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Nutritional Support for Patients Suffering From Intestinal Graft-versus-Host Disease After Allogeneic Hematopoietic Stem Cell Transplantation

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Background: Patients who exhibit gastrointestinal (GI) involvement due to graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (SCT) are often recommended to withhold oral intake (NPO) to avoid further damage to the GI mucosa. However, it is possible that continuing oral intake could be beneficial in many patients compared to total parenteral nutrition (TPN).

Objective: The primary objective of this prospective study was to evaluate whether programmed step-ladder oral dieting (enteral nutrition; EN) is feasible and beneficial for these patients.

Methods: A total of 18 patients who exhibited GI-acute GVHD (stage I to III gut GVHD) after SCT received an EN dieting program, and changes in clinical and laboratory parameters were compared to those in a control cohort of 17 patients who were placed on NPO with TPN. Patients with GVHD were included prospectively and those with intestinal bleeding/obstruction, severe pancreatitis, and cytomegalovirus enterocolitis were excluded.

Results: None of the patients in the EN group experienced significant adverse events, including exacerbation of GI symptoms. Although there was no statistically significant difference in the volume or frequency of diarrhea or the time to complete dietary recovery, parameters including body weight and serum levels of total protein and albumin tended to improve faster in the EN group.

Conclusion: The EN diet is safely applicable to patients suffering from GI involvement by GVHD. Am. J. Hematol. 81:747–752, 2006. © 2006 Wiley-Liss, Inc.

Key words: graft-versus-host disease (GVHD); enteral nutrition; immunonutrition

INTRODUCTION

Graft-versus-host disease (GVHD) is a major complication of allogeneic hematopoietic stem cell transplantation (SCT) that influences the ultimate prognosis of patients [1]. Gut involvement due to GVHD particularly impairs the host nutritional status and QOL due to long-lasting diarrhea and anorexia. Hence, effective supportive care of patients suffering from GVHD should include attention to intense nutritional support and bone mineral retention, since many receive concomitant steroid therapy. Additionally, normal intestinal architecture and functions are required to prevent biliary stasis, retarded bowel movement, bacterial translocation, and resultant systemic infection [2,3]. With the development of gut GVHD, patients are often recommended to withhold oral intake (NPO, "bowel rest") to avoid further damage to the gastrointestinal (GI) mucosa. However, this raises a serious concern since NPO care can induce atrophic deficit of the GI mucosa and resultant dysfunction

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TABLE I. Grade of Programmed EN Dieting

Step	Staple food (form of rice)	Side dishes (approved foods and cuisines)	Nutritive value
0	Liquid	Juice (without grain, without oranges), electrolytic supplement solution	500–2000 ml
1	Liquid	Water gruel, starch gruel, clear soup, consomme, juice, miso soup	Calories 300–350 kcal Protein 5–7 g Fat 15–2 g Dietary fiber 15 g
2	Mush	Potato, vegetables, canned fruits, vegetable juices, noodles, tofu, whitefish	Calories 600–650 kcal Protein 20–25 g Fat 5–8 g Dietary fiber 1.5–8 g
3	Rice gruel	Eggs, breads, banana, apple	Calories 900–1000 kcal Protein 30–35 g Fat 10–13 g Dietary fiber 8–9 g
4	Boiled rice	Blue-skinned fish, oil (~3 g/day)	Calories 1200–1300 kcal Protein 40–45 g Fat 15–20 g Dietary fiber 9–10 g
5	Boiled rice	Chicken (low fat), yogurt, oil (~8 g/day)	Calories 1500–1600 kcal Protein 60–65 g Fat 30–35 g Dietary fiber 12–13 g

Note: A patient-oriented stepped-up dieting program was gradually applied over six steps that varied with regard to the solidity, intensity, and acceptability by the patient.

of the GI system. Moreover, it has recently been reported that enteral nutrition (EN) was more effective than parenteral nutrition for the nutritional support of patients with an injured intestine due to trauma or an invasive operation [4,5]. Taken together, these findings suggest that the current patient management procedure that includes the interruption of oral feeding to enforce "bowel rest" in SCT patients suffering from GVHD should be critically reevaluated. Furthermore, EN, if tolerable, may be a preferred route for maintaining digestive and absorptive function as intact as possible.

In those suffering from GI involvement of GVHD, such evaluation becomes more complex since diarrhea is very often multifactorial and includes secretory dysfunction, osmotic factors, and rapid passage. Hence, the establishment of a standard care procedure remains very difficult. To address these concerns, we conducted a controlled cohort study to evaluate the benefit of different nutritional support measures for patients suffering from acute gut GVHD after SCT. Our clinical hypothesis was that a programmed and controlled scheduled oral nutritional support with EN is beneficial for patients who have mild to moderately progressing acute symptoms of gut GVHD.

PATIENTS AND METHODS

Patients

Seventy patients who were treated at the National Cancer Center Hospital from January 2001 to December 2003 and who developed GI symptoms by GVHD were involved in this prospective study. Forty among those eligible patients met the following inclusion criteria: (i) pathologically diagnosed GVHD with biopsied specimens, (ii) presented symptoms within 100 days after SCT, and (iii) clinically diagnosed as stage I to III gut GVHD and grade II to III acute GVHD according to the clinical grading criteria [6,7]. Patients who had intestinal tract bleeding, intestinal obstruction, or severe pancreatitis were excluded from this analysis, since these pathophysiologies are considered contraindications for EN. Additionally, patients with pathologically diagnosed cytomegalovirus enterocolitis were also excluded, and thus a total of 35 patients were left for this study.

Methods

In the study periods, two different nutritional intervention procedures were applied; patients who developed gut GVHD before July 2002 ($n = 17$) were treated with NPO and total parenteral nutrition (TPN) (C group), while the remaining patients who developed gut GVHD after July 2002 ($n = 18$) were treated by programmed GVHD dieting intervention (EN group). The patients were consecutively registered to our database at National Cancer Center Hospital, and this prospective study was approved by the IRB. The programmed EN dieting consisted of six steps with regard to solidity, intensity, and acceptability for intestinal digestion, as shown in Table I. Each food and nutrient was made more solid and dense

in a step-up manner, after the confirmation of stable symptoms that lasted for a minimum of 3 days. Each step of programmed EN dieting was suitably stepped down when intolerance or exacerbation of gut GVHD symptoms developed. Patients were made NPO with the appearance of significant abdominal symptoms (nausea, vomiting, and abdominal pain). Patients in the EN group only received oral intake without enteral tube feeding. On the other hand, the patients in group C were adequately allowed to eat according to their symptoms with TPN.

We evaluated "time to complete dietary recovery," which was defined as the duration from the start of nutritional management (stopping oral intake or start of programmed EN dieting) to the restoration of a normal diet with the recovery of nutritional parameters. Nutritional parameters evaluated in this study included (1) clinical symptoms, including volume and frequency of diarrhea, and body weight and (2) laboratory data, including total serum protein and albumin. Body mass index (BMI) was calculated as BMI = {height (m)}2/body wt (kg).

Statistical Analysis

Our clinical hypothesis was that a programmed and controlled schedule of nutritional support with oral intake (EN dieting) could be effective in the support of patients suffering from acute gut GVHD with mild to moderately progressing symptoms. We evaluated "the time to complete dietary recovery," which was defined as the duration from the start of nutritional management (stopping oral intake or start of EN dieting) to the recovery to normal diet, various enteral symptoms, and nutritional parameters. The time to complete dietary recovery is shown with a time–event cumulative curve, and the log-rank test was used to compare groups C and EN. Nutritional parameters are given as the mean of each group by time course, and the data in groups C and EN were compared by an analysis of variance (ANOVA). A P value of less than 0.05 was considered significant.

RESULTS

Patients' Characteristics

The patients' clinical backgrounds are summarized in Table II, which shows that there are no essential differences between groups C and EN. Older patients tended to receive a reduced-intensity regimen more often than a conventional regimen.

Safety of Programmed EN Dieting

Throughout the study, no severe adverse events associated with nutritional intervention were observed, indicating that our procedure with gradual stepped-up or -down dieting was safe. No severe infectious episodes were observed in each group. EN dieting had to be terminated early in 2 of 18 cases due to prolonged GI symptoms and exacerbation of an underlying malignant disorder. There were 4 censored cases in group C, mainly due to recurrence of the basic malignant disorder.

TABLE II. Patients' Characteristics

	EN group (N = 18)	C group (N = 17)
Age median (range)	53 (22–64)	53 (23–69)
Sex male/female	12/6	14/3
Disease		
AML	6	8
MDS	3	2
ALL	4	2
CML	3	1
NHL	1	2
ATL	1	1
Solid tumors	0	1
Transplantation source		
BM	1	3
PBSC	17	14
Transplantation regimen		
Conventional	5	7
Reduced intensity	13	10
Donor HLA typing		
Full match	14	14
1 locus mismatch	4	0
2 loci mismatch	0	3
GVHD prophylaxis		
CSP alone	10	8
CSP + MTX	6	4
CSP + ATG	2	2
Others	0	3
Gut GVHD stage		
1	5	9
2	7	3
3	6	5
GVHD grade		
II	6	8
III	12	9
Onset day of gut GVHD (mean of day)	74	68

Note: Patients who underwent SCT and developed gut GVHD were enrolled in this study. Patients who developed gut GVHD before July 2002 (n = 17) were treated with no oral intake (C group), while the EN group (n = 18) was treated by programmed GVHD dieting. AMI, acute myelogeneous leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; CML, chronic myelogeneous leukemia; NHL, non-Hodgkin lymphoma; ATL, adult T-cell leukemia; BM, bonemarrow; PBSC, peripheral blood stem cell; CSP, cyclosporine; MTX, methotrexate; ATG, anti-thymocyte globulin.

