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III. 研究成果の刊行物・別冊

Original Contribution

A Pharmacokinetic Study Evaluating the Relationship Between Treatment Efficacy and Incidence of Adverse Events with Thalidomide Plasma Concentrations in Patients with Refractory Multiple Myeloma

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Abstract

Background: Multiple myeloma (MM) is a clonal disorder of plasma cells, accounting for 10% of hematologic malignancies. Relapsed or refractory MM has a poor prognosis. Thalidomide has been reported to be effective for these patients; however, high-dose thalidomide has induced many adverse events, including in the nervous, gastrointestinal, and hematopoietic systems in approximately 20%-50% of patients. Recently, low-dose thalidomide therapy has been used in many countries in order to reduce these adverse events. The objective of this study was to determine whether plasma concentration of thalidomide is related to the efficacy and the development of adverse events in patients with refractory MM treated with low-dose thalidomide plus low-dose dexamethasone. **Patients and Methods:** A total of 66 patients (age range, 40-74 years) presenting with progressive disease after previous treatments were treated with low-dose thalidomide and low-dose dexamethasone. Thalidomide was administered orally at 100 mg/day for the first week. When severe adverse events did not develop, the dose was increased to 200 mg/day in the second week and was continued until progression. Dexamethasone was administered at a dose of 4 mg/day for the first 4 weeks, then decreased by 1 mg every week, and finally maintained at 1 mg/day. Plasma trough concentration of thalidomide 3 days after thalidomide treatment was analyzed by high-performance liquid chromatography in 45 patients (age range, 42-74 years) who agreed to participate in this study of thalidomide concentration analysis. **Results:** The mean concentrations at 100 mg/day and 200 mg/day were 0.343 μg/mL (range, 0.05-1.45 μg/mL) and 0.875 μg/mL (range, 0.19-2.09 μg/mL), respectively. The overall response rate (near-complete response + partial response + minimal response) of this treatment was 73%. Five had stable disease, and 3 patients experienced progressive disease. There was no relationship between the concentration of thalidomide in the plasma and the efficacy ($P > .8$). Severe adverse events, including grade > 2 nonhematologic and grade > 3 hematologic adverse events, were observed in 21 patients (46.6%). There was no significant difference in the concentration of thalidomide between the patients with and without severe adverse events ($P > .843$). **Conclusion:** The thalidomide concentration in the plasma does not predict treatment efficacy and the development of adverse events.

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Keywords: Angiogenesis, Dexamethasone, M-protein, Plasma trough concentration

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Introduction

Multiple myeloma (MM) is one of the clonal disorders of plasma cells, accounting for 10% of hematologic malignancies. The median overall survival (OS) has been improved by high-dose chemotherapy with autologous stem cell transplantation (autoSCT).[1] However, the treatment of relapsed patients after conventional and/or high-dose chemotherapy with autoSCT remains unsatisfactory.

Thalidomide was introduced in the 1950s as a sedative. However, in 1961, Lenz disclosed a close relationship between the administration of thalidomide to pregnant women and the development of a peculiar deformity (a teratogenic effect) in their babies.[2] Biotransformation of thalidomide occurs by hydrolysis or by hepatic CYP450-mediated hydroxylation, and both types of products are generally referred to as metabolites.[3,4] In 1994, D'Amato et al showed that thalidomide inhibited angiogenesis in their experiments with rabbits and suggested thalidomide as a therapeutic agent for diseases that involve angiogenesis, particularly those of malignant tumors.[5] Furthermore, in 1994, Vacca et al reported that the bone marrow of patients with myeloma was rich in microvessels and that there was a positive relationship between the disease activity of MM and the density of bone marrow microvasculatures.[6] Based on these data, a clinical trial with thalidomide was initiated in many countries as a new therapeutic agent for patients with relapsed/refractory myeloma. Barlogie et al reported the efficacy of thalidomide as a single agent in patients with refractory myeloma. Thalidomide was administered nightly at 200 mg. The dose was increased by 200 mg every 2 weeks for 6 weeks, so that the final dose was 800 mg/day. Thalidomide exhibited a 37% overall response rate (ORR) comprised of minimal response and 2-year survival rates of 60% in 169 patients with refractory myeloma.[7] In addition, they showed that the RR and survival were improved by the escalating doses of thalidomide. Grade > 3 toxicities included sedation/somnolence in 25%, constipation in 16%, and mainly sensory neuropathy in 9% of the patients. However, others reported that high-dose thalidomide caused many adverse effects involving the nervous, gastrointestinal, and hematopoietic systems. Recently, low-dose thalidomide therapy has been used in many countries in order to reduce these adverse events.

In the current study, we examined relationships between the plasma concentration of thalidomide and the efficacy as well as the development of adverse events when the patients with refractory myeloma were treated with low-dose thalidomide plus low-dose dexamethasone.

