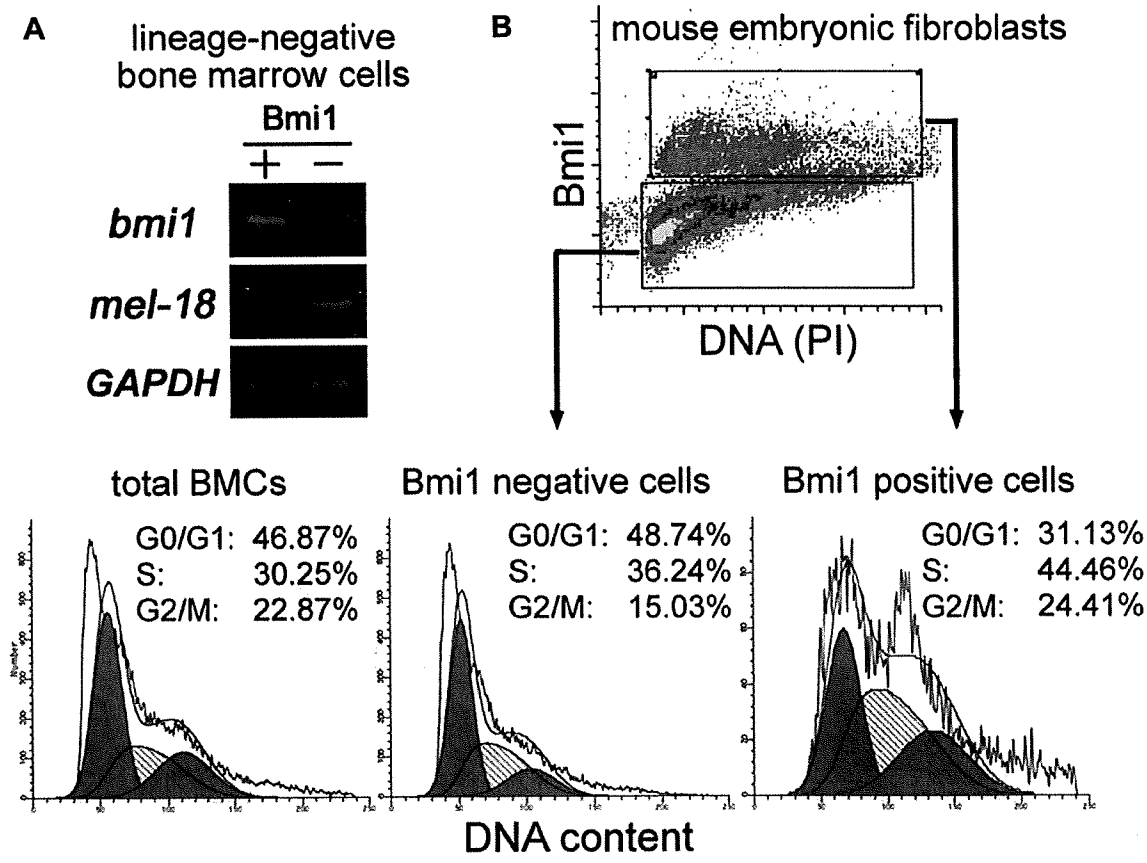


Figure 2. Bmi1-positive and Bmi1-negative fractions. (**A**) The Bmi1-positive and Bmi1-negative fractions were separated from the purified lineage-negative cells, and the expression patterns of *bmi1* and *mel-18* in their respective fractions were analyzed. The Bmi1-positive cells did not possess *mel-18* messenger RNA (mRNA), while the Bmi1-negative cells possessed *mel-18* mRNA. (**B**) Mouse embryonic fibroblasts were fixed and stained with anti-Bmi1 and propidium iodide. The percentage of Bmi1-positive cells in the S + G_2/M phase was greater than the corresponding overall value for bone marrow cells (BMCs). This suggests that Bmi1 was needed for cell proliferation. In contrast, a higher percentage of Bmi1-negative cells were in the G_0/G_1 phase.

positive cells in the S + G_2/M phase was higher than the percentage of Bmi1-negative cells in the same phase.

Our results showed that Mel-18 and Bmi1 rarely coexist in the nucleus of murine marrow cells. These results support the hypothesis that the unique expression of Bmi1 or Mel-18 in early hematopoietic progenitor cells may represent their different functions in hematopoiesis.

Knockdown of PcG gene expression by siRNA

The siRNA approach was applied to analyze the influence of the expression of *bmi1* and/or *mel-18* on murine hematopoietic cell function. Bone marrow cells were cultured for 48 hours, which was followed by the addition of siRNAs against *bmi1* and/or *mel-18*. Results of the introduction of siRNAs are shown in Figure 3A. Addition of siRNAs against *bmi1* caused approximately 60% to 70% decrease in *bmi1* expression, while that of siRNAs against *mel-18* did not affect the *bmi1* expression. Further, with regard to *bmi1* expression, the combined effect of siRNAs against *bmi1* and *mel-18* was similar to that of siRNAs against *bmi1*. In contrast, the addition of siRNAs against *mel-18* caused approximately 50% decrease in *mel-18* expression, while the addition of siRNAs against *bmi1* did not affect *mel-18* expression. Thus, the siRNA approach ensured that the expression of each *PcG* gene in murine bone marrow cells was limited to at least 50%.

The major *bmi1* target is the *ink4a/arf* locus, and the *ink4a* gene has been shown to be located downstream of *bmi1* [18]. Therefore, we analyzed the *ink4a* expression in the murine bone marrow cells that were exposed to each of the siRNAs. Exposure of the cells to the siRNAs against *bmi1* led to an increase in *ink4a* expression (Fig. 3B).

In vitro and in vivo assays of PcG gene-knockdown cells

The colony-forming activity of the murine bone marrow cells that had undergone knockdown of *PcG* gene expression was assessed in methylcellulose culture. The secondary CFC assay is used as an easy approach for investigating stem-cell functions [19]. Because the secondary CFCs are quite immature, we performed the assay by replating aliquots of cells obtained from complete primary colonies. The culture procedure is illustrated in

