Toxicities

All patients developed severe mucositis with oropharyngeal pain (WHO grade 3) after SCT. Only one patient had elevated transaminase levels greater than five times normal (case 5). All patients developed febrile neutropenia without a detectable pathogen, which subsided within 7 days by antibiotic treatment. No other acute toxicities associated with SCT were observed.

Patient survival

All three patients without CNS metastasis are alive disease-free at 113, 107 and 38 months, respectively, from the time of SCT (case 3–5). They are alive without complications, except for orbital growth retardation because of local irradiation and surgical enucleation. Two patients died of recurrent diseases 4 and 48 months, respectively, after SCT (case 1, 2). There was no second malignancy in this series.

Discussion

The prognosis of patients with metastatic retinoblastoma is poor with conventional chemotherapy and radiation therapy.[2,8] Honavar *et al*[9] have shown that postenucleation adjuvant therapy is safe and effective in significantly reducing the occurrence of metastasis in patients with retinoblastoma manifesting high-risk histopathologic characteristics.[9] Several centers have used conventional-dose chemotherapy and radiation therapy for hematogenously spread extraocular disease. Despite some reports of long-term event-free survival,[7,10] the bulk of the evidence suggests that the prognosis remains poor with such an approach.[11]

A limited number of studies and case reports have suggested that HDC with autologous stem cell rescue might be beneficial for patients with metastatic retinoblastoma (Table 3).[12 20] Namouni *et al*[14] conducted a study of HDC consisting of carboplatin, etoposide and cyclophosphamide (CARBOPEC) followed by autologous SCT in 25 patients, including 12 patients with distant metastases. Among eight children with bone and BM metastases, five survived between 11 and 70 months disease free, while three patients with CNS metastases relapsed in the CNS after HDC and died. Thus, the CARBOPEC regimen appeared to be effective only for patients with bone and/or BM involvement of retinoblastoma. Dunkel *et al*[16] reported four retinoblastoma patients with orbit and BM metastases who underwent HDC consisting of carboplatin and thiotepa with or without etoposide. All patients survived event-free for 46–80 months after the diagnosis of metastatic disease. They concluded that this treatment strategy is effective for metastatic retinoblastoma without CNS involvement. Rodriguez-Galindo *et al*[19] reported four retinoblastoma patients with bone and BM metastases, treated by intensive systemic therapy. Although they did not mention an effectiveness of HDC, they concluded that the use of intensive multimodal approach in patients with metastatic retinoblastoma without CNS involvement could achieve long-term survival.

The important component in HDC is the alkylating agents, which have favorable toxicity profile. There are some reports that thiotepa is effective for high-risk retinoblastoma and other malignancies.[16,19,21,22] As it penetrates well into the brain, as demonstrated by similar drug levels in CSF and in serum after intravenous injection bolus use, we should consider the high-dose thiotepa in the attempts of HDC in disseminated retinoblastoma, particularly with CNS involvement. However, we used not thiotepa but melphalan for HDC. High-dose melphalan and SCT have been used to treat neuroblastoma, rhabdomyosarcoma and Ewing's sarcoma in children.[23 26] In addition, Inomata and Kaneko[27] suggested that retinoblastoma was most sensitive to melphalan based on a colony assay on double agar layers. Kaneko treated six patients with intraocular retinoblastoma that recurred after irradiation therapy by injecting 40 mg/m^2 of melphalan into the ipsilateral intracarotid artery, and by applying ocular hyperthermia (45°C, 1 h).[5] Two patients were cured (no recurrence for more than 10 years) with a single treatment procedure while preserving adequate visual function. Based on their observation, we selected melphalan as a key drug for HDC. We should consider that not only thiotepa but also melphalan is an effective agent of HDC for retinoblastoma. As other agents, busulfan and nitrosurea drugs

Table 3 High-dose chemotherapy for retinoblastoma

Author (year)	*n*	*Marrow involvement (+/−)*	*Bone Metastasis (+/−)*	*CNS Metastatsis (+/−)*	*High-dose chemotherapy*	*Result*
Namouni *et al* (1997)[14]	12	1/11	7/5	4/8	CARBOPEC	6 alive
Dunkel *et al* (2000)[16]	4	3/1	4/0	0/4	CTE 3, TC 1	4 alive
Kremens *et al* (2003)[19]	5	4/1	2/3	0/5	CTE 4, BCyE 1	5 alive[a]
Rodriguez-Galindo *et al* (2003)[20]	4	4/0	4/0	0/4	CE 1, BuCyM 1, CyE 1, CyTopo 1	2 alive
Jubran *et al* (2004)[3]	4	1/3	2/0	1[b]/3	CTE	2 alive
Our cases	5	2/3	2/3	2/3	CDDP-CyM 2, MEC 2, TCyM 1	3 alive

[a]One alive after relapse.
[b]Pineal.
CARBOPEC = carboplatin + etoposide + cyclophosphamide; CTE = carboplatine + thiotepa + etoposide; TC = thiotepa + carboplatin; BcyE = busulfan + cyclophosphamide + etoposide; CE = carboplatin + etoposide; BuCyM = busulfan + cyclophosphamide + melphalan; CyE = cyclophosphamide + etoposide; CyTopo = cyclophosphamide + topotecan; CDDP-CyM = cisplatin + cyclophosphamide + melphalan; MEC = melphalan + etoposide + carboplatin; TCyM = thiotepa + cyclophosphamide + melphalan; DOD = dead of disease.

(nimustine, ranimustine), which are effective because of their capacity to cross the blood–brain barrier, have been used for retinoblastoma.[28,29]

We conclude that our treatment strategy that includes high-dose melphalan with autologous SCT and local irradiation is effective in patients with metastatic retinoblastoma without involvement of the CNS, although a wide variation in the HDC regimen made it difficult to judge the objective safety and efficacy of autologous SCT. A safer and more effective modality is required to better control CNS involvement. The possible risk of late sequelae secondary to additive toxicity by HDC and cranial radiation should be critically evaluated. Since metastatic retinoblastoma is a rare disease, a larger cooperative study is needed to clarify the safety and efficacy of this HDC strategy.

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Graft-versus-host disease of the kidney after rapid tapering of cyclosporin following reduced intensity hematopoietic stem cell transplantation

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Acute renal failure is a common problem after allogeneic hematopoietic stem cell transplantation (allo-SCT) and many factors contribute to its development.[1] Graft-versus-host disease (GVHD) rarely involves the kidney and most physicians believe that the kidney is not target of acute GVHD. However, several case reports have been published concerning chronic GVHD of the kidney and resulting nephrotic syndrome.[2,3] We describe a patient who developed renal failure attributable to acute GVHD after rapid tapering of cyclosporin following reduced intensity stem cell transplantation (RIST). A detailed description of his clinical courses provides important information on renal involvement of acute GVHD.

A 60-year-old man underwent RIST for acute myeloid leukemia, from an HLA-identical sister. Induction chemotherapy with cytarabine and idarubicin failed. Pretransplant evaluation showed normal renal function. Preparative regimen comprised fludarabine 30 mg/m^2 for 6 days and busulfan 4 mg/kg for 2 days. GVHD prophylaxis was cyclosporin 3 mg/kg, which was tapered rapidly from day 30 to day 45 to augment a graft-versus-leukemia effect. He achieved neutrophil engraftment on day 10. Regimen-related toxicities were minimal. His clinical course was uneventful until day 54, when he developed watery diarrhea. Blanching erythema appeared on day 60. Grade III acute GVHD was diagnosed based on histopathological examination, and cyclosporin was re-instated. Corticosteroids were avoided while GVHD was tolerable. His gastrointestinal GVHD worsened, and we initiated prednisolone 1.0 mg/kg on day 71. He responded to the immunosuppression, and the prednisolone and cyclosporin were tapered off by day 137. The creatinine clearance gradually decreased. Fractional excretion of sodium (FENa) was 2.3% on day 138. No hematuria, proteinuria or casts were detected on urinalysis. Renal ischemia had not occurred during his clinical course. Watery diarrhea started on day 150, but while the symptoms of gastrointestinal GVHD were mild, corticosteroids were withheld. When corticosteroids 0.5 mg/kg were resumed on day 178, the watery diarrhea immediately resolved. We tapered the corticosteroid off by day 209 but after discontinuation of corticosteroid, the watery diarrhea reappeared, and the renal dysfunction gradually worsened. His diarrhea was mild, and treated with supportive measures. Histopathological examination of the kidney on day 251 revealed tubulitis with tubular epithelial vacuolization (Figure 1). Glomerular change was minimal. Hemodialysis was initiated on day 270 for renal failure. We continued supportive treatment for the gastrointestinal GVHD. He died of septic shock on day 295.

The following suggest that the renal dysfunction was most likely due to acute GVHD. First, the renal dysfunction followed GVHD and improved with corticosteroid. Second, FENa was elevated when the renal dysfunction developed, supporting the concept that the renal dysfunction was not secondary to circulatory failure and that the kidney was primarily damaged. Third, the patient had received only one course of chemotherapy and had normal renal function at RIST, so the possibility that an underlying renal dysfunction was aggravated is unlikely. Finally, the duration of cyclosporin and antibiotic administration was short, and their potential for causing renal toxicity was minimized. The histopathological findings suggesting nephrotoxicity of calcineurin inhibitors such as increasing arteriolar hyalinosis, small-vessel narrowing, and progressive ischemic glomerulosclerosis were absent in the present patient.[4] Although the possibility cannot be excluded that renal toxicity from medications given during the peritransplant period aggravated an underlying renal dysfunction, it is unlikely to have been the primary cause. While conditioning regimen-related toxicities mostly develop within 28 days of transplantation,[5] our patient developed renal dysfunction more than 100 days after transplantation.

Renal biopsy revealed tubulitis. The pathological diagnosis of tubular involvement is consistent with the clinical course and changes in FENa. Seshi *et al* reported tubulitis in 17 of 26 patients who underwent allo-SCT and autopsy. The high incidence of tubulitis suggests an association between allo-SCT and tubulitis since the pathological diagnosis of tubulitis is rare. Although they found no significant association between GVHD and tubulitis, and discussed the issue that tubulitis is not a manifestation of renal GVHD, the small sample size obscured any significance, as indicated. The clinical course of the present patient supports an association between GVHD and tubulitis, requiring further investigation.

