CD10 Ab recognizes neutral endopeptidase, a 100-kD type II transmembrane glycoprotein, which is referred to as the common acute lymphoblastic leukemia antigen. CD10 antigen is expressed on some subsets of B- and T-lymphoid progenitors and germinal center cells. Short-term expansion of $CD33^+$ cells was observed within 2 weeks of culture (Fig. 2A, upper panel). $CD10^+$ cells started to appear at 2 weeks, and their numbers increased rapidly until 4 or 5 weeks of culture (Fig. 2A, middle panel). Cocultures on hMSC in the presence of SCF and FL led to generation of $1–5 \times 10^5$ $CD10^+$ cells from 2000 CB $CD34^+$ cells after 4 weeks of culture. Further addition of IL-7 to these cocultures had limited effect on $CD10^+$ cell production.

With regard to the transition into immature B cells, part of the generated $CD19^+$ cells began to express surface IgM after 4 weeks in coculture that included SCF and FL (Fig. 2A, lower panel; Fig. 2B). Surface phenotypes of the generated cells were analyzed after 6 weeks of culture (Fig. 2C), and most of the generated $CD10^+$ cells expressed CD19. Approximately 16.4% of $CD10^+$ cells expressed CD20. At that time, 32.5% of $CD10^+$ cells still showed surface expression of CD34. We did not detect any $CD3^+$ T-lineage cells or glycophorin A^+ erythroid cells.

Coculture of human $CD34^+$ cells on MS-5 stromal cells in the presence of SCF and G-CSF was reported to support human B lymphopoiesis [12]. Therefore, we compared B-lymphocyte production in hMSC coculture with added SCF plus FL vs SCF plus G-CSF. As shown in Figure 2D, the cocultures with SCF and FL generated B lymphocytes more efficiently than cocultures with SCF and G-CSF.

Therefore, we concluded that the coculture of CB $CD34^+$ cells on hMSC in the presence of SCF and FL was a suitable system for analyzing human B-lymphocyte development.

Screening for positive and negative regulators of human B lymphopoiesis

Because our coculture system was composed only of human-derived materials, except for FCS, we thought that it might be a suitable system for screening regulators of human B lymphopoiesis, and added several different low molecular weight inhibitors to the cocultures. Reactive oxygen species generation is related to induction of apoptosis in hematopoietic stem cells [33]. We confirmed that 100 μM N-acetylcysteine reduced apoptosis in murine hematopoietic stem cells by inhibiting reactive oxygen species generation. DUP697 is an inhibitor of Cox-2, which induces PGE_2 production. Endogenous PGE_2 was found in the supernatants of the cocultures and addition of 0.1 μM DUP697 blocked its production ($[PGE_2] = 0.74 \pm 0.40 \times 10^{-9}$ M without DUP697 and undetectable with DUP697). However, these inhibitors had no effect on human B-lymphocyte production (Fig. 3A). BIO is an inhibitor of a glycogen synthase kinase-3, which induces degradation of β-catenin [34]. Addition of BIO diminished the emergence of $CD10^+$ cells by approximately 30% (Fig. 3A), indicating that signals mediated by β-catenin inhibit human B-lymphocyte development. SB431542 is an inhibitor of ALK4/5/7, which are receptors for the TGF-β superfamily. Addition of SB431542 enhanced expansion of $CD10^+$ cells in a dose-dependent manner (Figs. 3A and B). Importantly, the influence of SB431542 on B-lymphocyte progenitors was greater than its influence on myeloid progenitors, because the percentage of $CD10^+$ cells increased significantly in the cocultures with added SB431542 (Fig. 3C).

Therefore, we determined that β-catenin and the TGF-β superfamily members act as negative regulators of human B-lymphocyte development in our coculture system.

Activin A and TGF-β1 negatively regulate human B lymphopoiesis

Among members of the TGF-β superfamily, TGF-β1 recognizes ALK-1 and -5, activin A binds to ALK-4, and BMP-4 uses ALK-2, -3, and -6 as receptors [24]. We added recombinant proteins as well as neutralizing Abs of these molecules to the cocultures. Production of human B lymphocytes decreased in a dose-dependent manner with the addition of TGF-β1, but not with activin A or BMP-4 (Fig. 4). A neutralizing Ab for activin A enhanced B-lymphocyte production approximately threefold, but neutralizing Abs for TGF-β1 and BMP4 had no effect (Fig. 5A). The physiological antagonist of activin A, follistatin, enhanced human B-lymphocyte production in a dose-dependent manner (Fig. 5B), and the percentage of $CD10^+$ cells in the generated cells increased markedly with the addition of follistatin (Fig. 5C), indicating that activin A downregulates human B lymphopoiesis more efficiently than myelopoiesis.

Therefore, both activin A and TGF-β1 inhibit human B-lymphocyte development, while BMP-4 has no apparent regulatory effect on human B-lymphocyte progenitor cells in our cocultures.

Both activin A and TGF-β1 inhibit early onset of human B lymphopoiesis

We next examined the effect of SB431542 on the frequency of B-lymphocyte progenitor cells. In limiting dilution culture with SB431542, the frequency of progenitor cells capable of generating $CD10^+$ cells increased significantly (Fig. 6A). Moreover, when a neutralizing Ab for activin A was added to cocultures derived from subpopulations of CB $CD34^+$ cells, the production of B lymphocytes, but not myeloid cells, was significantly enhanced (Fig. 6B and C). The enhancing effect on B-lymphocyte production was particularly great when the cocultures were started from $CD34^+CD38^-$ and $CD34^+CD38^+CD10^-$ subpopulations.

Therefore, both activin A and TGF-β1 inhibit early onset of human B lymphopoiesis.

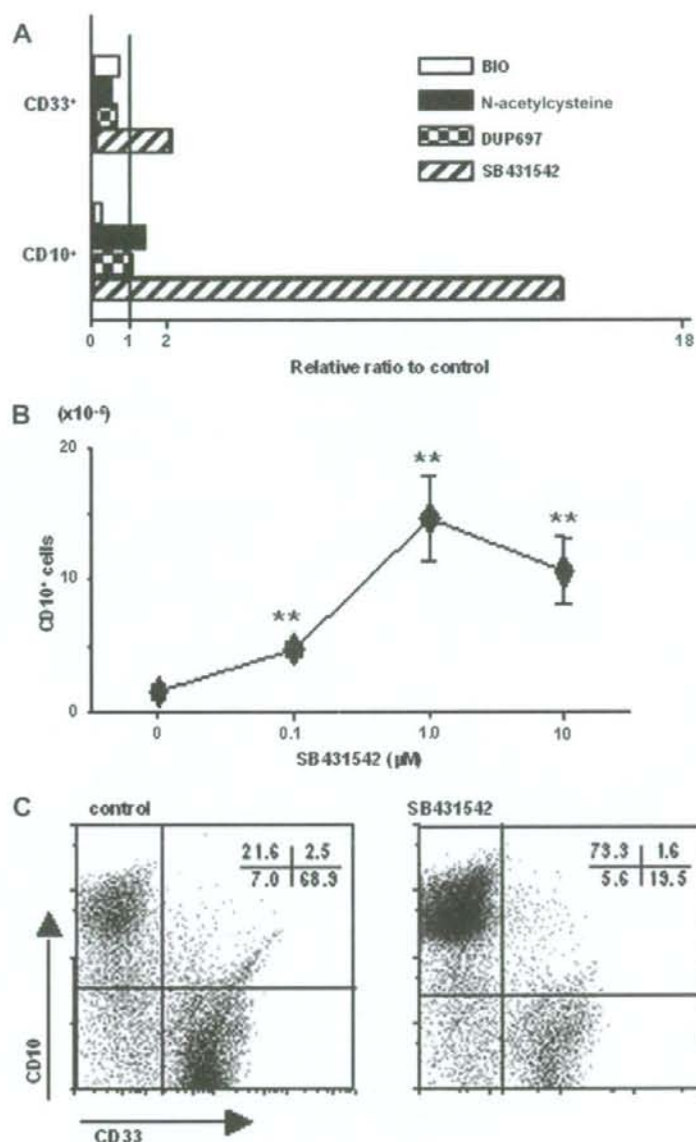


Figure 3. Addition of an inhibitor for activin receptor-like kinase [ALK]-4/5/7 to the cocultures selectively enhances human B-lymphocyte production. (**A**) 5 nM BIO (glycogen synthase kinase-3 inhibitor), 100 μM N-acetylcysteine (reactive oxygen species inhibitor), 0.1 μM DUP697 (Cox-2 inhibitor) or 10 μM SB431542 (ALK-4/5/7 inhibitor) was added to the cocultures of CB $CD34^+$ cells (2000 cells/well) on human mesenchymal stem cells (hMSC) in the presence of 10 ng/mL stem cell factor (SCF) and 5 ng/mL Flt3-ligand (FL). At day 28, the generated $CD33^+$ or $CD10^+$ cells were calculated from the recovered total cell numbers and the percentages of the positive cells confirmed by flow cytometry. Data are shown as mean in duplicated samples. Similar results were obtained in three independent experiments. (**B**) The indicated concentrations of SB431542 were added to the cocultures of CB $CD34^+$ cells (2000 cells/well) on hMSC in the presence of 10 ng/mL SCF and 5 ng/mL FL. Data are shown as mean ± standard deviation of numbers of the generated $CD10^+$ cells in triplicated samples. Statistically differences from control values (without SB431542) are shown with two ($p < 0.01$) asterisks. Similar results were obtained in two independent experiments. (**C**) Purified CB $CD34^+$ cells (2000 cells/well) were cultured on hMSC in the presence of 10 ng/mL SCF and 5 ng/mL FL with or without 1.0 μM SB431542. At day 28, the generated cells were stained with phycoerythrin (PE)-CD10 and fluorescein isothiocyanate (FITC)-CD33, and analyzed with flow cytometry. Similar results were obtained in four independent experiments.