Patients and Methods

Patients

Between December 2002 and December 2005, a total of 66 patients (age range, 40-74 years) who relapsed after and were refractory to previous treatments were treated with low-dose thalidomide and low-dose dexamethasone after obtaining written informed consent. The protocol was approved by the Japan Myeloma Study Group Institutional Review Board. The diagnosis of MM was made according to the Southwest Oncology Group criteria (Table 1). The following patients were excluded from the study: (1) pregnant women and female patients with the possibility of conception;

Table 1 Diagnostic Criteria for Myeloma Proposed by the Southwest Oncology Group

Major Criteria
I. Plasmacytoma on tissue biopsy
II. Bone marrow plasmacytosis with > 30% plasma cells
III. Monoclonal globulin spike on serum electrophoresis exceeding 3.5 g/dL for IgG peaks or 2.0 g/dL for IgA peaks, ≥ 1.0 g/day of κ or λ light chain excretion on urine electrophoresis in the absence of amyloidosis
Minor Criteria
a. Bone marrow plasmacytosis with 10%-30% plasma cells
b. Monoclonal globulin spike present but less than the levels defined above
c. Lytic bone lesions
d. Normal IgM < 50 mg/dL, IgA < 100 mg/dL, or IgG < 600 mg/dL
Diagnosis will be confirmed when any of the following features are documented in symptomatic patients with clearly progressive disease. The diagnosis of myeloma requires a minimum of 1 major + 1 minor criterion or 3 minor criteria that must include a + b:
1. I + b, I + c, I + d
2. II + b, II + c, II + d
3. III + a, III + c, II + d
4. a + b + c, a + b + d

Abbreviation: Ig = immunoglobulin

(2) patients who had received > 4 previous therapy regimens; (3) patients having another malignancy, hepatitis B infection, abnormal liver function (serum bilirubin > 2 mg/dL, aspartate aminotransferase > 2.5 times normal, or alanine aminotransaminase > 2.5 times normal), abnormal renal function (serum creatinine > 5 mg/dL), chronic respiratory diseases (PaO_2 < 60 Torr or SaO_2 < 90%), abnormal cardiac function (systolic ejection fraction < 50%), any severe drug allergy; (4) patients aged ≥ 75 years; and (5) patients with a poor Eastern Cooperative Oncology Group performance status of 4. Because myelosuppression was one of the most serious adverse events in Japanese reports, we also excluded the patients with a white blood cell count below 3×10^9/L and/or a platelet count below 7.5×10^9/L corresponding to grade 1 toxicity in the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE, version 2.0, 1998). All patients were enrolled 4 weeks after previous treatment.

Treatment

Thalidomide was administered orally at 100 mg/day before sleep for the first week. When the patients did not experience nonhematologic toxicities grade > 2 and hematologic toxicities grade > 3 according to the CTCAE, version 2.0, the dosage was increased to 200 mg/day in the second week and was continued until disease progression and revelation of unacceptable adverse events. Dexamethasone was administered at 4 mg/day for the first 4 weeks, then decreased by 1 mg every week, and finally maintained at 1 mg/day. Antithrombotic prophylaxis was not performed in this study.

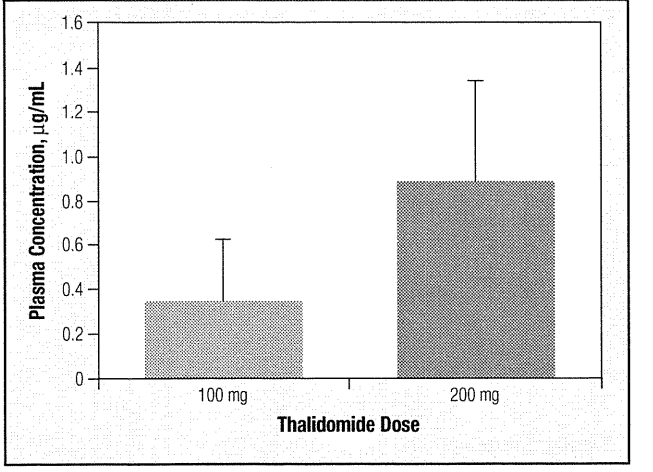
The primary endpoint of this study was RR. In Japan, immunofixation was not available in this study period, and

Table 2 Patient Characteristics

Characteristic	Value
Median Age, Years	64
Sex, n	
Male	17
Female	28
Number of Previous Therapies*	
1	26
2	8
3	11
Previous Therapy	
Conventional chemotherapy	30
Autologous stem cell transplantation	15
Ig Subtype	
IgG	35
IgA	4
IgD	1
Light chain only	5
Light Chain	
κ	29
λ	16
ECOG PS	
0	14
1	16
2	11
3	4
4	0

