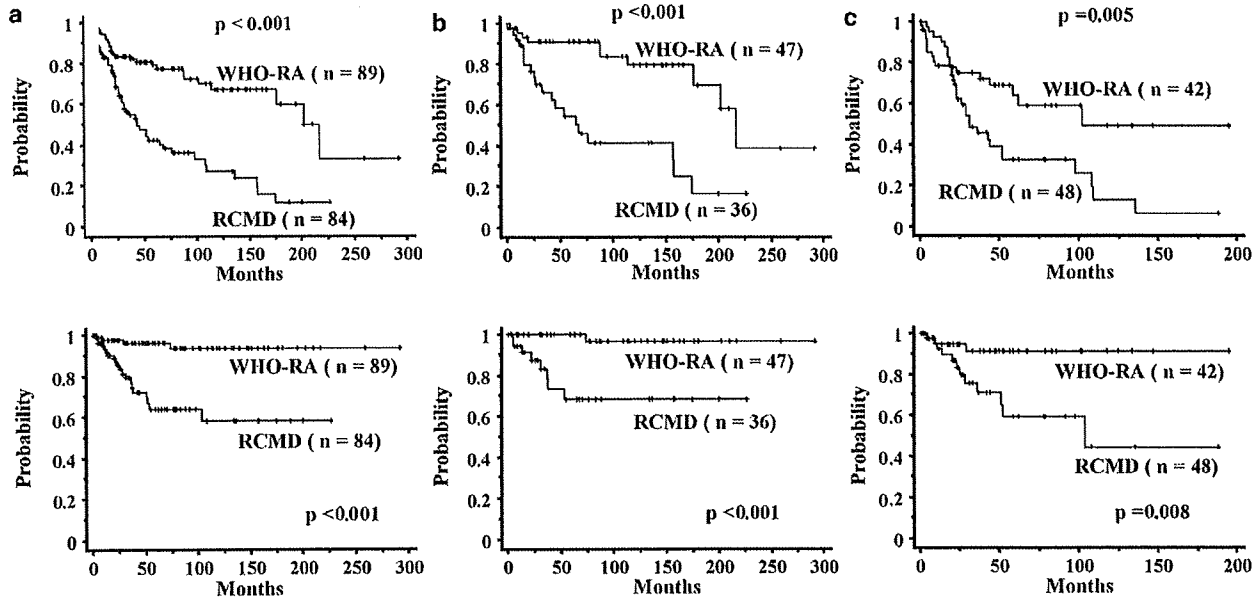

Figure 3 Cumulative overall survival and leukemia-free survival of WHO-RA and RCMD patients according to the modified WHO classification. (Top) Overall survival (OS). (Bottom) Leukemia-free survival (LFS). (**a**) Among all patients with FAB-RA, excluding 5q-syndrome, the modified RCMD patients had a more unfavorable prognosis than the modified WHO-RA patients (OS, $P<0.001$; LFS, $P<0.001$). (**b**) In patients aged 60 years or younger, the modified RCMD patients had a more unfavorable prognosis than the modified WHO-RA patients (OS, $P<0.001$; LFS, $P<.0.001$). (**c**) In patients aged older than 60 years, the modified RCMD patients had a more unfavorable prognosis than the modified WHO-RA patients (OS, $P=0.005$; LFS, $P=0.008$).

RCMD patients show a more unfavorable OS than the modified WHO-RA patients (Figure 3b-c). In the LFS, the modified RCMD patients were significantly more unfavorable than the modified WHO-RA patients (Figure 3a). For patients aged 60 years or less, the LFS of modified RCMD patients was significantly more unfavorable than that of the modified WHO-RA patients. For those older than 60 years, the modified RCMD patients show a more unfavorable LFS than the modified WHO-RA patients (Figure 3b-c).

Discussion

The patients in the present study were selected randomly, and the number of patients was smaller than our previous study.[12] However, the clinical features of Japanese patients were different from those of German patients as in the previous study. Japanese patients in present study were significantly younger than German patients (median age: Japan, 56 years; Germany, 62 years; $P=0.026$). Japanese patients had lower absolute neutrophil counts (median: Japan, 1.39×10^9/l; Germany, 1.82×10^9/l; $P=0.069$), lower hemoglobin concentrations (median: Japan, 8.2 g/dl; Germany, 10.3 g/dl; $P<0.001$), lower platelet counts (median: Japan, 34×10^9/l; Germany, 108×10^9/l; $P<0.001$), and a lower frequency of cytogenetic abnormalities (Japan, 27%; Germany, 64%; $P<0.001$) than German patients.

Previous reports from our Japanese and German MDS study group, as well as other investigators, confirmed that WHO-RA patients had a more favorable prognosis than RCMD patients.[7–9,12] In our previous report,[12] the concordance rate of morphological diagnosis according to the WHO classification between Japanese and German hematologists was 83.8%, and a significant concordance was achieved while using the WHO classification (κ 0.73, $P<0.001$). Therefore, we believe that the evaluation of the frequency of dysmyelopoiesis is comparable between Japanese and German hematologists. Moreover, the present final evaluations concerning dysplasia were reached by consensus among the Japanese and German groups by a joint review. We believe that the WHO classification based on morphological features is useful, at least, in our Japanese and German groups, if the morphological features reflect the prognosis. However, the previous study[12] was performed according to the criteria of a prior report from Germany.[7] The threshold of dys Mgk in this report was defined as 40%. In the criteria of RCMD according to the original WHO classification, the threshold of frequency for the degree of dysplasia in each lineage was defined as 10%. Still, the impact of this threshold (10%) in each lineage on prognosis has not been fully assessed. Nosslinger *et al.*[14] reported that WHO-RA patients did not show more favorable prognoses when compared to the RCMD patients. However, the threshold of dysplasia in their report was 50%. This demonstrates that the threshold of dysplasia for RCMD is still controversial. In the present study, all patients showed dys E$\geqslant$10%. Therefore, dys E$\geqslant$10% did not have a prognostic effect. Dys Mgk$\geqslant$10% was not an unfavorable prognostic factor for OS and LFS. On the other hand, dys G $\geqslant$10% and dys Mgk$\geqslant$40% were significant adverse prognostic factors correlated with OS and LFS. And, these threshold levels have similar prognostic effects between Japanese and German patients in uni- and multivariate analyses. We reported earlier that Pelger and mMgk were correlated with OS and LFS in Japanese patients.[10,11] However, when mature neutrophils had two lobes, the definition of 'Pelger' in this previous report was different from that in the present study. In this previous report, we defined mature neutrophils with the two lobes joined by a thin, hair-like bridge ('pince-nez type cells') as 'Pelger'. In contrast, we defined hypo-segmented mature neutrophils with

strikingly clumpy chromatin as 'Pelger' in the present study. Because of this difference of the definition, the frequency of Pelger in the present study was higher than that in this previous report. In the present study, Pelger+ and mMgk+ were significant adverse prognostic factors for OS and LFS. Again, these results were similar between the Japanese and German patients in uni- and multivariate analyses. The results of the present study support our previous results not only in Japanese patients, but also in German patients. Of note, the prognosis of dys G $\geqslant$10% with Pelger+ was not different from that of dys G $\geqslant$10% without Pelger+. In contrast, the prognosis of dys Mgk $\geqslant$40% with mMgk+ was worse than that of dys Mgk $\geqslant$40% without mMgk+.

We recently compared the clinical features of Japanese and German patients with FAB-RA and found some different prognostic factors, e.g. cytopenias according to IPSS publication were found to be useful for the assessment of prognosis in German FAB-RA patients, but not in Japanese FAB-RA patients.[12] In contrast, the prognostic relevance of the morphological features was similar in Japanese and German FAB-RA patients in the study presented here. However, in the multivariate analyses, there were slight differences between Japanese and German FAB-RA patients. For this reason, we speculate that the prognostic effects of the age category of Japanese patients may have an influence. In the present study, Japanese FAB-RA patients aged 60 years or less had a more favorable prognosis than German FAB-RA patients aged 60 years or less in OS ($P=0.001$) as in our previous study.[12] The degree of dysplasias was more severe in Japanese patients aged older than 60 years than those 60 years or younger. The frequency of RCMD according to our modified criteria was higher in Japanese patients aged older than 60 years than those 60 years or younger (48% vs 35%). Therefore, it seems that morphological features may not be significant independent prognostic factors due to the effects of age category in Japanese patients. In contrast, there were no differences in the frequency of RCMD according to our modified criteria between the German patients aged older than 60 years and those 60 years or younger (60% vs 62%). In addition, because the frequency of poor staining of the films was high in German patients, only a small number of cases could be judged in the morphological study ($n=58$) of German patients. It is expected that the significance as prognostic factors of morphological features becomes certain in multivariate analyses if we can examine more examples even in German patients. Concerning model C including cytogenetics, the number of patients with poor karyotype among 84 RCMD patients according to the modified definition was 17 (20%). In contrast, the number of patients with poor karyotype among 89 WHO-RA patients was only 4 (5%). We thought that the degree of dysplasias was related to the cytogenetic findings. Therefore, in model C, morphological features may not be significant independent prognostic factors.

