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LETTER TO THE EDITOR

Oral beclomethasone dipropionate as an initial treatment of gastrointestinal acute graft-versus-host disease after reduced-intensity cord blood transplantation

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Beclomethasone dipropionate (BDP) is a potent topically active corticosteroid. BDP is hydrolyzed to an active metabolite, beclomethasone 17-monopropionate, by esterase enzymes in the intestinal and bronchial mucosa. Systemic adverse effects are limited by incomplete mucosal absorption and rapid metabolism in the liver.[1] BDP had been developed as inhalant corticosteroid for the treatment of asthma and allergic rhinitis, then the efficacy of topical BDP was shown in the treatment of Crohn's or inflammatory bowel disease.[2,3] Oral BDP (6–10 mg/body/day) or BDP enema (3–5 mg/body/day) was used in these studies.

The pathological and clinical findings of gastrointestinal (GI) graft-versus-host disease (GVHD) are similar to those of inflammatory bowel disease. Three previous studies had shown the safety and efficacy of the topical BDP for GI GVHD. A phase I trial from the Fred Hutchinson Cancer Research Center (FHCRC) showed that 23 patients with mild to moderate GI acute GVHD were given oral BDP at a daily dose of 8 mg/body as an initial therapy. The response rate was 77%, and no patient presented with severe infection or adrenal insufficiency.[4] A randomized placebo-controlled trial from FHCRC demonstrated that response rates were significantly higher in patients given oral BDP and prednisone than those given prednisone alone as initial therapy for GI acute GVHD (71 versus 41%).[5] A phase II trial by Iyer *et al.*[6] showed that long-term use of oral BDP for the treatment of GI GVHD, which included chronic GVHD mainly, was safe and effective.

Infection is a significant problem, contributing to a majority of transplant-related mortality following cord blood transplantation (CBT). Systemic use of corticosteroid is a standard treatment of acute GVHD; however, it is associated with a high incidence of infection, impairing patients' survival.

The optimal treatment for immune reactions after CBT has not been established.[7] We have been undertaking clinical trials on CBT using reduced-intensity preparative regimen (reduced-intensity (RI)-CBT).[8] Since March 2004, we have used oral BDP as initial treatment of GI acute GVHD following RI-CBT instead of systemic corticosteroid. We will review medical, pathological and laboratory records of five patients with GI GVHD who were treated with oral BDP.

Between March 2003 and December 2004, 38 patients with hematological diseases or solid tumors underwent RI-CBT at Toranomon Hospital. Details of transplantation procedures and supportive cares were reported previously.[8] The preparative regimen mainly comprised fludarabine 125 mg/m^2, melphalan 80 mg/m^2 and 4 Gy total body irradiation. GVHD prophylaxis was cyclosporine 3 mg/kg or tacrolimus 0.03 mg/kg. CMV pp65 antigenemia was monitored weekly after engraftment. If CMV antigenemia exceeded 10/50 000, patients preemptively received ganciclovir 5 mg/kg or foscarnet 30 mg/kg intravenously twice a day. When patients developed pathologically proven GI acute GVHD with or without stage 1 and 2 skin involvement, patients received one enteric-coated cellulose capsule and an aqueous suspension by mouth every 6 h for a total daily dose of 8 mg. Both enteric-coated cellulose capsule and aqueous suspension containing 1 mg BDP powder (Wako Pure Chemical Industries, Ltd, Osaka, Japan) were prepared in our hospital. BDP in the form of aqueous suspension is activated in the stomach and the upper intestine. The enteric-coated capsule dissolves in the small intestine, delivering active drug into the small intestine and the colon. The duration of BDP was at the discretion of the primary physicians. All patients provided written informed consent in accordance with the requirements of the institutional review board. The other patients with grade II–IV acute GVHD were treated with systemic methylprednisolone 0.5–2.0 mg/kg. Response to BDP was determined according to the study by Martin *et al.*[9]

A total of 17 (44.7%) patients developed grade II–IV acute GVHD, and five of them were treated with oral BDP. Their clinical characteristics and outcomes were shown in Table 1. Responses to BDP included complete response (CR) ($n = 3$) and progression ($n = 2$). Acute GVHD had not recurred in the three patients (Cases 1–3) who achieved CR. None of these three patients developed either bacterial or fungal infection, whereas cytomegalovirus enterocolitis was diagnosed in a patient (Case 1). The remaining two patients (Cases 4 and 5) who had not responded to oral BDP were given methylpresonisolone 1–2 mg/kg as a second treatment, and all of them achieved CR. These two patients were complicated with cytomegalovirus enterocolitis during administration of methylprednisolone, and were successfully treated with ganciclovir or foscarnet. As of July 2006, one of the five patients (Case 1) is alive without disease progression. The other four patients died of recurrence (Cases 2, 3 and 4) and chronic GVHD (Case 5).

The present study showed that oral BDP is a useful agent for the treatment of GI acute GVHD following RI-CBT.

Table 1 Characteristics of BDP

	Sex	*Age (years)*	*Primary disease*	*Onset of gut GVHD*	*HLA match*	*GVHD stage and grade at diagnosis (skin/gut)*	*Duration of BDP (days)*	*Diarrhea at the end of BDP*	*Response*	*CMV disease*	*CMV antigenemia*	*BSI*	*Other infection*	*Survival (causes)*
Case 1	Female	32	Hodgkin's lymphoma	Day 45	4	(2/2), grade III	35	0	CR	Enterocolitis	Yes	No	No	Alive
Case 2	Male	68	ATL	Day 27	5	(1/3), grade III	60	0	CR	No	No	No	No	Dead (relapse)
Case 3	Male	45	AML	Day 29	5	(2/3), grade III	39	0	CR	No	No	No	No	Dead (relapse)
Case 4	Male	40	Malignant lymphoma	Day 53	4	(2/3), grade III	9	1196 ml/day	Progression	Enterocolitis	Yes	No	No	Dead (relapse)
Case 5	Male	59	AML	Day 26	4	(0/3), grade III	7	10 times/day	Progression	Enterocolitis	Yes	No	No	Dead (chronic GVHD)

AML = acute myeloid leukemia; ATL = adult T-cell leukemia; BDP = beclomethasone dipropionate; BSI = bloodstream infection; CMV = cytomegalovirus; CR = complete response; GVHD = graft-versus-host disease; HLA = human leukocyte antigen; RI-CBT = reduced-intensity cord blood transplantation.

