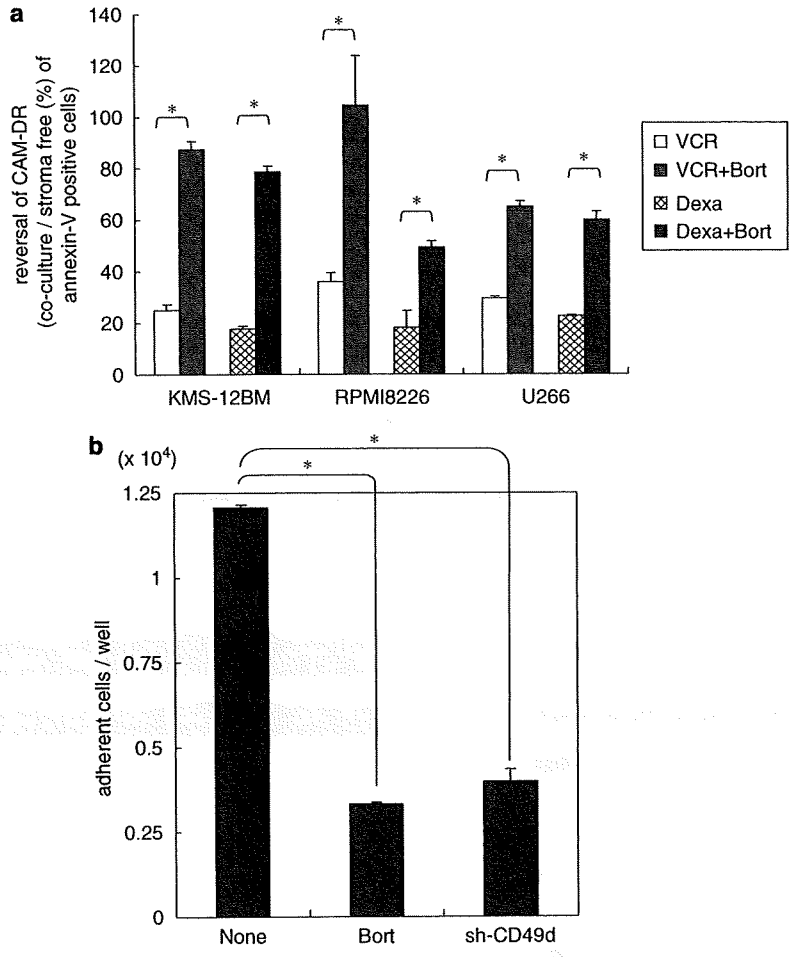

Figure 6 Bortezomib overcomes adhesion-mediated drug resistance (CAM-DR) to vincristine and dexamethasone in multiple myeloma (MM) cells. (**a**) Three myeloma cell lines were treated with 2 nM bortezomib for 24 h. After washing, we cultured cells with either vincristine (Bort + VCR) or dexamethasone (Bort + Dexa) in the absence or presence of stromal cells for additional 24 h. As controls, we carried out the same experiments without pretreatment of bortezomib (VCR and Dexa). The reversal of CAM-DR was determined as described in the legend of Figure 3. (**b**) Green fluorescent protein (GFP)-transduced KMS12-BM cells were cultured in the absence (None) or presence (Bort) of 5 nM bortezomib for 3 days. We used a KMS12-BM subline transfected with pLL3.7-sh-CD49d (sh-CD49d) as a control. Cells were then co-cultured with UBE6T-7 cells at 37 °C for 15 min. Non-adherent cells were washed off with phosphate-buffered saline (PBS), and resultant adherent cells were harvested with trypsin-EDTA. We determined GFP positivity (%) in non-adherent cells using flow cytometer. The means ± s.d. (bars) of three independent experiments are shown. The *P*-values were calculated by Student's *t*-test (*$P<0.05$).

did not downregulate the expression of CD49d in KMS-12BM cells, in which NF-κB is constitutively activated through the classical pathway (Keats *et al.*, 2007). The CD49d promoter contains canonical binding sites for Sp1, Ets and WT1 (Rosen *et al.*, 1994; Zutter *et al.*, 1997; Kirschner *et al.*, 2006). We are currently investigating whether these factors are implicated in bortezomib-mediated downregulation of CD49d mRNA expression.

The reversal of CAM-DR by bortezomib may underlie its broad range of synergy with other anticancer drugs as recently described (Horton *et al.*, 2006; Noborio-Hatano *et al.*, 2007). Unfortunately, severe pulmonary complications have been reported in Japanese patients treated with bortezomib (Miyakoshi *et al.*, 2006). Dose reduction by drug combination is one method to minimize side effects of bortezomib. Furthermore, it has been shown that the contact of MM cells with BM stromal cells through VLA-4/vascular cell adhesion molecule-1 (VCAM-1) interactions enhanced the production of osteoclastogenesis factors and that disruption of this cell-to-cell contact suppressed the development of osteoclastic osteolysis associated with MM (Michigami *et al.*, 2000; Pearse *et al.*, 2001). As bortezomib can suppress CD49d expression, this drug may also be effective for the amelioration of bone lesions in MM.

Although our findings provide rationale for safe and effective treatment strategies for refractory myeloma, further investigation is required to define suitable combinations involving not only conventional anti-myeloma drugs but also new drugs such as lenalidomide.

Previous studies revealed that both bortezomib and dexamethasone induced apoptosis primarily through caspase-9, whereas lenalidomide did so through caspase-8 (Mitsiades *et al.*, 2002). Indeed, we found that caspase-8 was not activated in MM cells by the drugs used in this study (data not shown). On the basis of these findings, the combination of lenalidomide with either bortezomib or dexamethasone is expected to trigger dual apoptotic signaling pathways and reverse DR, which results in the achievement of clinical responses in patients resistant to single agents. In support of this view, we have reported that bortezomib is effective for myeloma patients refractory to thalidomide therapy (Takatoku *et al.*, 2004). The combination of bortezomib with other anti-myeloma drugs, including thalidomide and its derivatives, is effective for overcoming CAM-DR and may improve the prognosis of MM patients.

Materials and methods

Cells and cell culture

We used four *bone fide* human MM cell lines, KHM-1B, KMS-12BM, RPMI8226 and U266, in this study (Drexler *et al.*, 2003). These cell lines were purchased from the Health Science Research Resources Bank (Osaka, Japan), and maintained in RPMI1640 medium (Sigma Co., St Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (Sigma Co.,) and antibiotics. Human BM-derived stromal cell line UBE6T-7 (Mori *et al.*, 2005), which was transduced with a human telomerase catalytic protein subunit, was kindly provided by Dr Akihiro Umezawa (National Research Institute for Child Health and Development, Tokyo, Japan), and used as stromal cells in co-culture experiments. Primary MM cells were isolated from the BM of patients at the time of diagnostic procedure. CD38-positive/CD45-low or negative ($CD38^+/CD45^{lo/neg}$) cells were purified from the BM of healthy volunteers, and used as normal plasma cells. Informed consent was obtained in accordance with the Declaration of Helsinki, and the protocol was approved by the Institutional Review Board.

Drugs and adhesion-blocking antibodies

Bortezomib (Velcade) was provided by Millennium Pharmaceuticals (Cambridge, MA, USA). We used vincristine (Shionogi Co. Ltd., Tokyo, Japan), doxorubicin (Meiji Co. Ltd., Tokyo, Japan) and dexamethasone (Sigma Co.) as conventional anti-myeloma drugs. All drugs were dissolved in RPMI1640 medium at appropriate concentrations and stored at −80 °C until use. We used the following adhesion-blocking antibodies in co-culture experiments: anti-CD44 (clone Hermes-1; Endogen, Rockford, IL, USA), anti-CD49d (2B4; R&D Systems, Minneapolis, MN, USA), anti-CD54 (Ab-2; Labvision Corp., Fremont, CA, USA), anti-CD184 (44717.111; R&D Systems), and mouse or rat IgG isotype controls (eBioscience, San Diego, CA, USA). Blocking experiments were performed as described earlier. In brief, 1×10^6 of MM cells were incubated with 10 μg/ml antibodies or isotype-matched controls in 100 μl of RPMI1640 medium for 1 h, then diluted to 2×10^5 cells/ml in complete medium, and added into culture wells preseeded with stromal cells (Matsunaga *et al.*, 2003).

Flow cytometric analysis of adhesion molecules

Cells were stained with specific monoclonal antibodies and analysed using BD-LSR or FACSaria flow cytometer (Becton Dickinson, Oxford, UK) as described earlier (Kikuchi *et al.*, 2005). The antibodies used were phycoerythrin (PE)-conjugated antibodies against CD11a (clone HI 111; BioLegend, San Diego, CA, USA), CD29 (4B4; Beckman Coulter, Fullerton, CA, USA), CD18 (6.7), CD22 (HIB22), CD29 (HUTS-21), CD49e (IIA1), CD56 (B159), CD138 (MI15) and CD184 (12G5) (all from Becton Dickinson), CD40 (5C3), CD44 (G44-26), CD49d (9F10) and CD54 (HA58) (all from eBioscience), allophycocyanine-conjugated antibodies against CD38 (HB7; Becton Dickinson) and PE-Cy7-conjugated antibodies against CD45 (2D1; Becton Dickinson).