In univariate analyses, dys G $\geqslant$10% was correlated with OS and LFS in all patients and in patients from each country. However, dys Mgk $\geqslant$10% was not correlated with OS and LFS in all patients or in patients from either country. In the present patients, RCMD patients diagnosed by using a uniform threshold of 10% for dys G and dys Mgk according to the original WHO classification did not show a worse prognosis than WHO-RA patients. We think that it may be necessary to revise the morphological definition of RCMD to improve the WHO classification. Therefore, we propose modified morphological criteria for RCMD.

This morphological analysis in MDS patients has several limitations. We held two meetings on BM morphology and made great efforts to achieve morphological consensus. However, the evaluation of dysplasias might be different among different observers. The number of evaluable cells in the megakaryocytic lineage is smaller than that in other lineages. Therefore, the concordance rate of frequency of dys Mgk among different observers might be lower than that of dys G or dys E. We think that the different morphological interpretation of megakaryocytes among different observers is one of the main causes of the disagreement in the diagnosis of WHO classification. For example, patients of FAB-RA in which three megakaryocytes among 25 megakaryocytes are judged to be dysplastic are classified as RCMD according to the original WHO classification. In contrast, patients of FAB-RA in which only two megakaryocytes among 25 megakaryocytes are judged to be dysplastic are classified as WHO-RA. Therefore, we think that this threshold (10%) of dys Mgk has problems not only for the assessing of the prognosis but also for the diagnosis of RCMD. It was reported that mMgk was specific dysplasias in MDS patients[15] and the concordance rates concerning mMgk was sufficient.[10] We think that the disagreement rate of morphological diagnosis might be decreased by using our modified criteria combining the frequency of dys Mgk and mMgk. The threshold (40%) of dys Mgk of our modified criteria is different from that (10%) of the original WHO criteria. The German group had already shown the usefulness of the WHO classification in several large-scale studies[7,13] using this threshold (40%). Therefore, we believe that the usefulness of this threshold shown in the present study is certain.

In conclusion, the present results showed that the degree of dysplasia in FAB-RA patients was related to OS and LFS, and the prognostic effect of dysplasia was similar between the Japanese and German FAB-RA patients. However, the thresholds of dysplasia influencing prognosis were different from the original threshold of the WHO classification. We propose to raise the threshold of dys Mgk in the criteria for RCMD from 10 to 40% and add mMgk+.

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ORIGINAL ARTICLE

Small number of HTLV-1-positive cells frequently remains during complete remission after allogeneic hematopoietic stem cell transplantation that are heterogeneous in origin among cases with adult T-cell leukemia/lymphoma

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) can provide long-term remission for patients with adult T-cell leukemia/lymphoma (ATLL) caused by human retrovirus, human T-lymphocyte virus (HTLV-1). To understand how HTLV-1-positive cells including ATLL cells were suppressed by allo-HSCT, we examined HTLV-1 provirus load and residual ATLL cells in peripheral blood of transplant recipients using PCR-based tests. We found that the copy number of HTLV-1 genome, called provirus, became very small in number after allo-HSCT; however, in most cases, provirus did not disappear even among long-term survivors. Tumor-specific PCR tests demonstrated that most of HTLV-1-positive cells that remained long after transplantation were not primary ATLL cells but donor-derived HTLV-1-positive cells. We also found a case having very low amount of residual disease in peripheral blood even long after transplantation. There was only one recipient in whom we failed to show the presence of HTLV-1 genome and antibody against HTLV-1 even with an extensive search, which strongly suggested the elimination of HTLV-1 after allo-HSCT. These results demonstrated that after allo-HSCT the small amount of residual HTLV-1-positive cells were heterogeneous in origin and that long-term disease control for ATLL could be obtained without the complete elimination of HTLV-1.
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Keywords: ATLL; transplantation; MRD; HTLV-1

Introduction

Adult T-cell leukemia/lymphoma (ATLL) is a peripheral T-cell lymphoma caused by a retrovirus, human T-lymphocyte virus (HTLV-1), which randomly integrates into the genome of infected T cells.[1–3] The HTLV-1 genome in T cells, called provirus, has been utilized for the diagnosis of the disease caused by or the carrier state of HTLV-1. For example, Southern blot analysis of HTLV-1, when it demonstrates a monoclonal proliferation of cells infected with HTLV-1, provides the strongest evidence for the diagnosis of ATLL.[4] Southern blot analysis usually detects a monoclonal population composed of 3–5% of total cells, which is generally enough to diagnose ATLL. On the other hand, polymerase chain reaction (PCR)-based tests detect HTLV-1 genome with much higher sensitivity than Southern blot analysis, allowing us to monitor a small amount of HTLV-1 provirus load.[5,6]

The clinical course of ATLL widely differs by clinical subtypes (acute, lymphoma, chronic and smoldering). The prognoses of acute and lymphoma types are very poor when treated with conventional or even high-dose chemotherapy;[7,8] however, with allogeneic hematopoietic stem cell transplantation (allo-HSCT), a long-term clinical remission (CR) is achievable as reported from several groups including ours.[9–11] For example, among cases with acute ATLL, allo-HSCT reduced the volume of tumor cells in the peripheral blood to undetectable level when tested by morphological examination or Southern blot analysis, suggesting that the reduction of ATLL cells was less than 5% of WBC, as we reported previously.[11]

In this study, as an extension of our previous report, to understand how small the population of HTLV-1-positive cells would become after allo-HSCT and to test whether HTLV-1 could be eradicated, we investigated HTLV-1 provirus load and the minimum residual disease (MRD) in 22 cases of ATLL using PCR-based gene amplification. Since PCR for HTLV-1 provirus picked up not only ATLL cells, but also all cells infected with HTLV-1, including polyclonal non-ATLL cells, we introduced a specific PCR method to detect ATLL cells utilizing a unique integration site of HTLV-1 in each ATLL case.

We found that cells carrying HTLV-1 existed at the very low level in peripheral blood of long-term survivors after allo-HSCT. Most of them were donor-derived cells, but MRD was simultaneously present only in one case. We also experienced a single case in which anti-HTLV-1 antibodies became negative with no HLTV-1 genome amplified with PCR-based tests, suggesting the eradication of HTLV-1.

Patients and methods

Clinical features of patients with ATLL

The diagnosis and classification of ATLL was based on the criteria proposed by the Lymphoma Study Group of Japan.[12] Twenty-two patients with the diagnosis of acute or lymphoma type ATLL who received allo-HSCT in three hospitals in Nagasaki, an endemic area of HTLV-1 in Japan, between September 1997 and May 2004 were included in this study.

Table 1 summarizes the clinical characteristics of these patients. Median age of the patients was 48 years. In 21 of all 22 cases, donor-derived hematopoiesis was obtained (Table 2).

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Table 1 Characteristics of patients and transplantation

Case no.	*Age at HSCT /sex*	*Disease status at HSCT*	*Donor α HTLV-1 Ab*	*Donor*	*Source*	*Conditioning Regimen*
1	44/M	NC	–	Related	BM	TBI-VP16-CA
2	48/M	PD	–	Related	BM	TBI-VP16-CA
3	43/F	CR	–	Unrelated	BM	TBI-VP16-CA
4	51/M	PR	–	Related	BM	BU-CY2
5	30/F	PR	+	Related	BM	BU-CY3
6	54/F	PR	+	Related	BM	BU-CY2
7	44/F	PR	–	Unrelated	BM	TBI-CY
8	48/F	CR	+	Related	BM	BU-CY3
9	35/M	PD	–	Related	PB	BU-CY2
10	39/M	PD	–	Related	PB	TBI-CY
11	41/F	NC	–	Unrelated	BM	TBI-CY
12	48/M	PR	–	Related	PB	TBI-CY
13	46/M	PR	+	Related	BM	TBI-CY
14	50/F	PR	–	Unrelated	BM	TBI-CY
15	63/M	PD	+	Related	PB	FLU-BU-ATG (RIST)
16	53/M	CR	–	Related	PB	FLU+L-PAM (RIST)
17	55/M	NC	–	Related	PB	FLU-BU (RIST)
18	63/M	NC	–	Related	PB	FLU-CY (RIST)
19	48/F	PR	+	Related	BM	FLU+L-PAM (RIST)
20	53/M	CR	+	Related	BM	FLU-CY (RIST)
21	56/M	PD	+	Related	PB	FLU+L-PAM+TBI (RIST)
22	62/M	PR	–	Related	PB	FLU-BU (RIST)

Abbreviations: BM, bone marrow; BU, busulfan; CA, Cytarabine; CR, complete response; CY, cyclophosphamide; FLU, fludarabin; HSCT, hematopoietic stem cell transplantation; L-PAM, melphalan; NC, no change; PB, peripheral blood; PD, progressive disease; PR, partial response; RIST, reduced-intensity conditioning transplantation; TBI, total body irradiation; VP16, Etoposide.

