blood

2012 119: 1036-1044
Prepublished online November 2, 2011;
doi:10.1182/blood-2011-06-361907

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

Enucleation of human erythroblasts involves non-muscle myosin IIB

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Mammalian erythroblasts undergo enucleation, a process thought to be similar to cytokinesis. Although an assemblage of actin, non-muscle myosin II, and several other proteins is crucial for proper cytokinesis, the role of non-muscle myosin II in enucleation remains unclear. In this study, we investigated the effect of various cell-division inhibitors on cytokinesis and enucleation. For this purpose, we used human colony-forming unit-erythroid (CFU-E) and mature erythroblasts generated from purified CD34$^+$ cells as target cells for cytokinesis and enucleation assay, respectively. Here we show that the inhibition of myosin by blebbistatin, an inhibitor of non-muscle myosin II ATPase, blocks both cell division and enucleation, which suggests that non-muscle myosin II plays an essential role not only in cytokinesis but also in enucleation. When the function of non-muscle myosin heavy chain (NMHC) IIA or IIB was inhibited by an exogenous expression of myosin rod fragment, myosin IIA or IIB, each rod fragment blocked the proliferation of CFU-E but only the rod fragment for IIB inhibited the enucleation of mature erythroblasts. These data indicate that NMHC IIB among the isoforms is involved in the enucleation of human erythroblasts. (*Blood.* 2012;119(4):1036-1044)

Introduction

During erythropoiesis, stem cells undergo lineage specific commitment and generate erythroid progenitor cells through cellular division events including nuclear (mitosis) and cytoplasmic (cytokinesis) division. These progenitor cells consist of immature and mature erythroid progenitors, the burst-forming unit-erythroid (BFU-E) and the colony-forming unit-erythroid (CFU-E), respectively. The BFU-E can be considered as a progenitor of the CFU-E. Indeed, after 6 to 7 days in culture, cells generated from human BFU-E have all the functional characteristics of CFU-E[1]. After an additional 6 to 7 days in culture, human CFU-E proliferate and differentiate into mature erythroblasts.[1] Terminally differentiated erythroblasts in mammals expel their nuclei via a process termed enucleation, becoming reticulocytes and subsequently mature erythrocytes. The nucleus separates from the remainder of the cell and is phagocytosed by reticular cells such as macrophages (for a review, see Chasis et al[2]).

Enucleation of erythroblasts is thought to occur through a process similar to cytokinesis. Several general principles apply to cytokinesis. Firstly, the microtubule cytoskeleton plays an important role in both the choice and positioning of the division site. Once this site is chosen, the local assembly of the actomyosin contractile ring remodels the plasma membrane. Finally, membrane trafficking to, and membrane fusion at the division site result in the physical separation of the daughter cells, a process termed abscission (for reviews, see Barr et al[3] and Glotzer et al[4]). Although modulation of the actomyosin cytoskeleton is crucial for proper cytokinesis, there is a paucity of information regarding how non-muscle myosin II contributes to enucleation.

Several investigations have studied the molecular mechanisms underlying the enucleation of mammalian erythroblasts. Koury et al used murine splenic erythroblasts infected with the anemia-inducing strain of Friend-virus (FVA cells), and demonstrated that filamentous actin (F-actin) accumulated in the contractile ring.[5] They also showed that the treatment of FVA cells with cytochalasin D blocked nuclear extrusion, while the addition of colchicine, vinblastine or taxol did not affect enucleation.[5] Based on these findings, they concluded that F-actin plays an important role in enucleation, while microtubules do not. It has also been shown that Rac 1 GTPases and their downstream effector mDia2 play important roles in the cytoskeletal reorganization that leads to the extrusion of the pycnotic nucleus from late-stage erythroblasts.[6] Recently, important roles for Myc,[7] Claudin 13[8] (a member of the Claudin family of tight junction proteins), histone deacetylase 2,[9] and membrane trafficking[10] have been reported in the regulation of terminal maturation in mammalian erythroid cells.

Non-muscle myosin II is a major cytoskeletal protein that interacts with actin to contribute to cellular processes such as cell migration,[11] cell adhesion,[12] and cytokinesis.[13] In mammals there are 3 non-muscle myosin II isoforms, each composed of one pair of heavy chains and 2 pairs of light chains. Three separate genes (*Myh 9, Myh10, and Myh 14*) encode the non-muscle myosin heavy chains (NMHCs; NMHC IIA, IIB, and IIC) in chromosomes 22q11.2, 17p13, and 19q13, respectively.[14-16] These isoforms share considerable homology and some overlapping functions, yet they exhibit differences in enzymatic properties, subcellular localization, molecular interaction and tissue distribution (for a review, see Even-Ram et al[17]). The mechanistic knowledge about non-muscle myosin II isoforms in enucleation may help us to explore the unknown NMHC abnormalities in hematologic disorders. Indeed, deficiency of non-muscle myosin II isoform can cause human disease. The 4 main autosomal dominant disorders are related to

Submitted June 17, 2011; accepted October 20, 2011. Prepublished online as *Blood* First Edition paper, November 2, 2011; DOI 10.1182/blood-2011-06-361907.

An Inside *Blood* analysis of this article appears at the front of this issue.

The online version of this article contains a data supplement.

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mutations in *Myh 9*, the gene for the NMHC IIA: May-Hegglin, Fechtner, Sebastian and Epstein (for a review, see Kunishima et al[18]). The common feature in all 4 syndromes is macrothrombocytopenia, and granulocyte inclusion bodies characterize the first 3 syndromes. Some patients later show onset of deafness, cataracts, and glomerulonephritis.[18]

In this study, we investigated the role of myosin in human erythroblast enucleation in association with other cytoskeletal molecules. The efficacy of inhibitors for cell division often varies depending on the species of the cell observed and their redundancy in the cells themselves.[19] Therefore, efficient inhibitors of cell division in human primary erythroid cells were selected using human CFU-E generated from purified $CD34^+$ cells to clarify the possible efficacy of these inhibitors on the enucleation of erythroblasts. Here we show that the inhibition of non-muscle myosin II ATPase by blebbistatin completely blocks enucleation of human erythroblasts. When the function of NMHC IIA or IIB was inhibited by an exogenous expression of myosin rod fragment, both rod fragments blocked the proliferation of CFU-E and the rod fragment for IIB inhibited the enucleation of mature erythroblasts. These data indicate that the enucleation of human erythroblasts involves non-muscle myosin IIB.

Methods

Reagents and antibodies

BSA, IMDM and propidium iodide (PI) were purchased from Sigma-Aldrich. FCS, penicillin and streptomycin were obtained from Flow Laboratories Inc. Insulin (porcine sodium, activity 28.9 U/mg) was obtained from Wako Pure Chemical Industries. IL-3 and SCF were kind gifts from Kirin Brewery Co Ltd, and erythropoietin (EPO) and G-CSF were from Chugai Pharmaceutical Co. Vitamin B_{12} was purchased from Eisai Co Ltd and folic acid was from Takeda Pharmaceutical Co Ltd. Triton X-100 was obtained from Wako Pure Chemical Industries. Diaminobenzidine-substrate chromogen and fuchsin-substrate chromogen systems were from Dako. RNase (Type III-A) was from Sigma-Aldrich. MACS MicroBeads for Indirect Magnetic Labeling was from Miltenyi Biotec. FITC-labeled and phycoerythrin (PE)–labeled antibodies for glycophorin A (GPA; JC159) were from Dako. Alexa Fluor 488– or Alexa Fluor 546–conjugated goat IgG directed against rabbit and mouse IgG were from Molecular Probes. Fc-blocking antibody (anti-CD16/32, clone: 93) was from eBioscience. Normal mouse and goat sera and rabbit immunoglobulins were from CellSignaling Technology. Alexa Fluor 488 phalloidin was from Invitrogen. Rabbit polyclonal antibody to non-muscle myosin IIA was from Sigma-Aldrich or Covance. Rabbit polyclonal antibody to non-muscle myosin IIB was from Abcam or Cell Signaling Technology. Mouse monoclonal antibody to tublin-α was from Neo Markers. Goat anti–mouse IgG-HRP was from Santa Cruz Biotechnology Inc. Anti–rabbit IgG, HRP-linked antibody and myosin light chain 2 antibody sampler kit were from Cell Signaling Technology. Amersham ECL Plus Western Blotting detection reagents were from GE Healthcare. Novex Sharp protein standard and NuPAGE Novex Bis-Tris mini gels were from Invitrogen.

Cell division inhibitors

Blebbistatin, a small molecule inhibitor that shows a high affinity and selectivity toward non-muscle myosin II ATPase,[20] and NSC23766, a specific inhibitor of Rac1 GTPases[21] that is known to inhibit enucleation,[6] were purchased from Calbiochem. Cytochalasin D, an inhibitor of actin polymerization that is known to inhibit enucleation,[5] and colchicine, an inhibitor of microtubules that are known to not be directly involved in enucleation[5] (for a review, see Wilson et al[22]), were purchased from Sigma-Aldrich. Y27632, a well-established inhibitor of ROCK that is a regulator of myosin phosphorylation,[23,24] was purchased from Enzo Life Sciences, Inc. Monastrol, an inhibitor of kinesin Eg5 which is a microtubule-based motor that plays a critical role in mitosis as it mediates centrosome separation and bipolar spindle assembly and maintenance,[25] was from Merck.

Cell preparations

G-CSF mobilized human peripheral blood $CD34^+$ cells were purified from healthy volunteers as described previously,[26] and stored in liquid nitrogen until required. Informed consent was obtained from each subject before their entry into this study, and the study was pre-approved by the Akita University Graduate School of Medicine Committee for the Protection of Human Subjects.

For the generation of erythroid progenitor cells, $CD34^+$ cells were thawed and prepared for culture as previously described.[27] Cells were cultured in IMDM erythroid medium containing 20% FBS, 10% heat-inactivated pooled human AB serum, 1% BSA, 10 μg/mL insulin, 0.5 μg/mL vitamin B_{12}, 15 μg/mL folic acid, 50nM β-mercaptoethanol (β-ME), 50 U/mL penicillin and 50 μg/mL streptomycin in the presence of 50 ng/mL IL-3, 50 ng/mL SCF and 2 IU/mL EPO. Cells were maintained at 37°C in a 5% CO_2 incubator as described previously.[27] After 7 days in culture, cells (day 7 cells, D7) were harvested and washed 3 times with IMDM containing 0.1% BSA and stored at 4°C until required. The maturation levels of the day 7 cells were found to be similar to that of the colony-forming unit-erythroid (CFU-E), a finding that has been reported elsewhere.[27,28] As a result, day 7 cells are described as CFU-E throughout this report.

Aliquots of CFU-E were also cultured in the erythroid medium with EPO alone, but without β-ME, SCF and IL-3, and then cultured for additional days to induce differentiation with or without various inhibitors of cell division. The volume of inhibitors, H_2O, PBS and DMSO vehicle solutions was fixed to 5% of the erythroid medium. The final concentration of DMSO in the erythroid medium was fixed to 0.2% (vol/vol) as DMSO over this concentration is known to be toxic for human erythroid progenitors. The cells were harvested at various time points, washed 3 times with IMDM containing 0.1% BSA, resuspended in IMDM containing 0.1% BSA and stored at 4°C until required.

For the evaluation of the enucleation ratio, the cells were spun onto slides using a Cytospin 3 (Shandon Lipshaw Inc) and stained with May-Grünwald-Giemsa or o-dianisidine (for hemoglobin staining) and hematoxylin. Enucleation was defined as the expulsion of the nucleus to the outside of the reticulocyte. Reticulocytes that are touching expulsed nuclei or that have thin, connecting strand of cellular material between the reticulocyte and the nucleus were also considered the earliest cell that has enucleated (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). The enucleation ratio of these cytospun cells was similar to that of cells prepared without mechanical force.[1] The enucleation ratio was calculated as [*Enucleation ratio* = erythrocytes/(erythrocytes+erythroblasts)] × 100% and by counting 300 cells including erythrocytes and erythroblasts on each slide. Triplicate cultures were used at each time point. The yield and viability were measured by dye exclusion using 0.2% trypan blue dye and a hemocytometer.