When patients with acute GVHD are given systemic corticosteroid, it involves a high risk of bloodstream infection (BSI). We previously reported that 32% of RI-CBT recipients developed BSI within 100 days of transplantation, and that it was associated with systemic use of corticosteroid (relative risk, 43.1).[10] However, none of the five patients developed BSI during and after administration of oral BDP in the present study. Oral BDP causes limited immunosuppression to the systemic organs other than the gut,[4,6] and the risk of BSI might not be elevated in patients given oral BDP for the treatment of GI acute GVHD.

Considering that three of the five patients achieved CR with oral BDP, it is reasonable to assume that systemic administration of corticosteroid is not requisite for GI acute GVHD, and that oral BDP is occasionally sufficient for its control. However, it should be noted that the remaining two patients failed to achieve CR, and that one of them finally died of chronic GVHD. The two patients with treatment failures (Cases 4 and 5) might have responded to short-course prednisone along with oral BDP, followed by a rapid prednisone taper as reported by McDonald *et al.*[5] These findings suggest that systemic corticosteroid might have been required for these two patients. Alternatively, oral BDP at a dose of 8 mg/day might have been suboptimal in these patients. Further studies are warranted to clarify the eligibility of oral BDP, and its optimal dose for the treatment of GI acute GVHD.

In conclusion, the present study demonstrated the feasibility of oral BDP in the treatment of GI acute GVHD following RI-CBT. It suppresses allogeneic immune responses in the gut without causing significant immunosuppression. However, this study is too small to draw a definite conclusion on oral BDP, and we are now planning a large-scale prospective study.

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bjh research paper

Graft failure following reduced-intensity cord blood transplantation for adult patients

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Summary

We reviewed the medical records of 123 adult reduced-intensity cord blood transplantation (RI-CBT) recipients to investigate the clinical features of graft failure after RI-CBT. Nine (7·3%) had graft failure, and were classified as graft rejection rather than primary graft failure; they showed peripheral cytopenia with complete loss of donor-type haematopoiesis, implying destruction of donor cells by immunological mechanisms rather than poor graft function. Three of them died of bacterial or fungal infection during neutropenia. Two recovered autologous haematopoiesis. The remaining four patients underwent a second RI-CBT and developed severe regimen-related toxicities. One died of pneumonia on day 8, and the other three achieved engraftment. Two of them died of transplant-related mortality, and the other survived without disease progression for 9·0 months after the second RI-CBT. In total, seven of the nine patients with graft failure died. The median survival of those with graft failure was 3·8 months (range, 0·9–15·4). Graft failure is a serious complication of RI-CBT. As host T cells cannot completely be eliminated by reduced-intensity preparative regimens, we need to be aware of the difficulty in differentiating graft rejection from other causes of graft failure following RI-CBT. Further studies are warranted to establish optimal diagnostic and treatment strategies.

Keywords: graft failure, graft rejection, chimaerism, second transplantation, graft-*versus*-host disease.

Graft failure is a serious complication after allogeneic stem cell transplantation (allo-SCT) (Georges & Storb, 2000). It comprises two clinical entities: primary graft failure and graft rejection (Hows, 1991). Primary graft failure implies that donor-derived haematopoiesis has not been obtained during a specific time interval after transplantation. Graft rejection implies the complete destruction of donor cells by an immunological mechanism (Georges & Storb, 2000). Three treatment options are available for primary graft failure: administration of haematopoietic growth factor (Nemunaitis *et al*, 1990), a booster infusion of donor haematopoietic stem cells (Bolger *et al*, 1986; Davies *et al*, 1994), and an infusion of previously harvested autologous haematopoietic stem cells (Mehta *et al*, 1996). In the management of graft rejection, another immunosuppressive conditioning is necessary before the second infusion of haematopoietic stem cells (Georges & Storb, 2000). It is critical to differentiate graft rejection from other causes of graft failure since the treatment approaches are different according to the aetiology.

Some recent reports have demonstrated the feasibility of cord blood transplantation (CBT) using reduced-intensity regimens (RI-CBT) for adult patients with advanced haematological diseases (Barker *et al*, 2003; Miyakoshi *et al*, 2004). While graft failure is probably a significant complication in RI-CBT as well as in CBT using myeloablative preparative regimens (Laughlin *et al*, 2004; Rocha *et al*, 2004), little information is available and its optimal diagnostic procedures and treatments remain unknown. We investigated the clinical features and determined the incidence of graft failure following RI-CBT.

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Patients and methods

Study patients

Between January 2002 and August 2004, 123 patients with haematological diseases or solid tumours underwent RI-CBT at Toranomon Hospital (Table I). All patients had incurable diseases by conventional treatments, and were not candidates for conventional allo-SCT because of the lack of a suitable sibling or unrelated donor, age over 50 years, and/or organ dysfunction (often attributable to previous intense chemotherapy and/or radiotherapy). The RI-CBT procedures have been reported previously (Miyakoshi *et al*, 2004). All patients provided written informed consent in accordance with the requirements of the Institutional Review Board.

Table I. RI-CBT patient characteristics.

Variable	Number
Age (years), median (range)	55 (17–79)
Sex (male/female)	70/53
Primary diseases	
Acute lymphoblastic leukaemia	18
Acute myeloid leukaemia	35
Chronic myeloid leukaemia	3
Adult T-cell leukaemia	15
Myelodysplastic syndrome	13
Malignant lymphoma	27
Multiple myeloma	4
Solid tumour	2
Aplastic anaemia	6
Risk of underlying diseases (high/low)	93/30
Preparative regimens	
Flud 125 mg/m^2 + L-PAM (80–140 mg/m^2) + TBI (2–8 Gy)	111
Flud 150 mg/m^2 + BU 8 mg/kg + TBI (4–8 Gy)	9
Flud 150 mg/m^2 + BU 8 mg/kg	1
Flud 150 mg/m^2 + L-PAM 140 mg/m^2	2
Number of infused nuclear cells, median (range), $\times 10^6$/kg	28 (17–52)
Number of infused CD34$^+$ cells, median (range), $\times 10^6$/kg	0·074 (0·001–0·33)
HLA match	
6/6	2
5/6	20
4/6	101
GVHD prophylaxis (cyclosporine alone/ tacrolimus alone)	89/34

Flud, fludarabine; L-PAM, melphalan; BU, busulphan; TBI, total body irradiation; GVHD, graft-*versus*-host disease.
The following conditions were defined as low/high risk: acute leukaemia in complete remission, chronic myelogenous leukaemia in chronic phase, malignant lymphoma in complete remission, multiple myeloma in complete remission, myelodysplastic syndrome in refractory anaemia (RA), and aplastic anaemia as low/high risk. All other conditions were considered as high risk.