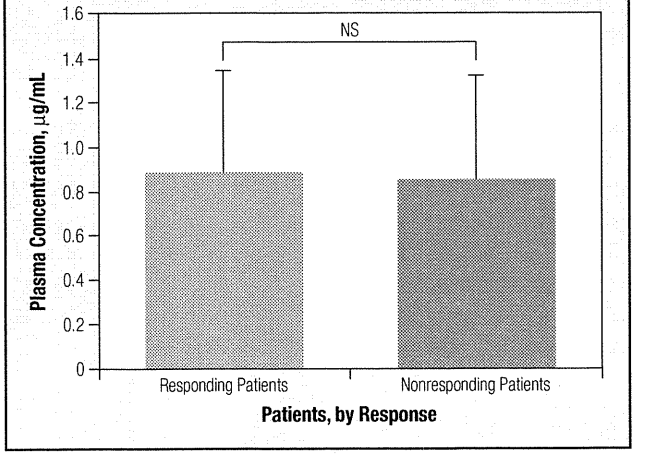
*The number of treatments in patients treated with autologous stem cell transplantation was counted as 1.
Abbreviations: ECOG = Eastern Cooperative Oncology Group; Ig = immunoglobulin; PS = performance status

Figure 1 Trough Levels of Thalidomide in Plasma



The mean concentrations at 100 mg/day (45 cases) and 200 mg/day (42 cases) were 0.343 μg/mL (95% CI, 0.258-0.428) and 0.875 μg/mL (95% CI, 0.732-1.018), respectively.

Figure 2 Plasma Thalidomide Concentration and Treatment Response



At 200 mg/day, the mean trough concentrations in the 30 responding patients and 12 nonresponding patients were 0.886 μg/mL (95% CI, 0.715-1.058) and 0.848 μg/mL (95% CI, 0.543-1.154), respectively. There was no significant difference between the 2 groups ($P > .8$).

complete response (CR) was not defined. A near-CR (nCR) was defined as the lack of detectable M-protein in serum/urine protein electrophoresis. A partial response (PR) was defined as a decrease of > 50% in serum M-protein and/or > 90% reduction of urinary M-protein, a minimal response (MR) as a decrease of 25%-49% in serum M-protein and/or 50%-89% in urinary M-protein, stable disease (SD) as no change in M-protein, and progressive disease (PD) as an increase of 25% in M-protein. These responses were evaluated at the maximum reduction of M-protein, and on 2 separate occasions, ≥ 6 weeks apart. Secondary endpoints were the incidence of adverse events and OS and progression-free survival.

The assessment of adverse events was also performed according to the CTCAE, version 2.0.

Measurement of Thalidomide Concentration in Plasma

The plasma was harvested within 1 hour of obtaining samples. The trough concentration of thalidomide immediately before the administration was determined at day 3 of the initial thalidomide dose and 3 days after dose escalation. Thalidomide concentration in the plasma was determined with the use of high-performance liquid chromatography (HPLC) by a modified version of the methods of Figg et al and Simmons et al.[8,9] Plasma was immediately separated from the blood samples, and the equal volume of 10% sulfuric acid was added to the plasma. A solution of phenacetin in methanol was added as an internal standard. After extraction with 2.5 mL of a solution of dichloromethane and n-hexane (1:1 v/v), followed by centrifugation (1700 g) for 10 minutes, the organic layer was removed and evaporated with a centrifugal evaporator. The residue was dissolved in the HPLC mobile phase buffer (methanol:water, 3:7 v/v), and the solution thus obtained was loaded onto an HPLC column. The pump was a Waters Alliance 2690 separation module, and the column (4.6 mm × 150 mm) contained TSKgel ODS-80TM (TOSHO, Tokyo, Japan). The column temperature was

Table 3 The Relationship Between Thalidomide Concentration in Plasma and the Incidence of Adverse Events Caused by Thalidomide

Adverse Event	Revelation	Number of Cases	Mean Plasma Concentration of Thalidomide 200 mg/day (μg/mL)	*P* Value
Severe Adverse Events (Grade > 2 Nonhematologic and Grade > 3 Hematologic)	+	21	0.89	.843
	–	24	0.86	
Drowsiness (Grade > 1)	+	9	1.16	< .05
	–	36	0.81	
Peripheral Neuropathy (Grade > 1)	+	16	0.94	.478
	–	29	0.84	
Skin Eruption (Grade > 1)	+	6	0.66	.265
	–	39	0.91	
Constipation (Grade > 1)	+	12	1.07	.103
	–	33	0.81	
Deep Vein Thrombosis (Grade > 1)	+	1	0.59	Not evaluated
	–	44	0.56	
Leukopenia (Grade > 2)	+	18	0.83	.603
	–	27	0.91	
Thrombocytopenia (Grade > 2)	+	9	1.04	.204
	–	36	0.83	

kept at 40°C. The flow rate was 1.0 mL/min. The absorbance at 220 nm was detected using a dual-wavelength ultraviolet absorption detector (Waters, model 2487). The retention time of thalidomide was approximately 6 minutes, and the retention time of the internal standard was about 12 minutes. There was no observed measurement obstruction by the biologic sample. Measurement was possible in the range of 50 μg/mL to 10 μg/mL, with a coefficient of variation of ≤ 10% and a deviation of ≤ 10%.