Cell cycle distribution

Cells were harvested, washed with cold PBS and fixed in 70% ethanol. The cells were then stored at −20°C until analysis. The fixed cells were centrifuged at 200*g*, washed with cold PBS twice, and RNase A was added at a final concentration of 0.5 mg/mL. The cells were then incubated for 10 minutes at 37°C. Next, 25 μg/mL PI was added and the cells were incubated for 30 minutes at room temperature in the dark. The cells were analyzed using a FACS Calibur instrument (BD Biosciences) equipped with CellQuest 3.3 software as reported previously.[29] Multicycle Version 4.0.0.4 cell cycle analysis software (Beckman Coulter) was used to determine the percentages of cells in the different cell cycle phases.

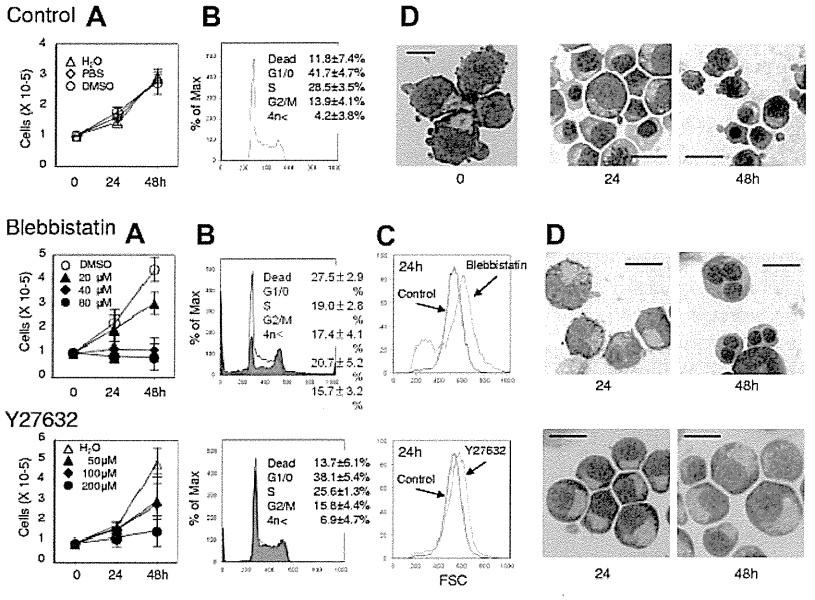

Figure 1. Myosin inhibitors block cell division of human CFU-E. Human CFU-E generated from purified CD34$^+$ cells were cultured for the indicated periods in the presence of EPO with or without various concentrations of inhibitors for non-muscle myosin II ATPase (blebbistatin) and a myosin activator Rho kinase (Y27632). (A) Effects of inhibitors and vehicle on the proliferation of CFU-E. Results are presented as the mean ± SD of 3 independent experiments. (B) Cell cycle analysis of cells cultured for 24 hours with (red areas) or without (solid lines) 80μM blebbistatin and 200μM Y27632. A representative result of 3 independent experiments is shown and is presented as the mean ± SD. (C) Size distribution analysis of CFU-E before (0 hours) and after 24 hours culture (red) with 80μM blebbistatin (green, middle panel) and 200μM Y27632 (green, bottom panel). A representative result of 3 independent experiments is shown. (D) May-Grünwald-Giemsa staining of cells cultured for 24 and 48 hours with or without 80μM blebbistatin and 200μM Y27632. A representative result of 3 independent experiments is shown. Scale bar = 10 μm.

Confocal microscopy

Fluorescence staining was imaged using a Confocal Laser Scanning Microscope 510 (LSM510; Carl Zeiss Microscope Systems) equipped with a 100× objective lens and a 10× camera lens (Carl Zeiss Microscope Systems) at zoom 3, as reported elsewhere.[29] Fluorochromes were excited using an argon laser at 488 nm for Alexa 488. Detector slits were configured to minimize cross talk between channels and processed using a software package (LSM510 Version 3.2) and Adobe Photoshop (Adobe Systems).

Construction of plasmid DNA

The myosin II tailpiece determines its paracrystal structure, filament assembly properties, and cellular localization.[30] To study the role of non-muscle myosin II isoforms in enucleation of human erythroblasts by the exogenous expression of their rod fragments, we constructed the plasmids encoding the rod fragments of the 2 NMHC-II isoforms as N-terminal GFP-fused protein. DNA fragments encoding Leu 1666 - Glu 1961 of NMHC IIA (ARF296) and Phe 1672–Glu 1976 of NMHC IIB (BRF305) were amplified by PCR as described elsewhere.[31,32] Each of them was subcloned into the *Hin*dIII-BamH1 sites of pEGFP-C3 (Clontech) to generate the pEGFP-ARF296 and pEGFP-BRF305, respectively.

Transfection

Transfected cells were obtained with the Amaxa nucleofection method. The Amaxa Nucleofector system (Lonza Cologne AG) was used as described by the manufacturer. Briefly, a pellet of 2-5 × 10^6 cells in 100 μL of Amaxa Human CD34$^+$ Cell Nucleofector solution wasmixed with 5 μg of pEGFP plasmid and subjected to nucleofection with a specific predefined program (Program U-008). After 24 hours of incubation, the cells were harvested and washed twice with IMDM containing 0.3% BSA. The dead cells were removed using an Annexin V MicroBead Kit (Miltenyi Biotec) and then incubated in the erythroid medium. GFP-positive cells were collected using a cell sorter (Dako Cytomation MoFlo).

Western blot analysis

CD34$^+$ cells were incubated in erythroid medium for the indicated days (day 7, 10-12 cells). Western blot analysis was carried out according to the manufacturer's protocol (Invitrogen).

Statistical analysis

Statistical analysis was performed using the Student *t* test for parametric data and the Mann-Whitney test for nonparametric data. A 2-tailed *P* value < .05 was accepted as statistically significant.

Results

Myosin inhibitors block cell division of human CFU-E

In this study, efficient inhibitors of cell division in human primary erythroid cells were selected using human CFU-E generated from purified CD34$^+$ cells (Figures 1-2). As cellular division consists of both mitotic and cytokinetic events, and given that the inhibition of either step should block cell proliferation, efficient inhibitors were defined as those that blocked the proliferation of CFU-E.

Figure 1A presents a time course analysis of the total cell number when human CFU-E were cultured with or without various concentrations of blebbistatin and Y27632. Blebbistatin and Y27632 were found to inhibit CFU-E proliferation in a dose-dependent manner. Cell cycle analysis demonstrated that blebbistatin decreased the number of cells in the G1/G0/S phase and resulted in the accumulation of cells in the G2/M phase, a finding that was accompanied by an increase in dead cells (Figure 1B). In addition, an increase in cells with high DNA content (Figure 1B) and an increase in larger erythroid progenitors compared with control cells (Figure 1C) were observed, suggesting the presence of multinucleated cells. This finding was confirmed by morphologic analysis (Figures 1D and 3). These data indicate that non-muscle myosin II ATPase is essential for the cellular division of CFU-E.

In contrast, Y27632 slightly increased the number of cells in the G2/M phase, generated larger cells compared with the control cells (Figure 1C) and produced multinucleated cells from 0.7 ± 1.2% to 5.7 ± 1.2% during 48 hours of incubation (n = 3, $P < .01$; Figures 1D and 3), albeit to a lesser extent than that observed in the presence of blebbistatin (from 0.7 ± 1.2% to 71.0 ± 6.1%; n = 3, $P < .01$; Figure 3). Western blot analysis showed that Y27632 does not affect the myosin light chain 2 (MLC2) expression, a downstream molecule of ROCK (a regulator of myosin phosphorylation[23,24]), but reduces the phosphorylation of MLC2 at Thr18/Ser19 in CFU-E, with a statistical significance (supplemental Figure 2). Given that the blocking of non-muscle myosin II ATPase and ROCK showed complete inhibition of the proliferative capacity of

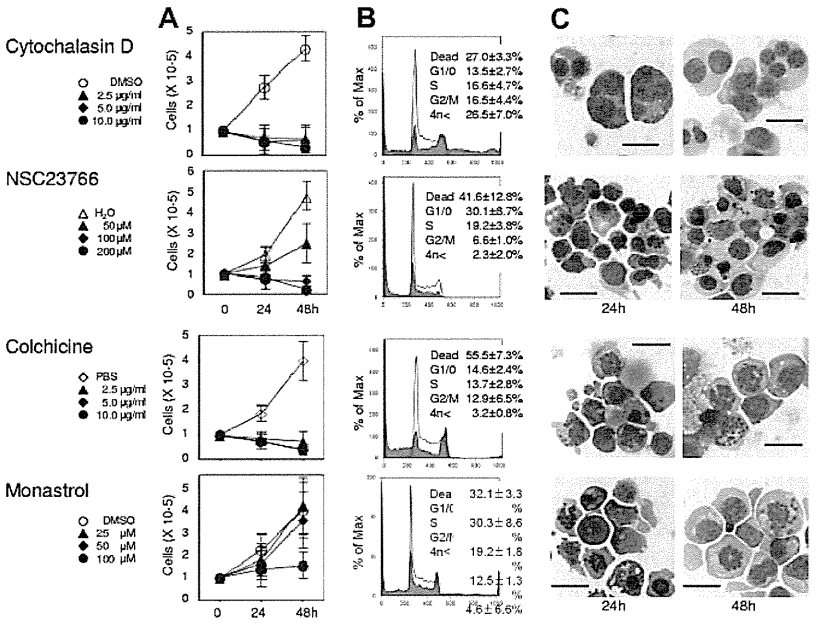

Figure 2. Actin, tubulin, and Eg5 inhibitors block cell division of human CFU-E. Human CFU-E were cultured for the indicated periods in the presence of EPO with or without various concentrations of inhibitors for actin polymerization (cytochalasin D and NSC23766), tubulin (colchicine) and Eg5 (monastrol). (A) Effects of inhibitors and vehicle on the proliferation of CFU-E. Results presented are the mean ± SD of 3 independent experiments. (B) Cell cycle analysis of cells cultured for 24 hours with (red areas) or without (solid lines) 10 μg/mL cytochalasin D, 200μM NSC23766, 10 μg/mL colchicine and 100μM monastrol. A representative result of 3 independent experiments is shown and is presented as the mean ± SD (C) May-Grünwald-Giemsa staining of cells cultured for 24 and 48 hours with or without 10 μg/mL cytochalasin D, 200μM NSC23766, 10 μg/mL colchicine and 100μM monastrol. A representative result of 3 independent experiments is shown. Scale bar = 10 μm.

CFU-E, non-muscle myosin II is suggested to be involved in cell division of human erythroid progenitor cells.

Actin, tubulin, and Eg5 inhibitors block cell division of human CFU-E

Although Koury et al have clearly shown that F-actin plays an important role in enucleation in murine FVA cells while microtubules do not,[5] the efficacy of inhibitors for cell division often depends on the species of the cell observed and their redundancy in the cells themselves.[19] We therefore reevaluated the efficacy of actin and tubulin/kinesin inhibitors on human CFU-E and erythroblasts. As illustrated in Figure 2, cytochalasin D, an inhibitor of actin polymerization, NSC23766, a specific inhibitor of Rac1 GTPases[21] that regulate actin polymerization, colchicine, an inhibitor of microtubules,[22] and monastrol, an inhibitor of kinesin Eg5, completely blocked CFU-E proliferation. Cytochalasin D decreased the number of cells in the G1/G0/S phase resulting in the accumulation of cells in the G2/M phase (Figure 2B), and produced multinucleated cells (Figures 2C and 3). Interestingly, NSC23766 dramatically increased the total number of dead cells and decreased the number of cells in the mitotic fraction (Figure 2B), and did not produce multinucleated cells (Figures 2C and 3). These data indicate that cytochalasin D and NSC23766 block the cell division of human CFU-E.