Definition

Graft failure comprises two clinical entities: primary graft failure and graft rejection. We defined the former as the combination of peripheral cytopenia and marrow hypoplasia for >60 d of RI-CBT, with the existence of a donor-type haematopoiesis (mixed or complete donor chimaerism). The latter was defined as the complete loss of donor-type haematopoiesis occurring anytime after transplantation. Peripheral cytopenia was defined as an absolute neutrophil count (ANC) $<0{\cdot}5 \times 10^9$/l and platelet count $<20 \times 10^9$/l. Engraftment was defined as an ANC $> 0{\cdot}5 \times 10^9$/l with mixed or complete donor chimaerism for three consecutive days. Regimen-related toxicity (RRT) was evaluated by the Common Terminology Criteria for Adverse Events version 3·0 (CTCAE v3·0) (Trotti *et al*, 2003). Transplant-related mortality (TRM) was defined as any death without progression of underlying diseases.

Diagnosis and management of graft failure

Chimaerism was assessed using fluorescent *in situ* hybridisation in sex-mismatched donor-recipient pairs. In sex-matched pairs, polymerase chain reaction (PCR) for variable numbers of tandem repeats was used with donor cells detected at a sensitivity of 10% (Thiede *et al*, 1999). Whole blood CD3$^+$ cells or marrow was assessed for chimaerism at the time of granulocyte engraftment. When engraftment was delayed, chimaerism was assessed before day 30. Patients with graft rejection generally underwent a second RI-CBT. When donor-type haematopoiesis was documented, granulocyte colony-stimulating factor (G-CSF) was given until day 60 or engraftment.

Statistical analysis

We used a univariate analysis to compare the differences between the patients with graft failure and those who achieved engraftment. *P*-values of <0·05 were considered significant.

Results

Engraftment

Ninety-six patients achieved engraftment at a median day 20 (range, 11–53 d). The other 27 patients had not achieved engraftment. One patient with adult T-cell leukaemia relapsed before engraftment (day 35). Graft failure was diagnosed in nine patients (7·3%). All of them were classified as having graft rejection. The other 17 patients died of TRM on a median of day 19 (range, 4–47). The causes of death included sepsis ($n = 12$), haemorrhage ($n = 2$), pneumonia ($n = 1$), invasive aspergillosis ($n = 1$) and multiple organ failure ($n = 1$).

Chimaerism was assessed before engraftment in 23 of 123 patients. Complete donor chimaerism was documented in 12 patients, of whom seven died before engraftment and five

achieved engraftment. Two patients had mixed chimaerism; one achieved engraftment and the other died before engraftment. The other nine had complete host chimaerism or graft rejection.

Clinical features of graft failure

Characteristics of the nine patients with graft failure and those who achieved engraftment are shown in Table II. There was no significant difference in patient characteristics between the two groups.

Of the nine with graft failure, three patients died of bacterial or fungal infection during neutropenia 28, 31, and 35 d after RI-CBT; two had sepsis and one had pneumonia.

Four patients underwent the second RI-CBT following fludarabine 125 mg/m^2, either melphalan 80 mg/m^2 or busulphan 8 mg/kg, and total body irradiation (TBI) 2–4 Gy. The median interval between the first and second RI-CBT was 46·5 d (range, 32–58 d). All of them developed grade 3–5 RRT. One died of pneumonia on day 8, and three achieved engraftment at median day 17 (range, 15–32 d) after the second RI-CBT. Of these, two died of TRM (thrombotic microangiopathy on day 57, and sepsis on day 160). The other patient survived without disease progression 9·0 months after the second RI-CBT.

Autologous haematopoiesis recovered in two patients. One patient finally died of disease progression 6·0 months after RI-CBT, and the other, who had refractory acute myeloid leukaemia, was alive in remission 15·4 months after RI-CBT.

As of May 2005, seven of the nine with graft failure had died, and the median survival of the nine patients was 3·8 months (range, 0·9–15·4 months).

Discussion

This study demonstrated that graft failure is a significant problem in RI-CBT, and is associated with a high mortality. It is important to differentiate graft rejection from other causes of graft failure, given that there are different treatment options for these conditions. Genetic markers have been used to make the distinction in allo-SCT using myeloablative preparative regimens, as they permit the determination of whether the marrow cells, peripheral blood neutrophils, and T cells are of donor or host origin (Georges & Storb, 2000). However, such a strategy may not be helpful in the diagnosis of graft failure following RI-CBT, where host T cells are not completely eliminated by preparative regimens. Our chimaerism study suggested that considerable amounts of host T cells might remain even in patients with graft failure because of poor graft function. Thus, we cannot technically rule out graft rejection in patients with primary graft failure after reduced-intensity stem cell transplantation (RIST). This is the dilemma in treating graft failure after RI-CBT.

Incidence of graft failure in our patients was comparable with that in myeloablative CBT (Laughlin *et al*, 2001). The high incidence of graft failure might be because of the features of cord blood rather than the conditioning regimens. The infused cell dose was reportedly a major determinant of neutrophil recovery (Laughlin *et al*, 2001; Wagner *et al*, 2002). However, the infused cell dose in the patients with graft failure was not significantly different from those in the patients who engrafted. The observations suggest that other risk factors might be associated with the development of graft failure rather than infused cell dose in our patients. However, it remains unknown whether the conventional risk factors for graft failure after allo-SCT, such as virus infections (Georges & Storb, 2000), could significantly affect graft failure in RI-CBT. Future studies are warranted.

The treatment of graft failure after RI-CBT has not been established. In cases of poor graft function that is unresponsive to haematopoietic growth factors, re-transplantation of another cord blood unit is necessary as a haematopoietic stem cell booster is unavailable in CBT. The selection of conditioning regimens is difficult. Most physicians believe that a conditioning regimen may be unnecessary for patients with primary graft failure because of poor graft function (Davies *et al*, 1994), while some conditioning regimens are essential to suppress graft rejection because of an immunological mechanism (Storb *et al*, 1987; Kernan *et al*, 1989). It is difficult to differentiate the two types of graft failure following RI-CBT. Since the important factor affecting the prognosis is opportunistic infection during prolonged neutropenia (McCann *et al*, 1994), our primary goal in the second RI-CBT was to ensure engraftment. We, therefore, adopted a conditioning regimen to suppress residual host T cells despite the possibility of increasing RRT.

