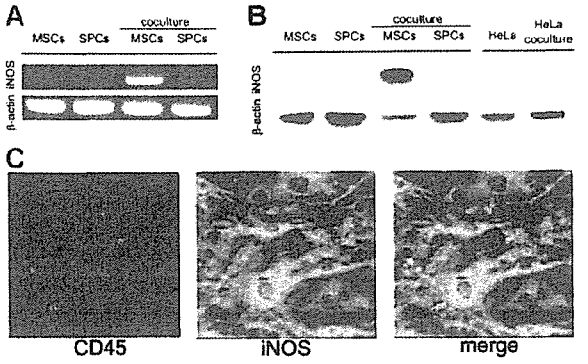

Figure 5. Induction of iNOS in MSCs. Total RNA and cell lysates were collected from MSCs alone, splenocytes alone, MSCs cocultured with activated T cells, or activated splenocytes cocultured with MSCs. MSCs were harvested just after washing out activated T cells with PBS. (A) RT-PCR analysis of iNOS mRNA. β-actin is shown as a control. (B) Western blot analysis of iNOS protein. Each lane contains 20 μg protein. β-actin is shown as a loading control. HeLa cells were used as negative control because the antibody also reacts with human iNOS protein. (C) Immunofluorescence of iNOS protein. Left panel, confocal immunofluorescent image of CD45 protein. Middle panel, confocal immunofluorescent image of iNOS protein. Right panel, merged confocal immunofluorescent images of CD45 protein and iNOS protein. Splenocytes (1×10^6) were activated with Con A in the presence of 1×10^5 MSCs for 48 hours. Images were visualized using a Nikon Eclipse TE300 microscope (Nikon, Tokyo, Japan) equipped with a 100×/1.40 numerical aperture oil objective lens, Nikon CFI Plan APO (Nikon). Images were acquired using Lasersharp software version 2.1 (Bio-Rad).

Other candidates as mediators of suppression by MSCs

Because TGF-β, IDO, and PGE_2 were reported as mediators of T-cell suppression by MSCs,[13-15] we further compared the effects of L-NAME with inhibitors of each mediator. Indomethacin (inhibitor of PGE_2 production) but not 1-MT (inhibitor of IDO) or an anti–TGF-β–neutralizing antibody restored T-cell proliferation as effectively as L-NAME (Figure 7A); however, the effects of L-NAME and indomethacin were not additive, suggesting that the NO and PGE_2 share signaling pathways leading to T-cell suppression (Figure 7A).

MSCs from iNOS$^{-/-}$ mice have reduced activity in T-cell suppression

Finally, we used MSCs from iNOS$^{-/-}$ mice to confirm that NO is produced by MSCs and that NO suppresses T-cell proliferation. MSCs from iNOS$^{-/-}$ mice were less effective than MSCs from wild-type mice at suppressing T-cell proliferation, suggesting that NO produced by MSCs is a major mediator of this effect (Figure 7B, left panel). We also confirmed that MSCs from iNOS$^{-/-}$ mice do not produce NO even in the presence of activated T cells (Figure 7B, right panel).

Discussion

Here, we demonstrate for the first time that the production of NO is involved in the suppression of T cells by MSCs. We also showed that NO inhibits Stat5 phosphorylation. Although NO was already known to suppress T-cell proliferation, NO has not been previously reported to mediate T-cell suppression by MSCs.[29] Our hypothesis that NO is produced by MSCs and that it suppresses T-cell proliferation in part through Stat5 inhibition was supported by the following facts: (1) NO was readily detected in the medium in the presence of MSCs; (2) L-NAME restored T-cell proliferation as well as Stat5 phosphorylation; and (3) MSCs from iNOS$^{-/-}$ mice had markedly reduced abilities to suppress T-cell proliferation. This hypothesis was further confirmed by the finding that iNOS was detected only in MSCs.

Compared with experiments in which cells were in direct contact, experiments performed in transwells showed a lag in NO production, suggesting that T-cell–MSC contact is critical for the early and efficient production of NO and, thus, T-cell suppression. Whether a soluble factor is a main mediator of T-cell suppression by MSCs has been controversial because results from transwell systems have been inconsistent.[12,14,16-17] Our finding that the transwell reduces but does not abolish T-cell suppression (Figure 4B) may help explain these conflicting reports. Although we could not define the mechanism by which NO production is suppressed in the transwell system, the amount of NO production appears to correspond with the extent of T-cell suppression.

Under stringent conditions, in which a lower number of MSCs was used, the restoration of T-cell proliferation by L-NAME reached up to approximately 80%, suggesting that NO is one of the major mediators; however, 100% restoration was never attained, suggesting that other factor(s) contribute to the suppression. Because TGF-β, IDO, and PGE_2 have been considered as possible mediators of T-cell suppression by MSCs,[13-15] we examined the effect of specific inhibitors of each. We found that indomethacin (inhibitor of PGE_2 production) restores T-cell proliferation as previously reported[15] but that neither 1-MT (inhibitor of IDO) nor the TGF-β antibody had an effect. Also, the effects of indomethacin

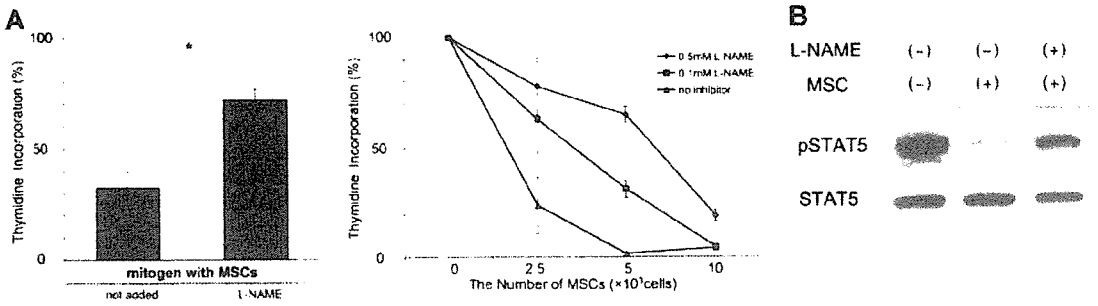

Figure 6. A specific inhibitor of NOS restores T-cell proliferation and Stat5 phosphorylation. (A) Effect of L-NAME on thymidine incorporation. Top panel, splenocytes (1×10^5) were activated with Con A in the presence or absence of 2.5×10^3 irradiated MSCs and in the presence or absence of 1 mM L-NAME. The incorporation of [^{3}H]-thymidine is shown relative to that in the absence of MSCs. The values are the means ± SD from 3 independent experiments. *$P < .05$. Bottom panel, dose-dependent restoration of T-cell proliferation by L-NAME. Splenocytes (1×10^5) were activated with Con A in the presence of the indicated number of irradiated MSCs for 48 hours. The concentrations of L-NAME are shown. Shown is a typical result of 3 independent experiments. (B) L-NAME restores Stat5 phosphorylation. Splenocytes (2×10^6) were activated with anti-CD3/CD28 beads in the presence or absence of 0.5×10^5 to 1×10^5 MSCs for 48 hours and in the presence or absence of 1 mM L-NAME. Western blotting for phosphorylated and total Stat5 was performed as described in Figure 2. Shown is a representative result from 5 independent experiments.

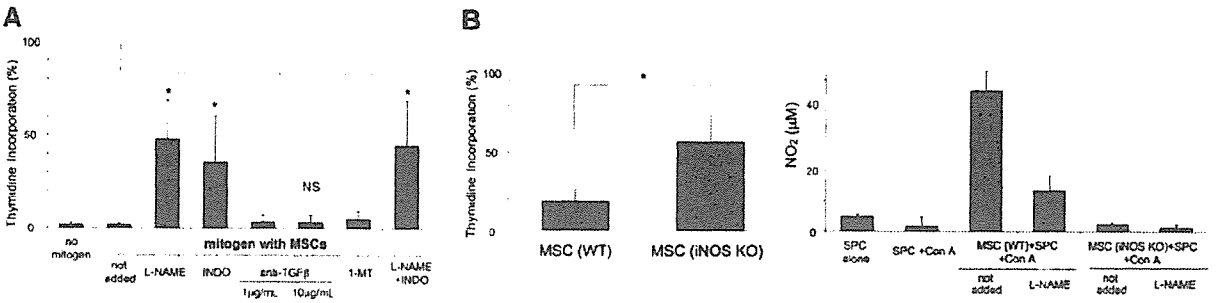

Figure 7. Effect of inhibitors and T-cell suppression by MSCs from iNOS$^{-/-}$ mice. (A) L-NAME and indomethacin (INDO) restored T-cell proliferation, but TGF-β antibody and 1-MT had no effect. Splenocytes (1×10^5) were activated with Con A in the presence or absence of irradiated MSCs (2.5×10^3), 1 mM L-NAME, 5 μM indomethacin,[14] 1 μg/mL or 10 μg/mL TGF-β antibody, and 1 mM 1-MT for 48 hours. The incorporation of [^{3}H]-thymidine is shown relative to that in the absence of MSCs. Shown is the mean ± SD of 3 independent experiments. *$P < .05$. NS indicates $P > .05$. (B) MSCs from iNOS$^{-/-}$ mice have a reduced ability to inhibit T-cell proliferation. Splenocytes (1×10^5) were activated with Con A in the presence or absence of MSCs (2.5×10^3) from either wild-type or iNOS$^{-/-}$ mice. Left panel, incorporation of [^{3}H]-thymidine relative to that in the absence of MSCs. Right panel, production of NO. The values are the means ± SD from 3 independent experiments. *$P < .05$.

were not additive with those of L-NAME. These results, combined with previous reports,[31,32] suggest that NO acts upstream of PGE_2. Furthermore, our results imply that NO may be the central mediator of T-cell proliferation.

Under our standard conditions (1:10 ratio of MSCs to splenocytes), 1 mM L-NAME restored T-cell proliferation to approximately 25%. Similarly, 5 μM indomethacin also produced an approximately 25% recovery (data not shown). These results suggest that the restoration by L-NAME or indomethacin is not specific to the more stringent conditions (1:40 ratio of MSCs to splenocytes).