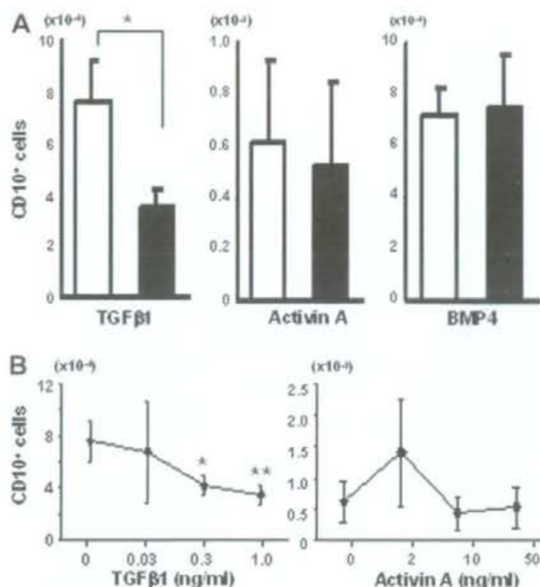


Figure 4. Transforming growth factor (TGF)-β1 negatively regulates human B lymphopoiesis in the cocultures. (**A**, **B**) Purified cord blood (CB) CD34$^+$ cells (2000 cells/well) were cultured on human mesenchymal stem cells (hMSC) in the presence of 10 ng/mL stem cell factor (SCF) and 5 ng/mL Flt3-ligand (FL) with control (open column), 1 ng/mL TGF-β1, 50 ng/mL activin A, or 50 ng/mL bone morphogenetic protein (BMP)-4 (closed column) (**A**), with the indicated concentrations of TGF-β1 or activin A (**B**). At day 28, the generated CD10$^+$ cells were calculated from total cell numbers and percentages of the positive cells confirmed by flow cytometry. Data are shown as mean ± standard deviation in triplicated samples. Statistically differences from control values are shown with one ($p < 0.05$) or two ($p < 0.01$) asterisks. Similar results were obtained in three independent experiments.

Effect of TGF-β superfamily members on the transition into immature B cells

As shown in Figure 2, part of CD19$^+$ cells develop into IgM$^+$ immature B cells after 4 weeks in culture. Therefore, we evaluated the effects of TGF-β superfamily members on the transition into immature B cells in coculture. Blocking ALK-4/5/7 by SB431542 failed to influence the percentage of the IgM$^+$ population in the cultured CD19$^+$ cells at 5 weeks of culture (Fig. 7A). Similarly, we did not detect any difference in the percentage of IgM$^+$ cells by the addition of recombinant TGF-β1, a neutralizing Ab for activin A, or follistatin (Fig. 7B, C, and D).

Therefore, we did not observe any influence by TGF-β superfamily members on the transition into immature B cells in our human B-lymphocyte coculture system.

Coculture production of activin A and TGF-β1

Using reverse transcription PCR, we confirmed that RNAs of activin A type I and type II receptors were expressed by both CB CD34$^+$ cells and CD34$^-$ cells (Fig. 8A). hMSC expressed RNAs of the TGF-β superfamily members (Fig. 8B). Supernatants from the cocultures contained 1700 ± 410 pg/mL activin A and 40.8 ± 19.4 pg/mL TGF-β1 at day 3 of culture, and 3200 ± 130 pg/mL activin A and 114.7 ± 16.1 pg/mL TGF-β1 at day 10 (Fig. 8C). When we examined BM sections from normal healthy individuals, we detected activin A- and TGF-β1–positive cells (data not shown).

Therefore, both activin A and TGF-β1 are produced in our cocultures and in human BM.

Discussion

We established a novel coculture system to analyze human B lymphopoiesis. In our system, hMSC could support the commitment and differentiation of CB CD34$^+$ cells into CD10$^+$ cells, followed by transition into IgM$^+$ immature B cells. hMSC retained their capacity for cell-to-cell contact inhibition; therefore, we could continue the cocultures for up to 6 weeks without passage of hMSC. Moreover, our coculture system is a biologically relevant model for human B-lymphocyte development in that it excludes the effects of xenograft materials. When IL-7 was added to the SCF and FL-containing cocultures, only a few IgM$^+$ cells appeared. Thus, adding the combination of SCF and FL enhanced the B-lymphocyte–supporting capacity of hMSC.

We found that the addition of SB431542, an inhibitor for ALK-4/5/7, enhanced the output of CD10$^+$ cells markedly. Follistatin, a physiological inhibitor of activin A, and a neutralizing Ab for activin A enhanced B-lymphocyte production, while a neutralizing Ab for TGF-β1 had no discernable effect. TGF-β1, but not activin A, suppressed B-lymphocyte production in a dose-dependent manner. The different effects of inhibitors and factors on human B-lymphocyte production seemed to be related to the fact that the culture supernatant contained a much higher concentration of activin A than of TGF-β1. Adding a neutralizing Ab for BMP-4 or recombinant BMP-4 protein itself had no effect on B-lymphocyte production. Therefore, the strength of the ability to suppress human B lymphopoiesis seemed to be activin A > TGF-β1 > BMP-4.

Members of the TGF-β superfamily are implicated in control of many biological processes, such as cell cycle, cell growth and differentiation, and lymphocyte development and function [25]. The importance of these regulatory cytokines on immune homeostasis is reflected by the phenotypes of TGF-β1–deficient mice that develop autoimmune diseases with production of autoantibodies [35], although suppression of self-reactive lymphocyte clones involves actions of TGF-β1 on both B and T lymphocytes [36]. There are several reports suggesting that the TGF-β superfamily can modulate B-lymphocyte proliferation, expression of surface antigen receptors, and Ab secretion [37–40]. In murine B lymphopoiesis, TGF-β [7] and activin

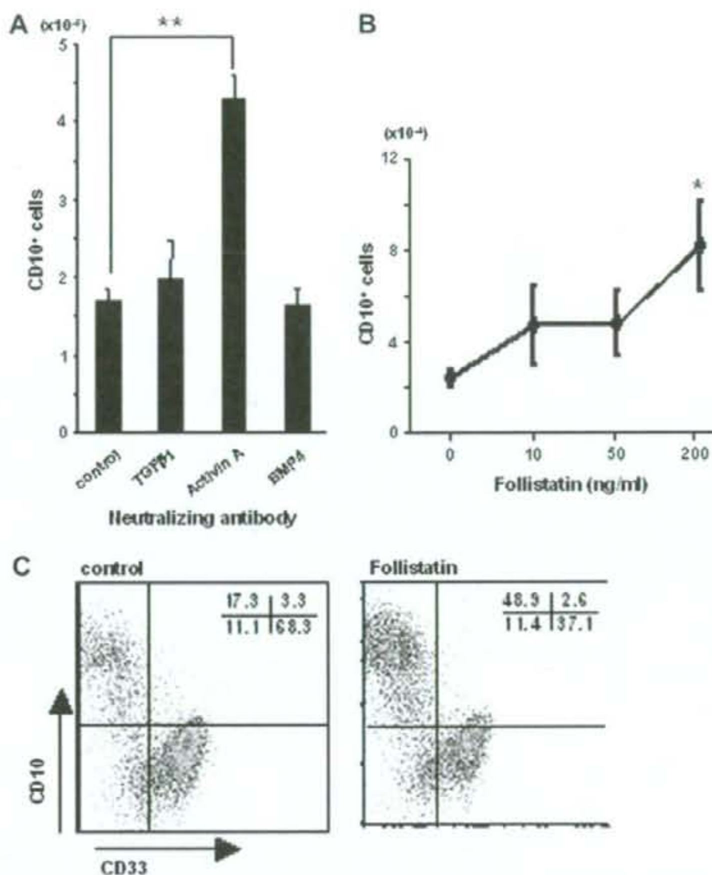


Figure 5. Inhibition of activin A enhances human B lymphopoiesis in the cocultures. (**A**, **B**) Purified cord blood (CB) CD34$^+$ cells (2000 cells/well) were cultured on human mesenchymal stem cells (hMSC) in the presence of 10 ng/mL stem cell factor (SCF) and 5 ng/mL Flt3-ligand (FL) with 10 μg/mL antibodies (Abs) for TGF-β1, activin A, or bone morphogenetic protein (BMP)-4 (**A**), or with the indicated concentrations of follistatin (**B**). At day 28, the generated CD10$^+$ cells were calculated from total cell numbers and percentages of the positive cells confirmed by flow cytometry. Data are shown as mean ± standard deviation in triplicated samples. Statistically differences from control values are shown with one ($p < 0.05$) or two ($p < 0.01$) asterisks. Similar results were obtained in three independent experiments. (**C**) Purified CB CD34$^+$ cells (2000 cells/well) were cultured on hMSC in the presence of 10 ng/mL SCF and 5 ng/mL FL with or without 200 ng/mL follistatin for 4 weeks. The generated cells were stained with phycoerythrin (PE)-CD10 and fluorescein isothiocyanate-CD33, and analyzed with flow cytometry. Similar results were obtained in three independent experiments.

