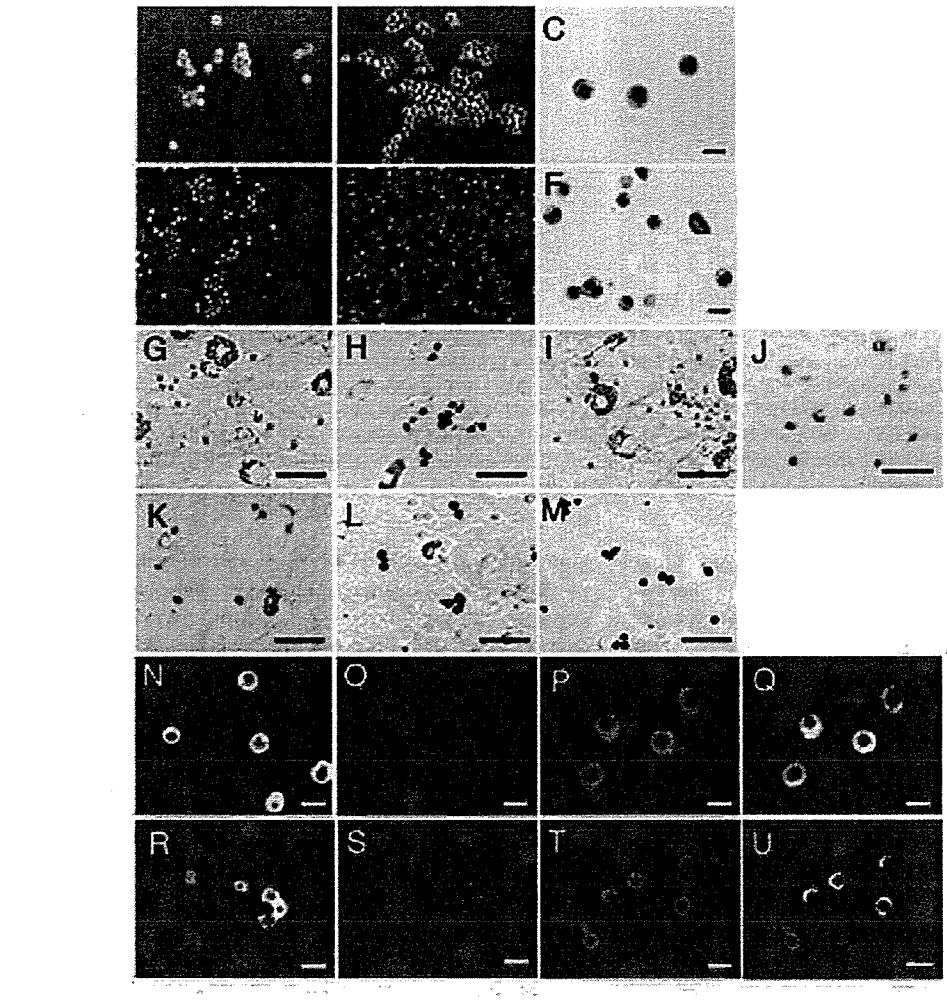

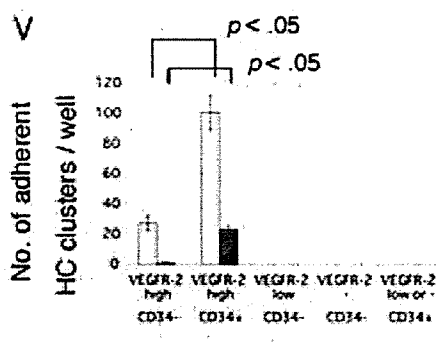

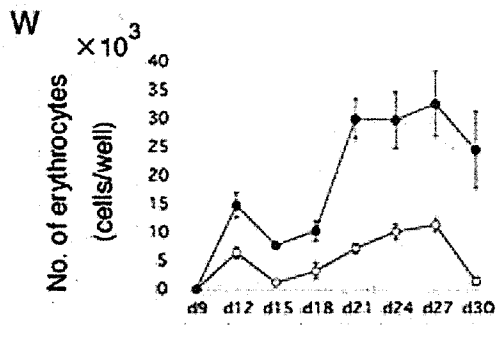

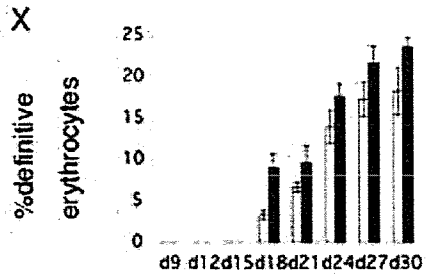

Figure 4. HC development from the VEGFR-2^{high} CD34$^-$ and VEGFR-2^{high} CD34$^+$ fractions. **(A, B):** Micrographs of adherent HC clusters generated on day 10 from the VEGFR-2^{high} CD34$^-$ fraction **(A)** and the VEGFR-2^{high} CD34$^+$ fraction **(B)**. **(C, F):** May-Giemsa staining of floating HCs on days 12 **(C)** and 27 **(F)**. **(D, E):** Micrographs of adherent HC clusters generated on day 27 from the VEGFR-2^{high} CD34$^-$ fraction **(D)** and the VEGFR-2^{high} CD34$^+$ fraction **(E)**. **(G–M):** Alkaline phosphatase detection of adherent HCs stained with antibodies to CD34 **(H)**, VEGFR-2 **(I)**, CD45 **(J)**, CD11b **(K)**, CD41 **(L)**, and Hbε **(M)**. The staining with the isotype control IgG1 is shown in **G**. **(N, R)** Immunostaining of hemoglobin (Hb) (fluorescein isothiocyanate [FITC[) and Hbγ (Cy3) in erythrocytes on days 12 **(N)** and 27 **(R)**. **(O–Q, S–U):** Immunostaining of Hb (FITC) and Hbε (Cy3) in erythrocytes on days 12 **(O–Q)** and 27 **(S–U)**. Merged images are shown in **N, Q, R,** and **U**. Nuclei were labeled with Hoechst 33342 **(N–U)**. The anti-human Hb Ab, which reacts with embryonic, fetal, and adult erythrocytes, was used to detect all erythrocytes among the floating cells during culture. The same staining results were obtained from both the VEGFR-2^{high} CD34$^-$ and VEGFR-2^{high} CD34$^+$ fractions. Original magnification, ×100 **(A, B, D, E, G–M)** and ×400 **(C, F, N–U)**. Scale bars = 100 μm **(A, B, D, E, G–M)** and 10 μm **(C, F, N–U)**. **(V):** The number of adherent HC clusters in the indicated fractions. Small clusters (white bars), which consisted of 20–49 round blast-like cells, and large clusters (black bars), which consisted of more than 50 cells, were counted on day 10. **(W):** Sequential analysis of the number of erythrocytes generated from the VEGFR-2^{high} CD34$^-$ (white circles) and VEGFR-2^{high} CD34$^+$ (black circles) populations. **(X):** Sequential analysis of the proportion of definitive erythrocytes (EryD) among all the erythrocytes generated by the VEGFR-2^{high} CD34$^-$ (white columns) and VEGFR-2^{high} CD34$^+$ (black columns) populations. EryD were defined as Hbε-negative, Hb- and Hbγ-positive erythrocytes, whereas primitive erythrocytes were Hb-, Hbγ-, and Hbε-positive. **(V–X):** Data represent the mean ± SD of triplicate wells, and representative results from one of three independent experiments are shown. Abbreviations: HC, hematopoietic cell; VEGFR, vascular endothelial growth factor receptor.