Table 2 Results of transplantation

Case no.	*Engraftment*	*Relapse*	*aGVHD*	*cGVHD*	*Outcome*
1	+	Day 3074	I	—	Alive with ATLL (day 3094+)
2	+	—	IV	NE	Died of GVHD on day 123
3	+	Day 144	II	—	Died of ATLL on day 165
4	+	Day 169	I	—	Died of ATLL on day 237
5	+	—	0	—	Alive in CR (day 1756+)
6	+	Day 833	II	—	Alive in 2nd CR (2nd CR after local irradiation, day 1679+)
7	+	Day 262	0	Extensive	Died of ATLL on day 1310
8	+	—	0	Extensive	Alive in CR (day 1497+)
9	+	—	III	—	Died of infection on day 137
10	+	—	0	Limited	Alive in CR
11	+	Day 78	I	Extensive	Died of ATLL on day 218
12	+	—	III	—	Alive in CR (day 254+)
13	+	—	IV	—	Died of TRM on day 120
14	+	—	0	—	Died of cerebral haemorrhage on day 216
15	+	—	0	Extensive	Died of GVHD on day 167
16	+	—	II	Limited	Alive in CR (day 1138+)
17	+	—	I	Extensive	Alive in CR (day 1087+)
18	+	—	III	Extensive	Died of infection on day 370
19	+	—	II	—	Alive in CR (day419+)
20	–	—	NE	NE	Alive in CR with recipient-derived hematopoiesis (day 580)
21	+	NE	0	NE	Died of infection on day 41
22	+	Day 73	I	Extensive	Alive with ATLL (day 184+)

Abbreviation: aGVHD, acute GVHD; cGVHD, chronic GVHD; NE, not eligible.

Only one patient (case 21) did not achieve CR after allo-HSCT and seven patients experienced a relapse of ATLL. At the time of analysis, 11 patients were alive and nine of these patients remained in CR.

Quantitative measurement of HTLV-I provirus load in peripheral blood

Peripheral blood samples were collected from the patients after they gave a written informed consent. Genomic DNA was extracted from mononuclear cells (MNC) of peripheral blood using the QIAGEN DNA Midi Kit (QIAGEN, Hiden, Germany) and from paraffin-embedded sample using DEXPAT (TAKARA BIO INC, Shiga, Japan). Quantitative measurement of HTLV-1 provirus was performed with real-time quantitative PCR (RQ-PCR) using the LightCycler System and DNA Master Syber Green I (Roche diagnostics, Mannheim, Germany) as reported previously.[13] In brief, 30 ng of genomic DNA was used as a template and the copy number of HTLV-1 provirus was assessed by the ratio of the amount of tax region of HTLV-1 and that of beta globin gene (tax copies/MNC = 2 × copy number of tax/copy number of beta-globin gene). The mean value of two

experiments was shown as the copy number of HTLV-1 provirus load. Figure 1 shows the correlation between the ratios of the positive control plasmid containing tax region in the irrelevant plasmids and the results of RQ-PCR tests in a log-scale graph. A statistically significant correlation was found ($r=0.89$, $P<0.001$). This system could quantify one copy of the tax gene in 5000 cells.

Detection of primary ATLL cells with inverse PCR

To detect the residual ATLL cells, we performed an inverse PCR as reported by Takemoto *et al.*[14] that amplified the integration site of HTLV-1 in the genome of tumor cells whose sequence was then utilized to establish case-specific PCR primers that amplified a part of HTLV-1 (LTR) and the franking region. Each PCR in this study could at least detect one primary ATLL cell among 10000 normal cells. PCR condition and the DNA sequence of the primer sets in nine cases tested are available upon request.

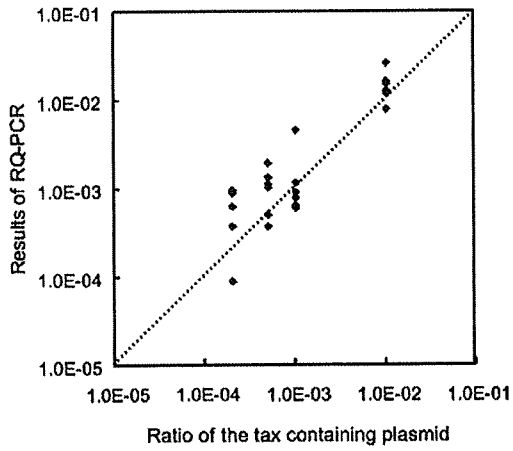

Figure 1 Correlation of the ratio of tax copy number between control plasmid and the quantification using RQ-PCR. Control plasmids containing the tax region of HTLV-1 were serially diluted with plasmids containing irrelevant sequence (beta-globin) and the ratio of target plasmid was quantified using the RQ-PCR method.

Colony formation and the expansion of HTLV-I-infected cells to test the origin of those cells

Previously, we established a method to clonally amplify HTLV-I-infected cells.[15] In brief, MNC in the peripheral blood were cultured in semisolid media containing 0.93% methylcellulose dissolved in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal calf serum (FCS) and 200 ng/ml of recombinant human interleukin (rhIL)-2 (TECHNE Corp., Minneapolis, MN, USA). After three weeks of culture, each colony grown in the semisolid media was picked up individually and transferred to liquid culture (IMDM with 20% FCS and 20 ng/ml of rhIL-2) for clonal expansion. All cell culture was performed at 37°C with 5% CO_2. The origin of cells (donor or recipient) was assessed by means of sex mismatch (using Y chromosome specific SRY gene detection) or the difference of the number in short tandem repeat (STR method).

Results

Quantitative measurement of HTLV-I provirus after allo-HSCT

A total of 86 samples in 22 patients were collected; samples per patient were from 1 to 10 (median 3.5 samples) with median sampling time of 6 months from transplant (0.5 month to 8.3 years). The copy numbers of HTLV-1 provirus in each case are shown in Figure 2a and b. Most of the samples contained a low amount of HTLV-1 provirus, except for two conditions: (1)

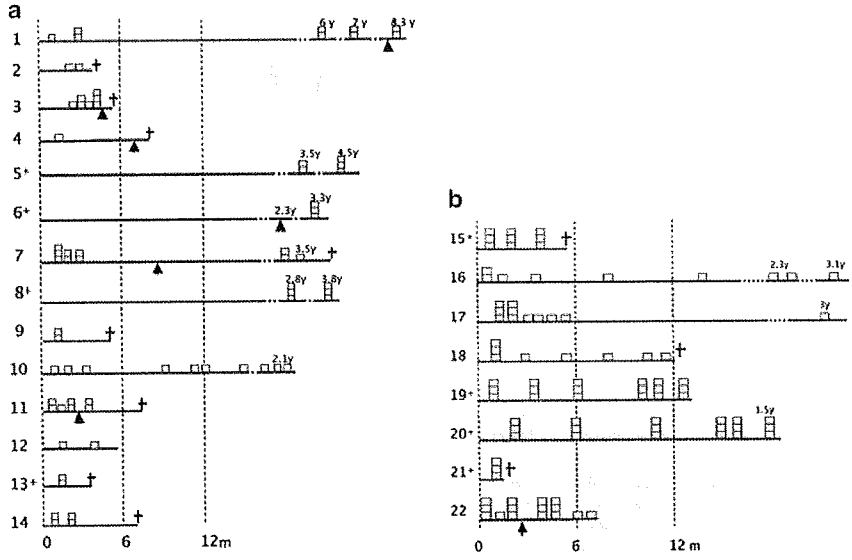

Figure 2 Quantification of HTLV-1 provirus load in the peripheral blood of recipients. Case number is on the left side of the figures. Case number with plus mark represents transplantation from a carrier donor. Copy number of provirus is shown as a gray or white box: three gray boxes represent virus load $\geqslant 10^{-2}$; two gray boxes, $10^{-2} \sim 10^{-3}$; one gray box, $10^{-3} \sim 10^{-5}$; white box, below detection level. Time after transplantation is described as month (m) or year (y). Cross mark represents death of the case and arrow indicates the time of relapse of ATLL. Cases treated with myeloablative conditioning are shown in (**a**) and those received RIST are in (**b**).

transplantation from a carrier donor and (2) right before (about 2 weeks) or after the clinical relapse of ATLL. In 22 samples transplanted from carrier donors, the provirus load was always 500 copies/10^5 cells or more despite the clinical disease status at sampling. The average copy number of HTLV-1 was significantly higher in patients transplanted from a carrier donor than from a noncarrier donor (mean value, 15 000 and 760 copy/10^5 cells, respectively, $P<0.0001$).