Although most of the results from our mouse MSC system are consistent with previous reports,[12,15,27] we did not find a clear correlation between T-cell suppression and the up-regulation of Kip1 or the down-regulation of cyclin D2. Instead, our results suggest that the inhibition of Stat5 phosphorylation is more important for T-cell suppression, at least under the conditions of our experiments. In the conditions studied here, after coculture with MSCs, T cells could respond to a second mitogenic stimulation (data not shown), whereas they could not respond to a second stimulation in a previous report,[27] suggesting that the status of T cells in our experiments is different than that in the previous report.

Our results provide new insight into how MSCs modulate immune function. Although it is known that the NO-Stat5 pathway is important for T-cell suppression by macrophages, this is the first report demonstrating that the NO-Stat5 pathway is also critical for T-cell suppression by MSCs. The physiologic role of NO produced by MSCs is unknown, and we are currently investigating the possibility that MSCs in bone marrow protect hematopoietic stem cells from T-cell–mediated destruction by inhibiting T-cell proliferation.

Acknowledgments

We would like to thank Dr Hitoshi Endo (Jichi Medical University) for technical assistance with immunofluorescence microscopy, Dr David Munn (MCG Immunotherapy Center, Medical College of Georgia, Augusta) for technical advice with dissolving 1-MT, and Dr Motohiro Matsuura (Jichi Medical University) for providing the RAW264.7 macrophage cell line.

This work was supported in part by grants from the Ministry of Health, Welfare, and Labor of Japan and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Technology of Japan.

Authorship

Contribution: K.S. performed the research and analyzed data; K. Ozaki designed the research and wrote the paper; K.H. performed Western blotting; I.O. carried out experiments regarding PMA plus ionomycin; T.N. provided technical advice; A.M. and K.M. provided some reagents and analyzed data; and K. Ozawa organized the research project.

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

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Leukemia & Lymphoma, August 2006; 47(8): 1613–1617

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ORIGINAL ARTICLE: CLINICAL

Pleocytosis after hemopoietic stem cell transplantation

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(Received 29 June 2005; accepted 17 September 2005)

Abstract

Frequency and clinical significance of cerebrospinal fluid (CSF) pleocytosis in hemopoietic stem cell (HSC) transplantation were surveyed. Cyclosporine (CSA)- or tacrolimus (FK506)-based regimens were used as graft-vs-host disease (GVHD) prophylaxis in allogeneic HSC transplantation. CSF pleocytosis with or without neurologic symptoms was detected in 12 of 25 patients receiving allogeneic HSC transplants but in none of 11 patients receiving autologous HSC transplants. Of the 12 patients with CSF pleocytosis, only one patient developed leukoencephalopathy later. There was a correlation between CSF cell numbers and trough levels of CSA but not with those of FK506. In patients receiving allogeneic HSC transplants, CSF pleocytosis may be relatively common and may reflect neurologic damage associated with calcineurin inhibitors.

Keywords: *Pleocytosis, hemopoietic stem cell transplantation, calcineurin inhibitors*

Introduction

Neurologic complications accompanied by CSF pleocytosis in patients receiving CSA or FK506 after allogeneic HSC transplantation or organ transplantation have been reported [1–4]. Mild symptoms including tremor, neuralgia, headache or peripheral neuropathy are common; however, some patients experience severe symptoms including psychoses, hallucinations, blindness, seizures, cerebellar ataxia, motoric weakness or leukoencephalopathy [5–7]. Generally, symptoms of CSA- and FK506-related neurotoxicity are reversed by substantially reducing the dosage of the drugs or discontinuing them [5]. We analysed the frequency and clinical significance of CSF pleocytosis in patients receiving a HSC transplant.

Materials and methods

Patients with hematologic malignancies who underwent HSC transplant between February 1990 and July 2004 at Jichi Medical School Hospital were retrospectively surveyed. Patients received lumbar puncture to inject methotrexate to prevent central nervous system disease both before and after HSC transplantation. Exclusion criteria in this study were as follows: central nervous system involvement of disease before or after HSC transplantation and death within 6 months after HSC transplantation. Patients received intrathecal methotrexate injection once just before HSC transplantation and up to four times according to the disease status after HSC transplantation. Since lumbar puncture was performed on the basis of the doctors' decisions, the day of it was different in each patient. CSF cell numbers were counted in all patients and CSF cell cytology was examined in the three samples from three patients. CSF pleocytosis was defined when the total cell number was more than 15 cells per 3 μl. CSA and FK506 were given intravenously at first and then they were given orally. Usually, in hospital, serum CSA and FK506 trough levels were monitored daily

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ISSN 1042-8194 print/ISSN 1029-2403 online © 2006 Informa UK Ltd.
DOI: 10.1080/10428190600625836

or every other day, while in out-patient clinic, they were monitored weekly or every 2 weeks. CSA and FK506 blood trough levels were assayed at the same time of CSF collection using the enzyme multiplied immunoassay technique. p-values below 0.05 were considered significant on Mann-Whitney's U test and χ^2 test.

Results

Thirty-six patients were evaluated (Table I): there were no differences in age, gender, intrathecal methotrexate injection and cranial irradiation between the allogeneic HSC transplantation group and the autologous HSC transplantation group. However, cases of acute myeloid leukemia were only included in the allogeneic HSC transplantation group and total body irradiation (TBI)-based regimens as conditioning such as 120 mg kg^{-1} of cyclophosphamide plus 12 Gy of TBI or 180 mg kg^{-1} of melphalan plus 12 Gy of TBI were performed in that group. Only chemotherapy-based regimens were used as conditioning in the autologous HSC transplantation group as reported previously [8]. In the allogeneic HSC transplantation group, most patients received allogeneic bone marrow transplants from HLA-matched unrelated donors. Four patients received allogeneic bone marrow transplants from HLA-identical siblings, one received an allogeneic peripheral blood stem cell transplant from a HLA-identical sister and one received an allogeneic peripheral blood stem cell transplant from a HLA-mismatched mother. High-titer anti-cytomegalovirus (CMV) gammaglobulin for CMV infection prevention was given to most patients of both groups. Antithymocyte globulin (ATG) for rejection and GVHD prevention was given only to seven patients receiving allogeneic HSC transplants. GVHD prophylaxis was provided by short-term methotrexate (MTX) plus CSA or short-term MTX plus FK506.

Table I. Characteristics of the patients.

	Auto	Allo	p-value
No. of Patients	11	25	
Age (years#)	23	22	0.3110
Gender			0.8247
Male	5	14	
Female	6	11	
Disease			0.0369
AML	0	8	
ALL	5	11	
CML	0	2	
NHL	6	4	
Conditioning			<0.0001
TBI-based	0	25	
Non-TBI-based	11	0	
GVHD prophylaxis			
MTX + CSA	ND	14	
MTX + FK506	ND	11	
Transplant			<0.0001
PBSC	11	2	
R-BM	0	4	
U-BM	0	19	
IT before transplantation#	2.0	4.0	0.0821
IT after transplantation#	4.0	2.0	0.5365
Time of IT after transplantation (days#)	167	179	0.6896
Cranial irradiation	1	1	0.5546
ATG	0	7	0.0505
CMV Ig	10	21	0.5546
Follow-up (days#)	2266	2059	0.1103
Outcome			0.1768
Alive	9	24	

Auto, autologous hemopoietic stem cell transplantation; Allo, allogeneic hemopoietic stem cell transplantation; PBSC, peripheral blood stem cell transplantation; R-BMT, related bone marrow transplantation; U-BMT, unrelated bone marrow transplantation; IT, intrathecal methotrexate injection; ATG, anti-thymocyte globulin; CMV, high-titer anticytomegalovirus gammaglobulin; ND, not done; #, median.

As shown in Figure 1, CSF cell numbers in the allogeneic HSC transplantation group were significantly higher than those in the autologous HSC transplantation group (20.9 ± 29.5 vs 3.2 ± 2.4, $p = 0.007$). In the autologous HSC transplantation group, there were no patients with CSF pleocytosis. CSF cells from three patients with pleocytosis were morphologically examined by cytospin preparations. As shown in Figure 2, most of the CSF cells showed normal

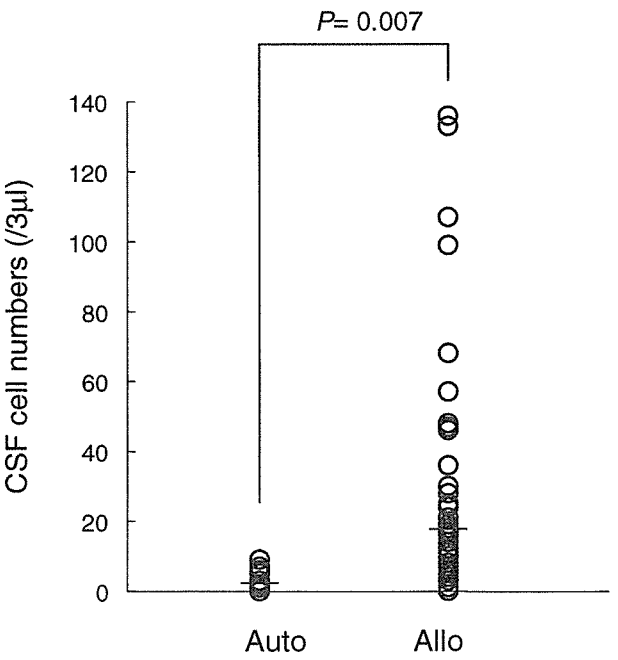

Figure 1. CSF cell numbers in autologous HSC transplantation and allogeneic HSC transplantation. The numbers of analysed CSF samples were 31 in autologous HSC transplantation and 63 in allogeneic HSC transplantation. Auto, autologous HSC transplantation; Allo, allogeneic HSC transplantation.