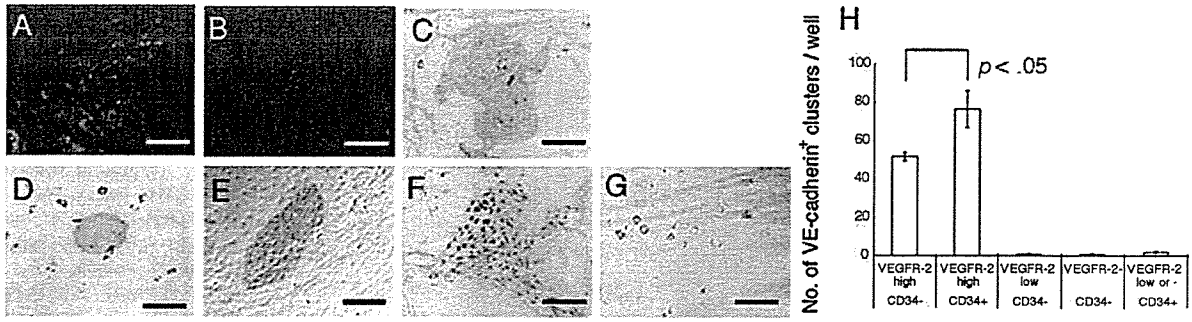

Figure 5. EC development from the VEGFR-2^{high} CD34^{-} and VEGFR-2^{high} CD34^{+} fractions. **(A–G):** Some green fluorescent protein-positive (GFP^{+}) embryonic stem cell-derived adherent cells formed sheet-like or cord-like endothelial cell (EC) clusters (**A**) that took up 1,1′-dioctadecyl-1,3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (**B**). Alkaline phosphatase detection of EC clusters stained with antibodies vascular endothelial cadherin (**C**), CD34 (**D**), VEGFR-2 (**E**), and vWF (**F**). The staining with the isotype control IgG1 is shown in **G**. Original magnification, ×100; scale bars = 100 μm (**A–F**). **(H):** The number of EC clusters in cultures of the indicated populations. The data represent the mean ± SD of triplicate wells, and representative results from one of three independent experiments are shown. Abbreviation: VEGFR, vascular endothelial growth factor receptor.

thereafter, constituting up to 20% of erythrocytes, in parallel with the second wave of erythropoiesis (Fig. 4X). Thus, the VEGFR-2^{high} CD34^{-} and VEGFR-2^{high} CD34^{+} fractions were capable of both primitive and definitive erythropoiesis, but the VEGFR-2^{high} CD34^{-} cells were less competent to differentiate than the VEGFR-2^{high} CD34^{+} cells. In contrast, the VEGFR-2^{low} CD34^{-} fraction produced few floating HCs, whereas the VEGFR-2$^{low\ or\ -}$ CD34^{+} and VEGFR-2^{-} CD34^{-} fractions failed to produce any HCs (data not shown).

EC Development from VEGFR-2^{high} Fractions

We also investigated the capacity of the various fractions sorted at day 6 to differentiate into ECs in the presence of exogenous VEGF. Some GFP-positive cells formed sheet-like or cord-like clusters that first appeared on the OP9 stromal layer on day 10 (Fig. 5A). These clusters took up DiI-Ac-LDL (Fig. 5B) and co-expressed VE-cadherin (Fig. 5C), CD34 (Fig. 5D), VEGFR-2 (Fig. 5E), vWF (Fig. 5F), and CD31 (data not shown), indicating that these cells are ECs. Immunostaining with a VE-cadherin mAb showed that 6 days after sorting, the VEGFR-2^{high} CD34^{+} fraction generated significantly more VEGFR-2^{high} VE-cadherin^{+} EC clusters than the VEGFR-2^{high} CD34^{-} fraction (Fig. 5H). Cluster formation was rare in the VEGFR-2^{low} CD34^{-} or VEGFR-2$^{low\ or\ -}$ CD34^{+} fraction, and no clusters were observed in the VEGFR-2^{-} CD34^{-} fraction. Thus, EC production was restricted to the VEGFR-2^{high} cell fractions, and the VEGFR-2^{high} CD34^{+} cells had more angiogenic potential than did the VEGFR-2^{high} CD34^{-} cells.