Efficacy of Programmed EN Dieting

Although there was a wide variation in each patient in diarrhea volume and frequency of diarrhea, we adapted ANOVA to evaluate whether there is a statistically significant difference between the two groups

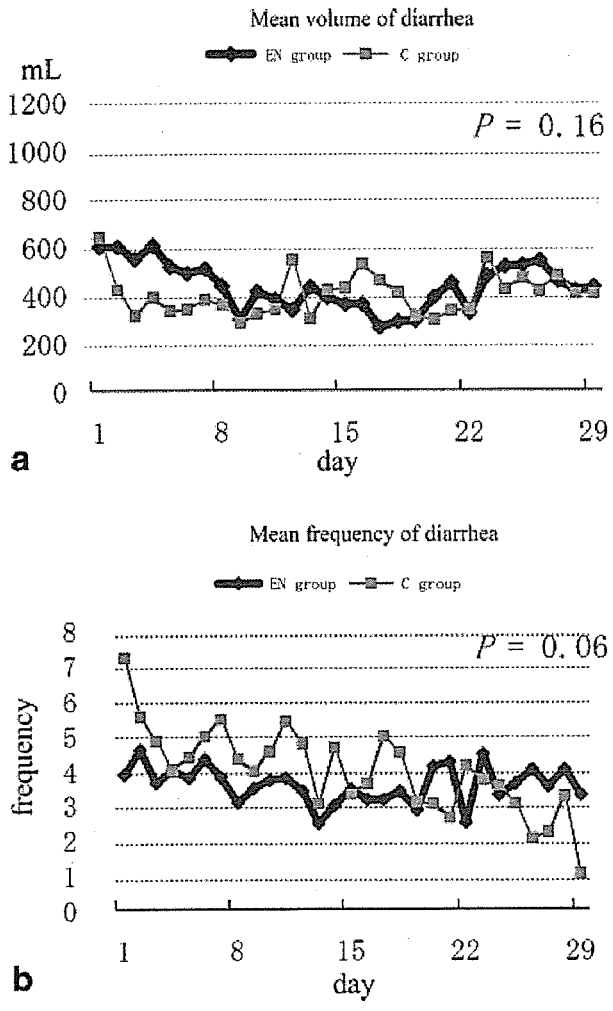

Fig. 1. Changes in mean volume and frequency of diarrhea. No difference was observed between the C and EN groups in the time-course of diarrhea as evaluated by volume ($P = 0.16$) (a) and frequency ($P = 0.06$) (b).

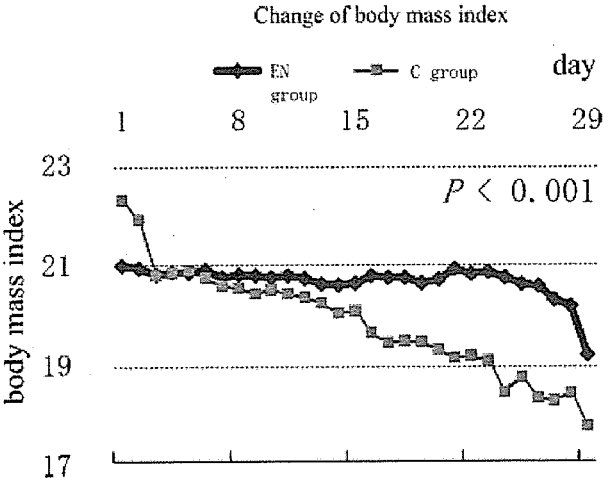

Fig. 2. Changes in BMI. The mean changes in BMI, with the first evaluation as a control, were compared between the two groups. A slower decrease in body weight tended to be observed in the EN group, while patients retained their BMI significantly better in the EN group than in the C group ($P < 0.001$). BMI was calculated as BMI = {height (m)}^{2}/body wt (kg).

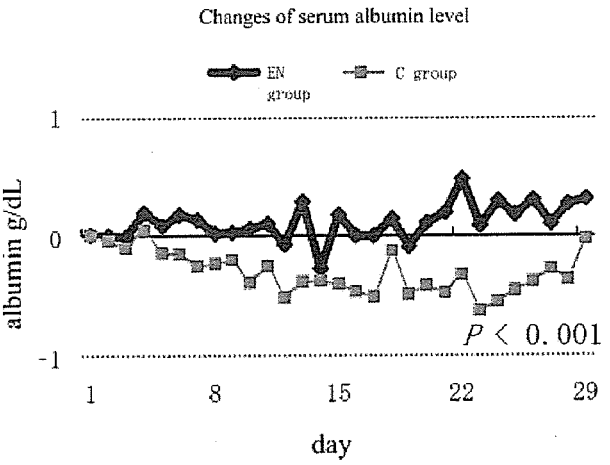

Fig. 3. Changes in albumin as nutritional parameter. One of the nutritional parameters, albumin (Alb), was evaluated between the C and EN groups. In the EN group, patients maintained significantly more stable levels of Alb ($P < 0.001$).

($P = 0.16$ and 0.06, respectively, Figure 1a and b). The mean body weight values in each group were compared by considering the absolute changes after adjusting by the value at the initial evaluation. In comparing the two groups, the decrease in body weight after the start of nutritional management was more obvious in group C than in group EN but this difference was not statistically significant ($P = 0.09$), since there was a wide interpatient variation. On the other hand, the change in BMI was significantly different between the two groups (Figure 2, $P < 0.001$).

Nutritional status was also estimated by laboratory parameters, including serum levels of total protein and albumin (Alb), which were determined as absolute changes by adjusting by the value at the first evaluation at the starting point of nutritional management, and a significantly slower decrease was noted in the EN group ($P < 0.001$) (Figure 3). These nutritional parameters remained higher in group EN than in group C. During the study period, no patient actually met with stopping rules mentioned above and consequently, the total number of days for NPO was not evaluated. The time to complete dietary recovery was compared between the two groups. While 38 days were required for the

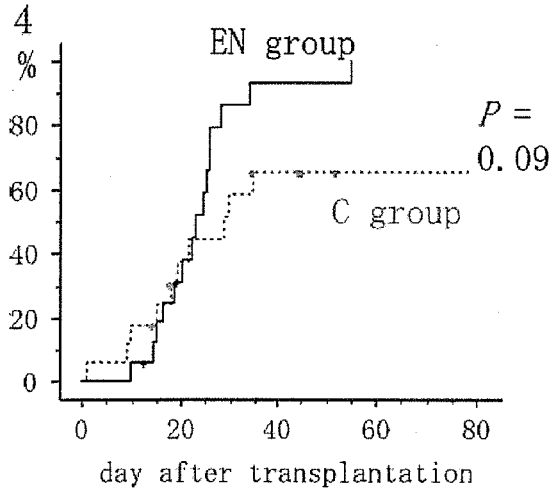

Fig. 4. Time to complete dietary recovery. The number of days required for return to a normal diet was 38 days in group C, while it was 31 days in group EN, with no statistically significant difference ($P = 0.09$).

recovery to a normal diet in group C, 31 days were required in group EN (Figure 4).

DISCUSSION

Since Weisdorf et al. reported that central venous parenteral nutritional support improved long-term survival in patients who underwent bone marrow transplantation (BMT) [8], intravenous TPN has been widely used in SCT. However, it has not yet been confirmed which procedure, enteral or parenteral nutrition, can provide more effective and safer nutritional support. In this study, we considered that the patients in the EN group may have preserved nutritional parameters better than the other group and ate sooner, although no differences were found in the time to complete dietary recovery. A clinical study group at Johns Hopkins University randomized BMT patients into two groups to receive different types of nutritional support, TPN or EN, and they did not observe any differences in nutritional parameters between the two groups [9]. In their study, patients who received TPN were allowed to eat anything they liked, while those with EN had few chances to receive TPN treatment. Moreover, those who had been receiving TPN were allowed to take oral intake and thus were not on strict NPO. Additionally, in our study, the two groups of patients were evaluated in different study periods, and there was a significant difference in the modality of the supportive measures. These points make a direct and strict comparison between the TPN and EN groups very difficult and unreliable. These biases, which are inherent to studies in this field, also existed in our study, which might explain why we failed to detect significant differences in clinical benefits.

We used to routinely advise patients to stop oral intake with the development of gut GVHD. Thereafter, they were encouraged to drink or eat gradually, since it has been suggested that inadequate nutritional support further deteriorates gut GVHD symptoms. To establish clearly defined subjective guidelines, we conducted this interventional cohort study. We found that both controlled and uncontrolled EN can be administered safely. Since the time to complete dietary recovery was almost comparable in the two groups, the results suggest that any EN program is acceptable and does not harm or degrade the QOL of patients suffering from GVHD. If this is confirmed, a restricted diet would not be necessary for those with moderately symptomatic gut GVHD. Nevertheless, the evaluation of nutritional parameters in this study suggested that controlled EN did a better job of maintaining body weight and serum nutritional status, compared to the results in the NPO group. The random administration of food intake may be inadequate compared to scheduled dieting, which attempts a gradual build-up of intestinal mucosa by the comprehensive supply of nutrients including glucose, protein, fat, fiber, etc. This may have a secondary advantage of keeping the mucosal barrier intact and preventing bacterial translocation through the GI tract.

Nevertheless, since the cause of diarrhea is multifactorial, it is inherently difficult to assess the effectiveness of and standardize nutritional intervention procedures. In the literature, four pathologies have been reported to be contraindications for EN since they cause undesirable bowel movement, i.e., presence of gastrointestinal bleeding, intestinal obstruction, severe pancreatitis, and intestinal perforation. The pathophysiology of diarrhea associated with gut GVHD includes osmotic and secretory diarrhea. Hypertonic EN is considered to further deteriorate symptoms of diarrhea. Hence, it is reasonable to suggest that dietary foods in EN adequately maintain an isotonic status as well as nutritional status to improve immunologic function. An intact GI system is vital for maintaining normal immune functions, and a novel concept of nutrition support, "immunonutrition," has been introduced, which focuses on the maintenance of the comprehensive biological protection system against external pathogens to maintain normal immune function [10]. Clinical benefits of immunonutrition, including improvement of nutritional parameters, decreased risk of infection, and shorter duration of hospitalization, have been reported in patients in the perioperation period and in those who required care in the ICU [11,12]. However, currently a precise evaluation

of the efficacy of each component of immunonutritional agents is difficult [13], and controversy still exists regarding the value of immunonutrition after SCT. This study did not evaluate this proposed immunonutrition, and to accomplish this in SCT practice, prospective monitoring of immune parameters would be required.

The serum level of albumin can be significantly affected by many variables including diarrhea associated with GVHD and, hence, would not be a very good marker for the evaluation of protein status in the HSCT population. However, in our experience, serum albumin decreased after SCT to suggest the possibility of the use in the estimation of patient's nutrition status at least for a short period of follow-up, when referring to the general description in the guideline by American Society for Parenteral and Enteral Nutrition, i.e., "low serum levels indicate which hospitalized patients are at increased risk of morbidity and mortality" [14].