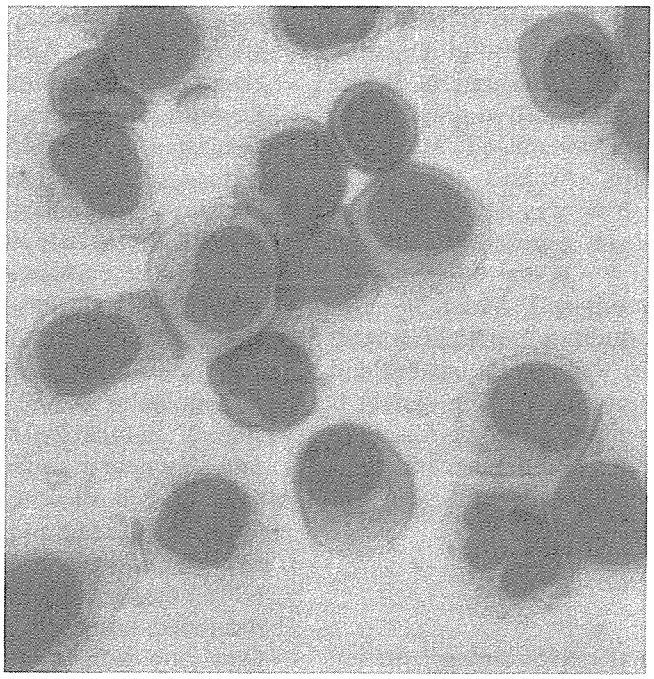

Figure 2. Cell morphology in CSF pleocytosis. Most cells were normal lymphocytes.

lymphocyte morphology, i.e. condensed round to oval nuclei without nucleoli and fine pale cytoplasm with a few azurophilic granules. CMV anti-genemia was detected in 10 patients receiving allogeneic HSC transplants but not in patients receiving autologous HSC transplants. Transplant-related complications were observed in the allogeneic HSC transplantation group as follows: sepsis (one); hemorrhagic cystitis due to adenovirus (one); bronchiolitis obliterans organizing pneumonia (one) and pseudomembranous colitis (two). None of the patients receiving autologous HSC transplants showed severe transplant-related complications. There were no patients having meningitis or encephalitis in both groups.

In allogeneic HSC transplantation, factors associated with CSF pleocytosis were analysed: CMV anti-genemia, acute GVHD, chronic GVHD and calcineurin inhibitor (CSA vs FK506) were not related to CSF pleocytosis (data not shown). Because calcineurin inhibitor blood concentrations may be associated with neurologic complications, we investigated the correlation between CSF cell numbers and CSA or FK506 blood concentrations. As shown in Figure 3, there was a positive correlation between CSF cell numbers and CSA trough levels but not FK506 trough levels.

There were no patients showing severe neurologic symptoms such as severe headache, blindness, seizures, cerebellar ataxia and confusion at the end of the last intrathecal methotrexate injection in the allogeneic HSC transplantation group. Some patients with CSF pleocytosis showed slight neurologic

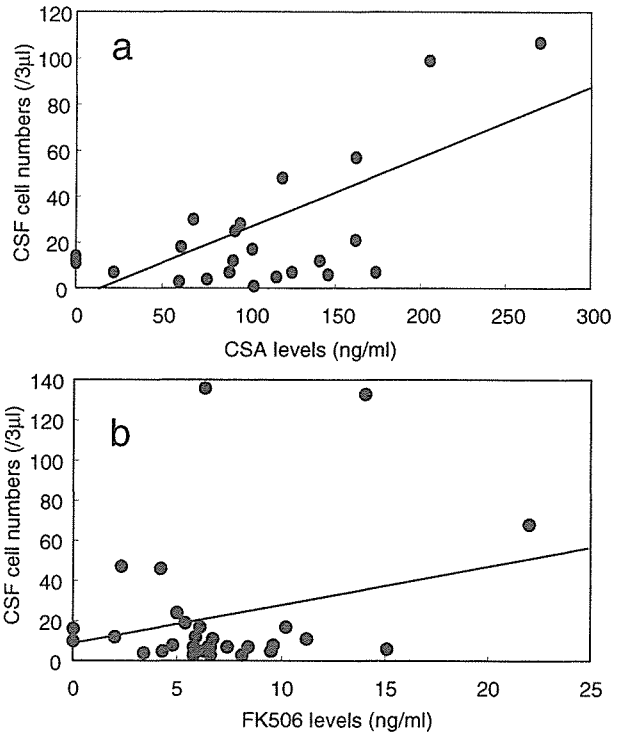

Figure 3. Relationship between CSF cell numbers and trough levels of calcineurin inhibitor. The numbers of analysed CSF samples were 24 in the CSA group and 31 in the FK506 group. CSF cell numbers correlated with CSA trough levels (a) $r^2 = 0.417$, $p = 0.0007$; but not with FK506 trough levels (b) $r^2 = 0.087$, $p = 0.1062$).

symptoms including tremor, nausea and headache at a mild level. Only one patient with lymphoblastic lymphoma developed leukoencephalopathy after

allogeneic bone marrow transplantation (BMT): a 15-year-old male who had been in second remission and had a history of meningeal infiltration. Before transplantation, the patient had received prophylactic cranial irradiation. On day 69 after BMT, MTX was injected intrathecally once. At this time, CSF pleocytosis (107 cells per 3 μl) was noted, although the patient had no neurologic symptoms. The CSA trough level was 270 ng ml^{-1}. On day 81, the patient complained of visual disturbance. Magnetic resonance imaging showed findings compatible with leukoencephalopathy. CSA was rapidly tapered and the symptom gradually disappeared. The patient is currently well without symptoms except for slight depression. Except for this patient, magnetic resonance imaging or computed tomography scan was not performed in other patients.

Discussion

Neurologic complications after HSC transplantation have been reported: these are caused by pre-transplant conditionings, metabolic disorders, viral infections, microangiopathy, complications induced by GVHD and calicineurin inhibitors [9,10]. de Brabander et al. [10] reported that severe neurologic complications are more frequent after BMT from alternative donors, compared with BMT for standard risk patients. They suggested that the increased incidence in alternative donor patients is associated with frequent lines of chemotherapy before BMT, intensified conditioning regimens including TBI and ATG used for the graft rejection prevention. The morbidity and profound immunosuppression caused by these treatments may lead to susceptibility to neurologic complications in patients receiving bone marrow transplants [10]. In our study, CSF pleocytosis was observed in allogeneic HSC transplantation but not in autologous HSC transplantation. Since TBI as conditioning and MTX as GVHD prophylaxis were used only in allogeneic HSC transplantation, these two treatments may have caused CSF pleocytosis in the patients [10–12]. In addition, unrelated donor HSC transplantation and ATG may have led to CSF pleocytosis associated with profound immunosuppression. Severe transplant-related complications occurred only in allogeneic HSC transplantation. Therefore, they may have been involved in CSF pleocytosis.

We suspect that CSF pleocytosis in allogeneic HSC transplantation may be associated with calcineurin inhibitors used for GVHD prophylaxis. Hauben [13] summarized neurotoxicity associated with CSA by the published literature: estimated the frequency of CSA neurotoxicity is 0.5–35% and risk factors of the toxicity include supratherapeutic blood concentrations of CSA, CSA-drug interactions and hypocholesterolemia. Associated abnormalities include elevated CSF protein and pleocytosis, electroencephalogram abnormalities and characteristic neuroimaging findings [13]. Similar neurotoxicity associated with FK506 has been reported and the incidence of the neurotoxicity is 5–30% in the literature [14], although the incidence and severity of FK506-related neurotoxicity are equal or higher than that of CSA-related neurotoxicity [4,15]. Postulated mechanisms of CSA neurotoxicity are vasculopathy based on CSA effects on endothelial cell synthesis of prostaglandin and release and uptake of endothelin as well as inhibition of mitochondrial steroid 26-hydroxylase [13]. CSA neurotoxicity is more frequent in patients with high CSA blood levels, even though these CSA levels are usually within therapeutic ranges [16]. We demonstrated that significant numbers of patients receiving allogeneic HSC transplants showed CSF pleocytosis and all of the patients had no or mild neurologic symptoms. Only one patient later developed leukoencephalopathy. These results suggest that CSF pleocytosis is relatively common in allogeneic transplantation and shows some brain damage. We observed a weak correlation between CSF cell numbers and CSA trough levels. Since CSA neurotoxicity is more frequent with high CSA blood levels [16], CSF cell numbers may reflect the degree of neurotoxicity. In contrast to CSA, there was no correlation between CSF cell numbers and FK506 trough levels. This may be due to the narrow therapeutic blood ranges of FK506. Since we did not measure CSA or FK506 concentrations in CSF, further studies are needed to identify a correlation between CSF cell numbers and calcineurin inhibitor concentrations in CSF. Although CSF pleocytosis is relatively common when allogeneic HSC transplantation is followed by calcineurin inhibitors for GVHD prophylaxis, we should carefully monitor such patients to detect early manifestations of leukoencephalopathy.

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INTERNAL MEDICINE

PICTURES IN CLINICAL MEDICINE

Bone Marrow Metastasis of Malignant Melanoma

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(DOI: 10.2169/internalmedicine.45.1616)

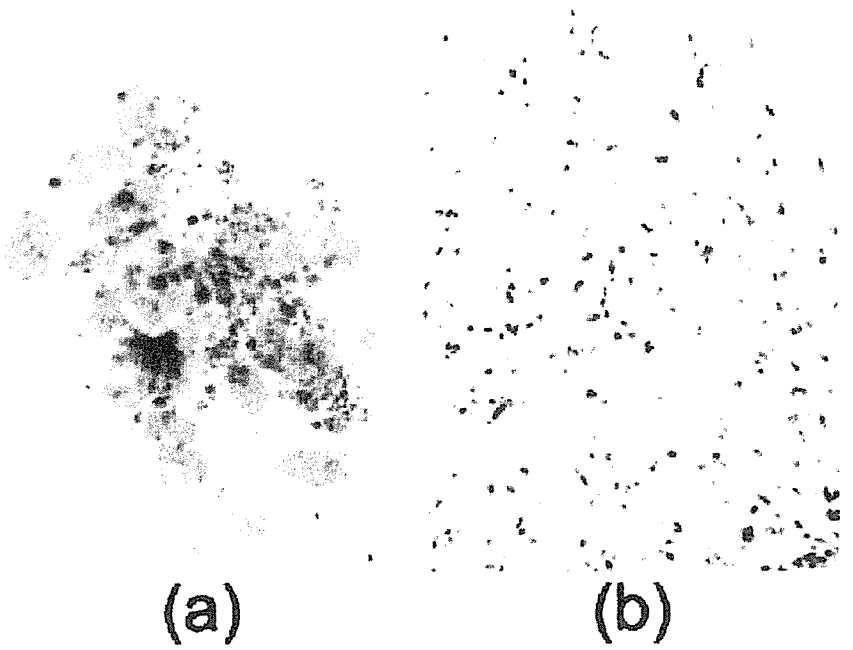

Figure 1. **Bone marrow was infiltrated with tumor cells including characteristic melanin pigments (a: aspirate, b: biopsy).**

A 67-year-old woman was admitted to our hospital with a 3-month history of lower back pain. One year earlier, the patient was diagnosed with malignant melanoma of the right maxillary bone and underwent an operation followed by adjuvant chemotherapy. A bone marrow aspirate revealed that the marrow was infiltrated by tumors composed of nests of poorly differentiated cells. These tumor cells were large and round shaped, and the cellular outlines within clumps of tumor cells were indistinct. Although these findings were not specific for any group of metastatic tumors, the cytoplasm of tumor cells was filled with numerous granules of melanin (Fig. 1). Thus, a diagnosis of malignant melanoma with bone marrow involvement was made. The primary site of metastatic tumors in bone marrow is difficult to determine by morphological examinations, including immunostaining. However, melanin pigments in cytoplasm are characteristic and definitive findings in malignant melanoma.