HC and EC Development from Single VEGFR-2^{high} CD34^{-} and VEGFR-2^{high} CD34^{+} Cells

Finally, we performed a single-cell analysis by using a single-cell deposition system (the Clon-Cyt system) to analyze whether the VEGFR-2^{high} CD34^{-} or VEGFR-2^{high} CD34^{+} fractions contain the common progenitor for both HC and EC lineages. Each well was observed by fluorescence microscopy 24 hours after cell deposition, and wells that contained more than one GFP-positive cell were excluded from further analysis. HC and EC clusters were produced by single cells from the VEGFR-2^{high} CD34^{-} and VEGFR-2^{high} CD34^{+} fractions. Immunostaining with anti-CD45, CD41, Hbγ, and VE-cadherin mAbs or by DiI-Ac-LDL incorporation assays confirmed the presence of HCs or ECs (Fig. 6A–6C, 6E, 6F). When a mixture of anti-CD45, CD41, and Hbγ mAbs was used for staining wells containing HC clusters, all round cells were positive (Fig. 6D). The concomitant development of both lineages of cells was also confirmed by immunostaining with a mixture of the three hematopoietic lineage mAbs and VE-cadherin mAb or with the anti-CD34 mAb (Fig. 6G, 6H). When 480 cells from each fraction were individually seeded, the potential for mono- or bipotential progenitor development was approximately 2-fold higher in the VEGFR-2^{high} CD34^{+} cell population than the VEGFR-2^{high} CD34^{+} cell population (the frequencies of HC development alone: 2.5% [12 wells] vs. 0.8% [four wells], $p < .05$; those of EC development alone: 15.4% [74 wells] vs. 7.9% [38 wells], $p < .05$; those of HC plus EC development: 2.3% [11 wells] vs. 1.0% [five wells], $p = .13$). Nevertheless, the data also strongly suggest that both VEGFR-2^{high} fractions contain the common hemoangiogenic progenitors, the "hemangioblasts."

Discussion

ES cells are pluripotent and can differentiate into multiple cell types, including derivatives of all three germ layers. Although their high potential for differentiation has been intensively examined in many murine ES cell culture systems [28–32], it is inevitable that the development of particular cells will be contaminated with that of cells from other lineages. Isolating cells of interest using FACS is a particularly useful approach to enrich for tissue-specific cells during in vitro ES cell differentiation. HC and EC development is a good model for such an approach because both lineages of cells have many well known common surface markers, as well as lineage-specific antigens [3, 4]. This enhances our ability to select live cells of interest. Indeed, these features have been used to identify the progenitors of both HC and EC lineages during murine ES cell differentiation [8, 31, 32].

The selection of tissue-specific stem cells or progenitors and the tracing of their fates by further culture are essential for preclinical research using monkey ES cells that aims to examine

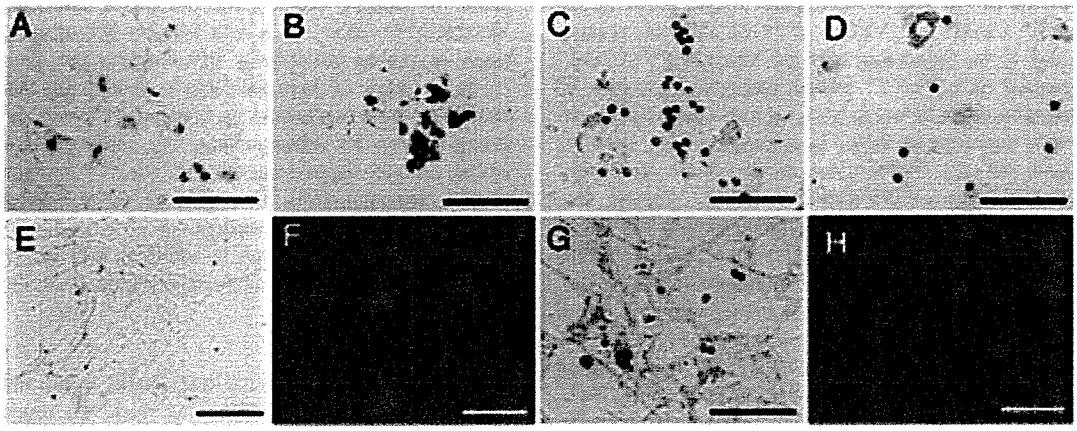

Figure 6. Single-cell deposition assay showing the hemoangiogenic potential of VEGFR-2^{high} CD34^{-} and VEGFR-2^{high} CD34^{+} cells. **(A–D):** VEGFR-2^{high} CD34^{-} and VEGFR-2^{high} CD34^{-} cells were sorted, and single cells were seeded onto an OP9 stromal layer in 96-well plates. Six days later, hematopoietic cell (HC) development was evaluated by immunostaining with monoclonal antibodies CD45 **(A)**, CD41 **(B)**, or Hbγ **(C)**. **(D):** When a mixture of all these monoclonal antibodies was used for staining wells containing HC clusters, all of them were positive. **(E, F):** EC development was evaluated by immunostaining with vascular endothelial cadherin mAb **(E)** or by the 1,1′-dioctadecyl-1,3,3,3′, 3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein incorporation assay **(F)**. **(G, H):** Concomitant development was confirmed by a mixture of CD45, CD41, and Hbγ (blue)/vascular endothelial cadherin (brown) double immunostaining **(G)** or immunostaining with CD34 mAb **(H)**. Original magnification, ×100; scale bars = 100 μm.