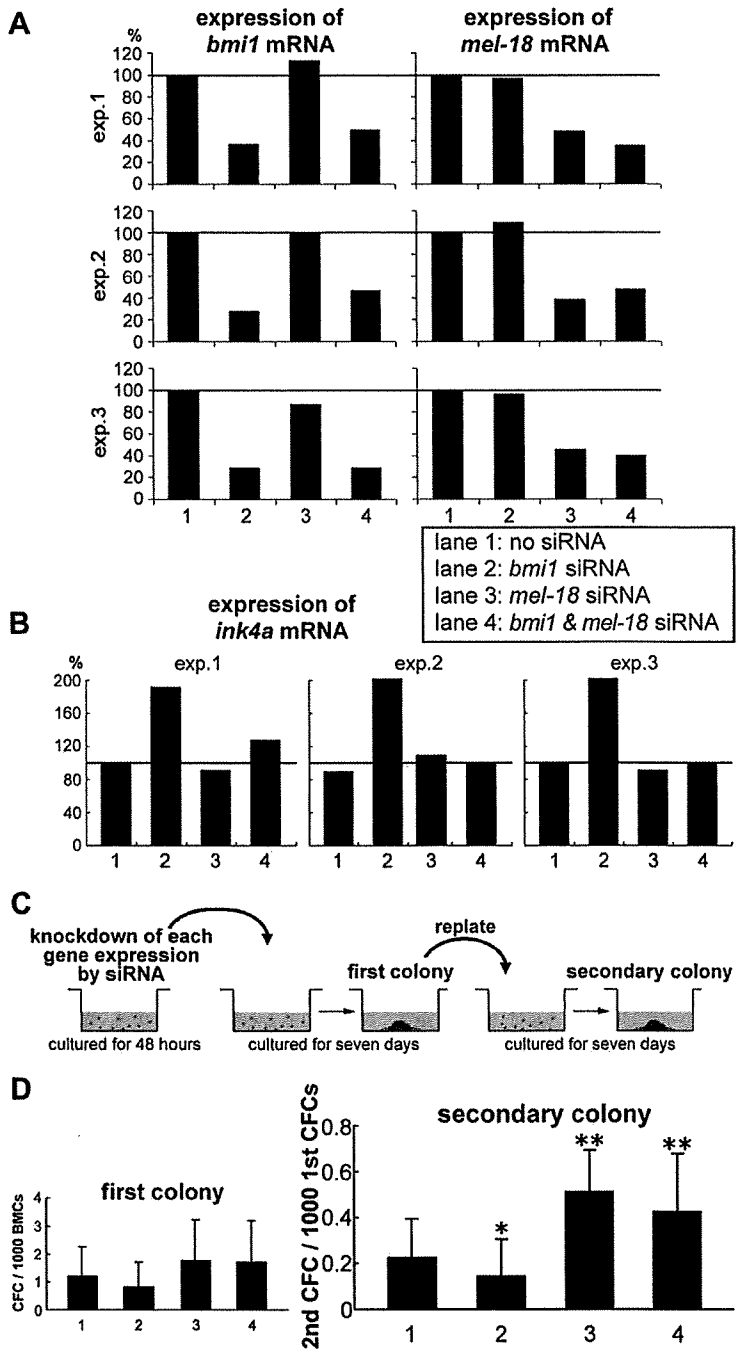


Figure 3. Knockdown of *PcG* gene expression and cell function in vitro. Lane 1, no small interfering RNA (siRNA); lane 2, *bmi1* siRNA; lane 3, *mel-18* siRNA; lane 4, *bmi1* and *mel-18* siRNAs. **(A)** Efficiency of gene knockdown was measured with quantitative reverse transcriptase polymerase chain reaction. Expression of the *GAPDH* gene was used as an endogenous control. The ratio of each gene knockdown is plotted on the y-axis, and the corresponding gene is plotted on the x-axis. Expressions of both *bmi1* (left panel) and *mel-18* (right panel) are shown. Gene expression in the marrow cells possibly decreased to at least 50% due to knockdown by siRNA. **(B)** siRNAs against *bmi1* increased the expression of the *ink4a* gene. **(C)** Basic experimental design of the siRNA experiments. siRNA agents were applied during the first 48 hours of culturing. **(D)** Methylcellulose colony assay was performed using marrow cells in which the expression of each gene was knocked down. There were no significant differences between the groups. However, the number of secondary colonies formed from *mel-18* knockdown cells was significantly higher than those formed from *bmi1* knockdown cells (0.51 ± 0.18 vs 0.23 ± 0.17 colonies per 1,000 primary colony-forming cells [CFCs]). The secondary colony-forming activity in *mel-18* knockdown cells was not restored by the addition of siRNAs against *bmi1*.

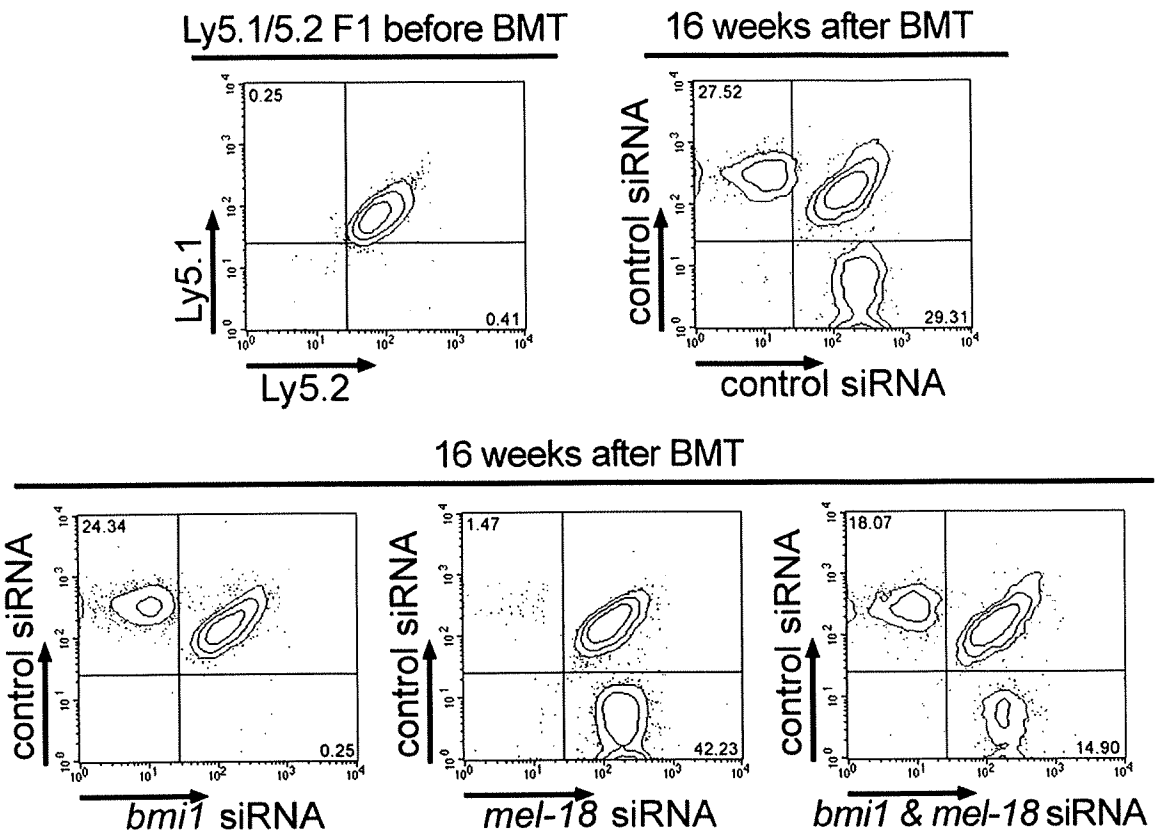


Figure 4. Knockdown of *PcG* gene expression and cell function in vivo. When no small interfering RNA (siRNA) agents were applied and equal numbers of Ly5.1 and Ly5.2 cells were transplanted, the percentages of Ly5.1- and Ly5.2-derived cells in the peripheral blood were equal. Equal numbers of the *bmi1* knockdown and *mel-18* knockdown cells (2×10^5 cells) were transplanted into mice irradiated with a dose of 9 Gy. Peripheral blood was collected 16 weeks after transplantation. The representative fluorescein-activated cell sorting profiles are shown. We observed a large percentage of cells derived from the *mel-18* knockdown marrow cells. BMT = bone marrow transplantation.

