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Proliferative effects of several hematopoietic growth factors on acute myelogenous leukemia cells and correlation with treatment outcome

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The response of human acute myelogenous leukemia (AML) cells to four different hematopoietic growth factors (granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin-1 β (IL-1β), interleukin-3 (IL-3), and stem cell factor (SCF)) and the relationship of the proliferative response of the AML cells to treatment outcome were studied. Proliferative responses were analyzed in 79 patients with *de novo* AML and 19 patients with AML arising from myelodysplastic syndrome (MDS). In *de novo* AML, a positive proliferative response (stimulation index >2) was seen in 65 to 75% of cases. AML cells arising from MDS had a much higher incidence of proliferative response to each growth factor (79 to 90%) and a much higher level of ^{3}H-TdR incorporation. The relationship to treatment outcome was evaluated in 79 patients with *de novo* AML. The patients whose leukemic cells had a positive proliferative response to any growth factor, especially IL-3 and SCF, had a poorer outcome, ie a lower complete remission (CR) rate, shorter CR duration, and shorter survival. The outcome was particularly poor in patients whose leukemic cells had proliferative responses to all four or any of the growth factors, compared to patients whose leukemic cells had no response. This increased response may be a marker of poor prognosis in patients with AML.

Keywords: hematopoietic growth factor; ^{3}H-TdR incorporation; AML; prognostic factor; CR duration; overall survival

Introduction

Hematopoietic growth factors are required for the proliferation and differentiation of hematopoietic cells. It is also known that these factors, either as single agents or in combination, are important for the *in vitro* proliferation of acute myelogenous leukemia (AML) cells in most cases. In some cases, however, the AML cells can become factor-independent through an autocrine or paracrine mechanism.[1–4] In evaluating the capacity of interleukin-1β (IL-1β) to serve as an autocrine growth factor for leukemic cells, we noticed that a high proliferative response of leukemia cells to exogenous IL-1β was an unfavourable prognostic factor in patients with AML in terms of the CR rate, the CR duration, and the overall survival.[5]

To complement that study, we evaluated the proliferative response of AML cells to four growth factors: granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and stem cell factor (SCF), in addition to IL-1β. All of these are known to have important roles in the early phases of maturation of myeloid precursor cells. We also analyzed the relationship of these proliferative responses to the treatment outcome of patients with AML.

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Materials and methods

Patients

A total of 98 patients with acute myelogenous leukemia (AML), 59 males and 39 females, aged 16 to 78 years (median 48 years) were included in this retrospective study. Of the 168 patients with AML who were treated at our hospital between 1983 and 1992, we selected only patients in whom: (1) the mononuclear cell fraction containing leukemic cells could be collected from the peripheral blood or the bone marrow and stored before the patients began chemotherapy; (2) leukemic cells accounted for more than 90% of the total mononuclear cells; and (3) the viability of freeze/thawed leukemic cells was more than 80%.

Ninety-eight patients met these criteria and all of these patients were included in this study. The diagnosis of AML was based on the clinical history and on the results of morphologic, cytochemical, and immunologic analyses of the leukemic cells. Seventy-nine patients presented with *de novo* AML and 19 with AML arising from myelodysplastic syndrome (MDS). Patients with *de novo* AML were classified according to the French–American–British (FAB) criteria;[6,7] the number of patients in each subtype was M0 ($n = 4$), M1 ($n = 15$), M2 ($n = 23$), M3 ($n = 15$), M4 ($n = 10$), M5 ($n = 8$), M6 ($n = 2$), and M7 ($n = 2$).

Collection and storage of leukemic cells

Upon admission to the hospital, mononuclear cells were collected from the peripheral blood or bone marrow of untreated patients using a Ficoll–Hypaque gradient. The leukemic cells were suspended in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 10% dimethylsulfoxide (DMSO) at a cell density of 1×10^7/ml. Two-milliliter aliquots of the leukemic cell suspension were dispensed into freezing tubes. A Cryo-Med programmable freezing system (Forma Scientific, Ohio, USA) was used to freeze these cells, which were stored in liquid nitrogen at –180°C.