the efficacy and safety of clinical applications using human ES cells [13, 33]. In this study, we have dissected the differentiation pathways by which primate ES cells generate HCs and ECs by analysis of HC and/or EC cell surface markers. Our results show that the surface markers associated with HC and EC development are expressed in a defined order during culture, consistent with earlier studies of murine ES cell differentiation [8, 31, 32]. Furthermore, we show here that it is possible to identify the progenitors of both lineages and to determine their fates by analyzing a combination of well known surface markers. These observations will facilitate further investigations on primate ES cells, including in vivo studies.

Disruption of the murine homologue of the VEGFR-2 (*Flk-1*) gene in murine embryos or ES cells resulted in a combined defect in HC and EC development, which may reflect the loss of a common progenitor, the hemangioblast [5, 6]. Furthermore, in vitro differentiation of murine ES cells shows that VEGFR-2^{+} cells indeed serve as hemangioblasts [7]. In primates, however, there is no direct evidence suggesting the presence of hemangioblasts during embryogenesis, although some studies have suggested that these cells are present in the fetus and during adulthood [34, 35]. Unlike murine ES cells, several studies have detected VEGFR-2 expression in undifferentiated primate ES cells [12, 19, 36–38]. We found that undifferentiated ES cells expressed low levels of VEGFR-2 and that this surface marker is down-regulated during culture of the ES cells in the OP9 coculture system. In addition, when VEGFR-2^{low} cells were sorted from the cultures on day 6 or from undifferentiated ES cells, little HC and EC differentiation was observed (Figs. 4 and 5). Moreover, markers of the undifferentiated state, such as TRA-1–60, Rex-1, and Oct-4, were expressed by the VEGFR-2^{low} cells (Fig. 2C, 2D, and data not shown), which suggests that the former fraction still contained undifferentiated components. In contrast, VEGFR-2^{high} cells emerged on day 6, immediately prior to HC and EC differentiation, and we showed here that these cells subsequently gave rise to both primitive and definitive HCs and ECs. It should be noted that the sorted VEGFR-2^{high} cell populations were not completely pure (purity ranged from 93.0%–99.7%). However, it is unlikely that the hemoangiogenic cells came from contaminating VEGFR-2^{low} or VEGFR-2^{-} cells, since the sorted VEGFR-2^{low} or VEGFR-2^{-} cells were not able to differentiate into either HC or EC lineages. The results of the single-cell culture assays also strongly suggest that the VEGFR-2^{high} fractions contain the common hemoangiogenic progenitors, the hemangioblasts. Recently, Wang et al. reported the identification of primitive endothelial-like cells derived from human ES cells by embryoid body (EB) formation [39]. Their observation that development of both lineages can be observed from a single ES cell-derived progenitor is in agreement with our own study. Furthermore, such progenitor cells expressed VEGFR-2, but not CD45, as has been observed in mesodermal differentiation of murine ES cells [7, 8]. In contrast, the VEGFR-2^{high} hemoangiogenic progenitors in the report of Wang et al. expressed VE-cadherin, generally considered to be an EC marker [39], whereas the progenitor cells in our study did not. Our results demonstrate that the appearance of hemoangiogenic progenitors, without any HC or EC lineage-specific properties, clearly precedes differentiation into either of these cell lineages. Furthermore, this is the first report to demonstrate that both primitive and definitive HCs, as well as ECs, were generated in the VEGFR-2^{high} fractions. These differences in hematopoietic and endothelial differentiation may be partially due to differences in the culture conditions (the EB and OP9 coculture system), and/or in the ES cells that were used for these studies.

Sequential FACS analysis with a combination of surface markers revealed that two distinct populations, VEGFR-2^{high} CD34^{-} and VEGFR-2^{high} CD34^{+} cells, were present on day 6. The potential of the VEGFR-2^{high} CD34^{+} cell to serve as a mono- or bipotential progenitor is approximately twice that of the VEGFR-2^{high} CD34^{-} cell, although both cell types produce equal proportions of HCs and ECs. Notably, when we analyzed

the expression of HC and/or EC lineage marker genes by the VEGFR-2^{high} CD34^{-} and VEGFR-2^{high} CD34^{+} cell populations on day 6, we found that only the VEGFR-2^{high} CD34^{+} cells expressed SCL. *Scl*$^{-/-}$ murine embryos show lack of blood formation and a defect in yolk sac angiogenesis, indicating that this transcription factor is essential for HC and EC development [40–42]. Furthermore, recent reports on the developmental kinetics of VEGFR-2 and SCL suggest that VEGFR-2^{+} SCL^{+} cells may be hemangioblasts [43, 44]. These observations together suggest that CD34 is expressed by the VEGFR-2^{high} cells during their differentiation into hemoangiogenic progenitors, concomitant with an upregulation of a set of factors that regulate the development of both lineages.

Recent studies, including our previous work, have reported that exogenous VEGF enhances early HC development [12, 45]. We also observed that ECs are generated more abundantly in the presence of VEGF (unpublished data). Unlike other reports with monkey or human ES cells [37, 39, 45], in our culture system, exogenous BMP-4 fails to induce hematopoietic differentiation, probably because it causes the OP9 stromal cells to differentiate and thereby impairs their interaction with ES cells [12]. We analyzed the effect of various concentrations of VEGF on the development of VEGFR-2^{high} cells by FACS and found that it increases the proportion of CD34^{+} cells in the VEGFR-2^{high} cell population in a dose-dependent manner. Taken together, the effect of VEGF on HC and EC development is mainly due to its ability to enhance the proliferation and/or differentiation of VEGFR-2^{high} CD34^{+} cells with a higher hemoangiogenic potential during the initial 6-day differentiation induction.

