preceding PIR or ES in 15 patients who displayed continuous symptoms of immune reactions. Acute GvHD developed after resolution of PIR or ES in 12 patients. Cumulative incidences of grade I to IV acute GvHD grouped by the presence or absence of PIR were 57% and 40%, respectively (P=0.16).

Organ involvement was as follows: skin stage 1 (n=6), stage 2 (n=6), stage 3 (n=9), and stage 4 (n=1); liver stage 1 (n=6), stage 2 (n=5), stage 3 (n=8), and stage 4 (n=7); gut stage 1 (n=3), stage 2 (n=13), and stage 3 (n=5). Among the 25 patients given corticosteroid to treat GvHD, responses were CR (n=15), PR (n=7), MR (n=1), and NC (n=2).

Nonrelapse Mortality

Thirty-two patients died without disease progression. Cumulative incidences of relapse and NRM at day 180 were 15% and 62%, respectively. Causes of NRM comprised acute GvHD (n=5), interstitial pneumonitis (n=2), thrombotic microangiopathy (n=3), heart failure (n=2), cytomegalovirus infection (n=2), invasive aspergillosis (n=2), miliary tuberculosis (n=1), cerebral hemorrhage (n=2), bacteremia (n=7), pneumonia (n=3), multiple organ failure caused by PIR (n=1), alveolar hemorrhage (n=1), and gastrointestinal bleeding (n=1).

Effect of PIR, ES, and GvHD on Relapse and NRM

Cumulative incidences of relapse were 18% and 5% in patients with and without PIR, respectively (P=0.32). Cumulative incidences of NRM were 60% and 65% in patients with and without PIR, respectively (P=0.35). Because development of ES was closely associated with PIR, these two reactions could not be separated from each other in evaluation of their effect on relapse and NRM. Development of GvHD was not a significant prognostic factor for relapse or NRM when PIR was treated as a time-dependent covariate.

DISCUSSION

We have demonstrated that most patients exhibited some immune reactions, whereas a certain proportion of findings were accounted for by infection and regimen-related toxicity. In particular, it is likely that diarrhea was in some cases caused by melphalan, which has dose-limiting gastrointestinal toxicity (*31*). However, the development of similar reactions in most patients suggests that these reactions are characteristic of RI-CBT. PIR developed during posttransplant myelosuppression. When compared with ES and GvHD, the higher CRP levels and fever observed in PIR suggest that the inflammation occurring in this reaction is intense. Although optimal treatment remains unknown, corticosteroid was administered at the discretion of the primary physician. Most patients responded to corticosteroid, although PIR occasionally progressed and merged with ES and GvHD despite immunosuppressive treatments. Because cytokine storm associated with PIR might trigger the development of ES or GvHD, suppression of PIR could be effective in reducing NRM. This small-sized study failed to show a prognostic impact of PIR, and the clinical significance of this reaction awaits further investigation.

The mechanism of PIR remains unknown. Pathologic examination of the skin obtained from six patients showed edema and vascular dilatation without lymphocytic infiltration. Interestingly, PIR occurred in patients who had not achieved engraftment, suggesting that the mechanism of PIR differs from that of ES/GvHD. The reaction is probably related to the response of adult recipients to transplanted cord blood rather than to the cord-blood engraftment. Antithymocyte globulin (ATG) and corticosteroid, which have strong immunosuppressive properties, were commonly used in CBT (*6, 8–11, 14, 17, 32–34*), whereas neither was used in this study. Immune reactions after CBT might therefore have manifested more easily with the present regimen. PIR could be attributed to a cytokine storm induced by massive proliferation of cells with a unique cytokine profile. Another possibility is homeostasis-driven proliferation of naive T cells in highly immunosuppressed individuals, as demonstrated in murine models (*35*). This reaction is associated with cytotoxic cytokines (*35*). However, fever as a transient response to contamination with maternal blood or cells during cord-blood collection cannot be excluded (*36*), and reactivation of virus such as human herpesvirus 6 might be associated with PIR (*37*).

The reaction at engraftment was similar to the reaction known as ES after autologous transplantation (*27*). The inflammation occurring in ES was less intense than that observed in PIR, as evidenced by less marked fever, weight gain, and CRP elevation (Table 2). In this regard, corticosteroid, which was given for PIR and continued during the manifestation of ES, might have masked the inflammatory reaction of ES. Surprisingly, five patients with ES developed central nervous system complications, with two diagnosed as having limbic encephalopathy. This type of neurologic complication has not been emphasized in allo-SCT using marrow or peripheral blood and might be characteristic of CBT (*38*). Fluid accumulation during this period might have accentuated the tendency for brain edema. Engraftment processes may differ between CBT and conventional allo-SCT.

Postengraftment reaction was characterized by a higher incidence of jaundice and a lower incidence of edema when compared with PIR and ES. Clinical manifestation was consistent with the immune reaction conventionally known as acute GvHD. Although the incidence of GvHD after CBT for adult patients has been reported to be low, the incidence of grade II to IV acute GvHD varies from 25% to 72% (*9–12, 21–24, 39*) and has not been thoroughly investigated. In the present study, the incidence of grade I to IV acute GvHD was 51%. GvHD is a significant problem in RI-CBT as well as in conventional myeloablative CBT. Cord blood might have the potential to elicit an intense graft-versus-host effect, creating a niche for early engraftment and GvL effects.

Few studies have described the immune reaction after CBT, and none have characterized PIR and ES in CBT. In the present study, there are several possible reasons for these immune reactions being distinct. First, we only enrolled adult patients because children develop GvHD less frequently than do adults (*5, 6, 8*). Second, the median nucleated cell dose in our study (2.9×10^{7} /kg) was greater than that reported in certain studies performed in Western countries (*9–12*). The low median body weight (53.8 kg) among the Japanese patients in this study might have favored engraftment and immune reactions. Third, 84% of our patients received cord blood from donors mismatched at tow to three HLA loci. The

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association between HLA disparity and the risk of GvHD remains unclear in CBT. Although most studies have failed to show a significant relationship between HLA disparity and the risk of GvHD (*5, 8, 14, 33*), a recent multivariate analysis of the largest series showed a significant association between acute GvHD and HLA disparity (*40*). Fourth, our conditioning regimen, which did not include ATG and used cyclosporine alone for GvHD prophylaxis, was mild, allowing the manifestation of immune reactions.

Although the present study provides an important description of the immune reaction after RI-UCBT, it contains certain limitations. This was a small retrospective study, and unrecognized bias caused by heterogeneous patient background might have influenced the results. Furthermore, the diagnostic criteria for immune reactions based on clinical and pathologic findings could not exclude infection or toxicity from various drugs including conditioning regimens. Therefore, it is possible that incidence of immune reactions was overestimated, particularly for PIR developing during neutropenia. In contrast, immunosuppressive treatments (mostly corticosteroid) for preceding complications could have masked the incidence and severity of ES and GvHD. ES has some similarities to acute GvHD, and it is sometimes difficult to make an accurate diagnosis of these complications. Further investigations are warranted to reveal the mechanism of immune reactions after RI-CBT and to develop a strategy of their control without reducing GVL effects.

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Featured Article

Successful Engraftment After Reduced-Intensity Umbilical Cord Blood Transplantation for Adult Patients with Advanced Hematological Diseases

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ABSTRACT

Purpose: **The purpose of this research was to evaluate the feasibility of reduced-intensity unrelated cord-blood transplantation (RI-UCBT) in adult patients with advanced hematological diseases.**

Experimental Design: **Thirty patients (median age, 58.5 years; range, 20–70 years) with advanced hematological diseases underwent RI-UCBT at Toranomon Hospital between September 2002 and August 2003. Preparative regimen composed of fludarabine 25 mg/m^2 on days −7 to −3, melphalan 80 mg/m^2 on day −2, and 4 Gy total body irradiation on day −1. Graft-*versus*-host disease prophylaxis was composed of cyclosporin alone.**

Results: **Twenty-six patients achieved primary neutrophil engraftment after a median of 17.5 days. Median infused total cell dose was 3.1 × 10^7/kg (range, 2.0–4.3 × 10^7/kg). Two transplant-related mortalities occurred within 28 days of transplant, and another 2 patients displayed primary graft failure. Cumulative incidence of complete donor chimerism at day 60 was 93%. Grade II-IV acute graft-*versus*-host disease occurred in 27% of patients, with median onset 36 days. Primary disease recurred in 3 patients, and transplant-related mortality within 100 days was 27%. Estimated 1-year overall survival was 32.7%. Excluding 7 patients with documented infection, 19 patients displayed noninfectious fever before engraftment (median onset, day 9). Manifestations included high-grade fever, eruption, and diarrhea. The symptoms responded well to corticosteroid treatments in 7 of 13 treated patients.**

Conclusion: **This study demonstrated the feasibility of RI-UCBT in adults.**

INTRODUCTION

Allogeneic hematopoietic stem-cell transplantation (allo-HSCT) is a curative treatment for refractory hematological malignancies. The therapeutic benefits are attributable to myeloablative radiochemotherapy and graft-*versus*-leukemia effects (1), whereas the severe regimen-related toxicity (RRT; Ref. 2) limited allo-HSCT to young patients without comorbidities.

Reduced-intensity stem-cell transplantation (RIST) using a nonmyeloablative preparative regimen has been developed to decrease RRT, whereas preserving adequate antitumor effects (3–5). Different pioneering conditioning regimens for RIST have been investigated, such as those including purine analogs (3–6) and total body irradiation (TBI). Although RIST has been attempted in various diseases (5, 6), suitable preparative regimens with adequate immunosuppression have yet to be established.