Colchicine and monastrol decreased the number of cells in the G1/G0/S phase, while cells accumulated in the G2/M phase, a finding that was accompanied by an increase in the number of dead cells (Figure 2B). Morphologic analysis showed that these inhibitors increased the number of cells undergoing mitosis (Figures 2C and 3). These data indicate that colchicine and monastrol block mitosis, and are efficient inhibitors of cell division in human CFU-E.

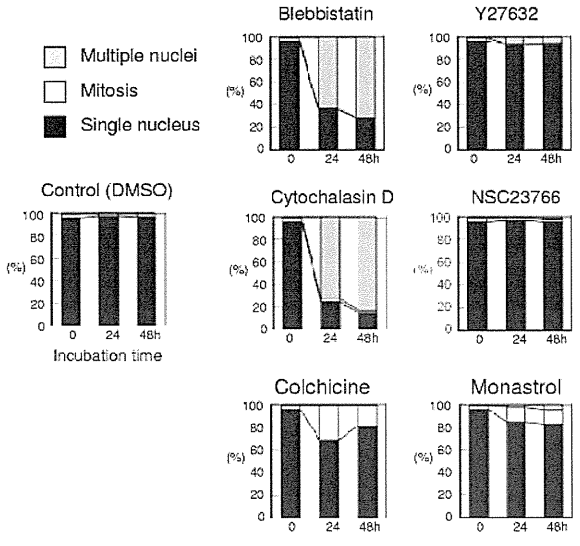

Figure 3. Morphologic analysis of cells cultured with or without inhibitors. Differential counts of cells with a single nucleus (closed bars), multiple nuclei (shaded bars), and cells in mitosis (open bars) are shown. Results presented are the mean of 3 independent experiments.

Myosin inhibitors block enucleation of human erythroblasts

Figure 4A presents a time course analysis of the enucleation ratio when day 11 human mature erythroblasts were cultured with or without various concentrations of blebbistatin and Y27632. During the 72-hour culture period, mature erythroblasts differentiated and started the enucleation process (Figure 4A and C top panel). After beginning the incubation, enucleation was rapidly initiated within 24 hours and continued until 72 hours. Both blebbistatin and Y27632 caused an immediate and significant inhibition of enucleation, in a dose dependent manner (Figure 4A and C middle and bottom panels). Western blot analysis showed that Y27632 reduces the phosphorylation of MLC2 at Thr18/Ser19 in these cells, with a statistical significance (supplemental Figure 2). In contrast to the significant effect on CFU-E (Figure 1B), neither blebbistatin nor Y27632 affects the cell cycle of day 11 mature erythroblasts (Figure 4B). These inhibitors did not affect the level of hemoglobinization of day 11 cells during 72 hours incubation (supplemental Figure 3). These results indicate that most of day 11 erythroblasts

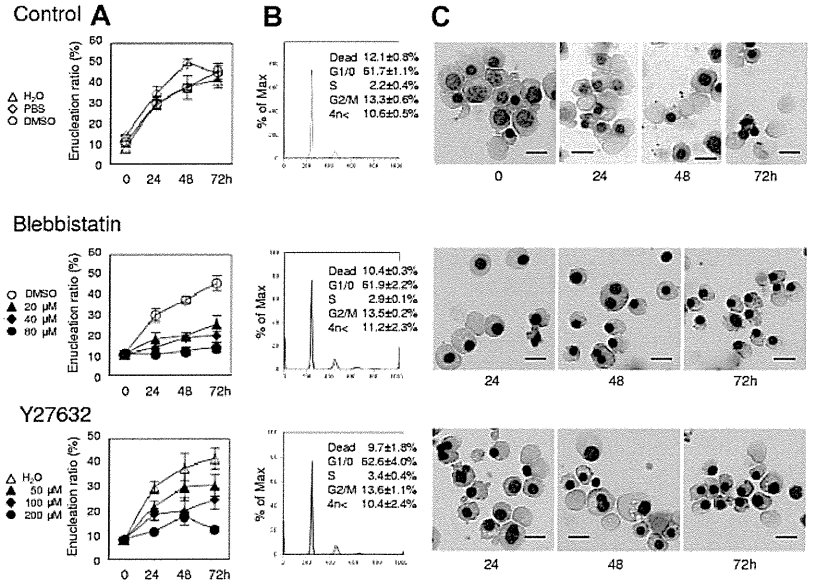

Figure 4. Myosin inhibitors block enucleation of human erythroblasts. Human CFU-E were cultured with EPO for an additional 4 days (day 11 cells) and differentiated to the level of mature erythroblasts incipient of enucleation. Mature erythroblasts were then cultured in the presence of EPO with or without various concentrations of inhibitors for non-muscle myosin II ATPase (blebbistatin) and a myosin activator Rho kinase (Y27632). (A) Effects of inhibitors and vehicle on the enucleation of day 11 mature erythroblasts. Results are presented as the mean ± SD of 3 independent experiments. (B) Cell cycle analysis of cells cultured for 24 hours with (red lines) or without (black lines) 80μM blebbistatin and 200μM Y27632. A representative result of 3 independent experiments is shown and is presented as the mean ± SD. (C) May-Grünwald-Giemsa staining of day 11 cells cultured for 24, 48 and 72 hours with or without 80μM blebbistatin and 200μM Y27632. A representative result of 3 independent experiments is shown. Scale bar = 10 μm.

consist of mature erythroblasts just before the stage of enucleation and that non-muscle myosin II is essential for the enucleation of human erythroblasts.

Actin inhibitors, but not tubulin/Eg5 inhibitors, block enucleation of human erythroblasts

Both cytochalasin D and NSC23766, which are known to inhibit enucleation,[5,6] caused an immediate and complete inhibition of enucleation of day 11 human mature erythroblasts (Figure 5 top 2 panels), indicating that actin polymerization is essential for the enucleation of human erythroblasts. In contrast, colchicine, an inhibitor of microtubules that are known to not be directly involved in enucleation,[5] did not block enucleation (Figure 5 bottom 3rd panel), indicating that day 11 human mature erythroblasts consist of postmitotic cells just before the stage of enucleation. In addition to the current confirmation of previous reports, we found for the first time that monastrol, an inhibitor of kinesin Eg5 which is a microtubule-based motor that plays a critical role in mitosis,[25] does not block enucleation of human erythroblasts (Figure 5 bottom panel).

Human erythroblasts express non-muscle myosin IIA and IIB

Mammalian cells have 3 isoforms of NMHC, termed IIA, IIB and IIC.[15,33,34] Western blot analysis showed that human CFU-E possess both NMHC IIA and IIB. The relative expression levels of NMHC IIA and IIB appeared to decrease as they matured, but the decreases were not statistically significant (Figure 6A). NMHC IIB was extremely low or undetectable in human PBMCs and NMHC IIC was not detected in either human erythroblasts or PBMC (data

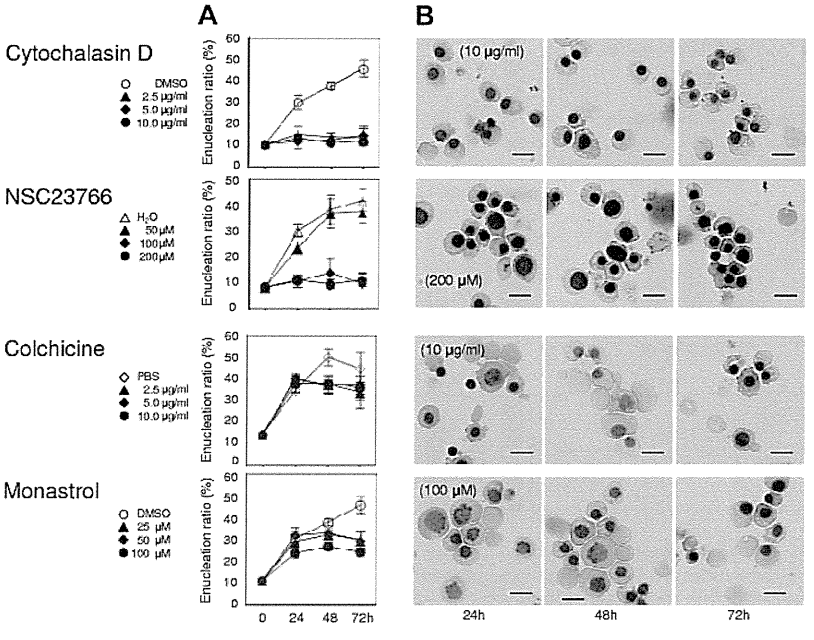

Figure 5. Actin inhibitors, but not tubulin/Eg5 inhibitors, block enucleation of human erythroblasts. Mature erythroblasts were cultured in the presence of EPO with or without various concentrations of inhibitors for actin polymerization (cytochalasin D and NSC23766), tubulin (colchicine) and Eg5 (monastrol). (A) Effects of inhibitors and vehicle on the enucleation of mature erythroblasts. Results are presented as the mean ± SD of 3 independent experiments. (B) May-Grünwald-Giemsa staining of cells cultured for 24, 48 and 72 hours with or without 10 μg/mL cytochalasin D, 200μM NSC23766, 10 μg/mL colchicine and 100μM monastrol. A representative result of 3 independent experiments is shown. Scale bar = 10 μm.

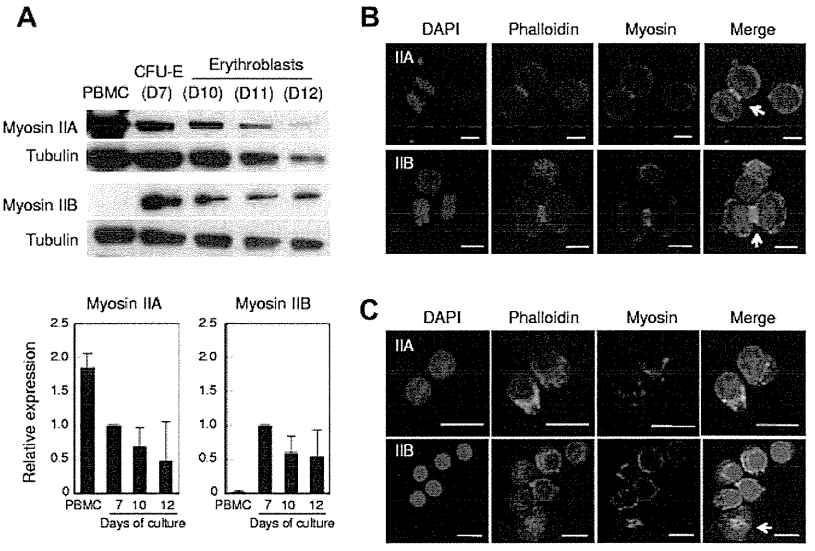

Figure 6. Human erythroblasts express non-muscle myosin IIA and IIB. (A) Western blotting of human PBMCs, CFU-E (day 7 cells, D7) and erythroblasts (D10-D12). D7-D12 indicates days of culture of purified human CD34$^+$ cells to induce erythroid differentiation. At indicated days, the cells were harvested and the protein obtained from 1×10^5 cells was applied to each lane. The relative expression levels of myosin II were normalized with tubulin expression and are the mean ± SD of 3 independent experiments. (B-C) Confocal microscopy of CFU-E (B) and day 12 enucleating mature erythroblasts (C) stained by DAPI, phalloidin and myosin IIA or IIB. NMHC IIA and IIB colocalized with actin (B arrows). In enucleating erythroblasts, NMHC IIB was present on the enucleated reticulocytes in the shape of a small ring colocalized with actin (C arrow).

not shown). The localizations of NMHC IIA and IIB were similar in CFU-E (Figure 6B), although IIB appeared to be more prominent in the contractile ring and colocalized with actin (Figure 6B arrow). In enucleating erythroblasts, NMHC IIA and IIB appeared to localize between the expelling nuclei and the reticulocytes (Figure 6C). Interestingly, NMHC IIB was present on the enucleated reticulocytes in the shape of a small ring that was colocalized with actin, which might indicate the expulsion route of the nuclei expelled from erythroblasts (Figure 6C arrow).

