is clearly a need for better combination chemotherapy to improve the survival.

Refractory and relapsed lymphoma may be viewed as the clinical consequence of drug resistance and rapid cell regrowth. The Norton-Simon model predicts that the total effect of therapy is related to the cell kill for each dose, the length of time drugs are administered, and the rate of tumor growth between each treatment, and that the most efficient way to treat heterogeneous cancer cells is to eradicate the numerically dominant, faster growing cells first, followed by the more slow-growing, resistant cells (11). The theoretical reason for the advantage afforded by dose-intensive treatment is that regrowth of resistant cells between cycles of chemotherapy is reduced by shortening the available time between doses and increasing the dose of drugs (11,12).

At the beginning of the 1990s, colony-stimulating factor (G-CSF) was introduced for clinical use, and it has made it possible to increase the dose intensity compared with dosages that can be delivered without G-CSF support. If so, more dose-intensive regimens than second- or third-generation regimens might improve the survival of patients with intermediate-grade NHL with poor prognosis features. Accordingly, a dose-intensive regimen (TCC-NHL-91) against advanced intermediate-grade lymphoma was started in 1991 in our center.

Here we report the long-term results of the TCC-NHL-91 regimen, which was employed before the incorporation of rituximab against advanced intermediate-grade lymphoma. Our results suggest that the dose-intensive sequential regimen may improve the survival of patients with advanced intermediate-grade NHL.

PATIENTS AND METHODS

Patient Eligibility

A total of 64 patients, treated with TCC-NHL-91 from February 1, 1991 to March 31, 2001 in our cancer center, were analyzed retrospectively. Patients' characteristics were as follows: age, 16–69 years; stage II bulky (≥7 cm), III , or IV disease as defined by the Ann Arbor staging criteria; no prior therapy; normal cardiac, renal, pulmonary, and hepatic function, unless abnormal because of disease involvement; no active double cancer. The study was conducted in accordance with the Helsinki declaration. The trial was approved by our institution's ethics committee, and informed consent for treatment was obtained from all patients.

Pretreatment Evaluations

Pretreatment evaluations included a thorough history, physical examination, blood cell count and differential blood chemistry tests, lumbar puncture, bone marrow aspiration, electrocardiography, chest X-ray, computed tomography of the chest and abdomen, abdominal echo, and gallium scans. Computed tomography of the head, gastrointestinal X-ray examination, endoscopy, and magnetic resonance imaging were performed as required clinically.

Treatment Strategy

The dose-intensive TCC-NHL-91 regimen was designed by reference to the CHOP and LNH84 (13) regimens, which at that time were considered as standard regimens for intermediate-grade lymphoma. Cyclophosphamide, doxorubicin (topoisomerase-II inhibitors), vincristine, and prednisone were "core" agents for the treatment of intermediate-grade lymphoma, and thus the total dose and dose intensity of these agents were not decreased in the regimen. The dose-intensive schedule was accomplished by using G-CSF to permit 2-week cycling of the drugs at their optimal dose levels rather than the conventional 3-week cycling. The consolidation phase referred to the application of the concept that multiple drugs are needed to perturb cancers maximally that are composed of cells of heterogeneous drug sensitivity. Drugs commonly used in salvage therapy, such as methotrexate (MTX), ifosfamide, cytarabine, and carboplatin, were included in this phase.

In experimental studies, prednisone, cyclophosphamide, doxorubicin, etoposide, bleomycin, mitoxantrone, and ifosfamide have shown additive effects with most antilymphoma agents [(14,15), unpublished data]. These agents can be combined simultaneously. MTX produced marked antagonistic effects with other antilymphoma agents in simultaneous exposure, while MTX followed by a variety of agents including vincristine and cytarabine produced synergistic effects [(16–18), unpublished data]. A schedule of MTX followed by vincristine was employed in this study. As MTX is highly time dependent (19), 24-h continuous infusion with late low-dose leucovorin rescue was used. Simultaneous exposure to cytarabine and mitoxantrone or carboplatin has shown synergistic effects and these agents were combined (15,17).

In advanced aggressive lymphoma, CNS relapse is not rare and the prognosis of secondary CNS lymphoma is extremely poor (20–26). To prevent CNS relapse, inthrathecal MTX, moderate-dose MTX, ifosfamide, and high-dose cytarabine, which cross the blood–brain barrier, were employed in the regimen.

Chemotherapy Regimen

The doses and schedule of the TCC-NHL-91 regimen are shown in Table 1. The induction phase regimen consisted of five cycles, each of a 2-week duration. Doxorubicin and mitoxantrone were administered by continuous

Table 1. TCC-NHL-91 Protocol (Minimum Seven Cycles)

Drug	Route	Dose	Treatment Day	Cycle (Interval) 1 (2W)	2 (2W)	3 (2W)	4 (2W)	5 (2W)	6 (3W)	7 (2W)	8 (3W)	
Cyclophosphamide	IV	1200 mg/m^2	1	•	•	•	•	•				
Ifosfamide	IV	1000 mg/m^2	1, 2, 3, 4							•		
Doxorubicin	CIV (24 h)	75 mg/m^2	1	•		•		•		•		
Mitoxantrone	CIV (24 h)	8 mg/m^2	1, 2				•					
	CIV (24 h)	8 mg/m^2	2, 3								•	X¶
Etoposide	IV	200 mg/m^2	1, 2, 3		•							R
Vincristine	IV	1.4 mg/m^2	1	•	•	•	•	•		•		T
	IV	1.4 mg/m^2	2, 9					•				
Bleomycin	IV	10 mg/m^2 500 mg/body	1	•	•	•	•	•		•		
Methylprednisolone*	IV	×2	1, 2, 3	•	•	•	•	•	•	•	•	
Methotrexate†	CIV (24 h)	500 mg/m^2 15 mg/body	1, 8							•		
Leucovorin‡	IV	×q6×6	3–4, 10–11						▽			
Cytarabine	IV	1 g/m^2 ×2	1, 2								•	
Carboplatin	IV	150 mg/m^2	1, 2, 3								•	
MTX§	IT	15 mg/body	1	•	•	•	•	•				
G-CSF	SC	1–2 mg/kg	variable	▽	▽	▽	▽	▽		▽	▽	

Until September 30, 1993, the fifth cycle of therapy was deleted for patients who entered in CR after two cycles of therapy.
*After June 1, 2000, dexamethasone 40 mg/body ×2 was delivered, because methylpredinosolone was not permitted for use in lymphoma by the Ministry of Health, Labour, and Welfare.
†When MTX level was higher than 1 μM at 48 h after the beginning of MTX administration, a higher dose and more frequent rescues were given.
‡Leucovorin started 36 h after the beginning of MTX administration.
§3–5 times in cycles 1–5.
¶For primary bulky mass and residual mass.

infusion for 24 h to prevent cardiotoxicity. The consolidation phase regimen, involving agents used for salvage therapies, consisted of the next three cycles: the sixth and eighth cycles of a 3-week duration, and the seventh cycle of a 2-week duration. During all cycles, patients received G-CSF (filgrastim, lenograstim, or nartograstim) at 1–2 μg/kg subcutaneously starting 2 or 3 days after the end of administration of cytotoxic agents until granulocyte recovery (>2000/μl). Chemotherapy subsequent to the first cycle was deferred until the absolute neutrophil count was greater than 2000/μl and the platelet count was greater than 10,000/μl. To prevent CNS relapse, 15 mg MTX and hydrocortisone at 50 mg/body were administered intrathecally in the first to fifth cycles of therapy as much as possible.

Involved-Field Radiation

Patients with primary bulky mass and/or patients with a residual mass after treatment received involved-field radiation (20–50 Gy). Radiotherapy was started within 2 months of the end of chemotherapy.

Cranial Radiation for CNS Prophylaxis. After September 1, 1997, patients with high LDH and/or massive bone marrow involvement received CNS prophylactic radiation (24 Gy in 12 fractions).

Supportive Care. All patients had an indwelling central venous catheter. Cotrimoxazole therapy (two tablets daily throughout the course of treatment except during the sixth cycle of therapy) was given to prevent pneumocystis carinii infection, and broad-spectrum antibiotics were given for a persistent undiagnosed fever ≥37.5°C.

Response Criteria

Response to treatment was assessed after every two cycles of therapy. The definition of complete remission (CR) used in the literature has been variable (27). We defined CR as the complete disappearance of all measurable and evaluable disease. Patients with a residual mass with no progression at 3 months after the end of therapy were also regarded as being in CR. Partial remission (PR) was defined as the diminution of >50% of all the initial masses.

Statistical Analysis

Overall survival (OS) was defined as the time from the beginning of treatment to any cause of death or the last follow-up. Progression-free survival (PFS) was defined the time from the beginning of treatment to lymphoma progression or death as a result of any cause or the last follow-up (28). The survival proportions were estimated with the Kaplan-Meier method. The log rank test was performed to test associations between patient characteristics and OS or PFS. Two-sided $p < 0.05$ was regarded as statistically significant. All statistical analyses were done by using SAS version 8.2 (SAS Institute, Cary, NC, USA).

RESULTS

Patients' Characteristics and Response to Therapy

Among the 64 patients treated with TCC-NHL-91, 5 patients were deleted from this study because they were diagnosed with lymphoblastic lymphoma ($n = 1$), follicular mix lymphoma ($n = 3$), or Hodgkin's lymphoma ($n = 1$) by an external pathological review. All these excluded patients are now alive in the first CR. The study was analyzed based on the clinical data of 59 patients on February 28, 2007. The main characteristics of the 59 patients and their response to therapy are listed in Table 2. There were 27 male patients (46%), with a median age of 48 (range 17–69 years), including 15 patients (27%) older than 60. Fifty-five patients (93%) had disease of stages III–IV. Forty-two patients (71%) had a bulky mass. Thirty-seven patients (63%) had two or more extranodal sites. Thirty-six patients (61%) had elevated serum lactate dehydrogenase (LDH). We retrospectively analyzed patients using the age-adjusted International Prognostic Index (aa-IPI) criteria (30). Sixteen patients (27%) had L- or LI-risk factors and 43 (73%) had HI- and H-risk factors. Fifty-six of the 59 patients entered CR (95%), 1 patient PR (2%), and 2 patients stable disease (3%). There was no significant association between any of the validation IPI risk factors, phenotype, or histological type, and response.