Figure 3C. As shown in Figure 3D, there were no significant differences between the numbers of primary colonies formed. However, the number of secondary colonies formed from *mel-18* knockdown cells was significantly higher than those formed from *bmi1* knockdown cells (0.51 ± 0.18 vs 0.23 ± 0.17 colonies per 1,000 primary CFCs). The effect of secondary colony-forming activity in the *mel-18* knockdown cells was not restored by the addition of siRNAs against *bmi1*. Because the effects of gene knockdown by siRNA do not persist for more than a few days, we considered that the fate of the bone marrow cells was decided during the 48-hour incubation with siRNAs.

For functional evaluation of HSCs, murine bone marrow cells that were exposed to siRNAs against *bmi1* and/or *mel-18* for 48 hours were transplanted into irradiated recipient mice. The Ly5.1 and Ly5.2 mice and the F1 mice, which are hybrids of the Ly5.1 and Ly5.2 mice, have been used for HSC-transplantation experiments [20–23]. F1 mice were used as the recipient mice. After transplantation, we determined the number of blood cells derived from the mice. Peripheral blood samples were collected 16 weeks after transplantation, and the percentage of donor cells was evaluated using flow cytometry. The representative fluorescein-activated cell sorting profiles are shown in Figure 4. When equal numbers of Ly5.1 and Ly5.2 cells exposed to control siRNA were transplanted, similar percentages of Ly5.1- and Ly5.2-derived cells were detected in the peripheral blood. However, the transplantation of *PcG*-knockdown cells yielded intriguing data. When the bone marrow cells exposed to siRNAs against *bmi1* were transplanted, we observed a small percentage of cells derived from the *bmi1* knockdown cells. In contrast, when the bone marrow cells exposed to siRNAs against *mel-18* were transplanted, we observed a relatively large percentage of cells derived from the *mel-18* knockdown cells. These results suggest that expression of the *PcG* genes—*bmi1* and *mel-18*—is involved in the reconstitution activity of hematopoietic cells.

Balance in Bmi1 and Mel-18 in human bone marrow cells

Expression of Bmi1 and Mel-18 proteins in human bone marrow cells obtained from healthy volunteers was examined by flow cytometry. Figure 5A shows a representative

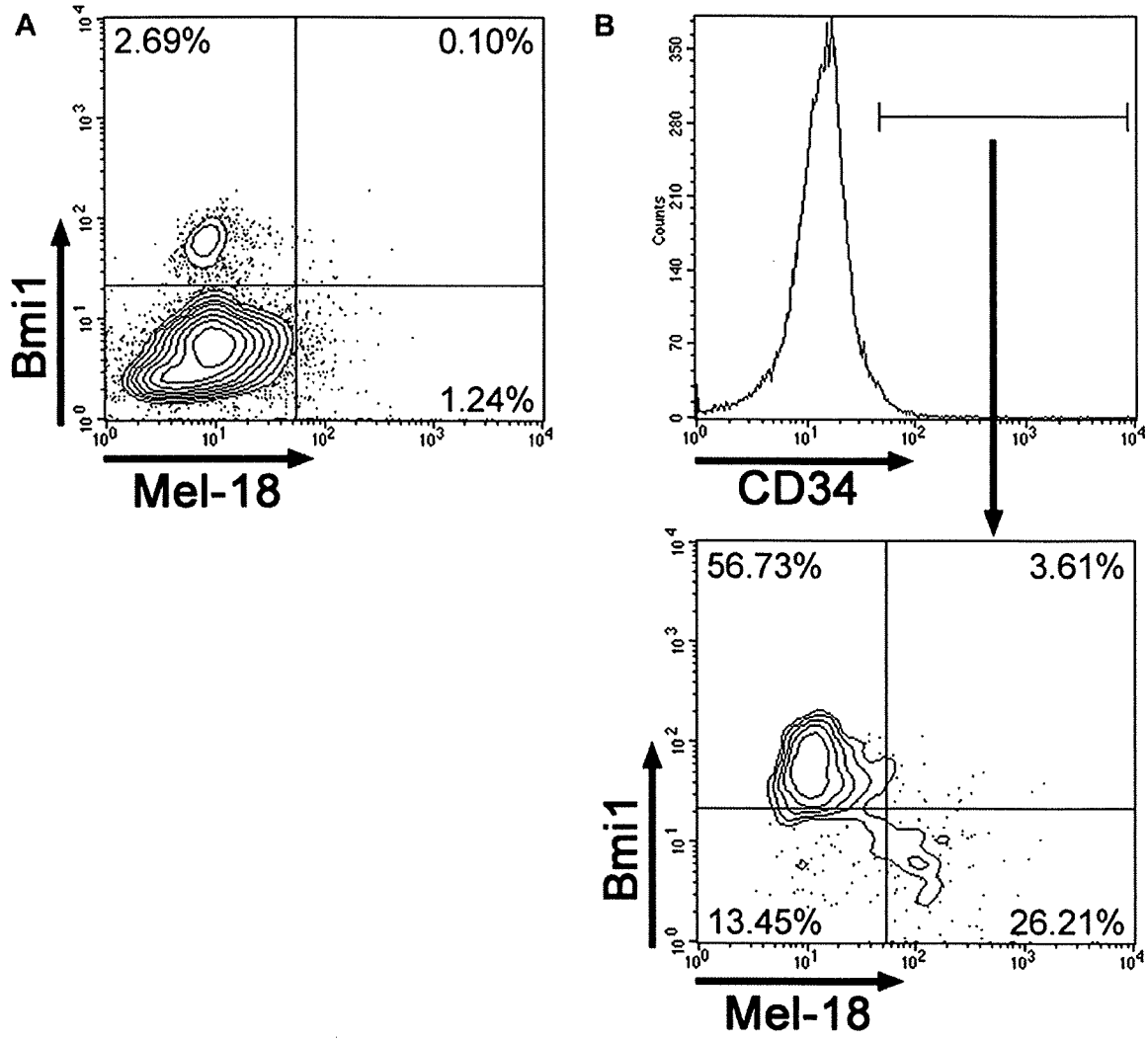


Figure 5. Balance between Bmi1 and Mel-18 expression in human bone marrow cells. (A) Typical flow-cytometric profiles of human bone marrow cells stained with Bmi1-phycoerythrin (PE) and Mel-18-Alexa Fluor 488 are shown. (B) In the CD34-positive fraction, the Bmi1-positive and Mel-18–negative cells were distinguishably separated from the Mel-18–positive and Bmi1-negative cells.

flow-cytometric analysis of human bone marrow cells stained with Bmi1-PE and Mel-18-Alexa Fluor 488. We observed that only a few human cells were positive for both Bmi1 and Mel-18, which was similar to the observations in case of mice cells. To study the expression of Bmi1 and Mel-18 in primitive hematopoietic cells, the human bone marrow cells were gated for CD34-positive cells. As shown in Figure 5B, the CD34-positive cells had either Bmi1 or Mel-18. In the CD34-positive fraction, the percentages of double-positive cells and double-negative cells were quite low.