Within 6 months from transplantation, the provirus load became undetectable at least once in eight out of 15 cases (case numbers 2, 3, 4, 10, 12, 16, 17 and 18). However, in all seven cases tested later, the copy number of HTLV-1 provirus became detectable again. At the time of the last follow-up, provirus load was below the detection level in only two cases (case numbers 16 and 17). The provirus load during the early period following transplantation was not related to the type of conditioning regimen, disease status before the transplantation or the duration of survival. There was no statistically significant association between provirus loads and the development of severe acute GVHD (data not shown). No specific pattern in the kinetics of virus load was noticed among long-term survivors or among patients that experienced relapse.

Analysis of MRD in the peripheral blood

As a low level of HTLV-1 provirus load was detected in the peripheral blood of most patients, we tested whether primary ATLL cells remained as MRD using specific PCR for primary ATLL cells, which amplified a unique franking genomic region of the HTLV-1 integration site in each case. In nine cases (cases 1, 5, 9, 10, 15, 18, 19, 21 and 22), 34 samples were analyzed with this method (Table 3 and Figure 3). Although the sensitivity of the inverse PCR varied from case to case, the amount of MRD that could be detected by this method was always below the provirus load quantified by RQ-PCR in every sample (data not shown).

Eighteen out of 19 samples collected after this period were negative for MRD regardless of the presence of HTLV-1 provirus. An exception was the sample taken at the time of relapse that took place 8.3 years after transplantation in case 1. CR was continuously maintained in this case and the peripheral blood samples at 6 and 7 years from transplantation were negative in the MRD test. A subcutaneous tumor, which developed at relapse, consisting mostly of CD4-positive cells, had the same integration site of HTLV-1 as primary ATLL cells, demonstrating that the primary ATLL cells had persisted for more than 8 years as MRD.

Analysis of the origin of cells carrying HTLV-1 provirus

Although most of the cells carrying provirus were HTLV-1-infected cells and were not derived from ATLL clones, these findings raised the question of whether these infected cells derived from recipients or donors. To answer this question, we cultured peripheral blood MNC in semisolid media in the presence of rhIL-2 to clonally expand cells infected with HTLV-1. Among 10 cases that maintained CR more than a year, samples were obtained from eight cases. In five out of eight cases, we could establish 30 cell lines (Table 4). Each cell line contained HTLV-1 provirus (data not shown).

In case 20, in which the graft was rejected after transplantation, all eight cell lines were derived from the recipient cells. Among other four cases, 22 out of 23 cell lines were found to originate from the donor cells including one cell line of case 1 that received transplantation from a noncarrier donor. In case 5, despite long-term CR (4.5 years) and complete donor chimerism in the peripheral blood, there was one cell line (one of seven cell lines) that derived from a recipient. By using the established cell line of recipient origin, we determined the franking genomic sequence of HTLV-1 integration site and set up the inverse-PCR. It was applied retrospectively to the genomic DNA extracted from a paraffin-embedded lymph node, which was a biopsy sample for the initial diagnosis in case 5. The lymph node

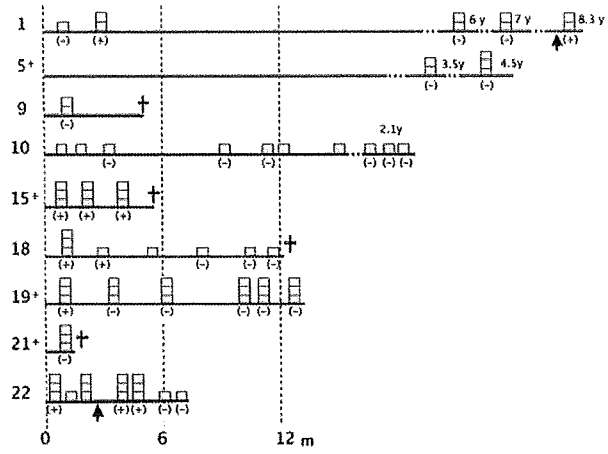

Figure 3 MRD of ATLL after transplantation. MRD of ATLL was assessed using case-specific inverse PCR method. Results of the inverse PCR are shown under the boxes that represent the copy number of provirus. Marks in this figure are the same as in Figure 2.

Table 3 DNA sequence of the franking region of HTLV-1 integration site

Case no.	*DNA sequence of the integration site*
1	AAATTTAGTACACAatatactatgacatataaagtatatgaggt...
5	AAATTTAGTACACAcagatctttccaggaaagataactttaaaa...
9	AAATTTAGTACACAtgcattaagttgaaagctggaaaaattaaa...
10	AAATTTAGTACACAaaaatgtacccaggatttgttttaatcagt...
15	AAATTTAGTACACAaggcataagccagatttacattataaatgc...
18	AAATTTAGTACACAaaatgtaaaaagcctcaagaaattgtaagc...
19	AAATTTAGTACACAgtttctaatcttattttgctgtgcaagctg...
21	AAATTTAGTACACAcatatgaactttaaagtagtttttccaat...
22	AAATTTAGTACACAggcacccagcctaaacccactgctacctga...

DNA sequence of the part of the 3′ region of HTLV-1 integration sites are shown.
Upper cases indicate the sequence of LTR.

Table 4 Origin of colony-forming cells in recipients

Case no.	*Anti-HTLV-1 Ab in the donor*	*Time (year) after HSCT at sampling*	*Number of IL-2-dependent cell lines*		
			Total	*Donor derived*	*Recipient derived*
1	–	7	1	1	0
5	+	4.5	7	6	1
8	+	3.8	5	5	0
10	–	2.1	0	—	—
16	–	2.3 and 3.1	0	—	—
18	–	1	0	—	—
19	+	1.1	9	9	0
20*	+	1.5	8	0	8

*Graft was rejected in case 20.

Table 5 Serial tests for anti HTLV-1 antibody and provirus in case 16

Time after transplantation	*3 weeks*	*1.5 months*	*0.5 year*	*1 year*	*1.6 years*	*2.3 years*	*3.1 years*
Anti-HTLV-1 antibody (PA assay)	NT	NT	x 16	UD	NT	UD	UD[a]
Proviral load	1.86×10^{-3}	UD	UD	UD	UD	UD	UD
Nested PCR test for pX region	NT	UD	UD	Positive	UD	UD	UD
PCR test for gag region	NT	NT	NT	NT	UD	UD	UD
PCR test for env region	NT	NT	NT	NT	UD	UD	UD
IL-2-dependent CFC	NT	NT	NT	NT	2 colonies	0	0

Abbreviations: CFC, colony-forming cell; NT, not tested; UD, undetectable.
[a]Undetectable with three different methods; Western blotting, particle agglutination and fluorescent antibody test.

sample had the same integration site of HTLV-1 as the cell line established 4.5 years after transplantation. Although two peripheral blood samples taken 4.5 years after transplantation were negative for this inverse-PCR, the colony-formation method could detect MRD in the same sample in case 5.

Negative results in the tests for HTLV-1 infection in case 16

In cases 16 and 17, at the time of the last follow-up, HTLV-1 provirus load was below the sensitivity of PCR (1 provirus/10^5 cells). However, the test for antibody against HTLV-1, which is widely used to demonstrate the infection with HTLV-1, was found to be negative only in case 16 (Table 5). Three different methods (Western blotting, particle agglutination and fluorescent antibody test) failed to demonstrate antibodies against HTLV-1 in this case. PCR tests for other parts apart from tax of HTLV-1, gag and env regions, were also negative. All extensive searches for HTLV-1 infection became negative 2.3 years after transplantation and remained negative 8 months later, 3.1 years from transplantation when this manuscript was written.