Number of Treatment Cycles and Dose Delivery

Forty-six patients (78%) received either seven (17 patients) or eight (29 patients) cycles of therapy (Table 3). Dose reduction was not performed in 55 patients. Dose reduction was performed in three patients over 60 and one patient with hepatic damage during the course of therapy. Thirteen patients (22%), including 10 patients over 60, stopped TCC-NHL-91 treatment before the seventh cycle of therapy for the following reasons: nine patients due to toxicity (one hemorrhagic cystitis; two interstitial pneumonitis; one ischemic heart disease; five intolerance), two due to refusal of further intensive treatment, and two due to non-treatment-related complications (idiopathic thrombocytopenic purpura and hypothyroidism). These patients were treated with other regimens after that. In the sixth cycle of therapy, six patients received only one cycle of therapy because of MTX toxicity. Intrathecal MTX was not sufficiently delivered with intensive chemotherapy (median, 3 times).

Involved-Field Radiation and Cranial Radiation

After chemotherapy, 43 of the 59 patients (64 sites) received involved-field radiation against the initial bulky mass and/or a residual mass without significant toxicity. The radiation dose ranged between 20 and 50 Gy (median dose of 30.6 Gy), varying with initial tumor size, extent, adjacent organs, response to chemotherapy, and the patient's physical condition. Fourteen patients received cranial radiation for CNS prophylaxis (24 Gy).

Toxicity

Table 3 details the treatment-related toxicity produced by this regimen. Grade 4 neutropenia occurred in 100% of patients and persisted for 1–8 days in the first to seventh cycles of therapy and for 1–15 days in the eighth cycle of therapy. Thrombocytopenia was less severe, but grade 4 toxicity was observed in 39% and 93% of patients in the sixth and eighth cycles of treatment, respectively. Infection associated with neutropenia was the other major complication. Of 403 total cycles of chemotherapy delivered, febrile neutropenic episodes (>38°C) occurred in 72 cycles (18%). Because continuous MTX infusion for 24 h and minimal leucovorin rescue were used, MTX toxicity was not ignorable. More than 50% of patients showed transient liver dysfunctions. Six patients received one but not two treatments of MTX because of severe stomatitis (four patients) or delayed MTX clearance (serum MTX concentration $>1 \times 10^{-6}$ M at 48 h) (two patients).

One patient developed hemorrhagic cystitis and two developed drug-induced interstitial pneumonitis, but all three patients recovered. Two patients died during chemotherapy. One patient died of pseudomonas sepsis during the therapy, and the other patient, who dropped out from the regimen after the first cycle, died of sepsis after CHOP therapy. One patient died of secondary leukemia after high-dose therapy with autologous peripheral blood stem cell transplantation in the third CR (11 years after the onset of lymphoma).

Relapse

Among the 56 CR patients, 17 patients relapsed. Four of them showed isolated CNS relapse, nine local relapse,

Table 2. Patients Characteristics and Outcome

Characteristic	No. of Pts (%)	CR (%)	% PFS at 5 Years	% PFS at 10 Years	*p*-Value	% OS at 5 Years	% OS at 10 Years	*p*-Value
All cases	59	56 (95)	68	61		78	76	
Median age (years, range 17–69)	48							
≤60	44 (75)	42 (95)	70	62	0.781	84	81	0.156
>60	15 (25)	14 (93)	60	60		60	60	
Gender								
Male	27 (46)	25 (93)	62	57	0.715	66	62	0.024
Female	32 (54)	31 (97)	72	65		88	88	
B symptoms								
(–)	25 (42)	23 (92)	72	62	0.683	76	76	0.632
(+)	34 (58)	33 (97)	64	60		79	76	
Stage								
II (bulky)	4 (7)	4 (100)	100	100	0.123	100	100	0.206
III–IV	55 (93)	52 (95)	65	58		76	74	
Bulky disease								
(–)	17 (29)	16 (94)	47	41	0.044	65	59	0.200
(+)	42 (71)	40 (95)	76	70		83	83	
ECOG PS								
0–1	29 (49)	28 (97)	69	57	0.565	79	76	0.804
2–4	30 (51)	28 (93)	66	66		76	76	
LDH								
Normal	23 (39)	22 (96)	65	56	0.604	78	78	0.466
High	36 (61)	34 (94)	69	65		78	74	
Extranodal sites								
0–1	22 (37)	21 (95)	82	77	0.128	86	86	0.244
2–	37 (63)	35 (95)	59	50		73	69	
Phenotype (T/B)								
T	9 (15)	9 (100)	44	44	0.165	78	67	0.735
B	50 (85)	47 (94)	72	64		78	78	
Histological type (WF)								
F. large cell	4 (7)	4 (100)	100	75	0.672	100	100	0.514
D. small cleaved cell	3 (5)	3 (100)	33	33		67	67	
D. mixed	11 (18)	11 (100)	55	55		64	55	
D. large cell	37 (66)	34 (92)	70	62		78	78	
T unclassified	2 (3)	2 (100)						
B unclassified	2 (3)	2 (100)						
aa-IPI								
L/LI	1/15 (27)	1/15 (100)	69	55	0.691	81	81	0.345
HI/H	20/23 (73)	19/21 (93)	67	64		77	74	

PFS, progression free survival; OS, overall survival; aa-IPI, age-adjusted International Prognostic Index. Thee *p*-values derived from log rank test.

and four generalized relapse. Two patients had suffered relapse from the radiation sites (total dose 20 and 30 Gy, respectively). By August 1997, 8 of the 32 patients had relapsed. Among them, four patients (50%) showed CNS relapse. Two patients with CNS relapse received seven cycles and the other two patients received eight cycles of therapy. Two patients received two treatments of intrathecal MTX and the other two patients received four treatments. These results suggested that the regimen was insufficient for CNS relapse. Then CNS prophylactic radiation (24 Gy) was added for 14 patients with high LDH and/or bone marrow involvement among the 27 patients. After that, no patient showed CNS relapse.

Survival

The median duration of follow-up has been 128 months (range 71–174 months). Sixteen of the 59 eligible patients have died (27%). Thirty-six patients are now in first CR, six patients in second CR, and one patient in stable disease. There was no significant association between any of the IPI risk factors and OS or PFS. Fig-

Table 3. Treatment Cycles and Toxicities

	Cycle							
	1 ($n = 59$)	2 ($n = 57$)	3 ($n = 57$)	4 ($n = 56$)	5* ($n = 45$)	6 ($n = 50$)	7 ($n = 46$)	8 ($n = 29$)
Interval (days)	12–20	12–24	12–27	12–33	13–29	10–33	14–51	
Median	16	15	16	18	17	23	19	
Neutropenia (<500/μl) (%)	86	79	54	98	90	29	74	100
Duration (days)	1–8	1–7	1–7	1–12	1–11	1–7	1–8	1–15
Median (days)	3	4	3	5	5	3	4	7
Thrombocytopenia (<20,000/μl) (%)	7	5	5	21	20	39	13	93
Anemia (Hb <8 g/dl) (%)	14	9	26	49	45	55	39	90
Liver dysfunction (GOT, GPT > 2.5 N) (%)	19	10	11	11	8	51	11	14
Fever >38°C (%)	14	12	5	20	14	32	15	45

The *n* value in parentheses represents number of patients.
*Five patients deleted the fifth cycle of therapy.

ure 1 shows the Kaplan-Meier estimate of the survival distribution of all patients. The OS and PFS at 5 years were 77.8% (95% CI 67.2–88.5%) and 67.6% (95% CI 55.6–79.6%), and those at 10 years were 75.9% (95% CI 64.9–86.9%) and 61.2% (95% CI 48.3–74.1%), respectively (Fig. 1).

Figures 2 and 3 show the OS and PFS by aa-IPI subgroups, respectively. The OS at 10 years was 81.3% (95% CI 62.1–100%) and 73.8% (95% CI 60.5–87.2%), for the patients in the L/LI- and HI/H-risk groups, respectively (Fig. 2). The PFS at 10 years was 54.7% (95% CI 29.5–79.9%) and 63.7% (95% CI 48.9–78.6%) for the patients in the L/LI- and HI/H-risk groups, respectively (Fig. 3). The detailed characteristics of all 59 patient and their outcomes are shown in Table 2. There was no significant difference in OS or PFS between/among the subgroups of patients, except regarding sex (OS at 10 years: male/female 62%/88%, $p = 0.024$).

Among 37 patients with diffuse large-cell lymphoma, 32 patients were B-cell type (DLBCL), and 5 patients T-cell type. At present, 23 patients with DLBCL and 4 patients with T-cell type are alive. We analyzed the data

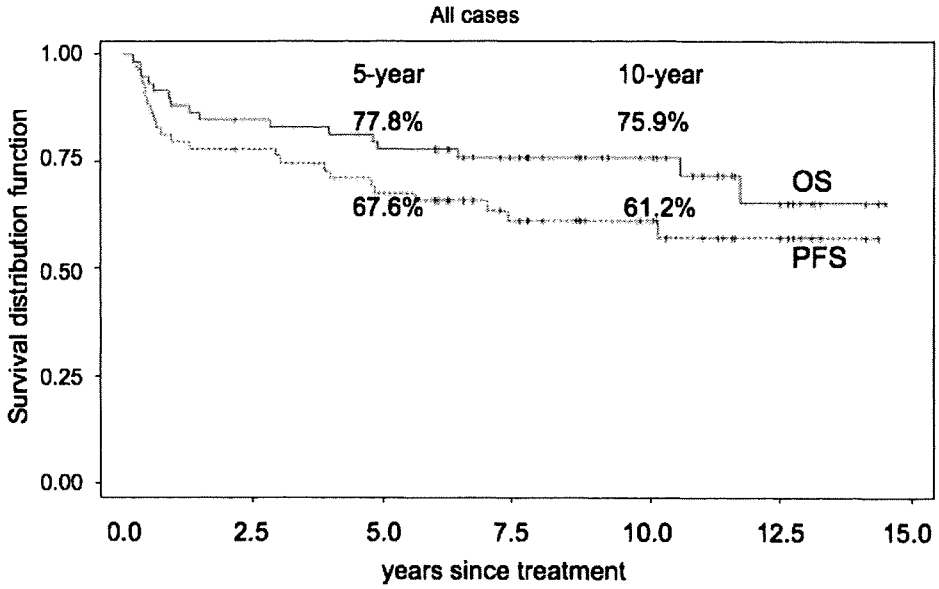

Figure 1. Kaplan-Meier estimate of OS and PFS of the 59 patients treated with TCC-NHL-91. OS at 5 years and 10 years was 77.8% and 75.9%, respectively. PFS at 5 years and 10 years was 67.6% and 61.2%, respectively.