Statistical Analysis

Response and serum concentration of thalidomide were compared by Student *t* test. The adverse events and serum concentration of thalidomide were also compared by Student *t* test.

Results

Plasma concentration of thalidomide was analyzed in 45 patients (age range, 42-74 years) who agreed to participate in this study of thalidomide concentration analysis. Male:female ratio and median age were 0.6 (17:28) and 64 years, respectively. The immunoglobulin (Ig) subtypes were as follows: IgG, 35 patients; IgA, 4 patients; IgD, 1 patient; and light chain only, 5 patients. A total of 15 patients had relapsed after previous autologous peripheral blood SCT, and the remaining 30 patients were refractory to previous conventional chemotherapies (Table 2).

Concentration of Thalidomide in Plasma

The trough levels of thalidomide in the plasma according to the different doses of thalidomide are shown in Figure 1. Dose escalation to 200 mg was unfeasible in 3 cases because of drug eruption, peripheral neuropathy, and constipation. The mean trough concentrations at doses of 100 mg/day (45 cases) and 200 mg/day (42 cases) were 0.343 μg/mL (95% CI, 0.258-0.428) and 0.875 μg/mL (95% CI, 0.732-1.018), respectively. The differences in the trough levels among the individuals varied considerably. However, in all but 1 patient, the trough levels of thalidomide 200 mg/day were significantly higher than those of thalidomide 100 mg/day ($P < .0001$).

Relationship Between the Response and Plasma Concentration of Thalidomide

The average duration of thalidomide was 9 months. Near-CR, PR, and MR were obtained in 2, 12, and 19 patients, respectively. The ORR of the present treatment (nCR + PR + MR) was 73% (median follow-up, 16.7 months). In these responding patients, 3 had response at 100 mg/day without dose escalation to 200 mg/day. In another 5 patients, there was no change in the M-protein levels; in another 3 patients, there was a progressive disease. In the remaining 4 patients, the effect of treatment was not evaluated, because of early death and the short duration of thalidomide administration (< 1 week). One patient died as a result of sepsis caused by severe neutropenia. Grade 4 neutropenia occurred in 2 patients, and grade 3 liver dysfunction occurred in 1 patient.

In the 30 patients with response ≥ MR at 200 mg/day and in the 12 patients with response < SD, the mean trough concentrations of thalidomide 200 mg/day were 0.886 μg/mL (95% CI, 0.715-1.058) and 0.848 μg/mL (95% CI, 0.543-1.154), respectively, and there was no significant difference between the 2 groups ($P > .8$; Figure 2). This result indicated that there was no relationship between the efficacy and the concentration of thalidomide in the plasma.

Relationship Between the Development of Adverse Events and the Plasma Concentration of Thalidomide

The frequent adverse events observed during the treatment are listed in Table 3, along with the concentration of thalidomide in the plasma. Severe adverse events, including grade > 2 nonhematologic and grade > 3 hematologic, were observed in 21 patients (46.6%). There was no significant difference in the concentration of thalidomide between the patients with and without severe adverse events except for drowsiness. Drowsiness, peripheral neuropathy, skin eruption, constipation, and deep vein thrombosis were observed in 9, 16, 6, 12, and 1 patient, respectively. Drowsiness, peripheral neuropathy, skin eruption, constipation, and deep vein thrombosis grade > 2 were observed in 4, 4, 4, 2, and 1 patient, respectively. The incidence of deep vein thrombosis was much lower in the current study than the reports from the United States and Europe. On the other hand, the incidences of leukopenia and thrombocytopenia were higher in the current study than the reports from the United States and Europe. Leukopenia and thrombocytopenia grade > 3 were observed in 5 patients and 1 patient, respectively. However, there was no relationship between the incidence of these 2 adverse events and the concentration of thalidomide.

Hyperglycemia, depression, and sleep disturbance, which might have been induced by dexamethasone, were observed in only 4, 2, and 2 patients, respectively.