Evaluation of growth factor-induced proliferation of leukemic cells in vitro

Human recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF), human recombinant interleukin 3 (IL-3), and human recombinant stem cell factor (SCF) were produced by the Kirin Brewery, Tokyo, Japan, and human recombinant interleukin 1-β (IL-1β) by the Ohtsuka Pharmaceutical, Tokushima, Japan. These growth factors were donated for use in this study.

Frozen leukemic cells were thawed rapidly at 37°C, washed, and resuspended in RPMI-1640 medium sup-

plemented with 10% AB(+) male serum. The density of the leukemic cells was adjusted to 5×10^5/ml, and 0.2-ml aliquots were added to the wells of a 96-well microtiter plate in triplicate. The cultures were incubated with or without growth factors at 37°C in an atmosphere of 5% CO_2 in air for 72 h. The concentrations of the growth factors used in this study were 100 ng/ml of GM-CSF, 1 ng/ml of IL-1β, 10 ng/ml of IL-3, and 10 ng/ml of SCF, because a preliminary study had shown that these concentrations produced the optimal proliferative response in most leukemic cells. One microcurie per well of tritiated thymidine (^{3}H-TdR) was added to the cultures during the final 8 h of incubation. The labeled cells were harvested onto glass-fiber filters with a multiple sample harvester, and the amount of radioactivity (c.p.m. per 1×10^5 cells) was determined in a liquid scintillation counter. The stimulation index (SI) was defined as the counts per minute (c.p.m.) in a stimulated culture divided by the c.p.m. in the corresponding unstimulated control. We noticed in a preliminary study that the proliferative response of frozen leukemic cells to growth factors was generally lower than that of fresh leukemic cells, but the SI was comparable between fresh and frozen leukemic cells, provided the viability of freeze/thawed cells was well maintained. Frozen cells have an advantage of being studied repeatedly. Therefore we used freeze/thawed leukemic cells in this retrospective study.

Treatment of AML

Over the past 12 years, we have administered combination chemotherapy with DCMP (daunomycin, cytosine arabinoside, 6-mercaptopurine, and prednisolone) and its modifications to induce remission in patients with AML.[8] Once a patient achieved a complete remission (CR) on DCMP, that regimen was continued for two to three additional cycles for consolidation. The patient was then placed on intermittent maintenance or intensification chemotherapy with either DCMP, VEMP (vincristine, cyclophosphamide, 6-mercaptopurine, and prednisolone), or mitoxantrone plus etoposide. If the CR persisted for 2 years, the treatment was discontinued and the patient was followed without chemotherapy, although the entire duration of treatment has become shorter in recent years.

In this study, we evaluated the treatment outcome in 79 patients with *de novo* AML according to the rate of CR, duration of CR and overall survival. Four of the 79 patients who received bone marrow transplants (BMT) were censored from analysis of the duration of CR and overall survival at the time of BMT.

The results were tested for statistical significance by χ^2 analysis. To evaluate the predictive factors of CR rate, multivariate analysis was performed with the logistic procedure. The durations of the CR and overall survival were analyzed by the Kaplan–Meier method and compared by the Cox–Mantle test. A level of $P < 0.05$ was accepted as statistically significant.

Results

Growth factor-induced proliferation of AML cells

In the absence of an exogenous growth factor, the mean ^{3}H-TdR incorporation by leukemic cells obtained from the 98 patients was 4382 c.p.m. per 1×10^5 cells (range 1544 to 83 732 c.p.m.).