In summary, we have been able to identify and characterize hemoangiogenic progenitors by sequential phenotypic analysis during primate ES differentiation. Our observations after cell sorting strongly suggest that the VEGFR-2^{high} fraction of cells contains hemangioblasts. The approach we have taken in this study will contribute to investigations of early developmental steps in human biology and, in addition, will provide a cell source for regenerative medicine applications in the future.

ACKNOWLEDGMENTS

We thank Tanabe Seiyaku Co. Ltd. (Osaka, Japan) for help in preparing the primate ES cells. This work was supported by grants from the Science Research on Priority Areas and the Creative Science Research programs. It was also supported by the Japan Society for the Promotion of Science; by the Ministry of Education, Culture, Sports, Science and Technology of Japan; and by the program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan.

DISCLOSURES

The authors indicate no potential conflicts of interest.

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Identification and Characterization of Hemoangiogenic Progenitors During Cynomolgus Monkey Embryonic Stem Cell Differentiation

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Stem Cells 2006;24;1348-1358; originally published online Jan 12, 2006;
DOI: 10.1634/stemcells.2005-0165

Leukemia (2006) 20, 2041–2079

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LETTERS TO THE EDITOR

Quantification of marrow CD34+ cells: an important tool for distinguishing between hypoplastic myelodysplastic syndromes and aplastic anemia

Leukemia (2006) **20,** 2041. doi:10.1038/sj.leu.2404393; published online 14 September 2006

The article by Matsui *et al.*[1] comes 9 years after our own study dealing with the same topic, that is, the value of CD34 enumeration in bone marrow (BM) to separate hypoplastic myelodysplastic syndrome (h-MDS) and aplastic anemia (AA).[2] The results that the authors report in their study are remarkably similar to ours. Differences in the microwave retrieval process may explain the slightly different percental values obtained by immunohistochemistry. Similar to the authors, we also found a statistically significant difference between the two groups (h-MDS vs AA).

The paper by Matsui *et al.*[1] is, however, highly inaccurate in the way it quotes our previous study. The authors seem to imply that we did not observe significant differences between the two groups and they stress the 'overlapping' results obtained by several groups and by us (see authors' reference 10). This is totally false. Our results speak for themselves. Our conclusions were the following: 'Bone marrow specimens of h-MDS cases showed statistically higher values of proliferating cell nuclear antigen (PCNA) and CD34 than did those of the AA cases: mean values (±s.d.) of CD34-positive cells in h-MDS, 0.94%±1.1; AA, 0.04%±0.1 ($P=0.0002$); PCNA-positive cells in h-MDS, 43.59%±13.3; AA, 14.80%±6.4 ($P<0.0001$)'. Our study confirms that AA is characterized by low expression of PCNA in bone marrow and reduced CD34 frequency compared with h-MDS and supports the concept of an early deficiency of stem cells in the former disorder. The results also illustrate how immunostaining permits a simple distinction of these conditions in routinely processed BM biopsy specimens'. The use of PCNA in this diagnostic context was previously reported by Kitagawa *et al.*[3]

Additionally, the comments in regard to our database, which are found in the discussion section (last page of the discussion, sentence beginning with 'Although *Orazi et al...*') are also quite inappropriate. Our cases were diagnosed in accordance with the French–American–British guidelines and were not 'misclassified'. Marrow karyotype was normal in only 28% of our cases of MDS. Contrary to what the authors seem to imply, a large proportion of patients with h-MDS do have indeed normal cytogenetics and an indolent clinical course. The separation of AA and h-MDS is problematic only in these cases and not in the group of MDS with an increased number of blasts (i.e. refractory anemia with excess of blasts). The utility of a correct separation between the two conditions lies in the need for a different conditioning regimen when bone marrow transplant is considered, particularly in younger patients with AA, and not necessarily in its prognostic value.

It is truly discouraging to note how some of our younger colleagues are eager to 'reinvent the wheel'.

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Reply to 'Quantification of marrow CD34+ cells: an important tool for distinguishing between hypoplastic myelodysplastic syndromes and aplastic anemia' by A Orazi

Leukemia (2006) **20,** 2041–2042. doi:10.1038/sj.leu.2404394; published online 14 September 2006

The intent of our study[1,2] was not to displace previous reports (including the one authored by Dr Orazi),[3] but rather to provide further evidence that quantification of CD34+ cells can serve as a useful discriminator between aplastic anemia (AA) and hypoplastic myelodysplastic syndrome (hMDS). Dr Orazi is, in fact, misquoting our paper when he states that we imply that his group did not observe significant differences between the two groups. We acknowledge throughout our paper that the study of Orazi *et al.*[2] demonstrated a significant difference in the mean percentage of CD34+ cells from patients classified as having either hMDS or AA. Further, our statement that there is overlap in the range of values given for the hMDS group is quite accurate, with a lower limit (0.1%) in these patients that is equal to the upper value given for those in the AA group. A potential confounding factor in their analysis may have come from reliance on the FAB guidelines to accurately diagnose patients, as those patients with normal cytogenetics and erythroid dysplasia as the sole morphologic abnormality may have in fact have had AA.