Discussion

The RR of single-agent thalidomide in patients with refractory myeloma has been reported as 30%-40%. Barlogie et al analyzed the efficacy according to various doses of thalidomide in a phase II study. They found that patients who took higher doses of thalidomide in a 3-month period had a greater M-protein reduction rate and a longer survival than others who took smaller doses of thalidomide, in high-risk patients as well as in low-risk patients.[7] On the other hand, Durie reported that, with a thalidomide dose of only 200 mg/day, responses ≥ MR was obtained in 44%, and the survival rate at 2 years was 22%.[10] Johnston and Abdalla also reported that PR was obtained in 42% of patients with resistant/refractory myeloma with low-dose thalidomide therapy (median dose, 175 mg/day), with a lower incidence of adverse events.[11]

When thalidomide was administered with dexamethasone, the RR was increased to 50%-60%.[12-14] Preclinical studies have demonstrated the synergic effects of thalidomide and dexamethasone. Thalidomide has induced glucocorticoid receptor expression on myeloma cells.[15] In addition, dexamethasone has increased the antiproliferative effect of thalidomide on myeloma cells in a dose-dependent manner.[16] In the United States and Europe, high-dose dexamethasone has been administered together with thalidomide. Low-dose thalidomide plus high-dose dexamethasone therapy showed the same efficacy as high-dose thalidomide plus high-dose dexamethasone therapy.[17,18] However, many adverse events have been observed in association with high-dose dexamethasone therapy, including hyperglycemia, depression, sleep disturbance, and fluid retention. Myers and colleagues reported that thalidomide plus low-dose dexamethasone therapy was effective in patients with refractory myeloma.[19] They used dexamethasone 4 mg/day, reducing the dose slowly over a few months according to the reports of Tiplady and Summerfield.[20] According to these reports, we decided to administer low-dose thalidomide plus low-dose dexamethasone in the present phase II study.

Figg et al reported the pharmacokinetics of thalidomide in patients with prostate cancer.[8] They reported that the half-life of thalidomide was 6.52 hours after a single dose of 200 mg, 7.08 hours after continuous daily dose of 200 mg, 18.25 hours after a single dose of 800 mg, and 16.19 hours after continuous daily dose of 800 mg. They also reported that, with the continuous daily dose of thalidomide, the concentration of thalidomide in plasma increased as the dose increased and that the mean maximum concentration of thalidomide 200 mg was 1.81 μg/mL. In the current study, the mean trough plasma concentration of thalidomide also increased as the dose increased.

The relationship between the efficacy and the plasma concentration of thalidomide has not been established. Biotransformation of thalidomide occurs by hydrolysis in blood and/or by hepatic CYP450-mediated hydroxylation.[21-24] The enantiomers of thalidomide undergo spontaneous hydrolysis and fast chiral interconversion at physiologic pH. After hydrolysis, 12 metabolites have been identified in humans. It is theoretically possible that hydroxylation made > 100 metabolites and degradation products. Several investigators have reported that the thalidomide metabolites, especially hydroxylated derivatives, have antiangiogenic effects and anticancer activities.[25,26] These reports suggest that the efficacy depends on the concentration of thalidomide metabolites rather than the concentration of thalidomide itself. In addition, polymorphism of the *CYP2C19* gene is reported to be associated with the efficacy to thalidomide in MM. According to these data, the interindividual difference of efficacy might be induced by the difference of metabolic activities.

We have reported that thalidomide concentration in plasma might be a marker of adverse events in small study.[27] However, the adverse events (other than drowsiness) did not correlate with the plasma concentration of thalidomide in the current study using low-dose thalidomide. Thalidomide was launched as a safe sedative,[28] which would explain the high incidence of drowsiness in the patients with high plasma concentration of thalidomide. It has been reported that the teratogenic properties of thalidomide might require prerequisite biotransformation, and chirally stable hydrolysis metabolites might be implicated. Like the efficacy of thalidomide, the development of adverse events with thalidomide seems to depend on the plasma concentration of thalidomide metabolites rather than on the concentration of thalidomide itself.

Further studies about the metabolism of thalidomide are needed to predict the response and the development of adverse events.

The incidence of adverse events induced by dexamethasone, including hyperglycemia, depression, and sleep disturbance, was low in the current study.

Conclusion

Low-dose thalidomide plus low-dose dexamethasone therapy was effective in patients with refractory MM. The incidence of adverse events other than leukopenia was low, compared with the reports from the United States and Europe. The thalidomide concentration in the plasma does not predict the treatment efficacy and the development of adverse events.

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Disclosures

The authors have no relevant financial relationships to disclose.

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ORIGINAL ARTICLE

PU.1 induces apoptosis in myeloma cells through direct transactivation of TRAIL

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We earlier reported that PU.1 was downregulated in myeloma cell lines and myeloma cells in a subset of myeloma patients, and that conditional PU.1 expression in PU.1-negative myeloma cell lines, U266 and KMS12PE, induced growth arrest and apoptosis. To elucidate the molecular mechanisms of the growth arrest and apoptosis, we performed DNA microarray analyses to compare the difference in gene expression before and after PU.1 induction in U266 cells. Among cell cycle-related genes, cyclin A2, cyclin B1, CDK2 and CDK4 were downregulated and p21 was upregulated, although among apoptosis-related genes, tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) was found highly upregulated. When TRAIL was knocked down by small interference RNAs, apoptosis of PU-1-expressing cells was inhibited, suggesting that TRAIL has a critical role in PU.1-induced apoptosis in both U266 and KMS12PE myeloma cells. In both U266 and KMS12PE cells expressing PU.1, PU.1 directly bound to a region 30 bp downstream of the transcription start site of the *TRAIL* gene. Upregulation of PU.1-induced transactivation of the *TRAIL* promoter in reporter assays, and disruption of the PU.1-binding site in the *TRAIL* promoter eliminated this transactivation. Therefore, we conclude that PU.1 is capable of inducing apoptosis in certain myeloma cells by direct transactivation of TRAIL.
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Keywords: myeloma; PU.1; TRAIL; apoptosis; p21