Expression of bmi1 and mel-18 genes in each lineage

It has been reported that *bmi1* expression is upregulated in the earlier stages of human and murine hematopoietic cell differentiation, while the expression of *mel-18* increases in the later stages of differentiation [24,25]. We examined the expression of *bmi1* and *mel-18* in each stage of hematopoietic cell differentiation by using real-time RT-PCR. As indicated in the previously mentioned reports, the expression of *bmi1* increased in primitive hematopoietic cells, and the expression of *mel-18* increased in the differentiated hematopoietic cells (Fig. 6A).

In order to determine the clinical application of these results, we examined the expression ratio of *bmi1* and *mel-18* in the hematopoietic transplantation source. We used the residual cells in the blood bags obtained in 22 cases of allogeneic bone marrow transplantation at our hospital. Each case is summarized in Table 1. The expression patterns of *bmi1* and *mel-18* indicated that use of transplantation sources with a low *bmi1* to *mel-18* ratio was associated with significantly early hematopoietic recovery

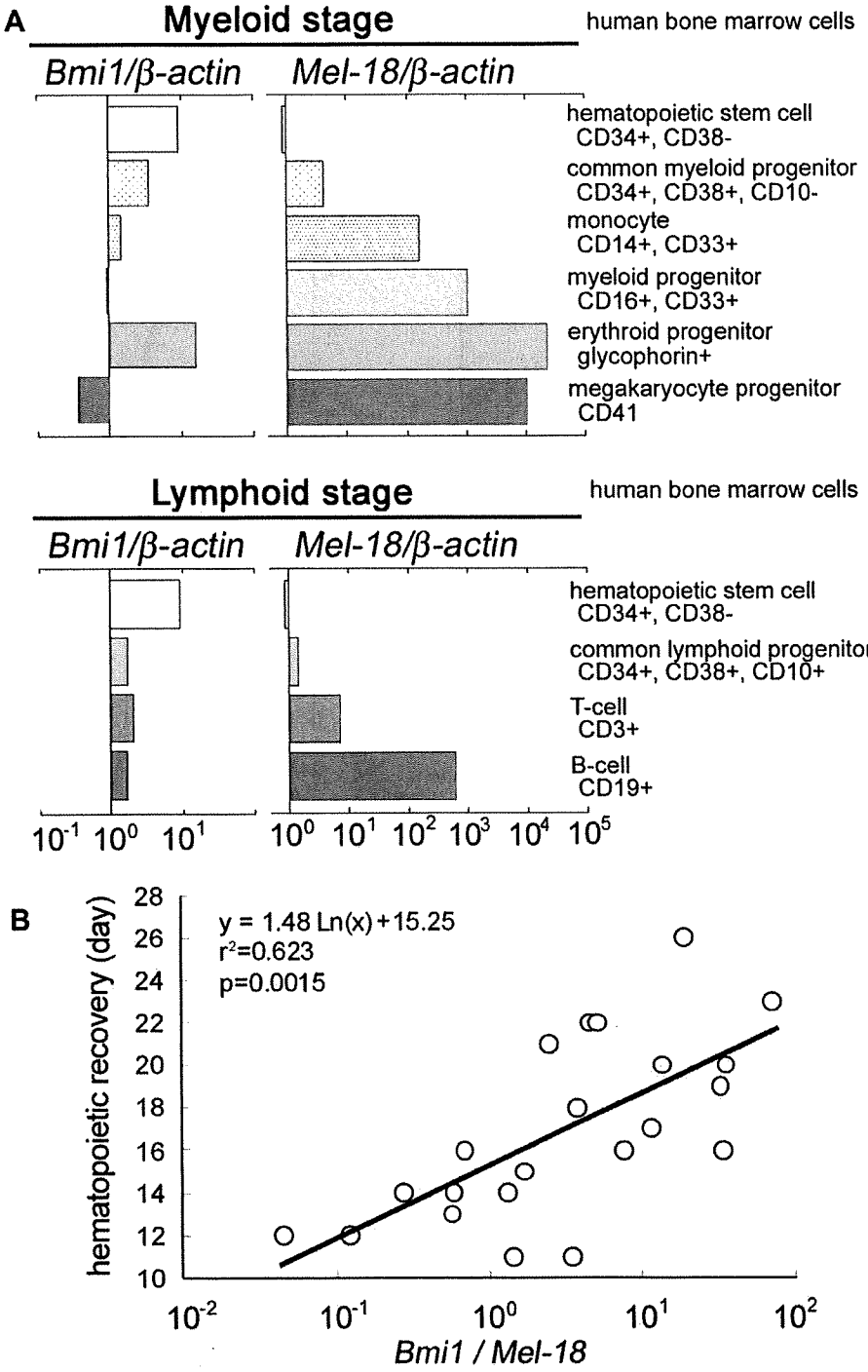


Figure 6. Expression of human *PcG* genes in each lineage and transplantation source. **(A)** Expression of Bmi1 increased in primitive hematopoietic cells, except in the erythroid lineage, and the expression of Mel-18 increased in differentiated hematopoietic cells. **(B)** The relation between hematopoietic recovery after transplantation and the Bmi1 to Mel-18 ratio is shown. The Bmi1 to Mel-18 ratio is plotted on the x-axis, and the day of hematopoietic recovery from hematopoietic stem cell transplantation is plotted on the y-axis. Hematopoietic recovery was significantly early when using transplantation sources with a low Bmi1/Mel-18 ratio.

($p = 0.0015$) (Fig. 6B). According to our data, expression patterns of the *PcG* genes are more reliable than the number of CD34-positive cells for predicting hematopoietic recovery (Suppl. Fig. S2).

Discussion

The *mel-18* and *bmi1* genes are members of the mammalian *PcG* gene family, and their protein products, together with M33, Rae28/Mph-1, and Scmh1, are constituents of

Table 1. The diseases and conditioning regimens of the patients who received bone marrow transplantation[a].

Diseases	n	%	Conditioning
Aplastic anemia	6	27	Flu, CPM, ATG, TLI
Acute lymphocytic leukemia	6	27	L-PAM, AraC, CPM, TBI
Acute myeloid leukemia	3	14	L-PAM, TBI
Chronic granulomatous disease	4	8	Flu, CPM, ATG, TLI
Familial hemophagocytic lymphohistiocytosis	1	5	Flu, L-PAM
Myelodysplastic syndrome	1	5	Flu, L-PAM
Rhabdomyosarcoma	1	5	TEPA, L-PAM
Total	22	100	

AraC = cytarabine; ATG = antithymocyte globulin; CPM = cyclophosphamide; Flu = fludarabine; L-PAM = melphalan; TBI = total body irradiation; TEPA = triethylene thiophosphoramide; TLI = total lymphoid irradiation.
[a]Median age: 13 years (range, 0 – 29 years); gender: 13 male and 8 female.

mammalian PRC1 [9–13,26]. Studies on early development in *Drosophila* have suggested that a transition from PRC2 to PRC1 may occur during development [27]. The PcG complexes are associated with and directly involved in the modification of the histone tails and the chromatin structure [28]; further, they play a role in the silencing and stabilization of chromatin [29,30]. While *PcG* regulates the inactive state of the homeotic gene, the *Trithorax* group (*TrxG*) regulates the active state of the gene [31–33]. In other words, *TrxG* and *PcG* act reciprocally in the transcriptional regulation of gene function in cells, with the former performing a positive function and the latter, a negative function. Bmi1 is the direct biological counterpart of the mixed lineage leukemia gene, which is a member of the TrxG group. When the Bmi-knockout mice are crossed with mixed lineage leukemia–heterozygote mice, the haploinsufficiency phenotype is completely reverted [34].