Enucleation of human erythroblasts involves non-muscle myosin IIB

Figure 7A illustrates the rod fragments, ARF296 and BRF305, which are encoded by Leu 1666–Glu 1961 of NMHC IIA and Phe 1672–Glu 1976 of NMHC IIB, respectively.[31,32] When ARF296 or BRF305 was exogenously expressed to CFU-E, these rods localized in cytoplasm (Figure 7B) and blocked the proliferation of CFU-E (Figure 7C). To investigate the function of myosin IIA and IIB in the enucleation of erythroblasts, mature erythroblasts (day 10 cells) were transfected with ARF296 and BRF305. The transfected cells were sorted by flowcytometry and cultured for an additional 72 hours (Figure 7D). Confocal microscopy showed that transfected cells maintain GFP-expression during the culture period (Figure 7D). As illustrated in Figure 7E, the enucleation of mature erythroblasts was inhibited by the exogenous expression of BRF305, with statistical significance, while only a slight inhibitory effect of ARF296 was apparent. These data indicate that enucleation of human erythroblasts involves non-muscle myosin IIB.

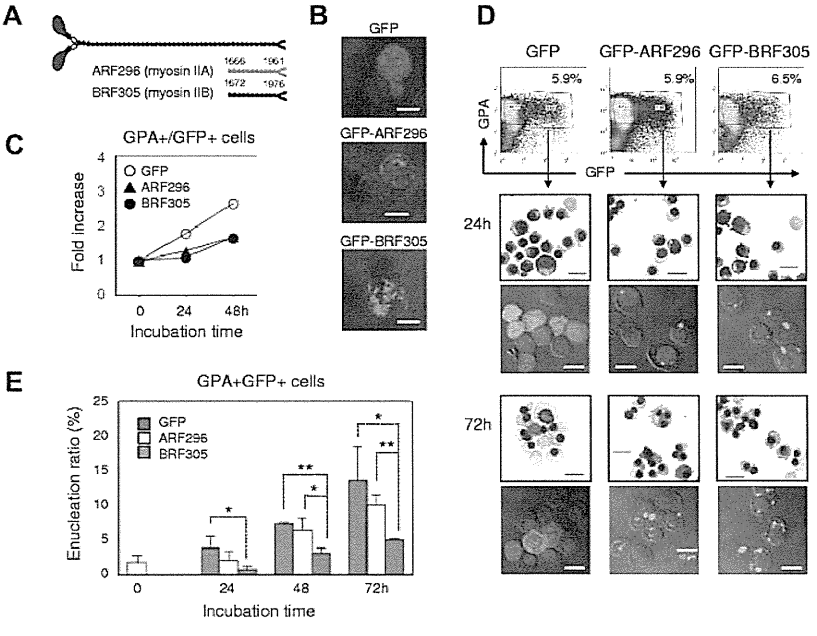

Figure 7. Enucleation of human erythroblasts involves non-muscle myosin IIB. (A) A schematic presentation of rod fragments: ARF296 (for myosin IIA) and BRF305 (for myosin IIB). (B) Confocal microscopy of CFU-E transfected by pEGFP-ARF296 and pEGFP-BRF305. Scale bar = 10 μm. (C) CFU-E were transfected with pEGFP-ARF296 and pEGFP-BRF305. After 24 hours of incubation, the dead cells were removed and then incubated in the erythroid medium. After culture for the indicated period, the cells were harvested, counted and GPA$^+$/GFP$^+$ cells were analyzed by flowcytometry. Representative data for 2 independent experiments are shown. (D) Mature day 10 erythroblasts were transfected with pEGFP-ARF296 and pEGFP-BRF305. GPA$^+$/GFP$^+$ cells were sorted and cultured in the erythroid medium. After culture for the indicated period, the cells were harvested and stained with May-Grünwald-Giemsa. An aliquot of the cells were observed by confocal microscopy. Scale bar = 10 μm. (E) GPA$^+$/GFP$^+$ cells were cultured in the erythroid medium. At the indicated period, the cells were harvested and counted. Results are presented as the mean ± SD of 3 independent experiments. *$P < .05$ and **$P < .01$.

Discussion

In this study, we demonstrate for the first time that blebbistatin and Y27632 inhibit the enuclation of human erythroblasts. Blebbistatin is a small molecule inhibitor that shows a high affinity and selectivity toward non-muscle myosin II ATPase,[20] while Y27632 is well-established inhibitor of ROCK.[23,24] Collectively, these data suggest that the enucleation of human erythroblasts involves non-muscle myosin II. This is of particular interest given that the precise role of myosin in enucleation has remained unclear, even though the formation of the contractile actin ring (CAR) and furrow formation are known to occur on the plasma membrane of enucleating erythroblasts.[5,6] As non-muscle myosin II is one of the central mechanisms involved in cytokinesis and is required for furrow formation,[22,24,35] our study also suggests that myosin shares a similar mechanistic function between cytokinesis and the enucleation of human erythroblasts.

Although we confirmed that Y27632 inhibits the phosphorylation of MLC2 at Thr18/Ser19, several lines of evidence indicate that activation of myosin II by ROCK is redundant with the action of other kinases such as citron kinase and that Y27632 blocks neither the initiation nor completion of cytokinesis of Hela cells, although it slows cleavage contraction.[36,37] Reportedly, ROCK-dependent phosphorylation of the mDia2 is an important determinant of mDia2 activity and this signaling mechanism affects actin polymerization.[38] Although further studies are needed, considering that Y27632 produces a limited number of multinucleated cells in the culture of CFU-E, the main mechanism of Y27632 in inhibiting cell division and enucleation may involve the Rac 1 GTPases and their downstream effector mDia2.

In addition to the previous report showing that human erythroblasts express both NMHC IIA and IIB,[39] our study demonstrates that human erythroid progenitor cells such as CFU-E also express both NMHC IIA and IIB. As shown in Figure 6A, PBMC that comprise monocytes and lymphocytes also exclusively express the IIA isoform. Platelets and granulocytes exclusively express the IIA isoform.[18] Taken together, the presence of IIB isoform may be restricted to the erythroid lineage among hematopoietic cells, which may be an intriguing distribution of myosin II isoforms considering the characteristic feature of enucleation of erythroblasts.

We demonstrated that the proliferation of CFU-E was inhibited by the exogenous expression of the rod fragments for NMHC IIA or IIB, which suggests that the expressions of the rod fragments of NMHC IIA and IIB are functional in the inhibition of cell division of CFU-E. We further showed that the enucleation of mature erythroblasts was inhibited by the transfection of the rod fragment for myosin IIB. Because filament formation is necessary for myosin II to function, exogenous expression of rod fragments containing the critical regions for assembly could exhibit a dominant negative effect, preventing the normal assembly of endogenous myosin II. In fact, it was shown that a 72-kDa rod fragment of NMHC IIB acts as a dominant-negative form and induced aberrant cell shape.[40] We also demonstrated that the myosin IIB rod fragment, BRF305 (Phe 1672–Glu 1976), can inhibit the function of endogenous myosin IIB by inhibiting normal filament assembly of MRC-5 SV1 TG1 cells, the SV40-transformant of human embryonic lung fibroblast MRC-5.[32] Thus, our findings indicate that enucleation of human erythroblasts involves non-muscle myosin IIB. Information concerning the specific functions of myosin IIB has been increased by the studies of cells isolated from NMHC IIB knockout mice. Ablation of NMHC IIB resulted in survival to E14.5, but with marked abnormalities in the heart including a ventricular septal defect, abnormal positioning of the aorta, and abnormalities including hydrocephalus and the abnormal migration of certain groups of neurons.[41-43] A defect in cytokinesis was observed in NMHC IIB$^{-/-}$ cardiac myocytes,[42] possibly because myosin IIA is absent in these cells.

There is a possibility that the relative expression level of the rod fragment, ARF296, against endogenous myosin IIA is not sufficient to show an inhibitory effect[32] in the enucleation of mature erythroblasts. We thus do not exclude the possibility that myosin IIA is also involved in enucleation of human erythroblasts. Interestingly, the clinical manifestations[12] of MYH9-related diseases involve the lens, causing cataracts.[18] The specific role of myosin IIA in maintaining transparency of the lens is unknown; however, Maddala et al[44] showed that inhibition of myosin light chain kinase resulted in the development of nuclear lens opacity and abnormal fiber cell organization, suggesting an association between myosin IIA and lens cell enucleation.

Keerthivasan et al previously reported that blebbistatin failed to block enucleation, and suggested that contraction of the actomyosin ring is not essential for the nuclear expulsion of murine erythroblasts.[10] However, we and others have demonstrated the presence of actomyosin contractile rings in the erythoblasts with incipient enucleation,[5,6] which strongly suggests that the actomyosin ring is functional in enucleation. The reason for this discrepancy in the effects of blebbistatin between mouse and human erythroblasts remains unclear. The efficacy of inhibitors for cell division often varies depending on the species of the cell observed.[19] Moreover, there are phenotypic differences in non-muscle myosin IIA deficiency between humans and mice. In humans, various mutations in the *Myh9* gene that encodes the NMHC IIA cause autosomal dominant disease, whereas in mice the complete deficiency is embryonic lethal but heterozygous mice are nearly normal.[18,45] One hypothesis might be that the degree of inhibition of non-muscle myosin II is more strictly required in mice to observe any effect of this molecule on the enucleation of erythroblasts.

Using human erythroblasts, we confirmed that the actin polymerization inhibitor cytochalasin D[5] and the Rac-specific inhibitor NSC23766[6] completely blocked enucleation. Tubulin/kinesin inhibitors blocked cell division of CFU-E but did not inhibit the enucleation, which suggests that the process of erythroblast enucleation is not entirely the same as that of cytokinesis. In addition, this study contributes the novel finding that the Eg5 inhibitor monastrol also does not inhibit the final step of enucleation in human erythroblasts. Monastrol is a reversible, cell-permeable, non-tubulin-interacting inhibitor of the mitotic kinesin Eg5 motor protein.[46,47] Eg5 is a member of the Kinesin-5 (BimC) subclass of kinesins and influences microtubules that form and organize the mitotic spindle in dividing cells.[46,47] Mammalian cells exposed to monastrol in vitro progress normally through the S and G2 phases of the cell cycle, but are temporarily delayed during mitosis. This delay is accompanied by faulty chromosome separation and the presence of a monoastral spindle composed of a radial array of microtubules surrounded by a ring of chromosomes.[48] Eg5 independent enucleation of human erythroblasts suggests that this is a process independent of the centrosome.

In conclusion, this study shows that the inhibition of ROCK and non-muscle myosin II ATPase completely blocked enucleation of human erythroblasts, demonstrating for the first time that non-muscle myosin II is required for human erythroblast enucleation. Further, we demonstrated that myosin IIB is involved in human

erythroblast enucleation. An increased body of mechanistic knowledge about non-muscle myosin II isoforms in enucleation may help us to explore the unknown NMHC abnormalities in hematologic disorders.

Acknowledgments

The authors are grateful to Prof Mohandas Narla for helpful discussions and comments and to Keiko Iwamoto, Hiromi Kataho and Etsuko Kobayashi from the Department of Hematology, Nephrology, and Rheumatology, Akita University Graduate School of Medicine for their valuable technical assistance.

This study was supported in part by Grants-in-Aid (23591412), funds from the "Global Center of Excellence Program (COE)" of the Ministry of Education, Science, Technology, Sports, and Culture of Japan, and a research grant from the Idiopathic Disorders of Hematopoietic Organs Research Committee of the Ministry of Health, Labor and Welfare of Japan.

Authorship

Contribution: K.U. designed and performed experiments, analyzed data, and wrote the manuscript; Y.-M.G., M.T., M.H., Y.M., M.N., H.T., N.T. and A.K. performed experiments and helped to write the manuscript; W.N. and Y.T. analyzed and interpreted the data and helped to write the manuscript; and K.S. designed the study, interpreted data and helped to write the manuscript.

