileum was hardly changed by AMB. On the other hand, the protein level of CYP3A2 in the liver was significantly elevated compared with the control level, while the levels in the duodenum and ileum showed no significant change.

Discussion

In order to understand why the blood concentration of CyA in a patient receiving immunotherapy decreased following coadministration of AMB, the effect of repeated i.v. administration of AMB (1.5 or 3.0 mg/kg) for 4 days on the disposition kinetics of CyA was examined in rats after i.v. or p.o. administration (Figures 2, 3). A dose of 1.5 or 3.0 mg/kg was used in rats because the total clearance of AMB in rats is about five times higher than that in humans (0.5 mg/kg) [21]. As it was previously established that dexamethasone [9] and cyclophosphamide [10] induced P-gp and CYP3A2 within 4 days of coadministration, in this study AMB was coad-

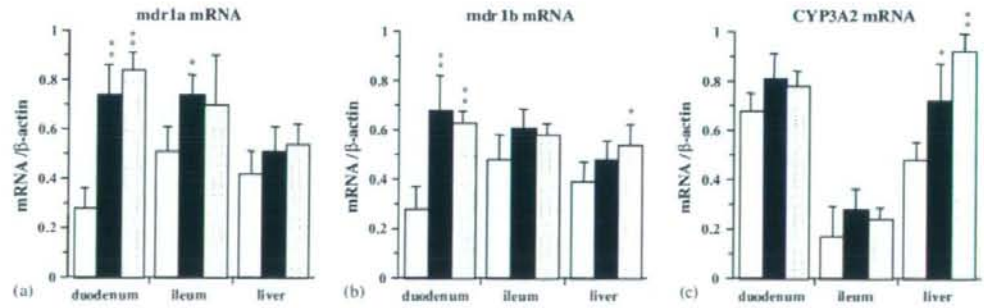

Figure 4. Effect of AMB on the expression of mdr1a, mdr1b and CYP3A2 mRNAs in duodenum, ileum and liver. The data represent the relative expression of the mRNAs obtained as mRNA/β-actin mRNA ratios in the control group (□), the low-AMB (1.5 mg/kg, ■) and high-AMB (3.0 mg/kg, ⬚) groups at 24 h after the last AMB treatment for 4 days. *,**Significant difference from the control group at $p<0.05$ and $p<0.01$, respectively

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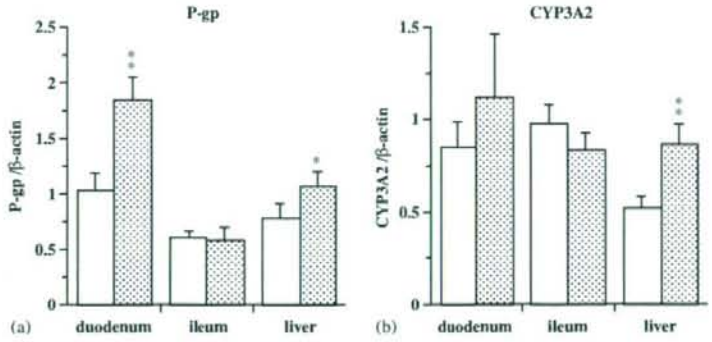

Figure 5. Western blot analysis of P-gp (a) and CYP3A (b) proteins in the duodenum, ileum and liver of rats with (□) or without (▨) the high-dose AMB (3.0 mg/kg) for 4 days, at 24 h after the last AMB treatment. *,**Significant difference from the control group at $p<0.05$ and $p<0.01$, respectively

ministered intravenously to rats for 4 days. The blood concentration of CyA after i.v. administration was significantly, though not dose-dependently, decreased in both AMB groups compared with the control. After p.o. administration, there was a significant, dose-dependent decrease of the blood concentration of CyA in both AMB groups. The oral bioavailability of CyA was clearly decreased by AMB treatment (Table 1).