Bmi1 is essential for HSC self-renewal. The expression of *ink4a* and *arf* is increased in the hematopoietic cells of *bmi1*-deficient mice [5]. In a previous study, double deletion of *bmi1* and *ink4a/arf* partially rescued the phenotypes observed in *bmi1*-deficient mice [18], suggesting that Ink4a, Arf, and p53 are downstream effectors of Bmi1 that are involved in regulating the proliferation and survival of HSCs during self-renewing cell divisions [35]. Moreover, the *bmi1/ink4a/arf*-mutant mice exhibit substantial recovery of hematopoietic cells [36]. In contrast, it is known that Mel-18 regulates cell cycle in breast cancer cells via cyclin D2 [37]. Recently, it has been reported that although Mel-18 is a PRC1 component similar to Bmi1, it regulates the cell cycle without associating with the Ink4a/Arf signals [38]. Moreover, in our study, a decrease in *bmi1* expression in murine bone marrow cells, which was induced by the addition of siRNAs against *bmi1*, was associated with an increase in *ink4a* expression, but a decrease in *mel-18* expression did not lead to any changes in the *ink4a* expression. Mel-18 is an important molecule involved in the differentiation of HSCs, and HSC differentiation is limited to *mel-18*–deficient mice [8]. Bmi1 and Mel-18 share the same structure, including the RING finger motif [14]. Bmi1 and Mel-18 may compete when they are incorporated in complexes. Recently, it has been reported that Mel-18 is a physiological regulator of Bmi1 expression in fibroblasts and epithelial cells [39]. The authors have suggested that this relationship between Bmi1 and Mel-18 expression may persist in other cell types. In the present study, we noted the existence of a reciprocal relationship between Bmi1 and Mel-18 in HSCs.

In addition to these transcriptional regulators, several systems with important factors, such as the Wnt, bone morphogenetic protein, Sonic hedgehog, and Notch pathways, have been proposed to be involved in the regulation of HSC self-renewal [40–43]. Signal transduction from the extracellular molecules is an indispensable factor in the self-renewal of HSCs. A partial crosslink may exist between the Notch and Wnt pathways [44]. In addition, there may be several other unknown cross-links in cellular signal transduction. We believe that the reciprocal expression of PcG proteins and the abovementioned signal transductions from extracellular molecules overlap at some point and regulate the fate of HSCs.

The findings of *PcG* gene balance in human bone marrow cells have considerable clinical significance. These findings can form the basis for assessments of hematopoietic recovery after bone marrow transplantation. For example, a high Bmi1 to Mel-18 ratio of the sample cells will increase the time required for complete engraftment. This could be attributed to the comparatively higher number of undifferentiated cells and the lower number of differentiating cells in such a sample. Conversely, a low Bmi1 to Mel-18 ratio in the bone marrow cells will correspond to early hematopoietic recovery because of the relatively higher number of differentiated cells. Thus, regular examination of *PcG* gene expression in transplantation sources may provide crucial information for clinical bone marrow transplantation.

Acknowledgments

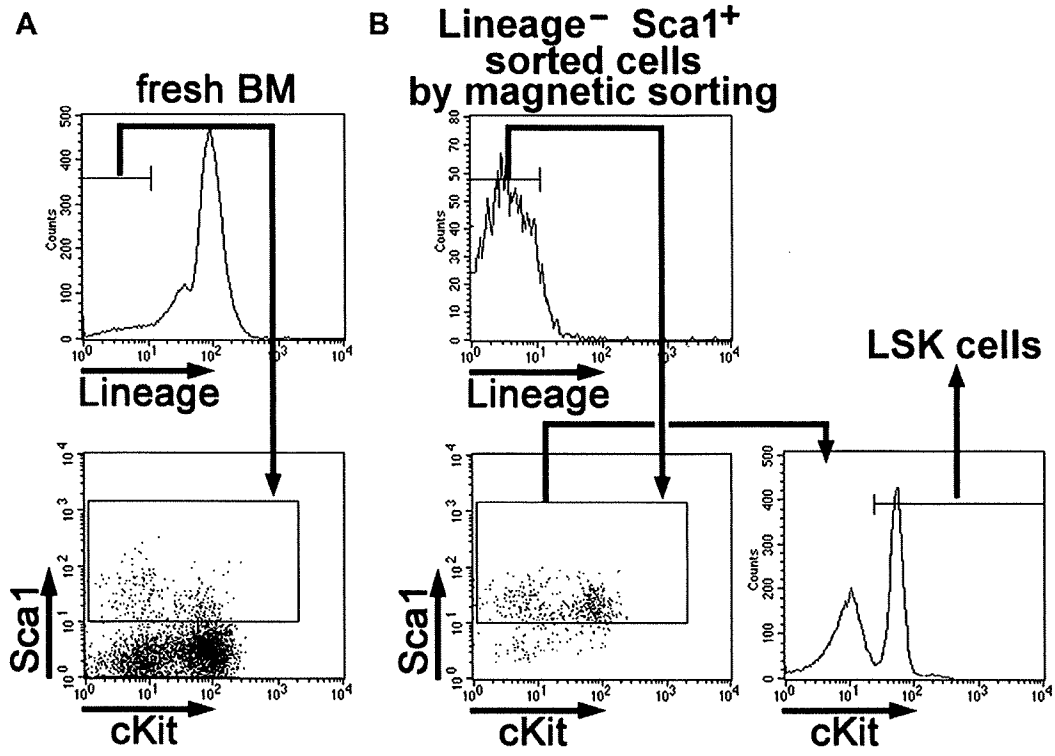
This work was supported by a Grant-in-Aid for Scientific Research (17790706) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (T. K.). We thank S. Nishimura and T. Sato for their helpful discussion. We thank the Analysis Center of Life Science, Hiroshima University, for allowing the use of their facilities. No financial interest/relationships with financial interest relating to the topic of this article have been declared.