In conclusion, the current study is hampered by preexisting biases including a small number of studied patients, a cohort analysis in different periods, and a lack of adequate measures for data evaluation. Nevertheless, it appears that patients supported by programmed EN experienced no exacerbation of gut GVHD symptoms, with a suggested benefit of enhanced maintenance of nutrition status. Further study is warranted to prospectively evaluate the value of various nutrients including arginine, o-3 fatty acid, and nucleic acid [13] and various clinical outcomes including the cost, complications, and QOL in an attempt to improve the nutritional and immune status of transplanted patients.

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bjh research paper

Pleiotropic role of histone deacetylases in the regulation of human adult erythropoiesis

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Summary

Histone acetylation and deacetylation play fundamental roles in transcriptional regulation. We investigated the role of histone deacetylases (HDACs) in human adult haematopoiesis, using the structurally distinct HDAC inhibitors FK228 (depsipeptide) and Trichostatin A. When $CD34^+$ cells were cultured with interleukin (IL)-3 or stem cell factor (SCF) + IL-3, FK228 (0·5 ng/ml) specifically enhanced the generation of immature erythroid cells with a $CD36^+$ glycophorin A $(GPA)^{low}$ phenotype. In semisolid cultures, FK228 promoted the formation of erythroid colonies by $CD34^+$ cells with IL-3 and SCF + IL-3. Furthermore, upon exposure to FK228, $CD34^+$ cell-derived $CD36^+GPA^-$ cells were induced to form erythroid colonies with IL-3 alone. Conversely, FK228 inhibited the generation of $CD36^+GPA^{high}$ relatively mature erythroid cells from $CD34^+$ cells in the presence of erythropoietin (EPO) and SCF + EPO. FK228 suppressed the EPO-mediated survival of $CD36^+GPA^{low/-}$ and $CD36^+GPA^{high}$ cells and induced their apoptosis. Similar effects were observed for trichostatin A in the generation of erythroid cells in IL-3- and EPO-containing cultures. These data suggest that HDACs negatively regulate the IL-3-mediated growth of early erythroid precursors by suppressing their responsiveness to IL-3, while playing an important role in EPO-mediated differentiation and survival of erythroid precursors. Our data revealed that HDACs have diverse functions in human adult erythropoiesis.

Keywords: histone deacetylase, erythropoiesis, interleukin-3, erythropoietin, inhibitor.

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Histone acetylation and deacetylation play key roles in the regulation of gene transcription through remodeling of chromatin structure (Gregory *et al*, 2001; Kramer *et al*, 2001; Eberharter & Becker, 2002; Felsenfeld & Groudine, 2003). Histone acetylation weakens histone-DNA contacts, thereby increasing the accessibility of transcription factors to their target DNA sequences, and inducing gene expression. In contrast, deacetylation of histones represses transcription of genes through the compaction of the chromatin structure. Acetylation of histones is mediated by a series of proteins with histone acetyltransferase activity (Gregory *et al*, 2001), while their acetylation is reversed by specific enzymes, histone deacetylases (HDACs) (Kramer *et al*, 2001). The acetylation status of histones is controlled by a balance between these competing enzymatic activities (Eberharter & Becker, 2002).

Haematopoiesis is a highly regulated and sequential process in which haematopoietic stem cells differentiate into lineage-restricted progeny, which in turn give rise to mature blood cells (Ogawa, 1993). During this process, a number of genes are orderly expressed in a lineage- and stage-specific manner. At the early stages of haematopoietic differentiation, various genes that are associated with self-renewal or multipotentiality, are expressed. Other genes, related to lineage-determination or differentiation are detected at the late stages of haematopoietic differentiation. Thus, not only the expression of proper genes but also the appropriate repression of unnecessary genes is crucial for normal haematopoiesis (Zhu & Emerson, 2002; Akashi *et al*, 2003; Kluger *et al*, 2004). Acetylation of histones has been implicated in the initiation of transcription of genes during

haematopoietic differentiation (Müller & Leutz, 2001). T cell (Huang & Muegge, 2001; Avni *et al*, 2002), granulocytic (Miyata *et al*, 2001), and erythroid (Harju *et al*, 2002; Bank, 2006) differentiation is accompanied by dynamic changes in histone acetylation of the regulatory regions of target genes. Moreover, transcription coactivators p300 and CREB-binding protein (CBP), which are involved in the proliferation and differentiation of various lineages of haematopoietic precursors, have been identified as histone acetyltransferases (Ogryzko *et al*, 1996; Blobel, 2000, 2002). On the other hand, a potential role of HDACs has been demonstrated in a subset of acute myeloid leukaemias where HDAC, aberrantly recruited by oncogenes, represses transcription of genes associated with myeloid differentiation, consequently causing a block in differentiation (Redner *et al*, 1999; Kramer *et al*, 2001; Redner & Liu, 2005). Therapeutic efficacy of HDAC inhibitors has been observed in acute myeloid leukaemia and other haematologic malignancies (Redner *et al*, 1999; Dokmanovic & Marks, 2005; Kuendgen *et al*, 2005; Monneret, 2005; Redner & Liu, 2005). Treatment of normal haematopoietic cells with HDAC inhibitors is reported to influence the proliferation of primitive haematopoietic progenitors (Bug *et al*, 2005; De Felice *et al*, 2005), the differentiation of cord blood-derived erythroid precursors (Fujieda *et al*, 2005), and the expression of γ-globin in adult erythroid cells (Perrine *et al*, 1993; Atweh *et al*, 1999; Cao, 2004). Nevertheless, the role of HDACs in normal haematopoiesis is still largely unknown.

The present study was conducted to examine the role of HDACs in human adult haematopoiesis, using the HDAC inhibitors FK228 (depsipeptide) and Trichostatin A (TSA) (Nakajima *et al*, 1998; Furumai *et al*, 2002; Dokmanovic & Marks, 2005; Kuendgen *et al*, 2005). FK228 is a naturally occurring polypeptide isolated from *Chromobacterium*, which is stable in culture medium because it is a pro-drug that is converted to the active form after incorporation into cells (Furumai *et al*, 2002). TSA, initially isolated as an antifungal agent from *Streptomyces hygroscopicus*, has been established as a specific HDAC inhibitor. These two HDAC inhibitors differ not only in structure, but also in specificity toward various HDACs (Furumai *et al*, 2002; Dokmanovic & Marks, 2005; Kuendgen *et al*, 2005). $CD34^+$ cells were obtained from granulocyte colony-stimulating factor-mobilised peripheral blood in adults. The present study found that FK228 promoted the generation of $CD36^+$ glycophorin A $(GPA)^{low}$ immature erythroid cells from $CD34^+$ cells in the presence of interleukin (IL)-3 and stem cell factor (SCF) + IL-3 by augmenting the growth of early erythroid precursors, while not significantly affecting the generation of myeloid cells. On the contrary, in the presence of erythropoietin (EPO) and SCF + EPO, FK228 suppressed the generation of $CD36^+GPA^{high}$ relatively mature erythroid cells from $CD34^+$ cells. FK228 induced apoptosis in $CD36^+GPA^{low/-}$ and $CD36^+GPA^{high}$ erythroid cells in the presence of EPO. Similarly, TSA stimulated the generation of erythroid cells from $CD34^+$ cells in IL-3-containing cultures but suppressed it in EPO-containing cultures. The results from the present study identified divergent roles for HDACs in the regulation of human adult erythropoiesis.

Materials and methods

Isolation of $CD34^+$ cells

Granulocyte colony-stimulating factor-mobilised peripheral blood was obtained from healthy adult donors after informed consent. $CD34^+$ cells were isolated from the peripheral blood, as described previously (Fujieda *et al*, 2005). Briefly, mononuclear cells were separated by centrifugation on Ficoll-Hypaque, and then washed and suspended in Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS) containing 0·1% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO, USA). $CD34^+$ cells were positively isolated from the mononuclear cells, using CD34 immunomagnetic beads (MACS; Miltenyi Biotec, Auburn, CA, USA), according to the manufacturer's instructions. The purity of $CD34^+$ cells routinely exceeded 95% after repeating the positive selection.

Recombinant growth factors and reagents

Recombinant human SCF, IL-3, and EPO were gifts from Kirin Brewery (Tokyo, Japan). Cytokines were used at the following concentrations: SCF, 50 ng/ml; IL-3, 10 ng/ml; EPO, 2 U/ml. HDAC inhibitor FK228 [(E)-(1S,4S,10S,21R)-7-[(Z)-ethylidene]-4,21-diisopropyl-2-oxa-12,13-dithia-5,8,20,23-tetraazabicyclo-[8,7,6]-tricos-16-ene-3,6,9,19,22-pentanone; FR901228, depsipeptide] was provided by Fujisawa Pharmaceutical Co. (Osaka, Japan; currently Astellas Pharma Inc., Tokyo, Japan). TSA was purchased from Wako Pure Chemical Industries (Osaka, Japan). FK228 and TSA were initially dissolved in 99·5% ethanol (Wako Pure Chemical Industries), stored at −80°C, and diluted with culture medium prior to use.

Flow cytometric analysis

Immunofluorescent staining was performed as reported previously (Fujieda *et al*, 2005). The following murine monoclonal antibodies were used: anti-CD14-phycoerythrin (PE), anti-GPA-PE (Becton Dickinson, San Jose, CA, USA); anti-CD15-fluorescein isothiocyanate (FITC), anti-CD36-FITC (BD Pharmingen, San Diego, CA, USA). Mouse IgM-FITC or IgG_{2b}-PE (BD Pharmingen) served as an isotype control. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA). Dead cells were excluded by propidium iodide (PI) (Sigma-Aldrich) staining. CellQuest software (Becton Dickinson) was used for data acquisition and analysis.

Cell culture

Suspension cultures were mainly performed in 96-well tissue culture plates (Nunc, Roskilde, Denmark) containing serum-free medium (StemSpan; Stem Cell Technologies, Vancouver, BC, Canada), 50 U/ml penicillin, 50 μg/ml streptomycin, and designated cytokines with or without FK228 or TSA, as described previously (Fujieda *et al*, 2005). On day 3–4, half of the culture medium was replaced with fresh medium containing the same cytokines and reagents. Viable cell numbers were counted by trypan blue dye exclusion.