Most physicians believe that the kidney is rarely involved in GVHD; however, *in vivo* imaging of mice with GVHD showed that several nonclassical organs are massively infiltrated by cytotoxic T-cell (CTL) during GVHD, including the brain, kidneys, and connective tissues.[6] Infiltration of CTL into the kidney explains the high response rates following RIST for metastatic renal cell carcinoma (RCC),[7] which derives from the proximal renal tubules. While tumor-specific antigens and minor histocompatibility antigens targeted by CTL have been cloned in allo-SCT for RCC,[8] the mechanism of the graft-versus-tumor effects remains unknown. Response is closely related to GVHD in allo-SCT for RCC,[7] and the sensitivity of RCC to alloimmunity can be explained by the hypothesis that the minor histocompatibility antigens expressed on proximal renal tubules are the target of alloimmunity and that RCC arising from the proximal renal tubules also expresses the antigen.

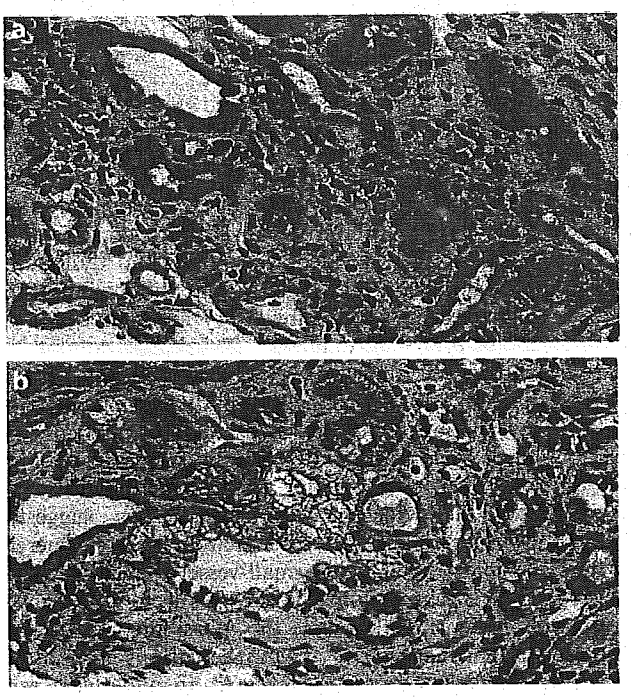

Figure 1 Pathology of the kidney (HE staining). (**a**) Histopathological examination of the kidney revealed tubulitis with massive infiltration of mononuclear cells. Glomerular change was minimal. (**b**) Tubular epithelial vacuolization.

Renal tubules, especially proximal renal tubules, are the target organ of rejection after renal transplantation.[9,10] Since recipients are often transplanted from HLA-mismatched and/or cadaver donors, renal tubular dysfunction after renal transplantation occurs more commonly than after allo-SCT. Proximal renal tubules can also be the target of alloimmunity. In contrast, renal tubular dysfunction after allo-SCT from an HLA-matched donor may be mild and underdiagnosed. In the present patient, the rapid tapering of cyclosporin may have contributed to the clinical manifestation of renal tubular dysfunction. This patient might also have had age-related renal dysfunction, his renal function being more susceptible to insults including GVHD. Alternatively, differences in the pathogenesis of acute GVHD between conventional myeloablative allo-SCT and RIST[11,12] might have contributed to the development of renal tubulitis in this patient.

The clinical course of this patient suggested that acute GVHD may lead to renal dysfunction and that the target tissue is the renal tubules. This hypothesis needs further evaluation by prospective studies and basic investigation of the underlying mechanism.

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Graft-versus-host disease

Optimal initial dose of oral cyclosporine in relation to its toxicities for graft-versus-host disease prophylaxis following reduced-intensity stem cell transplantation in Japanese patients

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Summary:

Since the introduction of reduced-intensity stem-cell transplantation (RIST), allogeneic stem-cell transplantation has become available for elderly patients. While pharmacokinetics of cyclosporine might differ according to age or other factors, cyclosporine is uniformly started at an oral dose of 6 mg/kg/day. We retrospectively reviewed medical records of 35 patients aged between 32 and 65 (median 52) years who had undergone RIST. Doses of cyclosporine were adjusted to the target blood trough level of 150–250 ng/ml. Cyclosporine dosages were changed in 33 patients (94%). Dose reduction was required in 32 patients because of high blood levels ($n=25$), renal dysfunction ($n=3$), hepatic dysfunction ($n=2$), and hypertension ($n=2$). Cyclosporine doses were increased in one because of the suboptimal level. The median of the achieved stable doses was 3.1 mg/kg/day (range, 1.0–7.4). Five patients sustained Grade III toxicities according to NCI-CTC version 2.0: renal dysfunction ($n=4$), hyperbilirubinemia ($n=2$), and hypertension ($n=2$). No patients developed grade IV toxicity. There was no statistically significant difference in the frequency and severity of cyclosporine toxicities between patients aged 50 years and above and those below 50 years. The initial oral cyclosporine dose of 6 mg/kg/day was unnecessarily high irrespective of age. The possible overdose of cyclosporine might have aggravated regimen-related toxicities.

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Cyclosporine is commonly started at a dose of 6 mg/kg/day via oral routes or 3 mg/kg/day via intravenous routes in reduced intensity stem cell transplantation (RIST) as well as conventional allogeneic hematopoietic stem cell transplantation (allo-HSCT).[1,2] However, its pharmacokinetics might differ according to various factors including age, and there is no solid evidence to support applying a fixed conventional starting dose of cyclosporine to every individual. Since the introduction of RIST, more elderly patients have become candidates for allo-HSCT. No reports have addressed optimal cyclosporine dose in elderly patients undergoing RIST. Only a few reports have been published concerning cyclosporine pharmacokinetics in elderly individuals.[3,4] Single dose studies have demonstrated that cyclosporine pharmacokinetics are not different in healthy elderly individuals compared to healthy young adults, nor is the between-subject variability in pharmacokinetic parameters more heterogeneous in healthy elderly individuals.[3] A population pharmacokinetic study of cyclosporine in organ transplant patients, including elderly allograft recipients up to 75 years of age, did not identify age as a covariate influencing cyclosporine pharmacokinetics.[3] These studies failed to show a significant association between cyclosporine disposition and the patients' ages. The association has never been investigated in the setting of allo-HSCT. The objective of this study was to evaluate the appropriateness of starting oral cyclosporine at a dose of 6 mg/kg/day in RIST in relation to target trough level and its toxicities especially in relation to age by comparing patients at the age of 50 years and above and those below 50 years.

Patients and methods

Patients

The medical records of all of the patients who underwent RIST at the National Cancer Center Hospital and Toranomon Hospital between January 2000 and September 2003 were reviewed. All patients and donors gave their written informed consent in accordance with the requirements of the Institutional Review Board. A total of 35

patients in whom cyclosporine was started orally at a dose of 6 mg/kg/day were enrolled. The median age of these patients was 52 (range, 32–65) years. Primary diseases consisted of acute myeloblastic leukemia ($n=8$), malignant lymphoma ($n=9$), myelodysplastic syndrome ($n=3$), acute lymphoblastic leukemia ($n=1$), other hematologic diseases ($n=3$) and solid tumors ($n=11$).

Transplantation procedures

Reduced intensity preparative regimens were busulfan at 4 mg/kg for 2 days and fludarabine at 25 mg/kg for 6 days ($n=30$), and busulfan at 4 mg/kg for 2 days and cladribine at 0.11 mg/kg. for 6 days ($n=5$).[5] Rabbit antithymocyte globulin (ATG, Thymoglobulin, Imtix-Sangstat, Lyons, France) at 2.5 mg/kg for 2 or 4 days, and total body irradiation 4 Gy were added to the preparative regimens in five and two patients, respectively.

The stem cell sources were an HLA-identical related donor ($n=32$), a one-locus mismatched related donor ($n=1$), and a matched unrelated donor (MUD) ($n=2$). Peripheral blood and marrow were transfused in RIST from related donors and that from a matched unrelated donor (MUD), respectively. All the patients received fluconazole at 200 mg for fungal prophylaxis until withdrawal of cyclosporine.

Administration of cyclosporine and monitoring of blood cyclosporine levels

All the patients received oral cyclosporine (NeoralTM, Novartis, Switzerland) at 3 mg/kg every 12 h, beginning on day −1. Short-term methotrexate was added to the GVHD prophylaxis in RIST from an HLA-mismatched related donor or MUD.

Blood levels of cyclosporine were monitored at least once a week. The blood samples were treated with EDTA anticoagulants, and the concentration of cyclosporine in whole blood was determined by fluorescence polarization immunoassay (FPI) with a specific monoclonal antibody.[6] Dosages of cyclosporine were adjusted according to its blood concentration and serum levels of creatinine.[7] The dose of cyclosporine and target therapeutic blood concentration vary widely in different centers.[8] In Japan, where incidence of acute GVHD is low compared with the Western countries,[9] the trough target range of cyclosporine 150–250 ng/ml is recommended by the Japan Society for Hematopoietic Stem Cell Transplantation http://www.jshct.com/guide_pdf/1999gvhv2.pdf.

Study end points and statistical analysis

The primary objective of this study was to evaluate the feasibility of oral cyclosporine starting at a dose of 6 mg/kg/day in RIST. We examined the proportion of patients in which cyclosporine was discontinued or its dosages were changed within 28 days of transplant. The other objective was to investigate the incidence of common cyclosporine toxicity: elevation of blood creatinine levels, hyperbilirubinemia, hyperkalemia, hypomagnesemia, and hypertension. These variables were compared between patients below age 50 years and those at 50 years and above. A univariate analysis using the χ^2 chi-square test and Mann–Whitney test were performed. Cumulative incidence of acute GVHD was calculated using Gray's method,[10] treating deaths without GVHD as a competing risk. Potential confounding factors considered in the analysis were age, sex, stem cell dose, HLA disparity, transplantation from an alternative donor, conditioning regimen and through levels of cyclosporine (below 150 ng/ml *vs.* higher than 150 ng/mL). Proportional hazard modeling was used to evaluate the influence of these factors on the incidence of acute GVHD using the proportional hazard modeling treating serum levels of cyclosporine as time-dependent covariates. *P*-values of less than 0.05 were considered statistically significant.

Results

Changes of cyclosporine dosages

Cyclosporine dosages were changed in 33 patients (94%) (Figure 1). Dose reduction was required in 32 patients (91%) because of high blood levels of cyclosporine ($n=25$), renal dysfunction ($n=3$), hepatic dysfunction ($n=2$), hypertension ($n=2$). Renal dysfunction progressed to acute renal failure in one patient after cessation of cyclosporine. Dosages of cyclosporine were increased in the other patient (3%), because blood levels of cyclosporine were below its target.