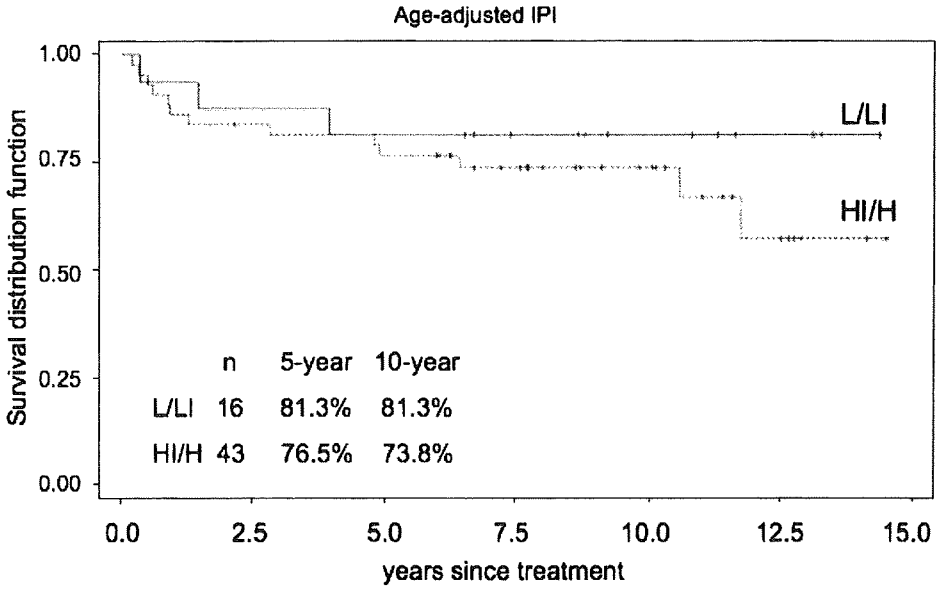

Figure 2. Kaplan-Meier estimate of OS by aa-IPI grouping. OS at 5 and 10 years was 81.3% and 81.3%, respectively, for the L/LI groups. OS at 5 and 10 years was 76.5% and 73.8%, respectively, for the HI/H groups.

for 32 patients with DLBCL. The OS and PFS at 10 years for patients with DLBCL were 78.1% (95% CI 63.8–92.5%) and 61.9% (95% CI 43.4–80.5%), respectively. Figures 4 and 5 show the OS and PFS in patients with DLBCL by aa-IPI subgroups, respectively. The OS at 10 years was 75.0% (95% CI 32.6–100%) and 78.6% (95% CI 63.4–93.8%) for the patients in the L/LI- and HI/H-risk groups, respectively. The PFS at 10 years was 37.5% (95% CI 0–93.6%) and 65.5% (95% CI 46.5–84.5%) for the patients in the L/LI- and HI/H-risk groups, respectively. There was no significant difference in OS or PFS between/among the subgroups of patients,

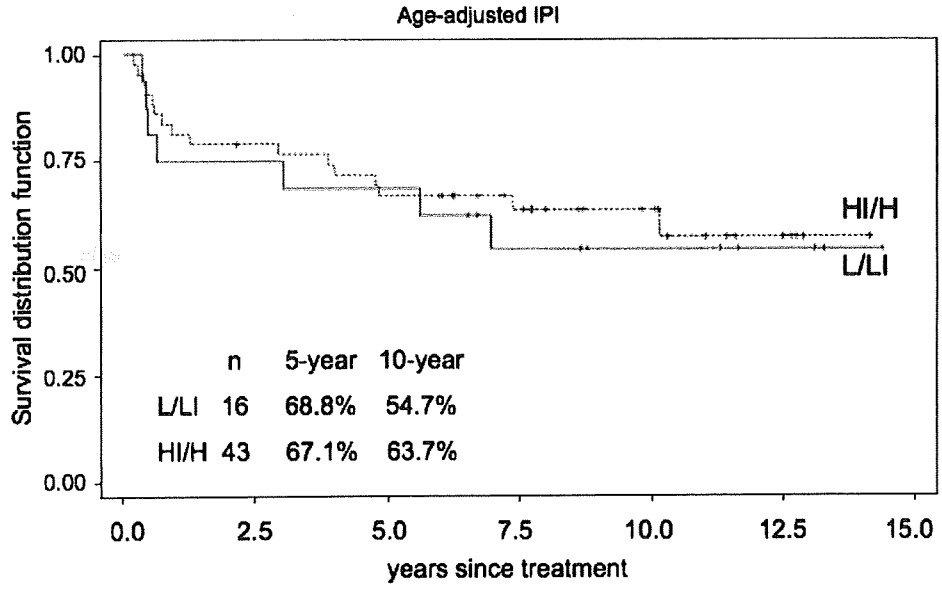

Figure 3. Kaplan-Meier estimate of PFS by aa-IPI grouping. PFS at 5 and 10 years were 68.8% and 54.8%, respectively, for the L/LI group. PFS at 5 and 10 years was 67.1% and 63.7%, respectively, for the HI/H group.

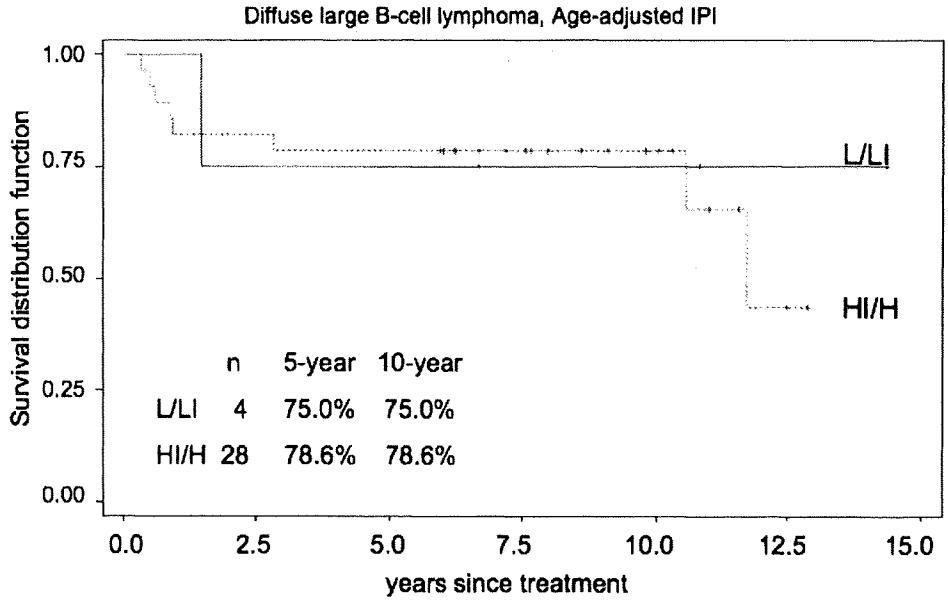

Figure 4. Kaplan-Meier estimate of OS by aa-IPI grouping of DLBCL. OS at 5 and 10 years was 75.0% and 75.0%, respectively, for the L/LI groups. OS at 5 and 10 years was 78.6% and 78.6%, respectively, for the HI/H groups.

but the number of patients for each subgroup was too small for reliable analysis.

DISCUSSION

This is a retrospective study conducted in a single institution involving 59 patients with advanced intermediate-grade lymphoma who underwent intensive chemotherapy with G-CSF support (TCC-NHL-91). We achieved a CR rate of 95% and a projected 10-year OS of 76% and projected 10-year PFS of 61%. The median age of our patient population was approximately 10 years lower than that in most cooperative group lymphoma studies,

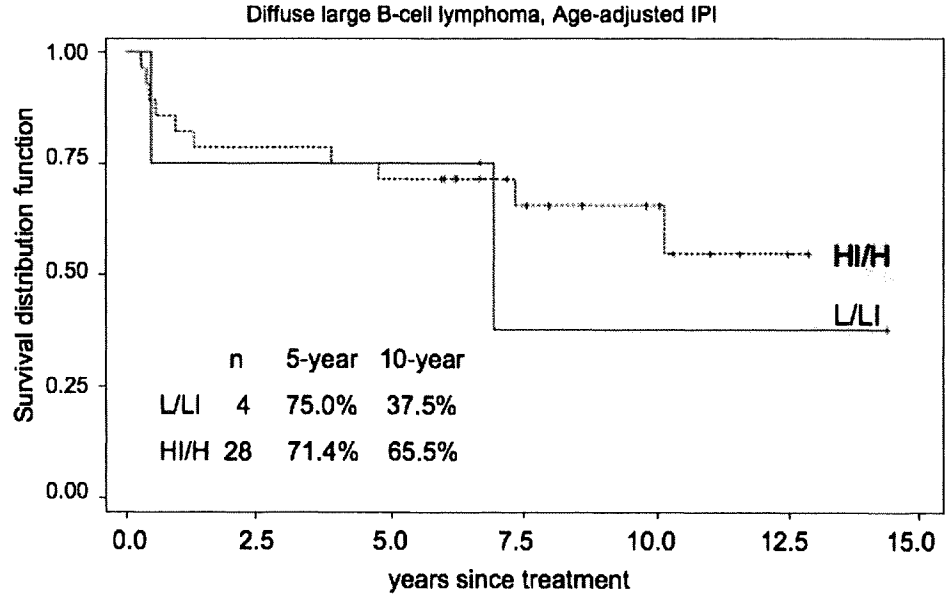

Figure 5. Kaplan-Meier estimate of PFS by aa-IPI grouping of DLBCL. PFS at 5 and 10 years was 75.0% and 37.5%, respectively, for the L/LI groups. PFS at 5 and 10 years was 71.4% and 65.5%, respectively, for the HI/H groups.

and younger age is a recognized favorable prognostic factor. However, considering that the patients were in advanced stages and had a median follow-up of 128 months, these results were encouraging.