Introduction

Multiple myeloma is an incurable hematological malignancy that is resistant to several chemotherapeutic agents as well as hematopoietic stem cell transplantation (Attal *et al.*, 2003). Recently, several new types of agents for myeloma, such as thalidomide, lenalidomide and the proteasome inhibitor bortezomib, have been analysed, but these agents do not lead to long-term complete remission of myeloma, although they are very effective and improve the survival duration of patients (Singhal *et al.*, 1999; Hideshima *et al.*, 2001, 2003). In contrast, the new types of molecular target agents for leukemia and lymphoma include imatinib, which targets bcr-abl tyrosine kinase and brings about striking improvement of the remission and survival rates of chronic myeloid leukemia patients, and rituximab, which targets CD20 antigen on the surface of B lymphoma cells and improves the remission and survival rates of lymphoma patients. These observations indicate that understanding the pathogenesis of a disease is very important for designing new types of molecular target agents. Nevertheless, in contrast to leukemia and lymphoma, the pathogenesis of multiple myeloma is still not well understood.

The PU.1 is an ETS family transcription factor that is important for both myelopoiesis and lymphopoiesis (Klemsz *et al.*, 1990; McKercher *et al.*, 1996). Gene expression generally requires long-range distal enhancer regions in addition to the promoter region (Yu *et al.*, 1999; Loots *et al.*, 2000; Radomska *et al.*, 2002; Okuno *et al.*, 2002a, 2002b). In the case of the PU.1 gene, the long-range distal enhancer region is located in a 14-kb 5′-upstream region in mice and a 17-kb 5′-upstream region in humans (Li *et al.*, 2001; Okuno *et al.*, 2005; Tatetsu *et al.*, 2007). In Friend leukemia, FEEV is integrated into the 14-kb 5′-upstream region of the *PU.1* gene locus and results in failure of PU.1 downregulation in erythroblasts, thereby leading to erythroleukemia in mice (Moreau-Gachelin *et al.*, 1988). We earlier reported that proper murine PU.1 gene expression requires the 14-kb 5′-upstream regulatory region, which consists of two highly conserved regions among different mammals, and that the FEEV integration site in Friend leukemia is located between these two conserved regions (Okuno *et al.*, 2005). Moreover, conditional knockout of this 14-kb 5′-upstream region led to downregulation of PU.1 to 20% of wild-type mice, and all of these mice developed acute myeloid leukemia (Rosenbauer *et al.*, 2004). These mice also developed T-cell lymphoma with upregulation of PU.1 in T lymphoma cells, because one of the two highly conserved regions in the 14-kb

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5′-upstream region has suppressor activity for PU.1 expression in the T-cell lineage through wnt signaling (Rosenbauer *et al.*, 2006). These data indicate that inappropriate regulation of the PU.1 gene, including failure of downregulation or upregulation in proper differentiation stages, leads to hematological malignancies in different hematological lineages (Tenen, 2003).

We recently reported that PU. 1 is downregulated in the majority of myeloma cell lines and freshly isolated myeloma cells from a subset of multiple myeloma patients (PU.1 low-to-negative subset), whereas normal plasma cells express relatively high levels of PU.1 (Tatetsu *et al.*, 2007). The PU.1 is downregulated by methylation of the 17-kb 5′-enhancer region and the promoter region in myeloma cell lines, whereas only the promoter region is methylated in T-cell lines (Amaravadi and Klemsz, 1999). Myeloma patients in the PU.1 low-to-negative subset may have a poor prognosis. In addition, conditional expression of PU.1 in PU.1-negative myeloma cell lines, U266 and KMS12PE, was found to induce cell growth arrest and apoptosis (Tatetsu *et al.*, 2007). These data suggest that downregulation of PU.1 may be an important genetic change for oncogenesis or growth maintenance of multiple myeloma cells. To elucidate the effects of PU.1 expression in U266 cells, we performed DNA microarray analyses and compared the differences in gene expression before and after PU.1 expression.