At the initial monitoring of cyclosporine, its median levels were 219 ng/ml (range, 80–403) in the whole population; 206 ng/ml (range, 93–403) and 232 ng/ml (range, 80–372) in patients below 50 years and those at 50 years and above, respectively ($P=0.50$). Changes of cyclosporine concentration were shown in Figure 1. Median interval between the initiation of cyclosporine and its initial dose adjustment was 15 days (range, 3–29). Stable dose of cyclosporine was 3.2 mg/kg/day (range, 1.0–7.4); 2.7 mg/kg/

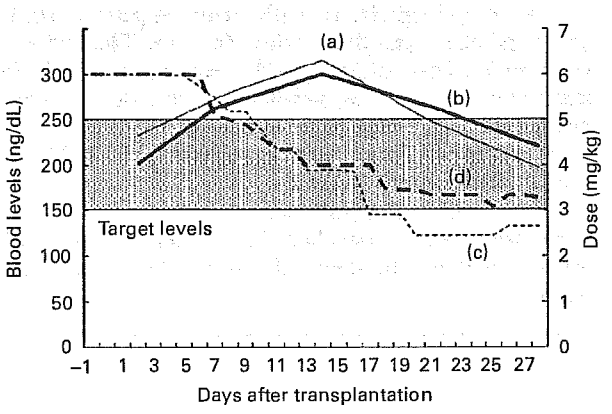

Figure 1 Serial changes of cyclosporine doses and concentration. There were no significant differences in cyclosporine doses and concentration between patients below 50 years and those at 50 years and above. (a) Cyclosporine concentration in patients below 50 years; (b) cyclosporine concentration in patients at 50 years and above; (c) cyclosporine doses in patients below 50 years; (d) cyclosporine doses in patients at 50 years and above.

Table 1 Cyclosporine-related toxicity within 28 days of transplant (NCI-CTC version 2.0)

Grade	*I*		*II*		*III*		*IV*	
Age (years)	*<50*	*≥50*	*<50*	*≥50*	*<50*	*≥50*	*<50*	*≥50*
Elevation of blood creatinine levels	1	4	8	2	1	3	0	0
Hyperbilirubinemia	4	10	3	4	0	2	0	0
Hypertension	0	0	12	15	0	2	0	0
Hypomagnesemia	7	8	3	3	0	0	0	0
Hyperkalemia	0	0	0	1	0	0	0	0

day (range, 1.0–7.4) and 3.3 mg/kg/day (range, 1.0–6.0) in patients below age 50 years and those at 50 years and above, respectively ($P=0.24$).

Toxicity

All the patients developed grade I toxicities or more (Table 1). Grade III toxicities were shown in five patients: renal dysfunction ($n=4$), hyperbilirubinemia ($n=2$), and hypertension ($n=2$). No patients developed grade IV toxicity. As shown in Table 1, there was no significant difference in frequency and severity of cyclosporine toxicities between patients at 50 years and above and those under 50 years. There was no significant difference in peak blood levels of cyclosporine within 28 days of transplant between patients with grade III or higher toxicities (median 369 ng/ml; range, 296–483) and those without them (median 361 ng/ml; range, 222–697) ($P=0.47$).

Acute GVHD

The cumulative incidence of grade I–IV acute GVHD was 43% (Figure 2). Median onset of GVHD was day 29 (range, 12–72). Median blood level of cyclosporine was 183 ng/ml (range, 25–340) at the onset of acute GVHD. Within 28 days of transplant, blood levels of cyclosporine below 150 ng/ml were documented in seven of 15 patients who developed acute GVHD within 35 days of transplant and seven of 20 patients who had not developed it ($P=0.51$). Any variables including blood levels of cyclosporine was not a significant risk factor for GVHD, when blood cyclosporine levels were treated as a time-dependent covariate.

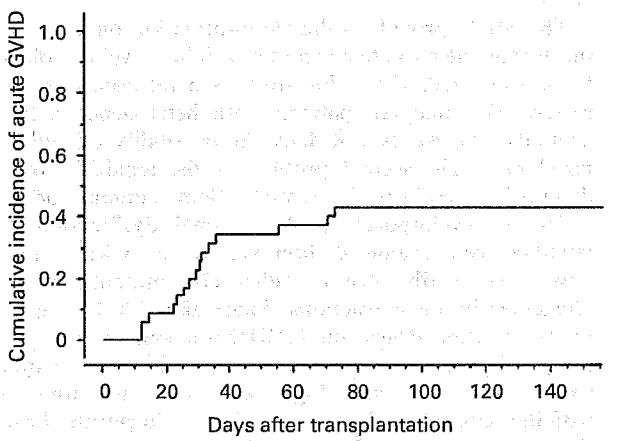

Figure 2 Cumulative incidence of grade I–IV acute GVHD at day 100 was 43%.

Discussion

This study urged us to reconsider the starting dose of cyclosporine in allo-HSCT recipients. At our institutions, cyclosporine is started at the two-divided dose of 6 mg/kg/day orally, and its trough levels are maintained between 150 and 250 ng/ml by FPI. This is a common practice in cyclosporine use in allo-HSCT in Japan. It is to be noted that the dose of cyclosporine was decreased in 33 of the 35 patients, and that the median dose of cyclosporine was settled at 3.1 mg/kg/day. These findings indicate that the starting dose of 6 mg/kg/day of oral cyclosporine is too high irrespective of age. It is reasonable to assume that an overdose of cyclosporine might have aggravated regimen-related toxicities following RIST. Most allo-HSCT recipients are given azole antifungals, which inhibit CYP enzymes, elevating blood cyclosporine concentration.

When we discussed the optimal starting dose of cyclosporine, we must consider clearance of cyclosporine, which decreases over time during the first 2 weeks after transplantation.[11,12] When the constant cyclosporine clearance during days 22–60 is 1.00, the clearance during days 0–7, 8–14, 15–21 are 1.46, 1.32, and 1.20, respectively.[11] The change of clearance in the natural course of transplant recipients may be related to our observation that cyclosporine levels were elevated in many patients, requiring dose reduction. However, the present study showed that cyclosporine levels exceeded the target levels within a week in some patients, necessitating dose reduction (Figure 1). The median day of cyclosporine dose reduction was day 14, when the decrease in its clearance was only 9.6%.[11] The change of clearance cannot fully explain the dose reduction requirement. When the target range of cyclosporine levels is determined at 150–250 ng/ml as in the Japanese standard, the starting dose of 6 mg/kg is probably unnecessarily high. The metabolism of CSP is still unclear and the target CSP levels vary among institutions.[8] Optimal target levels also remains unclear without convincing evidence. Further investigations to define target levels and the optimal starting dose are awaited.

While some conflicting reports have been published on cyclosporine toxicities in elderly patients,[3,13] the incidence and severity of cyclosporine toxicities were similar between

patients aged below 50 years and those at 50 years and above in this study. Previous studies demonstrate a reduction of *in vitro* and *in vivo* drug metabolism with age in humans,[14] and this tendency is prominent in patients above 70 years.[14] Lack of association between cyclosporine toxicity and patients' ages in this study might have been attributable to the fact that all of the patients enrolled in this study were younger than 70 years. Considering that most patients who receive RIST are younger than 70 years, the aging process itself might not have any apparent effect on the pharmacokinetics of cyclosporine or its variability in RIST.

This study provides valuable information on cyclosporine use for elderly patients; however, it has several problems to be discussed. First, this study is a retrospective chart review. We analyzed patients with heterogeneous backgrounds, and we cannot deny the possibility of unrecognized bias. The second problem is the reliability of the diagnosis of cyclosporine toxicity. Some common adverse events of cyclosporine such as renal dysfunction and jaundice were examined; however, these toxicities might have been attributable to either concomitant drugs or allogeneic immune reactions. Since allo-HSCT recipients receive multiple drugs, and GVHD is a common complication, it is theoretically impossible to differentiate cyclosporine toxicity from allogeneic immune reactions and toxicities caused by drugs other than cyclosporine. Last is the risk of acute GVHD, which rises as cyclosporine concentration falls.[15] Considering the low incidence of acute GVHD in Japanese allo-SCT recipients,[9] the low levels of cyclosporine targeted might have increased the incidence of acute GVHD in the present study. Further large-sized prospective evaluation is warranted to accurately evaluate cyclosporine toxicity and risk of GVHD in elderly patients.

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Identification of Serum Proteins Related to Adverse Effects Induced by Docetaxel Infusion from Protein Expression Profiles of Serum Using SELDI ProteinChip System

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Abstract. *Background: For the development of quick and easy methods for screening and identifying treatment-responsive proteins, we determined the protein expression profile of the serum after docetaxel infusion using a surface-enhanced laser desorption/ionization time-of-flight mass spectroscopy (SELDI TOF-MS) system. Materials and Methods: Blood from breast cancer patients was collected before and 4, 8, 24 and 48 hours after docetaxel infusion. The protein expression profile was determined by a SELDI TOF-MS system. The relative expression levels of target proteins were compared during the time-course after docetaxel injection. Results: We identified two representative proteins with molecular weights of 7790 Da and 9285 Da. The 7790 Da protein was high molecular weight kininogen, and the 9285 Da protein was apolipoprotein A-II. These two proteins had similar expression patterns in 5 patients, except one patient who experienced severe, acute, adverse effects. Conclusion: These results suggest that protein expression profiles determined by SELDI TOF-MS represent useful data for the identification of treatment-responsive proteins.*

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Key Words: SELDI TOF-MS, protein expression profile, adverse effect, ProteinChip System, HMW kininogen, apolipoprotein A-II.

Docetaxel is a key drug used to treat breast cancer. As docetaxel is hydrophobic, polysorbate 80 and ethanol are used as solvents. Some patients experience acute adverse effects, including anaphylactoid reactions, during docetaxel infusion and may develop shock. Several reports suggest that these adverse effects are caused by the solvent, which contains polysorbate 80 (1, 2). However, the mechanisms of these adverse effects are unknown. One approach to determine the mechanisms causing adverse effects is to analyze the treatment-responsive proteins.

Pharmacokinetics and pharmacodynamics are important areas of research in clinical pharmacology that analyze drug metabolism and host responses in order to predict the response and adverse effects of treatment.

Recently, DNA chip technology, such as DNA microarrays and cDNA arrays, has been used to predict responses to treatment. Some promising results have been reported in studies of irinotecan and other drugs (3-5), and there is no doubt that this genetic approach could be used to predict the potential response of a patient to treatment. However, genetic information alone cannot perfectly predict responses to treatment, because genes do not act by themselves. Genes exert their effect through proteins after transcription and translation. Thus, protein analysis is important to predict responses to treatment.