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Received for publication October 20, 2005; Accepted for publication January 11, 2006
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Leukemia Research
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Establishment and characterization of a new erythroblastic leukemia cell line, EEB: Phosphatidylglucoside-mediated erythroid differentiation and apoptosis

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Received 23 March 2005; received in revised form 20 October 2005; accepted 22 October 2005
Available online 5 December 2005

Abstract

A new erythroblastic leukemia cell line (EEB) was established from a patient with early erythroblastic leukemia. The cells had features of immature erythroblasts, including an agranular basophilic cytoplasm and CD36, CD71, CD175s (sialyl-Tn) and CD235a (glycophorin A) expression without CD41 expression, myeloperoxidase activity and platelet-peroxidase activity. The cells were confirmed to be of the erythroid lineage based on expression of the gamma-globin message. They were induced to differentiate into benzidine-positive cells by hemin and δ-amino levulinic acid (δ-ALA). An analysis of cell membrane lipids showed that EEB cells contain a type of glycerolipid, phosphatidylglucose (PhGlc), but not unbranched type 2 chains, i antigens. GL-7 which is a recombinant Fab fragment of GL-2 and binds to PhGlc, induced production of hemoglobin F (HbF) associated with accumulation of the gamma-globin (γ-globin) message in EEB cells. The GL-7-mediated erythroid differentiation was associated with apoptosis. These results suggest that direct signaling to PhGlc mediates erythroid differentiation and apoptosis in EEB cells.

Keywords: Erythroid cell line; Phosphatidylglycoside; Differentiation; Apoptosis; Glycosphingolipid; Microdomain

1. Introduction

Blood cells express several types of carbohydrate antigens during the course of differentiation of hemopoietic progenitors. Roles of carbohydrates on myeloid cells have been studied extensively: sialyl-Lewis x (CD15s), which is a ligand of E-selectin on activated endothelial cells, is expressed in neutrophils [1]. The interaction between sialyl-Lewis x and E-selectin mediates tethering and rolling of neutrophils to the vessel wall in the microcirculation, although two other selectins also contribute to the adhesion and the transendothelial migration of neutrophils into tissues [1,2]. In erythroid cells, dramatic changes in carbohydrates associated with development have been reported: a progressive branching of lacto-*N*-glycosyl carbohydrate chains linked to glycolipids and to band 3 protein occurs during the development of fetal to adult human erythrocytes [3,4]. Some of the linear structure represents i antigen, while some of the branched structure represents i antigen. Such changes in carbohydrate structure in the membrane coincide with the switch from fetal hemoglobin to adult hemoglobin. Previously, we showed that sialyl-Tn is expressed in colony-forming unit-erythroid to erythroblasts but not in erythrocytes, while sialyl-T and disialy-T are expressed in the entire course of erythroid differentiation [5,6]. Although carbohydrate profiles in erythroid

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0145-2126/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.leukres.2005.10.021

cells have been examined [7,8], little is known about the roles of carbohydrate antigens in erythroid cell differentiation. We established an erythroblastic leukemia cell line derived from a patient with early erythroblastic leukemia. Using this cell line, we found that erythroid differentiation and apoptosis are induced by GL-7, a recombinant Fab fragment reacting with PhGlc.

2. Materials and methods

2.1. Case history

The clinical course of the patient was previously reported [9]. A 49-years-old male was admitted to our hospital because of fever and gingival bleeding in November 1997. The peripheral blood showed a hemoglobin level of 7.3 g/dl, a platelet count of $2.6 \times 10^4\ \mu l^{-1}$, and a white blood cell count of $15{,}500\ \mu l^{-1}$ with 61% undifferentiated blasts. The serum lactate dehydrogenase level was markedly high (13,370 IU/l, normal <410 IU/l). A bone marrow aspirate demonstrated massive proliferation of blasts with undifferentiated features such as an extremely basophilic cytoplasm, a high nucleocytoplasmic ratio and one or more nucleoli. The blasts were negative for peroxidase, esterase (alpha-naphtyl butyrate and ASD-chloroacetate) and periodic acid-Schiff staining. Flow cytometry showed that the blasts were positive for CD36, CD71 and CD175s, but not for CD2, CD7, CD10, CD11b, CD13, CD15, CD19, CD33, CD34, CD41, CD45, CD38, CD235a or HLA-DR. The serum levels of sialyl-Tn and neuron-specific enolase activity were high. Karyotypic analysis of bone marrow cells showed 46,XY (37 cells/45 cells) and 47,XY,+8 (8 cells/45 cells). At first, the patient was suspected of having carcinocythemia of small cell carcinoma. Later, a final diagnosis of early erythroblastic leukemia was established based on the expression of the alpha-globin gene message. The patient died in December 1997 because of pulmonary bleeding after induction chemotherapy.

2.2. Cells and cell culture

Peripheral blood mononuclear cells were stored in a viable condition at −120 °C before the start of chemotherapy. Five months later, frozen peripheral blood mononuclear cells were thawed and cultured in YU-KLS medium, hormonally defined medium only containing 0.5% fetal calf serum (Yagai Research Center, Yamagata, Japan), supplemented with 10% fetal calf serum (FCS; Life Technologies Inc., Grand Island, NY) in 24-well plates at a concentration of $1 \times 10^6\ ml^{-1}$ at 37 °C in a 5% CO_2 incubator. After several weeks of culture, cells grew out and were maintained in the medium. To derive clones from the proliferating cells, single colonies grown in methylcellulose semi-solid media were picked up and cultured in the medium described above. A homogeneous population of cells was cloned by limiting dilution. The new erythroblastic leukemic cell line was designated EEB (early erythroblastic leukemia cell line) in October 1998.

The growth-supporting abilities of two hormonally defined media (STEMSPAN; StemCell Technologies, Inc., Vancouver, Canada, and EX-CELL Sp2/0, JRH Biosciences, Lenexa, KS) supplemented with 15% FCS and one standard medium (RPMI-1640; Invitrogen Co., Carlsbad, CA) supplemented with 15% FCS were examined. A polymerase chain reaction (PCR)-based method was used to test for contamination by mycoplasma and Epstein–Barr (EB) virus. Origins of the two types of cells were examined by Southern blot analysis.

CD34-positive cells were isolated by using the Mini-MACS CD34 isolation system (Miltenyi, Bergisch Gladbach, Germany) following the manufacturer's instructions. To obtain erythroblasts, clonal cell cultures were performed as follows: mononuclear cells were incubated in 1% methylcellulose, 30% fetal bovine serum, 1% bovine serum albumin, 3 U/ml erythropoietin, 50 ng/ml stem cell factor, 10 ng/ml granulocyte-macrophage colony-stimulating factor, 10 ng/ml interleukin-3, 10^{-4} M 2-mercaptoethanol and 2 mM L-glutamine (MethoCult GF H4434; StemCell Technologies Inc.). The cultures were incubated for 14 days at 37 °C in a 5% CO_2 incubator. On day 14 of culture, erythroid colonies were picked up and pooled.

2.3. Morphologic examination

Cells were stained with Wright–Giemsa, peroxidase, naphthol ASD-chloroacetate esterase, alpha-naphtyl butyrate esterase, acid phosphatase, Sudan black B and PAS solutions using standard methods. Hemoglobin-containing cells were detected using a specific reaction with benzidine/hydrogen peroxide solution (0.2% benzidine in 5 M glacial acetic acid and 10% H_2O_2) [10].

Electron microscopic examinations including analysis of morphology, platelet-peroxidase activity and myeloperoxidase activity, were described previously [11]. A platelet-peroxidase reaction was defined as positivity in the nuclear envelope and rough endoplasmic reticulum but negativity in the Golgi apparatus and cytoplasmic granules. A myeloperoxidase reaction was defined as positivity in the rough endoplasmic reticulum, nuclear envelope, Golgi apparatus and cytoplasmic granules.

2.4. Monoclonal antibodies

Commercially available fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated monoclonal antibodies used for the cell surface marker analysis were as follows: CD3 (OKT3; Ortho Diagnostic Systems, Beers, Belgium), CD4 (T4; Coulter Immunology, Hialeah, FL), CD8 (T8; Coulter), CD10 (OKBcALLa; Ortho), CD11b (Leu15; Becton Dickinson Immunocytometry Systems, Mountain View, CA), CD13 (MY7; Coulter), CD14 (Mo2;

Coulter), CD15 (80H5; Immunotech, Marseille, France), CD19 (B4; Coulter), CD33 (MY9; Coulter), CD34 (HPCA-2; Becton), CD117 (NU-c-kit; Nichirei Co., Tokyo, Japan), CD41 (P2; Immunotech), CD45 (Becton), CD71 (Becton), CD235a (anti-glycophorin A; Immunotech), HLA-DR (OKDR; Ortho) and anti-HbF (IQ products; Groningen, The Netherlands). Anti-sialyl-Tn (CD175s; TKH2), anti-sialyl-T (QSH1) and anti-disialyl-T (QSH2) monoclonal antibodies that react with erythoid cells were reported previously [5,6]. The human IgM monoclonal antibody GL-2 was originally established as an anti-i antibody and subsequently shown to also react with PhGlc [12,13]. GL-7, a recombinant Fab fragment of GL-2, reacts only with PhGlc [14].