For serum-free semisolid cultures, cells were plated in 35-mm culture dishes (Nunc) in a 1 ml mixture containing Iscove's modified Dulbecco medium (IMDM; Gibco-BRL, Gaithersburg, MD, USA), 20% BIT9500 (Stem Cell Technologies), 1·3% methylcellulose (Shinetsu Kagaku, Tokyo, Japan), 2 mmol/l L-glutamine (Gibco-BRL), 50 U/ml penicillin, 50 μg/ml streptomycin, 5×10^{-5} mol/l 2-mercaptoethanol (2-ME; Sigma-Aldrich), and designated cytokines with or without FK228. After 10–14 d in culture, erythroid clusters containing fewer than 50 cells and erythroid colonies consisting of 50 or more cells were scored. In some experiments, erythroid colonies were further classified according to their size. To determine the type of colonies, different colonies were picked and analysed for their phenotype. All images were captured using a Nikon Eclipse TE2000-U inverted microscope (Nikon Sankei, Tokyo, Japan), a Nikon digital DXM 1200 camera, and Nikon ACT-1 imaging software version 2·00. Images were resized, cropped, and assembled, using Adobe Photoshop Elements 2·0 (Adobe Systems, San Jose, CA, USA).

Cell sorting

$CD34^+$ cells (2×10^4/ml) were cultured in 24-well culture plates (Nunc) containing serum-free medium supplemented with SCF + IL-3. On day 3, half of the culture medium was exchanged with fresh medium containing the same cytokines. On day 7 of culture, resultant cells were stained with anti-CD36-FITC and anti-GPA-PE, and then $CD36^+GPA^-$ or $CD36^+GPA^{low/-}$ cells were sorted on a FACSVantage (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA). In some experiments, $CD36^+$ cells were isolated immunomagnetically, using anti-CD36-FITC and anti-FITC microbeads (Miltenyi Biotech).

Assessment of apoptosis

The annexin V/PI assay was performed using the Annexin V-FITC Apoptosis Detection Kit (BD PharMingen), as described previously (Ohishi *et al*, 2000). Briefly, cells were washed with PBS and incubated in binding buffer containing annexin V-FITC and PI for 15 min at room temperature according to the manufacturer's instructions. Apoptotic (annexin V^+PI^-) cells were distinguished from necrotic (annexin V^+PI^+) cells with a FACSCalibur flow cytometer. Apoptosis was also assessed by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end-labelling (TUNEL) method, using the *In situ* Cell Death Detection kit, Fluorescein (Roche Applied Science, Indianapolis, IN, USA). Briefly, cells were fixed in 2% PBS-buffered paraformaldehyde (Wako Pure Chemical Industries) and permeabilised with 0·1% TritonX-100 (Wako Pure Chemical Industries) dissolved in 0·1% sodium citrate. After washing, cells were resuspended in TUNEL reaction mixture containing FITC-dUTP and TdT, following the manufacturer's instructions. Fluorescein labels incorporated into DNA strand breaks were analysed by flow cytometry.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from cells using an RNeasy mini Kit (QIAGEN, Valencia, CA, USA) and cDNA was synthesised using the oligo-dT primer and ThermoScript transcription-polymerase chain reaction (RT-PCR) System (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The following primers were used: human EPO receptor forward, 5′-AGCTTCGTGCCCCTAGAGTT and reverse, 5′-GATCTCCACCCTCTGTACGC; human *GATA1* forward, 5′-AGGCCACTACCTATGCAACG-3′ and reverse, 5′-CCTGCCCGTTTACTGACAAT-3′; and human eukaryotic translation elongation factor 1 β 2 (*EEF1B2*) forward, 5′-CATGCCCTACGTTGGTATAATCAC-3′ and reverse, 5′-ACATCGGCAGGACCATATTTG-3′. Quantitative PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) and analysed with SDS software1·9 and Dissociation Curves1·0 (Applied Biosystems). The reaction conditions were as follows: an initial denaturation step at 95°C for 10 min was followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s. Transcript quantification was performed in duplicate for every sample. Gene expression was normalised using the endogenous *EEF1B2* gene expression as an internal standard. Relative gene expression was calculated as a fold induction compared with untreated $CD34^+$ cells.

Data analysis

Statistical comparisons were made using the Student *t*-test. Differences were considered significant at *P*-values <0·05.

Results

FK228 enhances the generation of $CD36^+GPA^{low}$ cells from $CD34^+$ cells in the presence of IL-3 and SCF + IL-3

It has been shown that IL-3 and SCF play crucial roles in proliferation and differentiation of early erythroid and myeloid precursors (Sonoda *et al*, 1988; Dai *et al*, 1991; Papayanno-

poulou *et al*, 1993; Broudy, 1997). We first examined the effect of increasing concentrations of FK228 on the generation of $CD36^+$ erythroid, $CD14^+$ monocytic, and $CD15^+$ granulocytic cells from $CD34^+$ cells in serum-free suspension cultures in the presence of SCF + IL-3. After 7 d in culture, FK228 increased the number of $CD36^+$ cells in a dose-dependent manner, with a maximum effect seen at 0·5 ng/ml (Fig 1A). Few or no $CD14^+$ cells were detected in all culture conditions. Although a small number of $CD15^+$ cells developed, FK228 did not significantly affect the generation of $CD15^+$ cells at concentrations of up to 0·5 ng/ml (Fig 1A). FK228 moderately suppressed generation of $CD15^+$ cells at 0·6 ng/ml (data not shown). No effects were observed with an equivalent volume of solvent vehicle ethanol (data not shown). We then tested the effect of 0·5 ng/ml of

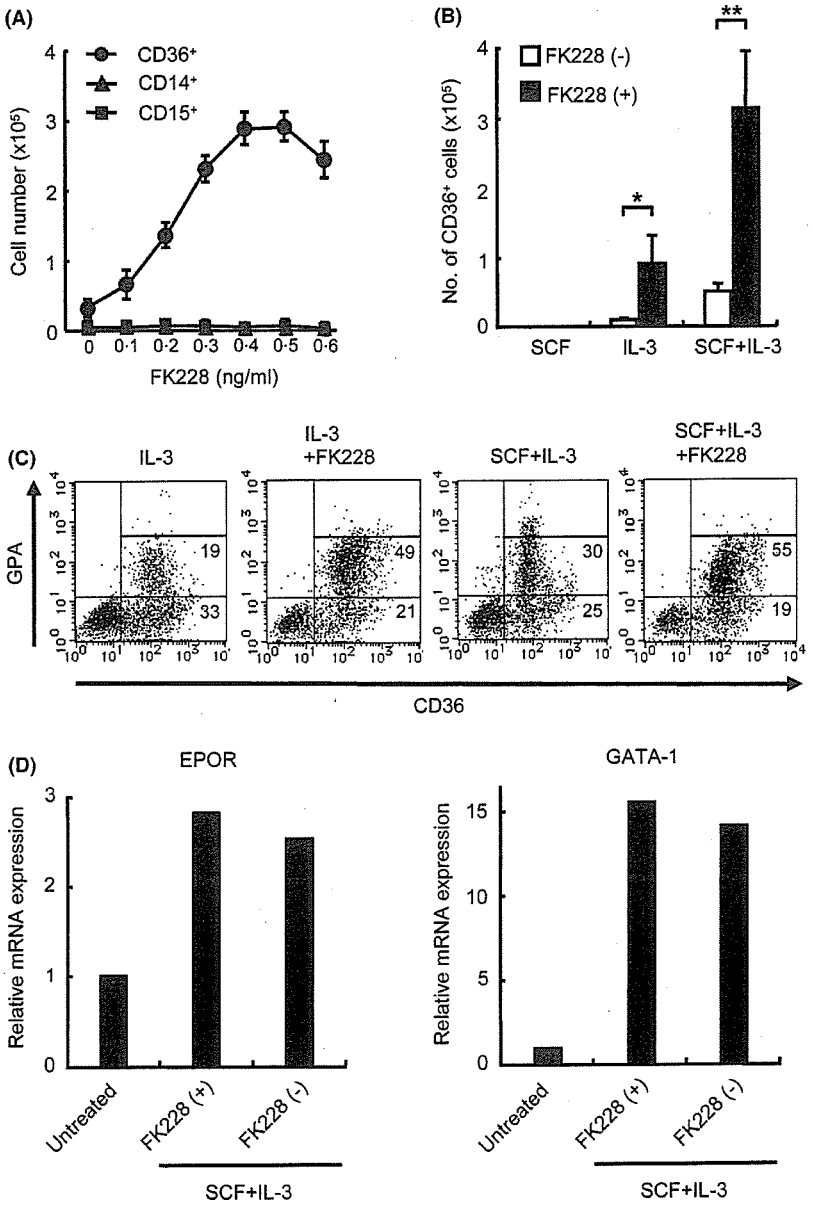

Fig 1. Stimulatory effect of FK228 on the generation of erythroid cells from $CD34^+$ cells with IL-3 and SCF + IL-3. (A) $CD34^+$ cells (3000 cells/well) were cultured for 7 d with SCF + IL-3 in the presence of various concentrations of FK228. The number of $CD36^+$, $CD14^+$, and $CD15^+$ cells was counted at day 7. Results are shown as mean ± standard deviation (SD) of triplicate cultures. (B) $CD34^+$ cells (3000 cells/well) were cultured for 7 d with SCF, IL-3, or SCF + IL-3 in the presence or absence of FK228 (0·5 ng/ml). The number of $CD36^+$ cells was estimated and shown as mean ± SD of triplicate cultures. $^*P < 0·05$ and $^{**}P < 0·01$ compared with control cultures. (C) The expression of CD36 and GPA on cells cultured with IL-3 or SCF + IL-3 in the presence or absence of FK228 (0·5 ng/ml) is shown. Each experiment was repeated at least three times with similar results. (D) Analysis of expression levels of *EPOR* (left column) and *GATA-1* (right column) in $CD34^+$ cells and in cells incubated for 7 d with SCF + IL-3 in the presence or absence of FK228. Data for each transcript are expressed relative to the corresponding values of uncultured $CD34^+$ cells.