Until recently, no ideal tools have been available to determine protein expression profiles. Although two-dimensional electrophoresis could be used for this purpose, it is labor-intensive and is not capable of high-throughput

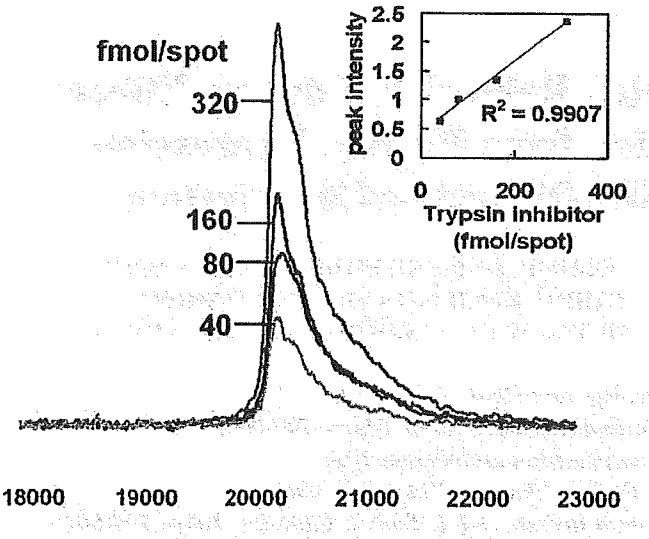

Figure 1. *Quantitative analysis. Various concentrations of trypsin inhibitor were studied with the SAX-2 (strong anion exchange) chip. Peak heights of trypsin inhibitor (molecular weight, approximately 20,000 Da) were clearly dose-dependent. The correlation coefficient calculated by Excel 2000 software was 0.99.*

analysis. Recent developments in mass spectrometry have been revolutionary in proteomic research. These new techniques are highly sensitive and have potential for various applications. Surface-enhanced laser desorption/ionization time-of-flight mass spectroscopy (SELDI TOF-MS) can be used to produce protein expression profiles. This system comprises ProteinChip arrays and time-of-flight mass spectrometry (TOF-MS). ProteinChip arrays possess varying chromatographic properties, such as anion exchange, cation exchange, metal affinity and reverse phase. The combination of the type of chip array and the washing conditions, such as the pH or salt concentration in the buffer, allows rapid analysis of protein profiles with only a small amount of sample. In the present study, we used the SELDI TOF-MS system to find docetaxel treatment-responsive proteins.

To select treatment-responsive proteins, it was necessary to compare the protein expression levels at several time-points after the treatment. However, no previous studies have indicated that mass spectrometry can be used for quantitative analysis of proteins. In the present study, we conducted a quantitative analysis of the targeted proteins. After selecting two representative treatment-responsive proteins, we used SELDI TOF-MS to determine the conditions for column purification of the proteins. We then determined the amino acid sequence of the purified proteins. The relationship between these proteins and docetaxel-induced shock is discussed, as well as the usefulness of the present approach in clinical pharmacology and clinical proteomics.

Table I.

Research ID	Gender	Protocol	Acute response
A	F	tri-week (60mg/m^2)	No
B	F	tri-week (60mg/m^2)	No
C	F	tri-week (60mg/m^2)	No
D	F	tri-week (60mg/m^2)	Shock
E	F	weekly (40mg/m^2)	No

Materials and Methods

Quantitative analysis. Various concentrations of trypsin inhibitor (Sigma-Aldrich, St. Louis, MO, USA) were studied using the SAX-2 chip (Ciphergen Biosystems, Fremont, CA, USA), and the heights of peaks were compared for the quantitative analysis.

Patients and blood samples. Ten breast cancer patients receiving docetaxel treatment were enrolled in this experiment, after providing informed consent in accordance with the guidelines of the National Shikoku Cancer Center Institutional Review Board, Japan. The results from 6 representative patients are described in the present report. Patients receiving docetaxel injections (60 mg/m^2) every 3 weeks and patients receiving weekly docetaxel injections (40 mg/m^2) were enrolled in this study. Dexamethasone (8 mg) was used as a premedication 30 min prior to docetaxel infusion. Docetaxel was administered as a 30-min infusion for patients on the weekly schedule and as a 60-min infusion for patients on the 3-week schedule. After the docetaxel infusions, oral dexamethasone (4 mg) was taken twice daily for one day for patients on the weekly schedule and for 2 days for patients on the 3-week schedule.

Blood (5 ml) was collected before docetaxel infusion and 4, 8, 24 and 48 h after docetaxel infusion. Serum was prepared quickly and stored at -80°C until analysis.

Medical information, such as adverse effects after docetaxel injection, was obtained from the medical records and entered into a database in accordance with the privacy policy of our institution.

Protein expression profiles. Protein expression profiles were determined using a SELDI TOF-MS system (Ciphergen Biosystems). IMAC3 (immobilized metal affinity capture), WCX-2 (weak cation exchange) and SAX-2 (strong anion exchange) ProteinChip arrays were used for analysis. The serum samples were centrifuged at 12,000 rpm using a microcentrifuge (TOMY Tech USA, Fremont, CA, USA). The supernatant was vigorously mixed with urea buffer (8 M urea:1% CHAPS/PBS, 1:1) for 10 min at 4°C and was diluted with $^{5x}_{1x}$ volumes of binding buffer (PBS for IMAC3, 50 mM phosphate buffer (pH 6) for WCX-2 and 50 mM Tris-HCl (pH 8) for SAX-2).

To immobilize copper onto the IMAC3 surface, 5 μl of 50 mM copper sulfate was loaded, and the chip was shaken for 5 min. Excess copper was removed under running deionized water, and the chip was shaken for 5 min with 10 μl of 50 mM sodium acetate (pH 4). The chip was rinsed under running deionized water and was then ready to be used for the analysis step.

The following procedure was used for chip analysis: (i) each spot was equilibrated with 150 μl of binding buffer twice on a shaker for 5 min, and excess buffer was removed; (ii) diluted samples (50 μl per

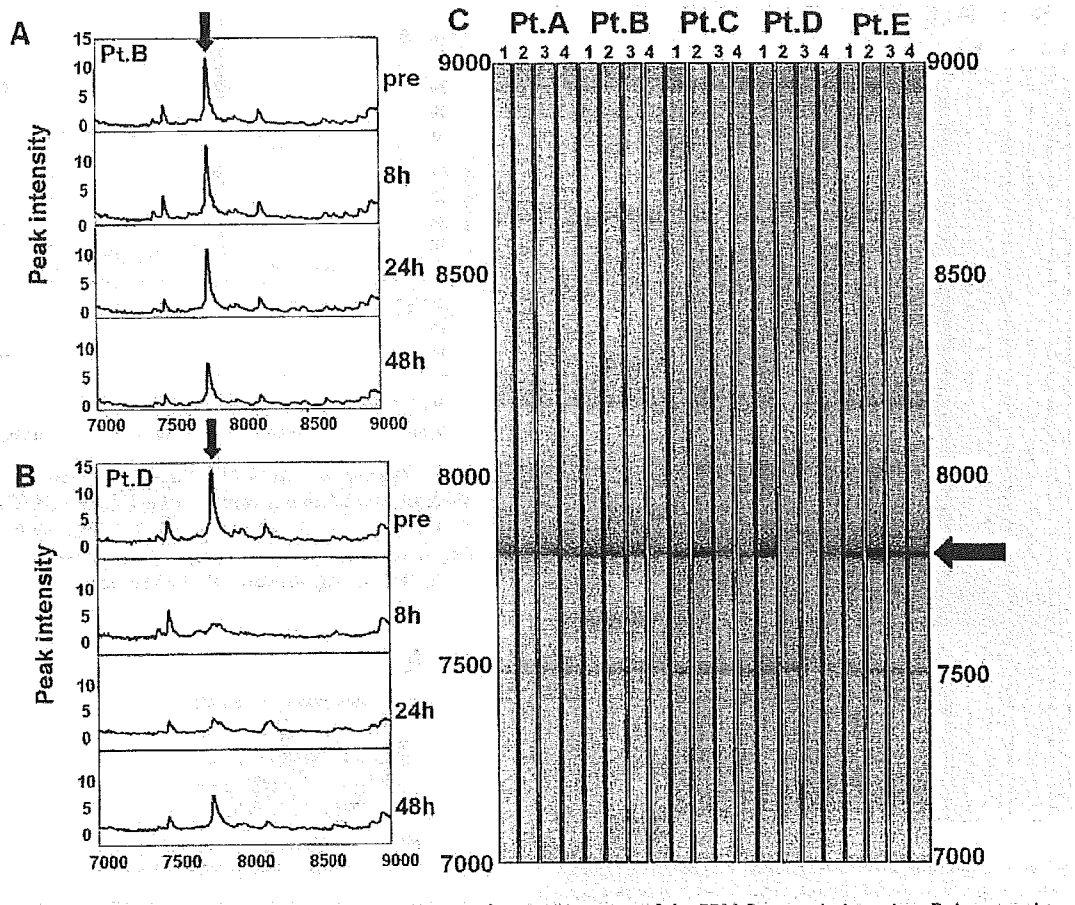

Figure 2. *Expression of 7790 Da protein at different times. (A) Typical response pattern of the 7790 Da protein in patient B, in trace view. (B) Specific response pattern in patient D, in trace view. (C) Gel view of 5 samples (Pt. A-Pt. E). Lane 1, pre-injection; lane 2, 8 h after injection; lane 3, 24 h after injection; and lane 4, 48 h after injection. In patient D, the protein suddenly disappeared and slowly reappeared over a period of days.*

spot) were loaded, and the chip was incubated on a shaker for 20 min at room temperature; (iii) the chip was washed 3 times with 150 μl per spot of binding buffer; (iv) the chip was rinsed with distilled water and dried; and (v) each spot was treated with 0.5 μl of saturated sinapinic acid prepared in aqueous solution containing 50% acetonitrile and 0.5% trifluoroacetic acid. Captured proteins were directly detected using a PBS II ProteinChip Reader (Ciphergen Biosystems).

Screening of docetaxel-responsive proteins. Quantitative analysis of proteins was performed using Peaks 3.0 software (Ciphergen Biosystems). Expression levels of the proteins were compared over the time-course of the experiment. Two representative proteins, that had similar expression patterns in patients, were selected.

Protein purification and amino acid sequencing. To determine the optimal pH and salt concentration of the buffer for purification of the target proteins, IMAC3, WCX-2 and SAX-2 assays were performed. The target proteins were fractionated by CM Sepharose Fast Flow (Amersham Biosciences, Piscataway, NJ, USA) and separated by 16% SDS polyacrylamide gel electrophoresis using the method reported by Schagger and Jagow (6). Electroblotting to PVDF membrane was performed using a TEFCO electroblotting system (TEFCO. Co., Ltd., Tokyo, Japan). Amino acid sequences of the purified proteins were analyzed according to the Edman method using a Procise 494 HT Protein Sequencing System (Applied Biosystems, Foster City, CA, USA). Database searches of the sequence data were performed using SWISS-PROT.