Although allo-HSCT from an HLA-identical sibling is promising, only 30% of the patients have an HLA-identical sibling donor. The value of unrelated cord-blood transplantation (UCBT) was confirmed for pediatric patients (7, 8). It has seen recent application in adult patients (9). Whereas the potential graft-*versus*-leukemia effects by cord-blood (CB) without severe graft-*versus*-host disease (GVHD; Ref. 10) has been reported, current questions include whether CB provides a sufficient number of stem cells for adults and suitable graft-*versus*-leukemia effects.

Reduced-intensity (RI)-UCBT (11, 12) represents a promising treatment for advanced hematological malignancies. Wagner *et al.* (12) reported recently the feasibility of RI-UCBT for pediatric patients. However, the feasibility in adult patients remains unclear. We report 30 adult patients with advanced hematological diseases who underwent RI-UCBT after fludarabine, melphalan, and 4 Gy TBI since October 2003 at our institution.

PATIENTS AND METHODS

Study Patients and Donors. Thirty patients with hematological diseases underwent RI-UCBT at Toranomon Hospital between September 2002 and August 2003. All of the patients had hematological disorders that were incurable with conventional treatments and were considered inappropriate for conven-

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tional allo-HSCT due to the lack of an HLA-identical sibling or a suitable unrelated donor, age >50 years old and/or organ dysfunction (generally attributable to previous intense chemo- and/or radiotherapy).

All of the patients provided written informed consent in accordance with the requirements of the Institutional Review Board.

HLA Typing and Donor Matching. An unrelated donor was searched through the Japan Marrow Donation Program (13) for patients without an HLA-identical sibling donor. When no appropriate donor was identified, the Japan Cord Blood Bank Network (14) was searched. CB units, which were ≥4 of 6 HLA-antigen matched and contained at least 2×10^7 nucleated cells/kg of recipient body weight before freezing were used. CB units were not depleted of T lymphocytes.

Preparative Regimen. The preparative regimen was composed of fludarabine 25 mg/m^2 on days −7 to −3, melphalan 80 mg/m^2 on day −2, and 4 Gy TBI in 2 fractions on day −1.

Supportive Cares. All of the patients were managed in reverse isolation in laminar airflow-equipped rooms and received trimethoprim/sulfamethoxazole for *Pneumocystis carinii* prophylaxis. Fluoroquinolone and fluconazole were administered for prophylaxis of bacterial and fungal infections, respectively. Prophylaxis of herpes virus infection with acyclovir was also given (15). Neutropenic fever was managed according to the guidelines (16, 17). Cytomegalovirus (CMV) pp65 antigenemia was monitored once a week. If positive results were identified, preemptive therapy with foscarnet was initiated. Hemoglobin and platelet counts were maintained at >7 g/dl and $>10 \times 10^9$/liter, respectively, with in-line filtered and irradiated blood transfusions.

Management of GVHD. GVHD was clinically diagnosed in combination with skin or gut biopsies after engraftment or attainment of 100% donor chimerism. Acute and chronic GVHD were graded according to the established criteria (18, 19).

GVHD prophylaxis was a continuous infusion of cyclosporin 3 mg/kg from day −1 until the patients tolerated oral administration. It was tapered off from day 100 until day 150. If grade II-IV acute GVHD developed, 1 mg/kg/day of prednisolone was added to cyclosporin and tapered from the beginning of clinical response.

Chimerism Analysis. Chimerism was assessed using fluorescent *in situ* hybridization in sex-mismatched donor-recipient pairs. In sex-matched pairs, PCR for variable numbers of tandem repeats was used with donor cells detected at a sensitivity of 10% (20).

Whole blood and CD3-positive cell chimerism was assessed at the time of granulocyte engraftment. When engraftment was delayed, chimerism was assessed on day 30. For those who died before engraftment, chimerism was assessed at least once during life.

Engraftment. Engraftment was defined as WBC counts $> 1.0 \times 10^9$/liter or absolute neutrophil counts $> 0.5 \times 10^9$/liter for 2 consecutive days. Granulocyte colony stimulating factor (Filgrastim) 300 μg/m^2/day was administered i.v. from day 1 until neutrophil engraftment.

Graft failure was defined as peripheral cytopenia and marrow hypoplasia occurring later than day 60, without detection of donor markers by cytogenetic and/or molecular techniques.

Table 1 Patient characteristics (n = 30)

Age (y), median (range)	58.5 (20–70)
Weight (kg), median (range)	52 (38–75)
Male/female	16/14
Diagnosis	
Malignancy	
Acute myeloid leukemia	14
Myelodisplastic syndrome	1
Acute lymphoblastic leukemia	3
Adult T-cell leukemia	5
Plasma cell leukemia	1
Chronic myeloid leukemia	1
Malignant lymphoma	1
Benign	
Severe aplastic anemia	4
Disease status at transplantation (malignancy)	
Remission	1
Refractory to previous chemotherapy	25

RRT and Transplantation-Related Mortality (TRM). RRT was defined as any nonhematological organ dysfunction from day 0 to day 28 and was graded according to the Bearman's criteria (2). TRM was defined as death without the primary disease progression.

Endpoints and Statistical Analysis. Primary end points were composed of the rates of durable engraftment and TRM within day 100. Secondary end points were the rates of RRT, acute and chronic GVHD, infections, event-free survival (EFS), and overall survival (OS).

Acute GVHD was analyzed for engrafted patients. Chronic GVHD was analyzed for patients who survived ≥100 days.

EFS was defined as the duration of survival after transplantation without disease progression, relapse, graft failure, or death. The probabilities of OS and EFS were shown by the Kaplan-Meier method as of January 31, 2004. Surviving patients were censored on the last day of follow-up. Cox regression analysis was used to determine the effect of various variables on OS.

RESULTS

Patient Characteristics. Median age was 58.5 years (range, 20–70 years), and median weight was 52 kg (range, 38–75 kg; Table 1). All of the patients were CMV-seropositive.

The malignancies of 25 patients were refractory to cytotoxic chemotherapies except acute myeloblastic leukemia (n = 1) in first CR. The remaining 4 patients had transfusion-dependent severe aplastic anemia.

CB Characteristics. Twenty-four and 6 patients received 4 of 6 and 5 of 6 HLA-antigen-matched CB, respectively. Twenty-one patient CB pairs were sex-mismatched. Median infused total nucleated cell dose and CD34-positive cell dose before freezing were 3.1×10^7/kg (range, $2.0–4.3 \times 10^7$/kg) and 0.74×10^5/kg (range, $0.17–2.5 \times 10^5$/kg), respectively.

Engraftment. Twenty-six patients [87%; 95% confidence interval (95% CI), 75–99%] achieved primary neutrophil engraftment, among whom median day of engraftment was 17.5 days (range, 10–54 days; Fig. 1). Their engraftment was durable

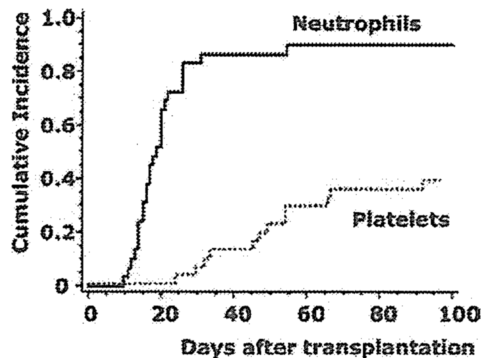

Fig. 1 Engraftment of neutrophils and platelets. Twenty-six (87%; 95% confidence interval, 75–99%) and 16 patients (40%; 95% confidence interval, 25–57%) achieved primary neutrophil and platelet engraftment, respectively.

Table 2 Neutrophil engraftment, chimerism, and overall survival

Variable	n	% (95% CI)[a]	P
Neutrophil engraftment			
Total cell dose			
≧3 × 10^7/kg	16	94% (82–100%)	
<3 × 10^7/kg	14	79% (57–100%)	0.25
HLA disparities			
HLA 5/6 match	6	67% (29–100%)	
HLA 4/6 match	24	92% (81–100%)	0.24
100% Donor chimerism			
Total cell dose			
≧3 × 10^7/kg	16	100%	
<3 × 10^7/kg	14	86% (67–100%)	0.63
HLA disparity			
HLA 5/6 match	6	83% (54–100%)	
HLA 4/6 match	24	96% (88–100%)	0.31
Overall survival			
Total cell dose			
≧3 × 10^7/kg	16	54% (24–83%)	
<3 × 10^7/kg	14	52% (6.6–87%)	0.70
HLA disparities			
HLA 5/6 match	6	63% (20–100%)	
HLA 4/6 match	24	51% (20–81%)	0.60

[a] CI, confidence interval.

without requiring readministration of Filgrastim. Two patients died of TRM within 28 days of transplant. Primary graft failure occurred in the remaining 2 patients, who underwent second RI-UCBT with the same preparative regimen and GVHD prophylaxis and achieved neutrophil engraftment and complete donor chimerism. No patients experienced a decrease in neutrophil <0.5 × 10^9/liter during the follow-up.

Platelet counts >20 × 10^9/liter were achieved by 16 patients (40%; 95% CI, 25–57%) on a median day of 39 days (range, 25–95 days). No other patient achieved platelet recovery until the last day of follow-up.

No significant association was found between neutrophil engraftment and either infused cell dose or HLA disparity (Table 2).