References

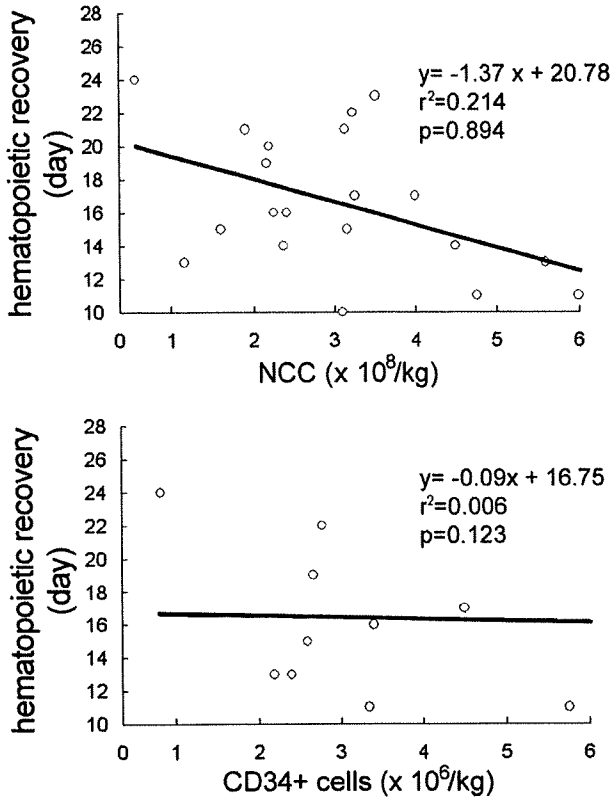
1. Antonchuk J, Sauvageau G, Humphries RK. HOXB4 overexpression mediates very rapid stem cell regeneration and competitive hematopoietic repopulation. Exp Hematol. 2001;29:1125–1134.
2. Antonchuk J, Sauvageau G, Humphries RK. HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. Cell. 2002;109:39–45.
3. Krosl J, Austin P, Beslu N, Kroon E, Humphries RK, Sauvageau G. In vitro expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein. Nat Med. 2003;9:1428–1432.
4. Ohta H, Sawada A, Kim JY, et al. *Polycomb* group gene *rae28* is required for sustaining activity of hematopoietic stem cells. J Exp Med. 2002;195:759–770.

5. Park I-K, Qian D, Kiel M, et al. *Bmi-1* is required for maintenance of adult self-renewing haematopoietic stem cells. Nature. 2003;423:302–305.
6. Iwama A, Oguro H, Negishi M, Kato Y, Nakauchia H. Epigenetic regulation of hematopoietic stem cell self-renewal by Polycomb group genes. Int J Hematol. 2005;81:294–300.
7. Iwama A, Oguro H, Negishi M, et al. Enhanced self-renewal of hematopoietic stem cells mediated by the Polycomb gene product Bmi-1. Immunity. 2004;21:843–851.
8. Kajiume T, Ninomiya Y, Ishihara H, Kanno R, Kanno M. *Polycomb* group gene *mel-18* modulates the self-renewal activity and cell cycle status of hematopoietic stem cells. Exp Hematol. 2004;32:571–578.
9. Alkema MJ, Bronk M, Verhoeven E, et al. Identification of Bmi1-interacting proteins as constituents of a multimeric mammalian Polycomb complex. Genes Dev. 1997;11:226–240.
10. Gunster MJ, Satijn DPE, Hamer KM, et al. Identification and characterization of interactions between the vertebrate Polycomb-group protein BMI1 and human homologs of polyhomeotic. Mol Cell Biol. 1997;17:2326–2335.
11. Hashimoto N, Brock HW, Nomura M, et al. RAE28, BMI1, and M33 are members of heterogeneous multimeric mammalian Polycomb group complexes. Biochem Biophys Res Commun. 1998;245:356–365.
12. Shao Z, Raible F, Mollaaghababa R, et al. Stabilization of chromatin structure by PRC1, a Polycomb complex. Cell. 1999;98:37–46.
13. Francis NJ, Saurin AJ, Shao Z, Kingston RE. Reconstitution of a functional core Polycomb repressive complex. Mol Cell. 2001;8:545–556.
14. Freemont PS, Hanson IM, Trowsdale J. A novel cysteine-rich sequence motif. Cell. 1991;64:483–484.
15. Ikuta K, Weissman IL. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. Proc Natl Acad Sci U S A. 1992;89:1502–1506.
16. Li CL, Johnson GR. Murine hematopoietic stem and progenitor cells, I: enrichment and biologic characterization. Blood. 1995;85:1472–1479.
17. Li CL, Wu L, Antica M, Shortman K, Johnson GR. Purified murine long-term *in vivo* hematopoietic repopulating cells are not prothymocytes. Exp Hematol. 1995;23:21–25.
18. Jacobs JJ, Kieboom K, Marino S, et al. The oncogene and Polycomb-group gene *bmi-1* regulates cell proliferation and senescence through the *ink4a* locus. Nature. 1999;397:164–168.
19. Buske C, Feuring-Buske M, Abramovich C, et al. Deregulated expression of HOXB4 enhances the primitive growth activity of human hematopoietic cells. Blood. 2002;100:862–868.
20. Sudo K, Ema H, Morita Y, Nakauchi H. Age-associated characteristics of murine hematopoietic stem cells. J Exp Med. 2000;192:1273–1280.
21. Ema H, Takano H, Sudo K, Nakauchi H. *In vitro* self-renewal division of hematopoietic stem cells. J Exp Med. 2000;192:1281–1288.
22. Ema H, Nakauchi H. Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. Blood. 2000;95:2284–2288.
23. Larsson J, Blank U, Klintman J, Magnusson M, Karlsson S. Quiescence of hematopoietic stem cells and maintenance of the stem cell pool is not dependent on TGF-β signaling *in vivo*. Exp Hematol. 2005;33:592–596.
24. Lessard J, Baban S, Sauvageau G. Stage-specific expression of *Polycomb* group genes in human bone marrow cells. Blood. 1998;91:1216–1224.
25. Hosen N, Yamane T, Muijtjens M, Pham K, Clarke MF, Weissman IL. Bmi-1-green fluorescent protein-knock-in mice reveal the dynamic regulation of *bmi-1* expression in normal and leukemic hematopoietic cells. Stem Cells. 2007;25:1635–1644.
26. Tagawa M, Sakamoto T, Shigemoto K, et al. Expression of novel DNA-binding protein with zinc finger structure in various tumor cells. J Biol Chem. 1990;265:20021–20026.
27. Schumacher A, Faust C, Magnuson T. Positional cloning of a global regulator of anterior-posterior patterning in mice. Nature. 1996;383: 250–253.
28. Orlando V. *Polycomb*, epigenomes, and control of cell identity. Cell. 2003;112:599–606.
29. Orlando V, Paro R. Chromatin multiprotein complexes involved in the maintenance of transcription patterns. Curr Opin Genet Dev. 1995;5: 174–179.
30. Pirrotta V. Chromatin complexes regulating gene expression in *Drosophila*. Curr Opin Genet Dev. 1995;5:466–472.
31. Lewis EB. A gene complex controlling segmentation in *Drosophila*. Nature. 1978;276:565–570.
32. Kennison JA, Tamkun JW. Dosage-dependent modifiers of Polycomb and Antennapedia mutations in *Drosophila*. Proc Natl Acad Sci U S A. 1988;85:8136–8140.
33. Paro R. Imprinting a determined state into the chromatin of *Drosophila*. Trends Genet. 1990;6:416–421.
34. Hanson RD, Hess JL, Yu BD, et al. Mammalian Trithorax and polycomb-group homologues are antagonistic regulators of homeotic development. Proc Natl Acad Sci U S A. 1999;96:14372–14377.
35. Park I-K, Morrison SJ, Clarke MF. *Bmi1*, stem cells, and senescence regulation. J Clin Invest. 2004;113:175–179.
36. Oguro H, Iwama A, Morita Y, Kamijo T, van Lohuizen M, Nakauchi H. Differential impact of *Ink4a* and *Arf* on hematopoietic stem cells and their bone marrow microenvironment in *Bmi1*-deficient mice. J Exp Med. 2006;203:2247–2253.
37. Chun T, Rho SB, Byun HJ, Lee JY, Kong G. The Polycomb group gene product Mel-18 interacts with cyclin D2 and modulates its activity. FEBS Lett. 2005;579:5275–5280.
38. Lee JY, Jang KS, Shin DH, et al. Mel-18 negatively regulates *INK4a/ARF*-independent cell cycle progression *via* Akt inactivation in breast cancer. Cancer Res. 2008;68:4201–4209.
39. Guo WJ, Datta S, Band V, Dimri GP. Mel-18, a Polycomb group protein regulates cell proliferation and senescence *via* transcriptional repression of Bmi-1 and c-Myc oncoproteins. Mol Biol Cell. 2007; 18:536–546.
40. Reya T, Duncan AW, Ailles L, et al. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature. 2003;423:409–414.
41. Bhardwaj G, Murdoch B, Wu D, et al. Sonic hedgehog induces the proliferation of primitive human hematopoietic cells *via* BMP regulation. Nat Immunol. 2001;2:172–180.
42. Zon LI. Self-renewal *versus* differentiation, a job for the mighty morphogens. Nat Immunol. 2001;2:142–143.
43. Stier S, Cheng T, Dombkowski D, Carlesso N, Scadden DT. Notch1 activation increases hematopoietic stem cell self-renewal *in vivo* and favors lymphoid over myeloid lineage outcome. Blood. 2002;99: 2369–2378.
44. Espinosa L, Ingles-Esteve J, Aguilera C, Bigas A. Phosphorylation by glycogen synthase kinase-3β down-regulates Notch activity, a link for Notch and Wnt pathways. J Biol Chem. 2003;278:32227–32235.