Discussion

In the present study, we measured HTLV-1 provirus load, detected MRD and determined the origin of HTLV-1 positive cells in the peripheral blood in 22 cases with ATLL treated with allo-HSCT. The HTLV-1 provirus load was reduced at least once to low levels (less than 1000 copies/10^5 cells) in most cases even among those who were transplanted in the status other than CR or those who received a reduced-intensity conditioning. These results showed a strong anti-ATLL effect of allo-HSCT in the short period after transplantation. The average dose of HTLV-1 provirus was significantly higher among cases transplanted from HTLV-1 carrier donors, suggesting the carryover of the virus positive cells from the donors. However, the level of provirus load after transplant did not always correlate to the final clinical outcome. Surprisingly, among most of the patients who survived more than 2 years, HTLV-1 provirus was detectable, although at a lower level, by PCR in their peripheral blood. Contrary to our results, Hishizawa *et al.*[16] using a quantitative PCR method similar to ours, reported the kinetics of HTLV-1 provirus load after allo-HSCT in five cases with ATLL, and they showed that HTLV-1 provirus load was undetectable in two cases in continuous CR. Major differences between their report and ours are the length of the follow-up period (1–15 and 1–84 months) and the number of patients (five and 22 cases). The longer observation periods and larger case number in our study might have facilitated the notice of the reappearance of HTLV-1-positive cells after allo-HSCT.

In contrast with the frequent positive results of provirus load, MRD of primary ATLL was rarely detectable after transplantation. In particular, after 6 months from transplantation, all samples of five cases tested during remission were negative for the MRD test despite the detectable level of provirus load, clearly demonstrating the presence of HTLV-1-positive cells other than ATLL in the peripheral blood of these patients.

HTLV-1-positive cells present in the recipients after allo-HSCT could be theoretically categorized into four groups: (1) MRD of primary ATLL cells, (2) non-ATLL cells of a recipient carrying HTLV-1 (e.g. T lymphocytes at the carrier state), (3) donor-derived cells infected with HTLV-1 in the host after transplant and (4) infused donor cells in the case of transplantation from a carrier of HTLV-1. Based on the results of colony-formation experiments, although the number of clones tested was not large, we demonstrated that there was difference in the origin of cells with HTLV-1 provirus. We found MRD in case 5 (as defined in group 1), donor-derived HTLV-1-positive cells in case 1 (group 3) and examples of group 4 in cases 5, 8 and 19. Non-ATLL cells of recipients were shown in case 20 (group 2). In some cases, we assumed that donor CD4-positive T cells were infected *de novo* with HTLV-1 in the recipient's body after transplantation as observed in case 1. Virus transmission into donor lymphocytes was described previously and our observation supported this report.[17]

In case 1, the MRD tests in the peripheral blood were negative in both samples taken at 6 and 7 years from transplantation; however, ATLL relapsed clinically as a subcutaneous tumor after 8 years of continuous CR. With the same integration sites of HTLV-1 in the primary and relapsed tumor cells, it was apparent that the primary ATLL cells remained somewhere in the body for more than 8 years after allo-HSCT and that negative tests for MRD in the peripheral blood did not necessarily indicate eradication of ATLL even long after transplantation.

On the other hand, in case 16, even with the extensive search for HTLV-1 provirus by PCR for various parts of HTLV-1 genome, we failed to demonstrate its presence in the peripheral blood. The antibody against HTLV-1 also became negative only in this case. So far, there has been no evidence to show the presence of HTLV-1 in this case for more than 8 months. There was a previous report of the eradication of HTLV-1 from a carrier who received allo-HSCT for pure red cell aplasia.[18] The tests for the virus performed in case 16 were almost the same as used in this report, suggesting that HTLV-1 was cleared off from the body after allo-HSCT in this case, indicating eradication of both ATLL cells and carrier T cells of HTLV-1 simultaneously by allo-HSCT.

Recently, we reported that allo-HSCT would bring about graft-versus-ATLL (GvATLL) effect even without clinically obvious graft-versus-host disease (GVHD).[10] GvATLL could be achieved when a specific immune response targeting HTLV-1 was initiated, such as cytotoxic T cells for tax protein as Harashima

et al.[19] reported. It is also possible that allogeneic immune reaction against recipient cells contributed to GvATLL effect even without HTLV-1-specific immune reactions as seen in transplantations from carrier donors. As most long-term survivors were positive for HTLV-1 provirus and anti-HTLV-1 antibody, our observation suggested that GvATLL had an effect on ATLL cells but not HTLV-1 provirus in most cases. Allogeneic immune reaction without clinically apparent GVHD might be enough to suppress ATLL cells in these situations.

In summary, allo-HSCT for ATLL profoundly reduced provirus load of HTLV-1 in recipients; however, small amounts of HTLV-1-positive cells that remained in long-term survivor were heterogeneous in origin. We also experienced the single case in which HTLV-1 seemed to be eradicated with allo-HSCT. Thus, it was suggested that the way allo-HSCT suppressed and controlled ATLL and HTLV-1 itself was not simple but heterogeneous from case to case. Further analysis is necessary to understand how ATLL is controlled by allo-HSCT through GvATLL effect, and to find how this effect be controlled and enhanced.

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Donor-derived DNA in fingernails among recipients of allogeneic hematopoietic stem-cell transplants

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To examine whether donor-derived cells could exist in nonhematopoietic tissues of recipients after allogeneic hematopoietic stem-cell transplantation, we examined the patterns of the short tandem repeat (STR) of DNA extracted from fingernail clippings of recipients so that the contamination of blood cells was excluded. All 21 patients reached donor-derived hematopoiesis after transplantation and 20 of them were in remission of the primary diseases at the time of sampling. Compared with the STRs of donor cells, among 9 of 21 patients, DNA extracted from fingernail samples showed coexistence of the donor pattern of the STRs, sharing from 8.9% to 72.9% of total STR areas. Time from transplantation to sampling was from 305 to 2399 days among positive cases. These results demonstrate for the first time the existence of stable contribution of donor cells in fingernails among recipients of allogeneic hematopoietic stem cells. (Blood. 2007;110: 2231-2234)

Introduction

Among recipients of allogeneic hematopoietic stem cells (allo-HSCs), donor-derived cells in nonhematopoietic tissues have been observed by several groups.[1-4] For example, in biopsy specimens such as skin, gastrointestinal mucosa, or liver that were taken mostly for the diagnosis of graft-versus-host disease (GVHD), cells carrying the Y chromosome were shown in nonblood cells (eg, epithelium of gastrointestinal tract) of female recipients who received transplants from male donors. These observations and reports of other tissues demonstrated that the cells existed in bone marrow or mobilized peripheral blood that could differentiate into (or contribute to) cells in organs or tissues other than those of hematopoietic lineage (eg, gastrointestinal mucosa, skeletal muscle, buccal mucosa, liver, and skin, etc).[1-7] On the other hand, there were also reports against the contribution, at least major contribution, of donor cells in nonhematopoietic cells in human and animal models.[8-10] One of the reasons for the discrepancy of these reports seemed to originate from methodological limitations.[8,11] For example, in histologic analysis using a fluorescent DNA probe technique such as fluorescence in situ hybridization (FISH), coexistence of blood cells in the tissue samples made it difficult to distinguish true donor-derived cells from recipient cells that 3-dimensionally overlapped the donor-derived blood cells. If samples are taken from a biopsy specimen for the diagnosis of GVHD, they are small in general, which makes it difficult to perform a full analysis of chimerism. To overcome these problems and to examine the existence of donor-derived cells in nonhematopoietic tissue, we investigated the contribution of donor-derived cells in fingernails, the tissue without blood cells, among recipients of allo-HSCs. This is the first report to examine the donor-derived DNA in fingernails.

Patients, materials, and methods

The protocol of this study was approved by the Internal Review Board of Nagasaki University. Twenty-one recipients of allo-HSCs participated in this study and were treated and followed at the Department of Hematology, Nagasaki University Hospital. To examine the existence of donor-derived cells in fingernails, the short tandem repeat (STR) of genomic DNA extracted from fingernail clippers was compared with STRs of donor and recipient cells. After obtaining written informed consent in accordance with the Declaration of Helsinki, fingernail samples of 10 fingers (21 cases) and peripheral blood of participants (9 cases) were collected. A new nail cutter was used for every collection of fingernail samples in each patient to avoid contamination. All pieces of nail from each patient were subjected to extraction of total genomic DNA with proteinase K and phenol/chloroform. From nonseparated total peripheral blood, total genomic DNA was extracted and used to examine the chimerism of hematopoiesis at the time of fingernail sampling in 9 cases. The STR of 10 regions was determined using AmpF/STR SGM Plus (Applied Biosystems, Foster City, CA). Polymerase chain reaction (PCR) was performed using GeneAmp PCR system 9600 (Applied Biosystems), and the difference of STRs was detected using an ABI PRISM 310 Genetic Analyzer and Genotyper software version 2.5 (Applied Biosystems). These procedures followed the instructions of the manufacturer. The contribution of donor-derived cells was described as a percentage of an average of donor peak areas divided by total peak areas of every different STR site (except for sex chromosome). The minimum sensitivity of this method was 5%. The Mann-Whitney *U* test (age and time from HSC transplantation [HSCT] to sampling) and the chi-square test (other factors) were used to test the statistical relationship with the existence of chimerism in nail.