While less toxic, immunosuppressive conditioning regimens, such as cyclophosphamide and antithymocyte globulin (ATG), are recommended in re-transplantation for graft rejection after conventional allo-SCT (Storb *et al*, 1987), the use of ATG in RIST and RI-CBT is controversial (Fujisaki *et al*, 2004; Kusumi *et al*, 2004). ATG strongly suppresses host immunity as well as donor immune cells in the graft, as it remains in the recipient's circulation because of its long half-life (Bunn *et al*, 1996). Furthermore, ATG in RI-CBT may increase the risk of graft failure (Kusumi *et al*, 2004). Due to this consideration, we avoided ATG and selected agents with short half-lives, such as fludarabine and melphalan, in addition to TBI. Consequently, three of the four patients with graft failure achieved engraftment, and the other died of early infection. These findings suggest that re-transplantation is a promising treatment for graft failure after RI-CBT. However, two patients died of TRM within 100 d of the second RI-CBT. Three of the four patients who underwent the second RI-CBT developed grade 3–5 RRT, and all three engrafted patients developed graft-*versus*-host disease (GVHD). The safety of the second RI-CBT needs to be improved. Since the conditioning regimen for the second RI-CBT included melphalan and TBI, which have significant mucous membrane toxicity (Sarosy *et al*, 1988), this may have contributed to the RRT. Indications

Table II. Characteristics of patients with graft failure and primary engraftment.

Variable	Graft failure	Primary engraftment
Number of patients	9	96
Age (years), median (range)	57 (17–68)	55 (20–79)
Day of diagnosis, median (range)	22 (15–43)	20 (11–53)
Risk of underlying diseases (high/low)	6/3	71/25
Primary diseases		
Acute lymphoblastic leukaemia	2	13
Acute myeloid leukaemia	3	30
Chronic myeloid leukaemia	0	3
Adult T-cell leukaemia	0	10
Myelodysplastic syndrome	1	11
Malignant lymphoma	1	20
Multiple myeloma	0	4
Solid tumours	0	1
Aplastic anaemia	2	4
Preparative regimens		
Flud + L-PAM 80 mg/m^2 + TBI 2 Gy	0	5
Flud + L-PAM 80 mg/m^2 + TBI 4 Gy	7	78
Flud + L-PAM 80 mg/m^2 + TBI 8 Gy	0	2
Flud + BU 8 mg/kg + TBI 4 Gy	2	6
Flud + BU 8 mg/kg + TBI 8 Gy	0	1
Others	0	4
Number of infused nuclear cells, median (range), $\times 10^6$/kg	27 (22–34)	28 (17–52)
Number of infused CD34$^+$ cell, median (range), $\times 10^6$/kg	0·066 (0·031–0·16)	0·08 (0·017–0·33)
HLA matching		
6/6	1	1
5/6	1	16
4/6	7	79
GVHD prophylaxis (cyclosporine alone/tacrolimus alone)	8/1	67/29
Treatment		
Second RI-CBT	4	NA
Supportive cares	5	NA
Outcomes		
Engraftment after second RI-CBT*	3	NA
Autologous marrow recovery	2	NA
Death without engraftment	4	NA

RI-CBT, reduced-intensity cord blood transplantation; GVHD, graft-*versus*-host disease; Flud, fludarabine; L-PAM, melphalan; BU, busulphan; TBI, total body irradiation; NA, not applicable.

The following conditions were defined as low/high risk: acute leukaemia in complete remission, chronic myelogenous leukaemia in chronic phase, malignant lymphoma in complete remission, multiple myeloma in complete remission, myelodysplastic syndrome in refractory anaemia (RA), and aplastic anaemia as low risk. All other conditions were considered as high risk.

*One patient died of thrombotic microangiopathy on day 57, and one patient died of sepsis because of gram-positive rods on day 160.

and dosage of such agents need further investigation. GVHD prophylaxis could be intensified, as GVHD tends to be severe after the second transplantation (Davies *et al*, 1994).

The present study showed that graft failure is a significant complication of RI-CBT; however, it has several limitations that should be discussed. First, this is a small retrospective study, and it might have unrecognised biases. Secondly, it should be noted that 17 patients died of TRM before engraftment. Considering the results of chimaerism analysis showing complete donor chimaerism in the evaluable seven patients, we cannot deny the possibility that poor graft function might have caused delayed engraftment, leading to TRM. The present study might have underestimated the risk of graft failure, especially that because of poor graft function. While we believe that this study will provide valuable information on this fatal complication, further large-sized studies are warranted to establish its optimal management.

Optimal procedures to reduce the risk of graft failure in RI-CBT have not been established. Establishment of optimal conditioning regimens would be beneficial. Physicians are concerned that immunosuppressive agents, such as ATG and alemtuzumab, in conditioning regimens might suppress donor immune cell function in the graft (Fujisaki *et al*, 2004; Kusumi *et al*, 2004; Rao *et al*, 2005). In contrast, a high rate of

engraftment has been reported in CBT using ATG (Staba *et al*, 2004). Further studies on the use of these agents are warranted. Barker *et al* (2005) recently reported a high rate of engraftment in myeloablative CBT using double cord blood units. This approach might be useful in RI-CBT as well. *Ex vivo* expansion of cord blood (Shpall *et al*, 2002; Ballen, 2005), and combination of cord blood and haploidentical bone marrow (Fernandez *et al*, 2003) might be worth investigating in RI-CBT.

Conflicts of interest

None of the authors have any conflict of interest.

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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′-GTCTACAGTTCGTAATGCTGAC-GT-3′ and that of the reverse primer was 5′-TGTGA-TCCACGGACACTCCTAC-3′; the TaqMan probe used was 5′-CGCTGGTTTCGATGATGGAGTCA-TTG-3′. For LRP mRNA, the forward primer was 5′-CGCTGTGATTGGAAGCACCTA-3′ and the reverse primer was 5′-CGGGAGGCAGCTCTTTCTC-3′; the TaqMan probe was 5′-ATGCTGACCCAG-GACGAAGTCCT-3′. The sequence for the forward primer for survivin mRNA was 5′-TGCCTGGCAG-CCCTTTC-3′ and for the reverse primer was 5′-CCT-CCAAGAAGGGCCAGTTC-3′; for the TaqMan probe, it was 5′-CAAGGACCACCGCATCTCTA-CATTC-3′. For cIAP1 mRNA, the sequence for the forward primer was 5′-CAGCCTGAGCAGCTTG-CAA-3′ and for the reverse primer, it was 5′-CAAGC-CACCATCACAACAAAA-3′; for the TaqMan probe, it was 5′-TTTATTATGTGGGTCGCAATGATGAT-GTCAAA-3′. For cIAP2 mRNA, the sequences of the forward and reverse primer were 5′-TCCGTCAAGTT-CAAGCCAGTT-3′ and 5′-TCTCCTGGGCTGTCT-GATGTG-3′, respectively; and the sequence for the TaqMan probe was 5′-CCCTCATCTACTTGAACA-GCTGCTAT-3′. The forward and reverse sequences for NAIP mRNA were 5′-GCTTCACAGCGCA-TCGAA-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′-AGTGGTAGTCCTGTTTCAGCAT-CA-3′ and for the reverse primer was 5′-CCGCACGG-TATCTCCTTCA-3′; the sequence for the TaqMan probe was 5′-CACTGGCACGAGCAGGGTTTCTT-TATACTG-3′. Finally, the forward primer sequence for GAPDH mRNA was 5′-GAAGGTGAAGGTCG-GAGT-3′ and for the reverse primer was 5′-GAA-GATGGTGATGGGATTTC-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