FK228 on the generation of $CD36^+$ erythroid cells from $CD34^+$ cells in the presence of SCF, IL-3, and SCF + IL-3. After 7 d, no $CD36^+$ cells were observed in the cultures containing SCF or SCF + FK228. While only a small number of $CD36^+$ cells developed in the cultures with IL-3 alone, addition of FK228 to the cultures remarkably increased the number of $CD36^+$ cells. A combination of SCF and IL-3 supported the generation of higher numbers of $CD36^+$ cells compared with IL-3 alone. FK228 also increased the number of $CD36^+$ cells in SCF + IL-3 (Fig 1B). It has been shown that erythroid precursors acquire the expression of GPA and its expression levels increase along with their differentiation (Loken *et al*, 1987). To evaluate the effect of FK228 on erythroid differentiation, $CD34^+$ cells were cultured with IL-3 or SCF + IL-3 in the presence or absence of FK228, and resultant cells were analysed for the expression of CD36 and GPA (Fig 1C). Cultures containing IL-3 or SCF + IL-3 supported the differentiation of $CD34^+$ cells into $CD36^+$ cells expressing no or low levels of GPA ($CD36^+GPA^{low/-}$ cells), a phenotype corresponding to immature erythroid cells (Loken *et al*, 1987). FK228 increased the proportion of $CD36^+GPA^{low}$ cells in both culture conditions. However, FK228 did not promote their further differentiation into $CD36^+GPA^{high}$ more mature erythroid cells. Thus, in the presence of IL-3 and SCF + IL-3, FK228 augmented the generation of $CD36^+GPA^{low}$ immature erythroid cells from $CD34^+$ cells at a concentration that does not influence the generation of myeloid cells. To verify the erythroid nature of cultured cells, we examined the expression levels of EPO receptor (R) and its downstream transcription factor GATA-1 (Fisher, 2003) by real-time RT-PCR. Since only a few cells were obtained from cultures with IL-3 alone, $CD34^+$ cells and those cultured for 7 d with SCF + IL-3 in the presence or absence of FK228 were analysed. As shown in Fig 1D, expression levels of *EPOR* and *GATA1* were increased in both culture conditions, compared with uncultured $CD34^+$ cells. In agreement with these findings, $CD36^+$ cells generated from $CD34^+$ cells by incubation for 7 d with IL-3 or SCF + IL-3 in the presence or absence of FK228 proliferated and differentiated into $CD36^+GPA^{high}$ more mature erythroid cells in response to EPO alone (data not shown).

FK228 promotes the formation of erythroid colonies by $CD34^+$ and $CD36^+GPA^-$ cells in the presence of IL-3 and SCF + IL-3

To elucidate the action of FK228 on early erythroid precursors, $CD34^+$ cells were incubated in methylcellulose cultures with SCF, IL-3, or SCF + IL-3 in the presence or absence of FK228, and analysed for erythroid colony formation. The erythroid nature of colonies was verified by staining cells in colonies with antibodies against CD36 and GPA. After 10–14 d of culture, no colonies were detected in the cultures that contained SCF or SCF + FK228. IL-3 alone mainly supported the formation of erythroid clusters comprising <50 cells. However, addition of FK228 to the cultures with IL-3 led to a remarkable increase in the number of erythroid colonies (Fig 2A–B). These erythroid colonies consisted mainly of $CD36^+GPA^{low}$ cells (data not shown). A combination of SCF and IL-3 supported the formation of higher numbers of erythroid colonies. FK228 also stimulated erythroid colony formation by $CD34^+$ cells in SCF + IL-3 (Fig 2A–B). Moreover, we observed that FK228 considerably increased the size of individual erythroid colonies in these culture conditions (Fig 2B–C). These data suggest that FK228 promotes the growth of early erythroid precursors in the presence of IL-3 and SCF + IL-3 presumably by enhancing their responsiveness to IL-3.

To further assess the effect of FK228 on IL-3-mediated growth of more mature erythroid precursors, $CD34^+$ cells were incubated for 7 d with SCF + IL-3, and $CD36^+GPA^-$ cells were isolated (Fig 3A). When the cells were incubated in semisolid cultures containing IL-3 alone, neither clusters nor colonies were observed. However, a significant number of erythroid colonies and clusters were formed from the $CD36^+GPA^-$ cells when FK228 was present in the cultures (Fig 3B). This indicates that early erythroid precursors lose their responsiveness to IL-3 concomitantly with their differentiation, but it can be recovered and enhanced by HDAC inhibition.

FK228 inhibits the generation of $CD36^+GPA^{high}$ erythroid cells from $CD34^+$ and $CD36^+GPA^{low/-}$ cells in the presence of EPO and SCF + EPO

EPO or its combination with SCF has a central role in proliferation, differentiation, and survival of erythroid precursors (Koury & Bondurant, 1990; Wu *et al*, 1995; Fisher, 2003; Testa, 2004). In the next series of experiments, we investigated the effect of FK228 on the generation of erythroid cells in the presence of EPO and SCF + EPO. $CD34^+$ cells were cultured with SCF, EPO, or SCF + EPO in the presence of varying concentrations of FK228, and the numbers of $CD36^+GPA^{high}$, $CD36^+GPA^{low}$, and $CD36^+GPA^-$ cells were assessed. After 7 d of incubation, no erythroid cells were observed with SCF alone in the presence or absence of FK228 (data not shown). Cultures with EPO alone contained a small number of erythroid cells, the majority of which were $CD36^+GPA^{high}$ cells. FK228 decreased the number of $CD36^+GPA^{high}$ cells in a dose-dependent manner, and no erythroid cells were detected when FK228 was present at a concentration of 0·3 ng/ml and more (Fig 4A). A combination of SCF and EPO supported the generation of higher numbers of $CD36^+$ cells expressing high, low, or no levels of GPA (Fig 4B). By morphological examination, $CD36^+GPA^-$ cells consisted mainly of immature erythroid cells, with fine nuclear chromatin and basophilic cytoplasm. $CD36^+GPA^{high}$ cells contained cells that were smaller in size and possessed more condensed chromatin, a feature of more mature erythroid cells. The characteristics of these $CD36^+GPA^{high}$ cells were similar to $CD36^+GPA^{high}$ cells generated in the cultures with EPO alone (data not shown). $CD36^+GPA^{low}$ cells were intermediate in size and chromatin structure (Fig 4C). Although FK228 dose-dependently reduced the number of $CD36^+GPA^{high}$ relatively mature erythroid cells,

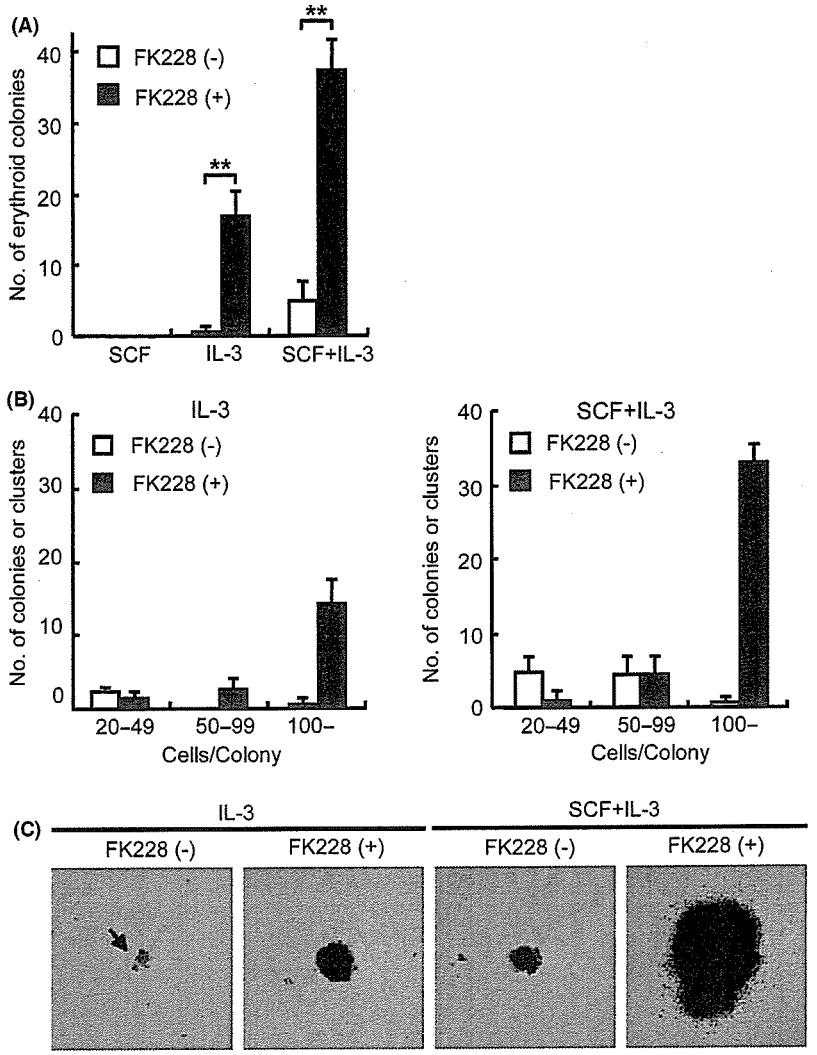

Fig 2. Effect of FK228 on erythroid colony formation by $CD34^+$ cells with IL-3 and SCF + IL-3. (A) $CD34^+$ cells (500 cells/well) were cultured in methylcellulose medium supplemented with SCF, IL-3, or SCF + IL-3 in the presence or absence of FK228 (0·5 ng/ml). The number of erythroid colonies was scored after 10–14 d of incubation. Data represent mean ± SD of quadruplicate cultures. $^{**}P < 0{\cdot}01$ compared with control cultures. (B) After incubation of $CD34^+$ cells in semisolid cultures with IL-3 or SCF + IL-3 in the presence or absence of FK228, the number of erythroid clusters, consisting of 20–49 cells, and erythroid colonies, comprising 50–99 cells or 100 and more cells, were scored and shown as mean ± SD of quadruplicate cultures. (C) Cultured cells were examined under an inverted microscope (original magnification, ×100). The arrow points to an erythroid cluster formed in the cultures containing IL-3 alone. Representative results are shown from three independent experiments.

the generation of $CD36^+GPA^{low}$ and $CD36^+GPA^-$ cells was not significantly influenced at a concentration of 0·3 ng/ml and below. The number of $CD36^+GPA^{low}$ and $CD36^+GPA^-$ cells decreased as the concentration of FK228 was further increased (Fig 4B). No effects were seen with the solvent vehicle ethanol at the same concentrations as FK228 (data not shown).

We further examined the effect of FK228 on the generation of $CD36^+GPA^{high}$ cells from $CD34^+$ cell-derived $CD36^+GPA^{low/-}$ cells in the presence of EPO and SCF + EPO. $CD36^+GPA^{low/-}$ cells were generated from $CD34^+$ cells cultured for 7 d with SCF + IL-3, and isolated by cell sorting (Fig 4C, left column). The cells were cultured for 7 d with SCF, EPO, or SCF + EPO in the presence or absence of FK228 (0·5 ng/ml). A considerable number of $CD36^+GPA^{high}$ cells developed with EPO alone, but no erythroid cells were observed in the cultures containing EPO + FK228. A combination of SCF and EPO produced higher numbers of $CD36^+GPA^{high}$ and $CD36^+GPA^{low}$ cells. FK228 almost completely inhibited the generation of $CD36^+GPA^{high}$ cells and decreased the number of $CD36^+GPA^{low}$ cells in SCF + EPO (Fig 4C, right column).