Table 1 shows the proliferative responses of the leukemic cells in the presence of the four different growth factors in the 79 patients with *de novo* AML and the 19 patients with AML arising from MDS. When the statistical analysis was done between the c.p.m. in the stimulated cultures and the c.p.m. in the corresponding unstimulated cultures, we observed that most cases with SI >2 were statistically significant. We therefore defined that an SI >2 was accepted as a positive proliferative response. A positive response to GM-CSF was seen in 52/79 (65.8%) patients with *de novo* AML, while those for IL-1β, IL-3 and SCF were 51/79 (64.6%), 59/79 (74.7%), and 54/79 (68.4%), respectively. The cells from patients with AML arising from MDS had a much higher ^{3}H-TdR incorporation (data not shown) and a much higher incidence of proliferation response (Table 1), than the cells from patients with *de novo* AML, although a statistically significant difference was seen only in response to GM-CSF. Median SI in response to four growth factors was higher in the cells from the patients with AML arising from MDS and a statistically significant difference was seen only in response to SCF.

Proliferative response to all four growth factors was observed in the leukemic cells from 40 of 79 (50.6%) patients with *de novo* AML and 15 of 19 (78.9%) patients with AML arising from MDS. In contrast, no proliferative response to any growth factor was observed in leukemic cells from 17 and two patients, respectively.

We then focused on the 79 patients with *de novo* AML, examining the distribution of the proliferative responses of their leukemic cells to growth factors according to the FAB subtype. We found no significant differences in the SI among the FAB subtypes of *de novo* AML, although the M1 patients had a slightly lower proliferative response to all the growth factors than the other subtypes (data not shown).

Response to chemotherapy

Because patients with AML arising from MDS generally respond poorly to chemotherapy and have a poor prognosis, we evaluated the clinical significance of growth factor-induced proliferation only in patients with *de novo* AML. Fifty-three of the 79 patients with *de novo* AML obtained a complete remission (CR) and the overall CR rate was 67.1%. Table 2 shows the relationship between the proliferative response of the AML cells to each growth factor and the CR rate. The CR rate decreased as the proliferative response to any of the growth factors increased. The CR rate was the lowest in the patients whose leukemic cells had an SI $\geqslant$10 to the growth factors, although a significant difference in the CR rate between those with leukemic cells with a SI $\leqslant$2 and those with an SI $\geqslant$ 10 was observed only in response to SCF.

Figure 1 shows the comparison of the duration of the CR between the patients whose leukemic cells had a positive proliferative response in ^{3}H-TdR incorporation in the presence of the four growth factors with those whose leukemic cells had no positive response. Because the number of patients whose SI was more than 10 was relatively small, those patients were included in the group with an SI >2. The duration of the CR was significantly shorter in the patients whose leukemic cells exhibited increased proliferation in response to any of the growth factors. It is particularly significant in response to IL-3 ($P = 0.0008$) and SCF ($P = 0.005$).

The overall survival of each group is shown in Figure 2. As

Table 1 Proliferative response of the leukemic cells to each growth factor

	No. patients	SI	Response to growth factor							
			GM-CSF		IL-1β		IL-3		SCF	
De novo AML	79	⩽2	27		28		20		25	
		2–10	42	) 65.8%	43	) 64.6%	34	) 74.7%	47	) 68.4%
		⩾10	10		8		25		7	
		median	3.3		2.7		5.5		2.7	
				$P = 0.04$						$P = 0.04$
MDS → AML[a]	19	⩽2	2		4		3		2	
		2–10	14	) 89.5%	10	) 78.9%	9	) 84.2%	13	) 89.5%
		⩾10	3		5		7		4	
		median	4.6		3.8		8.4		5.4	

[a]AML arising from MDS.

Table 2 Proliferative response to growth factors and the CR rate in 79 patients with *de novo* AML

SI	GM-CSF (%)	IL-1β (%)	IL-3 (%)	SCF (%)	
⩽2	21/27 (77.8)	23/28 (82.1)	15/20 (75.0)	21/25 (84.0)	
2–10	27/42 (64.3)	26/43 (60.5)	24/34 (70.6)	31/47 (66.0)	$P = 0.0004$
⩾10	5/10 (50.0)	4/8 (50.0)	14/25 (56.0)	1/7 (14.3)	