Chimerism Analysis. Chimerism data were obtained from all of the 30 patients. Cumulative incidence of complete donor chimerism at day 60 was 93% (95% CI, 84–100%), and median time to complete donor chimerism was 22 days (range, 13–56 days; Fig. 2). The 2 patients who died of TRM within 28 days had complete donor chimerism before neutrophil engraftment. All of the surviving patients were monitored for chimerism every 3 months, followed the cyclosporine tapering schedule from day 100 to day 150, and maintained complete donor chimerism during the follow-up even after the discontinuation of immunosuppressants.

No significant association was identified between complete donor chimerism and either infused cell dose or HLA disparity (Table 2).

RRT and TRM. Four patients (13%) developed grade III RRT. No patient had grade IV RRT. The most commonly involved organs were the gut and kidney (Table 3).

TRM within 100 days of RI-UCBT was 27%. Primary causes of death were interstitial pneumonitis (n = 2), acute GVHD (n = 2), gastrointestinal bleeding (n = 1), acute heart failure (n = 1), limbic encephalopathy (n = 1), and sepsis (n = 1).

GVHD. Grade II-IV and III-IV acute GVHD occurred in 27% (95% CI, 11–43%) and 23% (95% CI, 7.4–39%) of the patients, respectively. Median onset of grade II-IV acute GVHD was day 36 (range, day 17–66; Fig. 3).

Of the 13 patients who survived >100 days, 3 (23%) developed chronic GVHD.

Infection. Twelve patients developed infections: bacteremia (n = 8), invasive aspergillosis (n = 3), and pulmonary tuberculosis (n = 1). Nine of them had been treated with

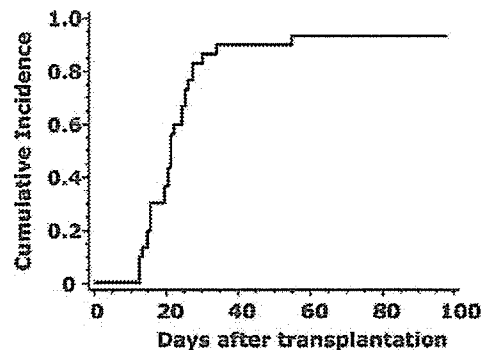

Fig. 2 Achievement of complete donor chimerism. Cumulative incidence of complete donor chimerism at day 60 after reduced-intensity unrelated cord-blood transplantation (*RI-UCBT*) was 93% (95% confidence interval, 84–100%), and median time to complete donor chimerism was day 22 (range, day 13–56).

Table 3 Regimen-related toxicity within 28 days (Bearman's score)

Score	Diarrhea	Kidney	CNS[a]	Liver	Lung
Grade 0	18	18	26	22	27
Grade 1	8	5	0	3	2
Grade 2	4	6	1	4	0
Grade 3	0	1	3	1	1
Grade 4	0	0	0	0	0

[a] CNS, central nervous system.

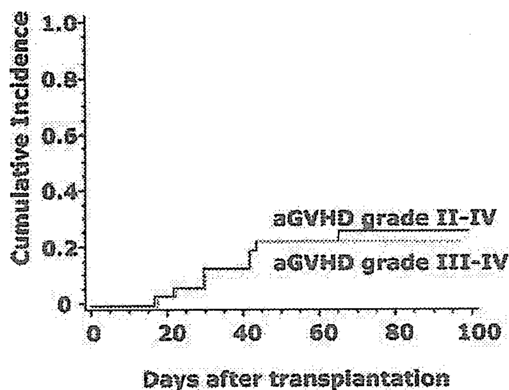

Fig. 3 Development of acute graft-*versus*-host disease (GVHD). Grade II-IV and III-IV acute GVHD developed in 27% (95% confidence interval, 11–43%) and 23% (95% confidence interval, 7.4–39%) of the patients, respectively. Median onsets of grade II-IV and III-IV acute GVHD were day 36 (range, day 17–66) and day 30 (range, day17–44), respectively.

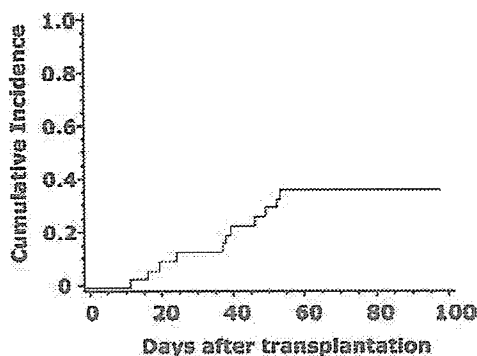

Fig. 4 Development of cytomegalovirus reactivation. Reactivation of cytomegalovirus was documented in 11 patients (37%) on a median of day 40 (range, day 13–55).

corticosteroids at the onset of infections. Reactivation of CMV was documented in 11 patients (37%) on a median of day 40 (range, day 13–55; Fig. 4). Eight of them had been treated with corticosteroids at the onset of CMV antigenemia. None of them developed CMV-related diseases. One patient developed hemorrhagic cystitis with adenovirus and BK virus infection.

Pre-Engraftment Noninfectious Fever. Seven patients with documented infection before engraftment were excluded from the analysis of pre-engraftment reaction (Table 4). Eighteen patients developed noninfectious fever before neutrophil engraftment (Fig. 5). Noninfectious high-grade fever often coexisted with eruption, diarrhea, and weight gain, starting on a median of day 9. Pathological examination of eruption from 8 patients revealed nonspecific inflammatory reactions and was not compatible with GVHD.

Survival. As of January 2004, a total of 11 patients remained alive. Median follow-up of the survivors and all of the enrolled patients were 238 days (range, 169–485) and 125 days (range, 26–485), respectively. Primary diseases recurred in 3 patients. Estimated 1-year OS and EFS were 32.7% (95% CI, 14.3–51.1%; Fig. 6) and 22.2% (95% CI, 5.9–38.5%; Fig. 7), respectively. Neither cell dose nor HLA disparity was associated with OS (Table 2).

DISCUSSION

Because CB contains a small amount of hematopoietic stem cells and stem cell boost or donor lymphocyte infusion is not available after UCBT, graft failure has been a major concern in adult UCBT. The present study demonstrated the feasibility of RI-UCBT for adult patients, in addition to pediatric patients (21). In this study, 26 of the 30 patients (87%) achieved durable engraftment, and 28 patients achieved complete donor chimerism by day 60, including 2 patients who died before engraftment. Interestingly, 4 patients with severe aplastic anemia, which has been associated with a high incidence of graft rejection (22), achieved complete chimerism after our reduced-intensity regimen. These findings suggest that the combination of fludarabine, melphalan, and low-dose TBI might be more immunosuppressive than conventional myeloablative regimens, creating niche for CB to engraft. Alternatively, CB may exert a strong graft-*versus*-host effect, making room for stable engraftment of stem cells.

Delayed hematopoietic recovery and infection during neutropenia are the significant concerns in adult UCBT. Laughlin *et*

Table 4 Characteristics of pre-engraftment reaction (n = 23)

Temperature	
38.0–38.9°C	2
39.0–39.9°C	10
≥40.0°C	7
Day of peak body temperature	9 (5–12)
Serum levels of CRP[a] (mg/dl)	13.8 (0.5–18.9)
Day of peak serum levels of CRP	10 (8–16)
Diarrhea	11
Eruption	10
Jaundice	5
Use of corticosteroid	13
Good response to corticosteroid	7

[a] CRP, C-reactive protein.

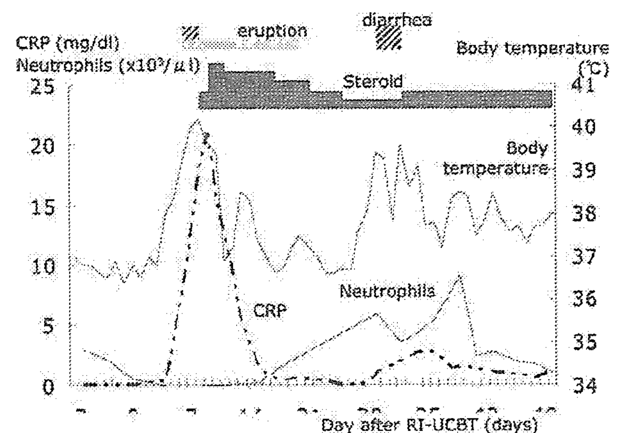

Fig. 5 Clinical course of a patient who developed pre-engraftment fever. Immune-reactions display two peaks, at around day 9 and day 18.

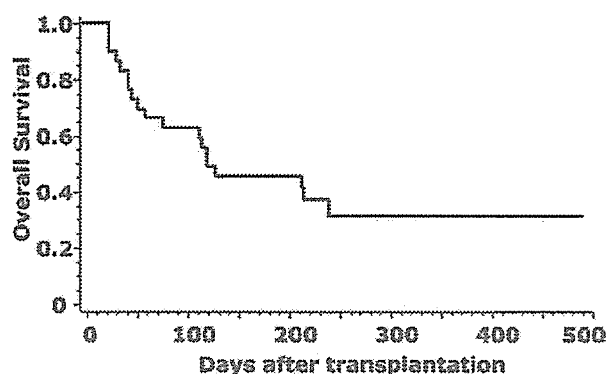

Fig. 6 Probability of overall survival after reduced-intensity unrelated cord-blood transplantation. Estimated 1-year overall survival was 32.7% (95% confidence interval, 14.3–51.1%).

al. (23) reported neutrophil recovery in 90% of patients by a median of 27 days after UCBT, which was significantly delayed compared with allo-HSCT. The delay has been attributed to the limited cell dose in the reports on myeloablative UCBT. The median nucleated cell dose in our study (3.1×10^7/kg) was greater than those in some reports from Western countries (2.1×10^7/kg; Ref. 9). The low median body weight (52 kg) in the Japanese population may favor neutrophil engraftment, whereas our results showed no association between the cell dose and engraftment in the small sample size. In the present study, median time to engraftment was 17.5 days (range, 10–54 days), which was much faster than that reported in previous studies on myeloablative UCBT (7–9). Our results were comparable with the report on adult RI-UCBT by Barker *et al.* (21). Their results showed neutrophil engraftment on a median of 26 days after busulfan/fludarabine/TBI 2 Gy and 9.5 days after cyclophosphamide/fludarabine/TBI 2 Gy. Whereas the reason for the difference remains unclear, these findings suggest that fludarabine-based reduced-intensity regimens enable rapid and stable engraftment.