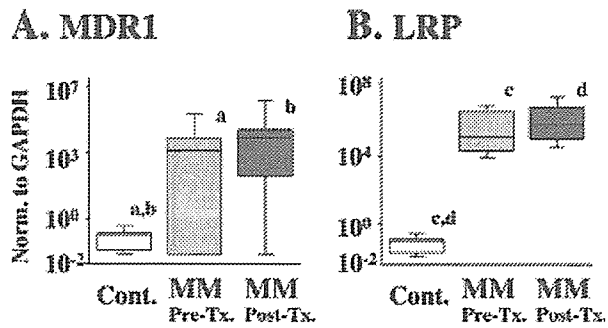

Fig. 1. Quantitative RT-PCR analysis of MDR1 (A) and LRP (B) in bone marrow samples from control cases (Cont.) ($n = 7$, white box) and MM patients before (Pre-Tx., light gray box) and after chemotherapy (Post-Tx., dark gray box) ($n = 26$). The relative intensity was calculated as [intensity of reaction 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 ($^aP < 0.01$) and after chemotherapy ($^bP < 0.01$), LRP expression in the controls and MM patients before chemotherapy ($^cP < 0.01$), the controls and MM patients after chemotherapy ($^dP < 0.01$) 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).

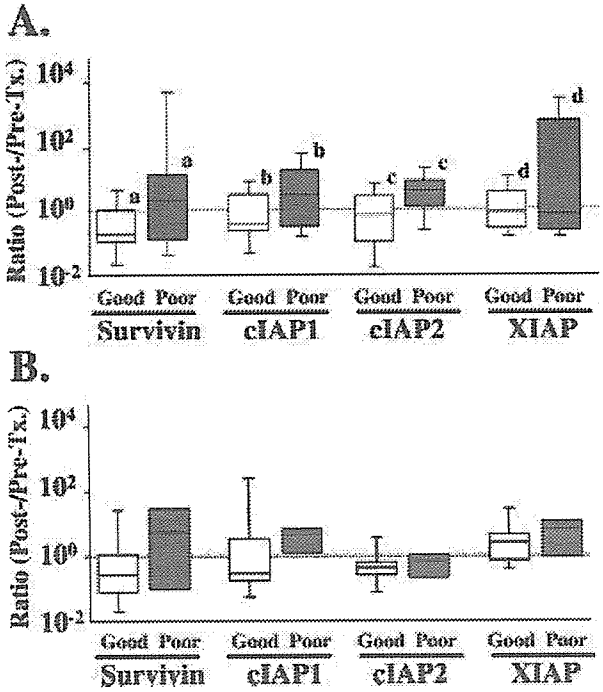

Fig. 2. Quantitative RT-PCR analysis of IAP family proteins in bone marrow samples from MM patients with increased (A, $n = 14$) or reduced (B, $n = 12$) expression of MDR1 after chemotherapy. The values are indicated as the ratio: [IAP expression after chemotherapy (Post-Tx.)/IAP expression before chemotherapy (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).

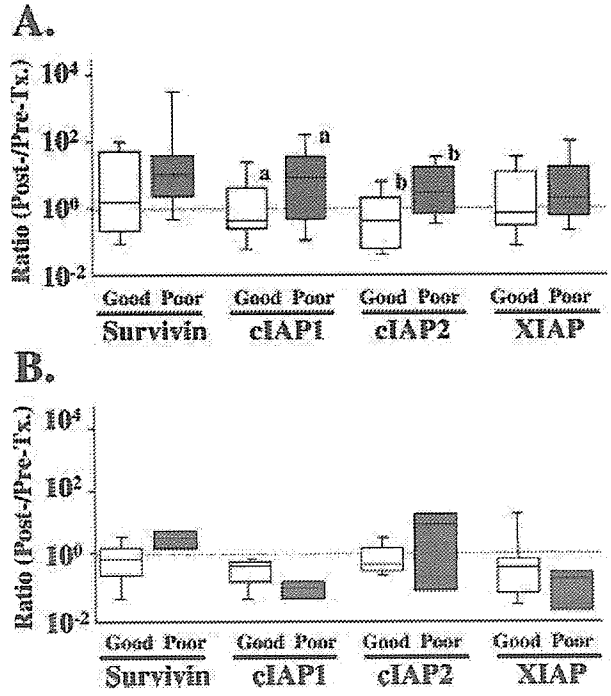

Fig. 3. Quantitative RT-PCR analysis of IAP family proteins in bone marrow samples from MM patients with increased (A, $n = 14$) or reduced (B, $n = 12$) expression of LRP after chemotherapy. In MM patients with increased LRP expression (A), the ratio of cIAP1 and cIAP2 expression was significantly higher 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

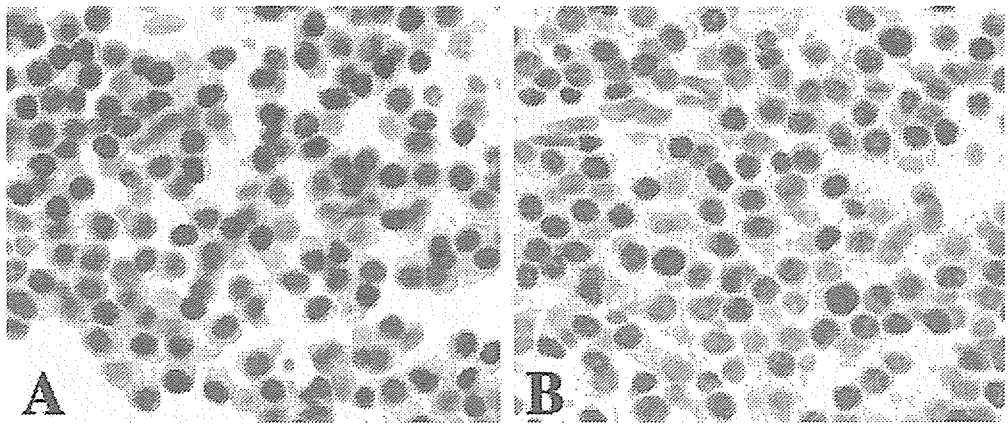

Fig. 4. Immunohistochemical localization of survivin in MM cells of the bone marrow before (A) and after chemotherapy (B). Note that the nuclei of more than half of the MM cells are positively stained although a few MM cells exhibit weak cytoplasmic staining. Differences in the ratio of survivin-positive 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 (pre-chemotherapy, 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.