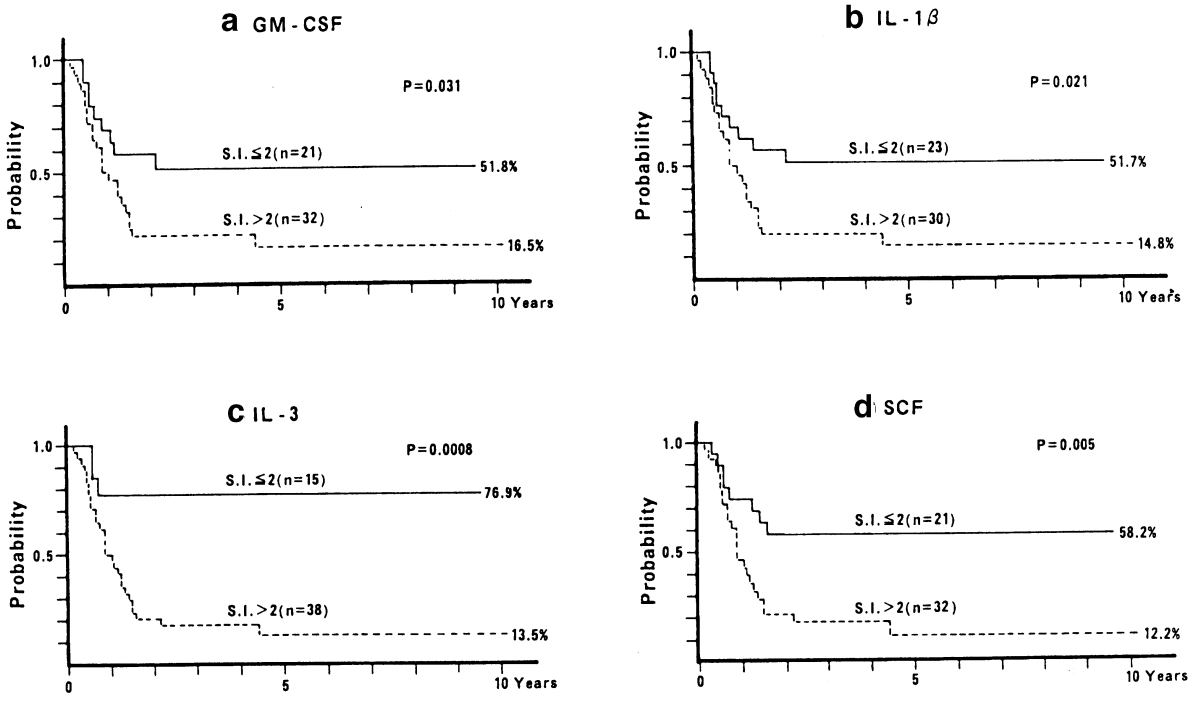

Figure 1 Duration of CR according to the proliferative response of the leukemic cells to four growth factors. (—) No positive proliferative response in ^{3}H-TdR incorporation (SI ⩽ 2); (– – –) with positive proliferative response (SI > 2).

with the duration of CR, the overall survival was shorter in the patients whose leukemic cells had an increased proliferative response to any of the growth factors. A significant difference was seen in response to IL-1β ($P = 0.021$), IL-3 ($P = 0.035$), and SCF ($P = 0.005$).

Table 3 shows the relationship between the number of growth factors which induced a positive proliferation of the leukemic cells and the clinical features of the patients. Whereas a CR was obtained in 15/17 (88.2%) patients whose leukemic cells had no proliferative response to any of the four growth factors (group A), the groups in which the leukemic cells proliferated in response to one to three growth factors (group B), or to all four growth factors (group C) had lower CR rates: 13/22 (59.1%) in group B and 25/40 (64.0%) in group C. The differences in the CR rate between those in group A and those in group B as well as between those in group A and