A [26,41] inhibit early onset of B-lymphocyte development. Because production of CD10$^+$ cells from CB CD34$^+$ cells was suppressed by both activin A and TGF-β1, our results from the coculture experiments coincide well with previous reports about the negative regulatory effects of these molecules on murine B-lymphocyte development. Notably, production of CD10$^+$ cells was influenced by the inhibition of TGF-β superfamily members even when the CD34$^+$CD38$^-$ stem cell population was used to initiate cocultures, and the influence was greater in the cocultures derived from CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$CD10$^-$ cells than in cocultures derived from CD34$^+$CD38$^+$CD10$^+$ cells. These data suggest that the TGF-β superfamily inhibits early onset of human B lymphopoiesis. Furthermore, production of CD10$^+$ cells was also influenced when the inhibitor was added after 2 weeks of coculture (data not shown), indicating that the TGF-β superfamily might suppress the proliferation of relatively differentiated B-lymphocyte progenitors. Thus, members of the TGF-β superfamily are likely to suppress human B lymphopoiesis at a wide range of differentiation stages. This hypothesis is supported in part by our reverse transcription PCR data showing that both CD34$^+$ cells and CD34$^-$ cells express receptors for the TGF-β superfamily. Although a number of investigators have reported regulatory effects of the TGF-β superfamily on class switching

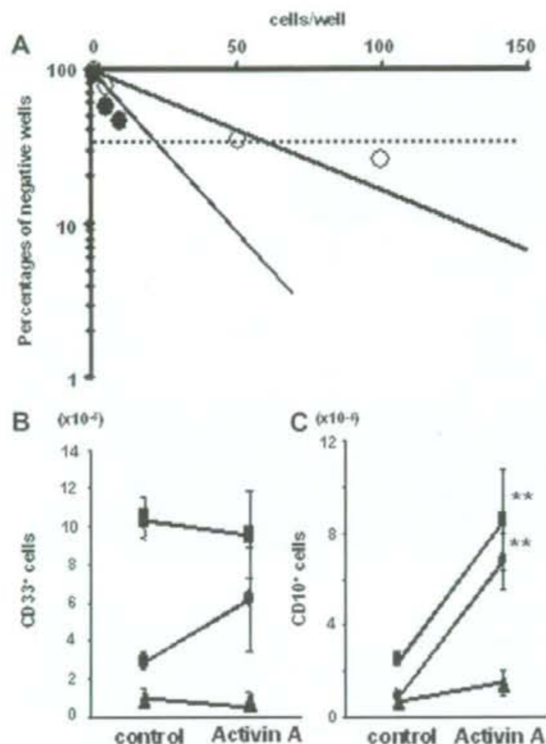


Figure 6. Transforming growth factor (TGF)-β superfamily inhibit early onset of human B lymphopoiesis. (A) Limiting dilution assays were performed in 96-well plates. Cord blood (CB) CD34⁺ cells were cultured on human mesenchymal stem cells (hMSC) at indicated concentrations in the presence of 10 ng/mL stem cell factor (SCF) and 5 ng/mL Flt3-ligand (FL) with (closed circle) or without 10 μM SB431542 (open circle). After 28 days of coculture, wells with cell expansion were scored. The expanded cells were analyzed by flow cytometry and calculated the well number of cultures generated CD10⁺ cells. Similar results were obtained in three independent experiments. (B, C) Purified CB CD34⁺ cells were stained with allophycocyanin (APC)-CD34, fluorescein isothiocyanate (FITC)-CD38, and phycoerythrin (PE)-CD10. CD38⁻ cells (square), CD38⁺ CD10⁻ cells (circle), and CD38⁺ CD10⁺ cells (triangle) were then sorted with FACSAria. The sorted cells (2000 cells/well) were cultured on hMSC in the presence of 10 ng/mL SCF and 5 ng/mL FL with or without 10 μg/mL antibodies for activin A for 4 weeks. Numbers of the generated CD33⁺ cells (B) or CD10⁺ cells (C) were evaluated. Data are shown as mean ± standard deviation in triplicated samples. Statistically differences from control values are shown with two ($p < 0.01$) asterisks. Similar results were obtained in two independent experiments.

and immunoglobulin secretion, little information about the influence on the transition from pre-B to immature B cells is available. We found that the percentage of surface IgM⁺ cells in CD19⁺ cells was not influenced by manipulating the TGF-β superfamily, while the production of CD19⁺ cells was affected. Therefore, the transition into immature B cells is unlikely to be influenced by the TGF-β superfamily.

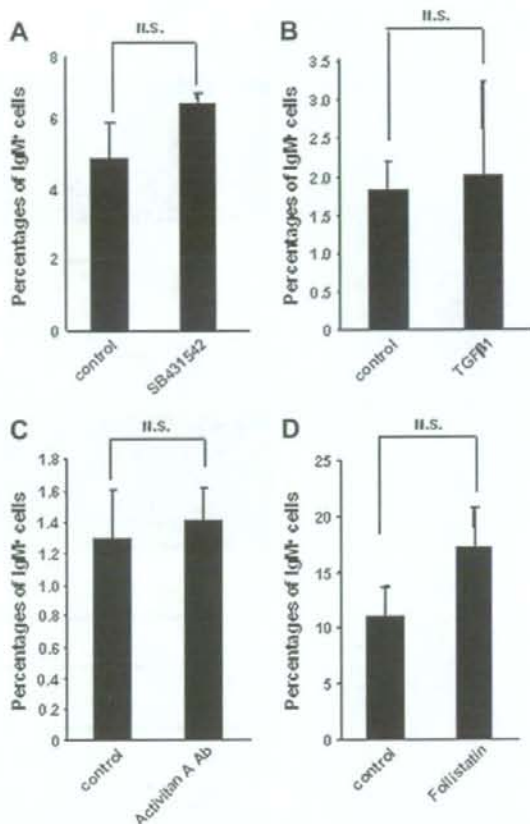
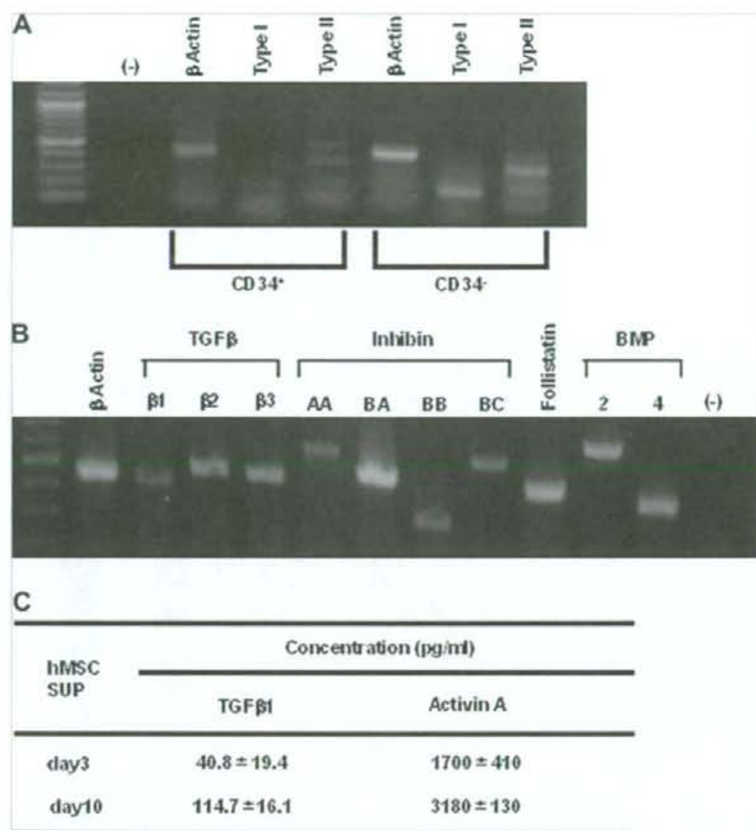


Figure 7. Transition from pre-B cells into immature B cells is not influenced by manipulating the transforming growth factor (TGF)-β superfamily in the cocultures. (A–D) Purified cord blood (CB) CD34⁺ cells (2000 cells/well) were cultured on human mesenchymal stem cells (hMSC) in the presence of 10 ng/mL stem cell factor (SCF) and 5 ng/mL Flt3-ligand (FL) with 1 μM SB431542 (A), with 0.3 ng/mL TGF-β1 (B), with 10 μg/mL an anti-activin A (C), or with 200 ng/mL follistatin (D). At day 42, the generated cells were stained with fluorescein isothiocyanate (FITC)-IgM and phycoerythrin (PE)-CD19, and analyzed with flow cytometry. Similar results were obtained in two independent experiments. NS = not significant.