Cell proliferation assay

Cell proliferation was monitored using Cell Counting Kit-8 (Dojin Chemical, Tokyo, Japan). In brief, cells were seeded in 96-well flat-bottomed microplates at a density of 5×10^4 per well and incubated with or without anti-myeloma drugs for 48 h at 37 °C. After incubation, 10 μl of WST-8 solution was added to each well at a final concentration of 1 μg/ml. Cells grown in complete medium alone were used as controls. After incubation at 37 °C for 4 h, absorbance was measured at a wavelength of 450 nm using a microplate reader.

Co-culture of MM cells with stromal cells and the assessment of cell death

For co-culture experiments, we established GFP-expressing MM cell lines by transfecting GFP expression plasmids to distinguish MM cells from stromal cells. UBE6T-7 stromal cells were seeded in 48-well plates to near confluence. After stromal cells were washed once with fresh medium, GFP-transduced MM cells were added to the plates and cultured in the absence or presence of anti-myeloma drugs. After 48 h, the cells were harvested by pipetting, washed with phosphate-buffered saline, and stained with PE-conjugated annexin-V (annexin-V/PE) (Biovision, Mountain View, CA, USA). Cell death/apoptosis was judged by annexin-V reactivity in GFP-positive populations using flow cytometer (Yanamandra *et al.*, 2006).

Construction and production of lentiviral shRNA/siRNA expression vectors

We used a lentiviral shRNA/siRNA expression vector pLL3.7. for knockdown of adhesion molecules. siRNA target sequences were designed to be homologous to wild-type cDNA sequences. Oligonucleotides were chemically synthesized, annealed, terminally phosphorylated and inserted into pLL3.7 vector. Oligonucleotides containing siRNA target sequences were shown in Supplementary Table S1. Ineffective sequences were used as sh controls. These vectors were co-transfected into 293FT cells with packaging plasmids (purchased from Invitrogen, Carlsbad, CA, USA). Infectious lentiviruses in culture supernatants were harvested, concentrated and infected as described earlier (Kikuchi *et al.*, 2007). Lentiviruses were then added into cell suspensions in the presence of 8 μg/ml polybrene, and transduced for 24 h. Transduction efficiencies were monitored by GFP expression using a flow cytometer.

Immunoblotting

Immunoblotting was carried out according to the standard method using the following antibodies: anti-CD49d (Novus Biologicals, Littleton, CO, USA), anti-CD54 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-caspase-8, anti-caspase-9 (Becton Dickinson) and anti-GAPDH (Santa Cruz Biotechnology) (Odgerel *et al.*, 2008).

PCR

We performed semiquantitative RT–PCR and real-time quantitative RT–PCR to estimate the expression of CD49d at mRNA levels. For the former, PCR amplification was carried out with 1 μl of cDNA solution (corresponding to 50 ng total RNA) in a 50-μl reaction mixture containing 5 U of *Taq* polymerase, 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$ and 100 mM dNTPs in the presence of specific primer pairs (200 nM each) as follows; CD49d, forward: 5′-GGATGT GAACAGAAAGGCAGA-3′, reverse: 5′-GCCAGTGTTGA TAACATGGAAA-3′; GAPDH (internal control), forward: 5′-CCACCCATGGCAAATTCCATGGCA-3′, reverse: 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. PCR products were resolved on 2% agarose gels, and visualized by staining with ethidium bromide. The results of 40 amplification cycles are shown. For the latter, we used the same primer pairs in the SYBR Green PCR system (Applied Biosystems, Foster City, CA, USA). Data quantification was carried out by the $2^{-\Delta\Delta C_t}$ method (Pfaffl, 2001).

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ORIGINAL ARTICLE

A phase II trial of weekly chemotherapy with paclitaxel plus gemcitabine as a first-line treatment in advanced non-small-cell lung cancer

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Abstract
Purpose The efficacy and toxicity of combined paclitaxel (PTX) and gemcitabine (GEM) was evaluated as a protocol for first-line chemotherapy in 40 patients with advanced non-small-cell lung cancer (NSCLC).
Methods Paclitaxel, 100 mg/m^2, was administered intravenously (IV) as a 1-h infusion, followed by GEM, 1,000 mg/m^2, IV over 30 min on days 1 and 8 of a 21-day cycle. The median age of patients was 66 years with a range of 33–75 years. Nearly all patients (39/40) had an ECOG performance status of 0 or 1. Thirteen patients (32%) had initial stage IIIB disease and 27 patients (68%) had stage IV disease. Histological subtypes were adenocarcinoma (73%) and squamous cell carcinoma (25%).
Results Twenty-two patients (55%) achieved a partial response and none achieved a complete response, giving an overall response rate of 55% (95% confidence interval: 38.2–71.8%). Disease stability was achieved in 14 patients (35%), and 4 patients (10%) had progressive disease. The median survival time was 11.9 months (95% CI: 10.3–14 months), with a 1-year survival rate of 47.5%. Grade 3 or 4 hematological toxicities observed included neutropenia in 37.5%, anemia in 2.5%, and thrombocytopenia in 5.0% of these patients. Non-hematologic toxicities were mild, with the exception of grade 3 and 4 pneumonitis. There were no deaths due to toxicity.
Conclusion Weekly chemotherapy with PTX plus GEM is effective and is acceptable for the first line treatment of advanced NSCLC.

Keywords Non-small-cell lung cancer · First-line chemotherapy · Weekly chemotherapy · Gemcitabine · Paclitaxel

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Introduction

Lung cancer ranks among the most commonly occurring malignancies and currently is the leading cause of cancer-related deaths worldwide [21]. In Japan lung cancer is responsible for approximately 55,000 cancer-related deaths per year [5]. Even though the clinical usefulness of first-line chemotherapy has been established for the cases of advanced non-small-cell lung cancer (NSCLC), the prognosis is still extremely poor.

A number of new agents have become available recently for the treatment of unresectable and metastatic NSCLC in Japan, including the taxanes, gemcitabine (GEM), and vinorelbine. In randomized phase III trials, these agents in combination with a platinum compound have been associated with improved survival of patients having advanced NSCLC [8, 17, 23, 24]. However, a platinum compound is associated with a greater toxicity than other drugs used to treat NSCLC. In addition to nausea and vomiting, it causes neuropathy, profound fatigue, and renal toxicity. Some

patients are unable to tolerate the drug toxicity and terminate treatment early. Based on these observations, non-platinum regimens have been proposed as an alternative to the platinum-based combinations for treatment of advanced NSCLC [13].

Paclitaxel (PTX) and GEM are new anti-cancer agents having significant single-agent activity against advanced NSCLC. A recent clinical phase II study of 122 patients with previously untreated, unresectable stage III or IV NSCLC receiving a 3-h infusion of PTX at a dose of 210 mg/m^2 showed a good response rate of 35% [25]. Although PTX is usually given once every 3 weeks, Chan et al. [10] demonstrated that weekly administration of PTX at a dose of 80–90 mg/m^2 provides similar tolerability and a possible increase in efficacy.

Gemcitabine, a novel deoxycytidine analog, had a response rate of 20% with a single weekly administration in previously untreated advanced NSCLC [4]. As a first-line treatment, single-agent GEM has been shown to have antitumor activity equal to that of cisplatin/etoposide, resulting in less toxicity and a slightly better quality of life [27].

These agents have different mechanisms of action, and their toxicities are partially non-overlapping. Although the usual administration of PTX is once every 3 weeks, a weekly administration can increase efficacy with good tolerability [1, 2]. We demonstrated that weekly administration with PTX and GEM is a tolerable and active regimen for patients with advanced NSCLC previously treated with platinum-containing chemotherapy regimens [20]. Based on these findings, we designed a phase II trial to examine the efficacy and tolerance of the non-platinum-based combination of PTX and GEM administered weekly for patients with untreated advanced NSCLC.

Patients and methods

Patient selection

All patients with histologically or cytologically confirmed advanced NSCLC were eligible for this phase II trial. The subjects of this study were patients with clinical stage IV NSCLC or stage III with unresectable disease or for whom radiotherapy with curative intent is not possible. Patients with unresectable disease or radiotherapy with curative intent is not possible include those with pleural effusion and dissemination, those with intrapulmonary metastasis within the ipsilateral lobe, those with an irradiation field exceeding one-half of one lung, those with metastasis to the contralateral hilar lymph nodes, and those with reduced lung function. Other eligibility criteria included: age older than 20 years and younger than 76 years; Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2; measurable lesions; life expectancy $\geq$12 weeks; adequate bone marrow reserve with a WBC count $\geq$4,000 per mm^3; platelet count $\geq 10 \times 10^4$ per mm^3; and hemoglobin level $\geq$9.0 g/dL; liver function with a AST and ALT $\leq 2.5 \times$ upper normal limit, unless as a result of liver metastases; and adequate renal function with a serum creatinine level $\leq$1.5 mg/dL. No prior radiotherapy treatment was allowed if the irradiated area was not the site of measurable lesion and the therapy was completed at least 2 weeks before enrollment into the study.

Patients were excluded for the following indications: $\geq$76 years of age (vinorelbine as single agent treatment), severe cardiovascular or cerebrovascular disease, uncontrolled diabetes or hypertension, active infection, pulmonary fibrosis, massive pleural effusion or ascites, active peptic ulcer, and severe neurological disorders. Patients were also excluded in case of previous malignancy and any evidence or history of hypersensitivity or other contraindications for the drugs used in this trial. Written informed consent was obtained from all patients.