TRM within 100 days was 27% in this study, which is lower than those reported on myeloablative UCBT (Refs. 7, 9, 24; 32–51% in pediatric patients and 56–63% in adults). Given the relatively old age (median, 58.5 years) and advanced stages of the primary diseases, our reduced-intensity preparative regimen probably decreased TRM. Our TRM within 100 days is comparable with that of 28% in adult RI-UCBT by Barker *et al* (21).

All of the patients tolerated our preparative regimen without grade IV RRT (Bearman's criteria; Ref. 2). Four patients developed grade III RRT with common involvements of the gut, kidney, and liver (Table 3). We used melphalan, which has dose-limiting toxicities of the gut and liver (25). These remained mild without hepatic veno-occlusive disease. Because renal toxicities of fludarabine, busulfan, and TBI 4 Gy are reportedly minimal, the high incidence of renal toxicity might be attributable to concomitant administration of nephrotoxic agents such as cyclosporin and antibiotics. Elderly patients might be susceptible to RRT. We plan to investigate optimal dosages of cyclosporin in RIST for elderly patients. Because TBI, even at a low dose, sometimes causes significant late toxicities in the lung (22), long-term follow-up is required.

Little information on GVHD after RI-UCBT is available. In the present study, the incidences of grade II-IV and III-IV acute GVHD and chronic GVHD were 27%, 23%, and 23%, respectively, whereas some reported those to be 33–44%, 11–22%, and 0–25%, respectively, in myeloablative UCBT (7, 8, 26). There are no significant differences in the incidences of GVHD between myeloablative UCBT and RI-UCBT. This is similar to the GVHD incidences in myeloablative allo-HSCT and RIST (27). Median onset of acute GVHD was 36 days (range, 17–66 days) in the present study, which was comparable with that of myeloablative UCBT (7, 8, 26). In contrast, the achievement of complete donor chimerism and the onset of acute GVHD are delayed in RIST compared with myeloablative allo-HSCT (27, 28). CB might have a potential of intense graft-*versus*-host effect, allowing niche for early engraftment. The characteristics of GVHD after RI-UCBT remain to be investigated, including different organ involvements and response to immunosuppressive treatment.

Interestingly, 20 patients developed inflammatory reactions before engraftment (Table 4). These reactions included noninfectious high-grade fever, eruption, diarrhea, and jaundice, starting on a median of day 9. Because the reactions preceded engraftment (median, day 17.5), we speculated that some form of immune reaction that is not categorized as acute GVHD occurs after RI-UCBT without achieving engraftment. The pre-engraftment fever has been reported on rare occasions in previous reports of UCBT and might be similar to those observed after haploidentical transplantations. Antithymocyte globulin and corticosteroids, which have strong immunosuppressive properties, were commonly used in previous studies on UCBT (9), whereas neither was used in the present study. Immunosuppressive treatment with corticosteroids was effective for the pre-engraftment fever. These findings support that immune-mediated reactions after UCBT might manifest easily with the present regimen. The doubling time of cultured CB $CD34^+$ cells is 7–10 days, which is several hundred-fold faster than that of cultured adult marrow cells (29). Mononuclear cells from CB display a unique cytokine profile such as comparable levels of

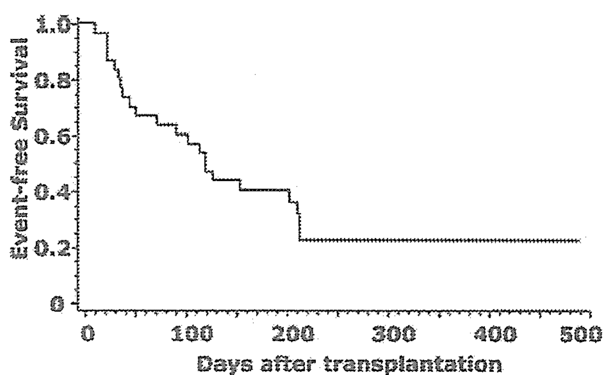

Fig. 7 Probability of event-free survival after reduced-intensity unrelated cord-blood transplantation. Estimated 1-year event-free survival was 22.2% (95% confidence interval, 5.9–38.5%).

interleukin (IL) 2, IL-6, and tumor necrosis factor α, reduced levels of IFN-γ and IL-10, and complete absence of IL-4 and IL-5 (30, 31). Pre-engraftment fever is possibly attributable to a cytokine storm induced by massive proliferation of cells with a unique cytokine profile. Another possibility is homeostasis-driven proliferation of naive T cells in highly immunosuppressed individuals, as demonstrated in murine models (32, 33). This reaction is reportedly associated with cytotoxic cytokines (32, 33). Fever as a transient response to contamination with maternal blood or cells during CB collection cannot be excluded (34). Reactivation of human herpesvirus 6 might be associated with this complication (35). If pre-engraftment fever exerts some antitumor effects, it is reasonable that patients with advanced and chemorefractory hematological diseases achieved long-term remission after RI-UCBT in the present study.

Infection is a common and significant problem in myeloablative UCBT (8, 9, 24), but little is known in RI-UCBT. The present study demonstrated that infection is also problematic in RI-UCBT. Twelve patients developed infection in this study, 9 of whom had been on corticosteroid therapy. Eight of 11 patients with CMV antigenemia had received corticosteroids. Delayed immunological reconstitution with or without GVHD, pre-engraftment fever, and corticosteroids may be risk factors for infection. Appropriate management of GVHD and pre-engraftment fever warrants additional investigation.

One-year OS was 35% in the present study, showing that some patients with advanced hematological malignancies can achieve durable remission after RI-UCBT. Contrary to our prediction, primary diseases recurred only in 3 patients. The candidates for RI-UCBT have extremely poor prognosis with conventional salvage chemotherapy. These findings suggest that RI-UCBT exerts strong antitumor activity and is promising for patients with refractory hematological malignancies without an HLA-identical sibling or an unrelated donor. In contrast, it is premature to apply RI-UCBT to low-risk diseases.

In conclusion, our study demonstrated the feasibility of RI-UCBT for adult patients with advanced hematological diseases, although the limitations included the small sample size and short follow-up. If CB is feasible for adults as an alternative stem cell source, RI-UCBT may become the choice of treatment for patients with advanced hematological diseases that are incurable with conventional treatments. RI-UCBT is particularly appealing for patients who require urgent treatments. Although RI-UCBT is currently associated with a high TRM, this study provided a rationale for continuing our clinical trials. Additional investigations need to focus on minimizing adverse effects including RRT, GVHD, and pre-engraftment immune reactions, whereas preserving graft-*versus*-leukemia effects.

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IAP Family Protein Expression Correlates With Poor Outcome of Multiple Myeloma Patients in Association With Chemotherapy-induced Overexpression of Multidrug Resistance Genes

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Multidrug-resistant (MDR) multiple myeloma (MM) patients who fail chemotherapy frequently express MDR1 protein, which serves as an efflux pump that protects neoplastic cells. The expression of lung resistance protein (LRP), which mediates intercellular and nucleocytoplasmic transport, is also correlated with chemotherapy resistance and shorter survival of MM patients. Here, we investigated the chemotherapy-induced change of MDR expression in MM patients using quantitative RT-PCR. Overall expression levels of MDR1 and LRP in MM patients were significantly higher than those in control subjects and increased after chemotherapy. More than half of the patients exhibited increased expression of MDR1 (14/26) or LRP (17/26) after chemotherapy. Also, the expression of inhibitor of apoptosis proteins (IAP) was determined in association with the prognosis of the patients. Among patients with increased MDR1-expression after chemotherapy, those with a poor outcome exhibited significant increases in survivin, cIAP1, cIAP2, and XIAP expression by chemotherapy compared with those with a good prognosis. Similarly, in the LRP expression-increased group, patients with a poor outcome showed significant increases of cIAP1 and cIAP2 expression compared with those with longer survival. In patients with reduced-MDR1 or LRP expression after chemotherapy, changes in the expression of IAPs induced by chemotherapy did not correlate with their prognosis. These findings indicate that IAP family proteins might play a role in worsening the prognosis of MM patients in association with chemotherapy-induced overexpression of MDR1 or LRP. Am. J. Hematol. 81:824–831, 2006. © 2006 Wiley-Liss, Inc.

Key words: multiple myeloma; MDR1; LRP; IAP; bone marrow

INTRODUCTION

The development of refractory disease in hematological malignancies such as multiple myeloma (MM) and acute myeloid (AML) or lymphoid leukemias (ALL) is frequently associated with the expression of one or several multidrug resistance (MDR) genes [1]. Clinical studies have established that MDR1 expression occurs in MM patients, and there is also clinical evidence of multidrug resistance [2]. In addition, the response rate to induction chemotherapy is significantly lower in patients with LRP expression than in patients without LRP expression [3]. Furthermore, MM patients with LRP expression have a shorter overall survival than those without it [4]. However, knockout mice experiments revealed that disruption of the LRP gene did not induce hypersensitivity to cytostatic agents [5]. Thus, the effects

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of LRP expression on the drug-resistance in MM patients are still controversial.