In conclusion, our coculture system of CB CD34⁺ cells on hMSC in the presence of SCF and FL is suitable for analyzing the regulatory mechanisms of human B-lymphocyte development. With this system, we showed that members of TGF-β superfamily, activin A and TGF-β1, are negative regulators of human B-lymphocyte development at a range of differentiation stages. We expect that our coculture system will be applicable to a variety of research and development processes, such as screening for regulatory molecules or drugs that influence human B-lymphocyte development, evaluating B-lymphocyte progenitors in patients with B-cell malignancies, and cloning human B-lymphocyte–supportive molecules.



hMSC SUP	Concentration (pg/ml)	
	TGFβ1	Activin A
day3	40.8 ± 19.4	1700 ± 410
day10	114.7 ± 16.1	3180 ± 130

Figure 8. Production of activin A and transforming growth factor-β1. (**A**, **B**) Expression of RNAs of type I and type II receptor of activin A in cord blood (CB) $CD34^+$ and $CD34^-$ cells (**A**) and of the various TGF-β superfamily members in human mesenchymal stem cells (hMSC) (**B**) was analyzed with reverse transcription polymerase chain reaction. (**C**) Purified CB $CD34^+$ cells were cultured on hMSC in the presence of 10 ng/mL stem cell factor (SCF) and 5 ng/mL Flt3-ligand (FL). Culture supernatant was collected just before medium change at day 3 and day 10, and subjected to enzyme-linked immunosorbent assay for activin A or TGF-β1, respectively. Data are shown as mean ± standard deviation (n = 3 each).

Acknowledgments

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Comprehensive Review

Proposals for a Grading System for Diagnostic Accuracy of Myelodysplastic Syndromes

Akira Matsuda,[1] Itsuro Jinnai,[1] Yasushi Miyazaki,[2] Masao Tomonaga[2]

Abstract

Despite recent advances in cytogenetics and molecular research, universal biomarkers for the diagnosis of the myelodysplastic syndromes (MDS) are still lacking. It is not easy to diagnose MDS by morphology alone, particularly in patients with < 5% blasts in the bone marrow (BM) and normal karyotype. Therefore, the possibility of misdiagnosis and discordance among observers can occur. In order to resolve these problems, we propose a grading system for diagnostic accuracy of MDS. The diagnostic accuracy of MDS is graded into "definite," "probable," or "possible" in addition to "idiopathic cytopenia(s) of uncertain significance (ICUS)." The criteria of grading for diagnostic accuracy are a combination of (1) the frequency of blasts in BM, (2) grade of dysplasia (high, intermediate, or low), and (3) division of cytogenetics (abnormal, normal, or unknown). For quantitative morphologic evaluation of dysplasias, we classified morphologic dysplastic changes into highly specific category A (pseudo–Pelger-Huet anomaly, degranulation of neutrophils, micromegakaryocytes, and ringed sideroblasts) and less specific category B (dysplasias other than those in category A). We believe that diagnostic problems would be reduced by using our grading system and repeating BM examination at suitable intervals for patients who are allocated into the "possible" or "ICUS" categories, and this will make the vague margin of MDS category clearer.

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Key words: Cytogenetics, Dyserythropoiesis, Idiopathic cytopenia of uncertain significance, Pelger-Huet

Introduction

Myelodysplastic syndromes (MDS) are acquired clonal stem-cell disorders characterized by ineffective hematopoiesis with myelodysplasia[1] and are associated with a high risk of progression to acute leukemias.[2] Despite recent advances in cytogenetics and molecular research, universal biomarkers for the diagnosis of MDS are still lacking. It is not easy to diagnose MDS, particularly in patients with < 5% blasts in the bone marrow (BM) and normal karyotype. In such patients, the diagnosis mainly depends on morphologic examinations. Minimal morphologic requirements to diagnose MDS are well established but might not be accurate or leave too much room for subjectivity. Herein, we propose a grading system for the diagnostic accuracy in an attempt to reduce misdiagnosis and improve concordance among observers.

Background for Proposals

Exclusion of nonclonal disorders[3-6] with some myelodysplasia is crucial to the diagnosis of MDS. However, in patients with < 5% blasts in the BM and normal karyotype, it is not easy to distinguish MDS from such nonclonal disorders by morphology alone. In addition, judgments of dysplasia are subjective to a certain extent. Therefore, misdiagnosis and discordance among observers are likely to occur. In patients with hypoplastic BM, it is important to distinguish hypoplastic MDS from aplastic anemia (AA). Dyserythropoiesis (Dys E) is often found in patients with AA and cannot be used alone to distinguish MDS from AA.[7]

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We previously reported that the presence of hyposegmented mature neutrophils (Pelger), degranulation of neutrophils (agranular or hypogranular neutrophils; Hypo-Gr), and micromegakaryocytes (mMgk) in BM or peripheral blood (PB) were found in 76%, 30%, and 74% of MDS cases, respectively, whereas there was no AA case with these dysplasias.[8] We confirmed the specificity of these dysplasias in a different case series showing that Pelger ≥ 10% or mMgk ≥ 10% in BM was not found in the AA group (presented at the Ninth International Symposium on MDS). We also showed that, among patients with refractory anemia (RA) according to the French-American-British (FAB) classification[9] (FAB-RA), excluding MDS associated with isolated deletion of chromosome 5q (5q-syndrome), the presence of Pelger ≥ 10% or mMgk ≥ 10% in BM (15% and 14% of RA cases, respectively) were significantly related to the shorter overall survival (OS) and leukemia-free survival (LFS). The median OS and LFS of cases with Pelger ≥ 10% were 29 months and 36 months, respectively, and were significantly shorter than those without Pelger ≥ 10% (158 months and not reached, respectively; $P < .001$ in both). Micromegakaryocytes ≥ 10% showed similar effect on OS and LFS (23 months vs. 153 months for OS [$P < .001$] and 51 months vs. not reached for LFS [$P < .001$]).[10] The concordance rates of Pelger and mMgk were reasonably high among observers.[11] These dysplasias are much easier to detect, not only for expert morphologists but also for clinical hematologists in general. We considered that misdiagnosis and discordance would be avoided by enumerating these MDS-specific dysplasias.

Idiopathic cytopenia of uncertain significance (ICUS) was first proposed by Mufti et al at the Eighth International Symposium on MDS in Nagasaki, Japan, in 2005. If patients with normal karyotype and < 5% BM blasts do not show morphologic dysplasia (ie, < 10% of any cell lineage) and all other diseases have been ruled out as a cause of cytopenia, the patients are diagnosed with ICUS. The cytopenia(s) should persist for ≥ 6 months without any other cause identified. The criteria for ICUS was proposed in a recent publication by Valent et al.[12] Idiopathic cytopenia of uncertain significance might be a useful category for patients with unexplained cytopenia who do not fulfill the criteria of MDS (either of the FAB classification or the World Health Organization [WHO] classification[13]). Extensive study for this category in terms of MDS pathophysiology, particularly a molecular aspect, will clarify the clinical and pathophysiologic features of the ICUS category.

We previously compared the morphologic features between FAB-RA, excluding 5q-syndrome AA at the Ninth International Symposium on MDS, held in Florence, Italy, in 2007. One hundred patients with FAB-RA, excluding 5q-syndrome, were diagnosed by a joint review of a Japanese and German collaboratory study.[10,14] Forty patients with AA who registered to the Japanese AA and MDS Registration System of the National Research Group on Idiopathic Bone Marrow Failure Syndromes, Japan were diagnosed by the Central Review Working Group. In all patients with FAB-RA, the frequency of dysplasia was ≥ 10% in ≥ 1 lineage. Some (17%) patients with AA showed Dys E ≥ 10% in BM; Hypo-Gr ≥ 10%, Pelger ≥ 10%, or mMgk ≥ 10% were found only in the FAB-RA group. In addition, dysplasia ≥ 10% in ≥ 2 lineages was found only in the FAB-RA group. The number of megakaryocytes was markedly decreased in all patients with AA. The presence (≥ 5%) of blasts in BM was never found in the patients with AA.

Table 1 Prerequisite Criteria

Criteria
A. Constant cytopenia (≥ 6 months) in ≥ 1 of the following lineages:
Hemoglobin < 11 g/dL
Absolute neutrophil count < 1.5×10^9/L
Platelet count < 100×10^9/L
B. Less than 20% blasts in PB or BM and absence of cytogenetic findings related with acute myeloid leukemia with recurrent cytogenetic abnormalities*
C. Less than 1×10^9/L monocytes in PB
D. Exclusion of all other hematopoietic or nonhematopoietic disorders as primary reason for cytopenia
E. Exclusion of aplastic anemia. In case of hypoplastic BM, exclusion of aplastic anemia needs to be considered using morphologic findings and cytogenetic data.

A-E must be fulfilled.
*t(8;21)(q22;q22); (AML1/ETO), t(15;17)(q22;q12); (PML/RARα), and inv(16)(p13;q22) or (t16;16)(p13;q22); (CBFβ/MYH11).

Recently, minimal diagnostic criteria for MDS have been proposed by Valent et al.[12] They did not show a list of dysplastic cells in their criteria. We think a clear and definite list of dysplastic cells is necessary for diagnostic criteria. We propose a grading system for diagnostic accuracy of MDS by combining the results of our morphologic study presented at the Ninth International Symposium on MDS with the criteria proposed by Valent et al.