Treatment

Paclitaxel, 100 mg/m^2, was administered IV during a 1-h infusion, followed by GEM, 1,000 mg/m^2, IV over 30 min on days 1 and 8 of 21-day cycle. Premedication for PTX consisted of dexamethasone 20 mg, diphenhydramine 50 mg, and ranitidine 50 mg IV for 30 min before PTX infusion. After the premedication for PTX was completed, a serotonin receptor antagonist was given as a 30-min infusion for prophylactic antiemetic therapy. Treatment was repeated every 3 weeks until maximum response plus two cycles or unacceptable toxicity. In stable disease, patients received a maximum of six cycles. At the investigator's discretion, patients were treated with up to eight cycles of the drug combination.

Dose modifications were planned according to hematologic and severe non-hematologic toxic effects. Once the doses were reduced, they were not increased. Patients who experienced grade 4 neutropenia, grade 4 thrombocytopenia, reversible grade 2 neurotoxicity, or liver dysfunction received reduced doses of both PTX, 75 mg/m^2, and GEM, 800 mg/m^2, for the next cycle. The next course of chemotherapy was started after 3 weeks when the leukocyte count was 3,000 per mm^3 or greater, the neutrophil count was 1,500 per mm^3 or greater, the platelet count was 75,000 per mm^3 or greater, serum creatinine was less than 1.5 mg/dL, GOT and GPT were less than twice the upper limit of the normal range, and the neurotoxicity was grade 1 or less. If hematologic recovery was not achieved by day 35 of treatment, the patient was withdrawn from the study.

Evaluation of responses and toxicity

Responses and toxicity were evaluated on the basis of tumor images obtained by computerized tomography (CT), laboratory results, subjective/objective symptoms, signs before, during, and after administration of the study drugs and during the period from completion of treatment to the final analysis. Measurable disease parameters were determined every 4 weeks by various means such as CT. Evaluation was performed in compliance with the response evaluation criteria in solid tumors (RECIST) guidelines for anti-tumor activity. Adverse events were assessed using the Common Terminology Criteria for Adverse Events version 3.0 (CTCAEv3.0). Patients were withdrawn from the study if evidence of tumor progression was observed. The institutional ethical review committee gave approval to the study.

Statistical analysis

The primary end point of the study was the response rate. Simon's two-stage design was used to determine sample size and decision criteria. It was assumed that a response rate of 40% in eligible patients would indicate potential usefulness, while a rate of 20% would be the lower limit of interest; $\alpha = 0.05$ and $\beta = 0.10$. Using these design parameters, the first stage of the study was to enroll 24 patients, and the regimen was rejected if fewer than five patients had an objective response. If six or more patients responded, the accrual was continued until 45 patients were enrolled (45 patients were required because of anticipated percentage of dropout cases). Combination therapy was considered effective if $\geq$14 of the 45 patients showed a response in the final analysis. Secondary end points were toxicity and overall survival. Response and survival rates were both calculated on an intent-to-treat basis. Overall survival and time to progression were measured from the start of this treatment until time of death or the date of the last follow-up clinical assessment. Survival curves were constructed using the Kaplan–Meier method (Fig. 1).

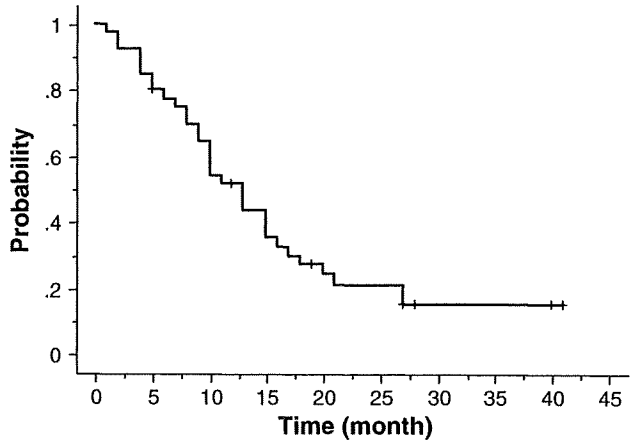

Fig. 1 Kaplan–Meier estimated overall survival curves. Median survival time, 11.9 months; 1-year survival rate, 47.5%

Results

Patient characteristics

A total of 40 patients were enrolled in the study between September 2001 and July 2004. The majority of patients were treated as outpatients. The clinical characteristics of the patients are listed in Table 1. The median age was 66 years with a range of 33–75 years. Nearly two-thirds of the patients were men. Twenty-four patients had an PS of 0, and 15 had PS of 1. Histological subtypes were 73% (29/40) adenocarcinoma and 25% (10/40) squamous cell carcinoma.

Table 1 Patient characteristics

Eligible patients	40
Gender	
Male	26
Female	14
Age (years)	
Median	66
Range	33–75
Performance status	
0	24
1	15
2	1
Histology	
Adenocarcinoma	29
Squamous cell	10
Large cell	1
Stage	
III	13
IV	27
Number of metastatic sites	
Median	2
Range	0–3
Location of metastases	
Bone	12
Lung nodules	10
Liver	9
Lymph nodes	8
Adrenals	6
Brain	3
Subcutaneous	1

Toxicities

The toxicities observed during this study are provided in Table 2. Hematological toxicities were the most common, but grade 3–4 toxicities, including neutropenia (37.5%), thrombocytopenia (5.0%), and anemia (2.5%) were relatively modest. There were only two cases of febrile neutropenia (5.0%). Grade 1 nausea, fatigue, alopecia, neuropathy, and angialgia occurred with a greater frequency than the non-hematologic toxicities. Grade 3–4 non-hematologic toxicities were not seen except in cases of pulmonary toxicity. Two patients (5.0%) developed interstitial pneumonitis (grade 3; one patient, grade 4; one patient), and were responsive to steroid therapy.

Efficacy of treatment

The median number of cycles administered per patient was 4, and the number of cycles ranged from 1 to 8. Twenty-two patients exhibited a partial response. The overall response rate was 55% (22/40) [95% confidence interval (CI): 38.2–71.8%]. Stable disease was achieved in 14 patients (35%), and 4 patients (10%) had progressive disease. All 40 patients were included in the survival analysis. The overall median survival time was 11.9 months (95% CI: 10.3–14 months). The 1-year survival rate was 47.5% (19/40). The median time to disease progression was 6.4 months. Thirty patients (75%) received chemotherapy, and 4 patients (10%) received thoracic irradiation as second-line treatment.

Discussion

Although a standard regimen of first-line chemotherapy for advanced NSCLC is being established, it is important to develop a more active and well-tolerated regimen. Several published randomized studies reported that non-platinum-based chemotherapy in advanced NSCLC was as effective and less toxic than platinum-based regimens [13, 15, 18, 29]. Georgoulias et al. [13] compared the combination of a cisplatin and docetaxel regimen with the GEM and docetaxel regimen. Objective response rates were similar in the two groups, with 32.4% in the former and 30.2% in the latter. The two groups did not differ in the overall survival or 1- or 2-year survival rates. They concluded that both drug combinations had comparable activity and the non-platinum-based regimen had the more favorable profile.

Generally, non-cisplatin-containing treatment does not require supplemental hydration as does standard cisplatin-based chemotherapy. This may be advantageous for elderly patients, patients with poor PS, and patients with renal or cardiac impairment. Recchia et al. [22] conducted a trial of PTX plus GEM in advanced NSCLC patients with a low PS. The chemotherapy regimen consisted of 200 mg/m^2 PTX on day 1 plus 1,000 mg/m^2 GEM on days 1 and 8, repeated every 3 weeks, for a maximum of eight cycles. They achieved a reasonable response rate of 41.3%. Median overall survival time was 13.6 months; the authors concluded that a satisfactory clinical benefit could be obtained with GEM plus PTX regimen in NSCLC patients with a poor PS.

Thus, non-platinum-based chemotherapy may be used as alternative to platinum-based regimens. We conducted a phase II trial was designed to examine the efficacy and tolerance of the non-platinum-based combination of weekly PTX and GEM for patients with untreated advanced NSCLC. Results including an overall response rate of 55%, a median survival time of 11.9 months, and a 1-year survival probability rate of 47.5% suggested that this regimen might have anti-tumor activity equal to that of platinum-based regimens.

Weekly chemotherapy for lung cancer has recently been carried out at several facilities, and favorable results were reported [9, 16, 26, 30]. Compared to standard chemotherapy with administration of drugs at intervals of 3–4 weeks, weekly chemotherapy appears acceptable for the reduction of a single dose level of anti-cancer drugs with fewer side effects. In addition, weekly dose level is more easily adjusted according to the general clinical condition of individual patients or if hematologic toxicity develops. Belani et al. [6] conducted a randomized phase II trial of a 3-week schedule of GEM plus PTX (ArmA) versus a weekly schedule of GEM plus PTX (ArmB) in the treatment of NSCLC. It was concluded that a weekly schedule resulted in improved survival and lower hematologic toxicity than the 3-week schedule.