Shipp et al. developed a model, IPI, for predicting outcome in patients with aggressive lymphoma on the basis of patient characteristics before treatment, in which most patients received the CHOP regimen (29). The aa-IPI criteria, based on tumor stage, LDH level, and PS, were used to identify four risk groups: L, LI, HI, and H risk, with predicted 5-year survival rates of 83%, 69%, 46%, and 32%, respectively. Expressing our data according to aa-IPI, the OS at 10 years was 81%, and 74% for patients in the L/LI- and HI/H-risk groups, respectively. These findings suggest that the TCC-NHL91 regimen showed a significant benefit in the patients in the HI/H-risk groups, and overcame poor prognostic factors.

Dose reduction was not performed in 55 patients, while it was performed in three patients over 60 and one patient with hepatic dysfunction. Fourty-six patients received seven or eight cycles of therapy (Table 3), but 13 patients, including 10 of 15 patients over 60 years old, and 3 of 44 patients at ≤60 years old, received six or fewer cycles of therapy. Many patients over aged 60 could not tolerate this regimen. The TCC-NHL-91 regimen is not recommended for elderly patients.

The TCC-NHL-91 regimen was toxic and the predominant toxicity was hematological (Table 3). Grade 4 hematotoxicity (neutropenia) was observed in 100% of the patients in spite of G-CSF support. However, recovery of neutropenia was rapid and the median durations of neutropenia (<500/μl) in the first to the seventh cycles of therapy was 3–5 days, and no patient died of severe infection. In the eighth cycle of therapy, the median duration of neutropenia (<500/μl) was 7 days (1–15 days) and one patient died of pseudomonas sepsis. Although our approach adds considerably to the acute toxicity and expense of lymphoma treatment, significant improvement of OS and PFS justify its use.

Radiotherapy has been reported to improve the survival in patients with limited-stage lymphoma (30), but its use for consolidation therapy for primary bulky sites or residual mass against advanced aggressive lymphoma are still controversial, because the sites of relapse are variable in advanced lymphoma (31). In North America, radiotherapy has rarely been considered for advanced aggressive lymphoma (30).

We performed involved-field radiation in 43 patients. Only 2 of the 17 relapsed patients relapsed from the radiation sites. Wilder et al. reported that 86% of patients treated with chemotherapy alone developed a recurrence at the presenting site of the bulky mass whereas only 12% of the patients who were treated with chemotherapy followed by radiotherapy developed disease recurrence within the radiotherapy field (32). Our findings show striking similarity with Wilder et al.'s findings. Recent randomized trials suggest that adjuvant radiotherapy against primary bulky mass or residual mass may significantly improve the relapse-free survival and OS of patients with advanced aggressive lymphoma (33,34). Considering these clinical findings and our data, radiotherapy is considered to have made a significant contribution to the outcome of our study.

In our study, the patients with bulky disease showed better OS and PFS than those without bulky disease. The OS at 10 years was 83% (95% CI 72–95%) and 59% (95% CI 35–82%) in these groups, respectively ($p = 0.20$). The total number of treatment cycles was similar in the two groups. These results are in conflict with previous findings that bulky disease showed worse prognosis (13,32,35). However, the difference is not statistically significant.

CNS prophylaxis is not usually included in the treatment regimens because of the relatively low incidence of CNS relapse and the toxicity of the prophylactic therapy. However, several studies have suggested that CNS relapse is not always rare in patients with advanced intermediate-grade NHL: the cumulative risk of CNS relapse in these patients was 4–20% and systemic relapse occurred rapidly after CNS relapse, resulting in a median survival time after CNS relapse of only a few months (20–26). Risk factors for CNS relapse have include advanced stages, high LDH, more than one extranodal site, high IPI, and involvement of paranasal sinus, orbital cavity, testis, or bone marrow, etc. van Besien et al. reported that high LDH and >1 extranodal sites are most important factors for CNS relapse and these patients had a 17% probability of CNS recurrence at 1 year after diagnosis (24).

CNS prophylaxis could be important for improving the outcome of high-risk patients, but there is no consensus regarding indications for prophylaxis or a standard CNS chemoprophylaxis regimen (36). The CNS prophylaxis of our therapy including intrathecal MTX, intermediate-dose MTX, ifosfamide, and high-dose cytarabine was insufficient. Chua et al. reported that intrathecal chemotherapy alone was inadequate CNS prophylaxis in patients with intermediate-grade non-Hodgkin's lymphoma (37). Our data support their findings.

After August 1997, we initiated prophylactic cranial radiation (24 Gy) in new patients ($n = 14$) with high LDH and/or massive bone marrow involvement, because at that time, these seemed important risk factors for CNS relapse in patients with advanced lymphoma (20–23). These included nine patients with both elevated serum LDH and more than one extranodal site. After that, no CNS relapse was observed and no patients

Table 4. Comparison of Conventional Chemotherapy and Dose-Intensive Regimens With G-CSF Support Against Advanced Aggressive Lymphoma

Author	Regimen	Radiotherapy	Patient	No. of Patients	Median Age (Range)	aa-IPI (L+LI/HI+H) (%)	OS		Dose Delivery
							Years	% (All)	
Shipp (29)	CHOP etc.		aggressive lymphoma	1,274	<60	54/46	5	46/32	
Gordon et al. (40)	200% PRO-MACE-CytaBOM	–	advanced aggressive lymphoma	74	43 (19–65)	54/46	4	73*	56/74 patients received 6 or more cycles of therapy (total 8 cycle)
Gisselbrecht et al. (41)	ACVBP	–	advanced aggressive lymphoma	181	46 (15–60)	3/98	5	60†	
Wilson et al. (42)	Dose-adjusted EPOCH	–	diffuse large-B-cell lymphoma	50	46 (20–88)	48/52	5 + 2 months	73‡	39/50 patients received 6 or more cycles of therapy (total 8 cycle)
Blayney et al. (43)	Dose-intensified 2W-CHOP	–	advanced aggressive lymphoma	88	53 (19–77)	64/37§	5	60¶	81/88 received 6 cycles of therapy (total 6 cycle)
Present study	TCC-NHL-91	+	advanced intermediate-grade lymphoma	59	48 (17–69)	27/73	5	78	50/59 patients received 6 or more cycles of therapy (total 8 cycle)

*52% in H-risk group.
†52% in HI- and H-risk groups.
‡55% in HI-risk group, 100% in H-risk group.
§IPI but not aa-IPI.
¶66% in HI-risk group, 53% in H-risk group.

receiving cranial radiation developed neurocognitive deficits. Although the number of patients was too small to allow analysis, prophylactic low-dose cranial radiation in addition to intrathecal MTX, etc., might contribute to preventing CNS relapse and improving OS. Recently, prophylactic low dose of cranial radiation (24–30 Gy) was introduced for CR patients with small-cell lung cancer because it decreased the incidence of brain metastases and prolonged survival, and this can be achieved with a tolerable level of acute and delayed neurotoxicity using modern techniques (38,39). Further evaluation and prospective studies of prophylactic cra-

nial radiation appeared to be warranted for high-risk lymphoma patients with CNS relapse.

The use of dose-intensive chemotherapy with G-CSF support against advanced intermediate-grade NHL has been controversial. However, the results of several regimens included better outcomes (40–43) (Table 4). Although the patients' backgrounds in each study were slightly different, the 5-year OS achieved by this approach could be about 10–30% better than that by conventional chemotherapy. Interestingly, in these trials, the outcome of the patients in the HI- and H-risk groups was improved significantly and there was no significant difference in OS between the L-/LI- and HI/H-risk groups. The results of our study are in agreement with those of these previous studies.

The introduction of rituximab and its combination with CHOP or CHOP-like regimens resulted in a high remission rate and OS against aggressive B-cell lymphomas (7–9). Five-year OS for patients with DLBCL was improved significantly (10–20%) by the addition of rituximab to standard regimens, but the treatment outcome is still unsatisfactory for especially high-risk patients. The toxicity profile of rituximab is excellent and the absence of relevant hematotoxicity allows its combination with dose-intensive regimens. In addition, the lymphoma cell populations sensitive to rituximab may be different from those sensitive to conventional antilymphoma agents (44). The combined use of rituximab with TCC-NHL-91 may improve the OS and PFS in advanced intermediate grade B-cell lymphoma.

In conclusion, TCC-NHL-91 achieved high CR, OS, and PFS in patients with advanced intermediate-grade NHL. The outcome was not found to be dependent on aa-IPI. TCC-NHL-91, combined with appropriate radiotherapy, could be very effective for younger patients with advanced intermediate-grade lymphoma. However, as this study was a single-institution study with retrospective analysis, it is not possible to reach definite conclusions. Further evaluation and prospective studies of the TCC-NHL-91 appear to be warranted.

ACKNOWLEDGMENTS: We thank Dr. Shigeo Nakamura (Nagoya University, Nagoya Japan) and Masabumi Abe (Fukushima Medical School, Fukushima, Japan) for their external pathological review.

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Oncogene (2009) 28, 231–242

www.nature.com/onc

ORIGINAL ARTICLE

Bortezomib overcomes cell adhesion-mediated drug resistance through downregulation of VLA-4 expression in multiple myeloma

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Multiple myeloma (MM) is incurable, mainly because of cell adhesion-mediated drug resistance (CAM-DR). In this study, we performed functional screening using short hairpin RNA (shRNA) to define the molecule(s) responsible for CAM-DR of MM. Using four *bona fide* myeloma cell lines (KHM-1B, KMS12-BM, RPMI8226 and U266) and primary myeloma cells, we identified CD29 (β1-integrin), CD44, CD49d (α4-integrin, a subunit of VLA-4), CD54 (intercellular adhesion molecule-1 (ICAM-1)), CD138 (syndecan-1) and CD184 (CXC chemokine receptor-4 (CXCR4)) as major adhesion molecules expressed on MM. shRNA-mediated knockdown of CD49d but not CD44, CD54, CD138 and CD184 significantly reversed CAM-DR of myeloma cells to bortezomib, vincristine, doxorubicin and dexamethasone. Experiments using blocking antibodies yielded almost identical results. Bortezomib was relatively resistant to CAM-DR because of its ability to specifically downregulate CD49d expression. This property was unique to bortezomib and was not observed in other anti-myeloma drugs. Pretreatment with bortezomib was able to ameliorate CAM-DR of myeloma cells to vincristine and dexamethasone. These results suggest that VLA-4 plays a critical role in CAM-DR of MM cells. The combination of bortezomib with conventional anti-myeloma drugs may be effective in overcoming CAM-DR of MM.
Oncogene (2009) **28,** 231–242; doi:10.1038/onc.2008.385; published online 13 October 2008

Keywords: myeloma; bortezomib; drug resistance; cell adhesion; VLA-4

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Received 2 June 2008; revised 19 August 2008; accepted 5 September 2008; published online 13 October 2008

Introduction

Despite recent advances in treatment strategies using dose-intensified regimens and new molecular-targeted compounds, multiple myeloma (MM) remains incurable (Kyle *et al.*, 2003). Most patients with MM eventually become resistant to the treatment and die of disease progression within 10 years. To improve the prognosis of myeloma patients, it is essential to overcome drug resistance (DR).