A Grading System for Diagnostic Accuracy of Myelodysplastic Syndrome

Exclusion of disorders with constant cytopenia(s) and some morphologic dysplasia(s) other than MDS is a prerequisite for diagnosing MDS. We propose that dysplasia(s) be divided into category A (high specificity) and category B (low specificity) for assessment of the frequency of dysplasia(s). A quantitative morphologic evaluation of category A or A + B is essential to start diagnosis of MDS. We then suggest a grading of dysplasia based on the enumeration and a division of cytogenetic findings. The criteria for grading of diagnostic accuracy are a combination of the frequency of blasts in BM, grade of dysplasia, and divisions of cytogenetics. The grades of diagnostic accuracy are divided into "definite," "probable," or "possible" in addition to "ICUS." Patients who are diagnosed as "definite," "probable," or "possible" should be classified according to the WHO classification for MDS.

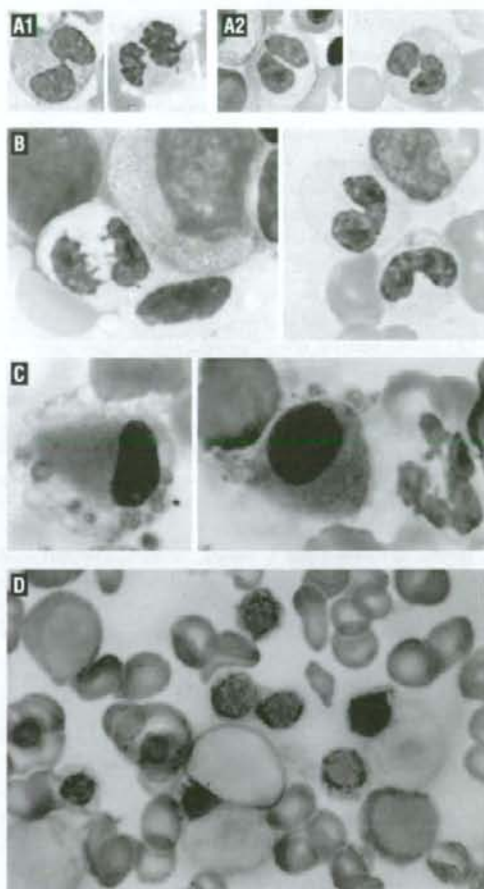
Step I: Exclusion Diagnosis of Disorders Other Than Myelodysplastic Syndrome

We modified the excellent prerequisite criteria proposed by Valent et al.[12] Table 1 shows our prerequisite criteria, consisting of the definition of constant cytopenias (≥ 6 months) and exclusion of disorders with constant cytopenias or some myelodysplasia. Acute myeloid leukemia (AML) should be excluded by frequency of blasts and cytogenetic findings. Bone marrow differential counts should be performed on 500 cells. Counting the number of monocytes in PB is necessary for the exclusion of chronic myelomonocytic

Table 2 Classification of Dysplasia

- **Category A**
  - Granulocytic series
    - Hyposegmented mature neutrophils (Pelger)
    - Degranulation (agranular or hypogranular neutrophils; Hypo-Gr)
  - Megakaryocytic series
    - Micromegakaryocytes
  - Erythroid series
    - Ringed sideroblasts
- **Category B**
  - Granulocytic series
    - Small size
    - Hypersegmentation
    - Pseudo–Chediak-Higashi granule
  - Megakaryocytic series
    - Nonlobulated nuclei
    - Multiple, widely separated nuclei
  - Erythroid series
    - Nucleus
      - Budding
      - Internuclear bridging
      - Karyorrhexis
      - Multinuclearity
      - Megaloblastoid change
    - Cytoplasm
      - Vacuolization

Figure 1 Dysplasia of Category A



(A) Hyposegmented mature neutrophils (Pelger). Two lobes are connected with a fine (1) or thin (2) filament. **(B)** Degranulation of neutrophils (agranular or hypogranular neutrophils; Hypo-Gr). **(C)** Micromegakaryocytes. **(D)** Ringed sideroblasts. **(A-D)** Provided by the National Research Group on Idiopathic Bone Marrow Failure Syndromes, Japan.

leukemia. To exclude nonclonal disorders (Table 1D), laboratory studies (eg, serum iron, ferritin, cobalamin, and folic acid levels; Coombs test; anti-nuclear antibody; thyroid function tests; etc) and abdominal ultrasonography are necessary.

For evaluations of BM cellularity, specimens of BM trephine biopsy must be examined. A BM biopsy of good quality and adequate length (≥ 1.5 cm) is necessary. Often, repeat BM examination is required to confirm the diagnosis when there is doubt about initial BM examination or if an inadequate sample was taken.[15] Because BM cellularity is highly age-dependent, hypocellularity is defined as < 30% in patients aged < 60 years or < 20% in patients aged ≥ 60 years.[16,17] In hypocellular BM, microscopic examinations for the assessment of dysplasias should be performed with ≥ 2 BM films, if necessary.

In patients with hypocellular BM, it is absolutely necessary to exclude AA to diagnose hypoplastic MDS (Table 1E). Dysplasia of BM cells, the percentage of blasts in BM, and abnormal localization of immature precursors (ALIP) are useful markers for this differentiation. As mentioned earlier, significant dysplasia in ≥ 2 lineages and > 10% of Hypo-G, Pelger, or mMgk strongly suggest MDS rather than AA. Abnormal localization of immature precursors is usually not found in AA but is found in MDS, and blasts in BM are usually < 5% in AA cases. In this regard, it is very difficult to diagnose hypoplastic RA cases that show dysplasia only in erythroid lineage. Morphologic evaluation alone might not be enough for the diagnosis, and other data such as cytogenetics will provide further useful information when they show MDS-specific karyotype (see Step VI). However, in some cases with hypoplastic BM, in particular those having dysplasia in a single lineage or at low percentage, careful course observation is necessary to make a diagnosis. On the other hand, the presence of paroxysmal nocturnal hemoglobinuria–type cells[18] or dysplasia in erythroid lineage alone does not support the diagnosis of MDS alone.

Step II: Classification of Dysplasia

Table 2 shows a classification of dysplasias into category A (highly specific) and category B (less specific), which is the thrust of our proposal. Dysplasias in Table 2 are modified from those described in the WHO classification, except for the periodic acid-Schiff (PAS) reaction for erythroid cells because the PAS reaction is no longer used routinely

in hematologic laboratories. As described earlier, Pelger, Hypo-Gr, and mMgk are highly specific to MDS when present at levels ≥ 10%. In addition, we think that the diagnostic value of ringed sideroblasts (RS) is similarly specific when present at a level of ≥ 15%. Dysplasias other than Pelger, Hypo-Gr, mMgk, and RS are less specific for MDS but, if present at ≥ 10%, are sufficient to suggest a diagnosis of MDS. Therefore, we think that the classification of dysplasias for the diagnosis of MDS is necessary and helpful for clinical hematologists in general. Quantitative assessment of category A or category B dysplasias is a basis for grading the accuracy of diagnosis of MDS. Four types of category A dysplasias are shown in Figure 1 (A-D) and Table 2. Category B dysplasias are shown in Table 2.

Pelger are hyposegmented (dumbbell-shaped) mature neutrophils. Two lobes are connected with a fine or thin filament (Figure 1A), and their chromatin structure is abnormally coarse. Hypo-Gr are neutrophils with a total or > 80% loss of neutrophilic granules in the cytoplasm (Figure 1B). Micromegakaryocytes are mono- or bi-nulceated megakaryocytes with a size less than that of normal promyelocytes and abundant platelet granule formation (Figure 1C). Ringed sideroblasts are erythroid cells with perinuclear siderotic granules occupying > one third of the nuclear margin or > 5 distinct siderotic granules in the perinuclear region (Figure 1D).

Step III: Assessment of Category-A Dysplasias

For the assessment of Pelger and Hypo-Gr, ≥ 100 mature neutrophils should be examined on BM films. The frequencies of Pelger and Hypo-Gr should be evaluated individually, not the sum of Pelger or Hypo-Gr. Because BM films frequently fail to stain optimally for neutrophil granules, observation of PB films is very helpful in confirming degranulation. In particular, when Hypo-Gr is the sole dysplasia in the absence of other dysplastic features, the assessment of Hypo-Gr should not be evaluated as a positive finding unless confirmed as mentioned earlier. Concerning the frequency of mMgk, ≥ 25 megakaryocytes should be examined on multiple BM films. When the megakaryocyte number is markedly reduced, detection of ≥ 3 mMgks is sufficient to regard this category-A dysplasia as ≥ 10%. In almost all patients with AA, megakaryocytes are absent or very few in number. For RS, ≥ 100 erythroblasts of all stages should be examined. Independent assessment of category-A dysplasias is necessary for grading of diagnostic accuracy of MDS.

Step IV: Assessment of Dysplasia A + B in Each Lineage

Concerning the frequencies of dysplasia A + B in each lineage, we suggest the microscopic methods as follows: ≥ 100 mature neutrophils, ≥ 25 megakaryocytes, and ≥ 100 erythroblasts in BM should be examined. The frequency of dysplasia in each lineage is evaluated by total dysplastic cells (%) showing category A or B. The frequency of Dys E is evaluated by the sum of frequency of RS on iron-stained films and that of category B on May-Giemsa–stained films. For example, when the frequency of RS and that of category B in erythroid lineage are 5% and 10%, respectively, the frequency of Dys E is calculated as 15%. The microscopic examinations for the assessment of dysplasia should be performed with multiple BM films if necessary. If the megakaryocyte number is markedly reduced, detection of ≥ 3 dysplastic megakaryocytes is sufficient to regard dysplasia A + B as ≥ 10%.