The clinical outcomes of weekly PTX and GEM therapy found in the literature [3, 6, 7, 11, 12, 14, 19, 28] and in our results are summarized in Table 3. The response rate ranges were from 23.1 to 55%; overall median survival time was 4.9–11.9 months; and 1-year survival rates were 26–53%. Most adverse reactions were hematologic (such as leukope-

Table 2 Maximum toxicity over 40 patients

	CTCAE v 3.0 grade (no. of patients)		Grade 3 or 4 (%)
	Grade 3	Grade 4	
Leukopenia	11	1	12 (30)
Neutropenia	11	4	15 (37.5)
Febrile neutropenia	2	0	2 (5.0)
Anemia	1	0	1 (2.5)
Thrombocytopenia	2	0	2 (5.0)
Pneumonitis	1	1	2 (5.0)

CTCAE v 3.0: Common Terminology Criteria for Adverse Events version 3.0

Table 3 PG regimens used as first-line treatment of advanced NSCLC

First author (ref.)	No. of patients		Regimen and schedule	Response rate (%)	Survival median	One-year (%)
Belani et al. [6]	50	Arm A	P 200 mg/m^2 day 1 q3w G 1 g/m^2 days 1, 8 q3w	28.2	7.5	34
	50	Arm B	P 100 mg/m^2 days 1, 8 q 3w G 1 g/m^2 days 1, 8 q3w	26.8	9.6	42
Bhatia et al. [7]	39		P 110 mg/m^2 days 1, 8, 15 q 4w G 1 g/m^2 days 1, 8, 15 q4w	38.2	4.9	26
De Pas et al. [12]	54		P 100 mg/m^2 days 1, 8, 15, 22 q 4w G 1 g/m^2 days 1, 8, 15, 22 q4w	46	9.6	53
Akerley et al. [3]	39		P 85 mg/m^2 days 1, 8, 15, 22, 29, 36 q 8w G 1 g/m^2 days 1, 8, 15, 22, 29, 36 q8w	23.1	7.5	32
Gillenwater et al. [14]	39		P 100 mg/m^2 days 1, 8, 15, 21 q 4w G 1 g/m^2 days 1, 8, 15, 21 q4w	35	4.9	35
Kosmidis et al. [19]	225		P 200 mg/m^2 day 1 q 3w G 1 g/m^2 days 1, 8, q3w	31	9.3	42
Treat et al. [28]	312		P 200 mg/m^2 day 1 q 3w G 1 g/m^2 days 1, 8, q3w	43.6	8.4	33
Our study	40		P 100 mg/m^2 days 1, 8, q 3w G 1 g/m^2 days 1, 8 q3w	55	11.9	47.5

NSCLC non-small-cell lung cancer, *P* paclitaxel, *G* demcitabine

nia and neutropenia of grade 3 or greater occurrence) in 28–53%. Variable toxicities may be due to population-related pharmacogenomics [11]. Overall, the non-hematologic toxicity was mild, and there were few adverse reactions of grade 3 or greater. A few patients had pneumonitis which was not responsive to steroid therapy. The treatment in our current study was reasonably tolerated, especially in the area of non-hematologic toxicity. Nausea, vomiting, and fatigue, which are often seen in cisplatin-containing regimens, were relatively mild; no patients developed renal toxicity.

In conclusion, weekly chemotherapy with PTX and GEM is a well-tolerated and effective regimen for previously untreated patients with advanced NSCLC. Further studies are expected for the application of this regimen to the elderly, and patients with a poor PS or suspected vulnerability to platinum compound toxicity.

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Conflict of interest statement None.

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ORIGINAL ARTICLE

Expression of myeloperoxidase enhances the chemosensitivity of leukemia cells through the generation of reactive oxygen species and the nitration of protein

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Myeloperoxidase (MPO), a pivotal lineage marker for acute myeloid leukemia (AML), has been also shown to have a prognostic value: a high percentage of MPO-positive blasts correlates to favorable prognosis. To understand the relationship between the expression of MPO in leukemia cells and the response to chemotherapeutic agents, we established MPO-expressing K562 leukemia cell lines and then treated them with cytosine arabinocide (AraC). Cells expressing wild-type MPO, but not mutant MPO that could not mature, died earlier of apoptosis than control K562 cells. Reactive oxygen species (ROS) were generated more in leukemia cells expressing MPO, and the generation was abrogated by MPO inhibitors or antioxidants. Tyrosine nitration of cellular protein also increased more in MPO-expressing K562 cells than control cells after treatment with AraC. In clinical samples, CD34-positive AML cells from high-MPO cases showed a tendency to be sensitive to AraC in the colony-formation assay, and the generation of ROS and the nitration of protein were observed only when the percentage of MPO-expressing cells was high. These data suggest that MPO enhances the chemosensitivity of AML through the generation of ROS and the nitration of proteins.

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Keywords: myeloperoxidase; reactive oxygen species; chemosensitivity; nitrotyrosine; acute myeloid leukemia

Introduction

It is widely accepted that the expression of myeloperoxidase (MPO), a microbiocidal protein, is a golden marker for the diagnosis of acute myeloid leukemia (AML) utilized by the French–American–British and WHO classifications[1,2] to determine the hematopoietic lineage of immature blasts as myeloid. Apart from its role in the diagnosis of AML, MPO has also been shown to have a prognostic value by several groups including ours.[3–5] These reports demonstrated that the percentage of MPO (or Sudan black B)-positive blasts assessed by cytochemical methods was related to the prognosis of AML patients; those with a higher percentage of MPO-positive blasts had better survival rates. Our previous report[5] showed significant differences in complete remission rate, disease-free survival and overall survival using multivariate analysis. However, so far there is no clear explanation as to how the expression of MPO relates to the prognosis of AML.

Chemotherapeutic agents create various reactions in leukemia cells when administered. One of the effects triggered by chemotherapy is the production of reactive oxygen species (ROS).[6,7] ROS are known to modulate the regulators of a wide variety of cellular biological processes including calcium signaling, protein phosphorylation, gene expression, cell growth and differentiation, and chemotaxis.[8,9] They also induce cellular damage associated with lipid peroxidation and alteration of proteins and nucleic acids.[10] Mainly on the basis of *in vitro* studies, it is believed that ROS produced by chemotherapeutic agents play a role in the induction of apoptosis in target cells, which could directly relate to the efficacy of chemotherapy.[7] MPO catalyzes the production of hypochlorous acid using hydrogen peroxide (H_2O_2) as a substrate.[11] Since hypochlorous acids are highly toxic for cells, it is presumed that higher amounts of hypochlorous acids produced by MPO would result in higher toxicity for cells. For example, in the HL60 leukemia cell line, the amount of MPO in cells was directly related to cytotoxicity elicited by chemotherapeutic agents.[12] MPO in HL60 cells was also demonstrated to be involved in the induction of apoptosis by H_2O_2.[13]

The clinical and experimental importance of MPO in the cytotoxicity of chemotherapeutic agents prompted us to directly evaluate the influence of MPO on the efficacy of cytosine arabinocide (AraC), an important antileukemia drug for AML, on leukemia cells. We generated MPO-expressing K562 leukemia cell lines that were originally negative for MPO expression to test for changes in sensitivity to AraC. In this report, we show that the activity of MPO directly enhanced the cytotoxicity of AraC by producing increased amounts of ROS and nitrated tyrosine residues in cellular proteins. In accordance with the observation on leukemia cell line, in samples from AML patients, AraC inhibited colony formation of AML cells more efficiently when MPO expression was high. The production of ROS and nitrated tyrosine was also partly related to the percentage of MPO-positive blasts in clinical samples. These observations suggest important roles for MPO in the cytotoxicity of chemotherapeutic agents during the treatment of AML.

Materials and methods

Vectors, cDNA constructs and mutagenesis

Full-length cDNA for human *MPO* (kindly provided by Dr Nagata, Institute of Medical Science, University of Tokyo)[14] was cloned into pCI-neo, a mammalian expression vector (Promega,

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Madison, WI, USA). The R569W mutation of the *MPO* gene (arginine at the 569th amino-acid position was changed into tryptophan) was generated by PCR-based methods that replaced the C nucleotide at the 1868 bp position by T. Mutagenesis was confirmed by using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and ABI PRISM3100 Genetic analyzer (Applied Biosystems). All PCR experiments were performed using the GeneAmp PCR System9700 and GeneAmp High Fidelity Enzyme Mix (Applied Biosystems).

Cell culture and electroporation

The human leukemia cell line, K562, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA; CCL-243) was maintained in Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (SAFC biosciences, Lenexa, KA, USA) and antibiotics at 37 °C under 5% CO_2. In some experiments, cytosine arabinoside (Sigma, St Louis, MO, USA), H_2O_2 (Wako Pure Chemical Industries, Osaka, Japan), *N*-acetylcysteine (Sigma) or 4-aminobenzoic hydrazide (Sigma) were added alone or in various combinations into the culture medium. Peroxynitrite tetramethylammonium (Alexis Biochemicals, San Diego, CA, USA) was used as a source of reactive nitrogen species (RNS). Cell growth was assessed using the Premix WST-1 Cell Proliferation Assay System (Takara Biochem., Tokyo, Japan). pCI-neo carrying normal or mutated cDNA for MPO was transfected into log-phase K562 cells by electroporation. In brief, cells suspended at the concentration of 1×10^7 cells per ml in Nucleofector solution (Amaxa biosystems, Gaithersburg, MD, USA) were mixed with 1 μg of plasmid DNA and then electroporation was performed with Nucleofector (program T-16; Amaxa biosystems). Stable lines that were transfected with various plasmids were selected as a single clone in the presence of 800 μg ml^{-1} of G418 (Sigma).

Flow cytometry analysis

For the detection of Annexin V, cells were stained with an Annexin V Fluos staining kit (Roche, Mannheim, Germany). To measure the mitochondrial membrane potential, cells were incubated with the J-aggregate-forming cationic dye, JC-1 (Molecular Probes, Karlsruhe, Germany), at a concentration of 10 μg ml^{-1} for 10 min at 37 °C. ROS in cells were measured by flow cytometry using 2-[6-(4′-amino)phenoxy-3*H*-xanthen-3-on9-yl]benzoic acid (APF; Daiichi pure chemicals, Tokyo, Japan) fluorescence and 2-[6-(4′-hydroxy) phenoxy-3*H*-xanthen-3-on9-yl] benzoic acid (HPF; Daiichi pure chemicals) fluorescence. APF reacts with hydroxyl radicals, peroxinytrite and hypochlorous acid. HPF reacts with hydroxyl radicals and peroxinytrite, but not with hypochlorous acid. For the detection of nitric oxide, diaminofluorescein-2 diacetate (Daiichi pure chemicals) was used. All flow cytometric measurements were performed with a FACScan flowcytometer (Becton Dickinson, San Jose, CA, USA). Data were analyzed using CellQuest software (Becton Dickinson).