Results and discussion

Table 1 shows the characteristics of donors and recipients, transplantation-related factors, and the percentages of donor-derived DNA in fingernails. All cases demonstrated complete donor chimerism in

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Table 1. Characteristics of hematopoietic stem-cell transplant recipients

Case no.	Diagnosis	Age at HSCT, y	Type of donor	HLA allele compatibility	Conditioning regimen for HSCT	Source of HSCs	aGVHD	cGVHD	Time from HSCT to sampling, d	cGVHD at sampling	Immunosuppressive therapy at sampling	Percentage of donor area
1	ALL	26	Unrelated	Match	CSI+CY+TBI	BM	II	Limited	521	Negative	None	17.2
2	AML	53	Related	Match	BU+CY	BM	None	Limited	2529	Positive	None	0
3	AML	42	Related	Match	BU+CY	BM	II	Limited	569	Negative	None	0
4	AML	23	Unrelated	Match	TBI+CY	BM	None	Limited	1259	Negative	None	39.2
5	AML	45	Related	Match	BU+CY	BM	I	Limited	1657	Negative	None	23.8
6	MDS	18	Related	Match	BU+CY	PB+BM	None	Extensive	1811	Positive	CsA	0
7	SAA	23	Related	Match	BU+CY	BM	I	Extensive	2287	Positive	FK	15.1
8	CML	25	Related	Match	BU+CY	PB	I	Extensive	2252	Positive	PSL	0
9	ALL	55	Unrelated	2 locus mismatch	TBI+Flu+L-PAM	CB	I	Limited	467	Negative	None	0
10	ALL	17	Related	Match	TBI+CY	BM	I	Extensive	999	Positive	CsA+PSL	72.9
11	AML	45	Unrelated	1 locus mismatch	TBI+CY	BM	None	Limited	550	Positive	None	0
12	AML	33	Unrelated	Match	CSI+CY+TBI	BM	II	Extensive	1711	Negative	None	0
13	AML	26	Related	Match	BU+CY	BM	None	Extensive	305	Positive	CsA+PSL+MMF	8.9
14	AML	30	Related	Match	BU+CY	BM	II	Extensive	1580	Positive	FK+PSL+MMF	0
15	ALL	20	Related	Match	BU+CY	BM	I	Limited	1111	Negative	None	69.1
16	ATL	40	Related	Match	TBI+CY	PB	None	Extensive	1518	Positive	CsA	31.2
17	AML	49	Unrelated	Match	TBI+CY	BM	None	Extensive	426	Positive	FK+PSL	0
18	ALL	28	Unrelated	4 locus mismatch	CY+Flu+BU	CB	II	Extensive	851	Positive	None	0
19	AML	17	Related	Match	TBI+CY	PB	II	Extensive	2107	Positive	FK	0
20	AML	42	Related	Match	BU+CY	PB	I	Extensive	1209	Positive	FK+PSL	0
21	CML	46	Related	Match	BU+CY	BM	None	Limited	2399	Negative	None	56.5

ALL indicates acute lymphoblastic leukemia; CSI, craniospinal irradiation; CY, cyclophosphamide; TBI, total body irradiation; BM, bone marrow; AML, acute myelogenous leukemia; BU, busulfan; MDS, myelodysplastic syndrome; PB, peripheral blood; CsA, cyclosporine A; SAA, severe aplastic anemia; FK, tacrolimus; CML, chronic myelogenous leukemia; PSL, prednisolone; Flu, fludarabine; L-PAM, melphalan; CB, cord blood; MMF, mycophenolate mofetil; and ATL, adult T-cell leukemia.

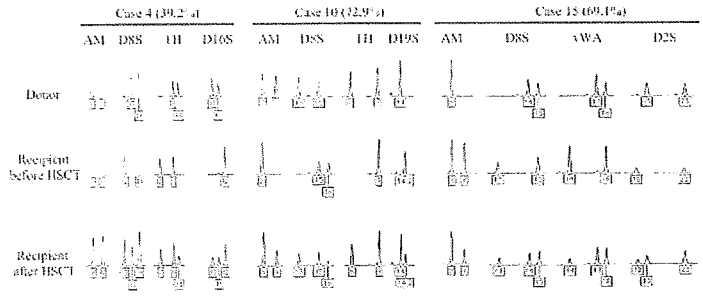

Figure 1. Representative STR patterns of donor blood cells (top), those of recipients before HSCT (middle), and those of nails in recipients after HSCT (bottom) are shown (cases 4, 10, and 15). The percentages in parenthesis were calculated by dividing the donor-derived short tandem repeat (STR) areas by the total STR peak areas in nails of recipients. The numbers of the fragment repeats are indicated below each STR. AM indicates amelogenin gene; D8S, D8S1179; TH, TH01; D16S, D16S539; D19S, D19S433; vWA, von Willebrand factor intron A; and D2S, D2S1338.

bone marrow cells within 100 days from transplantation, and there was no unexplained cytopenia in 17 cases. Among 4 cases (cases 2, 3, 11, and 21) that experienced relapse of primary diseases, 3 of them were in complete donor-derived hematopoiesis at the time of sampling. No case showed abnormal appearance of fingernails, including bleeding, when collected. In case 2, which later died of primary disease, fingernails were collected during partial remission. The other 20 patients were alive and in complete remission when this manuscript was written. In 9 of 21 cases, donor-derived STR peaks were detected in DNA samples of fingernails that shared from 8.9% to 72.9% of total peak areas. Representative STR peaks are shown in Figure 1. There was no statistically significant relationship between the presence of donor-derived STR peaks with mismatch of sex, disparity of blood type, or all factors listed in Table 1. However, because of the small number of cases in this study, we could not deny any relationship among the factors above. The only case that underwent transplantation after reduced-intensity conditioning showed no donor-derived STR in fingernails. So far there is 1 report that used PCR-based genotyping to show the positive contribution of donor-derived cells in nonhematopoietic tissue, buccal epithelial cells.[4] However, the detection and the comparison of STRs of blood or bone marrow samples are established and widely accepted methods for the analysis of chimerism after allo-HSCT, and fingernails have also been used to extract DNA for this type of analysis.[12,13] We did not observe any other peaks than those of recipients or donors in any STR test, demonstrating no contamination of other DNA samples. Taking advantage of the integration of HTLV-1 in T cells among adult T-cell leukemia/lymphoma (ATLL) patients, the existence of HTLV-1 in DNA of fingernails was examined by the genomic PCR of the pX region among 9 ATLL patients, resulting in no amplification in all cases (Figures S1, S2, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). It suggested that no white blood cell (WBC)–derived DNA existed in DNA extracted from fingernails (data not shown). We believe there was little chance of false-positive results in our analysis.

The percentages of donor-derived STR areas were correlated to other reports,[1-4] even a long time after transplantation, demonstrating the stable contribution of donor-derived cells in fingernails. Since nail is a tissue that regenerates continuously throughout life maintained by the stem cells of the nail matrix, the stable contribution of donor-derived DNA in nails suggested the existence of donor-derived cells in the stem-cell system of nails. Based on the clinical observation, when patients are treated with myeloablative conditioning regimen for transplantation, nail stem cells would be damaged to a certain extent so that transient growth retardation or arrest occurs. This harmful condition might facilitate the high contribution of donor-derived cells to nails.[14] In our approach, there is no data available regarding how donor-derived cells resided in these patients: donor-derived nail stem cells existed or the fusion of certain donor cells occurred to nail stem cells of the recipients. We also have no data on the difference of the contribution of donor cells in each finger.

Recently, the absence of donor-derived cells was reported in the hair bulb of allo-HSC recipients (115 cases) where there was no contamination of blood cells either.[9] In spite of the similar biologic features of hair and nail such as continuous regeneration or the same origin from the ectoderm, the contribution of donor-derived cells was clearly different between these 2 tissues after allo-HSCT. There is no clear explanation for the difference; however, biologic characteristics of stem cells and niche circumstances might contribute to these results. This report for the first time demonstrated the long-term, stable contribution of donor-derived cells in nails among recipients of allo-HSCs.