Supplementary data associated with the article can be found in the online version, at doi:10.1016/j.exphem.2009.04.011



Supplementary Figure S1. Purification of primitive hematopoietic cells. Bone marrow (BM) cells were enriched for lineage-negative and Sca1-positive cells, and the cells were then gated by cKit-allophycocyanin staining.



Supplementary Figure S2. Relationship between the number of infusion cells and hematopoietic recovery after transplantation. The number of infused marrow cells or infused CD34-positive cells is plotted on the x-axis, and the day of hematopoietic recovery from hematopoietic stem cell transplantation is plotted on the y-axis. There was no significant correlation. Although the use of a large number of transplantation source cells is advantageous, the expression patterns of the *PcG* genes are more reliable for predicting hematopoietic recovery.

Ela2 Mutations and Clinical Manifestations in Familial Congenital Neutropenia

Masaaki Shiohara, MD, Tomonari Shigemura, MD,* Shoji Saito, MD,* Miyuki Tanaka, MD,* Ryu Yanagisawa, MD,* Kazuo Sakashita, MD,* Hiroshi Asada, MD,† Eizaburo Ishii, MD,‡ Kazutoshi Koike, MD,§ Motoaki Chin, MD,‖ Masao Kobayashi, MD,¶ and Kenichi Koike, MD**

Purpose: Three familial cases of each of severe congenital neutropenia (SCN) and cyclic neutropenia (CN) in addition to 3 sporadic cases of SCN were analyzed for neutrophil elastase (*Ela2*) gene mutation. The contents of the neutrophil-specific granule proteins cathelicidin antimicrobial peptide and neutrophil gelatinase-associated lipocalin were also analyzed in SCN.

Methods: Genomic DNA was extracted from the patients' peripheral blood or bone marrow, and the coding sequence of the *Ela2* gene was amplified by polymerase chain reaction and subjected to direct sequencing. The contents of antimicrobial peptides were analyzed by flow cytometry.

Results: Three cases of familial SCN (P13L, R52P, and S97L), 2 of familial CN (W212stop and P110L), and 1 of sporadic SCN (V72M) were shown to have heterozygous mutations in the *Ela2* gene. W212stop found in a familial CN case was a novel mutation of *Ela2*. Prophylactic treatment for growth factors or antibiotic prophylaxis against bacterial infection was useful for lowering the frequency of infectious episodes. Adult patients tended to have less frequent infections compared with minors in the same family. The contents of both cathelicidin antimicrobial peptide and neutrophil gelatinase-associated lipocalin were significantly reduced in SCN compared with healthy controls.

Conclusions: Prophylaxis by growth factor or antibiotics is useful for decreasing risks of bacterial infections in SCN and CN. Adults were likely to have less frequent infections than children in familial cases of SCN and CN with the same mutation of *Ela2*.

Key Words: neutropenia, neutrophil elastase, neutrophil-specific granule protein

(*J Pediatr Hematol Oncol* 2009;31:319–324)

Congenital neutropenia consists of several disorders with or without other symptoms and signs. Severe congenital neutropenia (SCN, OMIM no. 202700) and cyclic neutropenia (CN, OMIM no. 162800) are the 2 major primary genetic types where neutropenia is the main component.[1,2] Peripheral blood (PB) neutrophil count is < 500/μL in the most severe type of SCN. Granulocytic maturation is usually arrested at the promyelocyte to myelocyte stage and numbers of more mature cells are severely reduced in bone marrow (BM) in SCN. In CN, the neutrophil count is not reduced except in the neutropenic period, which occurs at approximately 21-day intervals. In many cases, slight anemia and also thrombocytosis, probably as a consequence of infection, are observed.[3] Severe and frequent bacterial infections are inevitable from infancy in both diseases. Linkage analysis of families with CN led to identification of the neutrophil elastase gene (*Ela2*) (GenBank accession number: Gene ID 1991, cDNA NM_001972.2) as a cause of CN.[4] A subsequent study revealed *Ela2* mutations to be involved in about 60% of sporadic and autosomal dominant SCN.[5–8] It has not been clearly elucidated how heterozygous mutations in the *Ela2* gene cause both CN and SCN.

Bacterial infection is usually severe from infancy in both diseases, but the transition of clinical manifestations from childhood to adulthood is unclear. Familial cases with the same genetic alterations will provide useful insight into these diseases. In this study, 6 familial cases of SCN and CN and 3 sporadic SCN were analyzed for *Ela2* mutation. Clinical characteristics, including the effects of prophylaxis against bacterial infection or differences in the frequency of infectious episodes in familial cases, are discussed. Furthermore, the contents of neutrophil antimicrobial peptides (AMPs) in SCN were also analyzed.

PATIENTS AND METHODS

Patients

Three cases of each of familial (SCN-1: brothers; SCN-2 and SCN-3: father and daughter) and sporadic SCN (SCN-4, SCN-5, and SCN-6) and 3 cases of familial CN (CN-1: father and daughter; CN-2, and CN-3: father and son), a total of 15 samples, were analyzed (Tables 1, 2). The diagnosis of SCN was made according to the following criteria: an absolute neutrophil count of < 500/μL and granulocytic maturation arrested at promyelocyte to myelocyte stage in BM. CN was diagnosed when an approximately 21-day cycle of neutropenia (absolute neutrophil count < 500/μL) was observed. Neutrophil count was normal between the neutropenic periods in CN. Some patients had additional complicating diseases and SCN or CN: father of SCN-2, Crohn disease; SCN-4, leiomyoma; SCN-5, immunoglobulin A deficiency and chorea; father of CN-2, hyperthyroidism; and CN-3, febrile convulsions. None of the patients in the present study

Received for publication July 16, 2008; accepted November 27, 2008.
From the *Department of Pediatrics, Shinshu University School of Medicine, Matsumoto, Japan; †Sogo Iwaki Kyouritsu Hospital, Iwaki, Fukushima; ‡Nagano Children's Hospital, Azumino, Nagano; §Ibaragi Children's Hospital, Mito, Ibaragi; ‖Department of Pediatrics, Nihon University School of Medicine, Itabashi, Tokyo; and ¶Department of Pediatrics, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Hiroshima, Japan.
Reprints: Masaaki Shiohara, MD, Department of Pediatrics, Shinshu University School of Medicine, 3-1-1, Asahi, Matsumoto 390-8621, Japan (e-mail: shiohara@shinshu-u.ac.jp).