MM is characterized by the infiltration and growth of malignant plasma cells in the bone marrow (BM) microenvironment. MM cells localize within the BM through the interaction of adhesion receptors with their ligands on BM stromal cells and extracellular matrix proteins (Hideshima *et al.*, 2007). It has been demonstrated that MM cells in the BM microenvironment are much less sensitive to chemotherapeutic agents (Damiano *et al.*, 1999; Nefedova *et al.*, 2003). This type of DR has been termed cell adhesion-mediated DR (CAM-DR), which is believed to play a crucial role in both *de novo* and acquired DR in MM patients (Damiano *et al.*, 1999). Despite extensive investigations, the adhesion molecules critical for CAM-DR in MM have not been identified yet.

The proteasome inhibitor bortezomib (Velcade, formerly known as PS-341) has shown a clinical activity in patients with relapsed MM (Richardson *et al.*, 2003, 2005), and will be applied for the treatment of other hematologic malignancies and solid tumors in the near future (Fisher *et al.*, 2006; Davies *et al.*, 2007). Bortezomib is a reversible inhibitor of the 26S proteasome complex, which catalyses ubiquitin-dependent protein degradation. Inhibition of this complex ultimately leads to modulation of the abundance and functions of many intracellular proteins in bortezomib-treated cells (Hideshima *et al.*, 2001). Among them, the multifunctional transcription factor nuclear factor-kappa B (NF-κB) is considered the most relevant target in MM, because recent genome-wide approaches revealed that this factor is frequently activated in MM cells by mutations of the components of the NF-κB signaling cascade (Annunziata *et al.*, 2007; Keats *et al.*, 2007). Given the wide spectrum of transcriptional

targets of NF-κB including adhesion molecules and the IAP family of apoptosis inhibitors (Dolcet *et al.*, 2005), it is reasonable to speculate that CAM-DR of MM is mediated by NF-κB and could be overcome by bortezomib. To date, however, such possibilities have not been investigated.

In this study, we first attempted to identify the adhesion molecules responsible for CAM-DR in MM. By functional screening using the lentiviral short hairpin/small interfering RNA (shRNA/siRNA) system, we identified VLA-4 as a critical molecule for the induction of CAM-DR in MM cells. Furthermore, we found a novel and unique property of bortezomib to overcome CAM-DR by downregulating the expression of CD49d, a subunit of VLA-4. These results suggest that bortezomib enhances the effects of conventional anti-myeloma agents by overcoming VLA-4-mediated CAM-DR, and bortezomib-based combination chemotherapy can improve the treatment outcome of patients with MM.

Results

Surface expression of adhesion molecules on MM cells

In an initial effort to identify the molecules responsible for CAM-DR, we screened for the expression of adhesion molecules on MM cells using flow cytometry. By referring to previous studies (Tatsumi *et al.*, 1996; Cook *et al.*, 1997), we selected the molecules to be checked as follows: CD11a (lymphocyte function-associated antigen-1 (LFA-1)), CD18 (β2-integrin), CD22, CD29 (β1-integrin), CD40, CD44 (homing-associated cell adhesion molecule (HCAM)), CD49d (α4-integrin, a subunit of VLA-4), CD49e (α5-integrin, a subunit of VLA-5), CD54 (intercellular adhesion molecule-1 (ICAM-1)), CD56 (neural cell adhesion molecule (NCAM)), CD138 (syndecan-1) and CD184 (CXC chemokine receptor-4 (CXCR4)). We examined the expression of these molecules in four *bona fide* human MM cell lines (KHM-1B, KMS12-BM, RPMI8226 and U266) and normal plasma cells from healthy volunteers. As shown in Figure 1a, MM cell lines readily expressed CD29, CD44, CD49d, CD54, CD138 and CD184, whereas CD22 was barely detectable. The expression of CD11a, CD18, CD40, CD49e and CD56 was highly variable among cell lines. Normal plasma cells expressed the same set of molecules as MM cell lines except CD22, but their expression levels were generally lower than those of MM cells. It is of note that RPMI8226 showed a slightly different pattern from other cell lines: it expressed CD29, CD44 and CD49d lower but CD49e higher. Overall, we identified CD29, CD44, CD49d, CD54, CD138 and CD184 as major adhesion molecules expressed on MM cell lines.

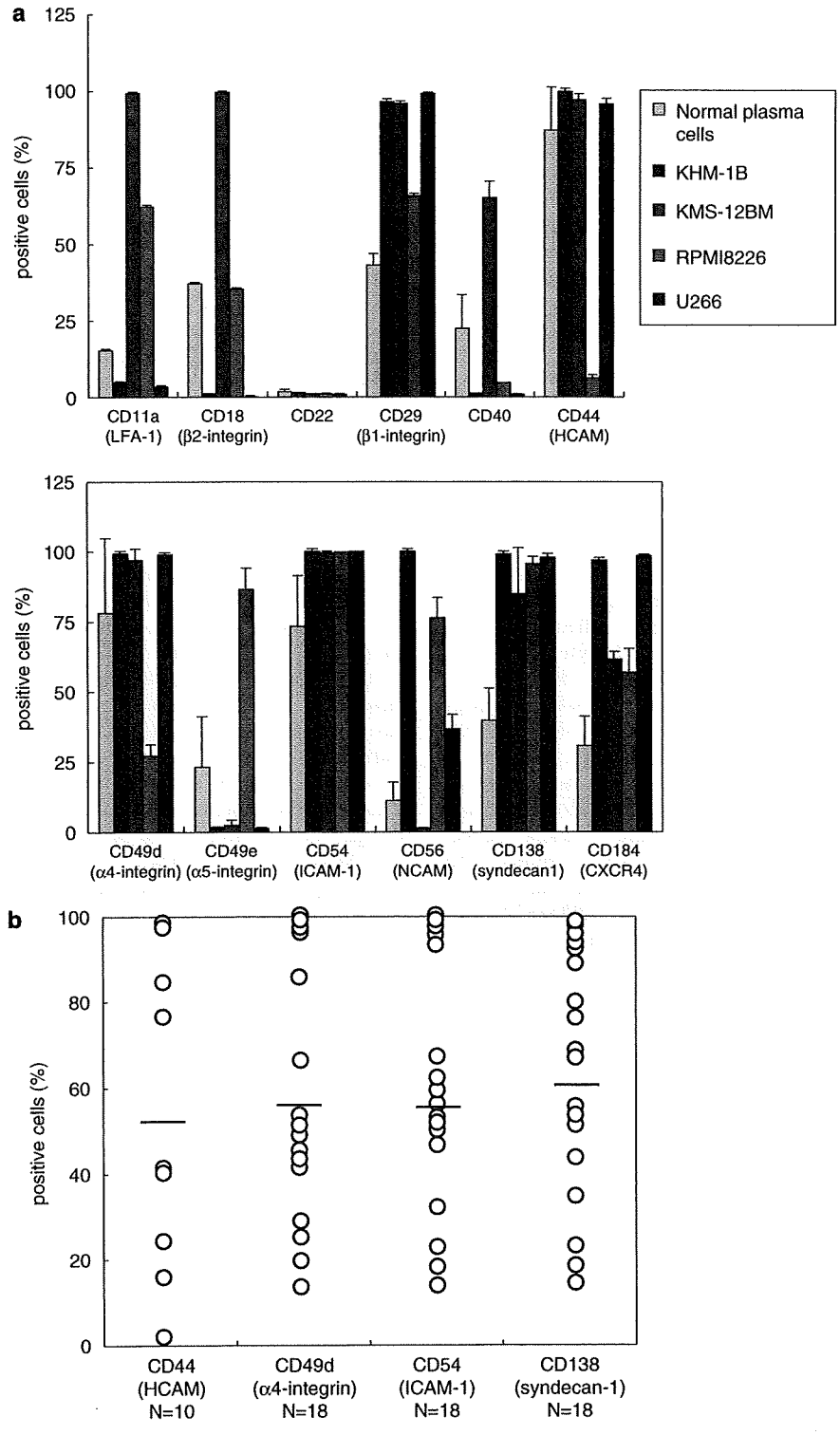
To further elucidate the expression pattern of adhesion molecules in MM, we screened for their expression on primary MM cells. As CD138 is commonly used as a specific marker for myeloma cells in BM specimens, we detected the expression of CD44, CD49d and CD54 in CD138-positive fractions in BM-mononuclear cells (MNCs) from 18 patients with MM by dual staining on flow cytometry. As shown in Figure 1b, CD44, CD49d and CD54 were moderately to markedly expressed in all patients involved in this study. The proportions of positive cells were 52.8 ± 37.7% for CD44, 57.0 ± 31.6% for CD49d and 56.8 ± 30.9% for CD54 in the CD138-positive fractions (CD138 positivity was 60.0 ± 31.0% in the entire fraction). This pattern closely resembled that of the cell lines. On the basis of these results, we focused on CD29 (β1-integrin), CD44 (HCAM), CD49d (α4-integrin), CD54 (ICAM-1), CD138 (syndecan-1) and CD184 (CXCR4) to determine the functional adhesion molecules in MM in further studies.