2.5. Antigen expression analysis

The method used for flow cytometric analysis was described previously [5,6,9]. Cells were incubated with FITC or PE-conjugated monoclonal antibodies for 30 min at 4 °C. Alternatively, cells were incubated with unconjugated monoclonal antibodies for 30 min at 4 °C and then FITC-conjugated anti-mouse or anti-human immunoglobulin for 30 min at 4 °C. Isotypic-matched antibodies were used as a negative control. The surface immunophenotype was assessed using a flow cytometer (Cytoron; Ortho Diagnostic Systems, Raritan, NJ). HbF content was evaluated using a previously reported method [15]. Briefly, cells were fixed by suspension in 1 ml of 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) for 10 min at room temperature. The cells were permeabilized by resuspending them in a 1-ml mixture of methanol:acetone (1:4, v/v) for 1 min. A PE-conjugated anti-HbF monoclonal antibody was added, and the mixture was incubated for 45 min at room temperature. Isotypic-matched IgG was used as a negative control. Stained cells were analyzed using a flow cytometer (Cyto ACE-150, JASCO Co., Tokyo).

The method used for the evaluation of erythropoietin receptors was reported previously [16]. Cells were incubated with biotinylated erythropoietin or, as controls, with biotinylated erythropoietin plus a 1000-fold excess of unlabelled erythropoietin at 37 °C for 1 h. The cells were then incubated with 2 μg/ml of streptavidin-RED670 conjugate (SARED670; Gibco BRL, Brand Island, NY) at 4 °C for 1 h. Stained cells were analyzed using a flow cytometer (FACScan, Becton, Mansfield, MA).

For immunostaining [14], cells were fixed with 3% paraformaldehyde for 20 min at room temperature, quenched with 50 mM NH_4Cl, and then blocked with 0.2% gelatin. Cells were incubated overnight with GL-2 at 4 °C and then with FITC-conjugated anti-human IgM and Alexa 594-conjugated cholera toxin (Molecular Probes, Inc., Eugene, OR). Cells were examined with a Zeiss LSM 510 confocal microscope equipped with a Plan-Apochromat ×100 oil DIC objective.

2.6. Chromosomal analysis

Karyotypes were analyzed with a G-banding method [17]. Briefly, the cells were incubated with 0.02 μg/ml of colcemid (Gibco BRL) for 2 h for metaphase arrest. Hypotonic treatment was carried out with 0.075 M KCL for 20 min. The cells were fixed with methanol–acetic acid (3:1), dropped onto glass slides, and air-dried. Metaphases were analyzed.

Multicolor-fluorescence in situ hybridization (FISH) on metaphase preparations was performed using Spectra Vysion probes according to the instructions of the manufacturer (Vysis, Downers Grove, IL). Images were visualized by an epifluorescence microscope (Zeiss, Oberhochen, Germany) and analyzed using an Applied Imaging CytoVision Work station (Newcastle, UK). Ten metaphase cells were analyzed.

2.7. Effects of agents

Cells were suspended in YU-KLS medium containing 10% FCS at a concentration of 1×10^5 ml^{-1} and incubated for 4 days at 37 °C after the addition of appropriate concentrations of several agents. The inducers used were 1.0% dimethylsulfoxide (DMSO; Sigma), 1×10^{-3} M δ-ALA (Sigma), 1×10^{-6} M butyric acid (BA; Sigma), 1×10^{-4} M hydroxyurea (HU; Sigma), 1×10^{-10} M actinomicine-D (AD; Sigma), 1×10^{-4} M hemin (Sigma), 1×10^{-7} M cytosine arabinoside (Ara-C; Sigma), 1×10^{-6} M retinoic acid (RA; Sigma) and 5×10^{-6} M phorbol 12-myristate 13-acetate (PMA; Sigma). In negative controls, only buffer was added. In other experiments, cells (1×10^6 ml^{-1}) suspended in YU-KLS medium containing 10% FCS were treated with either 10 μg/ml, 100 μg/ml or 200 μg/ml of GL-7 for 4 days at 37 °C. In negative controls, 10 μg/ml of human IgM (Chemicon International, Temecula, CA) was added. In another set of experiments, the following commercially supplied cytokines were used: erythropoietin (EPO; 5 U/ml; Kirin Brewery Co., Tokyo, Japan), granulocyte colony-stimulating factor (G-CSF; 10 ng/ml; Chugai Pharmaceutical Co., Tokyo, Japan), granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 ng/ml; Genzyme Diagnostics, Cambridge, MA), Interleukin-3 (IL-3; 10 ng/ml; Genzyme), IL-6 (5 ng/ml; R & D Systems), interferon-γ (IFN-γ; 10 U/ml; JCR Pharmaceutical Co., Kobe, Japan) and tumor necrosis factor-α (TNF-α; 10 ng/ml; Genzyme). Following treatment, erythroid differentiation and myeloid differentiation and apoptosis were evaluated.

2.8. Assessment of apoptosis

The apoptosis assay was carried out using a MEBCYTO apoptosis kit (MBL, Nagoya, Japan) according to the manufacturer's instructions [18]. In brief, cells were washed and resuspended in binding buffer. FITC-conjugated Annexin V and propidium iodide (PI) were added to the cell suspension, and then the mixture was incubated for

15 min in the dark at room temperature. Thereafter, the suspension was analyzed using a flow cytometer (FACScan, Becton).

2.9. Isolation of glycolipid and thin-layer chromatography (TLC) Immunostaining

Glycolipids were isolated from EEB cells as described previously [19]. The lipid extract was applied to a phenylboronate-agarose column (PBA-60, Millipore Co., Billerica, MA) and glycolipids were eluted with a chloroform/methanol/water (5:5:1, v/v/v) solution. For the isolation of GL-2-reactive lipids, TLC immunostaining was performed using GL-2 followed by horseradish peroxidase (HRP)-conjugated anti-human IgM [14]. The immunoreactive lipids were visualized using 3,3′-diaminobenzidine tetra hydrochloride (ICN Biochemical Inc., OH) as a chromogen [14].

2.10. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed as described previously [20]. Briefly, cDNA was synthesized using 4 μg of total RNA extracted with the RNeasy Mini Kit (QIAGEN) and Superscript II reverse transcriptase (Invitrogen Co., Carlsbad, CA). cDNA was amplified in 25 μl of 10 mM Tris–HCl, 2.5 mM $MgCl_2$ and 50 mM KCl (pH 8.3). Taq DNA polymerase (Roche Diagnostics, Basel, Switzerland) was added at 1 U/reaction mixture. The PCR profile was as follows: denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C and extension for 30 s at 72 °C (35 cycles). The reactions were run in a 9600R (Roche). The following primer sequences were used for reverse transcription-polymerase chain reaction: γ-globin forward primer, 5′-TGGCAAGAAGGTGCTGACTTC-3′; γ-globin reverse primer, 5′-TCACTCAGCTGGGCAAAGG-3′. These primers were purchased from Invitrogen.

2.11. Quantitative real-time PCR analysis

Quantitative real-time PCR analysis was performed as described previously [20]. Briefly, cDNA was synthesized using RNeasy Mini Kit-extracted (QIAGEN) total RNA, oligo(dT)12-18 primer and moloney murine leukemia virus reverse transcriptase (Invitrogen). PCR amplifications were performed in 25-μl reaction mixtures consisting of Taq Man Universal PCR Master Mix, No UNG (PE Biosystems, Warrington Cheshire, UK), 0.4 μM of the forward and reverse primers (Invitrogen), 0.25 μM of the TaqMan Probe (Applied Biosystems) and 5 μl of cDNA template. The PCR cycles were as follows: denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C and extension for 30 s at 72 °C (50 cycles). The reactions were run in an ABI PRISM 7700 Sequence Detection system using the software Sequence Detection System Version 1.6.3 (PE Biosystems). The following primer and probe sequences were used for quantitative real-time PCR: γ-globin forward primer, 5′-TGGCAAGAAGGTGCTGACTTC-3′; γ-globin reverse primer, 5′-TCACTCAGCTGGGCAAAGG-3′; γ-globin probe, 5′-FAM-TGGGAGATGCCATAAAGCACCTGG-TAMRA-3′. The fluorescent reporter and the quencher were 6-carboxyfluorescein and 6-carboxy-*N,N,N′,N′*-tetramethylrhodamine, respectively. For quantitative real-time PCR of the reference genes, we used the endogenous control human glyceraldehyde-3-phosphate dehydrogenase Assays-on-Demand Gene Expression Products (Applied Biosystems).

2.12. Immunoblotting

Immunoblotting for tyrosine-phosphorylated protein was performed using HRP-conjugated PY20 (Transduction Laboratories, Lexington, KY) as described previously [21]. After the incubation of EEB cells (2×10^7 ml^{-1}) for 4 days, 50-μl aliquots were transferred to microtubes, and an equal volume of GL-7 (20 μg/ml) was added. After 10 min, stimulation was terminated by adding 50 μl of SDS sample buffer. For a control, cells were treated with an unrelated Fab antibody (anti-HBs). Protein tyrosine phosphorylation was assessed by immunoblotting using HRP-conjugated PY20.

3. Results

3.1. Establishment of a new erythroblastic leukemia cell line

The EEB cell line has been maintained in YU-KLS medium supplemented with 10% FCS for more than 6 years since the cell culture of leukemic cells from the patient started. EEB cells grow as single cells in suspension without adhering to plastic dishes. The growth of EEB cells showed an exponential growth phase, which was followed by a plateau phase. The doubling time was 38 h. No specific proliferation parameters were identified. For aggressive proliferation, the initial cell concentration required was more than 5×10^6 cells per ml. EEB cells can be frozen under standard conditions using 70% medium, 20% FCS and 10% dimethylsulfoxide (Cellbanker; Nippon Zenyaku Co., Koriyama, Japan) and thawed cells easily grow in the same medium. EEB cells remain stable during this culture period and also after freezing and thawing. The two hormonally defined media, STEMSPAN and EX-CELL, similarly supported the growth of EEB cells, while the standard medium, RPMI-1640, hardly supported the growth of the cells (data not shown). Mycoplasma and EB virus were not detected in EEB cells. Southern blot analysis of genomic DNA extracted from EEB cells and the patient's leukemic cells showed the same bands, which suggested that EEB cells are derived from the patient's leukemic cells (data not shown). The DNA fingerprint analysis was planned to perform to exclude cell contamination in EEB cells later, however, it was impossible because original leukemic cells from the patient was no longer left.