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

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References

1. Hebiguchi M, Hirokawa M, Guo YM, et al. Dynamics of human erythroblast enucleation. *Int J Hematol.* 2008;88(5):498-507.
2. Chasis JA, Mohandas N. Erythroblastic islands: niches for erythropoiesis. *Blood.* 2008;112(3):470-478.
3. Barr FA, Gruneberg U. Cytokinesis: placing and making the final cut. *Cell.* 2007;131(5):847-860.
4. Glotzer M. The molecular requirements for cytokinesis. *Science.* 2005;307(5716):1735-1739.
5. Koury ST, Koury MJ, Bondurant MC. Cytoskeletal distribution and function during the maturation and enucleation of mammalian erythroblasts. *J Cell Biol.* 1989;109(6 Pt 1):3005-3013.
6. Ji P, Jayapal SR, Lodish HF. Enucleation of cultured mouse fetal erythroblasts requires Rac GTPases and mDia2. *Nat Cell Biol.* 2008;10(3):314-321.
7. Jayapal SR, Lee KL, Ji P, Kaldis P, Lim B, Lodish HF. Down-regulation of Myc is essential for terminal erythroid maturation. *J Biol Chem.* 2010;285(51):40252-40265.
8. Thompson PD, Tipney H, Brass A, et al. Claudin 13, a member of the claudin family regulated in mouse stress induced erythropoiesis. *PLoS One.* 2010;5(9).pii:e12667.
9. Ji P, Yeh V, Ramirez T, Murata-Hori M, Lodish HF. Histone deacetylase 2 is required for chromatin condensation and subsequent enucleation of cultured mouse fetal erythroblasts. *Haematologica.* 2010;95(12):2013-2021.
10. Keerthivasan G, Small S, Liu H, Wickrema A, Crispino JD. Vesicle trafficking plays a novel role in erythroblast enucleation. *Blood.* 2010;116(17):3331-3340.
11. Even-Ram S, Doyle AD, Conti MA, Matsumoto K, Adelstein RS, Yamada KM. Myosin IIA regulates cell motility and actomyosin-microtubule crosstalk. *Nat Cell Biol.* 2007;9(3):299-309.
12. Conti MA, Even-Ram S, Liu C, Yamada KM, Adelstein RS. Defects in cell adhesion and the visceral endoderm following ablation of nonmuscle myosin heavy chain II-A in mice. *J Biol Chem.* 2004;279(40):41263-41266.
13. Pollard TD. Mechanics of cytokinesis in eukaryotes. *Curr Opin Cell Biol.* 2010;22(1):50-56.
14. Berg JS, Powell BC, Cheney RE. A millennial myosin census. *Mol Biol Cell.* 2001;12(4):780-794.
15. Golomb E, Ma X, Jana SS, et al. Identification and characterization of nonmuscle myosin II-C, a new member of the myosin II family. *J Biol Chem.* 2004;279(4):2800-2808.
16. Leal A, Endele S, Stengel C, et al. A novel myosin heavy chain gene in human chromosome 19q13.3. *Gene.* 2003;312:165-171.
17. Even-Ram S, Yamada KM. Of mice and men: Relevance of cellular and molecular characterizations of myosin IIA to MYH9-related human disease. *Cell Adh Migr.* 2007;1(3):152-155.
18. Kunishima S, Saito H. Advances in the understanding of MYH9 disorders. *Curr Opin Hematol.* 2010;17(5):405-410.
19. Narumiya S, Yasuda S. Rho GTPases in animal cell mitosis. *Curr Opin Cell Biol.* 2006;18(2):199-205.
20. Kovacs M, Toth J, Hetenyi C, Malnasi-Csizmadia A, Sellers JR. Mechanism of blebbistatin inhibition of myosin II. *J Biol Chem.* 2004;279(34):35557-35563.
21. Gao Y, Dickerson JB, Guo F, Zheng J, Zheng Y. Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. *Proc Natl Acad Sci U S A.* 2004;101(20):7618-7623.
22. Wilson L, Panda D, Jordan MA. Modulation of microtubule dynamics by drugs: a paradigm for the actions of cellular regulators. *Cell Struct Funct.* 1999;24(5):329-335.
23. Kimura K, Ito M, Amano M, et al. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science.* 1996;273(5272):245-248.
24. Uehata M, Ishizaki T, Satoh H, et al. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature.* 1997;389(6654):990-994.
25. Sarli V, Giannis A. Targeting the kinesin spindle protein: basic principles and clinical implications. *Clin Cancer Res.* 2008;14(23):7583-7587.
26. Saito K, Hirokawa M, Inaba K, et al. Phagocytosis of codeveloping megakaryocytic progenitors by dendritic cells in culture with thrombopoietin and tumor necrosis factor-alpha and its possible role in hemophagocytic syndrome. *Blood.* 2006;107(4):1366-1374.
27. Oda A, Sawada K, Druker BJ, et al. Erythropoietin induces tyrosine phosphorylation of Jak2, STAT5A, and STAT5B in primary cultured human erythroid precursors. *Blood.* 1998;92(2):443-451.
28. Sawada K, Krantz SB, Kans JS, et al. Purification of human erythroid colony-forming units and demonstration of specific binding of erythropoietin. *J Clin Invest.* 1987;80(2):357-366.
29. Guo YM, Ishii K, Hirokawa M, et al. CpG-ODN 2006 and human parvovirus B19 genome consensus sequences selectively inhibit growth and development of erythroid progenitor cells. *Blood.* 2010;115(22):4569-4579.
30. Ronen D, Ravid S. Myosin II tailpiece determines its paracrystal structure, filament assembly properties, and cellular localization. *J Biol Chem.* 2009;284(37):24948-24957.
31. Mitsuhashi M, Sakata H, Kinjo M, Yazawa M, Takahashi M. Dynamic assembly properties of nonmuscle myosin II isoforms revealed by combination of fluorescence correlation spectroscopy and fluorescence cross-correlation spectroscopy. *J Biochem.* 2011;149(3):253-263.
32. Sato MK, Takahashi M, Yazawa M. Two regions of the tail are necessary for the isoform-specific functions of nonmuscle myosin IIB. *Mol Biol Cell.* 2007;18(3):1009-1017.
33. Shohet RV, Conti MA, Kawamoto S, Preston YA, Brill DA, Adelstein RS. Cloning of the cDNA encoding the myosin heavy chain of a vertebrate cellular myosin. *Proc Natl Acad Sci U S A.* 1989;86(20):7726-7730.
34. Takahashi M, Kawamoto S, Adelstein RS. Evidence for inserted sequences in the head region of nonmuscle myosin specific to the nervous system. Cloning of the cDNA encoding the myosin heavy chain-B isoform of vertebrate nonmuscle myosin. *J Biol Chem.* 1992;267(25):17864-17871.
35. Straight AF, Cheung A, Limouze J, et al. Dissecting temporal and spatial control of cytokinesis with a myosin II Inhibitor. *Science.* 2003;299(5613):1743-1747.
36. Kosako H, Yoshida T, Matsumura F, Ishizaki T, Narumiya S, Inagaki M. Rho-kinase/ROCK is involved in cytokinesis through the phosphorylation of myosin light chain and not ezrin/radixin/moesin proteins at the cleavage furrow. *Oncogene.* 2000;19(52):6059-6064.
37. Madaule P, Eda M, Watanabe N, et al. Role of citron kinase as a target of the small GTPase Rho in cytokinesis. *Nature.* 1998;394(6692):491-494.
38. Staus DP, Taylor JM, Mack CP. Enhancement of mDia2 activity by Rho-kinase-dependent phosphorylation of the diaphanous autoregulatory domain. *Biochem J.* 2011;439(1):57-65.
39. Marini M, Bruschi M, Pecci A, et al. Non-muscle myosin heavy chain IIA and IIB interact and colocalize in living cells: relevance for MYH9-related disease. *Int J Mol Med.* 2006;17(5):729-736.
40. Ben-Ya'acov A, Ravid S. Epidermal growth factor-mediated transient phosphorylation and membrane localization of myosin II-B are required for efficient chemotaxis. *J Biol Chem.* 2003;278(41):40032-40040.
41. Ma X, Kawamoto S, Hara Y, Adelstein RS. A point

mutation in the motor domain of nonmuscle myosin II-B impairs migration of distinct groups of neurons. *Mol Biol Cell.* 2004;15(6):2568-2579.

42. Takeda K, Kishi H, Ma X, Yu ZX, Adelstein RS. Ablation and mutation of nonmuscle myosin heavy chain II-B results in a defect in cardiac myocyte cytokinesis. *Circ Res.* 2003;93(4):330-337.
43. Tullio AN, Accili D, Ferrans VJ, et al. Nonmuscle myosin II-B is required for normal development of the mouse heart. *Proc Natl Acad Sci U S A.* 1997; 94(23):12407-12412.
44. Maddala R, Skiba N, Vasantha Rao P. Lens fiber cell elongation and differentiation is associated with a robust increase in myosin light chain phosphorylation in the developing mouse. *Differentiation.* 2007;75(8):713-725.
45. Matsushita T, Hayashi H, Kunishima S, et al. Targeted disruption of mouse ortholog of the human MYH9 responsible for macrothrombocytopenia with different organ involvement: hematological, nephrological, and otological studies of heterozygous KO mice. *Biochem Biophys Res Commun.* 2004;325(4):1163-1171.
46. Blangy A, Lane HA, d'Herin P, Harper M, Kress M, Nigg EA. Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation in vivo. *Cell.* 1995;83(7):1159-1169.
47. Wittmann T, Hyman A, Desai A. The spindle: a dynamic assembly of microtubules and motors. *Nat Cell Biol.* 2001;3(1):E28-E34.
48. Kapoor TM, Mayer TU, Coughlin ML, Mitchison TJ. Probing spindle assembly mechanisms with monastrol, a small molecule inhibitor of the mitotic kinesin, Eg5. *J Cell Biol.* 2000;150(4):975-988.

Ann. N.Y. Acad. Sci. ISSN 0077-8923

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Issue: *Hematopoietic Stem Cells VIII*

TIM-3 as a therapeutic target for malignant stem cells in acute myelogenous leukemia

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Acute myeloid leukemia (AML) originates from self-renewing leukemic stem cells (LSCs), an ultimate therapeutic target for AML. Recent studies have shown that many AML LSC–specific surface antigens could be such candidates. T cell immunoglobulin mucin-3 (TIM-3) is expressed on LSCs in most types of AML, except for acute promyelocytic leukemia, but not on normal hematopoietic stem cells (HSCs). In mouse models reconstituted with human AML LSCs or human hematopoietic stem cells, a human TIM-3 mouse IgG2a antibody with complement-dependent and antibody-dependent cellular cytotoxic activities eradicates AML LSCs *in vivo* but does not affect normal human hematopoiesis. Thus, TIM-3 is one of the promising targets to eradicate AML LSCs.

Keywords: leukemic stem cell; AML; TIM-3

Introduction

Acute myeloid leukemia (AML) is a clonal malignant disorder derived from a small number of leukemic stem cells (LSCs). The concept of LSCs has been proposed based on the finding in the 1980s that only a small fraction of AML blasts can form colonies *in vitro*. In 1996, Dick *et al.* showed that the $CD34^+CD38^-$, but not other fractions of bone marrow cells from AML patients, can reconstitute human AML in immunodeficient mice, demonstrating direct evidence for the presence of LSCs.[1] LSCs can self-renew and generate a large number of clonogenic leukemic blasts.[1–3] Although recent studies have suggested that LSCs are present in either in the $CD34^+CD38^+$ fraction[4] or the $CD34^-$ blastic fraction, at least in some types of AML[5,6] the $CD34^+CD38^-$ population represents highly-enriched LSCs in the vast majority of cases.[7] The $CD34^+CD38^-$ phenotype is, however, shared with normal human hematopoietic stem cells (HSCs) that have long-term reconstitution activity.[8,9]

In the clinic, conventional chemotherapies can currently achieve complete remission in ~90% of AML cases. However, a considerable fraction (~60%) of AML patients still relapse after intensive chemotherapy. The recurrence of AML in these patients could be caused by regrowth of the remaining LSCs. Therefore, LSCs could be the ultimate therapeutic target to achieve cure in AML patients. Thus, to selectively kill AML LSCs, sparing normal HSCs, one of the most practical approaches is to target the AML LSC–specific surface or functionally indispensable molecules. To achieve specificity for LSCs, the target molecule should be expressed on LSCs at a high level and not on normal HSCs.[10] It should not matter whether the molecule is expressed in normal blood cells or normal progenitor cells, because normal HSCs that are spared would replenish all mature blood cells after treatment.