Morphological analysis

Cells spread on slide glasses were stained with standard May–Grunwald Giemusa staining and the diaminobenzidine (DAB) method for the detection of MPO activity. For analysis of MPO activity with electron microscopy (JEM-1210 electron microscope; JEOL, Tokyo, Japan), cells fixed with 1.25% glutaraldehyde were incubated with DAB.

Western blot analysis

After disruption in lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P40, 1 mM EDTA, 10 μg ml^{-1} of aprotinin, 10 μg ml^{-1} of leupeptin and 1 mM phenylmethylsulfonyl fluoride), samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). Target proteins were visualized using a rabbit polyclonal antibody against MPO (DakoCytomation, Carpinteria, CA, USA), a rabbit polyclonal antibody to nitrotyrosine (Chemicon, Temecula, CA, USA), mouse monoclonal antibody to β-actin (Abcam, Cambridge, UK) or to heat-shock protein 90α/β (Santa Cruz Biotechnology, Santa Cruz, CA, USA) with peroxidase-labeled secondary antibodies (Amersham Bioscience, Buckinghamshire, UK) and an enhanced chemiluminescence system (ECL Advance Western Blotting Detection Kit; GE Healthcare Bio-Sciences, Buckinghamshire, UK).

Patients' samples for ROS and nitrotyrosine detection

Peripheral blood or bone marrow samples were obtained from 14 AML patients prior to treatment with informed consent. CD34-positive (+) cells were selected using an immunomagnetic column (Miltenyi Biotech, Auburn, CA, USA). The purity of CD34+ cells was assessed by flow cytometry, demonstrating that more than 95% of cells was CD34-positive after selection. In six cases, CD34-positive AML cells (1×10^5 per well in a 24-well culture plate) with or without 20 nM AraC were cultured in semisolid media (MethoCalt GF H4434; StemCell Technologies, Vancouver, BC, Canada). The number of colonies containing 30 or more cells was counted 7–14 days after plating. In other eight cases, cells were cultured in Iscove's modified Dulbecco's medium with 10% fetal bovine serum incubated with 10 μM AraC or saline as a control for 6 h with or without H_2O_2, and then analyzed for the detection of ROS. Using four out of eight samples treated with 10 μM AraC up to 6 h, the nitration of tyrosine residues was assessed by western blot analysis with anti-nitrotyrosine antibody as mentioned above. Quantification of bands of western blot was performed using FluoChem IS-8800 and AlphaEase FC Software (Alpha Innotech Corp., san Leandro, CA, USA). The intensity of bands was shown as an average value (AVG). The pixel value and area of each band were counted; then AVG was calculated as follows: AVG = [Σ(each pixel value−background)]/area, which was suggested by the system manual. Expression of MPO in CD34+ cells was examined by flow cytometry.

Statistical analysis

Results are presented as the mean ± s.d. of three independent experiments. Differences between experimental groups were compared using one-way analysis of variance followed by the Scheffe's multiple comparison procedure. Statistical significance was considered at a *P*-value of 0.05.

Results

Establishment of cell lines expressing wild-type or mutant MPO

K562 cell lines expressing wild-type and mutant (R569W) MPO were established as single clones. R569W mutation of the MPO

protein,[15] originally found in an MPO-deficient person, resulted in a defective maturation process. MPO protein with the R569W mutation attains apopro-myeloperoxidase status but cannot mature further; it remains in the non-functional stage. Western blot analysis demonstrated the presence of immature MPO protein (apopro-myeloperoxidase, 89 kDa) in both wild-type and mutant MPO-expressing cell lines (MPO-21 and R569W-2, respectively; Figure 1). On the other hand, as expected, the α-subunit of mature MPO protein at 64 kDa and the β-subunit at 14 kDa were detected only in MPO-21 cells since these subunits are generated at the late maturation process of MPO. No apparent difference in the morphological features of MPO-21 and R569W-2 were detected by May-Grunwald Giemusa staining (Figure 2a). Cytochemical analysis using light microscopy detected MPO activity in MPO-21 but not R569W-2 cells (Figure 2a). Other two MPO-expressing lines (MPO-6 and MPO-18) also had the same-size MPO protein as MPO-21 and showed the MPO activity (Supplementary data, Figure 1). Electron microscopy revealed that enzymatically active MPO protein was localized to the cytoplasm (Figure 2b).

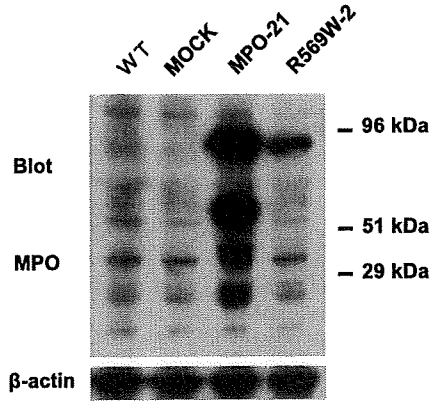

Figure 1 Western blot analysis of myeloperoxidase (MPO) protein in K562 cells. Immature MPO protein (apo-pro MPO, 89 kDa) was detected in K562 cells expressing wild-type MPO (MPO-21) and mutant MPO (R569W-2). Mature MPO protein (64 and 14 kDa) was seen only in MPO-21 cells. MOCK, K562 cells transfected with control plasmid; WT, wild-type K562 cells.

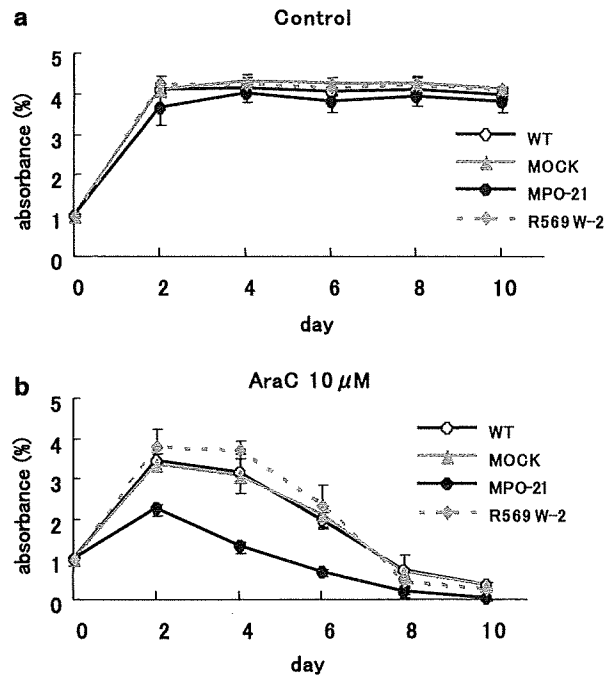

Figure 3 WST-1 analysis of K562 cells. Wild-type K562 (WT), MOCK (transfected with control vector), MPO-21 (wild-type myeloperoxidase (MPO)-expressing K562) and R569W-2 (mutant MPO-expressing K562) cells showed a similar growth pattern in the steady state (**a**). In the presence of cytosine arabinocide (AraC), MPO-21 cells demonstrated an earlier decline than other three cell lines (**b**). The mean value of three independent experiments at each point is shown with the standard deviation.

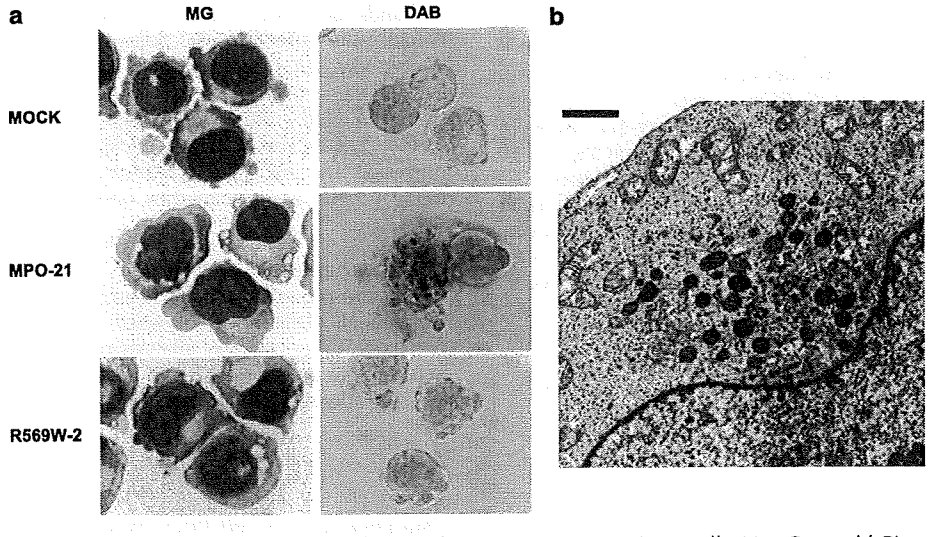

Figure 2 Morphological and cytochemical features of myeloperoxidase (MPO)-expressing K562 cells. May-Grunwald Giemusa (MGG) staining and diaminobenzidine (DAB) staining of K562 cells transfected with control vector (MOCK), wild-type MPO (MPO-21) and mutant MPO (R569W-2) (**a**). DAB was observed only in MPO-21 cells. (**b**) DAB-positive granules (dark granules) were seen in the cytoplasm of MPO-21 cells using electron microscope (scale bar represents 1 μm).