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Authorship

Contribution: D.I., H.S., and R.Y. performed experiments with H.T., and Y.S. and J.T. collected samples. T.F., S.Y., and T.H. collected clinical data. Data were analyzed by D.I. and Y.M. Y.M. organized this study and wrote the manuscript with M.T.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Brief Report

Karyotype at diagnosis is the major prognostic factor predicting relapse-free survival for patients with Philadelphia chromosome-positive acute lymphoblastic leukemia treated with imatinib-combined chemotherapy

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ABSTRACT

To identify factors associated with relapse-free survival (RFS), 80 patients with newly diagnosed Philadelphia chromosome-positive acute lymphoblastic leukemia, enrolled in a phase II study of imatinib-combined chemotherapy, were analyzed. The median follow-up of surviving patients was 26.7 months (maximum, 52.5 months). Twenty-eight out of 77 patients who had achieved CR relapsed. The probability of RFS was 50.5% at 2 years. Multivariate analysis revealed that the presence of secondary chromosome aberrations in addition to t(9;22) at diagnosis constitute an independent predictive value for RFS (p=0.027), and increase the risk of treatment failure by 2.8-fold.

Key words: acute lymphoblastic leukemia, Philadelphia chromosome, BCR-ABL, imatinib, karyotype.

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Introduction

The treatment for Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph$^+$ ALL) has changed dramatically since imatinib, a selective inhibitor of the ABL tyrosine kinase, was introduced.[1,2] Combined with chemotherapy, or even as a single agent, it can produce complete remission (CR) rates of 90% or higher in newly diagnosed patients.[3-9] We previously reported the results of a phase II study by the Japan Adult Leukemia Study Group (JALSG) to test the efficacy and feasibility of imatinib-combined chemotherapy for newly diagnosed Ph$^+$ ALL.[6] The rate of CR reached 96%, and that of BCR-ABL negativity in bone marrow 71%. However, despite a relatively short follow-up period, relapse occurred in a subset of the patients who had achieved CR.

On the other hand, remarkable progress is being made with the development of novel tyrosine kinase inhibitors with more potent *in vitro* and *in vivo* activities than imatinib.[10,11] Given this, we investigated factors associated with relapse-free survival (RFS).

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Design and Methods

Patients and treatments

Eligibility criteria included newly diagnosed Ph+ ALL, age between 15 and 64 years, an Eastern Co-operative Oncology Group performance status between 0 and 3, and adequate liver, kidney and heart function. Written informed consent was obtained from all patients prior to registration.

For remission induction therapy, imatinib was administered from day 8 to day 63 in combination with daunorubicin, cyclophosphamide, vincristine (VCR) and prednisolone (PSL). Consolidation therapy consisted of an odd course (C1) comprising high-dose methotrexate, high-dose cytarabine and methylprednisolone, and an even course (C2) with single-agent imatinib for 28 days. C1 and C2 were alternated for 4 cycles each. After completion of the consolidation therapy, patients received maintenance therapy consisting of VCR, PSL and imatinib for up to 2 years from the date CR had been achieved.[6] The daily dose of imatinib used in this study was 600 mg. Allogeneic hematopoietic stem cell transplantation (HSCT) was recommended if a matched sibling donor was available, and was allowed from an alternative donor.

The protocol was reviewed and approved by the institutional review board of each of the participating centers and was conducted in accordance with the Declaration of Helsinki.

Cytogenetic and molecular analysis

At diagnosis, bone marrow samples were examined for cytogenetic abnormalities with standard banding techniques. Karyotypes were classified according to the International System for Human Cytogenetic Nomenclature.[12] The number of BCR-ABL copies in bone marrow was determined at a central laboratory with the real-time quantitative RT-PCR test according to the previously described method.[13]

Statistical analysis

Kaplan-Meier survival analysis was performed to estimate the probabilities of RFS, event-free survival (EFS), and overall survival (OS), with differences between the groups compared by the log-rank test. Cumulative incidences of relapse were calculated with non-relapse mortality considered as a competing risk, and differences between the groups were compared with the Gray's test. For risk factor analysis, a Cox proportional hazards model was constructed. In multivariate analysis, variables with p values of <0.10 determined by univariate analysis were included in the final model. A hazard ratio (HR) was calculated in conjunction with a 95% confidence interval (CI).

Results and Discussion

A total of 80 patients were recruited between September 2002 and January 2005. The median age was 48 years (range 15-63), with 49 males and 31 females. CR was achieved by 77 (96.2%) patients. During a median follow-up of 26.7 months (maximum 52.5 months), 28 patients relapsed. Of the 17 relapses observed during the consolidation therapy, 13 occurred during the imatinib course. The probabilities of EFS and OS were 48.5±5.7% and 58.1±5.7% at 2 years (Figure 1). For patients who had achieved CR, the probability of RFS was 50.5±5.9% at 2 years. Allogeneic HSCT was performed for 60 patients, including 24 from a sibling donor, 1 from a related donor other than a sibling, 25 from an unrelated donor, and 10 from unrelated cord blood. Disease status at the time of transplantation was first CR for 44 patients, second CR for 4 and non-CR for 12. The 2-year RFS for those who had undergone allogeneic HSCT during first CR was 62.6±7.5% and 62.1±12.3% for those who had not undergone allogeneic HSCT. When allogeneic HSCT was considered as a time-dependent covariate, it was shown to have no significant effect on RFS (HR, 1.03; 95% CI, 0.51-2.09; p=0.934). Major and minor BCR-ABLs were detected in 23 and 56 patients respectively. The transcript type of the remaining patient could not be determined because fluorescent *in situ* hybridization analysis was used instead of the PCR test. Neither transcript types nor copy numbers at diagnosis were associated with RFS (p=0.763 and 0.912). Pre-treatment cytogenetic results were not available for 4 patients because analysis was not performed (n=2) or was not successful (n=2). Of the remaining 76 patients, 22 showed only t(9;22) or variant translocations, 51 showed additional chromosome aberrations, and 3 showed normal karyotype. Additional aberrations exceeding a frequency of 10% comprised +der(22)t(9;22) in 17 patients, abnormalities involving the short arm of chromosome 9 [abn(9p)] in 17, monosomy 7 in 10, and trisomy 8 in 10. Figure 2 compares RFS for patients with and without additional chromosome aberrations. The presence of additional aberrations was significantly associated with shorter RFS (p=0.003). The relapse rate was also higher in patients with additional aberrations (41% *vs.* 20% at 2 years, p=0.0414). Analyses of the 4 recurrent abnormalities mentioned above demonstrated a statistically significant negative impact on RFS for +der(22)t(9;22) and abn(9p) (p<0.001 and p=0.005). Even after allogeneic HSCT, patients with additional aberrations appeared to have a trend for shorter RFS than those without (p=0.080), but this might reflect a larger proportion of transplantation beyond first CR in the former (31% *vs.* 17%). In patients allografted during first CR, there was no difference in cumulative incidences of relapse dated from the day of transplantation between the 2 groups

(16.5% *vs.* 12.5% at 2 years, p=0.546). Variables that showed a significant effect on RFS in the univariate Cox model included additional chromosome aberrations (p=0.005), peripheral blood blasts % (p=0.024) and sex (p=0.03). Results of multivariate analysis are shown in Table 1. The presence of additional chromosome aberrations was identified as the only independent prognostic factor for RFS (p=0.027). These updated data strongly support recent reports showing the feasibility and remarkable efficacy of imatinib-combined chemotherapy for newly diagnosed Ph+ ALL.[3-9,14,15] The main objective of this report was to identify factors affecting RFS, an issue of rapidly increasing importance given the development of novel tyrosine kinase inhibitors which are expected to further expand the treatment options for this disease. Our data indicated that additional chromosome aberrations, particularly +der(22)t(9;22) and abn(9p), were associated with shorter RFS. It is well known that additional chromosome aberrations are seen frequently in Ph+ ALL. Before the imatinib era, some groups reported the prognostic relevance of additional aberrations.[16-18] By contrast, from a large series of 204 patients, Moorman *et al.*[19] recently showed no significant effect of specific additional aberrations, including +der(22)t(9;22) and del(9p), on survival. In this study, analyzing patients treated with imatinib-combined chemotherapy, the 2-year RFS rate exceeded 80% for those without additional aberrations, whereas outcomes for those with additional aberrations were relatively unfavorable.