Thus, FK228 strongly inhibited the generation of $CD36^+GPA^{high}$ relatively mature erythroid cells from $CD34^+$ and $CD36^+GPA^{low/-}$ cells in the presence of EPO and SCF + EPO, while its inhibitory effect on the generation of immature erythroid cells was comparatively lower in the presence of SCF + EPO.

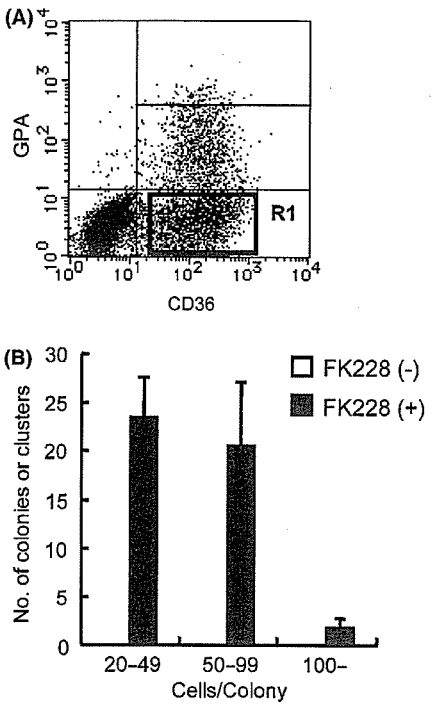

Fig 3. Effect of FK228 on erythroid colony formation by $CD34^+$ cell-derived $CD36^+GPA^-$ cells in the presence of IL-3. (A) After 7 d of culture of $CD34^+$ cells (2×10^4 cells/ml) in the presence of SCF + IL-3, $CD36^+GPA^-$ cells (R1) were isolated. (B) The cells (1000 cells/well) were incubated in semisolid cultures supplemented with IL-3 in the presence or absence of FK228. The number of erythroid clusters and colonies was scored after 10–14 d in culture. Data represent mean ± SD of quadruplicate cultures and are representative of three separate experiments.

Suppressive effect of FK228 on the EPO-mediated survival of $CD36^+GPA^{low/-}$ and $CD36^+GPA^{high}$ erythroid cells

As observed in Fig 4C, there were no erythroid cells left in the cultures following incubation of $CD36^+GPA^{low/-}$ erythroid cells with EPO in the presence of FK228. To examine the effect of FK228 on their EPO-mediated survival more precisely, the $CD34^+$ cell-derived $CD36^+GPA^{low/-}$ cells were incubated with EPO in the presence or absence of FK228, and viable cell numbers were serially counted. Without FK228, the number of viable cells started to increase after 2 d. Conversely, a significant loss of cell viability was evident at day 2 in the FK228-containing cultures (Fig 5A). No effects were again seen with a corresponding volume of vehicle solution (data not shown). To address the mechanism for the reduction of viable cells, $CD36^+GPA^{low/-}$ cells were cultured with EPO in the presence or absence of FK228, and analysed for annexin V binding and PI staining. Only a small portion of cells was positive for annexin-V in both cultures at 24 h (data not shown). After a 36-h-incubation, the proportion of annexin V^+PI^- apoptotic cells rapidly increased in the FK228-containing cultures, and at 48 h, the majority of cells became either annexin-V^+ PI^- apoptotic or annexin-V^+ PI^+ necrotic (Fig 5B). These data suggest that FK228 induces apoptotic cell death in $CD36^+GPA^{low/-}$ erythroid cells in the presence of EPO. To confirm apoptosis, the $CD34^+$ cell-derived $CD36^+GPA^{low/-}$ cells were cultured for 36 h with EPO in the presence or absence of FK228, and were processed for the TUNEL assay, which detects double-stranded DNA breaks. Results from the TUNEL assay were consistent with those from the annexin V/PI assay; a substantially higher percentage of cells was positive for TUNEL-staining in the FK228-containing cultures, compared to control cultures (Fig 5C).

We then investigated the effect of FK228 on the survival of $CD36^+GPA^{high}$ more mature erythroid cells in various culture conditions. To generate $CD36^+GPA^{high}$ cells, the $CD34^+$ cell-derived $CD36^+GPA^{low/-}$ cells were incubated for 7 more days with SCF + EPO. Resultant cells were found to be mainly $CD36^+GPA^{high}$ cells (Fig 6A). These cells were cultured with SCF, EPO, or SCF + EPO in the presence or absence of FK228, and the number of viable cells was counted. At day 2, few viable cells were detected in the cultures with SCF in the presence or absence of FK228. While a significant number of $CD36^+GPA^{high}$ cells were alive in the cultures containing EPO or SCF + EPO, FK228 almost completely suppressed their survival in both cultures (Fig 6B). To evaluate whether FK228-exposed $CD36^+GPA^{high}$ cells underwent apoptotic cell death, cultured cells were stained with annexin V and PI, and the percentage of annexin V^+PI^- apoptotic cells was assessed. After 30 h of culture, a significant portion of cells cultured with SCF in the presence or absence of FK228 was annexin V^+PI^-. Although the percentage of annexin V^+PI^- cells was decreased in the presence of EPO and SCF + EPO, FK228 increased the proportion of annexin V^+PI^- cells to a similar extent in these cultures (Fig 6C).

These results indicate that FK228 inhibits EPO-mediated survival of $CD36^+GPA^{low/-}$ and $CD36^+GPA^{high}$ cells and induces their apoptotic cell death. Most $CD36^+GPA^{high}$ cells are suggested to undergo apoptosis, even in the presence of SCF + EPO, presumably because they had lost the responsiveness to SCF and their survival was fully dependent on EPO.

Effect of TSA on the generation of erythroid cells from $CD34^+$ cells in IL-3- and EPO-containing cultures

We further tested whether the structurally different HDAC inhibitor TSA has a similar effect on the generation of erythroid cells. When $CD34^+$ cells were cultured for 7 d with SCF, IL-3, or SCF + IL-3 in the presence or absence of 5 ng/ml of TSA, the generation of $CD36^+$ erythroid cells was enhanced by TSA in the cultures containing IL-3 or SCF + IL-3 (Fig 7A). TSA mainly increased the population of $CD36^+GPA^{low}$ cells in these cultures (data not shown). We then incubated $CD34^+$ cells for 7 d with SCF, EPO, or SCF + EPO with or without the same concentration of TSA. TSA inhibited the generation of $CD36^+$ erythroid cells from $CD34^+$ cells in the presence of EPO

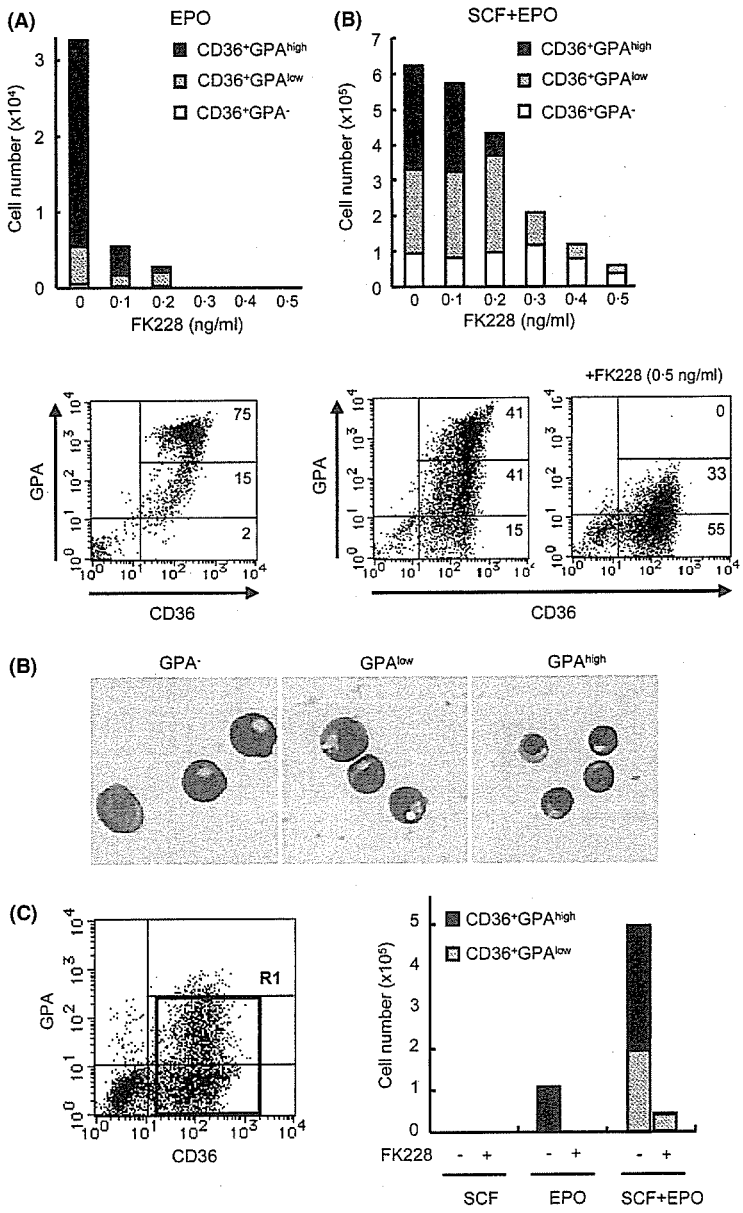

Fig 4. Inhibitory effect of FK228 on the generation of erythroid cells in the presence of EPO and SCF + EPO. (A) $CD34^+$ cells (3000 cells/well) were cultured for 7 d with EPO in the presence of various concentrations of FK228. The numbers of $CD36^+GPA^{high}$, $CD36^+GPA^{low}$, and $CD36^+GPA^-$ cells are shown as mean of triplicate cultures (upper panel). The expression of CD36 and GPA on cells cultured with EPO is shown (lower panel). (B) The effect of various concentrations of FK228 on the generation of erythroid cells from $CD34^+$ cells (3000 cells/well) in the presence of SCF + EPO was similarly analyzed (upper panel). The phenotype of cells cultured with SCF + EPO with or without FK228 (0·5 ng/ml) is shown (lower panel). (C) Morphological examination of $CD36^+GPA^{high}$, $CD36^+GPA^{low}$, and $CD36^+GPA^-$ cells in the cultures containing SCF + EPO (original magnification, ×400). (D) $CD34^+$ cells (2×10^4 cells/ml) were incubated with SCF + IL-3, and $CD36^+GPA^{low/-}$ cells (R1) were sorted at day 7 (left). The cells (3000 cells/well) were cultured with SCF, EPO, or SCF + EPO in the presence or absence of FK228 (0·5 ng/ml). On day 7 of culture, the number of $CD36^+GPA^{high}$ and $CD36^+GPA^{low}$ cells was counted (right). All experiments were repeated at least three times with similar results.

and SCF + EPO (Fig 7B). The number of $CD34^+GPA^{high}$ cells was markedly suppressed by TSA in both culture conditions (data not shown). Thus, as observed for FK228, TSA exerted stimulatory and inhibitory effects on the generation of erythroid cells in IL-3- and EPO-containing cultures, respectively.