Recently, two papers have reported the T cell immunoglobulin mucin-3 (TIM-3) as a surface molecule expressed in LSCs of most AML types.[11,12] Here, we discuss the potential usefulness of TIM-3 in eradicating AML LSCs, leaving normal HSCs intact.

Expression and functions of TIM-3 in normal hematopoiesis

TIM-3 was originally identified as a surface molecule expressed in interferon (IFN)-γ–producing $CD4^+$ Th1 cells and in $CD8^+$ T cytotoxic type 1 (Tc1) cells[13] in mouse hematopoiesis. TIM-3 plays an important role in limiting and controlling

doi: 10.1111/j.1749-6632.2012.06550.x

Ann. N.Y. Acad. Sci. 1266 (2012) 118–123

Th1-dependent immune responses and in inducing immune tolerance.[13–15] The ligand of TIM-3 in lymphocytes is galectin-9, an S-type lectin with two distinct carbohydrate recognition domains that can bind to carbohydrate chains on the TIM-3 IgV domain. Engagement of TIM-3 by galectin-9 induces Th1 cells to undergo apoptosis and inhibits their production of IFN-γ.[16] Thus, TIM-3 is a negative regulator of Th1- and Tc1-driven immune responses.

TIM-3 is also expressed in myeloid cells, including $CD11b^+$ macrophages, $CD11c^+$ dendritic cells (DCs), and mast cells, and it recognizes apoptotic cell–expressed phosphatidylserine (PS) through the TIM-3 IgV domain.[17–20] The binding of PS to TIM-3 does not interfere with binding of galectin-9 to TIM-3, as the binding sites of these molecules are located at opposite sides of the IgV domain. In TIM-3–expressing DCs, recognition of PS by TIM-3 enhanced phagocytosis of apoptotic cells and cross-presentation of apoptotic cell–associated antigens to $CD8^+$ T cells.[18]

In human steady-state hematopoiesis, TIM-3 is expressed mainly in monocytes and a fraction of NK

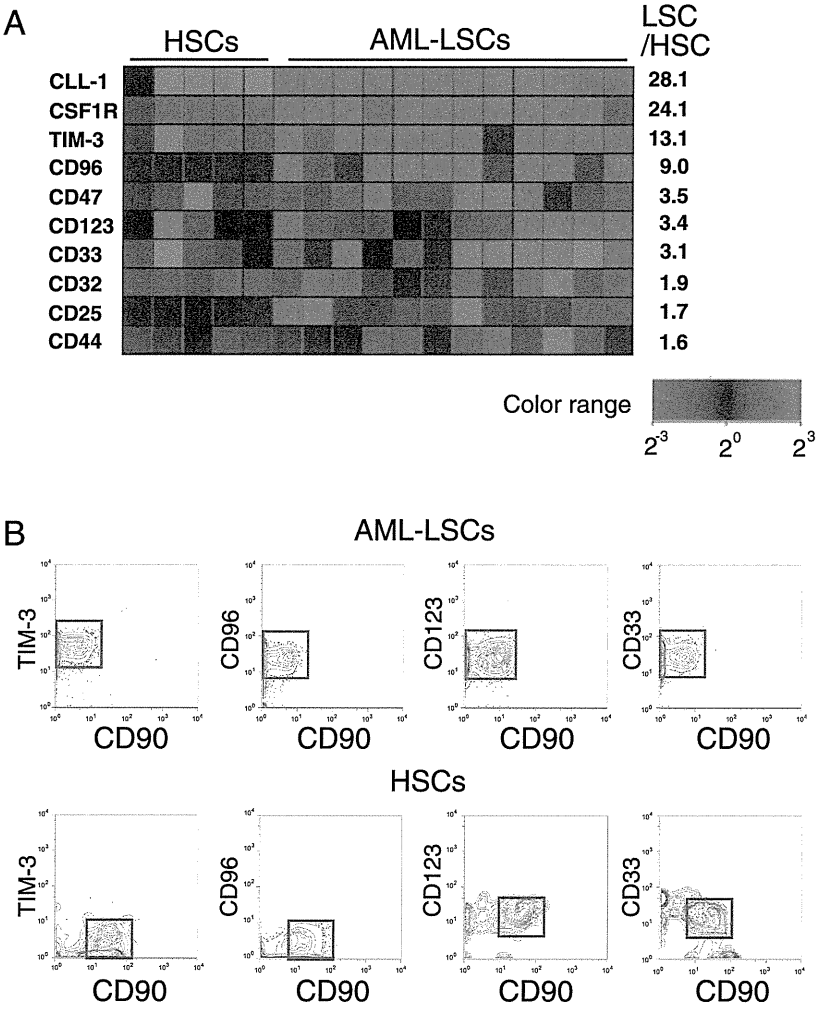

Figure 1. The expression of LSC-specific surface molecules in AML. (A) $CD34^+CD38^-$ HSCs and $CD34^+CD38^-$ AML LSCs were purified and analyzed on cDNA microarray. The surface molecules that are expressed strongly in AML LSCs compared to normal HSCs are listed. (B) The expression of representative surface markers in AML LSCs and normal HSCs.

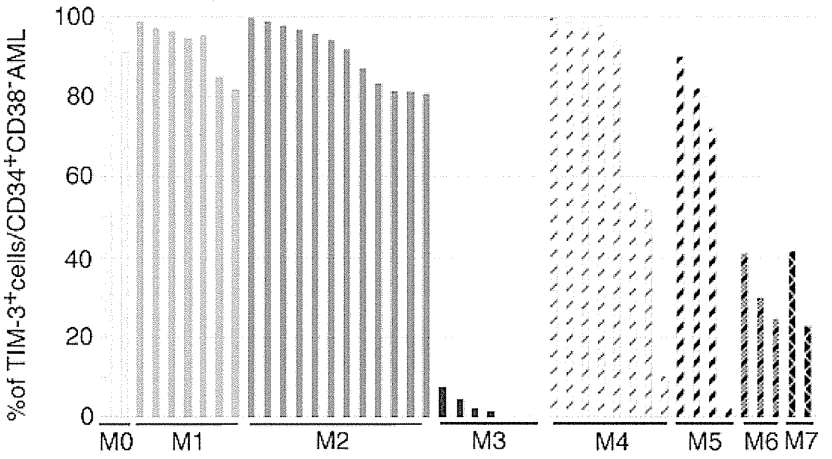

Figure 2. The frequency of TIM-3$^+$ cells in the CD34$^+$CD38$^-$ fraction in each AML subtype. TIM-3 protein is expressed in the vast majority of the CD34$^+$CD38$^-$ LSC population in AML types M0, M1, M2, and M4.

cells, but not in granulocytes, T cells, or B cells.[11] In the bone marrow TIM-3 is not expressed in normal HSCs or the vast majority of the CD34$^+$CD38$^+$ progenitor population. But within the CD34$^+$CD38$^+$ fraction, TIM-3 was expressed in a fraction of granulocyte/monocyte progenitors (GMPs) at a low level, while it is not in common myeloid progenitors (CMPs), megakaryocyte/erythrocyte progenitors (MEPs), or common lymphoid progenitors (CLPs). The vast majority of purified TIM-3$^+$ GMPs gave rise to CFU-M, suggesting that upregulation of TIM-3 mainly occurs in concert with the monocyte lineage commitment at the GMP stage.[11]

TIM-3 expression in human AML

TIM-3 has been identified as an AML LSC–specific marker based on the comparison between expression profiles of CD34$^+$CD38$^-$ AML cells and normal HSCs (Fig. 1A). While TIM-3 protein is not expressed in normal HSCs (Fig. 1B), it is expressed at a high level in the vast majority of CD34$^+$CD38$^-$ LSCs and the CD34$^+$CD38$^+$ progenitor fraction in AML M0, M1, M2, and M4 types in virtually all cases (Fig. 2) and in all cytogenic subgroups.[11,12] In AML M5, M6, and M7, a considerable fraction of CD34$^+$CD38$^-$ cells express TIM-3. However, TIM-3 expression was not seen in the CD34$^+$CD38$^-$ population in M3 cases (Fig. 2). TIM-3 expression tends to decline at the CD34$^-$ leukemic blast stage. Of note, the expression level of TIM-3 was found to be high in AML with core-binding factor translocations or with mutations in CEBPA.[12]

Strikingly, the TIM-3$^+$ population in the bone marrow contains all AML LSCs, and normal HSCs are always included in patients' TIM-3$^-$ populations. TIM-3$^+$ and TIM-3$^-$ AML populations have been transplanted into sublethally-irradiated immunodeficient mice and only mice transplanted with TIM-3$^+$ AML cells developed human AML.[11] In another experiment, the TIM-3$^-$ fraction did not include LSCs but normal HSCs, as evidenced by the fact that normal human hematopoiesis was frequently reconstituted in mice transplanted with the TIM-3$^-$ fraction.[12]

Targeting AML LSCs by TIM-3-specific antibodies in xenograft models

To utilize TIM-3 to target AML LSCs, it is critical to establish human TIM-3 antibodies that can kill TIM-3–expressing cells *in vivo*. In terms of an antibody-based treatment, knowing the antibody-dependent cell-mediated cytotoxicity (ADCC) and the complement-dependent cytotoxicity (CDC) activities is critical in order to eliminate target cells.[21] A TIM-3 monoclonal antibody (IgG2b) was obtained by immunizing Balb/c mice with L929 cells stably-expressing human TIM-3 and soluble TIM-3 protein.[11] For this antibody, the variable portion of the VH regions of the cloned hybridoma that

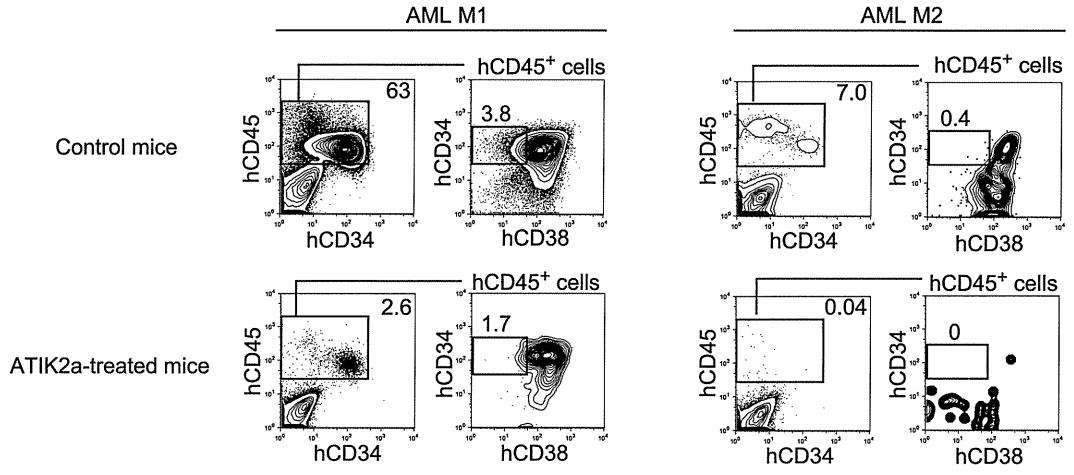

Figure 3. Potent antileukemic effects of the TIM-3 monoclonal antibody in a xenograft model. Mice were transplanted with LSCs purified from patients with AML M1 (left) or M2 (right) and were then treated with ATIK2a or control antibodies. In both experiments, ATIK2a treatment significantly reduced the human $CD45^+$ AML cells and $CD34^+CD38^-$ AML LSCs *in vivo*. Representative results of bone marrow analysis four weeks after treatment are shown.

recognize TIM-3 were grafted onto IgG2a Fc regions because the IgG2a subclass is the most efficient at inducing ADCC activity in mice.[22,23] The established clone, ATIK2a, was effective at killing TIM-3–expressing cell lines via both CDC and ADCC.[11]

The effect of ATIK2a on normal and malignant AML hematopoiesis was tested in xenograft models. NOD-SCID mice transplanted with 10^5 $CD34^+$ cord blood cells with or without ATIK2a injection developed almost equal percentages of human cells. In mice injected with ATIK2a, however, human $TIM\text{-}3^+$ mature monocytes were not found, suggesting that while targeting TIM-3 does not affect hematopoiesis, it does eliminate normal monocytes.