Results

Interferon-stimulated genes are mainly upregulated in the U266 myeloma cell line after PU.1 induction

As earlier reported (Tatetsu *et al.*, 2007), we generated a stable U266$^{tetPU.1}$ cell line derived from the PU.1-negative myeloma cell line U266 with a tet-off conditional expression system of PU.1. RNA was purified from U266$^{tetPU.1}$ cells before (day 0) and at days 1 and 3 after PU.1 induction and subjected to DNA microarray analyses (Illumina:Sentrix Human-6 Expression BeadChip). The expression levels of genes were analysed using the GeneSpring7.2 software, and compared among days 0, 1 and 3. A total of 47 296 human genes were analysed, among which 21 565 genes were found to be expressed and 25 731 genes were not expressed. Among the 21 565 genes expressed, 479 genes were upregulated by more than twofold and 1697 genes were downregulated by more than 50% on either day 1 or day 3 after PU.1 induction. The 30 genes showing the highest upregulation after PU.1 induction are shown in Table 1 (day 1) and Supplementary Table S1 (day 3). IFIT1, IFITM1, IFIT2, IFIT4, IFI27, G1P2, C1orf29, LY6E, LAMP3 and G1P3, which are known as IFN-stimulated genes (de Veer *et al.*, 1998), were upregulated by more than 20-fold at day 1 and more than fivefold at day 3. These upregulations were confirmed by semi-quantitative PCR of IFIT4 and IFI27 (data not shown). These data indicate that PU.1 induction activated the expression of many IFN-stimulated genes in U266 myeloma cells. In contrast, the 30 most downregulated genes at day 1 and day 3 after PU.1 induction are shown in Supplementary Table S2 and Supplementary Table S3. Among these downregulated genes, Syndecan 1, which is a marker of plasma cells and known as CD138, was the most downregulated gene 3 days after PU.1 induction.

Table 1 Thirty genes showing the highest upregulation at day 1 after PU.1 induction

Gene	*Fold expression*
IFIT1	167.8
IFITM1	155.2
IFIT2	126.8
IFIT4	98.2
IFI27	83.3
TNFSF10	76.5
G1P2	71.0
IFI44L	55.3
LY6E	44.7
ISG20	43.5
TRIM22	41.6
IFI44	37.5
IFITM3	36.3
OASL	35.7
RSAD2	35.1
PRIC285	28.0
MX1	27.8
LAMP3	25.6
CMPK2	25.5
IRF7	24.8
MT2A	24.7
PARP12	24.7
CXCL10	22.8
USP18	20.8
LOC285510	19.2
IL1RN	18.7
SLC24A1	18.2
SP110	17.6
G1P3	17.2
SP110	16.9

Cell cycle arrest induced by PU.1 may be partially related to upregulation of p21

Next, we analysed the mechanisms of the cell growth arrest of U266 cells induced by PU.1 by comparing the gene expression profiles of cell cycle-related genes. Most of the cyclin genes, particularly cyclin A2, B1, B2, D1, D2 and E2, were all continuously downregulated from day 1 to day 3 after PU.1 induction, and E2F1 and E2F2 were also downregulated (Table 2). Regarding the CDK families, CDK2 and CDK4 were downregulated to 77 and 40%, respectively. We earlier confirmed downregulation of cyclin D1 at the protein level (Tatetsu *et al.*, 2007). In addition, cyclins, CDKs, and E2F were analysed by western blot. We confirmed that cyclin A2 and B1, CDK2 and CDK4 were also downregulated at the protein levels (Figure 1), whereas E2F2 protein level was not changed (data not shown). The downregulation of these cyclins and CDK family members was consistent with the growth arrest of U266$^{tetPU.1}$ cells after PU.1 induction. We further evaluated the expression profiles of growth inhibitory genes, such as the tumor suppressor genes p53, p15 and p16, and found that only p21 was upregulated by 3.4-

Table 2 Changes in cell cycle-related genes after PU.1 induction

Gene symbol	*Day 1/Day 0 (fold)*	*Day 3/Day 0 (fold)*
CCNA1	1.35	1.07
CCNA2	0.47	0.36
CCNB1	0.65	0.86
CCNB2	0.54	0.48
CCNB3	0.93	0.97
CCND1	0.60	0.57
CCND2	0.64	0.34
CCND3	1.94	0.71
CCNE1	0.91	0.84
CCNE2	0.39	0.37
CCNH	1.14	0.70
E2F1	0.49	0.61
E2F2	0.22	0.26
RB1	1.08	1.07
CDK2	0.77	0.81
CDK4	0.40	0.77
CDK6	0.95	0.99
CDK7	0.62	1.10
p15	1.31	1.59
p16	1.09	1.01
p18	0.77	0.64
p19	0.92	0.93
p21	3.40	2.16
p27	0.90	0.80
p53	0.82	1.04
p57	0.79	1.80

fold at day 1 and 2.2-fold at day 3 after PU.1 induction (Table 2). The protein level of p21 was also increased by 4.3-fold (Figure 1). Therefore, we next evaluated the effect of upregulated p21 on the growth arrest of U266$^{tetPU.1}$ cells expressing PU.1 by knocking p21 using stably expressed small interference RNAs (siRNAs) (Figure 2a). Suppression of p21 partially restored growth of U266$^{tetPU.1}$ cells expressing PU.1, suggesting that p21 may be partially involved in the growth arrest of U266$^{tetPU.1}$ cells induced by PU.1 (Figure 2b).