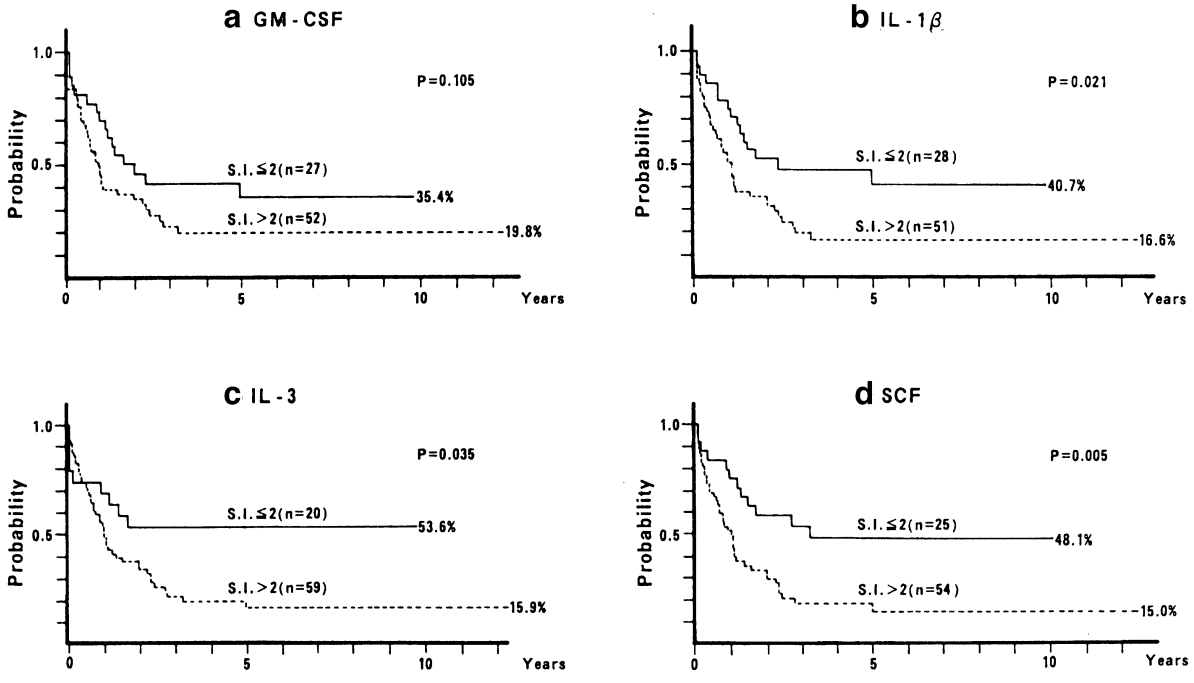

Figure 2 Overall survival according to the proliferative response of the leukemic cells to four growth factors. (—) SI ≤ 2; (– – –) SI > 2.

Table 3 Number of growth factors which induced a positive proliferative response and clinical features

No. of growth factors which induced a proliferative response		*No. patients*	*CR (rate)*			*Median age*	*Median initial WBC (range,/μl)*		*Median spontaneous ^{3}H-TdR incorporation (range, c.p.m.)*		
A	0	17	15 (88.2%)	$P = 0.04$	$P = 0.05$	45	35 700 (1200–296 600)	$P = 0.09$	1988 (991–26 398)	$P = 0.36$	$P = 0.002$
B	1–3	22	13 (59.1%)			53	12 300 (600–207 900)	$P = 0.06$	2298 (708–28 500)	$P = 0.05$	
C	4	40	25 (64.0%)			48	33 400 (700–425 400)		7320 (707–46 642)		

those in group C were statistically significant. The patients' age and initial WBC count were not significantly different in these three groups. However, the spontaneous incorporation of ^{3}H-TdR by the leukemic cells from the patients in group C was significantly higher than that of the patients in groups A and B. In this study, the median spontaneous ^{3}H-TdR incorporation did not correlate with the achievement of a CR. There were 11 patients whose leukemic cells had a high spontaneous level of ^{3}H-TdR incorporation (more than 10 000 c.p.m.). A CR was obtained in six of these patients (54.5%) compared to 47 of 68 patients (69%) with a spontaneous ^{3}H-TdR incorporation of less than 10 000 c.p.m. (statistically not significant). The duration of the CR in these patients was generally short; most patients had a relapse by 9 months. All patients except for one who had BMT died by 30 months, and the overall survival was significantly shorter in these patients ($P = 0.007$). The patients' age, initial WBC count and spontaneous ^{3}H-TdR incorporation, as well as the number of growth factors which induced a positive proliferative response (group A *vs* group B *vs* group C) were evaluated using multivariate analysis (Table 4). We found the CR rate was significantly influenced by age. It was also influenced by the number of growth factors which induced a positive proliferative response, although a statistically significant difference was not seen ($P = 0.10$).