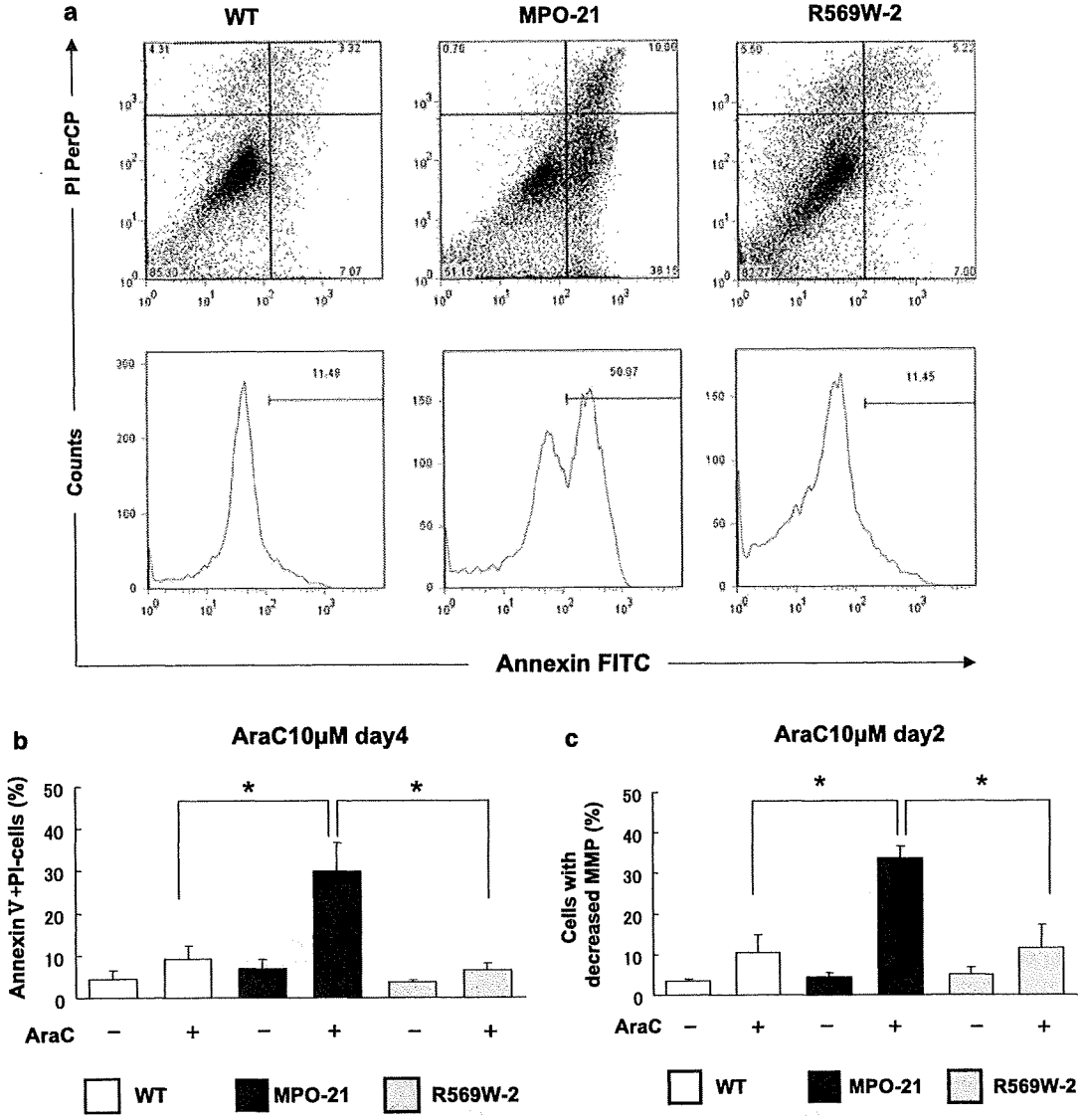

Figure 4 Apoptosis is accelerated in MPO-21 cells. Histogram of Annexin V expression on day 4 of cytosine arabinocide (AraC) treatment (**a**). A larger proportion of MPO-21 cells expressed Annexin V in a propidium iodide (PI)-negative fraction than wild-type K562 (WT). Data of three independent experiments are shown (**b**). There was a statistically significant difference (*$P<0.05$). Change of mitochondria membrane potential (MMP) was probed by JC-1 on day 2 of AraC treatment and the results of three independent experiments are shown (**c**). The decrease of MMP was significant in MPO-21 cells (*$P<0.05$).

MPO activity enhanced the cytotoxic effect of AraC by inducing apoptosis

Proliferation of wild-type K562 (WT), control K562 transfected with an empty vector (MOCK), MPO-21 and R569W-2 cells was similar as assessed by WST-1 assay, keeping maximum absorbance that showed a log phase in growth after day 2 of culture (Figure 3a). However, when cells were treated with AraC at 10 μM, MPO-21 showed an earlier decline than others (Figure 3b). MPO-6 also showed similar pattern as MPO-21 (Supplementary data, Figure 2). Since it is known that AraC induces apoptosis in leukemia cells,[16] we next analyzed whether the introduction of MPO in K562 cells accelerated this process or not. As shown in Figure 4a, on day 4 of AraC treatment, a larger proportion of MPO-21 cells (38%) were found to have Annexin V (and propidium iodide-negative) on their surface than wild-type K562 cells (7%). On the other hand, no change was observed between wild-type K562 and R569W-2. Data from three independent experiments showed statistical differences in the expression of Annexin V between MPO-21 and wild-type K562 or R569W-2 ($P<0.05$, Figure 4b). An earlier marker for apoptosis, the change of mitochondrial membrane potential detected using JC-1, was also significantly increased in MPO-21 cells than other two lines on day 2 ($P<0.05$, Figure 4c).

MPO enhanced the generation of ROS

Since MPO catalyzes the formation of hypochlorous acid, an ROS, we examined whether the generation of ROS was enhanced by the expression of MPO using fluorescent markers

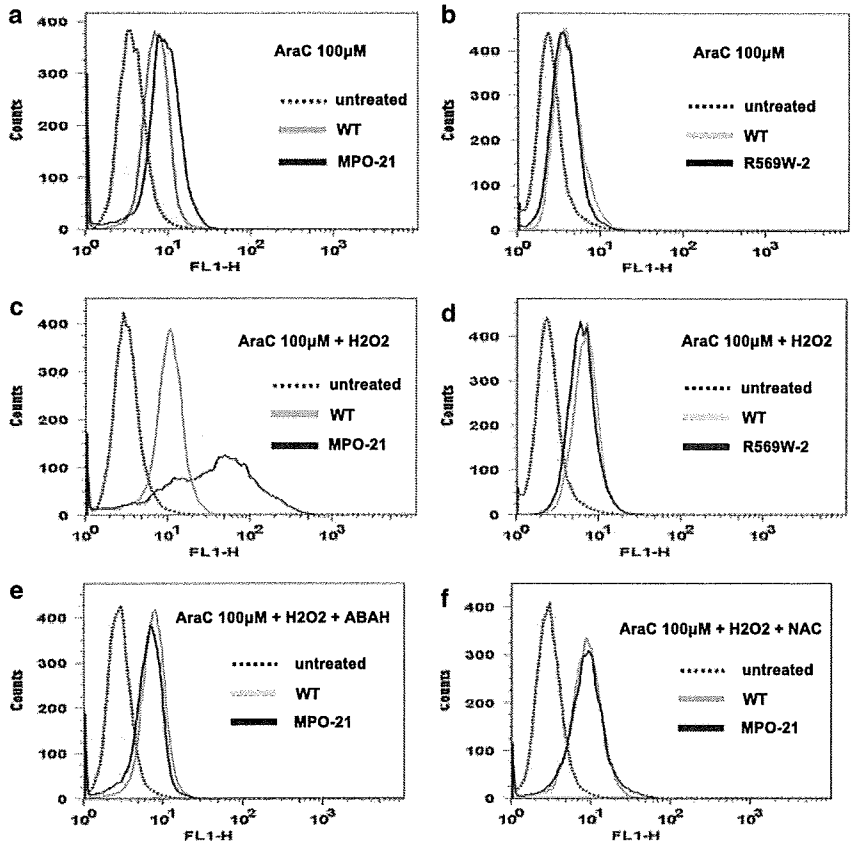

Figure 5 Reactive oxygen species (ROS) generation in K562 cells expressing myeloperoxidase (MPO). After treatment with cytosine arabinocide (AraC), ROS production probed with APF was increased in wild-type K562 (WT) cells, and was still stronger in MPO-21 cells (**a**). There was no difference in ROS production between WT and R569W-2 treated with AraC (**b**). In the presence of H_2O_2, ROS generation was enhanced more strongly in MPO-21 cells than WT or R569W-2 cells (**c** and **d**). These changes were completely abrogated by 4-aminobenzoic hydrazide (ABAH), an inhibitor of MPO (**e**), and *N*-acetylcysteine (NAC), an antioxidant (**f**). FL1-H represented the fluorescent intensity of APF.

for ROS. After treatment with AraC, the amount of ROS detected by APF but not by HPF was increased in MPO-21 cells when compared to wild-type K562 cells or R569W-2, suggesting the production of hypochlorous acid among ROS (Figures 5a and b). To clarify differences in ROS production, H_2O_2 was added into the culture medium to enhance MPO-dependent ROS production. ROS production was increased with H_2O_2 alone (Supplementary data, Figures 3a and b); however, combining H_2O_2 (40 μM) with AraC significantly enhanced the generation of ROS in MPO-21 cells but not in wild-type K562 cells or R569W-2 (Figures 5c and d). ROS were also generated in other MPO-expressing cell lines, MPO-6 and MPO-18 (Supplementary data, Figures 3c–f). The increase of ROS was completely abolished by 4-aminobenzoic hydrazide (100 μM), an inhibitor of MPO (Figure 5e) or by *N*-acetylcysteine (1 mM), a thiol antioxidant (Figure 5f). These results suggested that the activity of MPO was directly related to the production of ROS when cells were treated with AraC. We did not observe any change in the fluorescent intensity of diaminofluorescein-2 diacetate, a probe for nitric oxide, even after treatment with H_2O_2 and AraC (data not shown).