Table 3 Grade of Dysplasia

Dysplasia Grade
High (Defined as 1 or 2)
1. Pelger ≥ 10% or Hypo-Gr ≥ 10% plus mMgk ≥ 10%
2. RS ≥ 15%
Intermediate
Dysplasia (category A or B) ≥ 10% in 2-3 lineages
Low
Dysplasia (category A or B) ≥ 10% in 1 lineage
Minimal
Dysplasia (category A or B) 1%-9% in 1-3 lineages

Step V: Grade of Dysplasia

As shown in Table 3, the grade of dysplasia is divided into high, intermediate, low, or minimal. High is defined as follows: (1) when Pelger ≥ 10% or Hypo-Gr ≥ 10% plus mMgk ≥ 10% in granulocytic and megakaryocytic lineages or (2) when RS ≥ 15% in erythroid series. In order to classify a case as high by the existence of RS ≥ 15% alone, other sideroblastic anemias such as alcoholic anemia must be excluded. Intermediate is defined as dysplasia A + B ≥ 10% in 2-3 lineages. Low is defined as dysplasia A + B ≥ 10% in a single lineage. Minimal is defined as 1%-9% of dysplasia A + B in 1-3 lineages.

Step VI: Division of Cytogenetic Findings

The divisions of cytogenetic findings are abnormal, normal, or unknown. Abnormal is defined as typical clonal abnormal karyotypes recurrently found in MDS (del[5q], –7/7q–, +8, del[20q], complex, and others) with high frequency as reported by Haase et al.[19] This definition is similar to that of typical chromosome abnormalities proposed by Valent et al.[12] t(8;21)(q22;q22), t(15;17)(q22;q12), inv(16)(p13;q22), and t(16;16)(p13;q22) are not included in the abnormal division even when the blast percentage is < 20%. Patients with these cytogenetic abnormalities are diagnosed with AML with recurrent cytogenetic abnormalities according to the WHO classification. Normal is defined as normal karyotype by analyzing > 10 metaphases. When cytogenetic findings are not available because of poor samples or an absence of metaphases, cases are labeled unknown.

Step VII: Grade of Diagnostic Accuracy

Table 4 shows the criteria for grading the diagnostic accuracy. These criteria are a combination of the frequency of blasts in BM, grade of dysplasia, and division of cytogenetics. The grade of diagnostic accuracy is divided into definite, probable, possible, and ICUS. The reliability of the diagnosis as MDS is high in the following order: definite, probable, and possible. In patients diagnosed as possible or ICUS, the diagnostic accuracy is low; thus, re-examination at suitable intervals is required to confirm the diagnosis. In such cases, the diagnosis might become more accurate when re-examination provides a result of definite or probable or remains possible or ICUS for a long period. The observation of the clinical course of patients with possible or ICUS will provide important information on the pathophysiologic similarity or dissimilarity between these diagnostic groups based on diagnostic grading.

Table 4 Grade of Diagnostic Accuracy for Myelodysplastic Syndromes

Grade	Blasts in BM (%)	Grade of Dysplasia	Division of Cytogenetics
	5-19	High, intermediate, low	Any
MDS Definite	0-4	High, intermediate, low	Abnormal
	0-4	High	Any
MDS Probable	0-4	Intermediate	Normal or unknown
MDS Possible	0-4	Low	Normal or unknown
ICUS	0-4	Minimal or none	Normal or unknown

Step VIII: Subtyping According to the World Health Organization Classification

Patients who are diagnosed as definite, probable, or possible should be classified according to the WHO classification. Patients classified in the possible category in our system are diagnosed as RA- or MDS-unclassified (refractory neutropenia or refractory thrombocytopenia) according to the WHO classification. However, the diagnosis of these patients should be tentative. The diagnosis according to the WHO classification of these patients must be decided by re-examination of BM at suitable intervals.

Discussion

Diagnosis of MDS must be as accurate and consistent as that of acute leukemia. However, the judgment of morphologic dysplasias has the inherent problem of the subjective nature of the morphology, and the objectivity of the evaluation has long been problematic. For the elimination or reduction of these problems, we propose a grading system for diagnostic accuracy of MDS. Category-A dysplasias are much easier for clinical hematologists to detect on routine BM diagnosis. Category-B dysplasias are sufficiently reliable when observed along with category A. Therefore, quantitative, morphologic evaluation by using this system will facilitate the routine diagnosis of MDS.

Exclusion of nonclonal disorders with minimal or no morphologic dysplasia is extremely important for the differential diagnosis of MDS as described in Step I. We believe our system is also useful in this respect. If there is no certain evidence for this exclusion diagnosis of non-MDS disorders despite the careful performance of other laboratory examinations, the possibility of misdiagnosis would likely be markedly reduced by using this grading system for diagnosis. Repeat BM examination at suitable intervals for patients graded as possible or ICUS will make clearer the still-vague margin of MDS as a clinical entity. It is also important to identify differences if present in responses to new drugs such as lenalidomide and hypomethylating agents.

Of course, our diagnostic schema still requires validation and demonstration of reliability, hopefully in 2 populations or a split-sample cohort. Long-term observation of MDS cases diagnosed with our proposal is also necessary for the evaluation of this proposal. Recent techniques in the detection of genetic abnormalities such as fluorescence in situ hybridization and single nucleotide polymorphism arrays[20] expand cytogenetic data of MDS. Although universal biomarkers for the diagnosis of MDS are still lacking, new data on genetic abnormality of MDS will be quite useful for accurate diagnosis and understanding the biology of MDS. In conclusion, until the discovery of universal biomarkers for entire MDS or subtypes of MDS, this diagnostic grading system could be useful for clinical routine work.

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Diagnosis and classification of myelodysplastic syndrome: International Working Group on Morphology of Myelodysplastic Syndrome (IWGM-MDS) consensus proposals for the definition and enumeration of myeloblasts and ring sideroblasts

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ABSTRACT

The classification of myelodysplastic syndromes is based on the morphological criteria proposed by the French–American–British (FAB) and World Health Organization (WHO) groups. Accurate enumeration of blast cells, although essential for diagnosis of myelodysplastic syndrome and for assignment to prognostic groups, is often difficult, due to imprecise criteria for the morphological definition of blasts and promyelocytes. An International Working Group on Morphology of Myelodysplastic Syndrome (IWGM-MDS) of hematopathologists and hematologists expert in the field of myelodysplastic syndrome reviewed the morphological features of bone marrows from all subtypes of myelodysplastic syndrome and agreed on a set of recommendations, including recommendations for the definition and enumeration of blast cells and ring sideroblasts. It is recommended that (1) agranular or granular blast cells be defined (replacing the previous type I, II and III blasts), (2) dysplastic promyelocytes be distinguished from cytologically normal promyelocytes and from granular blast cells, (3) sufficient cells be counted to give a precise blast percentage, particularly at thresholds that are important for diagnosis or prognosis and (4) ring sideroblasts be defined as erythroblasts in which there are a minimum of 5 siderotic granules covering at least a third of the nuclear circumference. Clear definitions and a differential count of a sufficient number of cells is likely to improve precision in the diagnosis and classification of myelodysplastic syndrome. Recommendations should be applied in the context of the WHO classification.

Key words: myelodysplastic syndrome, myelodysplastic syndrome, myeloblast, ring sideroblast.

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Introduction

The term myelodysplastic syndrome (MDS) is used to describe a heterogeneous group of disorders that are characterized by clonal and ineffective hematopoiesis, morphological dysplasia, peripheral blood cytopenias and progressive bone marrow failure. MDS transforms to acute myeloid leukemia (AML) in approximately 30% of cases. Survival following a diagnosis of MDS varies from a few months to more than ten years (comparable to age/sex matched normal popu-

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lations).[1] This highly variable prognosis underscores the importance of a classification system, supplemented by a prognostic index, to predict the survival of patients with MDS and the likelihood of transformation to AML. With the recent development and introduction of several effective treatment options for MDS,[2,3] the need for a classification system to predict responsiveness to treatment and clinical outcomes for individual patients has become even more important.

During the past 20 years, several MDS classification and prognostic scoring systems have been proposed.[4-7] Several of these systems have gained acceptance with the French-American-British (FAB) classification as modified by the World Health Organization (WHO), the International Prognostic Scoring System (IPSS) being the most widely used. In addition, the recent identification of transfusion burden and a modification of the IPSS by Malcovati and co-workers,[8] the so-called WPSS (World Prognostic Scoring System), have surfaced as an important component of our understanding of the natural history of MDS. Refinements in classification are needed as research continues to advance our knowledge of the etiology and the pathogenesis of MDS.

To address these issues, a panel of experts in the classification of MDS, the International Working Group on Morphology of MDS (IWGM-MDS) convened on three occasions in 2005/06 to review and refine the morphological criteria for the classification of MDS. This group consisted of both clinical hematologists and hematopathologists. The latter attended all three meetings and participated actively in the review and characterization of many individual cases (slide review). The former provided clinical input as to the relevance of the precise determination of morphological cell types in the assessment of patients with MDS. This model has been utilized with success in the development of the 2008 WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues.