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).

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).

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-

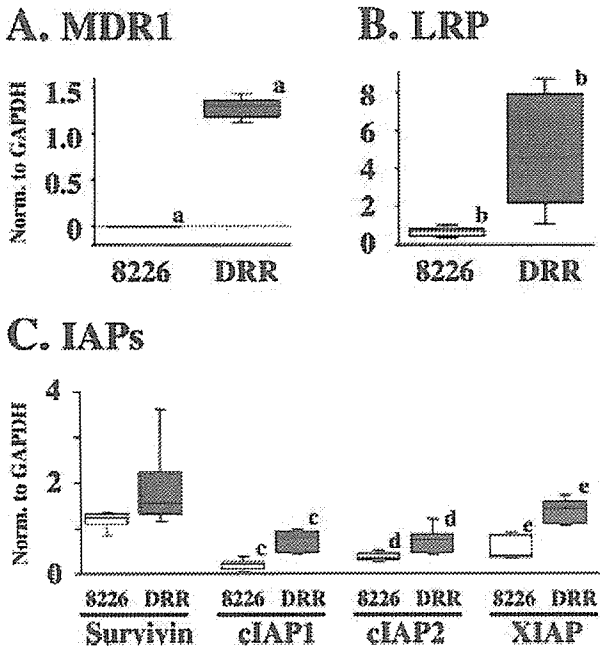

Fig. 5. Quantitative RT-PCR analysis of (A) MDR1, (B) LRP, and (C) IAPs in the MM cell line RPMI8226 (8226, white box) and doxorubicin-resistant variant RPMI8226 (DRR, gray box). As expected, the DRR cells expressed significantly higher levels of MDR1 (A) and LRP (B) than did the original RPMI8226 cells ($^{a,b}P < 0.05$, 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 ($^{c-e}P < 0.05$, 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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Monitoring of WT1-specific cytotoxic T lymphocytes after allogeneic hematopoietic stem cell transplantation

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Donor-derived cytotoxic T lymphocytes (CTL) that respond to tumor antigens emerge after hematopoietic stem cell transplantation (HSCT), particularly in association with the status of immune recovery. To analyze the frequency of CTL against PR1, PRAME and WT1 after HSCT, a tetramer-based analysis was performed in 97 samples taken from 35 patients (9 AML, 11 MDS, 2 CML, 4 ALL, 7 lymphoma and 2 renal cell carcinoma [RCC]) with the HLA-A02 phenotype. Regarding PR1, only 1 sample showed the presence of tetramer-positive cells (0.04%/lymphocyte). Similarly, in PRAME, only 10 of 97 samples were sporadically positive with low titers. For WT1, positive results were detected in 39 of 97 samples and 7 (2 CML, 1 ALL, 2 lymphoma and 2 RCC) patients clearly showed positive results more than once. On the basis of these results, we performed serial analyses of WT1-specific CTL during the clinical course in 2 patients with RCC, who underwent HSCT with a reduced-intensity regimen, to examine the precise correlation between the kinetics of CTL, the occurrence of GVHD and the observed clinical response. A higher positive rate for WT1-specific CTL and a correlation with the clinical response suggest that WT1 may be a useful antigen for a wider monitoring application.

Key words: WT1; cytotoxic T lymphocytes; allogeneic hematopoietic stem cell transplantation; renal cell carcinoma

Allogeneic hematopoietic stem cell transplantation (HSCT) is considered to be the most powerful and distinguished immunotherapy since the concept of graft-*versus*-leukemia/tumor (GVL/GVT) effect has been established.[1] However, the underlying mechanism of the anticancer effect is not yet clear. Although the generation of donor-derived cytotoxic T lymphocytes (CTLs) in response to tumor antigens is considered to be the primary reason,[2,3] the potential of any tumor-specific antigens to induce CTL should be critically evaluated in correlation with the clinical response. It is very likely that the immunogenic antigen, which plays the predominant role in the GVT effect, will be a potent candidate antigen for clinically realistic immunotherapy, including tumor vaccine, dendritic cell therapy and adoptive CTL infusion, to treat malignant disorders. WT1, PR1 and PRAME have been attractive targets for immunotherapy because of their expression in a wide variety of tumors, with a relative lack of expression in most normal tissues.[4–6]

One of the Wilms' tumor genes, WT1, encodes a zinc finger transcription factor,[7] and binds to the early growth response-1 DNA consensus sequence in growth factor gene promoters.[8] WT1 is expressed at a high level in most types of leukemia[9] and various types of solid tumors, including melanoma, renal cell carcinoma (RCC), and lung, breast, testicular and ovarian cancer.[10,11] Although WT1 is expressed at low amounts in the nuclei of some normal cells, it is limited to very few tissues, including splenic capsule and stroma, some gonadal cells and hematopoietic precursor cells.[12] It has been reported that MHC class I-restricted, WT1-specific CTLs were generated from human peripheral blood mononuclear cells (PBMC) by *in vitro* stimulation with WT1 peptide.[13–15] Additionally, an anticancer effect of WT1-specific CTL and the rejection of WT1-expressing tumor cells have been reported.[16–18]

The other candidates include PR1, which is derived from proteinase 3, a myeloid tissue-restricted serine protease present in azurophil granules in myeloid cells.[5] PR1 is overexpressed in some leukemia cells, and it has been reported that PR1-specific CTL selectively lyses chronic myelogenous leukemia (CML).[19] PRAME (preferentially expressed antigen in melanoma) is encoded by genes that are overexpressed in a wide variety of tumors, including melanoma (95% of patients), RCC (41%), lung cancer (50%), acute leukemia (30%) and multiple myeloma (52%).[20] PRAME is not expressed in normal tissue, except for testis, and very low levels are found in endometrium, ovaries and adrenals.[6,20–23]

In this study, we assessed the frequency of CTL against WT1, PR1 and PRAME by tetramer assay in peripheral blood taken from patients who underwent HSCT, to identify the best candidate antigen for clinically applicable immunotherapy.