In contrast, ATIK2a exerted profound effects on leukemia development. In mice transplanted with human AML of M0, M1, and M4 types, ATIK2a treatment significantly reduced the human $CD45^+$ AML cell burden, as well as the $CD34^+CD38^-$ LSC cell numbers *in vivo* (Fig. 3). Retransplantation into secondary recipients of the remaining AML cells from primary recipients treated with ATIK2a never gave rise to human AML, indicating that the ATIK2a treatment successfully eradicated the LSCs in the primary recipients. These data suggest that eliminating AML LSCs by using TIM-3–"killing" antibodies may be a practical approach to curing human AML.

Perspective

To use surface markers for targeting AML LSCs, specificity as well as sensitivity are critical. TIM-3 has several advantages over other candidate markers. First, TIM-3 protein is not detectable in normal HSCs or in other myelo-erythroid or lymphoid progenitors, although monocyte-lineage committed progenitors begin to upregulate TIM-3. Second, TIM-3 could mark all LSCs that can reconstitute human AML in immunodeficient mice in the majority of M0, M1, M2, and M4 AML cases, and its expression level is sufficient to eradicate LSCs by antibody-based treatment. The expression level of other candidate molecules, including CD25,[24] CD32,[24] CD44,[25] and CD47,[26] in LSCs was only two- to threefold higher at the mRNA level compared with normal HSCs (Fig. 1A), and in some AML cases, LSCs did not express these molecules. CD33 and CD123 proteins are expressed at relatively high levels in normal HSCs (Fig. 1B) and myeloid progenitors, including CMPs and GMPs,[27] suggesting that targeting these molecules would harm normal hematopoiesis. In fact, prolonged cytopenia has been observed in AML patients treated with gemtuzumab, a recombinant humanized anti-CD33 monoclonal antibody conjugated with the cytotoxic antibiotic calicheamicin.

In contrast, CLL-1,[28] CSF1R,[29] and CD96[30] are molecules specifically expressed by LSCs. CLL-1 is

a transmembrane glycoprotein;[31] the proportion of CLL-1–expressing $CD34^+CD38^-$ AML cells is highly diverse in cases.[28] CD96 is a member of the Ig gene superfamily; it is expressed on activated T cells.[32] Of note, similar to the case of TIM-3, $CD96^+$, but not $CD96^-$, AML cells efficiently reconstitute AML in the immunodeficient mice,[30] suggesting that CD96 can mark all functional AML LSCs. The expression level of CD96 protein is also high enough to clearly separate AML LSCs and normal HSCs (Fig. 1B). However, the sensitivity of TIM-3 is likely to be the highest among these molecules, at least for AML M0, M1, M2, and M4.

To apply our findings to clinic, we have developed a chimeric TIM-3 monoclonal antibody by fusing the variable regions of ATIK2a to the human IgG constant region. Thus far we have injected this antibody to cynomolgus monkeys, which showed no significant adverse effects (unpublished data). A clinical study with further intensive treatment is being planned.

It is important to understand the function of molecules in the maintenance and/or reconstitution capabilities of LSCs. For example, it was shown that anti-CD44 monoclonal antibodies reduced leukemic burden and blocked secondary engraftment in a NOD-SCID model.[25] This effect on LSCs was mediated in part by the disruption of LSC–niche interactions.[25] Anti-CD47 antibodies can block LSC reconstitution in a NOD-SCID model,[26] and this might be due to the activation of phagocytosis by macrophages through inhibition of interaction of CD47 with SIRPA. Since the pathway for eradication of LSCs by anti-CD44 or anti-CD47 treatment is different from that of anti-TIM-3, the combination of these antibodies might be critical for future treatments targeting AML LSCs.

Conflicts of interest

The authors declare no conflicts of interest.

References

1. Lapidot, T. *et al.* 1994. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* **367:** 645–648.
2. Bonnet, D. & J.E. Dick. 1997. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat. Med.* **3:** 730–737.
3. Hope, K.J., L. Jin & J.E. Dick. 2004. Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. *Nat. Immunol.* **5:** 738–743.
4. Taussig, D.C. *et al.* 2008. Anti-CD38 antibody-mediated clearance of human repopulating cells masks the heterogeneity of leukemia-initiating cells. *Blood* **112:** 568–575.
5. Martelli, M.P. *et al.* 2010. CD34+ cells from AML with mutated NPM1 harbor cytoplasmic mutated nucleophosmin and generate leukemia in immunocompromised mice. *Blood* **116:** 3907–3922.
6. Taussig, D.C. *et al.* 2010. Leukemia-initiating cells from some acute myeloid leukemia patients with mutated nucleophosmin reside in the CD34(-) fraction. *Blood* **115:** 1976–1984.
7. Ishikawa, F. *et al.* 2007. Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nat. Biotechnol.* **25:** 1315–1321.
8. Bhatia, M. *et al.* 1997. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc. Natl. Acad. Sci. USA* **94:** 5320–5325.
9. Ishikawa, F. *et al.* 2005. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor {gamma} chain(null) mice. *Blood* **106:** 1565–1573.
10. Krause, D.S. & R.A. Van Etten. 2007. Right on target: eradicating leukemic stem cells. *Trends Mol. Med.* **13:** 470–481.
11. Kikushige, Y. *et al.* 2010. TIM-3 is a promising target to selectively kill acute myeloid leukemia stem cells. *Cell Stem Cell.* **7:** 708–717.
12. Jan, M. *et al.* 2011. Prospective separation of normal and leukemic stem cells based on differential expression of TIM3, a human acute myeloid leukemia stem cell marker. *Proc. Natl. Acad. Sci. USA* **108:** 5009–5014.
13. Monney, L. *et al.* 2002. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature* **415:** 536–541.
14. Sanchez-Fueyo, A. *et al.* 2003. Tim-3 inhibits T helper type 1-mediated auto- and alloimmune responses and promotes immunological tolerance. *Nat. Immunol.* **4:** 1093–1101.
15. Sabatos, C.A. *et al.* 2003. Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. *Nat. Immunol.* **4:** 1102–1110.
16. Zhu, C. *et al.* 2005. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat. Immunol.* **6:** 1245–1252.
17. Anderson, A.C. *et al.* 2007. Promotion of tissue inflammation by the immune receptor Tim-3 expressed on innate immune cells. *Science* **318:** 1141–1143.
18. Nakayama, M. *et al.* 2009. Tim-3 mediates phagocytosis of apoptotic cells and cross-presentation. *Blood* **113:** 3821–3830.
19. Nakae, S. *et al.* 2007. TIM-1 and TIM-3 enhancement of Th2 cytokine production by mast cells. *Blood* **110:** 2565–2568.
20. Dekruyff, R.H. *et al.* 2010. T cell/transmembrane, Ig, and mucin-3 allelic variants differentially recognize phosphatidylserine and mediate phagocytosis of apoptotic cells. *J. Immunol.* **184:** 1918–1930.
21. Nimmerjahn, F. & J.V. Ravetch. 2007. Antibodies, Fc receptors and cancer. *Curr. Opin. Immunol.* **19:** 239–245.
22. Nimmerjahn, F. & J.V. Ravetch. 2005. Divergent immunoglobulin g subclass activity through selective Fc receptor binding. *Science* **310:** 1510–1512.
23. Uchida, J. *et al.* 2004. The innate mononuclear phagocyte network depletes B lymphocytes through Fc

receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. *J. Exp. Med.* **199:** 1659–1669.
24. Saito, Y. *et al.* 2010. Identification of therapeutic targets for quiescent, chemotherapy-resistant human leukemia stem cells. *Sci. Transl. Med.* **2:** 17ra19.
25. Jin, L. *et al.* 2006. Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nat. Med.* **12:** 1167–1174.
26. Majeti, R. *et al.* 2009. CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. *Cell* **138:** 286–299.
27. Taussig, D.C. *et al.* 2005. Hematopoietic stem cells express multiple myeloid markers: implications for the origin and targeted therapy of acute myeloid leukemia. *Blood* **106:** 4086–4092.
28. van Rhenen, A. *et al.* 2007. The novel AML stem cell associated antigen CLL-1 aids in discrimination between normal and leukemic stem cells. *Blood* **110:** 2659–2666.
29. Aikawa, Y. *et al.* PU.1-mediated upregulation of CSF1R is crucial for leukemia stem cell potential induced by MOZ-TIF2. *Nat. Med.* **16:** 580–585, 581p following 585.
30. Hosen, N. *et al.* 2007. CD96 is a leukemic stem cell-specific marker in human acute myeloid leukemia. *Proc. Natl. Acad. Sci. USA* **104:** 11008–11013.
31. Bakker, A.B. *et al.* 2004. C-type lectin-like molecule-1: a novel myeloid cell surface marker associated with acute myeloid leukemia. *Cancer Res.* **64:** 8443–8450.
32. Wang, P.L. *et al.* 1992. Identification and molecular cloning of tactile. A novel human T cell activation antigen that is a member of the Ig gene superfamily. *J Immunol.* **148:** 2600–2608.

blood

2012 120: 2454-2465
Prepublished online June 27, 2012;
doi:10.1182/blood-2012-03-420489

Revised International Prognostic Scoring System for Myelodysplastic Syndromes

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

Revised International Prognostic Scoring System for Myelodysplastic Syndromes

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The International Prognostic Scoring System (IPSS) is an important standard for ssessing prognosis of primary untreated adult patients with myelodysplastic syndromes (MDS). To refine the IPSS, MDS patient databases from international institutions were coalesced to assemble a much larger combined database (Revised-IPSS [IPSS-R], n = 7012, IPSS, n = 816) for analysis. Multiple statistically weighted clinical features were used to generate a prognostic categorization model. Bone marrow cytogenetics, marrow blast percentage, and cytopenias remained the basis of the new system. Novel components of the current analysis included: 5 rather than 3 cytogenetic prognostic subgroups with specific and new classifications of a number of less common cytogenetic subsets, splitting the low marrow blast percentage value, and depth of cytopenias. This model defined 5 rather than the 4 major prognostic categories that are present in the IPSS. Patient age, performance status, serum ferritin, and lactate dehydrogenase were significant additive features for survival but not for acute myeloid leukemia transformation. This system comprehensively integrated the numerous known clinical features into a method analyzing MDS patient prognosis more precisely than the initial IPSS. As such, this IPSS-R should prove beneficial for predicting the clinical outcomes of untreated MDS patients and aiding design and analysis of clinical trials in this disease. (*Blood*. 2012;120(12): 2454-2465)

Introduction

The myelodysplastic syndromes (MDS) consist of a heterogeneous spectrum of myeloid clonal hemopathies. The International Prognostic Scoring System (IPSS) has been an important standard for assessing prognosis of primary untreated adult MDS patients.[1] However, since its publication in 1997, modification of existing parameters and additional prognostic systems have been suggested as providing meaningful differences for patients' clinical outcomes,[2-5] and the World Health Organization (WHO) added morphologic refinement of the French-American-British (FAB) classification.[6,7] In addition, the WHO Prognostic Scoring System (WPSS)[2,3] has provided new insights into prognostic variables, adding red blood cell (RBC) transfusion dependence along with IPSS cytogenetic classification and WHO dysplastic categories. Importantly, recent newer cytogenetic groupings are reported to be prognostically valuable and to refine those features used in the IPSS.[8] Additional variables suggested as providing prognostic information in MDS included serum lactate dehydrogenase (LDH),[9-11] ferritin,[12] and β_2-microglobulin[13,14] as well as marrow fibrosis[15-17] and patient comorbidities and performance status.[5,18-20]

To examine the prognostic impact of these new clinical and cytogenetic variables and attempt to refine the IPSS, coordination of investigators and coalescence of MDS databases from multiple international institutions provided a much larger combined database of patients by the International Working Group for Prognosis in MDS (IWG-PM) project.