It has become possible to reverse clinical multidrug resistance by blocking P-glycoprotein-mediated drug efflux. However, potential new approaches to treat refractory diseases by using MDR modulators have not yet generated promising results. Recently, emerging knowledge about the importance of overcoming anti-apoptosis and drug resistance in treating a variety of malignancies, including MM, has raised new hope for improving the treatment outcome for patients with cancer [6]. Several targeting therapies that aim to reverse the anti-apoptotic process in MM cells have been explored in a number of experimental systems and clinical studies [6]. Thus, the aim of the present study was to investigate anti-apoptotic mechanisms that are employed in bone marrow cells from MM patients with MDR expression.

IAP family proteins, including survivin, block apoptosis induced by a variety of apoptotic triggers [7,8]. Although the exact biochemical mechanism by which these proteins suppress apoptosis is under debate, survivin is known to directly bind to and inhibit caspase-3 and -7, which act as terminal effectors in apoptotic protease cascades [8,9]. Survivin is widely expressed in fetal tissues, but becomes restricted during development, and is negligibly expressed in the majority of terminally differentiated adult tissues [10,11]. However, analysis of the differences in gene expression between normal cells and tumor cells has revealed that survivin is one of the genes that is most consistently overexpressed in tumor cells relative to normal tissue [12]. In fact, survivin is prominently expressed in transformed cell lines and in many human cancers including hematopoietic cell tumors [13]. It can usually be detected in the cytoplasm of tumor cells, and is therefore widely regarded as being a cytoplasmic protein [10,14,15]. However, several studies have examined the nuclear accumulation of survivin in gastric cancer cells [16] and lung cancer cells [17]. We have recently shown that ALL cells principally exhibit nuclear localization of survivin, while CLL cells possess cytoplasmic survivin [18]. Thus, the significance of nuclear-cytoplasmic localization in tumor cells is still controversial.

In the present study, we examined MM patients by focusing on the contribution of IAPs to their prognosis. First, the expression of MDR1 and LRP was compared between bone marrow samples from MM patients before and after chemotherapy using quantitative RT-PCR. The patients were divided into two groups, one consisting of those with increased MDR1 or LRP expression after treatment and the other of those with reduced expression of MDR1 or LRP after treatment. Then, the expression of IAP family proteins including survivin, cIAP1, cIAP2, NAIP, and XIAP, which suppress apoptosis by caspase and procaspase inhibition [19–22], was also determined in both groups. In MM patients with chemotherapy-induced overexpression of MDR1 or LRP expression, the increased expression of several IAPs was significantly correlated with their prognosis. The implications of these findings regarding the multidrug resistance of MM cells and their clinical significance are discussed.

MATERIALS AND METHODS

Patients

Fresh frozen bone marrow samples from control (7 cases, age, median 55, max. 74, min. 43; male: female, 1:6) and MM (26 patients; male:female, 14:12; age, median 68, max. 85, min. 36) patients who received induction chemotherapy were collected. Melphalan-based regimens utilizing melphalan/prednisone or VMCP were administered to 20 of the MM patients. Also, four MM patients were treated with VAD, and two patients received VAD followed by high-dose melphalan. The prognosis of the patients in association with their responses to induction chemotherapy was assessed by their survival times. Patients with a survival time of more than 4 years were determined to be those with a good prognosis, while patients who died within 4 years were designated as those with a poor outcome. To rule out the influence of aging on bone marrow cells, age-matched control patients were analyzed. All MM samples were collected at the time of their initial aspiration biopsy and after chemotherapy. The patients were not infected with specific viruses including HTLV-1 and had not been treated prior to the study.

The procedures followed were in accordance with the ethical standards established by the ethics committee of Tokyo Medical and Dental University.

Preparation of RNA and Quantitative Assay for mRNA Expression of MDR1, LRP, and IAP Family Proteins Using TaqMan RT-PCR

RNA was extracted from frozen bone marrow samples of control subjects with no hematological disorders and MM samples before and after chemotherapy using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's directions. For quantitative RT-PCR, fluorescent hybridization probes and the TaqMan PCR Core Reagents Kit with AmpliTaq Gold (PerkinElmer Cetus, Norwalk, CT) were used with the ABI Prism 7900HT Se-

quence Detection System (PerkinElmer, Foster City, CA). Oligonucleotides as specific primers and TaqMan probes for the MDR1, LRP, and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized at a commercial laboratory (PerkinElmer Cetus). The primers and TaqMan probes were as follows. The sequence of the forward primer for MDR1 mRNA was 5′-GTCTACAGTTCGTAATGCTGACGT-3′ and that of the reverse primer was 5′-TGTGATCCACGGACACTCCTAC-3′; the TaqMan probe used was 5′-CGCTGGTTTCGATGATGGAGTCATTG-3′. For LRP mRNA, the forward primer was 5′-CGCTGTGATTGGAAGCACCTA-3′ and the reverse primer was 5′-CGGGAGGCAGCTCTTTCTC-3′; the TaqMan probe was 5′-ATGCTGACCCAGGACGAAGTCCT-3′. The sequence for the forward primer for survivin mRNA was 5′-TGCCTGGCAGCCCTTTC-3′ and for the reverse primer was 5′-CCTCCAAGAAGGGCCAGTTC-3′; for the TaqMan probe, it was 5′-CAAGGACCACCGCATCTCTACATTC-3′. For cIAP1 mRNA, the sequence for the forward primer was 5′-CAGCCTGAGCAGCTTGCAA-3′ and for the reverse primer, it was 5′-CAAGCCACCATCACAACAAAA-3′; for the TaqMan probe, it was 5′-TTTATTATGTGGGTCGCAATGATGATGTCAAA-3′. For cIAP2 mRNA, the sequences of the forward and reverse primer were 5′-TCCGTCAAGTTCAAGCCAGTT-3′ and 5′-TCTCCTGGGCTGTCTGATGTG-3′, respectively; and the sequence for the TaqMan probe was 5′-CCCTCATCTACTTGAACAGCTGCTAT-3′. The forward and reverse sequences for NAIP mRNA were 5′-GCTTCACAGCGCATCGAA-3′ and 5′-GCTGGGCGGATGCTTTC-3′, respectively; while the sequence for the TaqMan probe was 5′-CCATTTAAACCACAGCAGAGGCTTTAT-3′. The sequence of the forward primer for XIAP mRNA was 5′-AGTGGTAGTCCTGTTTCAGCATCA-3′ and for the reverse primer was 5′-CCGCACGGTATCTCCTTCA-3′; the sequence for the TaqMan probe was 5′-CACTGGCACGAGCAGGGTTTCTTTATACTG-3′. Finally, the forward primer sequence for GAPDH mRNA was 5′-GAAGGTGAAGGTCGGAGT-3′ and for the reverse primer was 5′-GAAGATGGTGATGGGATTTC-3′; the TaqMan probe sequence was 5′-CAAGCTTCCCGTTCTCAGCC-3′. The conditions for one-step RT-PCR were as follows: 2 min at 50°C (Stage 1, reverse transcription), 10 min at 95°C (Stage 2, RT inactivation and AmpliTaq Gold activation), and then 45 cycles of amplification for 15 sec at 95°C and 1 min at 60°C (Stage 3, PCR). The expression of MDR1, LRP, or IAP family proteins was quantitated according to a method described elsewhere [23]. Briefly, the intensity of the reaction was evaluated from the quantity of total RNA in Raji cells (ng) corresponding to the initial number of PCR cycles to reveal the linear increase in reaction intensity (threshold cycle) for each sample on a logarithmic standard curve. Data on the quantity of RNA (ng) for the MDR1, LRP, and IAPs were normalized using the data for GAPDH in each sample.

Identification of Apoptotic Cells

To identify apoptotic cells, the terminal deoxytransferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method was employed as previously described [24] using formalin-fixed paraffin-embedded bone marrow tissues from MM patients. Briefly, tissue sections were deparaffinized and incubated with proteinase K (prediluted, DAKO, Glostrup, Denmark) for 15 min at room temperature. After washing, TdT, fluorescein isothiocyanate (FITC)-dUTP, and dATP (Boehringer Mannheim, Mannheim, Germany) were applied to the sections, which were then incubated in a moist chamber for 60 min at 37°C. Anti-FITC-conjugated antibody-peroxidase (POD converter, Boehringer Mannheim) was employed for detecting FITC-dUTP labeling, and color development was achieved with DAB containing 0.3% hydrogen peroxide solution. Sections were then observed under a microscope and the proportion of TUNEL-positive cells was determined by dividing the number of positively stained cells by the total cell number after counting more than 1,000 cells.

Immunohistochemistry for Survivin and Proliferative Cells

Four micrometer-thick sections of formalin-fixed paraffin-embedded bone marrow tissues from MM patients were cut on slides covered with adhesive. Sections were deparaffinized, and endogenous peroxidase was quenched with 1.5% hydrogen peroxide in methanol for 10 min. Antibodies were then applied to identify survivin and to characterize proliferative cells. The primary antibodies included polyclonal rabbit antibody against human survivin (SURV 11-A, Alpha Diagnostic International, San Antonio, TX) and monoclonal antibody Ki-67 (DAKO). All sections were developed using biotin-conjugated secondary antibodies against rabbit IgG or mouse IgG followed by a sensitive peroxidase-conjugated streptavidin system (DAKO) with DAB as the chromogen. Negative control staining procedure was performed using rabbit or mouse immunoglobulin of irrelevant specificity substituted with the primary antibody for each staining. The Ki-67-positive cell ratio was determined by dividing the number of positively stained cells by the total cell number after counting more than 1,000 cells.