The goals of this Working Group were to (i) define minimal diagnostic criteria for MDS; (ii) develop standardized definitions for myeloblasts and promyelocytes; and (iii) propose a classification for sideroblasts. This article details the proposals of the IWGM-MDS for the definition of myeloblasts, promyelocytes and ring sideroblasts. Proposals for minimal diagnostic criteria and for dealing with cases of possible MDS that do not meet these criteria are dealt with in an accompanying paper.[9]

Background: current MDS classification systems

Several classification systems have been developed to predict survival or transition to AML following MDS diagnosis. The first of these, the FAB system, was introduced in 1982 and is based on the percentage of blasts and morphological dysplastic features of blood and bone marrow.[4] According to this system, patients are diagnosed with MDS when dysplastic bone marrow hematopoiesis is present and/or myeloblasts are between 5 and 30% of all bone marrow cells. The FAB system served as the standard for MDS classification for two decades and provided considerable prognostic information. Nonetheless, the clinical outcomes of patients assigned to the same MDS subgroup remain too variable to accurately predict survival or transformation to AML in individual patients.

The International Prognostic Scoring System[5,6] provided a prospective risk assessment from the initial diagnosis but was dependent on having both an accurate bone marrow blast assessment and cytogenetic analysis. Increasing blast percentages[5-10,11-20,20-30] indicated an increase in the risk of leukemic transformation and of death from all causes. Therefore, an accurate definition of the blast percentage and separation of blast cells from promyelocytes is critical.

In 2001, the WHO[7] proposed a revision of the FAB morphological approach. The revisions included lowering the threshold for the percentage of blasts required to make the diagnosis of AML from 30% to 20%, thus elimination of the MDS subcategory of refractory anemia with excess blasts in transformation (RAEB-T). In addition, chronic myelomonocytic leukemia (CMML) was reclassified from a subcategory of MDS to a subcategory of myelodysplastic/myeloproliferative disorder.

Of considerable importance was the introduction of a new subtype: refractory anemia with multilineage dysplasia without ring sideroblasts (RCMD) or with ring sideroblasts (RCMD-RS) with ≥10% of dysplasia in at least two cell lines, and refractory anemia (RA) or refractory anemia with ring sideroblasts (RARS) with dysplasia restricted to the erythroid lineage. The precise qualitative features of the dysplastic cells were described with several illustrations, particularly of the erythroid dysplasia.

Developing a classification system

All of the classification systems described above depend on an assessment of dysplastic changes in the marrow. In addition, recognition and enumeration of blast cells is of critical importance both in the diagnosis of AML and MDS, and for stratifying MDS patients into prognostic groups.[10,11] According to the IPSS, for example, patients with -10% bone marrow blast cells would be assigned to the intermediate 1 or 2 risk groups, and would have a worse prognosis than low-risk patients.[5]

It is often assumed that definitions of blast cells are applied uniformly by hematologists/pathologists worldwide, and that blast cells could be identified and counted very easily. Unfortunately this is not so. The FAB group defined type I and type II blast cells, both having a high nucleocytoplasmic ratio, a diffuse chromatin pattern and usually visible nucleoli; type I blast cells are agranular and type II have scanty granules.[4] Subsequently Goasguen and colleagues analyzed bone marrow smears obtained from 18 patients with MDS classified according to the FAB classification and defined a type III blast, with more than 20 fine azurophilic granules but otherwise with the characteristics expected of a blast cell.[12] In the FAB classification such cells were categorized as promyelocytes. The inclusion of type III blast cells in the blast cell count led to 7 patients (39%) being reclassified from refractory anemia with excess of blasts (RAEB) to RAEB-T and was found to give a better separation of survival curves of different FAB categories of MDS. Despite the ability of this classification system

to refine survival estimates for patients with MDS it was unclear how often type III blasts have been utilized in myelograms in typical clinical practice. Subsequent to this publication protocols for some clinical trials have included type III blast cells in the blast cell count but this has not been universal practice. The WHO classification does not give any specific recommendations for the definition of blast cells.[7]

In practice, although FAB type I and type II blasts can generally be readily distinguished from each other it has proved difficult to distinguish FAB type II blasts from type III blasts. In addition, the enumeration of promyelocytes, which are often abnormal in MDS, remains problematic and their separation from type II and type III blasts has remained imprecise.

Statistical analysis

Concordance was determined using the κ statistics.[18]

Results

The Working Group participants reviewed previous attempts to define blasts (agranular *vs.* granular) and promyelocytes. Each member of the group was asked to bring blood and bone marrow slides obtained from patients with various subtypes of MDS and/or AML that would serve as the basis for discussion of the identification of different types of blasts and promyelocytes. Myelograms were determined from these slides, and the data were captured and recorded electronically for subsequent statistical analysis. The starting point for developing definitions was the 1991 paper by Goasguen and colleagues.[12] In addition, criteria for separating granular blast cells from promyelocytes were developed.

Definition of myeloblasts

After a review of the literature, assessment of blood and bone marrow films individually and collectively, and much discussion, the participants arrived at a consensus regarding the definition of a myeloblast. Myeloblasts were defined in terms of several nuclear characteristics, including a high nuclear/cytoplasmic ratio, easily visible nucleoli and usually, but not invariably, fine nuclear chromatin. Nuclear shape is variable. Cytoplasmic characteristics include variable cytoplasmic basophilia; there may or may not be granules or Auer rods but no Golgi zone is detected (Figure 1). The exception to this last observation may be seen in cases of AML with t(8;21) where there may be blast cells with a small distinct Golgi, with or without an Auer rod, but with no other features of a promyelocyte. After reviewing all the available bone marrow smears, the IWGM group recommended that myeloblasts in MDS should be classified as agranular or granular. The agranular blasts correspond to the type I blasts of the FAB classification. Granular blasts are cells that have the nuclear features of blast cells but also have cytoplasmic granules. These cells will thus include type II blasts as defined by FAB, as well as type III blasts as defined by Goasguen *et al.*[12]

Granular blasts must be distinguished from promyelocytes (*see below*).

Promyelocytes

The group discussed the morphological features that define normal promyelocytes. Nuclear characteristics of normal promyelocytes included a central or eccentric nucleus and chromatin, which may still be fine or may be intermediate. The nucleolus is usually easily visible and prominent (Figure 1). The group determined that the principal distinguishing characteristic of the normal promyelocyte was the presence of a visible Golgi zone. Other cytoplasmic characteristics include uniformly dispersed azurophilic granules, and in most instances basophilic cytoplasm. Dysplastic promyelocytes have the recognizable features of a promyelocyte including a round, oval, or indented nucleus that is often eccentric, a Golgi zone (at least faintly visible) and a nucleus with fine or coarse chromatin and an easily visible nucleolus. Abnormal features that lead to recognition of promyelocytes as being dysplastic include reduced or irregular cytoplasmic basophilia, a poorly developed Golgi zone, hypergranularity, hypogranularity and irregular distribution (clumps) of granules.

The group agreed, therefore, on the following morphological categories: normal promyelocytes, blasts (which are differentiated as simply agranular or granu-

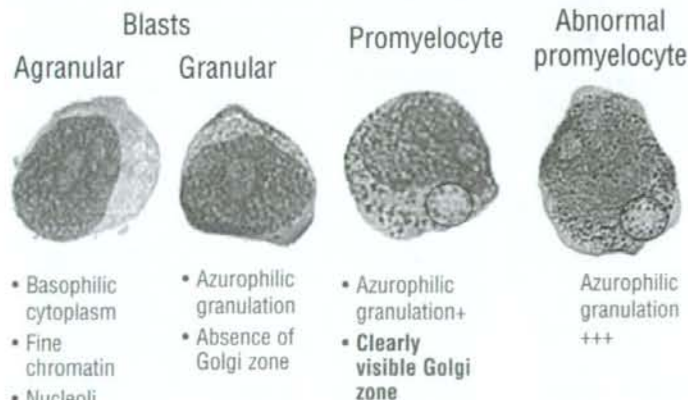

Figure 1. Blasts, promyelocytes, abnormal promyelocytes.

lar, irrespective of the number of granules) and dysplastic promyelocytes. To verify the reproducibility of these propositions five experts were asked to review 264 consecutive cells from one case of AML (FAB-M2). The pictures were captured utilizing a unique digital image, capable of merging multiple consecutive fields (600x800 pixels). Each observer performed the task on his/her own computer by downloading the file from a dedicated website of the MDS Foundation. A drop-down menu was provided with the following choices: blasts (agranular and granular), promyelocytes (normal or abnormal), mature granulocytes, others (Figure 2). Results were sent electronically to the MDS Foundation headquarters and analyzed by JG.[14]

Individual assessments can be seen in Table 1. If we consider that a very good concordance would be agreement of 5/5 or 4/5 experts, then an 89.4% concordance was achieved in separating blasts from promyelocytes. Examining the data with kappa statistics demonstrated a high concordance when viewing one observer versus another (Table 2).