Material and methods

Subjects

After we obtained written informed consent, peripheral blood samples were obtained from patients with an HLA-A02 serotype who had undergone allogeneic HSCT, with a conventional (CST) or reduced-intensity regimen (RIST), between August 2000 and May 2004 at National Cancer Center Hospital (Tokyo, Japan). Patients who received non-T-cell depleted-HSCT from a serologically full HLA-matched donor for hematological malignancy or RCC were eligible for this study.

Samples were taken at least 3 weeks after transplantation with confirmed hematological engraftment and more than 90% donor chimerism. Additional requirements for sampling included complete remission in leukemia patients and disease without bone marrow involvement in those with lymphoma or RCC.

Tumor antigen epitope peptides and cytomegalovirus peptide

The following >80% pure HLA-A*0201 binding peptides were obtained for the experiments, using high-performance liquid chromatography (Qiagen, Tokyo): WT1 peptide RMFPNAPYL (amino acids [AA] 126–134), PR1 peptide VLQELNVTV (AA 169–177) and PRAME peptide ALYVDSLFFL (AA 300–309); cytomegalovirus (CMV) pp65 peptide NLVPMVATV (AA 495–503) was used as a positive control.

Tetramer staining

Antibodies to CD4, CD8, CD19, CD13, CD45RA, CD45RO, CD27, CD57, CCR7, PE-conjugated CMV-tetramer and PR1-tetramer were purchased form Beckman Coulter (Fullerton, CA), and

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APC-conjugated CMV, WT1 and PRAME-tetramer were purchased from ProImmune (Oxford, UK). The antibodies and tetramer complexes were added to 200 μL heparinized whole blood or cell suspension, and incubated for 15 min at room temperature in the dark. For the quantification of antigen-specific CTL, whole blood samples were used, and the red blood cells were lysed with ammonium chloride-based lysing solution after antibody staining. After being washed twice with BSA-containing PBS, the cells were fixed and analyzed on a flow cytometer (FACS Calibur, Becton Dickinson). Analysis was performed using Cellquest software. For the quantification of antigen-specific-CTL, whole blood samples were used, and $CD4^-$, $CD19^-$, $CD13^-$, $CD8^+$ and $tetramer^+$ fraction of the lymphocyte gate were defined as antigen-specific CTL. Samples with more than 0.02% antigen-specific CTL per lymphocyte were defined as positive results. For immunophenotyping of the antigen-specific CTL, fresh PBMC, separated from heparinized blood by Ficoll-Hypaque (IBL, Japan) density gradient centrifugation, was used to acquire a higher number of lymphocytes (minimum of 10^5) per analysis. The cells were gated on $CD8^+$ and $tetramer^+$ fraction of the lymphocyte, and then the positive ratios for CCR7, CD45RA, CD45RO, CD27 and CD57 were analyzed.

Expansion of antigen-specific CTL

PBMC was diluted at 1×10^6 cells/mL in RPMI 1640 (Sigma) supplemented with 10% FBS, gentamicin and streptomycin (hereafter referred to as culture medium). PBMC (2×10^6 cells) were seeded in a 24-well plate, and the peptide was added to a final concentration of 5 μM on day 0. The peptide was diluted to 10 mg/mL in dimethyl sulfoxide (DMSO) prior to use, and the same amount of DMSO alone was used as a negative control. The culture was fed on days 4 and 7 by replacing half of the medium with a fresh culture medium containing 20 U/mL IL-2 and 10 μM peptide. Cells were cultured for 14 days.

Intracellular cytokine staining

The cultured cells were washed with culture medium, and 5×10^4 cells per test were suspended in 200 μL medium. Tetramer was added to the test samples, and incubated for 15 min at 37°C in the dark. For peptide-stimulated cells, specific peptides were added to a final concentration of 10 μM and incubated for 6 hr at 37°C. Breferdin A (Sigma; 10 μg/mL) was added during the last 4 hr of incubation. Positive and negative controls were obtained by stimulating the cells with 10 μg/mL staphylococcal enterotoxin B or PBS. Samples were washed, permeabilized and stained with anti-IFN-γ and anti-CD8 antibodies, and analyzed using a FACS Calibur.

Genotyping of the HLA-A02 locus

The DNA genotype of the HLA-A02 locus was examined using a Micro SSP allele-specific HLA class 1 DNA typing tray (One Lambda, CA, USA), according to the manufacturer's protocol. In brief, DNA was extracted from the lymphocytes, and added to a PCR reaction buffer containing dNTP and Taq polymerase. The sample-reaction mixture was applied to a 96 PCR tray that had been preloaded with allele-specific primers for HLA-A02. After the PCR reaction, the samples were electrophoresed on a gel, and photographed by an UV transilluminator. HLA-genotype was determined by the patterns of the allele-specific PCR product.

Results

Tetramer assay

Samples were obtained from 35 patients (9 AML, 11 MDS, 2 CML, 4 ALL, 7 lymphoma and 2 RCC): 10 had received CST and 25 received RIST. The stem cell source was peripheral blood stem cells from a related donor in 24, related bone marrow in 2 and unrelated bone marrow in 9. The genotype for the HLA-A02 allele was analyzed in 27 patients: 13 had A*0201, 12 had A*0206, 1 had A*0207 and 1 had both A*0201 and A*0206 genotypes.

TABLE I – DETECTION OF ANTIGEN-SPECIFIC CTL BY TETRAMER ASSAY

Antigen	Disease	No. of patients	CTL-positive patients	No. of samples	Samples with 0.02% ≤ CTL
WT1	AML	9	0	16	1
	MDS	11	0	17	2
	CML	2	2	8	4
	ALL	4	1	7	3
	NHL	7	2	14	7
	RCC	2	2	35	22
	Total	*35*	*7 (20)*[2]	*97*	*39 (40)*
PRAME	AML	9	0	16	2
	MDS	11	0	17	2
	CML	2	0	8	0
	ALL	4	0	7	1
	NHL	7	0	14	0
	RCC	2	1	35	5
	Total	*35*	*1 (2.8)*	*97*	*10 (10)*
PR1	AML	9	0	16	1
	MDS	11	0	17	0
	CML	2	0	8	0
	ALL	4	0	7	0
	NHL	7	0	14	0
	RCC	2	0	8	0
	Total	*35*	*0 (0)*	*70*	*1 (1.4)*

[1]When the patients showed 0.02% ≤ CTL at least twice, they were considered to be positive.–[2]Values in parentheses indicate percentages.

All samples from CMV-seropositive patients were positive for CMV-specific-CTL (CMV-CTL) with high titers (mean 2.3%), and the same result was obtained using CMV-tetramer purchased from Beckman Coulter or ProImmune. The frequency of CMV-CTL did not differ between the A*0201 and A*0206 genotypes, but the patient with the A*0207 genotype was negative for CMV-CTL, since he was seronegative for CMV.