The aims of this study were to refine the IPSS by reassessing the prior major predictive features, determining the impact of the newer clinical features for prognostic power, incorporating larger and more differentiated cytogenetic subgroups, and reevaluating

Submitted March 28, 2012; accepted June 17, 2012. Prepublished online as *Blood* First Edition paper, June 27, 2012; DOI 10.1182/blood-2012-03-420489.

The online version of this article contains a data supplement.

Presented in preliminary form at the 11th International Symposium of MDS, Edinburgh, Scotland, May 19, 2011.[50]

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

their prognostic impact. Statistically weighted clinical features were used to generate a prognostic categorization model. This larger combined database permitted better analyses of the specific impact of marrow blast percentage, depth of cytopenias, and of the less frequent features, particularly further evaluating the relatively rare cytogenetic subgroups. In addition, as some features had only been reported from single centers, this combined database extended such findings.

Methods

Under the aegis of the MDS Foundation, MDS databases of primary untreated MDS patients from multiple international institutions from 11 countries, including data from the Spanish, French, Piemonte (Italy) and Brazilian MDS Registries and that from the International MDS Risk Analysis Workshop (IMRAW), were submitted and evaluated by the IWG-PM project. Databases came from both university- and nonuniversity-based hospitals associated with the country's MDS-focused groups. Institutional review board approval was obtained from the respective institutions. After careful vetting for accuracy, a combined IWG-PM database of 7012 patients, classified morphologically by FAB (n = 7000) and, in most cases, by the WHO criteria (n = 5504),[6] was created. Inclusion criteria were: primary MDS patients whose disease had not been treated with disease-altering therapy during their MDS phase (ie, no hypomethylating agents, intensive chemotherapy, or hematopoietic stem cell transplantation). Marrow blasts were required to be $\leq 30\%$, peripheral blood blasts $\leq 19\%$, white blood count (WBC) $\leq 12 \times 10^9$/L, and absolute neutrophil count (ANC) $\leq 8 \times 10^9$/L). The patient's blood counts needed to demonstrate ≥ 2 months of stable disease. Marrow blasts and cytogenetic results, hemoglobin, ANC, and platelet levels at diagnosis were documented, and data regarding patient's survival and development of acute myeloid leukemia (AML) were obtained. The patient ages were ≥ 16 years. Data regarding use of erythropoiesis-stimulating agents or myeloid growth factors were not systematically collected.

The results of cytogenetic analysis of bone marrow were reviewed by the Cytogenetics Committee (D.H., Chair; J.S., C.F., M.M.L.B., F.S., and M.L.S.) using standard ISCN criteria.[21] Specific karyotypic abnormalities and their risk categories were used as per Schanz et al, which required ≥ 10 patients for inclusion as a specific abnormality. Parameters evaluated were cytogenetic risk category,[8] marrow aspirate blast percent, depth of cytopenias, degree of marrow fibrosis (0-1+ vs 2-3+), Eastern Cooperative Oncology Group performance status, serum LDH (normal values defined by each hospital), ferritin and β_2-microglobulin levels, RBC transfusion dependence, and patient age at diagnosis. The database of untreated primary MDS patients from the Medical University of Vienna was used as an external independent validation cohort.

Statistical methods

Modeling of prognostic risk was based on multivariate analysis of survival time and time to AML transformation. Functional relations of bone marrow blasts and cytopenias with prognostic risk were analyzed to define appropriate categories for score calculation.[22] Robust Cox models[23] for survival, time to transformation, and combination of both were built to derive the relative weights within the score. To compensate for possible heterogeneities, analyses were stratified by data source, year of diagnosis, and age. This led to generation of reference scores for these clinical outcomes.

The score was developed following a hierarchical approach. The main score was built based on the initially elaborated categories for bone marrow blasts and cytopenias together with the cytogenetic risk categories.[8] The categorizations of cytopenias were adjusted to clinically relevant cutpoints. To calculate a specific feature's added score, the proportional weight of the score was used. A "combined" score model was effectively used rather than having 2 separate models for survival and AML transformation. Separate specific score variants for survival and AML transformation were considered, but they provided very little gain. Therefore, for ease of communication and implementation, 1 unified model was preferred. This model approximated (statistically) both outcomes adequately, particularly for survival when age was included. Risk-scoring values were rounded to the nearest 0.5 unit with re-estimation of all statistics for the rounded scores. To ease interpretation, boundaries of the 5 risk categories of the final score were chosen to build a scale with approximately equal risk increments between 2 adjacent levels.

The effect of age was modeled as an optional additive feature for overall survival prediction by including age in a model with the already defined main scores. Additional potentially differentiating features were analyzed to estimate their incremental prognostic values, given the already defined main score.

As a measure of prognostic power, the Dxy coefficient for censored data[24] was used. Dxy is a concordance coefficient varying between -1 and 1, with 0 representing no predictive power and 1 perfect concordance of ascribed risk and survival and time to transformation, respectively. For a potentially additive feature to be considered clinically significant, both a $P < .05$ and a gain in prognostic power (Dxy) were required. Dxy's were internally validated by bootstrapping the related Cox models.[24] Two-sided P values $< .05$ were reported as significant. Correlations between ordered categorical variables were measured by Kendall tau. In line with the nature of the project, no adjustment for multiple testing was applied. All analyses were performed using the open source software R Version 2.14.1.[25,26] Kaplan-Meier curves were used to demonstrate clinical outcomes.

Results

Patient characteristics

Data from 7012 patients from multiple institutional databases in the combined IWG-PM database were evaluated. Their median age was 71 years, 77% were > 60 years, the male/female ratio 1.5:1, and median follow-up time 3.9 years. The 7012 patients obtained for evaluation were classified by FAB (n = 7000, 99.8%)[7] and additionally by WHO (n = 5504, 78.5%)[6] and/or WPSS (n = 2325, 33.2%).[9] Table 1 shows the individual clinical variables and outcomes (survival, AML evolution) for our patient cohort, with Dxy concordance coefficients (indicating prognostic power) and univariate P values. Bootstrap-validated Dxy values were almost identical with the sample results given in Table 1 (all differences were at most .01, except the value for β_2-microglobulin).

Identification of significant prognostic variables

As in the IPSS, marrow cytogenetic subset, marrow blast percentage, and cytopenias were considered as the basis of this new prognostic system (the Revised IPSS [IPSS-R]) given their statistical weight compared with the other variables analyzed herein using Cox proportional hazard regression analyses, with overall survival and AML transformation as outcomes. Multivariate analysis of these variables led to their relative statistical weighting, determining their impact on prognostic risk, using Cox proportional hazard regression. In descending order, these 5 major variables for evaluating clinical outcomes were: cytogenetic risk groups, marrow blast percentage, and depth of cytopenias (hemoglobin, platelet, and ANC levels, respectively). The novel components obtained in the current analysis included: 5 rather than 3 cytogenetic prognostic subgroups with specific classification of a number of less common cytogenetic subsets and alteration of others (Table 2)[8]; the $< 5\%$ marrow blast category was split between 0%-2% and $>$ 2-$< 5\%$, whereas all patients with $> 10\%$ blasts were grouped in the same category; depth of cytopenias at clinically and statistically relevant cutpoints rather

Table 1. Clinical variables of MDS patients

	No. of patients	% of patients	Survival years, median	Dxy (95% CI; *P**)	No. of patients	% of patients	AML/25% y	Dxy (95% CI; *P**)
Cytogenetics	7012	100		.25	6485	100		.27
Very good	255	4	5.4	(.23-.26)	255	3	NR	(.24-.31)
Good	5069	72	4.8		4657	72	9.4	
Intermediate	947	13	2.7		875	14	2.5	
Poor	283	4	1.5		276	4	1.7	
Very poor	458	7	0.7		452	7	0.7	
BM blasts	7012	100		.30	6485	100		.47
0-2%	3279	47	5.9	(.28-.32)	3004	46	NR	(.44-.50)
> 2- < 5%	1266	18	4.2		1172	18	8.5	
5-10%	1377	19	2.3		1263	20	2.2	
> 10%-30%	1090	16	1.3		1046	16	1.0	
> 10%-20%	(901)	(13)	(1.3)		(860)	(13)	(0.93)	
> 20%-30%	(189)	(3)	(1.4)		(186)	(3)	(1.0)	
Hemoglobin, g/dL	7012	100		.21	6485	100		.16
≥ 10	3377	48	5.5	(.19-.23)	3109	48	9.5	(.12-.19)
8-< 10	2464	35	2.9		2286	35	5.5	
< 8	1171	17	2.0		1090	17	2.4	
Platelets†	7012	100		.23	6485	100		.17
≥ 100	4195	60	5.1	(.21-.25)	3823	59	8.7	(.14-.21)
50- < 100	1469	21	2.8		1368	21	3.1	
< 50	1348	19	1.6		1294	20	3.1	
ANC†	7012	100		.11	6485	100		.16
≥ 0.8	5758	82	4.4	(.10-.13)	5303	82	9.2	(.13-.19)
< 0.8	1254	18	1.9		1182	18	1.9	
IPSS-R	7012	100		.43	6485	100		.52
Very low	1313	19	8.8	(.42-.45)	1212	19	NR	(.49-.55)
Low	2646	38	5.3		2395	37	10.8	
Intermediate	1433	20	3.0		1310	20	3.2	
High	898	13	1.6		857	13	1.4	
Very high	722	10	0.8		711	11	0.7	
Sex	7012	100		.07	6485	100		.04
Male	4243	61	3.3	(.05-.09)	3962	61	5.8	(.00-.07)
Female	2769	39	4.8		2523	39	8.0	(.030)
Age	7012	100		.05	6485			−.02
≤ 60 y	1582	23	5.7	(.03-.06)	1489	23	8.1	(−.05-.01)
> 60 y	5430	77	3.5		4996	77	6.1	(.082)
ECOG Performance Status	2496	36		.16	2489	38		.09
0	751	30	4.3	(.13-.18)	748	30	8.8	(.04-.15)
1	1477	59	2.2		1473	59	6.3	(.005)
2-4	268	11	1.6		268	11	3.5	
Serum ferritin	3049	43		.16	2747	42		.11
≤ 350 ng/mL	1602	53	6.3	(.13-.20)	1435	52	NR	(.05-.17)
> 350 ng/mL	1447	47	4.2		1312	48	14.5	(.004)
Serum LDH	4257	61		.12	4130	64		.12
Normal	3103	73	4.1	(.10-.14)	3007	73	9.2	(.08-.16)
High	1154	27	2.1		1123	27	3.2	
Serum β_2-microglobulin	1005	14		.14 (.10-.18)	1005	15		.02 (−.08-.11)
≤ 2 g/mL	263	26	3.8		263	26	6.7	(.498)
> 2 g/mL	742	74	1.7		742	74	4.6	
Marrow fibrosis	1323	19		.04	1183	18		.05
No	1158	88	5.2	(.01-.07)	1055	89	14.5	(−.01-.12)
Yes	165	12	3.2	(.004)	128	11	4.8	(.069)
RBC transfusion dependence	2933	42		.26	2645	41		.27
No	2003	68	6.9	(.23-.29)	1808	68	14.5	(.22-.32)
Yes	930	32	2.3		837	32	2.1	
IPSS	7008	100		.37	6481	100		.48
Low	2625	37	7.0	(.35-.39)	2394	37	NR	(.45-.51)
Intermediate-1	2778	40	3.6		2541	39	6.1	
Intermediate-2	1126	16	1.5		1074	17	1.2	
High	479	7	0.9		472	7	0.7	

AML/25% indicates time for 25% of patients to develop AML.

*All univariate P values not explicitly stated are $P < .001$.

†$\times 10^9$/L.