Cell Lines

The establishment and characterization of the human MM tumor cell line RPMI8226 was previously described [25]. The cells were obtained from the American type culture collection (ATCC, Rockville, MD) and routinely maintained in RPMI 1640 medium (Sigma, St Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Daiichi Seiyaku, Tokyo, Japan), 1% (v/v) penicillin at 100 units/ml (Invitrogen, Carlsbad, CA), and 1% (v/v) streptomycin at 100 units/ml (Invitrogen). The cells were grown in a humidified atmosphere at 37°C in 5% CO_2. We also generated the doxorubicin resistant variant of RPMI8226 cell line, designated DRR, according to a previously described method [25].

Statistical Analysis

Statistically significant differences in the quantitative analysis were determined using Wilcoxon's test for the comparison of paired MM samples before and after chemotherapy. Statistically significant differences for the quantitative analysis were determined using the Mann–Whitney's U test for comparison between control and MM samples, MM samples with good or poor prognosis, and the MM cell lines, RPMI8226 and DRR.

RESULTS

Expression of mRNA for MDR1 and LRP Determined by Real-Time Quantitative PCR

To quantitate the mRNA expression levels of MDR1 in MM bone marrow cells, real-time quantitative RT-PCR was performed using bone marrow samples from controls and MM samples before and after chemotherapy. As shown in Fig. 1A, the expression of MDR1 exhibited significant up-regulation in MM compared with the controls ($P < 0.01$). The overall expression in MM tended to increase after chemotherapy. More than half of the patients (14/26) exhibited up-regulated expression of MDR1 after chemotherapy in spite of the number of neoplastic cells possibly decreasing due to chemotherapy. Thus, MM cells in MDR1-up-regulated patients should express higher levels of MDR1 after chemotherapy. By contrast, 12 patients out of 26 with MM showed the reduced expression after treatment. This reflects the decrease in the number of MM cells in the bone marrow after chemotherapy.

Similarly, the expression of LRP exhibited significant up-regulation in MM compared with the controls ($P < 0.01$) (Fig. 1B). The expression of LRP tended to increase after chemotherapy. There were 17 patients with increased LRP expression, while

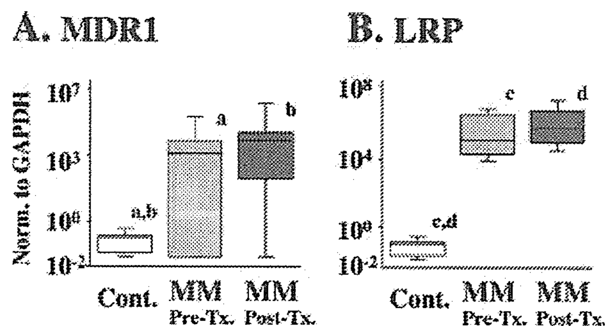

Fig. 1. Quantitative RT-PCR analysis of MDR1 (A) and LRP (B) in bone marrow samples from control cases (Cont.) (n = 7, white box) and MM patients before (Pre-Tx., light gray box) and after chemotherapy (Post-Tx., dark gray box) (n = 26). The relative intensity was calculated as [intensity of reaction of MDR1 (total Raji RNA, ng)]/[intensity of reaction of GAPDH (total Raji RNA, ng)]. The box plot graphs indicate the values for control and MM patients, where the bars indicate the 90 and 10% tile and the boxes indicate the 75–25% tile. Differences were significant between MDR1 expression in the controls and MM patients before chemotherapy ($^{a}P < 0.01$) and after chemotherapy ($^{b}P < 0.01$), LRP expression in the controls and MM patients before chemotherapy ($^{c}P < 0.01$), the controls and MM patients after chemotherapy ($^{d}P < 0.01$) seen by the Mann–Whitney's U-test.

decreased expression was observed in 9 after chemotherapy.

These results indicate that MDR1 and LRP are highly expressed in MM cells compared with control bone marrow cells and that this might be induced by chemotherapy in more than half of all patients. We thus focused on the analysis of patients with chemotherapy-induced up-regulation of MDR1 or LRP in the following study.

Expression of IAP Family Protein mRNA in MM Patients with Increased or Reduced Expression of MDR1 after Chemotherapy

Next, to quantitate the mRNA expression of IAP family proteins in MM bone marrow cells, real-time quantitative RT-PCR was performed using bone marrow samples from MM samples before and after chemotherapy. The values are indicated as the ratio postchemotherapy/prechemotherapy in Fig. 2. Among patients with increased expression of MDR1 after chemotherapy (14/26), the expression ratios for survivin, cIAP1, cIAP2, and XIAP were significantly higher in patients with poorer outcomes than in those with a good prognosis ($P < 0.05$, respectively) (Fig. 2A). In contrast, in patients with reduced MDR1 expression, no significant difference in the ratios of IAP expression was observed between patients with a good and poor prognosis (Fig. 2B).

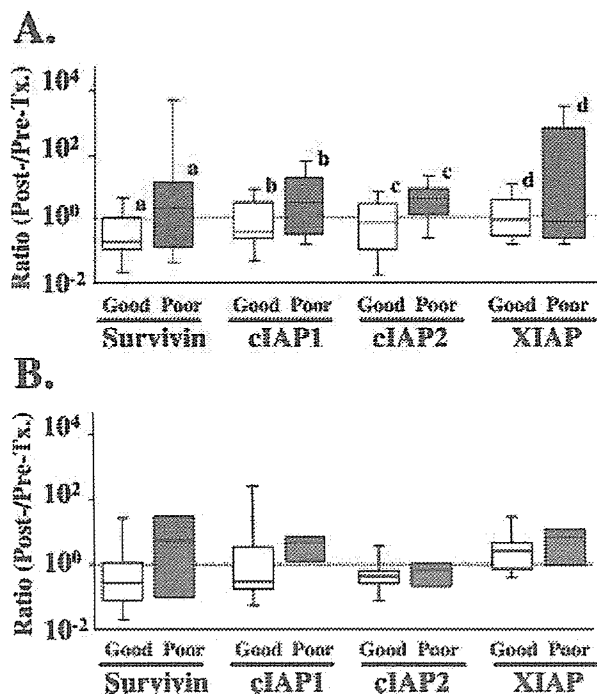

Fig. 2. Quantitative RT-PCR analysis of IAP family proteins in bone marrow samples from MM patients with increased (A, $n = 14$) or reduced (B, $n = 12$) expression of MDR1 after chemotherapy. The values are indicated as the ratio: [IAP expression after chemotherapy (Post-Tx.)/IAP expression before chemotherapy (Pre-Tx.)]. The box plot graphs compare the ratio of IAP expression between patients with a good prognosis (Good: white box) and those with a poor outcome (Poor: gray box). In MM patients with increased MDR1 expression (A), the ratios of expression of survivin, cIAP1, cIAP2, and XIAP were significantly higher in those with a poor outcome ($n = 7$) than in those with a good prognosis ($n = 7$) ($^{a-d}P < 0.05$, respectively, Mann–Whitney's U-test). In contrast, the expression levels of IAPs were not significantly different between patients with a good ($n = 3$) or poor prognosis ($n = 9$) in MM patients with reduced expression of MDR1 (B).

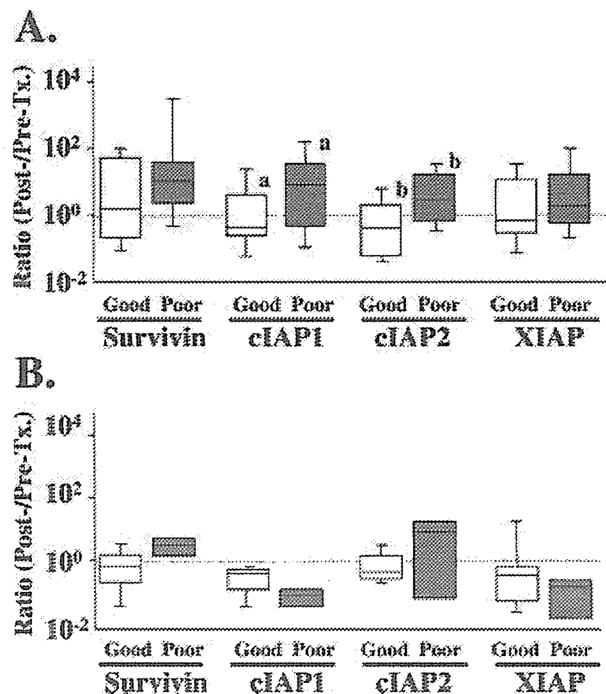

Fig. 3. Quantitative RT-PCR analysis of IAP family proteins in bone marrow samples from MM patients with increased (A, $n = 14$) or reduced (B, $n = 12$) expression of LRP after chemotherapy. In MM patients with increased LRP expression (A), the ratio of cIAP1 and cIAP2 expression was significantly higher in those with a poor outcome ($n = 9$) than in those with a good prognosis ($n = 8$) ($^{a,b}P < 0.05$, respectively, Mann–Whitney's U-test). In contrast, the ratios of expression of IAPs were not significantly different between patients with a good ($n = 2$) or poor prognosis ($n = 7$) in MM patients with reduced expression of LRP (B).