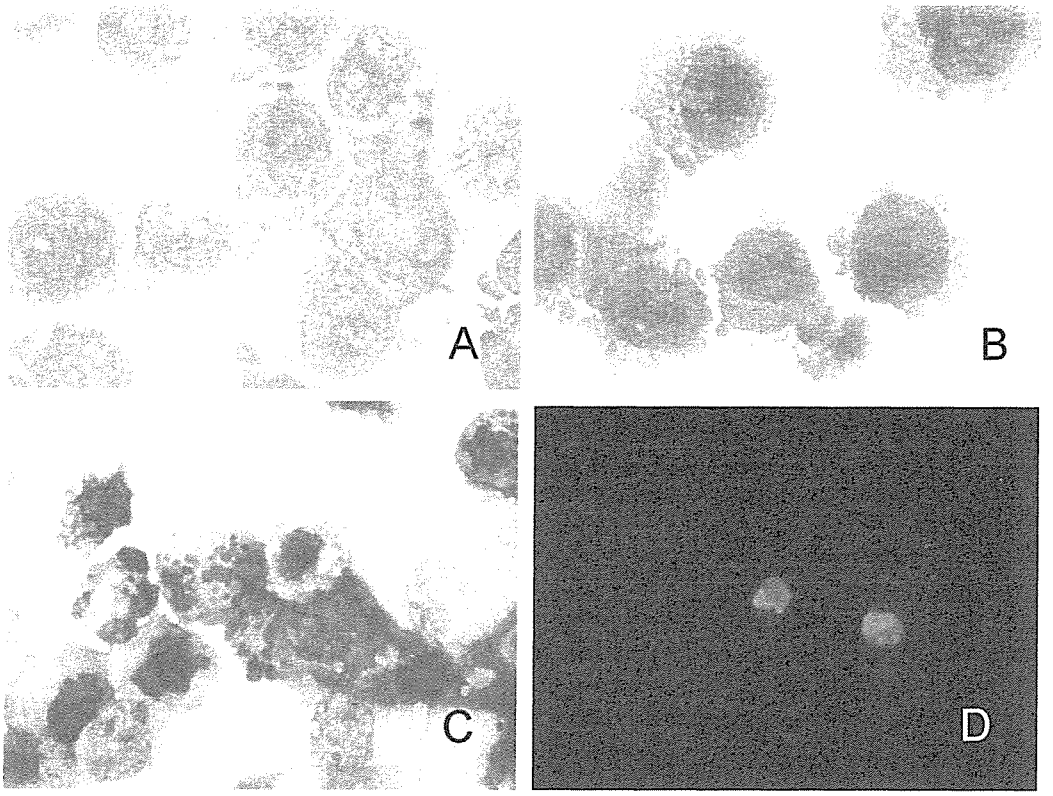

Fig. 1. Morphologic examination of EEB cells. EEB cells were immature erythroblastic cells (A) and showed a decrease in cell size and nuclear condensation after treatment with GL-7 at a concentration of 100 μg/ml for 2 days (B), while most underwent apoptosis after treatment with GL-7 at 200 μg/ml for 3 days (C). EEB cells were positively stained with GL-2 (D). (A–C) Wright–Giemsa staining; (D) immunostaining.

3.2. Morphologic analysis

EEB cells were medium to large and had round-shaped nuclei with several nucleoli and a basophilic agranular cytoplasm (Fig. 1A). The cells were negative for peroxidase, alpha-naphthyl butyrate esterase, naphthol-ASD-chloroacetae, Sudan black B and PAS stainings. A small percentage of the cells were positive for acid phosphatase staining. These features resemble those of the patient's leukemic blasts and immature erythroblasts. Although the original leukemic cells of the patient showed positive staining for neuron-specific enlolase activity, EEB cells had lost the activity (data not shown). The ultrastructural study showed that EEB cells had immature blastic features without myeloperoxidase activity, platelet-peroxidase activity or granules containing ferritin molecules (data not shown).

3.3. Immunophenotypic analysis

The cell surface marker profiles are summarized in Table 1. The surface markers of the EEB cells were almost the same as those of the original blasts. EEB cells were only positive for CD36, CD45, CD71, CD117, CD235a, and erythroid-associated carbohydrate antigens, including sialyl-T, disialyl-T and CD175s, at various expression levels. The cells were negative for myeloid antigens, including CD13, CD15 and

Table 1
Phenotypic characteristics

Antigen (CD)	EEB (%)	Patient (%)
CD2	0.5	0.8
CD3	0.4	ND
CD7	0.4	1.8
CD10	0.4	0.2
CD11b	0.5	12.7
CD13	0.8	14.4
CD15	1.6	4.4
CD19	0.3	5.2
CD20	1.0	11.6
CD33	11.7	8.0
CD34	0.2	1.5
CD36	99.9	99.9
CD41	0.8	0.8
CD45	97.0	13.9
CD71	48.7	77.3
CD117	36.4	ND
CD175s	88.8	88.8
CD235a	72.1	8.4
HLA-DR	0.5	6.4
Sialyl-T	95.3	87.0
Disialyl-T	35.6	41.0
GL-2	56.0	ND

CD175s, sialyl-Tn; CD235a, glycophorin A; ND, not determined. The values of EEB cells shown are the percent positive cells of representative data in three tests. Reactivity is defined as positive when more than 15% of cells are stained with a monoclonal antibody.

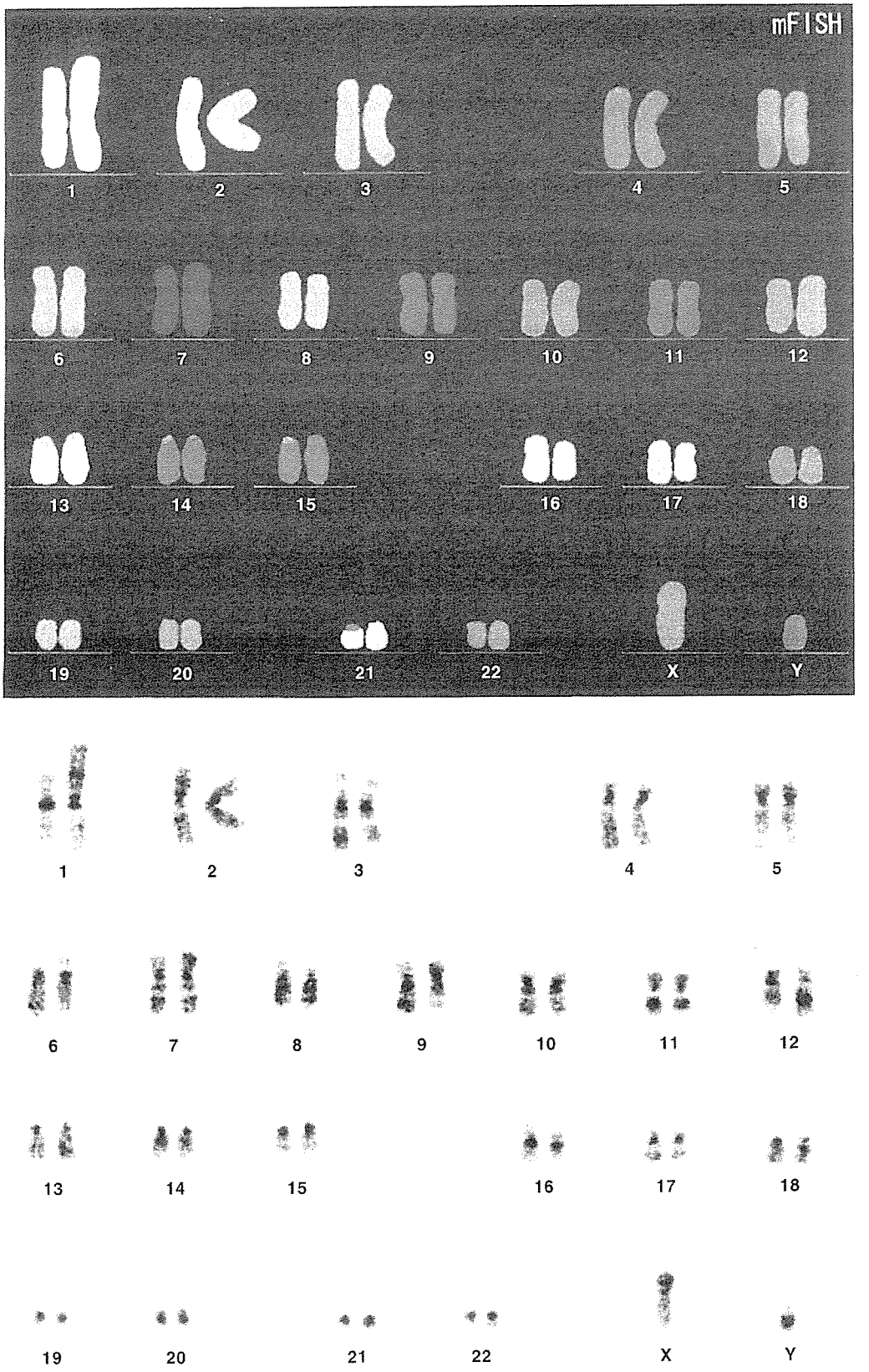

Fig. 2. Multicolor FISH analysis. EEB cells showed a normal karyotype.

CD33, lymphoid antigens, including CD2, CD3, CD7, CD10, CD19 and CD20, a stem cell antigen (CD34), and a platelet antigen (CD41). The cell surface antigen expression profiles showed that EEB cells belong to an erythroid lineage and are more differentiated than the original blasts. Erythropoietin receptor expression was analyzed using flow cytometry; EEB cells did not express erythropoietin receptors even though they were treated with PMA or DMSO (data not shown). Using this method, erythropoietin receptor expression was shown in an erythropoietin-responsive erythroid cell line, AS-E2 [22].

The flow cytometric analysis showed that about 56% of the EEB cells expressed an antigen(s) recognized by GL-2 (Table 1). Reactivity of the antibody with EEB cells was also confirmed using immunostaining: the membrane reaction was clustered showing a capping-like phenomenon (Fig. 1D).

3.4. Cytogenetic analysis

A chromosomal analysis of G-banding metaphases of EEB cells was performed three times, i.e., 1, 2 and 3 years after the establishment; EEB cells revealed that the karyotype was normal, 46,XY in all analyses (Fig. 2). The normal karyotype of EEB cells was confirmed by the multicolor FISH analysis; all 10 of the cells analyzed showed 46,XY (Fig. 2). EEB cells showed no p53 gene mutation, tandem duplication of the FLT3 gene, or FLT3 activation loop mutation (data not shown).