It should also be noted that when performing a marrow differential count, the myeloblast percentage should be determined by counting at least 500 nucleated cells, with the total including at least 100 nonerythroid cells to improve precision. The Working Group emphasized the use of this number to be extremely important for correct classification of patients with MDS, especially when cells of the erythroid lineage exceed 50%. Other methods of determining the myeloblast percentage may result in some patients being classified incorrectly. Counting an adequate number of cells is of critical importance for the classification of patients whose blast counts fall near the boundary between MDS categories of different prognostic significance. It is similarly essential to perform a 500-differential count on the blood film of patients with circulating blast cells since relatively small differences in the percentage of blast cells are of prognostic significance; the 2008 WHO classification assigns patients to different MDS categories with a blast count of less than 1%, 1%, 2-4% or 5%.[15]

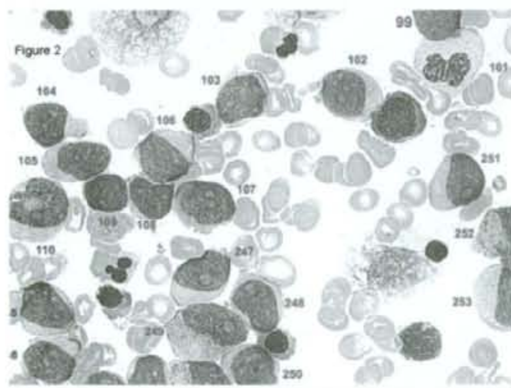

Figure 2. Example of individual cells counted by the experts.

Ring sideroblasts

The prognosis of patients with pure sideroblastic anemia may differ from that of patients with non-sideroblastic anemia; therefore, clear, standardized definitions of sideroblast types are necessary. Varying definitions of ring sideroblasts have led to confusion and controversy among clinicians. Early investigators defined ring sideroblasts as having iron granules in a perinuclear distribution surrounding the entire nucleus. Other investigators have required that perinuclear granules encircle at least one third of the perinuclear area, but not necessarily the entire nucleus.[16] Ringed sideroblasts were sometimes required to have a minimum of 5 granules and sometimes a minimum of 10 granules.

After a review of many cases of sideroblastic anemia, the group determined that ring sideroblasts should have at least 5 granules in a perinuclear distribution; that these granules could either surround the entire nucleus, be localized to portions of the perinuclear area or cover at least one third of the nucleus (Figures 3 and 4).

Table 1. **Agreement of the expert panel. (A) Light grey bar: Maturing Granulocytes. (B) Medium grey bar: Promyelocytes. (C) Black bar: Blast Cells.**

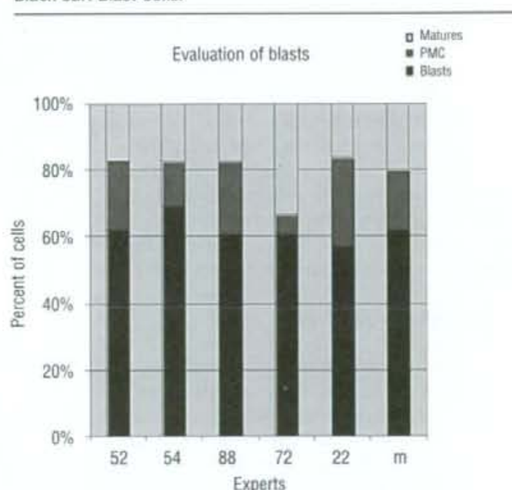

Table 2. **Degree of consistency among experts: percent agreement/unweighted κ coefficients for pairs of 5 readers based on 264 cells divided into 4 categories (blasts, promyelocytes, matures, others).**

Expert ref #	52	54	88	72	22
52	1	0.77/0.57	0.80/0.63	0.72/0.51	0.82/0.68
54	–	1	0.78/0.61	0.76/0.56	0.75/0.56
88	–		1	0.76/0.59	0.85/0.74
72	–	–	–	1	0.72/0.55
22	–	–	–	–	1

Consistency among readers is evaluated by percentage of agreement (first value) and the unweighted kappa coefficient (second value) for all pairs of readers. Conclusion: Percent agreement varies from 0.72 (pair (72×52) to 0.85 (pair 22×88) demonstrating a high concordance rate between experts. When adjusted for chance agreement, however, the κ values were somewhat lower indicating less than optimal agreement in some cases. The adjustment for chance agreement is influenced by small numbers of cells in two categories.

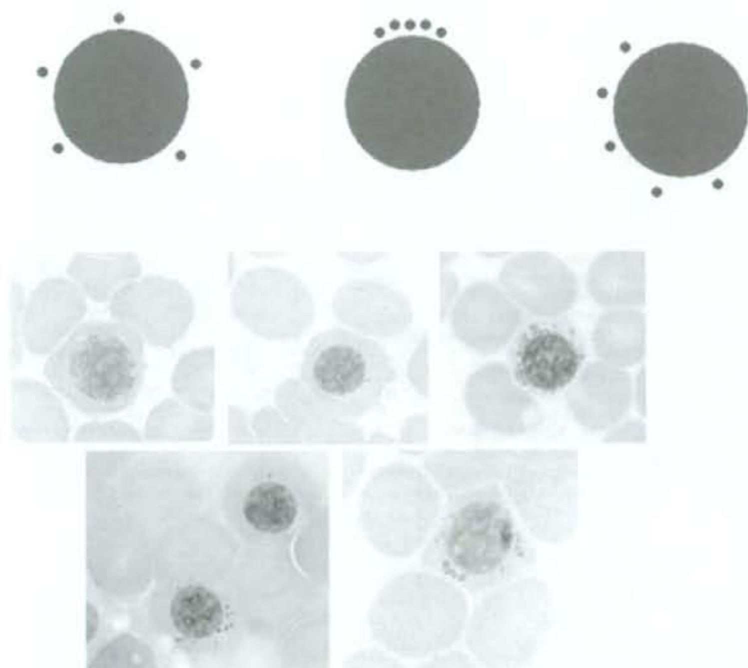

Figure 3. Perinuclear siderotic granules (cartoon of potential examples).

Figure 4. Prussian blue reaction of erythroid precursors. a. Upper Panel (left to right): no siderotic granules; type 1 sideroblast (1 granule); type 3 sideroblast (numerous granules). b. Lower panel (left to right): type 1 sideroblast (upper cell), type 2 sideroblast (lower cell); type 3 sideroblasts (lower right); Hematoxylin counter stain; Prussian Blue reaction.

The Working Group defined three types of sideroblast: Type 1 sideroblasts: fewer than 5 siderotic granules in the cytoplasm; Type 2 sideroblasts: 5 or more siderotic granules, but not in a perinuclear distribution; Type 3 or ring sideroblasts: 5 or more granules in a perinuclear position, surrounding the nucleus or encompassing at least one third of the nuclear circumference.

The group recommends that when counting ring sideroblasts all stages of erythroid precursors be counted and should include at least 100 nucleated erythroid precursors and for the definition of RARS the required number of ring sideroblasts remains at 15% as defined previously in the FAB and WHO classifications. The definition of a ring sideroblast proposed by the IWGMDS (an erythroblast with at least 5 siderotic granules covering at least a third of the circumference of the nucleus) has been incorporated into the 2008 WHO classification of Tumours of Haematopoietic and Lymphoid Tissues.[15]

The group also addressed the nuclear counterstain used to optimize the distinction of erythroid cells. The Working Group discussed the value of a number of stains such as neutral red, basic fuchsin, saffronin, hematoxylin, and light Giemsa, as well as staining for H-type ferritin and polyclonal antibody staining for siderotic granules. The group considered that further studies were needed to assess the value of these counterstains and methods but agreed that all had merits.

Only type 3 sideroblasts would qualify as *ring* sideroblasts to separate sideroblastic from non-sideroblastic anemia. This proposal will be tested in a similar manner to the blast definition by developing a web based digital image of multiple types of sideroblasts.

Discussion

In the absence of biological markers to stratify patients with MDS morphological assessment is essential for defining risk, regardless of which risk system is utilized. Because of the importance of determining the percentage of blasts as well as of ring sideroblasts, the IWGM-MDS focused on careful definitions that are illustrated and confirmed to be reproducible. The proposed definitions are intended to be used in conjunction with the WHO classification in order to make the categorization of patients with MDS more precise.

Authorship and Disclosures

GJM, JMB, JG, BJB, RB and TV reviewed the bone marrow preparations in the workshops and carried out the review of the digital images; GJM, JMB and BJB prepared the final manuscript; IB, MC, PF, UG, EH-L, IJ, AM, AkM, CMN, GS, MT and AY reviewed some of the material and contributed to the general discussions and all gave approval to the manuscript.

The authors reported no potential conflicts of interest.

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ICSH guidelines for the standardization of bone marrow specimens and reports

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Standardization, bone marrow, aspirate, trephine biopsy, report

SUMMARY

The bone marrow examination is an essential investigation for the diagnosis and management of many disorders of the blood and bone marrow. The aspirate and trephine biopsy specimens are complementary and when both are obtained, they provide a comprehensive evaluation of the bone marrow. The final interpretation requires the integration of peripheral blood, bone marrow aspirate and trephine biopsy findings, together with the results of supplementary tests such as immunophenotyping, cytogenetic analysis and molecular genetic studies as appropriate, in the context of clinical and other diagnostic findings. Methods for the preparation, processing and reporting of bone marrow aspirates and trephine biopsy specimens can vary considerably. These differences may result in inconsistencies in disease diagnosis or classification that may affect treatment and clinical outcomes. In recognition of the need for standardization in this area, an international Working Party for the Standardization of Bone Marrow Specimens and Reports was formed by the International Council for Standardization in Hematology (ICSH) to prepare a set of guidelines based on preferred best practices. The guidelines were discussed at the ICSH General Assemblies and reviewed by an international panel of experts to achieve further consensus.

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