Results

Quantitative analysis. To confirm that the SELDI TOF-MS system can be used for quantitative analysis, various concentrations of trypsin inhibitor were analyzed using the SAX-2 chip. The peak heights of trypsin inhibitor (molecular weight, approximately 20,000 Da) occurred in a dose-dependent manner (Figure 1) with a correlation coefficient of 0.99 calculated by Excel 2000 software. The SELDI TOF-MS system can therefore be used to quantitatively analyze protein profiles.

Patient characteristics and acute reaction related to docetaxel injection. Ten patients were enrolled in the study, and the data from 6 representative patients are presented in this report. The background of the patients, injection schedule and acute reactions recorded in the medical records are

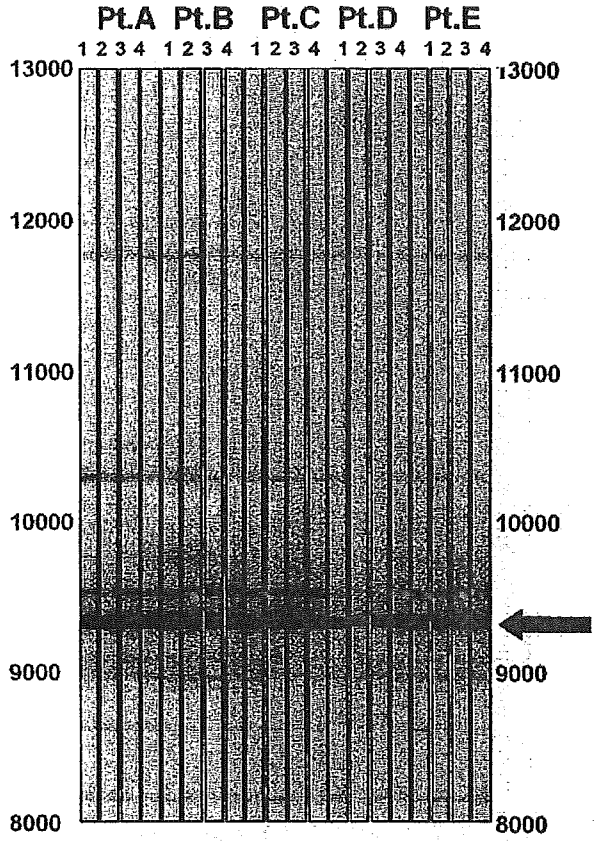

Figure 3. *Gel view of 9285 Da protein in 5 patients. In patient D, expression of the 9285 Da protein decreased transiently. The expression of this protein was unchanged in all other patients. Lane 1, pre-injection; lane 2, 8 h after injection; lane 3, 24 h after injection; and lane 4, 48 h after injection.*

summarized in Table I. Five of the 6 patients experienced no severe adverse effects, although slight flushing of the face was observed in two patients. Only one patient (patient D) experienced severe, acute, adverse effects and experienced signs and symptoms of shock. Severe flushing of the face, tachypnea with dyspnea, tachycardia and low blood pressure were observed in patient D. The docetaxel injection was stopped immediately and additional dexamethasone (8 mg) was injected. The patient recovered from shock, and docetaxel dissolved in simple saline (without polysorbate 80) was slowly infused. The infusion was completed without any additional adverse effects.

Protein profiles and docetaxel-responsive proteins. We compared serial profiles from the same patients according to the time-course of the infusion and selected two peaks of interest. The peaks occurred at 7790 Da and 9285 Da. Figure 2A shows data from patient B, and Figure 2B shows data from patient D. Figure 2C shows data from 5 patients and indicates that the expression of the 7790 Da protein disappeared suddenly only in patient D. Patient D, who

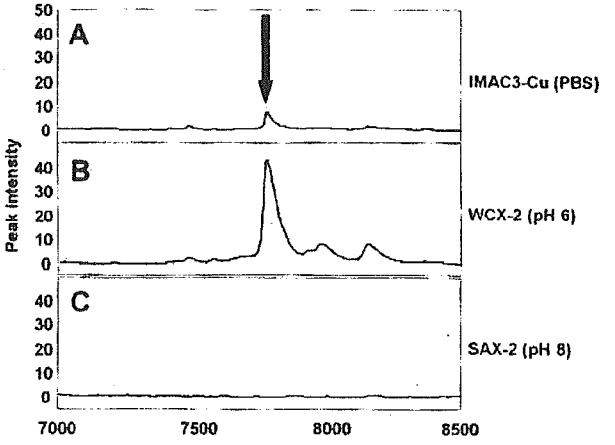

Figure 4. *Binding capacity of 7790 Da protein to three different chips. (A) Immobilized metal affinity capture using copper (IMAC3-Cu chip, PBS buffer), (B) weak cation exchange (WCX-2 chip, pH 6 buffer) and (C) strong anion exchange (SAX-2 chip, pH 8 buffer). The signal obtained from the WCX-2 chip displayed the highest intensity.*

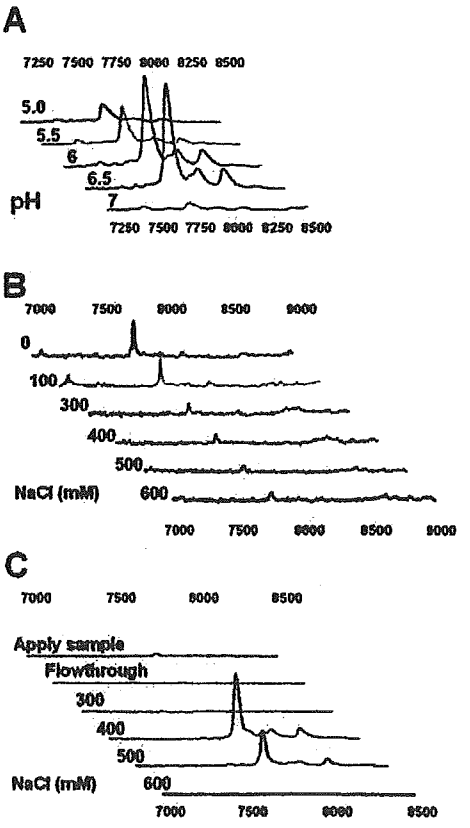

Figure 5. *Capture and elution conditions for 7790 Da protein. (A) Capture pH for the 7790 Da protein using the WCX-2 chip. (B) NaCl concentration for elution of the 7790 Da protein from the WCX-2 chip. (C) NaCl concentration for purification of the 7790 Da protein by CM sepharose affinity chromatography. These results suggest that the best conditions were pH 6 for capture and 300-400 mM NaCl for elution from the WCX-2 chip. In CM sepharose columns, buffer at pH 6 was used as a capture solution, and the optimal concentration of NaCl for elution was 400 mM.*

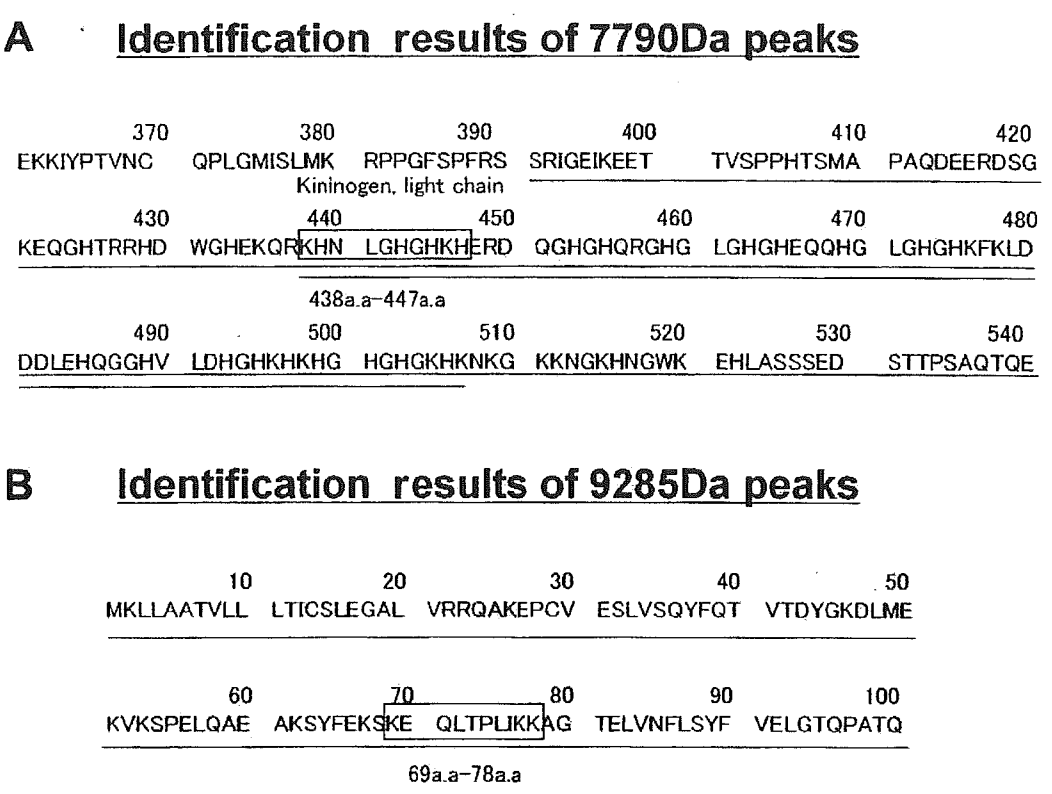

Figure 6. *Amino acid sequences of target proteins. (A) Ten amino acids from the NH3 terminal of the 7790 Da protein were analyzed according to the Edman method. The sequence was identical to amino acids 438-447 of high molecular weight kininogen. (B) Ten amino acids from a fragment of the 9285 Da protein were analyzed after in-gel digestion. The sequence was identical to amino acids 69-78 of apolipoprotein A-II.*

experienced severe adverse effects during docetaxel injection, displayed a rapid down-regulation of the 7790 Da protein, but the expression of this protein recovered over several days. Figure 3 shows gel view data of the 9285 Da protein from the same samples. The expression pattern of this protein was almost the same as the 7790 Da protein.

Purification of target proteins. To establish a procedure to purify the target proteins, we determined which chip is preferable to capture the target proteins, and then determined the optimal pH and salt concentration in the buffer using protein arrays. The results revealed that both proteins bound to the WCX-2 chip more strongly than to the other two chips (data from 7790 Da protein shown in Figure 4).