Establishment of the in vitro *culture system for the assessment of CAM-DR of MM cells*

To investigate the involvement of these adhesion molecules in CAM-DR of MM cells, we established a culture system recapitulating CAM-DR *in vitro*. As described in Materials and methods, green fluorescent protein (GFP)-expressing MM cells were added into culture dishes with (co-culture) or without (stroma free) a preseeded UBE6T-7 stromal cell line, and cultured for 2 days in the absence or presence of four anti-myeloma drugs. We determined the cytotoxic effects of the drugs on MM cells specifically by measuring annexin-V positivity in GFP-positive fractions on flow cytometry. Figure 2a shows the representative results of KMS-12BM cells treated with suboptimal doses of each drug determined in pilot experiments: bortezomib 5 nM, vincristine 1 nM, doxorubicin 100 nM and dexamethasone 50 nM (Supplementary Figure S1). All of them are lower than clinically achievable concentrations *in vivo* according to recent clinical trials (Fisher *et al.*, 2006; Davies *et al.*, 2007). These drugs were capable of

Figure 1 Surface expression of adhesion molecules on multiple myeloma (MM) cells. (**a**) We screened for surface expression of adhesion molecules on MM cells using four myeloma cell lines (KHM-1B, KMS-12BM, RPMI8226 and U266) and normal plasma cells. Cells were stained with phycoerythrin (PE)-conjugated antibodies against CD11a (LFA-1), CD18 (β2-integrin), CD22, CD29 (β1-integrin), CD40, CD44 (HCAM), CD49d (α4-integrin), CD49e (α5-integrin), CD54 (ICAM-1), CD56 (NCAM), CD138 (syndecan-1), and CD184 (CXCR4), and subjected to flow cytometry. To analyse normal plasma cells, BM-MNCs were triple-stained with allophycocyanine (APC)-conjugated anti-CD38, PE-Cy7-conjugated anti-CD45 and PE-conjugated antibodies against each adhesion molecule. Cells in the $CD38^{+}/CD45^{lo/neg}$ fraction were gated as normal plasma cells. The means ± s.d. (bars) of three independent experiments are shown. (**b**) The expression of adhesion molecules was detected in primary MM cells. BM-MNCs were double-stained with an FITC-conjugated anti-CD138 antibody and PE-conjugated antibodies against CD44, CD49d and CD54. Each circle represents the positivity (%) of CD44, CD49d, and CD54 in the CD138-positive fractions, and that of CD138 in the entire fraction of BM-MNCs of individual patients (N = sample numbers). Bars indicate the average values of each molecule.

inducing apoptosis in more than 20% of KMS-12BM cells under stroma-free condition. In addition, we stained cells with propidium iodide to estimate the contribution of other forms of cell death to the cytotoxicity of these drugs. The percentages of dead cells obtained with propidium iodide staining were almost equal to or slightly higher than those obtained with annexin-V staining, implying that the major form



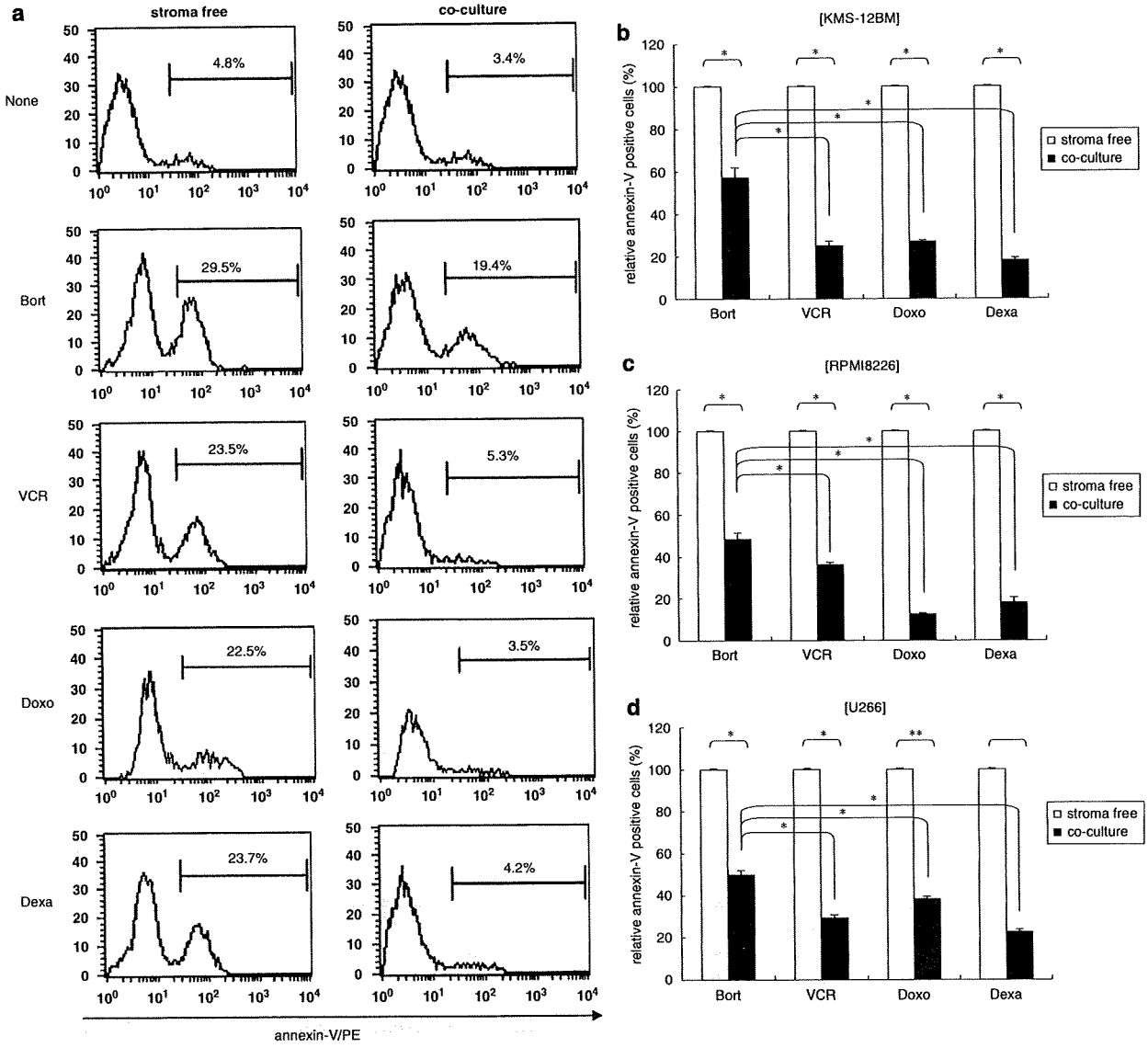


Figure 2 Establishment of the *in vitro* culture system for the assessment of cell adhesion-mediated drug resistance (CAM-DR) of multiple myeloma (MM) cells. (**a**) Green fluorescent protein (GFP)-transduced KMS-12BM cells were treated with either 5 nM bortezomib (Bort), 1 nM vincristine (VCR), 100 nM doxorubicin (Doxo) or 50 nM dexamethasone (Dexa) in the presence (co-culture) or absence (stroma free) of UBE6T-7 stromal cells for 48 h. Cell death/apoptosis was determined by reactivity with phycoerythrin (PE)-conjugated annexin-V (annexin-V/PE) in GFP-positive fractions on flow cytometry. Representative histogram plots are shown. Annexin-V positivity is indicated as a percentage in each histogram. (**b**) The *Y* axis shows the proportion of annexin-V-positive cells under co-culture condition with that under stroma-free condition setting at 100% in KMS-12BM cells treated with each drug. The means ± s.d. (bars) of three independent experiments are shown. The same experiments were carried out in RPMI8226 (**c**) and U266 (**d**). Drug concentrations were 2 nM for bortezomib, 1 nM for vincristine, 100 and 70 nM for doxorubicin, and 50 and 20 nM for dexamethasone in RPMI8226 and U266 cells, respectively. The *P*-values were calculated by Student's *t*-test. $*P<0.05$.

of cell death is apoptosis (Supplementary Figure S2A). The cytotoxic effects were markedly diminished under the co-culture condition, suggesting that CAM-DR was successfully reproduced in our system (Figure 2a; Supplementary Figure S2). DR was not acquired in KMS-12BM cells cultured with stroma cells in trans-wells, which preclude direct cell-to-cell interactions, indicating that direct contact is indispensable for CAM-DR of MM (data not shown). As shown in Figures 2b–d and Supplementary Table S1, CAM-DR was similarly observed in all three myeloma cell lines treated with all four drugs tested, although the extent of CAM-DR was relatively low for bortezomib (discussed later). Furthermore, CAM-DR was reproduced with different concentrations of the drugs (Supplementary Figure S2b). Using this system, we attempted to

determine which adhesion molecule(s) is important for CAM-DR in MM cells.

Reversal of CAM-DR by shRNA/siRNA- and blocking antibody-mediated knockdown of VLA-4 in MM cells
To investigate which adhesion molecule(s) is critical for the acquisition of CAM-DR in MM cells, we performed loss-of-function analyses for CD44 (HCAM), CD49d (α4-integrin), CD54 (ICAM-1), CD138 (syndecan-1) and CD184 (CXCR4) using the shRNA/siRNA lentivirus system (Kikuchi *et al.*, 2007). Because CD29 (β1-integrin) is heterodimerized with CD49d and functions as VLA-4 (α4β1-integrin) on MM cells, we could achieve loss of function of VLA-4 by solely targeting CD49d. As shown in Figure 3a, shRNA/siRNA expression vectors were constructed by inserting chemically synthesized oligonucleotides containing target sequences (Supplementary Table S2) into pLL3.7 vector, and their inhibitory activities were checked in KMS-12BM cells (data not shown). Constructs with the strongest activities were transfected into three MM cell lines along with sh controls, and a specific reduction of target expression was confirmed (Figure 3b; Supplementary Figure S3). Overall, we established 15 sublines in which the expression of individual adhesion molecules was markedly downregulated, and examined the levels of CAM-DR to four anti-myeloma drugs. To quantitatively assess the contribution of each molecule to CAM-DR, we defined the ratio of annexin-V reactivity of GFP-positive cells under the co-culture condition to that under the stroma-free condition as a reversal of CAM-DR. The reversal of CAM-DR to bortezomib was detectable in CD49d-knockdown sublines of all three cell lines, whereas no reversal was observed in sublines carrying shRNA/siRNA against other four adhesion molecules and inactive sh controls (Figure 3c; Supplementary Table S3). In addition, we performed the same experiments using vincristine and dexamethasone in KMS-12BM sublines. The CAM-DR to vincristine and dexamethasone was also reversed by knockdown of CD49d but not other four molecules (Figure 3d; Supplementary Table S4). It is of note that the reversal of CAM-DR to either vincristine or dexamethasone was at the almost equal level to that to bortezomib in CD49d-knockdown sublines (compare Figures 3c and d). In view of the fact that bortezomib is relatively resistant to CAM-DR (see Figure 2 and Supplementary Table S1), this implies that bortezomib modulates the expression of CD49d in MM cells (discussed later).