The duration of the CR in these three groups is compared

Table 4 Multivariate analysis for CR rate

	Relative risk	*P value*
Age	1.04	0.01
Initial WBC count	1.32	0.45
Spontaneous ^{3}H-TdR incorporation	0.48	0.28
Group A/group B/group C	1.75	0.10

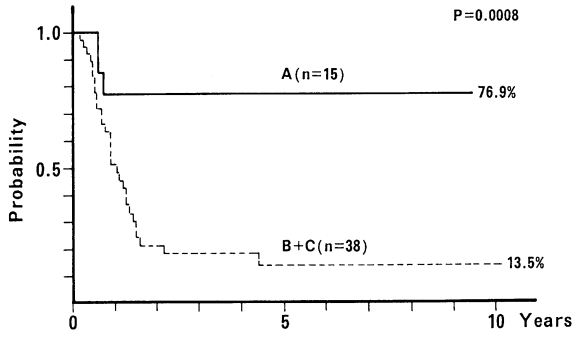

Figure 3 Duration of the CR according to the number of growth factors which induced a positive proliferative response of the leukemic cells. A, (—) no proliferative response to any of the four growth factors; B, proliferative response to one to three growth factors; C, proliferative response to all four growth factors. B + C, (– – –) were combined because they were not significantly different. CR, complete remission; SI, stimulation index.

in Figure 3. Because the duration of the CR and the overall survival in group B and group C were not statistically significant, the patients of group B and group C were combined. The actuarial relapse-free survival rates were 76.9% in group A and 13.5% in group B + C. A significant difference was seen in the duration of the CR between those in group A and those in group B + C ($P = 0.0008$).

Figure 4 shows the pattern of the overall survival in the three groups. The actuarial overall survival rate was 63.0% for patients in group A and 15.0% in group B + C. A statistically significant difference was observed in the overall survival between in group A and those in group B + C ($P = 0.0031$).

Discussion

In this study we evaluated the proliferation of leukemic cells in terms of ^{3}H-TdR incorporation in response to four different growth factors (GM-CSF, IL-1β, IL-3 and SCF) which are known to act in the early phase of differentiation of myeloid precursor cells and found that 65 to 75% of the leukemic cells from patients with *de novo* AML showed a positive proliferative response. There were no significant differences among the FAB subtypes of those with *de novo* AML. Several other *in vitro* studies have found that these factors stimulated leukemic

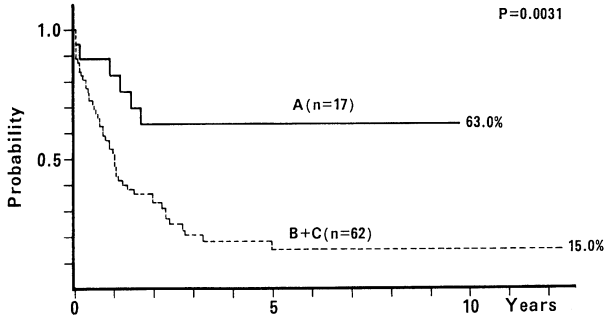

Figure 4 Overall survival according to the number of growth factors which induced a positive proliferative response of the leukemic cells. A, (—) no proliferative response to any of the four growth factors, B, proliferative response to one to three growth factors; C, proliferative response to all four growth factors. B + C, (– – –) were combined because they were not significantly different.

cells from more than half or nearly half of the patients with AML using either ^{3}H-TdR incorporation[9–14] or a clonogenic assay.[15–21] From these *in vitro* studies it has been suggested that these growth factors also may play an essential role in the *in vivo* proliferation of most AML cells.