Despite the paper published 9 years ago by Dr Orazi and coworkers, enumeration of CD34 in the marrow has not become a

Quantitative RT-PCR analysis of sphingolipid metabolic enzymes in acute leukemia and myelodysplastic syndromes

Leukemia (2006) **20,** 2042–2046. doi:10.1038/sj.leu.2404386; published online 7 September 2006

Sphingolipids and their metabolites recently appeared as a potent class of regulators of cell proliferation, survival as well as apoptosis.[1] The ceramide/S1P rheostat has been proposed as the model to determine the cell's fate.[2] This rheostat is being increasingly recognized as a critical element in tumor cell proliferation and chemotherapy. Sphingomyelinase is responsible for the first step of the sphingomyelin-catabolic pathway and produces ceramide, which figures mostly as the proapoptic

factor in response to various reagents including anti-cancer drug or radiation. On the contrary, sphingosine kinase (SPHK) is the enzyme that produces sphingosine 1–phosphate (S1P) from sphingosine. S1P binds to five G-protein coupled receptors called S1P receptors. S1P promotes cell survival or motion as the first or second cellular messenger in response to various agonists. Therefore, enzymes in this pathway provide potential targets for new anti-cancer drugs.

Interestingly, overexpression of SPHK1 is thought to be oncogenic, and renders transfected cells chemoresistant.[2] SPHK1 mRNA was significantly higher in various cancer tissues than in their normal counterparts.[3] In prostate cancer cell lines, we reported the inverse relationship between SPHK1 level and anti-cancer drug sensitivity.[4] The quantity of each cellular sphingolipid metabolite was thought to be determined by the complex balance between each metabolic enzyme activity and substrate. However, no analysis of gene expression of sphingolipid metabolizing enzymes including SPHK1 of acute leukemia or related diseases has been reported.

In the present study, we performed quantitative RT-PCR assay to measure the mRNA levels of nine major enzymes involved in the sphingolipid metabolic pathway including sphingosine kinase 1 (SPHK1), sphingosine kinase 2 (SPHK2), acid sphingomyelinase (ASMase), neutral sphingomyelinase 2 (NSMase2), acid ceramidase (ACDase), sphingosine 1–phosphate lyase (SPL), sphingosine 1–phosphate phosphatase 1 (SPP1), glucosyl ceramide synthase (GlcCer Syn), sphingomyelin synthase 1 (SM Syn). Multidrug-resistant gene (MDR) and BCL2 were also measured to examine the chemoresistance gene expression in the current samples.

Quantitative PCR was performed with Power SYBR Green master mix (Applied Biosystems, Foster City, CA, USA) in duplicate using primer sets described in Table 1, and ABI PRISM 7000 sequence detection systems (Applied Biosystems) were used for the measurement. ABL gene expression was measured as the internal control with Taqman probe as shown in Table 1 according to the recommendation by Beillard *et al.*[5] The specificity of PCR product was confirmed in the preliminary experiments using cell lines. Standard curve was created using cDNA fragment of each enzyme produced by the PCR method and then inserted into the cloning vector. The relative gene expression level was calculated as the ratio of each gene expression/ABL gene expression.

After obtaining informed consent, bone marrow cells were collected from 19 patients with acute leukemia and 60 patients with myelodysplastic syndromes (MDS) (28 RA (refractory anemia), 21 RAEB (refractory anemia with excessive blast) and 11 RAEB-t (RAEB in transformation) according to FAB classification) mostly at their initial diagnosis or before any treatment. For the normal control, bone marrow samples for the disease staging were used from 11 patients with non-Hodgkin's lymphoma without bone marrow invasion. Mononuclear cells were collected and RNA was extracted. The first strand cDNA was prepared using the Super Script First-Strand System (Invitrogen). CML-BC and Ph_1 + ALL samples were omitted because the ABL gene was used as the internal control of RT-PCR assay. Patient characteristics are provided as the Supplementary Data.

Figure 1 shows the message levels of sphingolipid metabolic enzymes as well as BCL2 and MDR. We mainly focused on the statistical difference between AL and normal control by using one-way factorial analysis of variance and multiple comparison test (Bonferroni/Dunn's method). Statistical analysis was performed using Microsoft Excel software and Stat view version 5 (SAS Institute Inc., Cary, NC, USA). We used RNA from total mononuclear cells of bone marrow aspirates instead of purified hematopoietic stem cells or blast cells. Therefore, the heterogeneity of bone marrow component might have affected our results.

Among enzymes examined, AL also showed noticeable increases of SPHK1 message as compared to normal population. Some AL showed more than 2 log order higher SPHK1 gene expression as compared to the normal control. SPHK2 did not show significant differences between groups analyzed. It is also of note that SPHK1 expression of RAEB-t is also significantly higher than normal, although we could not see significant differences in SPHK1 gene expression between RAEB and normal control. There was no correlation between the SPHK1 gene expression level and abnormal karyotypes with poor prognosis such as seven monosomy or complex abnormality (data not shown), suggesting that SPHK1 gene expression is independent from karyotype abnormality. Although we did not make a sequential analysis of the same MDS patient, SPHK1 might be a candidate of the surrogate marker of AL and MDS, because its expression gradually increased during the progression of MDS and high in AL.

Okazaki's group[6] reported the correlation between chemoresistance and the increase of glucosylceramide synthase and spingomyelin synthase in leukemia cell lines and a small clinical sample. The increase of these enzyme activities might decrease the cellular ceramide level. However, the significance of these findings has not been repeated by others. In our analysis, the message levels of these genes did not show any significant difference between AL and normal control. ASMase, ACDase and SPL were not different between AL and normal control.