To further analyze the effect of H_2O_2 on cell growth, we treated wild-type K562 and MPO-21 cells with H_2O_2 for a short period in the presence or absence of AraC. As shown in Figure 6a, after treatment with 40 μM H_2O_2 for 45 min, the value of WST-1 assay decreased in both wild-type K562 and MPO-21 cells transiently and recovered on day 2. However, in the presence of AraC, the same treatment with H_2O_2 suppressed cell growth more significantly in MPO-21 than in K562 cells (Figure 6b). In addition, only wild-type K562 cells recovered from the suppression. In this system, the combination of AraC and H_2O_2 was not enough to suppress the growth of leukemia cells; MPO was also necessary.

Generation of nitrotyrosine was enhanced by MPO

Since MPO was shown to catalyze the generation of not only ROS but also nitrotyrosine in the presence of nitrogen dioxide, we next examined whether the introduction of MPO in K562 cells also changed the amount of nitrotyrosine. Western blot analysis using an anti-nitrotyrosine antibody detected strong nitration of proteins in the positive control lysate of wild-type K562 cells incubated with RNS (Figure 7). Without AraC or RNS, wild-type K562, R569W-2 and MPO-21 cells showed similar patterns and intensities in the expression of nitrotyrosine, which were all much weaker than those of the positive control. After treatment with AraC, the intensity of bands only increased in MPO-21 cells.

Colony formation of AML cells in semisolid media

In six AML cases, colony formation of CD34+ AML cells was tested in the presence or absence of AraC (Table 1). CD34+ cells were selected from the bone marrow or peripheral blood to avoid the influence of MPO present in mature myeloid cells. The number of colonies generated was increased among three cases with low MPO (3, 6 and 10%) than three with high MPO positivity (90, 96 and 100%). AraC (20 nM in culture) suppressed colony formation in three cases with high MPO compared to low-MPO cases: the number of colonies decreased to 0–10% of control in the presence of AraC, whereas 33–89% of control in low-MPO cases.

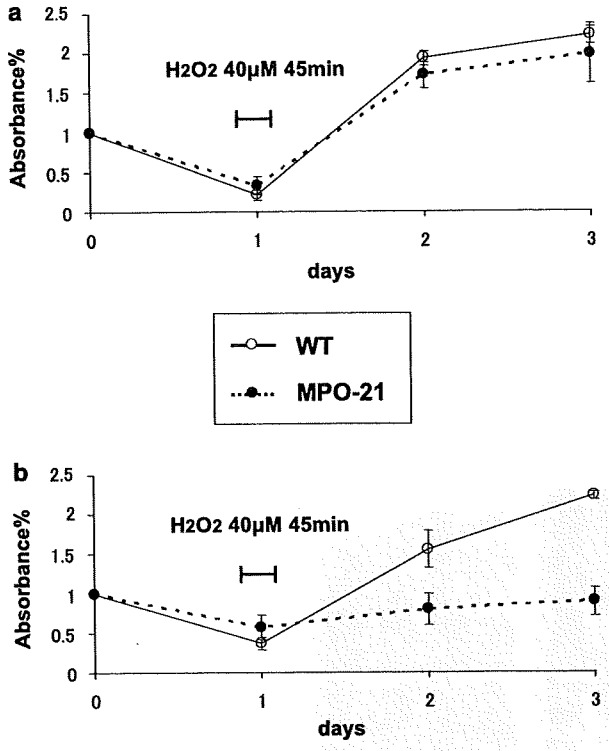

Figure 6 Combination of cytosine arabinocide (AraC), hydrogen peroxide (H_2O_2) and myeloperoxidase (MPO) showed the strong suppression of proliferation as measured by WST-1 assay. WT and MPO-21 cells were treated with H_2O_2 for 45 min with or without AraC. Transient treatment with H_2O_2 alone resulted in no difference between WT and MPO-21 (**a**). Addition of AraC suppressed proliferation of MPO-21 cells only (**b**). The mean value of three independent experiments at each point is shown with the standard deviation.

Production of ROS and nitrotyrosine in AML cells treated with AraC

We next examined whether the expression of MPO in CD34+ AML cells related to the production of ROS when treated with AraC. As shown in Figures 8a and b, in one out of eight samples tested, ROS production was increased by AraC in the presence of H_2O_2. The MPO positivity in this case was 98% by flow cytometry. In other seven cases, regardless of the percentage of MPO-positive cells (0, 0.4, 94, 1, 5.5, 96 and 25% among CD34+ cells), no ROS were detected. Figures 8c and d are the representative histograms of negative samples in which ROS were not detected in leukemia cells even after treatment with AraC, H_2O_2 or the combination of both.

Nitration of tyrosine residues was tested with western blot analysis among four cases of AML: two with high MPO (case 8,

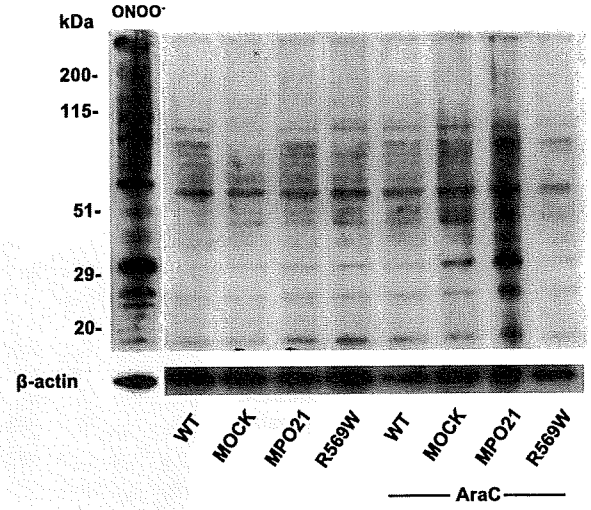

Figure 7 Nitration of tyrosine residues after cytosine arabinocide (AraC) treatment in MPO-21 cells. The generation of nitrotyrosine was examined in cells treated with reactive nitrogen species (RNS) or AraC. Positive control samples treated with RNS (peroxynitrite tetramethylammonium ($ONOO^-$)) and AraC-treated MPO-21 cells showed a clear increase in nitrotyrosine when compared to untreated controls. WT, wild-type control.

Table 1 Inhibition of colony formation of CD34-positive AML cells by AraC

Case number	*Diagnosis*	*% MPO+cells*	*Number of cells per well*	*Number of colonies per well*		*Percentage of colony number in AraC 20*
				Control	*AraC (20 nM)*	
1	M4	6	1×10^5	19	17	89
2	MDS/AML	3	1×10^5	63	55	87
			2×10^5	111	89	80
3	M5b	10	1×10^5	141	47	33
			2×10^5	TMTC	66	NA
4	M1	100	1×10^5	0	0	NA
			2×10^5	4	0	0
5	M2	96	1×10^5	61	6	10
6	M2	90	1×10^5	8	0	0

Abbreviations: AML, acute myeloid leukemia; AraC, cytosine arabinocide; MDS, myelodysplastic syndromes; MPO, myeloperoxidase; NA, not available; TMTC, too many to count.

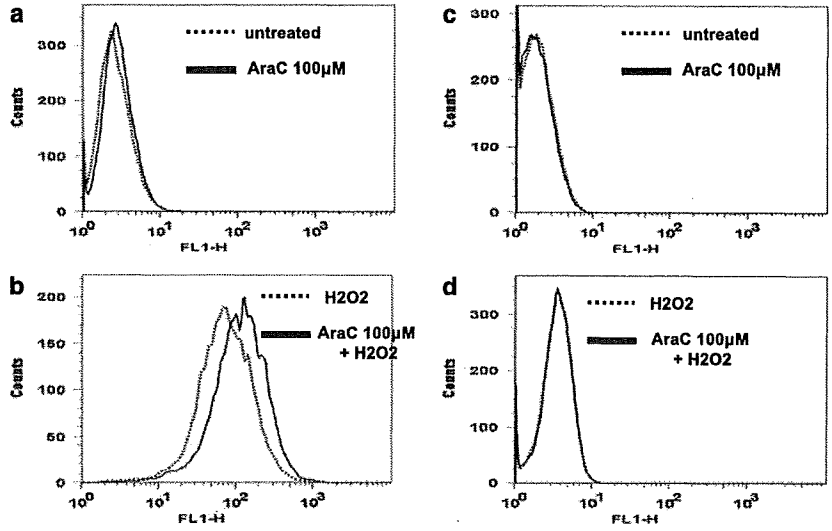

Figure 8 Generation of reactive oxygen species (ROS) in acute myeloid leukemia samples. CD34+ cells were selected and were treated with 100 μM cytosine arabinocide (AraC) and 40 μM hydrogen peroxide (H_2O_2) for 3 h. ROS generation was examined with flow cytometry. Histograms of a positive case (**a** and **b**, high myeloperoxidase (MPO) expression) and a negative case (**c** and **d**, low MPO expression) are shown.