The leukemic cells from the patients with AML arising from MDS had a greater proliferative response to all four growth factors than those from the patients with *de novo* AML, which may indicate that these growth factors can induce the proliferation of leukemic cells which may originate from a more primitive stem cell. Proliferative responses to all four growth factors were observed in the leukemic cells from 40 of 79 patients with *de novo* AML and 15 of 19 patients with AML arising from MDS. This may be explained by the homology in the extracellular domains of the receptors for growth factors, or it is possible that leukemic cells indeed express multiple growth factor receptors.[1]

In this retrospective study, the patients with *de novo* AML whose leukemic cells exhibited a positive proliferative response to any of the four growth factors had a lower CR rate, a shorter duration of CR, and a shorter overall survival. The CR rate was much lower in patients with an SI of more than 10.

There have been only a few studies examining the relationship between the proliferative response of leukemic cells to growth factors and patients' treatment outcomes. With regard to IL-1, Preisler *et al*[22] have found that the presence of IL-1β gene activity in AML is associated with a poor long-term prognosis. We have previously reported that a proliferative response to IL-1β in *de novo* AML is associated with a poor long-term prognosis in terms of the duration of the CR and overall surivival.[5] Some patients were re-evaluated as a part of this current study. Although the incidence of the increased proliferative response to IL-1β was slightly higher in this study, the significance of IL-1β in the prognosis of AML was essentially similar. With regard to SCF, Ashmar *et al*[23] and Lerner *et al*[24] have reported the possibility that high expression levels of c-kit in adult patients with AML may be associated with a poor prognosis. Thus, the expression of these growth factors in AML is generally considered to be a poor prognostic factor.

The patients whose leukemic cells proliferated in response to any of the four growth factors, as well as all four growth factors, showed poor outcomes in terms of the duration of the CR and overall survival, compared to those whose leukemic cells did not proliferate in response to any of these growth factors. The *in vivo* proliferation of AML cells may not necessarily be induced by a single growth factor. After a patient achieves a clinical CR, if the residual leukemic cells are responsive to any of these growth factors, it is likely that these cells will regrow in response to one or more factors, and that the patient will suffer a relapse.

Granulocyte colony-stimulating factor (G-CSF) or GM-CSF can recruit quiescent leukemic cells into the cell cycle to become more susceptible to cell cycle dependent antileukemic agents such as cytosine arabinoside (ara-C).[25,26] However, convincing benefit has not been obtained by priming with G- or GM-CSF. We observed CR in 13 (50%) of 26 patients with relapsed or refractory AML who were treated with G-CSF given 2 days before, during and after ara-C-containing remission induction chemotherapy (unpublished data). Interestingly, a CR rate was lower in patients whose leukemic cells showed a high proliferative response to G-CSF *in vitro* (unpublished data). This finding suggests that the response by G-CSF and ara-C-containing regimen might not be due to the sensitizing effects of G-CSF on leukemic cells, and that some

other effects of G-CSF on leukemic cells, such as change of pharmacokinetics of ara-C in leukemic cells, may participate.[27]

There have been several prognostic factors identified in patients with AML, such as the patient's age, initial WBC count, and cytogenetic abnormalities. Recently, Lowenberg *et al*[28] have reported that the capacity of leukemic cells for autonomous proliferation is associated with low rates of CR induction, disease-free survival and overall survival. Hunter *et al*[29] have reported that the presence of autonomous growth characteristics of blast cells was found to be the single most important indicator of CR and disease-free survival in patients with AML. We observed that the patients whose leukemic cells exhibited a high spontaneous level of ^{3}H-TdR incorporation (more than 10 000 c.p.m.) had a poor prognosis in terms of the duration of the CR and the overall survival. We also observed that the spontaneous incorporation of ^{3}H-TdR by the leukemic cells was significantly higher in the group whose leukemic cells proliferated in response to all four growth factors. It appears that those leukemic cells which had a high spontaneous rate of proliferation also generally had a positive proliferative response to growth factors. This response may be an important marker of poor prognosis in patients with AML. Further studies are needed to confirm this observation.

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