Interestingly, NSMase2 was decreased in AL, RAEB and RA samples as compared to normal control. Among SMases, NSMase2 has been cloned recently and was reported to play as a growth suppressor linking confluence to the G0/G1 cell cycle checkpoint.[7] The decrease of NSMase2 in AL is an unexpected and novel finding. Considering the sphingolipid rheostat model, either the increase of SPHK1 or the decrease of NSMase2 gene expression results in the decrease of ceramide/S1P ratio in leukemia blast cells, which might stimulate cell proliferation or survival. Although the regulatory mechanism of ASMase gene expression was reported in cell lines, the transcriptional regulation of NSMase2 has not been clarified yet, and is an interesting topic for future analysis. Statistical significance was also observed in SPP1 only between AL and normal control but not between MDS and normal control. As for SPL and SPP1, which convert S1P to phosphoethanolamine and palmitaldehyde, or sphingosine, respectively, we only observed the increase in SPP1 message but not SPL. The increase of SPP1 expression is also thought to modulate the ceramide/S1P rheostat, however, the significance of this finding remains to be determined.

As to other well-known chemoresistance genes, MDR showed no significant difference between AL and normal control. On the contrary, statistical significance was observed in BCL2 between AL and normal, but not between MDS and normal control. We analyzed the relationship between SPHK1 and MDR or SPHK1 and BCL2 gene expression. In AL, there was no relationship between SPHK1 gene expression level and two well-recognized genes of chemoresistance, MDR and BCL2, of the same sample (precise data not shown). This could suggest that SPHK1 is not always located downstream of BCL2 in AL.

Furthermore, it was also revealed that there are no correlationships between SPHK1 and NSMase2, between SPHK1 and SPP1 gene expression, or between SPHK1 and SPHK2 gene expression.

Table 1 Primer sets of quantitative RT-PCR

Hugo gene nomenclature	*chromosome*	*Alternate name*	*GenBank accession no.*	*Amplicon location relative to transcription start (bp)*	*% GC*	*Forward primer sequence*	*Reverse primer sequence*	*Annealing Temperature*
SPHK1	17	Sphingosine kinase 1	NM_021972	+821/+1060	58.6	TCCTGGCACTGCTGCACTC	TAACCATCAATTCCCCATCCAC	61.0
SPHK2	19	Sphingosine kinase 2	NM_020126	+26/+186	70.9	AGCAGCAGGACCAGAGGCCA	GGTGAGGGCAAAGCGTGGG	67.0
SPL	10	Sphingosine-1-phosphate lyase 1	NM_003901	+794/+934	51.5	TGGAGGTGGATGTGCGGGCAA	CCCAGACAAGCGTCGACATGAAG	62.0
SPP1	4	Sphingosine-1-phosphate phosphatase 1	NM_030791	+637/+772	44.9	ACCGCCATCCCCATTTCT	AGGAATCCAGCAATAATATCCAG	59.0
ASMase	11	Acid sphingomyelinase	NM_000543	+1073/+1220	56.0	AAGCCCTGCGCACCCTCAGAA	CCTGAAGCTCCCCCACCAGCC	64.0
NSMase2	2	Neutral sphingomyelinase II	NM_018667	+1535/+1645	60.0	ACTTTGATAACTGCTCCTCTGAC	TTCGTGTCCAGCAGAGTACC	63.0
ACDase	8	Acid ceramidase	NM_177924	+875/+968	47.8	GATATTGGCCCCAGCCTACTTT	ACCCTGCTTAGCATCGAGTTCA	60.0
GlcCer synthase	9	Glucosylceramide synthase	NM_003358	+144/+341	37.5	CAAGCTCCCAGGTGTCTCTCTTC	GATTAATGCCAACTTTTTTACCACCTA	64.0
SM synthase1	10	Sphingomyelin synthase 1	NM_147156	+757/+894	48.4	GAAGCCCAACTGCGAAGAATAA	AGAGTCGCCGAGG GGAATAC	60.0
MDR	7	Multidrug resistance 1	NM_000927	+1141/+1349	48.7	AGTGGGCACAAACCAGATAA	CTGTCCATCAACACTGACCA	63.0
BCL2	18	BCL2 (B-cell lymphoma type 2)	NM_000633	+337/+583	61.7	GCCGAGATGTCCAGCCAG	AGTTCCACAAAGGCATCCCA	62.0
ABL	22	PCR primer	Sense			CCCAACCTTTTCGTTGCACTGT		
			Antisense			CGGCTCTCGGAGGAGACGTAGA		
		Taqman probes	Sense			ACTAAAGGTGAAAAGCTCCGGGTC-FITC		
			Antisense			LCRed640-TAGGCTATAATCACAATGGGGAATGG		