3.5. Induction of differentiation

Benzidine-positive cells were counted after EEB cells had been treated with hemin, DMSO, δ-ALA, BA, HU, AD, hemin and Ara-C (Fig. 3). Untreated EEB cells were negative for the hemoglobin staining, while 40–50% of the cells treated with δ-ALA or hemine were positive for benzidine. After incubation with PMA, EEB cells loosely adhered to plastic dishes and exhibited a decrease in cytoplasmic basophilia. However, no cytochemical change, including alpha-naphthyl butyrate esterase activity or change in the expression of surface antigens (CD11b, CD13, CD14, CD15, CD33, CD36, CD41 and CD235a) was observed (data not shown). Similarly, RA did not obviously induce myeloid or erythroid differentiation of EEB cells (data not shown).

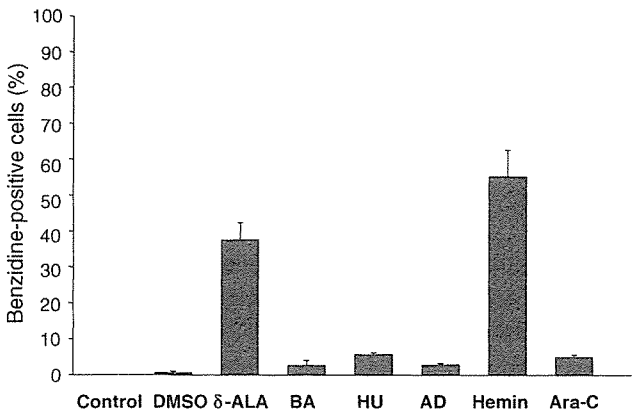

Fig. 3. Differentiation to benzidine-positive cells induced by various agents. EEB cells were cultured with DMSO, δ-ALA, BA, HU, AD, hemine, Ara-C and GL-7 at appropriate concentrations for 4 days. After incubation, benzidine-positive cells were counted. The addition of δ-ALA and hemin induced EEB cells to differentiate into benzidine-positive cells. Each value is the mean ± S.D. of three experiments conducted in duplicate.

Since GL-7 induces granulocytic differentiation of HL-60 cells [14], the effect of the Fab fragment on EEB cell differentiation was examined. As shown in Fig. 4A, GL-7 inhibited the growth of EEB cells in a dose-dependent manner. The growth inhibition induced by GL-7 was associated with erythroid differentiation and apoptosis. Following treatment of EEB cells with GL-7, the number of benzidine-positive cells increased: maximal erythroid differentiation occurred after a 2-day incubation with the addition of 100 μg/ml of GL-7 (Fig. 4B). In this setting, EEB cells showed a decrease in both cell size and cytoplasmic basophilia, and nuclear condensation (Fig. 1B). An increase in the production of HbF was

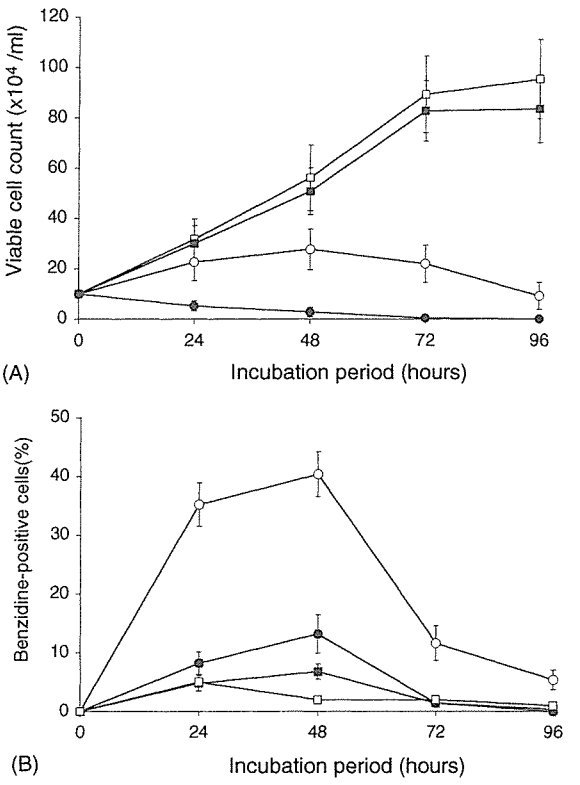

Fig. 4. Growth curve of EEB cells and differentiation to benzidine-positive cells induced by GL-7. Viable cells were counted following treatment of EEB cells with GL-7 or no treatment (A). (□) Control (human IgM); (■) 10 μg/ml of GL-7; (○) 100 μg/ml of GL-7; (●) 200 μg/ml of GL-7. Each value is the mean ± S.D. of three experiments conducted in triplicate. GL-7 induced the differentiation into benzidine-positive EEB cells in a dose-dependent and a time-dependent manner (B): maximal numbers of benzidine-positive cells were observed on treatment with 100 μg/ml of GL-7 for 2 days. (□) Control (human IgM); (■) 10 μg/ml of GL-7; (○) 100 μg/ml of GL-7; (●) 200 μg/ml of GL-7. Each value is the mean ± S.D. of three experiments conducted in triplicate.

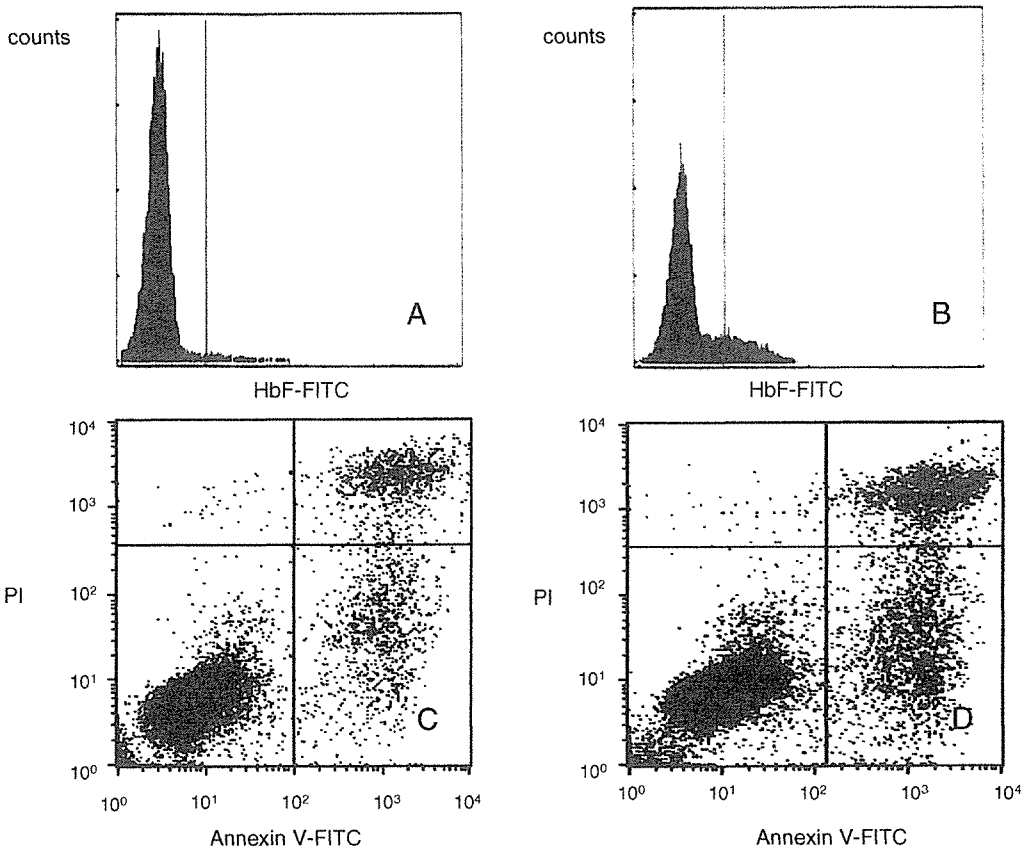

Fig. 5. Production of HbF and apoptosis induced by GL-7. The addition of 100 μg/ml of GL-7 for 2 days induced production of HbF in EEB cells (B) compared with the addition of control human IgM (A). However, the addition of 100 μg/ml of GL-7 for 3 days increased the number of apoptotic cells (D) compared with the addition of control human IgM (C). One representative experiment of three performed.

confirmed to have occurred using flow cytometry (Fig. 5B). The induction of erythroid differentiation of EEB cells by GL-7 was associated with apoptosis. The apoptosis induced by GL-7 was confirmed based on morphologic changes: EEB cells showed cytoplasmic membrane blebbing and nuclear fragmentation (Fig. 1C). GL-7-induced apoptosis was confirmed using flow cytometry utilizing Annexin V and PI staining (Fig. 5D). Following treatment with GL-7, the number of apoptotic cells increased: on the addition of 100 μg/ml of GL-7, apoptotic cells numbers increased after maximal erythroid differentiation on day 2 of the culture (Fig. 4A). In contrast, the addition of 200 μg/ml of GL-7 for 3 days caused the apoptosis of most cells with minimal erythroid differentiation (Figs. 1C and 4B).

GM-CSF and IL-6 slightly stimulated only the growth of EEB cells (145% and 150% versus 100% of control without cytokine-stimulation, respectively), while other cytokines including EPO, G-CSF, IL-3, IFN-γ and TNF-α did not affect the growth or the differentiation (data not shown).

3.6. Analysis of γ-globin message

The γ-globin message was detected by RT-PCR (Fig. 6A). EEB cells treated with GL-7 expressed the message. Because untreated EEB cells also expressed it, a quantitative real-time PCR analysis was conducted to identify induction of the γ-globin message in EEB cells treated with GL-7. As shown in Fig. 6B, GL-7-treated EEB cells showed a slight increase in γ-globin message content.

3.7. Analysis of PhGlc in EEB cells

GL-2 reacts with two carbohydrates, i and PhGlc [13]. Therefore, carbohydrate profiles of the EEB cell membrane were analyzed using TLC immunostaining: PhGLc but not i was detected in the EEB cell membrane (Fig. 7).