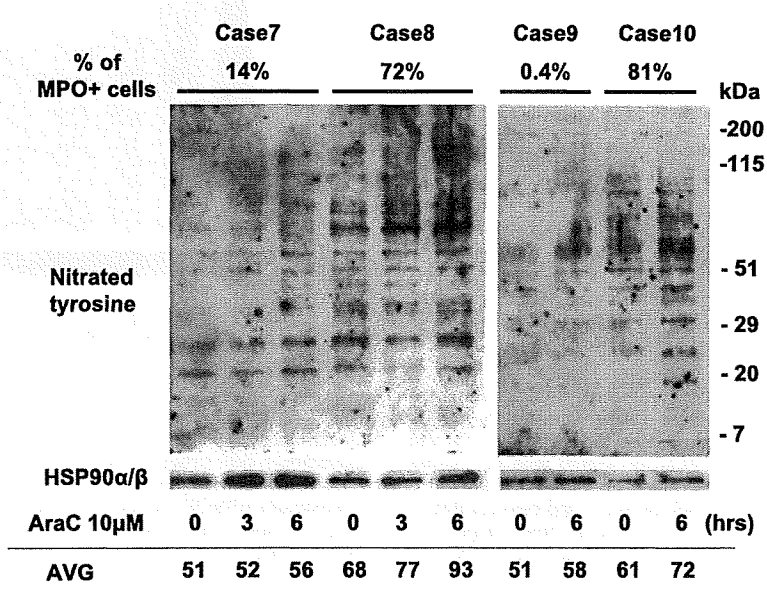

Figure 9 Nitration of tyrosine residues in acute myeloid leukemia (AML) samples. CD34+ cells from four AML cases were treated with 10 μM cytosine arabinocide (AraC) for up to 6 h. Two cases had high myeloperoxidase (MPO) expression (cases 8 and 10), and two had low MPO (cases 7 and 9). Nitrotyrosine was detected by western blot analysis. The intensity of bands in each lane was shown as average value (AVG) in this figure. The percentage of MPO-positive cells was assessed using flow cytometry.

72% of MPO positivity, and case 10, 81%) and two with low MPO (case 7, 0.4%, and case 9, 14%). The intensity of bands in each lane was measured as described in Materials and Methods, then shown as an average intensity (AVG) in Figure 9 (raw data of this procedure is in Supplementary data, Figure 4 and Table 1), which increased along with the incubation time with AraC. The increment of AVG after 6 h of treatment was larger among cases with high MPO (136 and 118% in cases 8 and 10, respectively) than among those with low MPO (110 and 113% in cases 7 and 9, respectively).

Discussion

In this study, we demonstrated that MPO-expressing K562 leukemia cells showed an increased sensitivity to AraC when compared to wild-type or non-functional MPO-expressing K562 cells. After treatment with AraC, these cells generated a higher amount of ROS and nitrated tyrosine residues, resulting in an earlier induction of apoptosis. These reactions were abrogated by inhibitors of MPO or ROS. The results above strongly suggested the relationship between the expression of MPO and

the production of ROS or tyrosine nitration in leukemia cells when treated with AraC. Since ROS and protein nitration were already shown to be toxic for target cells, it is likely that the active MPO protein itself worked with AraC to increase its cytotoxicity. Accordingly, using fresh AML cells, the inhibition of colony formation by AraC tended stronger in cases with high MPO than in those with low MPO expression. It is interesting that the number of colony in high-MPO cases was less than that in low-MPO cases inspite of the fact that the forced expression of MPO in K562 did not influence their proliferation. It seemed that MPO itself does not change growth of cells, but the characteristics of AML cells that express MPO might relate to one of the many factors that control their growth, at least, in some cases. The generation of ROS and the nitration of tyrosine residues, though not so apparent as in colony-formation experiments, were observed only when CD34+ blasts expressed MPO at high levels. It is conceivable that similar reactions were triggered by AraC in high-MPO AML cells as in MPO-expressing K562 cells. MPO did not enhance the fluorescence of diaminofluorescein-2 diacetate, which reacts with NO, in MPO-expressing K562 cells after AraC treatment; however, the nitration of tyrosine residues in these cells was observed by western blot analysis. It seemed that the ROS generated by MPO were involved in the nitration of tyrosine residues as reported previously.[17–19]

Clinical observation has repeatedly shown a significant impact of the percentage of MPO-positive blasts on the prognosis of AML patients.[3–5] From data in this study, we postulate that MPO itself could enhance the cytotoxicity of chemotherapeutic agents through the generation of ROS or the nitration of cellular proteins, and that it could contribute, at least in part, to favorable responses to chemotherapy. It is very interesting that AML cases with favorable karyotypes such as t(15;17), t(8;21) and inv(16) usually have a high percentage of MPO-positive blasts.[20,21] Recently, a polymorphism in the promoter region of the *MPO* gene was shown to relate to survival of breast cancer patients after chemotherapy:[22] patients having lower transcriptional activity of *MPO* (G to A conversion at the −463 nucleotide of the *MPO* gene) showed significantly worse prognosis. The authors of this report concluded, in concordance with our current observation, that the oxidative stress would modify prognosis after chemotherapy for breast cancer.

Leukemia stem cells that consist of a small fraction of the overall leukemia cell population have been reported to maintain leukemia.[23] It is highly possible that the chemosensitivity of leukemia stem cells is an important and vital factor for obtaining a good response to chemotherapy leading to a favorable prognosis. We previously reported that expression of the *MPO* gene in CD133-positive leukemia cells related to the prognosis of AML.[24] As the CD133-positive fraction of AML cells contained leukemia stem cells,[25] the results of the present study could be interpreted as events occurring in the growth fraction of AML cells.

Myeloperoxidase cannot be the sole marker of a good response to chemotherapy. For example, defenses against oxidative stress would also affect the response to ROS generated by anticancer drugs. In this regard, the results in Figures 8 and 9, the ROS and nitrotyrosine generation in clinical samples needed to be re-evaluated. It therefore is necessary to fully understand the biology of the immature (stem cell) fraction of leukemia, including the expression of MPO and defense mechanism against ROS and its relationship with other factors such as the karyotype of leukemia cells and other genetic abnormalities.

Acknowledgements

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

Proposals for a Grading System for Diagnostic Accuracy of Myelodysplastic Syndromes

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Abstract

Despite recent advances in cytogenetics and molecular research, universal biomarkers for the diagnosis of the myelodysplastic syndromes (MDS) are still lacking. It is not easy to diagnose MDS by morphology alone, particularly in patients with < 5% blasts in the bone marrow (BM) and normal karyotype. Therefore, the possibility of misdiagnosis and discordance among observers can occur. In order to resolve these problems, we propose a grading system for diagnostic accuracy of MDS. The diagnostic accuracy of MDS is graded into "definite," "probable," or "possible" in addition to "idiopathic cytopenia(s) of uncertain significance (ICUS)." The criteria of grading for diagnostic accuracy are a combination of (1) the frequency of blasts in BM, (2) grade of dysplasia (high, intermediate, or low), and (3) division of cytogenetics (abnormal, normal, or unknown). For quantitative morphologic evaluation of dysplasias, we classified morphologic dysplastic changes into highly specific category A (pseudo–Pelger-Huet anomaly, degranulation of neutrophils, micromegakaryocytes, and ringed sideroblasts) and less specific category B (dysplasias other than those in category A). We believe that diagnostic problems would be reduced by using our grading system and repeating BM examination at suitable intervals for patients who are allocated into the "possible" or "ICUS" categories, and this will make the vague margin of MDS category clearer.

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Key words: Cytogenetics, Dyserythropoiesis, Idiopathic cytopenia of uncertain significance, Pelger-Huet

Introduction

Myelodysplastic syndromes (MDS) are acquired clonal stem-cell disorders characterized by ineffective hematopoiesis with myelodysplasia[1] and are associated with a high risk of progression to acute leukemias.[2] Despite recent advances in cytogenetics and molecular research, universal biomarkers for the diagnosis of MDS are still lacking. It is not easy to diagnose MDS, particularly in patients with < 5% blasts in the bone marrow (BM) and normal karyotype. In such patients, the diagnosis mainly depends on morphologic examinations. Minimal morphologic requirements to diagnose MDS are well established but might not be accurate or leave too much room for subjectivity. Herein, we propose a grading system for the diagnostic accuracy in an attempt to reduce misdiagnosis and improve concordance among observers.

Background for Proposals

Exclusion of nonclonal disorders[3-6] with some myelodysplasia is crucial to the diagnosis of MDS. However, in patients with < 5% blasts in the BM and normal karyotype, it is not easy to distinguish MDS from such nonclonal disorders by morphology alone. In addition, judgments of dysplasia are subjective to a certain extent. Therefore, misdiagnosis and discordance among observers are likely to occur. In patients with hypoplastic BM, it is important to distinguish hypoplastic MDS from aplastic anemia (AA). Dyserythropoiesis (Dys E) is often found in patients with AA and cannot be used alone to distinguish MDS from AA.[7]

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