The capture pH and the NaCl concentration for elution of the 7790 Da protein were investigated using the WCX-2 chip (Figure 5). The results indicated that the best conditions were pH 6 for capture (Figure 5A) and 300-400 mM NaCl for elution (Figure 5B). We used these conditions in large-scale purification using CM sepharose column chromatography. We diluted 11.4 ml of serum to 100 ml with sodium phosphate and citrate buffer (pH 6) and applied it to a CM sepharose column (25 cm). After equilibrating with the same buffer, elution was performed using 300-600 mM NaCl stepwise. The target protein was eluted under the conditions indicated by the protein chip analysis (Figure 5C). After the 300-400 mM NaCl fraction had been dialyzed and concentrated, the sample was applied to SDS PAGE. The band containing the 7790 Da protein was removed from the gel. The purification procedure for the 9285 Da protein was determined by almost the same process, and the protein was purified (data not shown).

Amino acid sequencing of the target proteins. To determine the amino acid sequence of the target proteins, the proteins were transferred from a gel to PVDF membranes. The amino acid sequence of the purified protein was determined according to the Edman method. A sequence of 10 amino acids from the NH3 end of the 7790 Da protein was directly analyzed and determined to be identical to amino acids 438-447 of high molecular weight (HMW) kininogen (Figure 6A). This protein was also analyzed by simple MS and MSMS system, and was identified as kininogen (Figure 7). The amino terminal end of the 9285 Da protein was blocked and could not be analyzed directly. Analysis of the amino acid sequence was therefore performed after tryptic digestion, and a 10 amino acid internal sequence from the

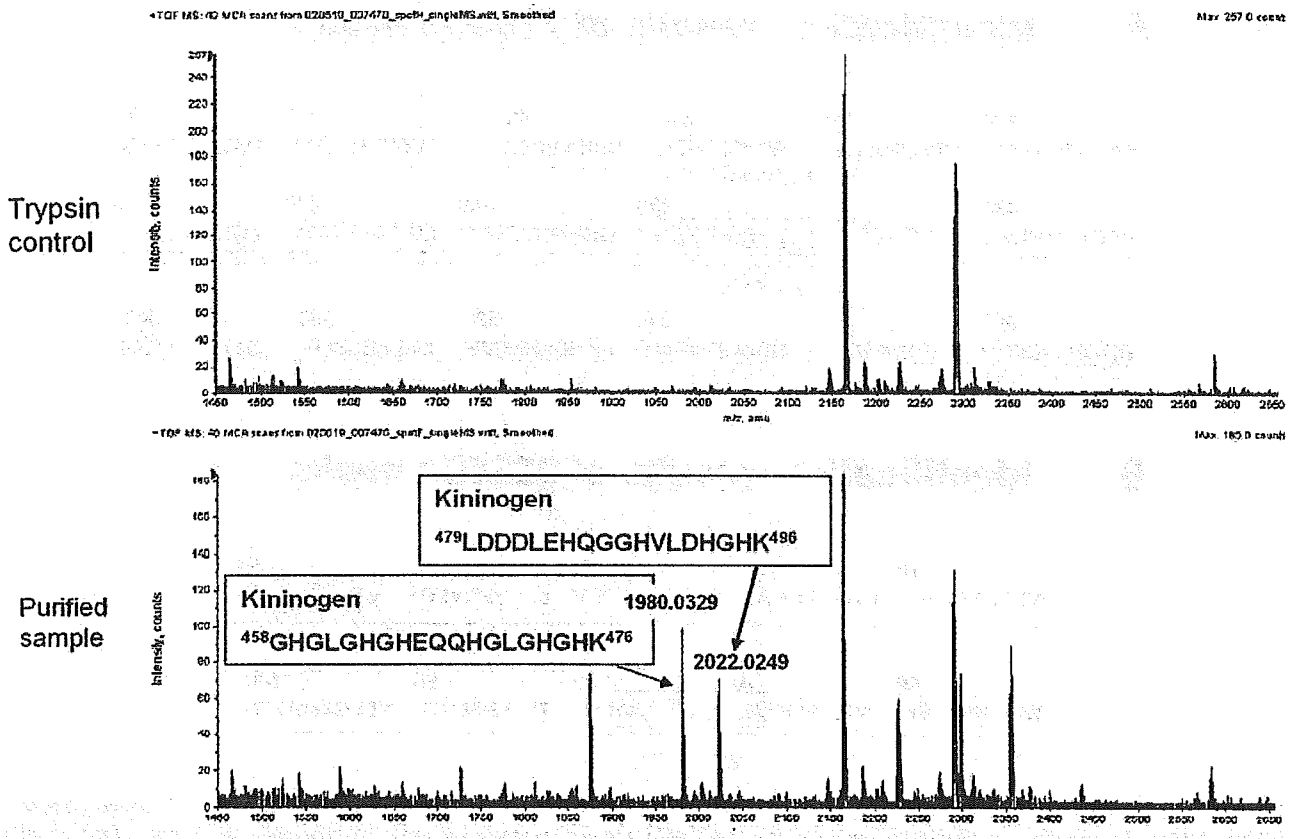

Figure 7. *Single MS and MSMS analysis of tryptic in-gel digestion sample. A piece of the gel containing 7790 Da proteins was treated with trypsin and the digested peptides were analyzed by MS and MSMS systems. Typical signals, which were considered to be fragments of kininogen, were detected.*

9285 Da protein was determined. The sequence was identical to amino acids 69-78 of apolipoprotein A-II (Figure 6B).

Discussion

Widespread expression analysis of DNA became available with the development of cDNA microarrays. Predictions of response and adverse effects were recently studied using DNA microarrays and cDNA expression arrays. However, it is clear that DNA and mRNA do not work by themselves, because mRNA must be translated into protein. This principle indicates the importance of protein analysis in post-genome projects. However, no ideal tool was previously available to determine protein expression profiles. Although two-dimensional electrophoresis could be used in this respect, the process is labor intensive and incapable of high-throughput analysis. In addition, two-dimensional electrophoresis requires skillful techniques to obtain reproducible data.

Mass spectrometry techniques have recently been used in the field of protein analysis. Liquid chromatography combined with TOF-MS offers very high sensitivity. However, this system cannot produce protein expression profiles. SELDI TOF-MS, which was recently developed, can determine protein expression profiles from crude samples, such as serum, urine and other body fluids. SELDI TOF-MS is constructed with TOF-MS and chip arrays, which have chromatographic properties on their surface. SELDI TOF-MS can be used to quickly develop protein profiles using only a small amount of sample. Several groups have found biological markers such as tumor markers using this system (7-13).

In the present study, we developed protein expression profiles from serum collected before and after docetaxel injection. We compared the protein expression profiles over the time-course of the infusion to find docetaxel-responsive proteins. We determined the amino acid sequences of the selected proteins. One of the docetaxel-responsive proteins was HMW kininogen, which is a factor in the kinin/kallikrein cascade of blood coagulation. This protein suddenly decreased in patient D, who experienced severe shock during docetaxel injection. No other patients displayed sudden decreases in this protein. Cochrane *et al.* reported a relationship between HMW kininogen and hypotensive shock (14), and Gallimore reported changes in HMW kininogen during lethal endotoxin shock (15). These reports suggest that HMW kininogen is a shock-related protein, although no previous reports have suggested that HMW kininogen is related to drug-induced

shock. The other docetaxel-responsive protein that we detected was apolipoprotein A-II, which is related to lipid and cholesterol metabolism. To the best of our knowledge, no previous reports have described an association between apolipoprotein A-II and shock.

The expression levels of HMW kininogen and apolipoprotein A-II in all patients (except for patient D) were unchanged or were slightly decreased and recovered quickly without treatment. No severe adverse effects were observed in these patients, although some experienced slight flushing of the face. These findings suggest that the homeostatic mechanisms of the body worked quickly, allowing these patients to recover from the effects of docetaxel infusion.

The roles of the proteins detected in our study in docetaxel-induced shock are unknown. Even if the changes in these proteins are the result of shock, rather than the cause of shock, this information may help elucidate the mechanisms of docetaxel-induced shock and may lead to measures for prediction of and protection from shock. Our ultimate goals are to find predictive markers for adverse effects in order to prevent/treat adverse effects, and to find molecular targets of the response. We have started to screen other proteins that respond to docetaxel treatment in order to identify proteins causing adverse effects.

Two noteworthy technical points from our experiments have not previously been reported. The first point is that we successfully used SELDI TOF-MS to compare protein expression levels in crude samples, which is an important step for screening responsive proteins. Previously, clear evidence that mass spectrometry can be used for quantitative analysis of protein from crude samples has been unavailable. Therefore, the SELDI ProteinChip System may be a key technology for protein expression analysis. The ProteinChip System can also perform high-throughput analysis. This technology is very useful for the discovery of biomarkers, particularly when a large number of samples need to be analyzed. The second noteworthy technical point is that we determined the procedure for protein purification in only one day using SELDI TOF-MS. The purification conditions determined by SELDI TOF-MS could be applied to large-scale column chromatography for both proteins. These results indicate that high-speed purification is possible using SELDI TOF-MS.

In conclusion, we found that the SELDI TOF-MS ProteinChip System can be used to compare protein expression levels in protein profiles and to quickly purify target proteins. These two points may lead to breakthroughs in clinical proteomics and clinical pharmacology.

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Allografting

Reduced-intensity hematopoietic stem-cell transplantation for malignant lymphoma: a retrospective survey of 112 adult patients in Japan

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Summary:

We conducted a nation-wide survey of 112 adult Japanese patients who underwent reduced-intensity stem cell transplantation (RIST) from 1999 to 2002. Underlying diseases included indolent ($n=45$), aggressive ($n=58$) and highly aggressive lymphomas ($n=9$). Median age of the patients was 49 years. A total of 40 patients (36%) had relapsed diseases after autologous stem cell transplantation and 36 patients (32%) had received radiotherapy. RIST regimens were fludarabine-based ($n=95$), low-dose total body irradiation-based ($n=6$) and others ($n=11$). Cumulative incidences of grade II–IV acute graft-versus-host disease (GVHD) and chronic GVHD were, respectively, 49 and 59%. Cumulative incidences of progression and progression-free mortality were 18 and 25%, respectively. With a median follow-up of 23.9 months, 3-year overall survival rates were 59%. A multivariate analysis identified three significant factors for progression, which are history of radiation (relative risk (RR) 3.45, confidential interval (CI) 1.12–10.0, $P=0.03$), central nervous system involvement (RR 6.25, CI 2.08–20.0, $P=0.001$) and development of GVHD (RR 0.28, CI 0.090–0.86, $P=0.026$). RIST may have decreased the rate of transplant-related mortality, and GVHD may have induced a graft-versus-lymphoma effect. However, whether or not these potential benefits can be directly translated into improved patient survival should be evaluated in further studies.
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Allogeneic stem cell transplantation (allo-SCT) is a curative treatment for advanced malignant lymphoma.[1,2] Initially, the benefit of allo-SCT was thought to be largely dependent on the intensity of the conditioning regimen prior to transplantation. Recently, an additional benefit of allo-SCT is derived from an allogeneic graft-versus-malignancy (GVM) effect that reduces the likelihood of disease relapse following transplantation.[3,6] With high regimen-related toxicity (RRT) and treatment-related mortality (TRM), high-intensity, myeloablative conditioning regimens are being replaced by reduced-intensity or nonmyeloablative conditioning regimens. The preliminary data suggest improved survival rates due to decreased TRM.[7] Reduced-intensity stem cell transplantation (RIST) is potentially a curative treatment for heavily pretreated, elderly patients; however, little information is available regarding the outcomes of RIST for malignant lymphoma. We retrospectively analyzed the outcome of RIST. The purpose of this study was to elucidate the treatment-related toxicity of RIST and to evaluate the impact of a potential graft-versus-lymphoma (GVL) effect.