3.8. Analysis of phosphorylation

To determine whether the erythroid differentiation induced by GL-7 is associated with protein tyrosine phosphorylation, early changes in protein tyrosine phosphorylation were analyzed. EEB cells treated with GL-7 showed no protein tyrosine phosphorylation (data not shown).

3.9. Expression profiles of GL-2-reactive blood cells

Profiles of i and PhGlc expression in normal blood cells are not well understood. Therefore, the reactivity of GL-2 with various blood cells was examined: GL-2 reacted with

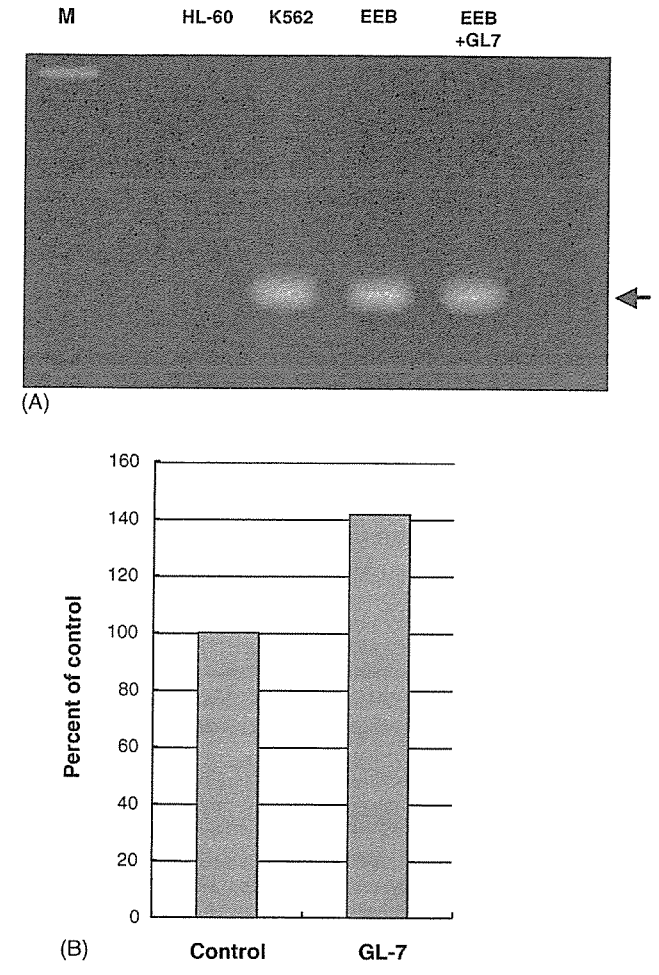

Fig. 6. Assessment of γ-globin gene expression in EEB cells. The γ-globin message was detected in K562 cells and in EEB cells treated or not treated with GL-7 but not in HL-60 cells by RT-PCR analysis (A). Quantitative RT-PCR analysis showed a slight increase in the γ-globin message in EEB cells treated with GL-7 for 48 h compared with cells treated with control human IgM for 48 h (B). One representative experiment of two performed.

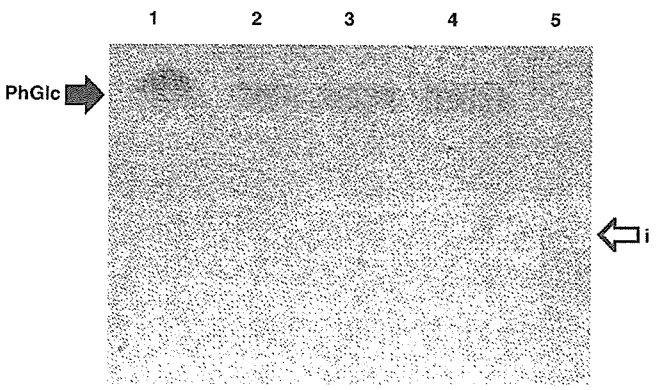

Fig. 7. TLC immunostaining. Lanes 1–5 indicate the application of 8 μl of PhGlc, 1 μl, 2 μl and 4 μl of glycolipid extracted from EEB cells, and 8 μl of i glycolipid, respectively. EEB cells contain PhGLc (the black arrow, the narrow band) but not i glycolipid (the white arrow, the wide band). One representative experiment of two performed.

cord blood erythrocytes (42%) and erythroblasts derived from both adult bone marrow (36%) and cord blood (64%) but not with CD34-positive cells (1%) or adult erythrocytes (1%).

4. Discussion

We established a human erythroblastic leukemia cell line, EEB, from a patient with early erythroblastic leukemia. Morphologic, cytochemical, immunophenotypic and gene expression analyses demonstrated that EEB has features of immature erythroid cells, including expression of CD36, CD71, CD175s, CD235, HbF and γ-globin message. Erythropoietin receptors were not detected in EEB cells, but that is not surprising. Generally, erythropoietin-independent erythroblastic leukemia cell lines show no erythropoietin receptors on the cell surface [23]. Since EEB cells do not have the megakaryocytic marker CD41 or platelet-peroxidase activity, they do not show megakaryocytic features. EEB cells show dim expression of CD117 and bright expression of both CD175s and CD235a. These features correspond to colony-forming unit-erythroid cells. The differential capacity of EEB cells is limited: they differentiated to benzidine-positive cells with the addition of δ-ALA and hemin. Neither TPA nor retinoic acid induced the differentiation of EEB cells to benzidine-positive cells, CD41-positive cells or myelomonocytic cells. The karyotype of EEB cells was normal and the cells had no P53 gene mutation, tandem duplication of the FLT3 gene, or FLT3 activation loop mutation. Since it seems that leukemic cell lines showing a normal karyotype are rare, it is necessary to perform chromosomal analysis of EEB cells in more detail. Moreover, molecular mechanisms of EEB cell growth remain to be clarified.

GL-2 was established as an antibody that reacts with an unbranched type 2 carbohydrate chain (i antigen). Thereafter, it was shown that GL-2 also reacts with a glycolipid, phosphatidylglucoside (PhGlc) [13]. Recently, GL-7, a recombinant Fab fragment of GL-2, has been prepared and shown to induce granulocytic differentiation of HL-60 cells [14]. Such granulocytic differentiation is associated with the rapid phosphorylation of Src family kinases, leading to an up- and down-regulation of CD38 and c-Myc expression, respectively. In our study, the binding of GL-2 to EEB cells was shown by flow cytometry and immunofluorescence microscopic analysis, respectively. We demonstrated that GL-7 induced production of HbF by EEB cells in a dose-dependent and a time-dependent manner. The accumulation of the γ-globin message in EEB cells treated with GL-7 was confirmed by quantitative real-time PCR analysis. These results suggest that GL-7 induces erythroid differentiation of EEB cells via the accumulation of γ-globin message. Since GL-2 binds both i antigen and PhGlc [12,13], glycolipid profiles of the EEB cell membrane were analyzed by TLC: EEB cells had PhGlc but not i antigen. Therefore, erythroid differentiation induced by GL-7 occurs via the interaction of GL-7 with PhGlc. Taken together, our results

and those of a previous study [14] suggest that PhGlc is involved in both myeloid and erythroid differentiation.

GL-7-mediated erythroid differentiation in EEB cells was found to be associated with apoptosis. This is not surprising: it is well known that erythroid differentiation of K562 cells is usually associated with terminal cell division [24,25]. Recently, Bianchi et al. demonstrated that tallimustine-mediated induction of erythroid differentiation of K562 cells is associated with apoptosis [26]. Tallimustine is a synthetic derivative of distamycin, which is a DNA-binding compound, and exhibits a very high level of anti-tumor activity [27]. Several antibodies cause the apoptosis of target cells: Rituximab, an anti-CD20 antibody, leads to direct signaling for apoptosis as well as complement activation and cell-mediated cytotoxicity in CD20-positive B cells [28], although it is not known whether the antibody induces lymphoid differentiation of the cells. Anti-carbohydrate monoclonal antibodies also induce apoptosis of target cells [14]: both DH59B, an anti-GM1 antibody, and Vj41, an anti-sphingomyelin antibody, induce apoptosis but not myeloid differentiation of HL-60 cells. GL-7-mediated erythroid differentiation of EEB cells may resemble tallimustine-mediated erythroid differentiation of K562 cells, because the two agents induce both erythroid differentiation and apoptosis in erythroid cells.

Glycosphingolipids are distributed in the outer leaf of the plasma membrane, forming clusters known as glycosphingolipid microdomains. The contribution of glycosphingolipid microdomains to signal transduction has been determined [29,30]: various ligands, including antibodies, toxins, lectins and complementary glycosphingolipids, bind to glycosphingolipid microdomains, leading to the activation or inhibition of transducer molecules associated with glycosphingolipids. Src-family kinases in the inner leaflet of glycosphingolipid microdomains can be activated via stimulation of surface glycosphingolipid microdomains. An anti-GM3 antibody inhibits Ras and MAP kinase activity, while it activates Rho and Lyn activity. Recently, it has been shown that PhGlc forms microdomains in HL60 cells [14]. The granulocytic differentiation of HL60 cells induced by GL-7 is associated with a rapid phosphorylation of Src family kinases [14]. Since GL-7 is a recombinant monovalent Fab fragment antibody, the granulocytic differentiation of HL60 cells induced by GL-7 does not depend on clustering of PhGlc like the interaction of galectin-1 and CD45 in T cells [31]. Therefore, PhGlc-mediated direct signaling leads to the granulocytic differentiation of HL60 cells via the phosphorylation of Src family kinases. In contrast, PhGlc-mediated erythroid differentiation in EEB cells was not associated with tyrosine phospholyration. PhGlc-mediated signaling may differ among cell lineages. EEB cells are useful for examining the association between signal transduction and erythroid differentiation mediated by PhGlc.

We showed that GL-2 reacted with erythroblasts developed from both adult bone marrow and cord blood but not with CD34-positive cells. Therefore, the i antigen and/or PhGLc might play a role both in fetal and in adult erythropoiesis.

In summary, the early erythroblastic leukemia cell line, EEB, was established from a patient with early erythroblastic leukemia. The cell line has a normal karyotype and retains erythroblastic features. The cell line can be available upon suitable request for further analysis.

Acknowledgements

The authors thank Dr. Eguchi for performing electron microscopic analysis, Dr. Inokuchi for performing gene mutation analysis, and technicians in Mitsubishi Kagaku Bio-Clinical Laboratories for performing multicolor FISH analysis.

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