Regarding WT1-specific CTL (WT1-CTL), 39 of 97 samples had more than 0.02% CTL per lymphocyte. Seven patients (2 CML, 1 ALL, 2 lymphoma and 2 RCC) showed positive results at least twice, and we defined them as WT1-CTL positive patients. Among those with positive WT1-CTL between days 40 and 520 postHSCT, 1 ALL patient and 1 CML patient received CST, while the other 5 patients received RIST. All of the WT1-CTL-positive patients had experienced skin involvement by graft-*versus*-host disease (GVHD) of grade 1–3 prior to the detection of WT1-CTL, except for 1 ALL patient. The other target organs of GHVD were the liver in 1 patient, and gut in 1 patient. The HLA-A*02 genotype in WT1-CTL-positive patients was A*0206 in 5, A*0201 in 1 and both the A*0201 and A*0206 genotypes in 1.

Regarding PR1, all 70 samples were 0%, except for 3 samples that showed 0.01 and 0.04%. The sample with 0.04% PR1-specific-CTL was taken from a patient with AML at day 925 postSCT. However, another sample taken at day 966 from the same patient was negative (0%), suggesting that the initial result was a false-positive one. Similarly, in PRAME, 87 of 97 samples were negative and 10 samples from 7 different leukemia, lymphoma or RCC patients were sporadically positive with low titers (<0.05%), but positive results were not found at different occasions. Only 1 RCC patient showed a positive result more than once. However, the staining of PRAME-tetramer was dull compared to the prominent positive staining of WT1-tetramer, which suggests that the result may have been false-positive (Table I).

Expansion of antigen-specific CTL

The samples taken from 7 patients (4 AML, 2 CML and 1 RCC) were cultured with WT1, PR1, PRAME, CMV peptide or DMSO. The frequency of antigen-specific-CTL was analyzed by tetramer assay before and 14 days after culture. The CMV-CTL expanded in all 7 samples by 2- to 50-fold, whereas none of the PR1 or PRAME

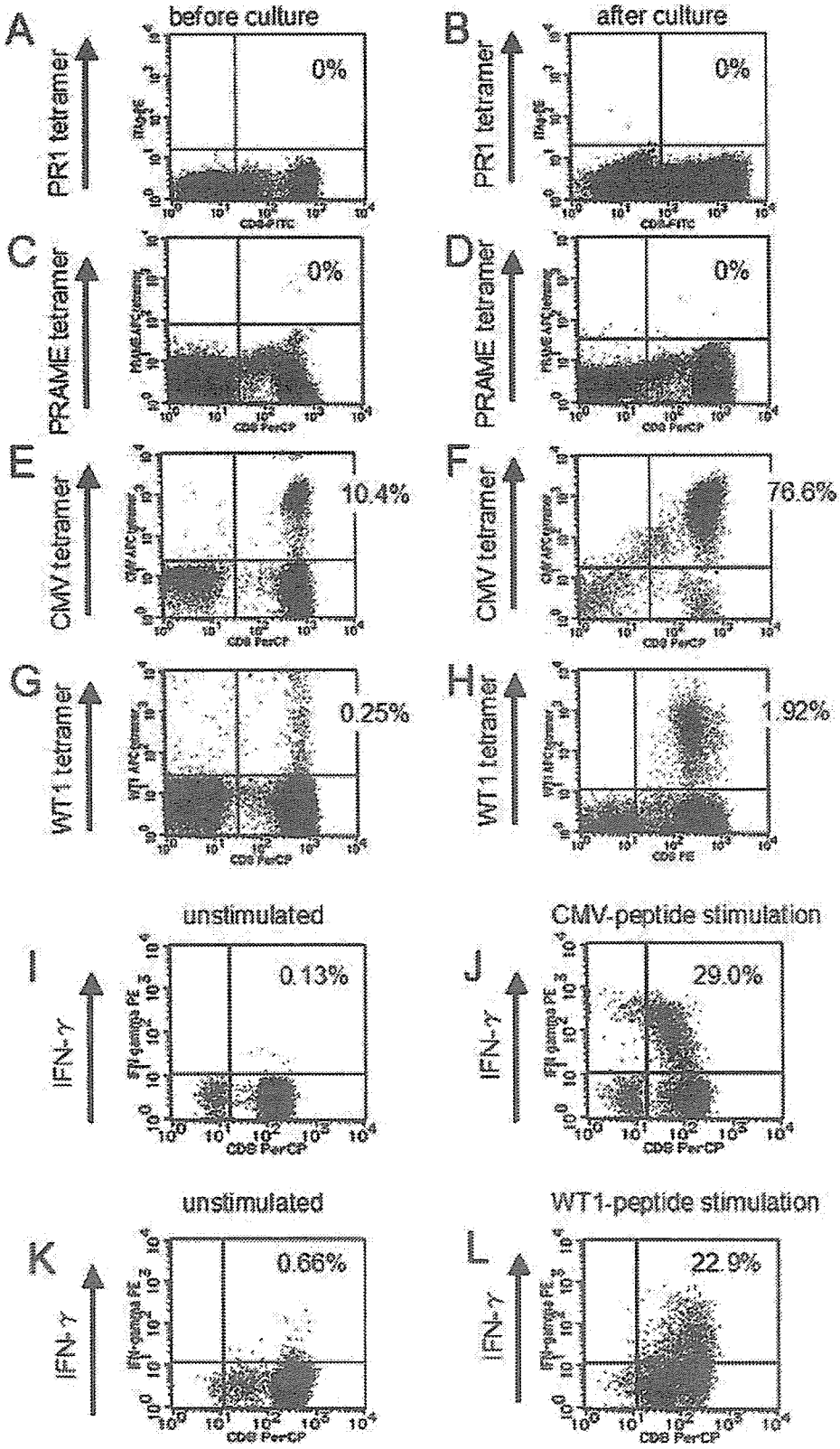

FIGURE 1 – Cell expansion of antigen-specific CTL and intracellular IFN-γ staining. PR1-CTL culture in CML patient (*a*, *b*) and PRAME-CTL culture in a RCC patient (*c*, *d*) remained in undetectable levels, even after expansion culture. CTL showed an expansion of CMV-CTL (*e*, *f*) and WT1-CTL (*g*, *h*). The intracellular IFN-γ staining showed that the CMV-CTL and WT1-CTL produced IFN-γ when stimulated with the peptide (*j*, *l*), while the unstimulated control did not (*i*, *k*). The cells are gated on a tetramer$^+$ fraction.