IRF7 shows the highest upregulation and IRF4 does strong downregulation among transcription factors after PU.1 induction

Among the genes upregulated after PU.1 induction, IRF7 was the only transcription factor that was highly upregulated at both day 1 (24.8-fold) and day 3 (6.0-fold) after PU.1 induction (Table 1 and Supplementary Table S1). The protein level of IRF7 was also highly upregulated, as evaluated by western blotting analysis (Figure 1). Therefore, we speculated that IRF7 may be a key molecule for activating the expression of IFN-stimulated genes. In contrast, IRF4, which is also known as MUM1 or Pip and is highly expressed in almost all myeloma cells (Iida *et al.*, 1997), was downregulated to 44% at day 1 and 36% at day 3. Protein level of IRF4 was also strongly downregulated to 1.8% at day 2 and 18% at day 3 after PU.1 induction (Figure 1). Other upregulated transcription factor in both mRNA and protein levels was STAT1, which binds to IRFs and translocates to the nucleus during interferon signal transduction. In conclusion, both the most upregulated transcription factor, IRF7, and the strongly downregulated transcription factor, IRF4, belong to the interferon signal transduction pathway.

We next evaluated the expression levels of genes involved in lineage commitment and differentiation. The critical transcription factors for lineage commitments were evaluated with the DNA microarray data, and RUNX1, which is essential for definitive hematopoiesis and directly regulates PU.1 (Huang *et al.*, 2008), was found to be downregulated to about 50% at days 1 and 3 after PU.1 induction, although no changes in expression were detected for GATA-2 and C/EBPα. To confirm protein expression levels of these transcription factors, we performed western blot analyses and found that protein levels of RUNX1 and C/EBPα were downregulated to 44 and 57% 3 day after PU.1 induction, respectively (Figure 1). In contrast, protein level of GATA-2 was not changed and there was no expression of GATA-1 in U266$^{tetPU.1}$ cells both before and after PU.1 induction by western blot analyses.

TRAIL may be a key molecule for apoptosis of U266$^{tetPU.1}$ and KMS12PE$^{tetPU.1}$ cells induced by PU.1

Among the apoptosis-related genes, *TRAIL (TNFSF10)*, a ligand for death receptors DR4 and DR5, was upregulated by 76.5-fold at day 1 (Table 1) and 1.7-fold at day 3 after PU.1 induction in U266$^{tetPU.1}$ cells, and these data were confirmed by real time–PCR (44.0-fold at day 2) (Figure 3a). Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) was also strongly upregulated (12.1-fold at day 2) after PU.1 induction in KMS12PE$^{tetPU.1}$ cells (Figure 3a). It is well known that TRAIL can induce apoptosis in myeloma cells (Mariani *et al.*, 1997; Mitsiades *et al.*, 2002). Therefore, to clarify whether TRAIL is a key molecule for the apoptosis of U266$^{tetPU.1}$ and KMS12PE$^{tetPU.1}$ cells induced by PU.1, we stably introduced siRNAs for TRAIL into both cell lines and obtained stable transformants showing strong knockdown of TRAIL (Figure 3a). Stable expression of siRNA targeting TRAIL inhibited apoptosis of U266$^{tetPU.1}$ and KMS12PE$^{tetPU.1}$ cells induced by PU.1, whereas scrambled siRNAs did not (Figure 3b), and the difference in apoptosis between siRNA for TRAIL and its scrambled counterparts was statistically significant (Figure 3c). Taken together, TRAIL may mainly induce apoptosis in U266$^{tetPU.1}$ and KMS12PE$^{tetPU.1}$ cells after PU.1 expression.

PU.1 directly binds to the TRAIL promoter in U266$^{tetPU.1}$ and KMS12PE$^{tetPU.1}$ cells

Next, we examined how TRAIL was induced after PU.1 expression. It was earlier reported that IRF3 upregulated the *TRAIL* promoter after paramyxovirus infection (Kirshner *et al.*, 2005). In our microarray data, IRF3 was not upregulated, whereas IRF7 was highly upregulated, after PU.1 induction in U266$^{tetPU.1}$ cells. Therefore, we performed chromatin immunoprecipitation assays of the *TRAIL* promoter with anti-IRF7 and anti-PU.1 antibodies, and unexpectedly found that PU.1 itself, but not IRF7, directly bound to the *TRAIL* promoter (Figure 4a). In case of KMS12PE$^{tetPU.1}$ cells