Furthermore, we confirmed the importance of CD49d in CAM-DR using adhesion-blocking antibodies instead of shRNA/siRNA introduction. We used specific antibodies against CD44, CD49d, CD54 and CD184 to revert CAM-DR, but were not able to test an anti-CD138 antibody because it is not commercially available. MM cells were pretreated with these antibodies, cultured with or without stromal cells in the presence of bortezomib, and subjected to flow cytometric analysis for annexin-V reactivity. As shown in Figure 4 and Supplementary Table S5, significant reversal of CAM-DR was achieved by treatment with anti-CD49d (α4-integrin) and anti-CD54 (ICAM-1) antibodies in KMS-12BM and U266 cells, albeit the effect of the former was much stronger. The effectiveness of anti-CD54 may stem from its effects on stromal cells, because previous studies revealed that CD54 was expressed on BM stromal cells (Corso *et al.*, 2005). In contrast, the other antibodies failed to revert CAM-DR in KMS-12BM and U266 cells. Unexpectedly, CAM-DR was not affected by any antibodies in RPMI8226 cells, probably due to the relatively low expression of CD49d. Although there was slight discrepancy between the results obtained with shRNA/siRNA and adhesion-blocking antibodies, our data clearly indicate that VLA-4, a heterodimer of CD49d and CD29, is the most important adhesion molecule for CAM-DR in MM cells.

Downregulation of CD49d expression by bortezomib
It is tempting to speculate that bortezomib modulates the expression of CD49d in MM cells from our two findings: the relative resistance of bortezomib to CAM-DR (see Figure 2) and equal reversal of CAM-DR to all three drugs on disruption of VLA-4 signaling (see Figure 3). In support of this view, Duechler *et al.* (2005) reported that bortezomib decreased the surface expression of CD23 in chronic lymphocytic leukemia. Therefore, we investigated the effect of bortezomib and other anti-myeloma drugs on the expression of adhesion molecules on MM cell lines. Figure 5a and Supplementary Figure S4 show the representative data of flow cytometric analysis of viable KMS-12BM cells before and after treatment with bortezomib. Untreated KMS-12BM cells strongly expressed CD29, CD44, CD49d, CD54, CD138 and CD184. Bortezomib did not affect the expression levels of CD29, CD44, CD54, CD138 and CD184, but decreased the expression of CD49d from 99.4 ± 0.1 to $34.5 \pm 0.9\%$ ($n=3$, $P<0.05$). Bortezomib-induced downregulation of CD49d expression was similarly observed in RPMI8226 and U266 cells: from 33.2 ± 0.6 to $12.4 \pm 1.9\%$ in RPMI8226 cells ($n=3$, $P<0.05$) and from 99.4 ± 0.04 to $74.3 \pm 4.1\%$ in U266 cells ($n=3$, $P<0.05$). Furthermore, we confirmed bortezomib-mediated reduction of CD49d expression by immunoblotting using whole-cell lysates (Figure 5b), semiquantitative reverse transcription (RT)–PCR (Figure 5c) and real-time quantitative RT–PCR (Figure 5d), suggesting that this phenomenon takes place at mRNA levels. In striking contrast, other anti-myeloma drugs, such as vincristine, doxorubicin and dexamethasone, did not affect the expression of CD49d (Figure 5e; Supplementary Figure S4) and other adhesion molecules (Supplementary Figure S5) in any cell lines examined. The specific reduction of CD49d expression by bortezomib may underlie the relative resistance of the drug to CAM-DR in MM cells.

Next, we investigated the mechanisms by which bortezomib suppresses the expression of CD49d mRNA. For this purpose, we directly inhibited NF-κB activity in KMS-12BM cells using SN-50 peptide, which interferes with nuclear translocation of p50 by binding to its nuclear localization sequence (Lin *et al.*, 1995). Surface expression of CD49d was not affected by the

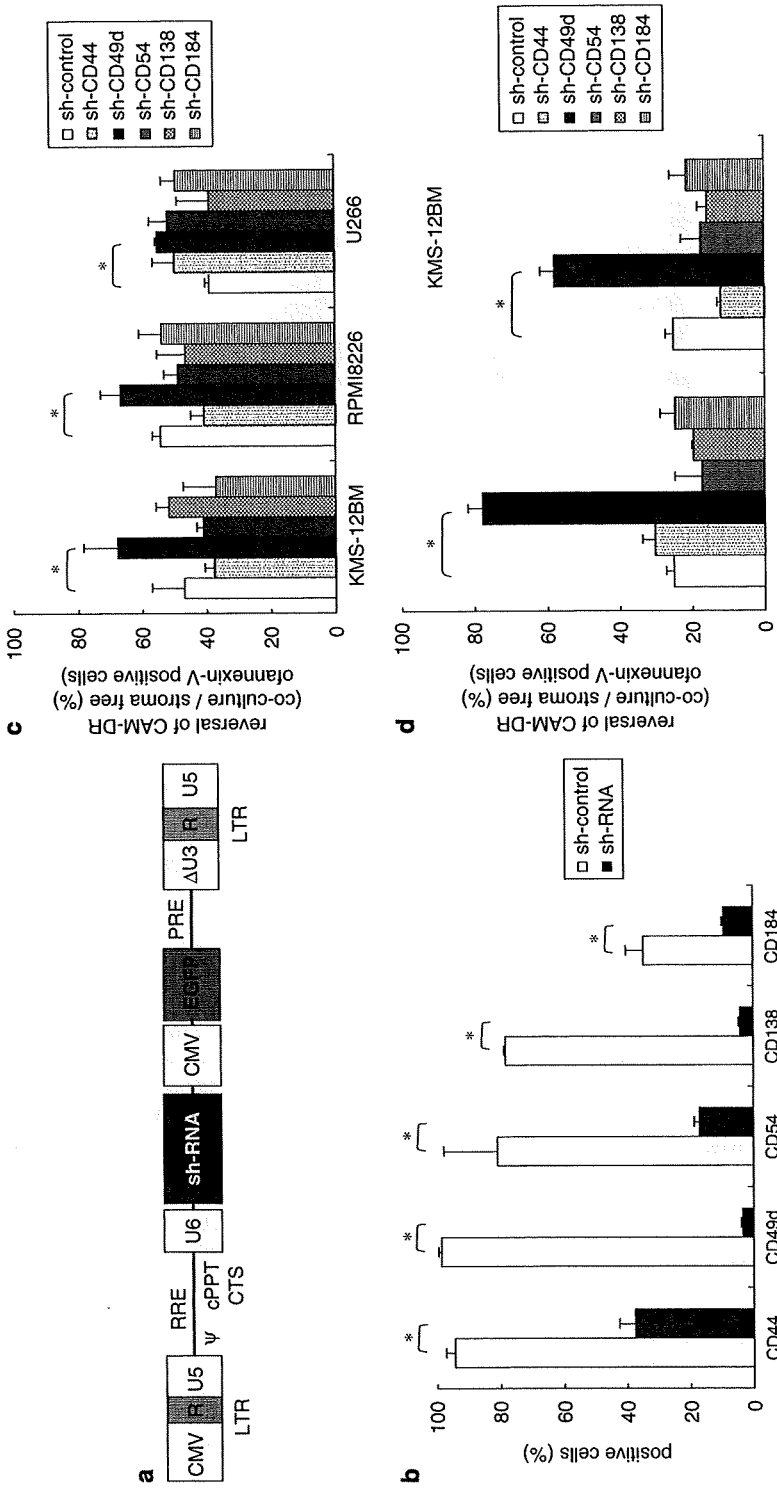


Figure 3 Effects of short hairpin RNA (shRNA)-mediated knockdown of adhesion molecules on adhesion-mediated drug resistance (CAM-DR) in multiple myeloma (MM) cells. (**a**) Schematic representation of a pLL3.7 lentiviral shRNA expression vector: U5 and U6 indicate U5 and U6 promoters, respectively; EGFP, enhanced green fluorescent protein; ψ, a packaging signal; RRE, rev-responsive element; cPPT, central polypurine tract; CTS, central termination sequence; CMV, cytomegalovirus promoter; PRE, wood-chuck hepatitis virus post-transcriptional regulatory element and LTR, long terminal repeat. See Materials and methods for details of construction. (**b**) KMS-12BM cells were transfected with either pLL3.7-sh-CD44, sh-CD49d, sh-CD54, sh-CD138, sh-CD184 or sh control vector. GFP-positive cells were collected by FACSaria flow cytometer, and stained with phycoerythrin (PE)-conjugated anti-CD44, anti-CD49d, anti-CD54, anti-CD138 and anti-CD184 antibodies, or PE-conjugated mouse and rat IgG isotype-matched controls. The means ± s.d. (bars) of three independent experiments are shown. The *P*-values were calculated by Student's *t*-test. $^*P<0.05$ against the sh control. (**c**) MM cell lines stably transfected with shRNA vectors were cultured with 2 nM bortezomib in the presence (co-culture) or absence (stroma free) of stromal cells. After 48 h, MM cells were harvested by pipetting, stained with annexin-V/PE, and subjected to flow cytometric analysis. The *Y* axis shows the reversal of CAM-DR as a ratio (%) of annexin-V positivity under co-culture vs stroma-free conditions. When annexin-V reactivity under co-culture condition is equal to that of stroma-free condition, the reversal of CAM-DR is 100%. The means ± s.d. (bars) of three independent experiments are shown. The *P*-values were calculated by Student's *t*-test. $^*P<0.05$ against the sh control. (**d**) KMS-12BM sublines stably transfected with shRNA vectors were cultured with 1 nM vincristine (VCR) or 50 nM dexamethasone (Dexa) in the presence (co-culture) or absence (stroma free) of stromal cells. After 48 h, the reversal of CAM-DR was examined as described above. $^*P<0.05$ against the sh control.