Patients and methods

Data collection

We conducted a nation-wide retrospective survey of 112 adult Japanese patients who underwent RIST from 1999 to 2002 in 32 participating hospitals. All of the RIST recipients who were eligible in this study were included in each hospital. In Japan, approximately 2000 transplants are performed annually. The types of transplantation are autologous (40%), myeloablative allogeneic (45%), and

reduced-intensity or nonmyeloablative allogeneic transplantation (15%).[8] Since 20% of RIST recipients had advanced malignant lymphoma,[8] approximately half of the patients with malignant lymphoma who underwent RIST in Japan were surveyed in this study.

Data were derived from questionnaires distributed to each participating center. Minimum data required for the inclusion of a patient in this study were age, histological diagnosis, prior treatment details, status at transplant, conditioning regimens, date of transplant, date of last follow-up, disease status at last follow-up, date of disease progression/death and causes of death. Information on rituximab use prior to RIST was not collected in this study.

Definition

Reduced-intensity regimens were defined as reported previously.[9,10] The upper limits of busulfan, melphalan, and TBI were 8 mg/kg, 140 mg/m^2, and 2 Gy, respectively, for consideration as reduced-intensity preparative regimens. Engraftment was defined as white blood cell counts $>1.0 \times 10^9$/l or absolute neutrophil counts $>0.5 \times 10^9$/l for two consecutive days. Graft-versus-host- disease (GVHD) was clinically diagnosed in combination with skin or gut biopsies. Acute and chronic GVHD were graded according to the established criteria.[11,12]

Histological diagnosis was based on institutional diagnosis. Discrepancies in nomenclature among centers were resolved according to the synonyms in the WHO classification.[13] Indolent, aggressive, and highly aggressive lymphomas were classified according to the report by Chan[14] with some modifications. Transformed low-grade lymphoma was classified into aggressive lymphoma. However, patients who had recurrent low-grade lymphoma rarely receive biopsy before transplant, and patients with transformed low-grade lymphoma might have been analyzed as low-grade lymphoma in this study. Adult T-cell leukemia/lymphoma was classified into a highly aggressive category, because its clinical course is aggressive and patients' median survival is as short as about 6 months. Chimerism was determined by short-tandem repeat PCR method or sex chromosome FISH, and disease status was evaluated with CT, MRI scan, bone marrow aspiration, or spinal tap in varying intervals from 1 month to 60 months according to each participating hospital's rule. Those with chemosensitive diseases included all patients who had shown a response to the last therapy prior to transplantation (partial remission (PR), complete remission (CR) unconfirmed, and CR); all the other patients were classified as having chemoresistant diseases. Progression-free survival (PFS) was measured as the time from the day of transplantation until disease relapse/progression or death from any causes. Both relapse and progression were defined as disease progression with transplantation-related deaths being censored. TRM is defined as all causes of deaths without disease progression at any time after transplant. RRT was defined as all nonhematological organ dysfunctions from day 0 to day 28, and were graded according to the Seattle criteria.[15]

Statistical analysis

The primary end point was 3-year PFS. Secondary end points included 3-year overall survival (OS), TRM, and disease progression rates. The cumulative incidences of progression and progression-free mortality were evaluated using the Gray's method,[16] considering each other's risk as a competing risk. OS and PFS were estimated using the Kaplan–Meier method. Potential confounding factors considered in the analysis were age, sex, donor types (an HLA-matched related donor and an alternative donor), stem cell sources (marrow, peripheral blood, and cord blood), performance status according to the Eastern Cooperative Oncology Group (ECOG) criteria,[17] serum levels of lactate dehydrogenase, intervals from diagnosis to transplantation, the number of prior chemotherapy regimens, history of autologous SCT, history of radiation, clinical stages, chemosensitivity, presence of extramedullary involvement (central nervous system, and marrow), presence of bulky mass, disease category (indolent, aggressive, highly aggressive), different conditioning regimens, and use of methotrexate as GVHD prophylaxis. Proportional hazard modeling was used to evaluate the influence of these factors on PFS and disease progression. The influence of the development of GVHD on PFS and disease progression was evaluated using the proportional hazard modeling treating the development of acute GVHD as a time-dependent covariate. Factors associated with at least borderline significance ($P<0.10$) in a univariate analysis were subjected to a multivariate analysis using backward stepwise proportional-hazard modeling. *P*-values of less than 0.05 were considered statistically significant.

Results

Patient characteristics and transplantation procedures

Patients' characteristics and transplantation procedures are shown in Table 1. None received *ex vivo* T-cell depleted transplantation.

Regimen-related toxicity

Information on RRT within 28 days of RIST was available in 106 patients and was graded according to Bearman's criteria (Table 2).

Engraftment

Four patients died before engraftment. None developed primary graft failure. Of the 108 patients who achieved primary engraftment, 91 patients were evaluable for chimerism. In all, 85 patients (93%) achieved complete donor-type chimerism within 100 days of transplant. Three subsequently achieved complete donor-type chimerism, one died of infection with mixed chimerism 164 days after transplant, and two remained alive with mixed chimerism (623 and 606 days after transplant). None received donor lymphocyte infusion (DLI) for engraftment.

Table 1 Patient characteristics and transplantation procedures

	Indolent lymphoma[a]	*Highly-aggressive*[b], *Aggressive lymphoma*[c]
Sex		
Male/female	21/24	41/26
Age		
Median (range)	48 (61–32)	50 (72–22)
Interval from diagnosis to transplantation (years)		
Median (range)	3.7 (0.1–15.1)	1.6 (0.3–12.1)
Numbers of prior chemotherapy regimens		
Median (range)	4 (1–15)	4 (1–14)
Prior local radiation therapy		
Yes/no	11/34	25/42
Previous history of HDT/ASCT		
Yes/no	10/35	30/37
Disease status at transplant		
CR/Non-CR/ND	1/40/4	6/56/5
I–II/III–IV/ND	9/31/5	12/44/11
Patients with bone marrow invasion	15	15
Patients with CNS invasion	2	9
Patients with bulky mass	6	4
Performance status at transplant		
0–1/2–4	40/3	50/14
Increased serum LDH level at transplant[d]		
Yes/no	19/26	34/33
Chemosensitivity at transplant		
Sensitive/ resistant	31/14	38/29
Conditioning regimens		
Fludarabine and busulfan	16	25
Fludarabine and cyclophosphamide	12	16
Fludarabine and melphalan	9	12
Fludarabine and 200 cGy total body irradiation	2	3
200 cGy total body irradiation	1	5
Other	5	6
GVHD prophylaxis		
Cyclosporin and methotrexate	16	25
Cyclosporin and mycofenolate mofetil	2	7
Cyclosporin alone	21	28
Tacrolimus and methotrexate	5	6
Tacrolimus alone	1	1
Use of anti-thymocyte globulin as preparative regimens		
Yes/no	9/36	9/58
Stem-cell sources		
Blood from an HLA-matched related donor	29	49
Blood from an HLA-mismatched related donor	3	5
Marrow from an HLA-matched related donor	1	5
Marrow from an HLA-matched unrelated donor	7	7
Mismatched cord blood	0	6

HDT/ASCT = high-dose therapy and autologous stem cell transplantation; CR = complete remission; ND = not described; LDH = lactate dehydrogenase; GVHD = graft-versus-host disease.
[a]Indolent lymphoma included follicular ($n=44$), marginal zone B-cell ($n=2$), small lymphocytic ($n=1$), lymphoplasmacytic ($n=1$), and cutaneous T-cell ($n=1$).
[b]Highly aggressive lymphoma included lymphoblastic ($n=3$), adult T-cell ($n=4$), and Burkitt ($n=2$).
[c]Aggressive lymphoma included diffuse large B-cell ($n=27$), peripheral T-cell, unspecified ($n=9$), mantle cell ($n=8$), NK-cell ($n=4$), anaplastic large cell ($n=4$), and angioimmunoblastic ($n=2$). Transformed low-grade lymphoma was treated as diffuse large B-cell lymphoma ($n=4$).
[d]Normal ranges of LDH were determined in each participating hospital.

Table 2 Regimen-related toxicity within 28 days according to the Bearman's criteria

Grade	*0*	*I*	*II*	*III*	*IV*
Mucosa	64	27	12	1	0
Central nervous system	99	0	1	4	0
Lung	93	3	4	4	1[a]
Kidney	84	13	3	4	0
Liver	74	15	14	1	1[b]
Bladder	100	4	0	0	0
Heart	95	3	5	1	0
Gut	74	20	6	4	0

[a]Idiopathic pneumonia syndrome.
[b]Hepatic veno-occlusive disease.

Graft-versus-host disease

Seven patients were not evaluated for acute GVHD, since four died before engraftment and three lacked the data regarding GVHD. In the remaining 105 patients, cumulative incidence of grade II–IV acute GVHD was 49% with a median onset of day 24 (range, 8–99). Of the 98 patients survived longer than 100 days after transplant, cumulative incidence of chronic GVHD was 59%.

Response to RIST

In all, 84 patients including 52 patients with chemosensitive diseases and 32 patients with chemoresistant diseases had measurable lesions prior to transplant, and were evaluated for response to RIST. A total of 72 patients (86%) responded to RIST (CR 63 and PR nine). As of February 2004, median duration of response was 22.5 months (range, 2.2–38.9). After initial response to RIST, primary disease recurred or progressed in four patients. Median interval between initial response and disease progression was 4.1 months (range, 1.4–11.2). Response to RIST was shown according to histological subtypes (Table 3). Five patients

Table 3 Response rates and outcomes of RIST according to histological subtypes

Chemosensitivity	*Indolent (n = 45)*		*Aggressive (n = 58)*		*Highly aggressive (n = 9)*	
	Sensitive	*Refractory*	*Sensitive*	*Refractory*[a]	*Sensitive*	*Refractory*
No. of patients	31	14	34	24	4	5
Response rate[b]	24/26 (92%)	11/11 (100%)	22/23 (97%)	11/17 (65%)	3/3 (100%)	1/4 (25%)
Progression after response	1	0	2	1	0	0
Progression-free survival at 3 years (%)	83	64	56	30	0	0
Total deaths	4	5	12	16	1	5
Causes of death						
Primary disease	1	0	3	6	0	3
GVHD	2	2	5	4	1	1
Infection	1	2	4	5	0	1
Other TRM	0	1	0	1	0	0

RIST = reduced intensity stem cell transplantation; GVHD = graft-versus-host disease; TRM = transplant-related mortality.
[a]Four patients with chemorefractory transformed low-grade lymphoma responded to RIST, and survived without disease progression with a median follow-up of 25.2 months (range, 16.1–32.4)
[b]Patients without measureable disease at transplant were excluded.