It is well known that renal failure is the main side effect of AMB in clinical practice. There are some reports indicating that the disposition kinetics of CyA is influenced by renal failure in rats. Shibata *et al.* [22] reported that CYP3A and P-gp in liver and intestine are not likely to be involved in lowering the oral bioavailability of CyA in gentamicin-induced acute renal failure, and that a change in bile function is responsible for the marked decrease. Huang *et al.* [23] reported that the expression level of P-gp in rats with glycerol-induced acute renal failure was increased 2.5-fold in the kidney, but was not increased in the liver or brain. Leblond *et al.* [24] reported that chronic renal failure is associated with a decrease in total liver CYP450 (mainly CYP2C11, CYP3A1 and CYP3A2) activity in rats, and this leads to a significant decrease in drug metabolism. In the rats, hepatic function was not impaired, as judged from the laboratory data, whereas increases in the values of serum creatinine and BUN indicated an appreciable impairment of renal function by the high-AMB treatment (Table 2). However, the increases of serum creatinine and BUN were not large compared with the increases of about 3- to 5-fold and 2- to 5-fold in model rats with gentamicin- or glycerol-induced renal failure, respectively [22–24]. Therefore, it was considered that the AMB-induced renal impairment was not great even in the high-AMB group, and may have had only a slight influence on the excretion of CyA, which is metabolized mainly by CYP3A.

It was found that in our model the expression of mdr1a and mdr1b mRNAs was characteristically induced about 3-fold in the duodenum compared with the control, but was only slightly increased in the ileum and liver by both AMB treatments (Figure 4). Also, the expression of CYP3A2 mRNA was increased about 2-fold over the control in the liver. These results are broadly consistent with the previous findings in mice treated with dexamethasone (DEX) [25,26]. Unlike DEX, AMB did not induce a substantial increase of CYP3A2 mRNA expression in intestine, possibly because the dose levels of AMB were relatively low. This seems consistent with the relatively minor renal impairment. The studies with DEX [9–11,26] indicated that increased mRNA levels of mdr1a, mdr1b and CYP3A2 mRNAs are well reflected in increased levels of P-gp and CYP3A2 proteins. In this study also, the induction of the mRNAs by AMB treatment resulted in correspondingly increased levels of P-gp and CYP3A2 proteins (Figure 5). Therefore, it was considered that the oral bioavailability of CyA was reduced in the present

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animal model as a result of increased efflux transport via P-glycoprotein in the duodenum and an increased first-pass effect of hepatic CYP3A2 induced by AMB. Interestingly, there was not a great difference in the $t_{1/2}$ values of CyA among the three groups. Therefore, it appears that the induction of CYP3A2 in the liver only slightly influences the disposition kinetics of CyA. Previously it was suggested that, under physiological conditions, the oral bioavailability of CyA is mainly controlled by CYP3A in the upper intestine, rather than in the liver, but when P-gp is induced by steroid, the intestinal absorption of CyA may be inhibited [26]. Therefore, it is considered that the decrease of bioavailability of CyA caused by AMB treatment is mainly due to a decrease of intestinal absorption resulting from the induction of P-gp.

In the case of our patient, the stable trough values of blood CyA concentration during repeated p.o. administration of CyA were decreased after repeated intravenous infusion administration of AMB was started, and the decrease continued for 3 weeks. During this period, the laboratory data indicated relatively minor impairment of hepatic and renal functions by AMB. The patient had also been receiving hydrocortisone since the transplantation, but the low dose of hydrocortisone (50–100 mg/day) appeared to have had little influence on the plasma level of CyA prior to the start of AMB treatment. Therefore, we think that the clinical observations can also be explained mainly in terms of the induction of P-gp in the intestine by AMB.