Discussion

By using the structurally distinct HDAC inhibitors FK228 and TSA, the role of HDACs in proliferation, differentiation, and survival of human adult erythroid precursors was addressed. We found that FK228 increased the generation of

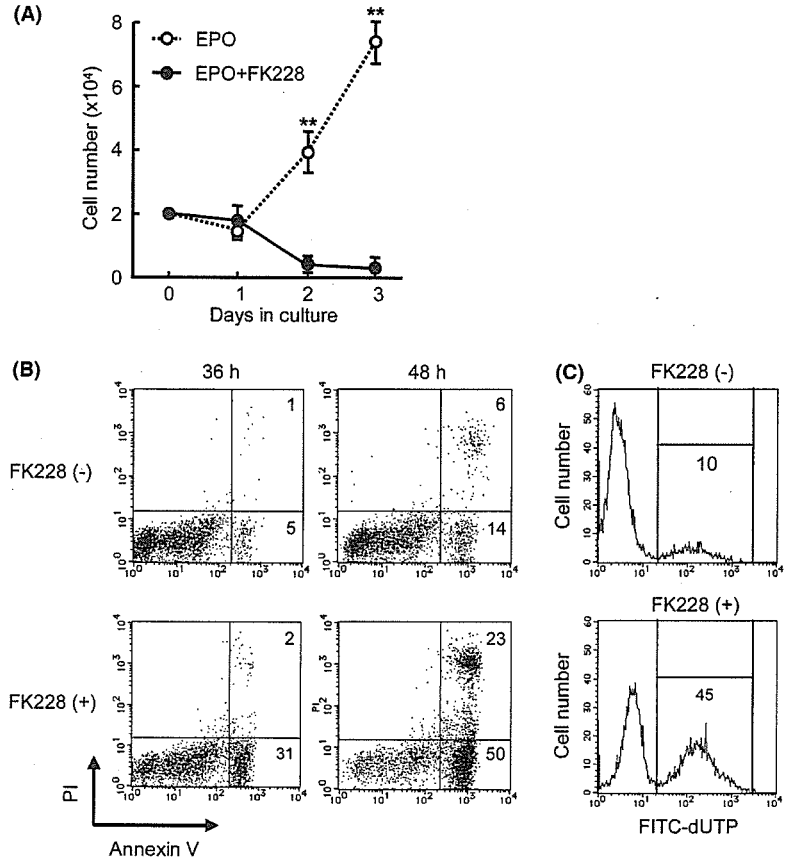

Fig 5. Effect of FK228 on the survival of $CD36^+GPA^{low/-}$ cells in the presence of EPO. (A) $CD34^+$ cell-derived $CD36^+GPA^{low/-}$ cells (2×10^4 cells/well) were incubated with EPO in the presence or absence of FK228 (0·5 ng/ml), and viable cell numbers were serially counted. $^{**}P < 0{\cdot}01$ compared with control cultures. (B) At 36 and 48 h, resultant cells were stained with annexin V-FITC and PI. (C) At 36 h, cultured cells were analysed by TUNEL assay.

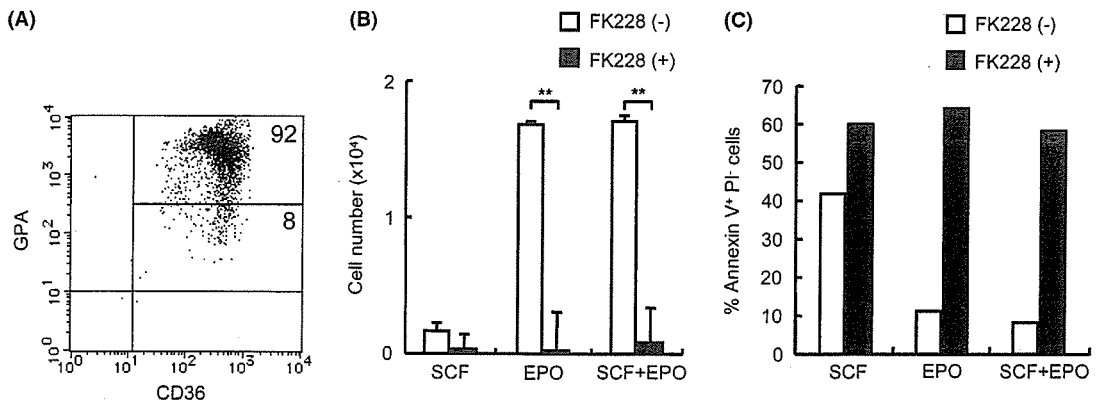

Fig 6. Effect of FK228 on the survival of $CD36^+GPA^{high}$ cells in the presence of EPO and SCF + EPO. (A) $CD34^+$ cell-derived $CD36^+GPA^{low/-}$ cells (2×10^4 cells/ml) were cultured for 7 d with SCF + EPO. The resultant cells were analysed for expression of CD36 and GPA. (B) The $CD36^+GPA^{high}$ cells (2×10^4 cells/well) were incubated for 2 d with SCF, EPO, or SCF + EPO in the presence or absence of FK228 (0·5 ng/ml), and viable cell numbers were counted at day 2. Results are shown as mean ± SD of triplicate cultures. $^{**}P < 0{\cdot}01$ compared with control cultures. (C) After a 30-h incubation, apoptosis was assessed by annexin V and PI staining. Data represent mean percentage of annexin V^+PI^- cells in triplicate cultures.

$CD36^+GPA^{low}$ immature erythroid cells from $CD34^+$ cells in the presence of IL-3 and SCF + IL-3 by promoting the growth of early erythroid precursors. In contrast, FK228 inhibited the generation of $CD36^+GPA^{high}$ relatively mature erythroid cells from $CD34^+$ cells in the presence of EPO and SCF + EPO. FK228 suppressed the EPO-mediated survival of

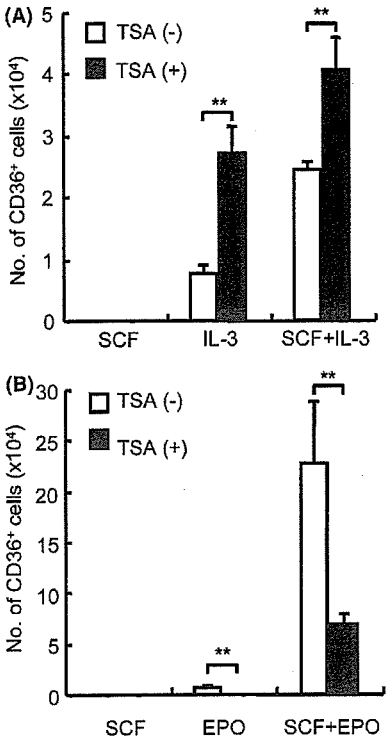

Fig 7. Effect of TSA on the generation of erythroid cells from $CD34^+$ cells. (A) $CD34^+$ cells (3000 cells/well) were incubated with SCF, IL-3, or SCF + IL-3 in the presence or absence of TSA (5 ng/ml). On day 7, the number of $CD36^+$ erythroid cells was counted and shown as mean ± SD of triplicate cultures. (B) Following the incubation of $CD34^+$ cells (3000 cells/well) with SCF, EPO, or SCF + EPO in the presence or absence of TSA (5 ng/ml), the number of $CD36^+$ cells was analysed. Mean ± SD of triplicate cultures is shown. $^*P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$ compared with control cultures. Data are representative of three independent experiments.

$CD36^+GPA^{low/-}$ and $CD36^+GPA^{high}$ erythroid cells and induced their apoptotic cell death. Similar effects were observed for TSA in the generation of erythroid cells from $CD34^+$ cells in IL-3- and EPO-containing cultures. These data indicate that HDACs negatively control the IL-3-mediated proliferation and differentiation of early erythroid precursors while playing a critical role in the EPO-mediated differentiation and survival of erythroid precursors. Our data demonstrate divergent functions of HDACs in human adult erythropoiesis.

In serum-free cultures containing IL-3 or SCF + IL-3, $CD34^+$ cells differentiated into $CD36^+GPA^{low/-}$ immature erythroid cells. This finding is in agreement with previous studies showing that IL-3 alone, or in combination with SCF, supports the differentiation of early erythroid precursors into β-globin$^+$ immature erythroid cells (Papayannopoulou *et al*, 1993). FK228 increased the generation of $CD36^+GPA^{low}$ immature erythroid cells from $CD34^+$ cells with IL-3 and SCF + IL-3 but did not evoke a further differentiation into $CD36^+GPA^{high}$ more mature erythroid cells. In semisolid cultures, FK228 promoted the growth of early erythroid precursors in response to IL-3 and SCF + IL-3, while no stimulatory effects were observed in the presence of SCF. These results indicate that HDAC inhibition by FK228 enhances the IL-3 responsiveness of early erythroid precursors, thereby stimulating their proliferation and differentiation into $CD36^+GPA^{low}$ immature erythroid cells in the presence of IL-3 and SCF+IL-3. Neither colonies nor clusters developed from $CD34^+$ cell-derived $CD36^+GPA^-$ cells in the presence of IL-3 alone. However, upon exposure to FK228, the $CD36^+GPA^-$ cells were induced to form a significant number of erythroid colonies in the presence of IL-3. This further suggests that, although IL-3 responsiveness of early erythroid precursors decreases during differentiation (Dai *et al*, 1991), it can be restored and enhanced by inhibition of HDAC activity during the early stages of erythroid differentiation. In general, sequence-specific DNA-binding repressors and HDAC form a repressor complex that suppresses the transcription of target genes (Pazin & Kadonaga, 1997; Kramer *et al*, 2001; Redner & Liu, 2005). It may be possible to speculate that such a repressor complex negatively controls the transcription of genes associated with IL-3-mediated growth of early erythroid precursors. In the presence of IL-3 and SCF + IL-3, FK228 and TSA had a similar stimulatory effect on the generation of erythroid cells, while the effect of FK228 appeared to be stronger than that of TSA. It has been shown that FK228 exclusively inhibits class I HDACs, while TSA suppresses both class I and class II HDACs (Furumai *et al*, 2002; Dokmanovic & Marks, 2005; Monneret, 2005). Our results suggest that class I HDACs are involved in the regulation of IL-3-mediated growth of early erythroid precursors.