Acquisition of resistance to imatinib is an emerging problem in the treatment of chronic myeloid leukemia. One of the most common mechanisms of resistance is the mutation involving the ABL kinase domain. Although it has not been confirmed whether such mutations compromise the clinical outcome of Ph+ ALL patients treated with imatinib-combined chemotherapy, our observation that most of the early relapses occurred during the consolidation courses consisting of imatinib alone implies possible imatinib resistance. If that is the case, switching from imatinib to other novel tyrosine kinase inhibitors based on the pre-treatment cytogenetic results soon after achieving CR or even earlier could be an alternative treatment approach for further improving outcome in Ph+ ALL. Lack of mutation analysis is a major limitation of this study. Recently, Pfeifer *et al.*[20] studied the ABL kinase domain mutation status in newly diagnosed Ph+ ALL patients who were treated with imatinib-combined chemotherapy, and showed that even before exposure to imatinib, mutations were detected in 38% of patients. Importantly, the frequency of the mutant allele was low in such patients. However, at the time of relapse, the same mutation was present as the dominant clone in 90% of the relapsing cases.[20] Altogether, further insights will be provided by investigating the association between karyotype and mutation status at diagnosis.

Despite such limitations, the analysis of 80 patients entered into a single trial identified karyotype at diagnosis as a significant prognostic factor for RFS in newly diagnosed Ph+ ALL patients treated with imatinib-combined chemotherapy. Although our results need to be confirmed regarding kinase domain mutation status, these findings may play a critical role in the future treatment of Ph+ ALL.

Table 1. Multivariate analysis of factors associated with relapse-free survival.

P-value	*HR (95% CI)**	*Factors*	
Additional [chromosome aberrations	0.027	2.84 (1.12-7.19) 1.00	Present Absent
Peripheral blood blasts%	0.051	1.12 (1.00-1.22)	Per 10% increase
Sex	0.148 1.00	1.73 (0.82-3.64) Female	Male

*HR, hazard ratio; 95% CI, 95% confidence interval. *Values higher than unity indicate higher risk for failure.*

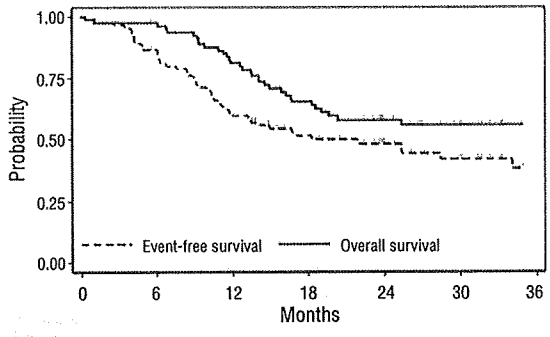

Figure 1. Kaplan-Meier curves for event-free and overall survival. The probabilities of event-free and overall survival at 2 years were 48.5% and 58.1% respectively (n=80).

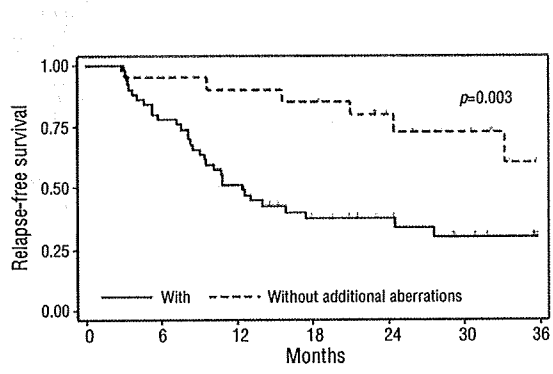

Figure 2. Relapse-free survival for patients with and without additional cytogenetic aberrations. Patients with additional cytogenetic aberrations (n=50) had significantly shorter relapse-free survival than those without (n=20).

Authorship and Disclosures

MY designed and co-ordinated the study, analyzed the data, and wrote the paper; JT, NU, FY, SM, and IJ designed the study, and provided patient sample and clinical data; IS, HA, KN, YU, MT, and AM provided patient sample and clinical data; HN co-ordinated the study, and revised the paper. YM provided patient sample and clinical data, and engaged in data management. SO designed the study, provided patient sample and clinical data, and engaged in data management; KM designed the study, and analyzed the data; TN chaired the study group, co-ordinated the study, and revised the paper; RO served as the principal investigator, chaired the study group, and revised the paper. All authors reviewed the paper, interpreted the results, and approved the final version. The authors reported no potential conflicts of interest.

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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).

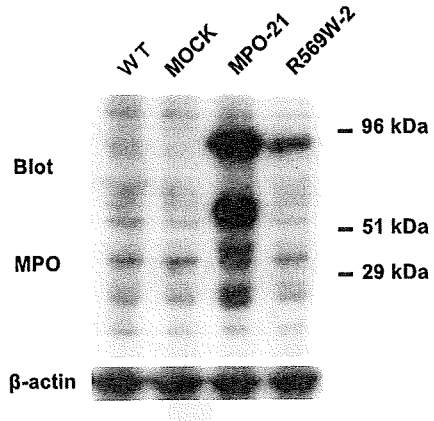

Figure 1 Western blot analysis of myeloperoxidase (MPO) protein in K562 cells. Immature MPO protein (apo-pro MPO, 89 kDa) was detected in K562 cells expressing wild-type MPO (MPO-21) and mutant MPO (R569W-2). Mature MPO protein (64 and 14 kDa) was seen only in MPO-21 cells. MOCK, K562 cells transfected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

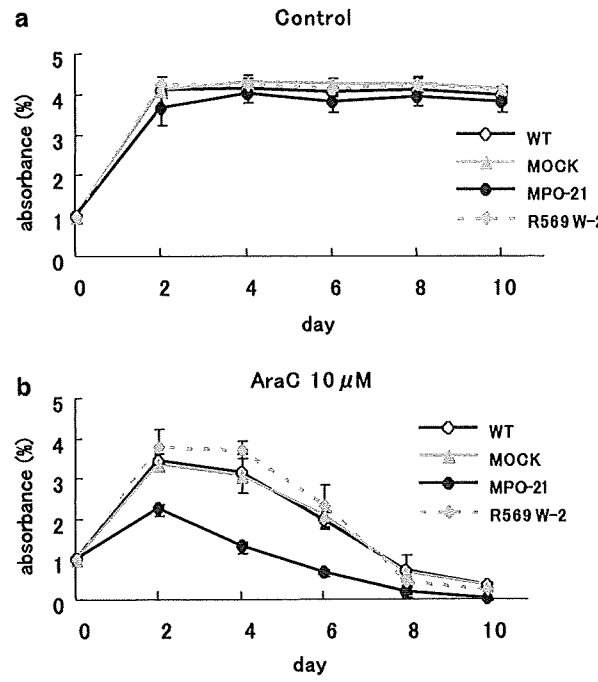

Figure 3 WST-1 analysis of K562 cells. Wild-type K562 (WT), MOCK (transfected with control vector), MPO-21 (wild-type myeloperoxidase (MPO)-expressing K562) and R569W-2 (mutant MPO-expressing K562) cells showed a similar growth pattern in the steady state (**a**). In the presence of cytosine arabinocide (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.

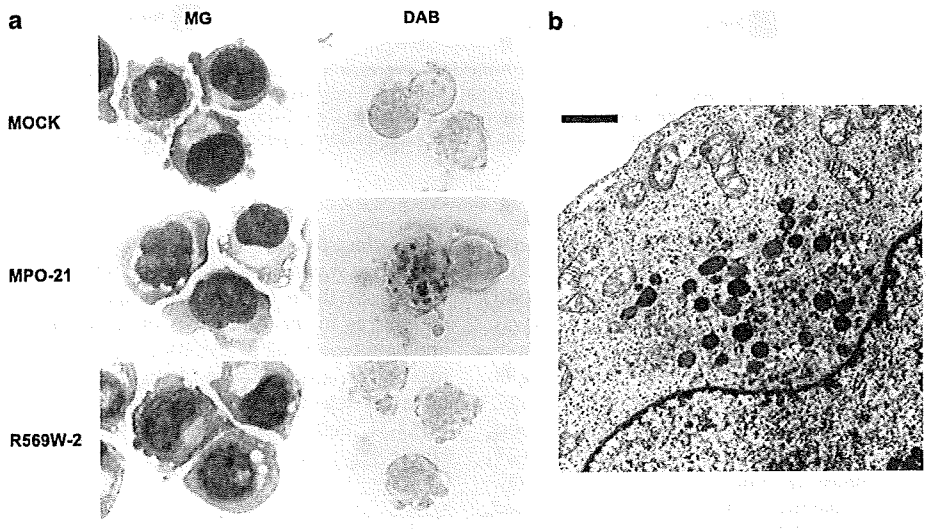

Figure 2 Morphological and cytochemical features of myeloperoxidase (MPO)-expressing K562 cells. May-Grunwald Giemusa (MGG) staining and diaminobenzidine (DAB) staining of K562 cells transfected with control vector (MOCK), wild-type MPO (MPO-21) and mutant MPO (R569W-2) (**a**). DAB was observed only 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).