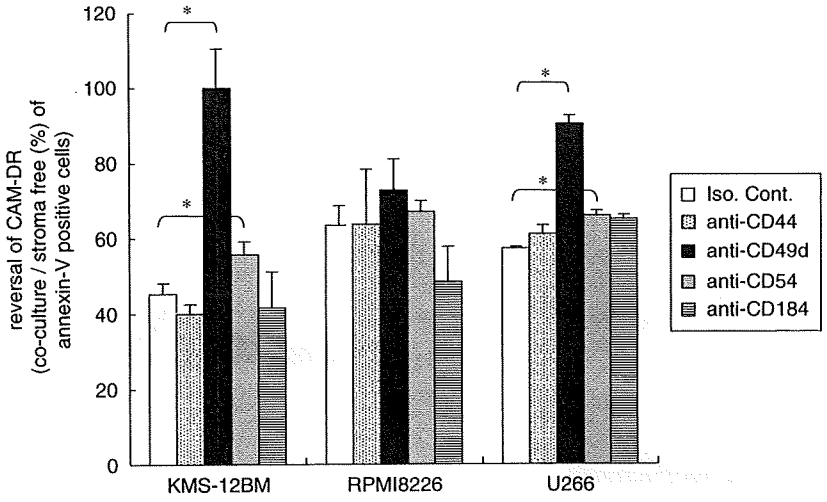


Figure 4 Effects of blocking antibodies against adhesion molecules on adhesion-mediated drug resistance (CAM-DR) in multiple myeloma (MM) cells. MM cell lines were treated with either antibodies against CD44, CD49d, CD54 and CD184 or isotype-matched controls (iso. cont.) at 10 μg/ml at 37 °C for 1 h. After treatment, cells were cultured with 2 nM bortezomib in the presence (co-culture) or absence (stroma free) of stromal cells for 48 h. The reversal of CAM-DR was determined as described in the legend of Figure 3. The means ± s.d. (bars) of three independent experiments are shown. The *P*-values were calculated by Student's *t*-test. $*P<0.05$ against isotype-matched controls.

p50 inhibitory peptide, suggesting that bortezomib-mediated downregulation of CD49d is not a direct consequence of the inhibition of NF-κB activity (Supplementary Figure S6).

The reversal of CAM-DR to conventional anti-myeloma drugs by pretreatment with bortezomib

Given that bortezomib decreases the expression of CD49d, which plays a crucial role in CAM-DR, pretreatment of MM cells with bortezomib could overcome CAM-DR to conventional anti-myeloma drugs. Finally, we tested this hypothesis using the co-culture system for CAM-DR. MM cells were pretreated with bortezomib for 24 h, followed by exposure to either vincristine or dexamethasone for additional 24 h in the presence or absence of stroma cells. Pretreatment with bortezomib significantly reversed CAM-DR to both vincristine and dexamethasone in all three cell lines tested (Figure 6a), which coincided with the detachment of myeloma cells (Figure 6b). In particular, CAM-DR to VCR was almost completely inhibited in KMS-12BM and RPMI8226 cells: reversal ratios were 87.4 ± 3.2 and 104.6 ± 19.4%, respectively. In U266 cells, the effects of bortezomib were moderate but significant. This may be attributable to the relatively weak effects of bortezomib on CD49d expression in U266 cells (see Figure 5d). It should be emphasized that the combination of bortezomib with either vincristine or dexamethasone at the doses used in this experiment did not show additive effects under stroma-free condition (data not shown).

Discussion

In this study, we have clearly demonstrated that VLA-4, a heterodimer of CD49d/CD29, plays a critical role in CAM-DR of MM using a unique strategy involving myeloma cell lines in which individual adhesion molecules were stably knocked down by the aid of shRNA. In support of our finding, several studies described the roles of VLA-4 in the pathophysiology of MM. For example, CD29-mediated adhesion of MM cells to fibronectin upregulated the expression of the CDK inhibitor p27 and induced NF-κB activation, both of which confer CAM-DR to MM (Chauhan *et al.*, 1996; Hazlehurst *et al.*, 2000; Landowski *et al.*, 2003). Two independent groups reported that administration of anti-CD49d antibody suppressed the growth of MM cells in murine xenograft models (Mori *et al.*, 2004; Olson *et al.*, 2005). In line with these experimental findings, Schmidmaier *et al.* (2006) found that MM patients with primary multidrug resistance showed significantly higher concentrations of serum VLA-4 and ICAM-1 than responders. The involvement of VLA-4 in CAM-DR was also demonstrated in AML by the seminal study of Matsunaga *et al.* (2003), in which the combination of cytosine arabinoside and anti-CD49d antibody achieved a 100% survival rate in mice transplanted with AML cells. These findings strongly suggest that VLA-4-mediated signaling is important for the development of DR in MM and AML cells both *in vitro* and *in vivo*.

In addition, we obtained evidence indicating that bortezomib can overcome CAM-DR by selectively downregulating CD49d expression in myeloma cells. This ability was specific for bortezomib and was not observed in other commonly used anti-myeloma drugs such as vincristine, doxorubicin and dexamethasone. Moreover, we have found that bortezomib represses the expression of CD49d at mRNA levels. Regarding the mechanisms of this phenomenon, the direct involvement of NF-κB is unlikely because the p50 inhibitory peptide

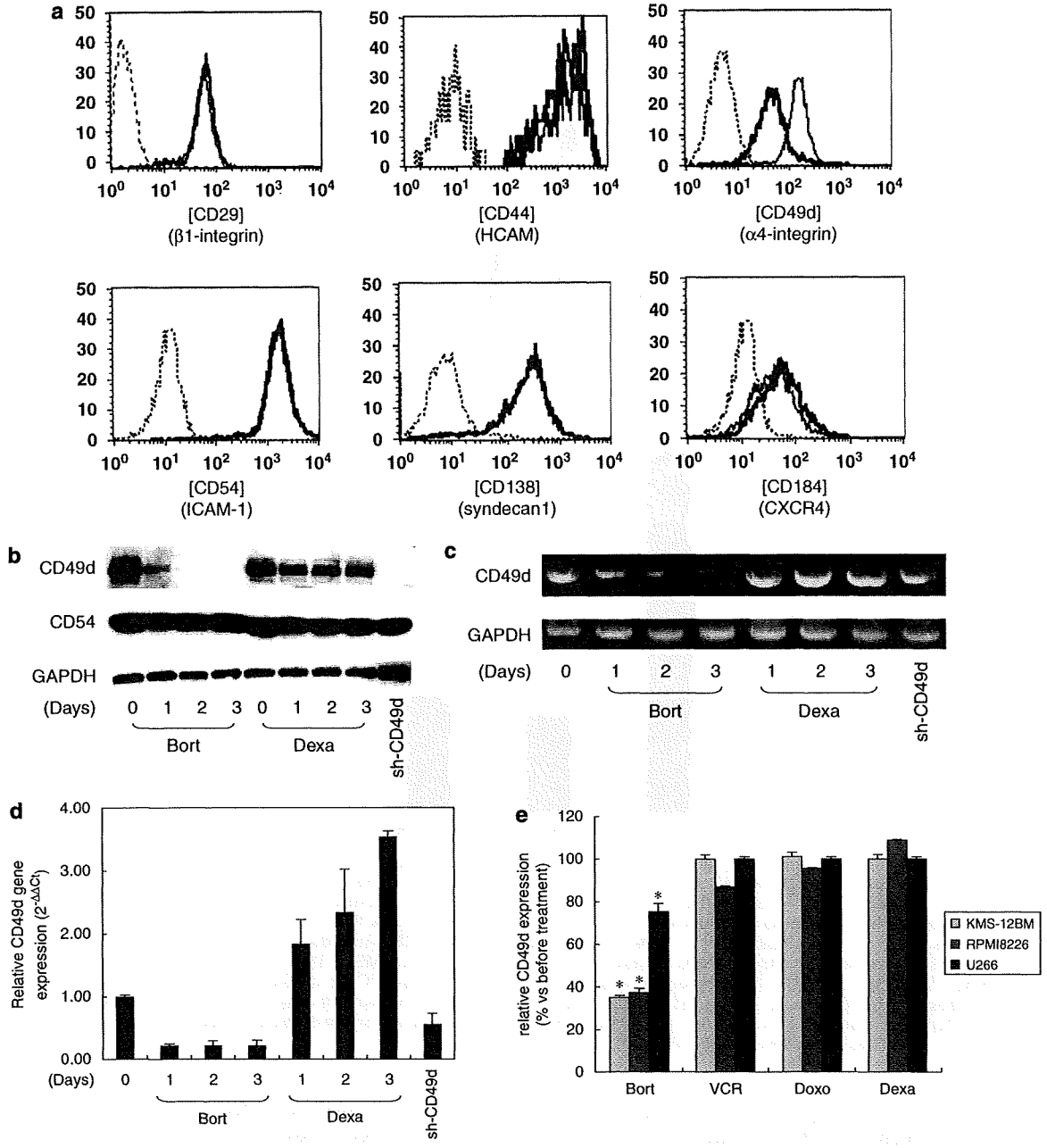


Figure 5 Effects of anti-myeloma drugs on the expression of adhesion molecules in multiple myeloma (MM) cells. (**a**) Surface expression of CD29, CD44, CD49d, CD54, CD138 and CD184 was detected by flow cytometry on KMS-12BM cells before and after treatment with 5 nM bortezomib for 48 h. Thin lines, bold lines and dotted lines show plots before treatment, after treatment and of isotype-matched controls, respectively. Representative histograms of three independent experiments are shown. (**b**) KMS12-BM cells were treated with either 5 nM bortezomib (Bort) or 50 nM dexamethasone (Dexa) for up to 3 days. We used a KMS12-BM subline transfected with pLL3.7-sh-CD49d (sh-CD49d) as a control. Cells were harvested at the indicated time points, and subjected to immunoblot analysis for the expression of CD49d, CD54 and GAPDH (loading control). (**c**) Total cellular RNA was isolated simultaneously at the experiments described in (**b**), and subjected to semiquantitative reverse transcription (RT)-PCR for the expression of CD49d and GAPDH (loading control). (**d**) Total cellular RNA was isolated simultaneously at the experiments described in (**b**), and subjected to real-time quantitative reverse transcription (RT)-PCR. The expression of CD49d was normalized to that of GAPDH and quantified by the $2^{-\Delta\Delta C_t}$ method. (**e**) The expression of CD49d was detected before and after bortezomib treatment in KMS-12BM, RPMI8226 and U266 MM cells by flow cytometry. The concentrations of bortezomib were 5, 2 and 2 nM for KMS-12BM, RPMI8226 and U266 cells, respectively. Data shown are the means ± s.d. (bars) of relative CD49d expression (ratio (%) of CD49d positivity after vs before treatment) of three independent experiments. The *P*-values were calculated by Student's *t*-test. $^*P<0.05$ against the values obtained before treatment.