Thus, chemotherapy induced the overexpression of MDR1 and IAPs in patients with a poor outcome.

Expression of IAP Family Protein mRNA in MM Patients with Increased or Reduced Expression of LRP after Chemotherapy

To determine whether the chemotherapy-induced overexpression of LRP influenced the expression of IAP in association with the prognosis of the patients, the ratios for the expression of IAPs postchemotherapy/prechemotherapy were compared between patients with a good or poor prognosis. As shown in Fig. 3A, the ratios of cIAP1 and cIAP2 expression were significantly higher in patients with a poor outcome than in those with a good prognosis ($P < 0.05$, respectively). Similar to MDR1, patients with reduced expression of LRP exhibited no significant difference regarding the ratio between patients with a good and poor prognosis (Fig. 3B).

Immunolocalization of Survivin in MM Cells

To examine the histological localization of survivin in MM cells, immunohistochemical staining was performed in bone marrow samples from patients with survivin overexpression. As shown in Fig. 4A and B, survivin was localized to the nucleus of MM cells, although in a few cells cytoplasmic staining was also observed.

Apoptotic and Proliferative Cells in MM Samples before and after Chemotherapy

To determine the apoptotic and proliferative cell frequency of MM cells, the TUNEL-positive cell ratio and Ki-67 positive cell ratio were analyzed only for bone marrow samples in which foci of MM

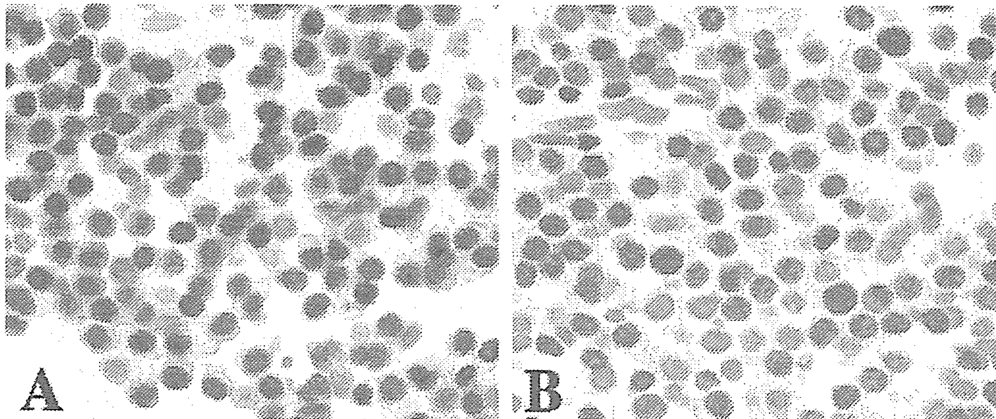

Fig. 4. Immunohistochemical localization of survivin in MM cells of the bone marrow before (A) and after chemotherapy (B). Note that the nuclei of more than half of the MM cells are positively stained although a few MM cells exhibit weak cytoplasmic staining. Differences in the ratio of survivin-positive MM cells were not remarkable before and after chemotherapy. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cells could be histologically identified. As summarized in Table I, the overall ratio of TUNEL-positive apoptotic cells exhibited a very mild increase after chemotherapy, although the difference was not significant. However, in cases with MDR1 up-regulation, the apoptotic cell ratio tended to decrease (prechemotherapy, median, 0.8; postchemotherapy, 0.4) after chemotherapy.

By contrast, the Ki-67-positive proliferative cell ratio significantly decreased due to chemotherapy ($P < 0.01$) (Table I). Differences were not significant between samples from MDR1 up-regulated patients and down-regulated patients as well as LRP up-regulated and down-regulated patients (data not shown). These results indicated that chemotherapy induced the reduction of proliferative MM cells and that the overexpression of MDR1 was associated with the reduction of apoptotic cells.

Expression of IAP Family Protein mRNA in Human MM Cell Lines with Increased MDR1 and LRP Expression

Finally, to test whether MDR1 or LRP overexpression was correlated with the up-regulated expression of IAPs in the human MM cell line, IAP family protein mRNA expression was compared between the MM cell line RPMI8226 (8226) and doxorubicin-resistant variant (DRR) of RPMI8226. As shown in Fig. 5A,B, DRR cells expressed significantly higher levels of MDR1 and LRP ($P < 0.05$, respectively) than the original RPMI8226 cells. The DRR cells were also resistant to apoptosis induced by doxorubicin (data not shown). As expected from the findings using clinical samples from MM patients after chemotherapy, the DRR cells tended to express higher levels of IAPs than did the original RPMI8226 cells (Fig. 5C). The differences were significant between 8226 and DRR cells in terms of cIAP1, cIAP2, and XIAP expression ($P < 0.05$, respectively).

TABLE I. Apoptotic and Proliferative Cell Ratio of MM Cells of the Bone Marrow before and after Chemotherapy

Cells	Pre-Tx. ($n = 11$) (%)	Post-Tx. ($n = 12$) (%)
TUNEL$^+$	0.8 (0.0–6.0)[a]	1.4 (0.2–6.6)
Ki-67$^+$	48.4 (18.1–64.5)[b]	18.9 (10.0–23.5)[b]

[a]Values given in parentheses are median (Min.–Max.) values.
[b]The difference was significant between the Ki-67$^+$ cell ratios of MM samples before chemotherapy (Pre-Tx.) and after chemotherapy (Post-Tx.) ($P < 0.01$ by the Mann–Whitney's U-test).

DISCUSSION

A strong correlation exists between MDR1 expression by tumor cells and previous chemotherapy in MM patients, and in particular is related to prior exposure to the natural agents vincristine and doxorubicin [26]. A in vitro study also reveals that exposure to doxorubicin selects for MDR1 expressing MM cell line cells [27]. We confirmed in the present study that the doxorubicin-resistant MM cell line, DRR, expressed higher levels of MDR1 as well as LRP than did the original RPMI8226 cells. The data from the clinical samples also supported these findings in the sense that the postchemotherapeutic induction of MDR1 as well as LRP overexpression was observed in more than half of the patients.

Regarding the prognosis, the MM patients who exhibited overexpression of MDR1 or LRP together with the up-regulation of IAPs exhibited a poor out-

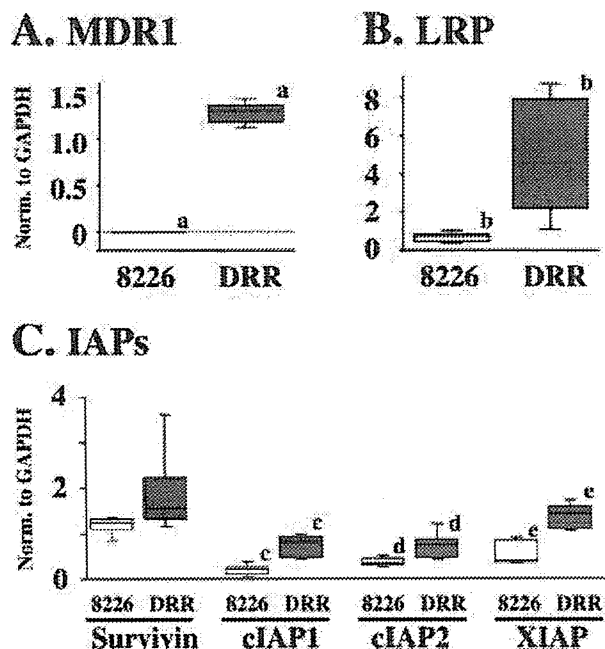

Fig. 5. Quantitative RT-PCR analysis of (A) MDR1, (B) LRP, and (C) IAPs in the MM cell line RPMI8226 (8226, white box) and doxorubicin-resistant variant RPMI8226 (DRR, gray box). As expected, the DRR cells expressed significantly higher levels of MDR1 (A) and LRP (B) than did the original RPMI8226 cells ($^{a,b}P < 0.05$, respectively, by Mann–Whitney's U-test). Note that the DRR cells tended to express higher levels of IAPs than did the original RPMI8226 cells (C). Differences were significant between cIAP1, cIAP2, and XIAP expression for 8226 and the DRR cells ($^{c\text{–}e}P < 0.05$, respectively, by Mann–Whitney's U-test).

come. It has been shown that the intracellular transport of IAP molecules is very important for the anti-apoptotic effects of these proteins [17]. Thus, the overexpression of MDR proteins mediates intracellular protein transport, might facilitate the transport of IAPs and introduce the hyper-function of these proteins in these MM patients. The apoptotic cell ratio was very low in the MM cells of these patients. Using the human HL60 leukemia cell line and its multidrug resistant line HL60R, Notarbartolo et al. [28] indicated that HL60R cells expressed much more MDR1 as well as survivin and cIAP2 than HL60. Treatment with doxorubicin strongly down-regulated survivin and XIAP in HL60 cells, and in contrast, the levels of these IAP mRNA were much less affected by the treatments in HL60R cells. Our data using the MM cell line also revealed that the DRR cells expressed higher levels of IAPs as well as MDRs than did the RPMI8226 cells. Western blot analysis confirmed that the expression dynamics of IAPs at the protein level was almost parallel with the dynamics at the mRNA level in MM (RPMI8226) cells after treatment with anti-cancer drug, although the levels of protein expression changed a little slowly (manuscript in preparation). These results suggest that IAPs might play a key role in tumor resistance to chemotherapeutic drugs in association with MDRs, although the interrelationships between IAPs and MDR gene products are far from being completely understood.