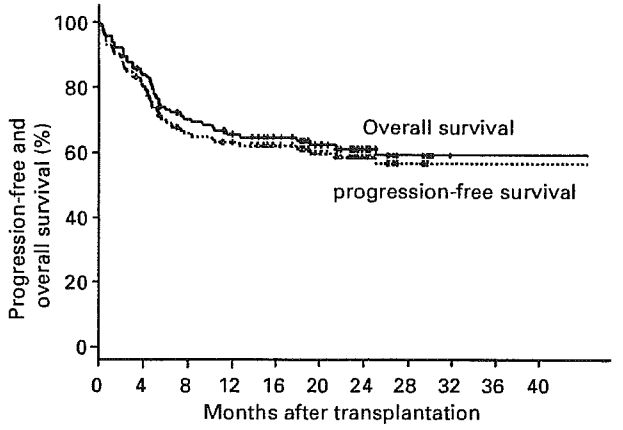

Figure 1 Overall survival (OS) and progression-free survival (PFS) following transplant. The 3-year OS and PFS were 59.0% (95% CI, 55.0–64.0%) and 56.5% (95% CI, 51.5–61.5%), respectively.

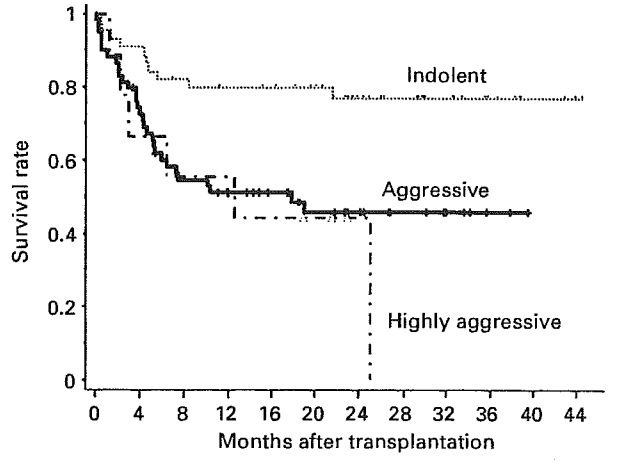

Figure 2 Overall survival (OS) following transplant according to the histological subtypes. The 3-year OS according to the histological subtypes was indolent 79% (95% CI, 67–91%), aggressive 48% (95% CI, 35–61%), and highly aggressive 0%; follicular 81% (95% CI, 69–92%), diffuse large B-cell 31% (95% CI, 13–49%), peripheral T-cell 56% (95% CI, 23–89%), and mantle cell 76% (95% CI, 45–100%).

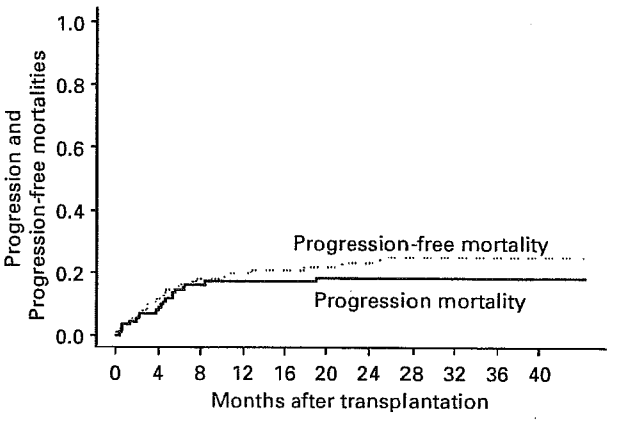

Figure 3 Cumulative incidences of disease progression mortality and transplant-related mortality (TRM). Cumulative incidences of disease progression mortality and TRM at 3 years were 18.3 and 25.2%, respectively.

received DLI for either disease progression or disease persistence following RIST. One showed objective disease response after DLI. The outcome in patients with CNS disease or whether they relapse in the CNS or outside the CNS was not collected.

OS, PFS and TRM

As of February 2004, 69 were alive with a median follow-up duration of 23.9 months (range, 3.4–44.5). The 3-year OS and PFS were 59.0% (95% CI, 55.0–64.0%) and 56.5% (95% CI, 51.5–61.5%), respectively (Figure 1). The 3-year OS according to the histological subtypes (Figure 2) was indolent 79% (95% CI, 67–91%), aggressive 48% (95% CI, 35–61%), and highly aggressive 0%; follicular 81% (95% CI, 69–92%), diffuse large B-cell 31% (95% CI, 13–49%), peripheral T-cell 56% (95% CI, 23–89%), and mantle cell 76% (95% CI, 45–100%). There was no difference in 3-year OS between T-cell and B-cell lymphomas ($P = 0.08$). The cumulative incidences of progression and progression-free mortality were 18.3 and 25.2%, respectively (Figure 3).

Since progression-free mortality was evaluated with relapse censored as a competing risk, it is apparently lower than an absolute incidence of 27%.

Primary causes of death were disease progression in 13, whereas 30 died without disease progression (Table 3) GVHD complicated with infection ($n=15$), infection ($n=13$), idiopathic pneumonia syndrome ($n=1$), and hepatic veno-occlusive disease ($n=1$). The causative organisms included Gram negative rods ($n=4$), Gram positive cocci ($n=4$), fungi ($n=3$), and unknown ($n=2$).

Prognostic factors for PFS

Results of univariate and multivariate analysis on relapse and PFS are shown in Tables 4 and 5, respectively. Three variables including history of any types of irradiation prior to RIST, CNS involvement at transplant, and absence of grade II–IV acute GVHD were adversely associated with disease progression (Table 4). Four variables including poor PS, short interval from diagnosis to transplant, nonmethotrexate-containing GVHD prophylaxis, aggressive-type histology were adversely associated with PFS (Table 5).

Discussion

Although the eligibility was decided according to different protocols at each participating hospital and the possibility of a selection bias cannot be excluded, this multicenter, retrospective analysis described the gross characteristics of RIST in Japan.

RRT has been a significant problem in allo-SCT for malignant lymphoma,[18 20] while only two patients (1.8%) died of RRT within 28 days of RIST. TRM was lower than those reported on conventional allo-SCT.[18 20] RIST might decrease RRT and provided better prognosis in short-term follow-up than conventional transplantation. The incidence of acute GVHD is lower in Japan than in Western countries because of the relative genetic homogeneity of the population;[21] however, 43 patients developed grade II to IV acute GVHD, which was fatal in 15 patients. The rate of acute GVHD was similar to those reported on myeloablative or reduced-intensity allo-SCT from Western countries.[19,20,22,23] The relatively high incidence of acute GVHD in the present study was probably associated with less intense GVHD prophylaxis in RIST than in conventional allo-SCT. The use of methotrexate beneficially affected PFS in our multivariate analysis. Additional methotrexate is probably beneficial especially in RIST because RIST recipients are elderly and with comorbidities, and GVHD is a higher risk of TRM.

A GVL effect is associated with GVHD in allo-SCT for hematologic malignancies.[3,24] While this trend is remarkable in acute leukemia,[3] it has been inconsistent in malignant lymphoma.[4,18,20,25] GVHD was associated with reduced disease progression; however, PFS was not improved in the present study. GVHD is sometimes fatal, and may offset patients' prognosis. Since the impact of GVHD on a GVL effect varies according to disease status and patients' conditions, management of GVHD should be tailored. Further studies are warranted to establish a proper GVHD prophylaxis.

Few reports are available on infections after RIST.[26 29] RIST seemed to be associated with less infections due to the shorter duration of neutropenia and less damage to mucosal barriers. However, we showed that opportunistic infection is the second leading cause of death in RIST. Most patients had received multiple courses of chemotherapy, and occult infections might have existed at RIST. These infections can be fatal in RIST recipients. Management of bacterial and fungal infections following RIST requires further investigation.

In the present study, PFS was significantly different according to histological subtypes (Figure 2), which is consistent with previous reports.[23,30] Indolent lymphoma has a low relapse rate, and the major causes of mortality are GVHD and infections (Table 3). Our study showed that chemotherapy-resistant indolent lymphoma can achieve good outcomes after RIST, and that the response to RIST is not associated with chemosensitivity before RIST (Table 3). These findings are comparable to previous reports.[19] RIST for indolent lymphoma needs to be reserved for those with advanced diseases, since RIST is associated with TRM. Intensification of GVHD prophylaxis and infection control may produce more promising results in RIST for indolent lymphoma.

In contrast, the outcomes of RIST for aggressive and highly aggressive lymphomas were poor.[23] Although allo-SCT has been considered ineffective for these lymphomas,[30] the present study showed that some can achieve remission after RIST (Table 3). However, the response rate of these lymphomas was not satisfactory in RIST for chemorefractory aggressive and highly aggressive lymphomas. Investigations are necessary to determine better timing and indications of RIST for these lymphomas. This study and others[31] revealed history of irradiation, central nervous system involvement and chemosensitivity at transplantation as significant prognostic factors (Table 4). These are useful to identify patients who would benefit from RIST. Another approach to improve the response rates of RIST for these lymphomas is intensification of preparative regimens as far as patients can tolerate without increasing RRT. Since the strength of GVL effect depends on the initial ratio between the number of tumor-specific immunocompetent cells in the graft and tumor cell burden of the recipient,[32] debulking of lymphoma cells by preparative regimens will be beneficial. The other problem in RIST for aggressive and highly aggressive lymphoma is the high rates of TRM. Most patients who achieved response after RIST remained progression-free (Table 3), suggesting a benefit of allogeneic immunity to suppress disease progression. Intensification of GVHD prophylaxis contributes to improve GVHD-related outcomes;[33 35] however, use of potent immunosuppressive agents might diminish a GVL effect,[35] and could increase the rate of serious infections.[34] Maintaining the fine balance between GVHD and GVL effects is important and frequently difficult in RIST for these lymphomas. Another promising approach is to reinforce a GVL effect without increasing GVHD. For example, monoclonal antibodies such as rituximab, tumor vaccines, and adoptive transfer of cytotoxic T-cells