On the other hand, FK228 inhibited the generation of $CD36^+GPA^{high}$ relatively mature erythroid cells from $CD34^+$ cells with EPO and SCF + EPO, and induced apoptosis in a majority of $CD36^+GPA^{low/-}$ and $CD36^+GPA^{high}$ erythroid cells in the presence of EPO. Nevertheless, when $CD36^+GPA^-$ erythroid cells were incubated with IL-3-containing cultures, FK228 exerted a substantial stimulatory effect. These data indicate that FK228 displays inhibitory effects on erythroid precursors specifically in the EPO-containing cultures. One of the principal functions of EPO is to prevent apoptosis in erythroid precursors (Koury & Bondurant, 1990; Wu *et al*, 1995; Fisher, 2003; Testa, 2004). Ablation of EPO or EPO receptors in mice causes embryonic death from severe anaemia due to apoptosis of late erythroid precursors and their progeny (Wu *et al*, 1995). We postulate that HDAC inhibition may block a signalling pathway from EPO, consequently inhibiting the generation of erythroid cells from $CD34^+$ cells in the presence of EPO and SCF + EPO and inducing apoptosis in erythroid cells in the presence of EPO. FK228 was less inhibitory regarding the generation of $CD36^+GPA^{low/-}$ immature erythroid cells compared with $CD36^+GPA^{high}$ more mature erythroid cells, probably because SCF-induced c-kit signalling prevented the immature erythroid cells from apoptotic cell death (Zeuner *et al*, 2003; Testa, 2004). Interestingly, in our previous study (Fujieda *et al*, 2005), an identical dose of FK228 (0·5 ng/ml) did not affect the proliferation of cord

blood-derived $CD36^+$ erythroid precursors in the presence of EPO, while their differentiation was reversibly suppressed. This further implies that the role of HDACs in the regulation of EPO-mediated erythropoiesis might be different in adults and neonates.

The present study shows that HDAC inhibition by FK228 exerts a strong influence on proliferation, differentiation, and survival of erythroid precursors at a concentration where the generation of granulocytic cells is not significantly influenced. In clinical trials of FK228, however, treatment with FK228 elicited a rapid neutropenia and thrombocytopenia, but only mild anaemia was observed (Sandor *et al*, 2002; Byrd *et al*, 2005). These findings lead us to consider that the effect of FK228 on erythropoiesis could be counterbalanced by its stimulatory and inhibitory effect on erythroid precursor cells *in vivo*. Our data suggest that, if a particular HDAC enzyme of class I HDACs, which are common targets of FK228 and TSA, is specifically associated with either IL-3- or EPO-mediated erythropoiesis, its selective inhibition may cause a more significant effect on erythropoiesis. It is also possible that HDAC inhibition more profoundly affects abnormal erythropoiesis in several haematological disorders where erythroid precursors are hypersensitive to haematopoietic growth factors (Tefferi & Spivak, 2005). In fact, it has been recently reported that hydroxamate derivatives of butyrate and propionate, which are known to be potent HDAC inhibitors, not only induce fetal globin production, but also stimulate erythropoiesis *in vivo* in β thalassaemia mice (Cao *et al*, 2005). The current study points to a pivotal role of HDACs in human adult erythropoiesis and a therapeutic potential of HDAC inhibitors as a modulator of erythropoiesis. As HDAC inhibitors have been shown to act in synergy with hypomethylating agents in human primitive haematopoietic progenitors and leukaemic cells (Milhem *et al*, 2004; Lubbert, 2005; Araki *et al*, 2006), it is intriguing to examine a combinatory effect of these reagents on erythroid precursors. Further studies with HDAC inhibitors will provide not only insights into epigenetic control mechanisms in human haematopoiesis, but also crucial information for the improvement and development of epigenetic therapies for various haematological diseases, such as β chain haemoglobinopathies.

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Early Detection of Plasma Cytomegalovirus DNA by Real-Time PCR after Allogeneic Hematopoietic Stem Cell Transplantation

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ONISHI, Y., MORI, S., HIGUCHI, A., KIM, S., FUKUDA, T., HEIKE, Y., TANOSAKI, R., MINEMATSU, T., TAKAUE, Y., SASAKI, T. and FURUTA, K. *Early Detection of Plasma Cytomegalovirus DNA by Real-Time PCR after Allogeneic Hematopoietic Stem Cell Transplantation.* Tohoku J. Exp. Med., 2006, **210** (2), 125-135 —— Cytomegalovirus (CMV) infection is an important cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation. Therefore, preemptive ganciclovir therapy based on early detection of CMV reactivation is widely used to prevent CMV disease. Real-time polymerase chain reaction (PCR) has been widely used for monitoring CMV reactivation as well as the antigenemia assay that detects CMV structural phosphoprotein with a molecular weight of 65,000 (pp65). We developed a real-time PCR assay system for CMV based on a double-stranded DNA-specific dye, SYBR Green I, and quantified DNA, which was extracted automatically from plasma. This real-time PCR assay and the pp65 antigenemia assay were compared in parallel with 357 blood samples obtained from 64 patients who underwent allogeneic hematopoietic stem cell transplantation (allo-HSCT). Real-time PCR assay results correlated with those of the pp65 antigenemia assay ($p < 0.0001$). It is noteworthy that the detection of CMV DNA by PCR preceded the first positive antigenemia by 14 days. In this study, 10 of 64 patients developed CMV disease. The antigenemia assay detected CMV reactivation earlier than the development of CMV disease only in four of 10 patients. In contrast, our real-time PCR detected CMV-DNA before the development of CMV diseases in eight of 10 patients. The real-time PCR with SYBR Green I as a detection signal is simple and readily performed, and may be a useful system for early detection of CMV reactivation after allo-HSCT. ——— cytomegalovirus; real-time PCR; SYBR Green I; allogeneic hematopoietic stem cell transplantation; ganciclovir

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Cytomegalovirus (CMV) continues to be a major cause of morbidity, and this occasionally leads to the death of patients after allogeneic hematopoietic stem cell transplantation (allo-HSCT) (Boeckh et al. 2003). Although ganciclovir is an effective agent for CMV infection and disease, the administration of ganciclovir causes various adverse effects, including myelosuppression and nephrotoxicity. Therefore, it is important to discriminate high-risk patients from others to avoid over-treatment. Approaches that ganciclovir is used only in high-risk patients based on monitoring of CMV reactivation are called preemptive therapies. Preemptive therapies based on the antigenemia assay which detects CMV structural phosphoprotein with molecular weight of 65,000 (pp65) as a guide for starting ganciclovir have been widely used in clinical settings (Boeckh et al. 1996; Kanda et al. 2002a, b). However, this assay requires processing within 8 hrs of sampling, is time-consuming and suffers from a lack of standardization (Boeckh et al. 1994). Moreover, it lacks the sensitivity needed to predict the occurrence of CMV gastroenteritis (Boeckh et al. 1996; Mori et al. 2004), and can not be used when the leukocyte count is very low (Boeckh et al. 1997).

The direct detection of CMV DNA based on PCR has been investigated as an alternative measure for monitoring CMV infection, but qualitative PCR may not be able to discriminate between clinically significant and insignificant reactivation in immunocompromised individuals. On the other hand, studies using quantitative PCR have been shown to be useful for detecting patients at high risk of developing CMV disease (Gor et al. 1998). Real-time PCR, one modality of quantitative PCR, is a simple, reliable, cost-effective, and time-saving alternative strategy (Holland et al. 1991). Many institutes have developed real-time PCR assays for monitoring CMV, and have reported encouraging results (Gault et al. 2001; Cortez et al. 2003; Li et al. 2003; Nitsche et al. 2003; Ikewaki et al. 2005), where a dual-labeled fluorogenic hybridization probe or two single-labeled probes have been used to monitor PCR product formation.

Recently, a novel real-time PCR technique using a fluorescence dye, SYBR Green I, which upon binding to double-stranded DNA exhibits fluorescence enhancement, has been developed. This is the simplest real-time PCR technique based on the detection of PCR products by DNA-intercalating dye of SYBR Green I (Karsai et al. 2002). The use of SYBR Green I dye provides great flexibility and reduced cost because no target-specific probes are required. We have designed a new real-time CMV PCR assay that incorporates this system (Higuchi et al. 2002). Since cell separation from whole blood and DNA extraction could strongly affect the assay's reproducibility, we used plasma instead of peripheral blood mononuclear cells (PBMC), and a MagNA Pure automated DNA extraction instrument to automatically extract DNA from plasma. These changes made our real-time CMV PCR system much simpler than previously reported. In this study, we compared the laboratory and clinical feasibilities of this newly developed real-time CMV PCR using SYBR Green I with the existing pp65 antigenemia assay, which has been widely used as a guide for starting ganciclovir after allo-HSCT.

Materials and Methods

Patients and samples

We tested 357 blood samples obtained from 64 consecutive patients who underwent allo-HSCT and achieved sustained engraftment in our center between April 2003 and January 2004. The blood samples for the CMV antigenemia and real-time PCR were collected once a week from day 5 - 9 after transplantation until leaving our hospital. The study was approved by the National Cancer Center Institutional Review Board. All patients gave their written informed consent. The characteristics of these patients are shown in Table 1.

Stem cell transplantation procedure

Conditioning regimens and graft-versus-host disease (GVHD) prophylaxis are shown in Table 1. Fludarabine- or cladribine-based reduced intensity regimens were used in 47 patients and conventional myeloablative regimens were used in 16. Another patient did not receive any conditioning treatment before transplantation because he had severe bone marrow suppression and bacterial