It would be also important to clarify the mechanisms responsible for the up-regulation of IAP family proteins in MM cells after chemotherapy. Insulin-like growth factor-1 (IGF-1) and interleukin-6 (IL-6) promote the proliferation of MM cells. IGF-1 stimulates the sustained activation of NF-κB and Akt and up-regulates a series of intracellular anti-apoptotic proteins including FLIP, survivin, cIAP-2, and XIAP. In contrast, IL-6 does not cause sustained NF-κB activation, induces less pronounced Akt activation, and increases the expression of only survivin [29]. We previously revealed that TNF-α is present locally in the bone marrow microenvironment and is associated with the regulation of cellular proliferation/apoptosis in hematological diseases [30]. TNF-α induces NF-κB nuclear translocation, cIAP-1 and cIAP-2 up-regulation, and proliferation in MM cells [31]. Thus, the expression of IAP is controlled by complex cellular signals. Further study is necessary to elucidate the mechanism of specific IAP induction in MM cells during chemotherapy by clarifying the genome-wide expression profiles of apoptosis-associated molecules using microarray technique.

Concerning the apoptotic process of MM cells, it has been shown that murine and human MM cell lines are sensitive to TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis [32]. Adriamycin sensitized this type of apoptosis in an adriamycin-resistant MM cell line [33]. The apoptotic signaling was dependent on a mitochondrial apoptotic pathway but not on a death receptor-mediated apoptosis pathway including that for the IAP family proteins cIAP-1, cIAP-2, and XIAP. By contrast, coexposure of human leukemia/MM cells to TRAIL and the cyclin-dependent kinase inhibitor flavopiridol (FP) increases mitochondrial injury and apoptosis [34]. TRAIL/FP induced no discernible changes in survivin expression, a modest decline in the level of cIAP, and resulted in the marked transcriptional down-regulation of XIAP. Thus, TRAIL/FP-induced apoptosis in human leukemia/MM cells disturbs XIAP-associated anti-apoptotic processes.

In conclusion, we showed that chemotherapy induced up-regulation of the expression of IAP mRNA and significantly worsened the prognosis of MM patients who exhibited chemotherapy-induced overexpression of MDRs. These results suggest that MDRs and IAPs might cooperate or interact to make MM

cells more resistant to chemotherapy, although analysis using a larger group of patients should be performed. In vitro data using RPMI8226 and DRR cell lines also suggested this association. Further studies using knocking down system of protein expression should clarify the mechanism responsible for MDRs as well as IAPs induction in MM cells under chemotherapy and a novel chemotherapeutic strategy should be considered for blocking IAPs in MDR-overexpressing MM cells.

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Expression of IAP-Family Proteins in Adult Acute Mixed Lineage Leukemia (AMLL)

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Inhibitor of apoptosis protein (IAP)-family proteins suppress apoptotic signaling in normal/neoplastic cells in various settings. To determine the apoptosis-resistant mechanism in adult acute mixed lineage leukemia (AMLL) with biphenotypic blasts responsible for resistance against chemotherapy, the expression levels of IAP-family proteins in AMLL bone marrow cells were analyzed by quantitative RT-PCR. The overall expression levels of IAPs were higher than those in control, AML, and ALL cells. A significant difference for the expression of survivin was observed between AMLL and AML ($P < 0.05$), and differences between AMLL and ALL were significant for the expression of survivin ($P < 0.05$), NAIP ($P < 0.05$), and XIAP ($P < 0.05$). These findings suggest that higher expression of various IAPs is associated with the chemotherapy-resistant nature of this specific type of leukemia. Am. J. Hematol. 78:173–180, 2005. © 2005 Wiley-Liss, Inc.

Key words: IAP; apoptosis; AMLL; AML; ALL; bone marrow

INTRODUCTION

The regulation of apoptotic cell death has a profound effect on the pathogenesis and progression of hematological malignancies. Acute mixed lineage leukemia (AMLL) is a relatively rare group of hematological malignancies that exhibits the expansion of biclonal or biphenotypic blasts in peripheral blood [1,2]. According to FAB criteria, AMLL may present as ALL or as one of the AML subtypes, often as M1 [2]. AMLL has a high incidence of clonal chromosomal abnormalities, the most common being the t(9;22)(q34;q11) (Ph chromosome) and structural abnormalities involving 11q23 [2]. Recently, molecular analysis revealed that the *mixed lineage leukemia* (*MLL*) gene rearrangement occurs in AMLL cases and also in a fraction of AML/ALL patients [3]. One characteristic feature of AMLL as well as *MLL* gene-rearranged leukemia is a poor patient prognosis associated with lower sensitivity to chemotherapeutic procedures [2,4]. Resistance against chemotherapy might result from the resistance to apoptosis-inducing drugs such as steroids and Ara-C [5,6]. Regarding the complicated mechanisms that regulate apoptosis in the bone marrow of acute leukemias and myelodysplastic syndromes (MDS), we previously showed that a variety of apoptosis-related molecules are active in hematopoietic cells [7–13]. However, the associated parameters and molecules involved in apoptosis in AMLL are unclear.

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IAP-family proteins, including survivin, block apoptosis induced by a variety of triggers [14,15]. Although the biochemical mechanism by which IAP-family members suppress apoptosis is under debate, survivin is known to bind directly to and inhibit caspase-3 and -7, which act as terminal effectors in apoptotic protease cascades [15,16]. The expression of survivin is ubiquitous in fetal tissues but is restricted during development and is negligible in the majority of terminally differentiated adult tissues [17,18]. However, an analysis of the differences in gene expression between normal and tumor cells reveals that survivin is a protein whose gene is most consistently overexpressed in tumor cells relative to normal tissue [19]. Survivin is prominently expressed in transformed cell lines and in many human cancers, including hematopoietic cell tumors [20]. It is also usually detected in the cytoplasm of tumor cells and is therefore widely regarded as a cytoplasmic protein [17,21,22]. However, several studies have shown the nuclear accumulation of survivin in gastric cancer cells [23] and lung cancer cells [24]. We recently reported that ALL cells principally exhibited the nuclear localization of survivin, while CLL cells exhibited cytoplasmic distribution [13]. Although the significance of this nuclear-cytoplasmic expression in tumor cells is still controversial, the subcellular localization of survivin should also be clarified for AMLL subjects.

We also reported that survivin exhibited higher levels of expression in acute lymphocytic leukemia (ALL) and that chronic lymphocytic leukemia (CLL) cases exhibited significant over-expression of survivin and cIAP2 [13]. In acute myelogenous leukemia (AML) cases, some of these IAP-family proteins, such as NAIP and XIAP, are expressed at significantly higher levels [25]. To focus on the contribution of IAPs to the expansion of blasts in AMLL, we examined cases of AMLL that exhibited biphenotypic proliferation of blasts. The expression levels of survivin tended to be high in AMLL samples compared with control bone marrow, AML, and ALL subjects. The expression of other IAPs, including cIAP1, cIAP2, NAIP and XIAP, which suppress apoptosis by inhibiting caspase and procaspase [26–29], was also observed in these samples. The significance of IAP-family proteins in resistance against chemotherapy in AMLL is discussed.

MATERIALS AND METHODS

Patients

Fresh-frozen and formalin-fixed paraffin-embedded bone marrow-aspirated samples from 13 individuals with no hematological disorders were used as normal controls (male/female 5:8; age, median 52 years, range: 25–84 years), 9 patients with AML (8 with M2 and 1 with M1 according to the FAB classification, male/female 5:3; age, median 41 years, range: 19–78 years), 7 patients with ALL (male/female 2:5; age, median 58 years, range: 46–87 years), and 8 patients with AMLL with biphenotypic blasts (male/female 4:4; age, median 50 years, range 17–73 years) were examined. To rule out the influence of aging on bone marrow cells, age-matched control cases were analyzed. Flow-cytometic analysis was routinely performed for CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD13, CD14, CD16, CD19, CD20, CD33, CD34, CD41a, CD56, and HLA-DR. Among them, the data for CD19, CD13, and CD33 were tabulated to demonstrate the biphenotypic nature of blastic cells in AMLL samples (Table I). Diagnoses were based on Catovsky's standard clinical and laboratory criteria [2] including cell morphology [30,31]. All samples were collected at the time of the initial aspiration biopsy and stored at −80°C. We selected the adult M1 or M2 AML samples and adult

TABLE I. Summary of Cases With Adult AMLL

Case no.	Age (years)	Sex	Blast (%)	Cell markers (%)			Chromosome abnormality
				CD19	CD13	CD33	
1	40	F	90.2	98.7	58.3	99.4	45,XX,der(12)t(12;22)(p13;q11) −22
2[a]	57	M	4.2	31.2	50.2	55.0	46,XY
3	67	M	94.4	95.2	67.8	0.9	36,XY,−3,−3,−5,−7,−9,−13,−15,−16,−17,−20
4	61	F	95.7	95.9	6.4	56.6	46,XX
5	17	M	96.4	99.8	53.8	50.6	46,XY
6	21	F	76.0	97.3	75.2	64.0	47,XX,+8
7	43	F	92.0	97.9	67.6	83.1	46,XX,i(8)(q10)del(9)(?q), der(9)del(9)(p22)t(9;22)(q34;q11),der(22)t(9;22)
8	73	M	69.2	89.2	45.4	13.5	46,XY,del(20)(q11.2)

[a]For case 2, material for flow-cytometric analysis was not sufficient at the time of initial diagnosis, although the diagnosis was confirmed as AMLL at the time of second biopsy. For the second biopsy sample, the blast count accounted for more than 90% of the bone marrow cells and consisted of more than 90% CD19-positive cells and more than 50% CD13/CD33-positive cells.