It was previously clarified that the induction of P-gp and CYP3A2 continues for 2 weeks after the final DEX treatment [27]. Here, the patient received repeated administrations of AMB for 40 days, so it was difficult to predict when the levels of P-gp and CYP3A2 expression would recover to the control values; therefore, we chose to increase the dose of CyA gradually. The level of blood CyA concentration began to increase 7 days after the dose of CyA was increased from 120 mg/day to 160 mg/day, and after the dose of CyA was further increased to 240 mg/day, the blood CyA concentration reached 210 ng/ml. Therefore, we speculate that the blood CyA concentration might have recovered to about 100 ng/ml within 40 days after the start of AMB treatment, if the dose of CyA had been kept at 120 mg/day throughout. However, there are species differences in hepatic metabolism and susceptibility to metabolic changes. Therefore, it would be desirable to examine further the influence of AMB treatment on the metabolic activity, uptake and efflux of CyA in the intestine by means of *in vitro* studies using human hepatocytes, human hepatic microsomes, intestinal membrane, Caco-2 cells, etc.

In conclusion, the results indicate that the oral bioavailability of CyA is decreased by coadministration of AMB because of an increase of expression of P-gp and CYP3A induced by AMB treatment. The results in this rat model were consistent with the clinical observations. Therefore, the blood concentration of drugs that are substrates of P-gp and CYP3A, such as CyA, should be carefully monitored in patients when AMB is coadministered in combination with such drugs.

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ERRATUM

Cord blood transplantation using minimum conditioning regimens for patients with hematologic malignancies complicated by severe infections

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Please note that there are errors in this article:

On the 2nd page on line 14 in the right column, "13 h" should be "23 h".

On the 3rd page on line 21 in the left column, "12 h" should be "22 h".

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False-positive *Aspergillus* galactomannan antigenaemia after haematopoietic stem cell transplantation

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***Objectives*: Although *Aspergillus* galactomannan (GM) antigen detection is widely applied in the diagnosis of invasive aspergillosis (IA), false-positive reactions with fungus-derived antibiotics, other fungal genera or the passage of dietary GM through injured mucosa are a matter of concern. The aim of this study was to investigate the cumulative incidence and risk factors for false-positive GM antigenaemia.**

***Patients and methods*: The records of 157 adult allogeneic haematopoietic stem cell transplantation (HSCT) recipients were retrospectively analysed. Episodes of positive GM antigenaemia, defined as two consecutive GM results with an optical density index above 0.6, were classified into true, false and inconclusive GM antigenaemia by reviewing the clinical course.**

***Results*: Twenty-five patients developed proven or probable IA with a 1 year cumulative incidence of 12.9%, whereas 50 experienced positive GM antigenaemia with an incidence of 32.2%. Among the total 58 positive episodes of the 50 patients, 29 were considered false-positive. The positive predictive value (PPV) was lower during the first 100 days than beyond 100 days after HSCT (37.5% versus 58.8%). Gastrointestinal chronic graft-versus-host disease (GVHD) was identified as the only independent significant factor for the increased incidence of false-positive GM antigenaemia (PPV 0% versus 66.7%, $P = 0.02$).**

***Conclusions*: GM antigen results must be considered cautiously in conjunction with other diagnostic procedures including computed tomography scans, especially during the first 100 days after HSCT and in patients with gastrointestinal chronic GVHD.**

Keywords: fungal infections, invasive aspergillosis, chronic GVHD, gastrointestinal tract, mucosal damage

Introduction

Invasive aspergillosis (IA) remains one of the leading infectious causes of death after allogeneic haematopoietic stem cell transplantation (HSCT), despite new antifungal agents that have become available in recent years.[1] The high mortality rate of IA was mainly attributed to the difficulty of diagnosis at the early stage of the disease, because histopathological examinations require invasive procedures and fungal cultures have low specificity and sensitivity in detecting IA.

Monitoring of the circulating *Aspergillus* galactomannan (GM) antigen by the sandwich enzyme-linked immunosorbent assay (ELISA) is a feasible non-invasive biological method for early diagnosis of IA.[2] The GM ELISA test has sensitivity of 67% to 100% and specificity of 81% to 99% in neutropenic patients and allogeneic transplant recipients,[3–6] and was introduced as microbiological evidence in the European Organization for Research and Treatment of Cancer and Mycoses Study Group (EORTC/MSG) criteria for opportunistic invasive fungal infection.[7] However, a concern is the false-positive reactions,

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