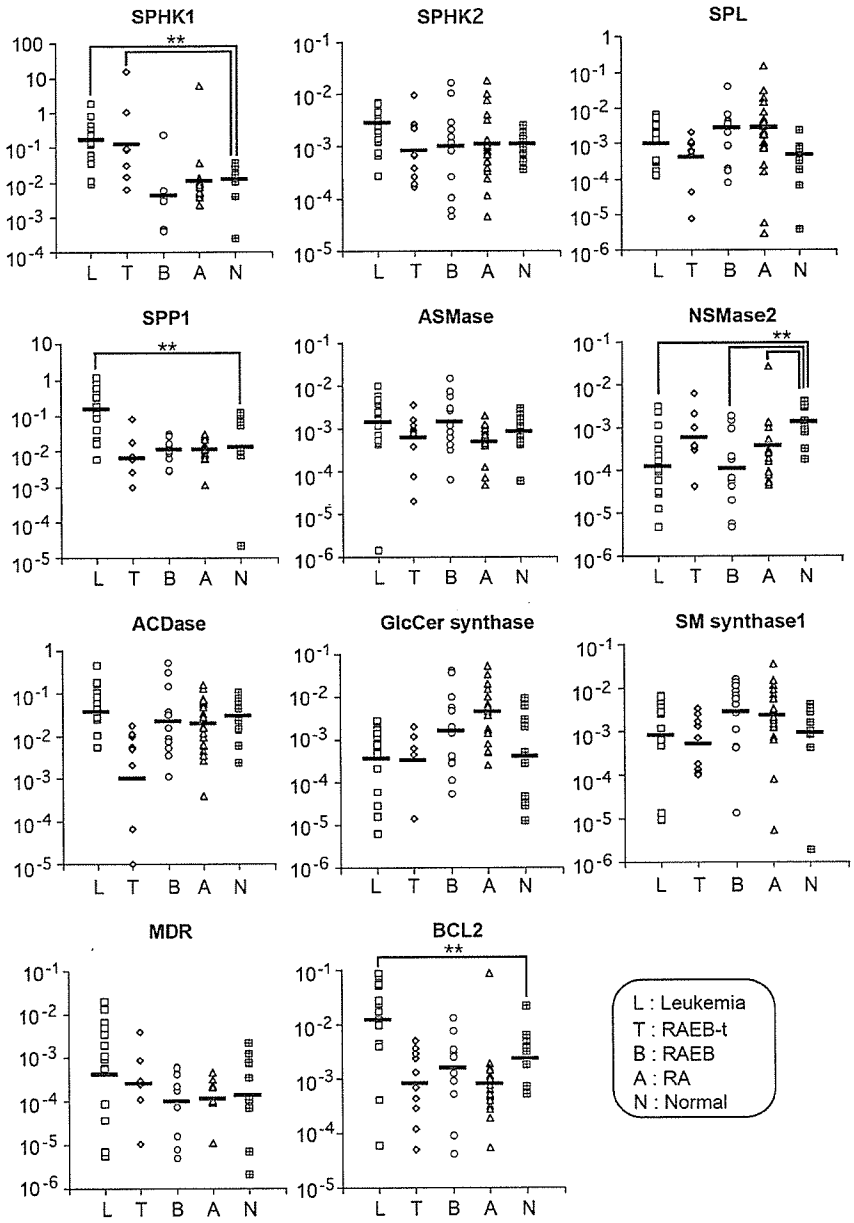

Figure 1 Relative message expression levels of sphingolipid metabolic enzymes in AL, MDS and normal control. Quantitative RT-PCR was performed with bone marrow RNA. The relative expression level was calculated with the enzyme gene expression/ABL gene expression level and was shown in the log scale. The classification of MDS was according to the FAB classification. Horizontal short bar denotes the mean value of the group. Statistical significances were analyzed by using one-way factorial analysis of variance and multiple comparison test (Bonferroni/Dunn's method). *Means $P<0.01$.

Apoptosis induction can be suitable for the treatment of diseases such as malignant tumors. The trials of novel inhibitors of SPHK1 are based on the hypothesis that the modification of ceramide/S1P rheostat induces apoptosis of malignant tumor cells and enhances chemosensitivity. Actually, a synthetic compound with SPHK inhibitor activity can induce apoptosis in tumor cells even with multidrug resistance.[3] As we[4] recently reported that SPHK1 activity is a chemotherapy sensor in prostate cancer cells, and overexpression of SPHK1 has been reported in solid tumors,[3] it is of interest to know whether this observation can be applied to hematological malignancies, especially acute leukemia and MDS. Such data are important because the relapse after intensive chemotherapy is still an unsolved problem in leukemia treatment, and new remedies are urgently required. Our data showed AL or RAEB-t cases with much higher SPHK1 gene expression compared to normal. Therefore, it is suggested that the development of new SPHK1 inhibitor is also beneficial for AL patients whose SPHK1 gene expression (and probably SPHK enzyme activity) is enhanced.

Bonhoure *et al.*[8] reported that sustained SPHK1 overexpresion can render HL60 cells chemoresistant by decreasing the cellular ceramide level and that a novel SPHK1 inhibitor, F-12509a, could recover chemosensitivity. Considering the molecular target of chemotherapy, enzyme inhibitors are more practical than agents for enzyme activation (NSMase2 in our case). Inhibitor of SPHK1 is almost at the stage of clinical investigation. In the present study, we could analyze only one time point of patients (mostly at their first diagnosis before chemotherapy) and could not measure enzyme activities due to the paucity of samples. The measurement of SPHK1 gene expression and/or SPHK enzyme activity of each patient might be necessary to assess the efficacy of enzyme inhibitors in future clinical settings. Sequential analysis of the same patients will also add further information.

The localization and activation of enzymes are also important factors to determine the final cellular ceramide/S1P rheostat. SPHK1 was reportedly activated by phosphorylation by agonists and translocated to membranes.[2] Therefore, further analysis of enzyme activation is necessary to conclude firmly that sphingolipid metabolizing enzymes such as SPHK1 are a novel and promising molecular target for acute leukemia chemotherapy.

Taken together, this is, to our knowledge, the first report of a gene expression profile of major sphingolipid metabolizing enzymes of AL and MDS using quantitative RT-PCR, It documents the increase of SPHK1 gene expression in AL and RAEB-t and the decrease of NSMase2 gene expression in AL and RAEB, suggesting that sphingolipid metabolizing enzymes such as SPHK1 could be a novel target for the chemotherapy of AL.

Acknowledgements

We express sincere thanks to Professor S Nakamura and other members of the Department of Laboratory Medicine, Nagoya University School of Medicine for their assistance. This work was partially supported by the Health and Labor Sciences Research Grants for the Research on Measures for Intractable Diseases from the Ministry of Health, Labor and Welfare.

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