TABLE 1. Primer Sequence of *ELa2*, *Gfi-1*, and *Hax-1* Genes

Genes	Forward Primer	Product Size	Reverse Primer
ELa2			
Exon 2	cggaggggcagagaccccgga	760	agaccgggacgcggggtccga
Exon 3	ctcgagcaccttcgccctcag	452	tcaacggcccatggcgggtat
Exon 4	tggtgacggctcccactctacagat	557	ctagcccggtgcctgttgctgcag
Exon 5	gactgcagcacaggcaccgtggctag	462	acaaaacagagaatgtttattgtgc
Gfi-1			
Exon 2	cacgtctccacctggttttc	363	aaaaacagcagcaaaaggga
Exon 3	ccttcagcaccctcagactc	423	agctgtccaagtcccagaga
Exon 4	gcacgcagtgttctacaagc	713	agagagaaggccgctgagag
Exon 5	ctctcagcggccttctctct	415	tctcgaggctcacagtggt
Exon 6	tttagggtcagctgaggggt	456	tggctctggggagaattatg
Exon 7	aacacgtcaccctccaagtt	481	gaggtaaggcgaaggaggag
Hax-1			
Exon 1	tcgaaatcgcttttcgtac	500	gcgacttctgtcctctctg
Exon 2	ctccgaccctctccctagc	529	atgggaaaggacttgggc
Exon 3	gggttggtgggtgaaataaa	457	ctctggtgatctgcccac
Exon 4, 5	caggaaggagtgtgtaaat	580	agtccccaccatattccg
Exon 6, 7	tggggtgagttgaaagaaa	619	tgaaatcaggttttagatg

developed myelodysplastic syndrome or acute myeloid leukemia. Methods of prophylaxis against bacterial infection were trimethoprim-sulfamethoxazole (TMP-SMX, 0.1 g/kg/d, maximum 3.0 g/d), 4 SCN and 5 CN; erythromycin (250 mg/d), 1 SCN; and no prophylaxis, 3 SCN and 1 CN. Granulocyte colony-stimulating factor (G-CSF) treatment (2 μg/kg by subcutaneous injection, 2 to 3 times/wk) was given simultaneously with TMP-SMX in SCN-1, SCN-4, CN-1, and CN-2. Early treatment given to all the patients in cases of bacterial infection consisted of intravenous administration of antibiotics. SCN-6 received cord blood transplantation because of neutropenia refractory to G-CSF and life-threatening bacterial pneumonia. Information regarding infections was obtained from both questionnaires and patient records, and the data were analyzed retrospectively.

Mutational Analysis of *Ela2* Gene

This study was approved by the Institutional Review Board for Human Genome Research of Shinshu University. Genomic DNA was extracted from the patients' PB or BM mononuclear cells using standard techniques. Four exons covering the coding sequence of the *Ela2* gene were amplified by polymerase chain reaction (PCR). Each product was prepared in a 25-μL reaction with 0.5 U of Taq DNA polymerase (Takara, Tokyo, Japan), PCR buffer, 200 mM dNTPs, and 0.5 mM of each primer. PCR was performed with an initial denaturation step at 94°C for 2 minutes followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, with a final extension step at 72°C for 5 minutes. PCR products were run on 1.5% agarose gels, excised, and purified using a commercial kit (Geneclean II; Qbiogene, Carlsbad, CA). Direct sequencing was performed in both directions using the same primers. An ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) using BigDye version 2 terminator chemistry was used for all sequencing. Sequencing of the coding region of growth factor independence (*Gfi*)-*1* genes (GenBank accession number: Gene ID 2672, cDNA NM_005263)[9] or *Hax-1* (GenBank accession number: Gene ID 10456, cDNA NM_006118.3)[10,11] was also performed. Primer sequences used for PCR and sequencing for *Ela2*, *Gfi-1*, and *Hax-1* genes in this study are listed in Table 1.

Flow Cytometric Analysis

Monoclonal antibodies (mAbs) for phycoerythrin-CD16, peridinin chlorophyll protein (PerCP)-CD45, and

TABLE 2. Clinical Details of SCN and CN Patients

Patient	Family Member	Sex	Age (y)	Neutrophil Count $\times 10^9$/L Before G-CSF	Maturation Arrest
SCN-1	B	M, M	21, 18	0, 0	mbl, pro
SCN-2	Fa, D	M, F	39, 6	0, 0	mye, mye
SCN-3	Fa, D	M, F	35, 5	0.3, 0	ND, pro
SCN-4	—	F	31	0	mye
SCN-5	—	M	19	0.1	mye
SCN-6	—	M	5	0	pro
CN-1	Fa, D	M, F	51, 9	0, ND	mye, mye
CN-2	Fa, S	M, M	42, 6	0, 0	ND, ND
CN-3	Fa, S	M, M	40, 9	0.5, 0.1	ND, ND

B indicates brother; CN, cyclic neutropenia; D, daughter; Fa, father; F, female; G-CSF, granulocyte colony-stimulating factor; M, male; mbl, myeloblast; mye, myelocyte; ND, not determined; pro, promyelocyte; S, son; SCN, severe congenital neutropenia; —, sporadic.

isotype-matched mouse immunoglobulin were purchased from BD Immunocytometry Systems (Mountain View, CA). Cathelicidine antimicrobial peptide (CAMP) and neutrophil gelatinase-associated lipocalin (NGAL) were purchased from HyCult Biotechnology (Uden, the Netherlands). For analysis of cytoplasmic CAMP or NGAL expression in PB neutrophils, samples of 1 to 2×10^6 whole blood cells were collected in polystyrene tubes and incubated with appropriately diluted PerCP-CD45, control mouse immunoglobulin, CAMP or NGAL monoclonal antibodies, and secondary fluorescein isothiocyanate-goat antimouse immunoglobulin, as described previously.[12] The cells were washed twice and analyzed with a FACScan flow cytometer using the Lysis II software program (BD Immunocytometry Systems). Viable cells were gated according to their forward light-scattering characteristics and side-scattering characteristics (SSC), and then the neutrophil population was gated according to their SSC and CD45 expression. The Mann-Whitney *U* test was used for statistical analysis using StatView software version 5.0 (SAS Institute, Cary, NC).

RESULTS

Ela2 mutations were found in all patients of familial SCN, 1 of 3 sporadic SCN, and 2 of 3 familial CN cases (Table 3), which spanned all coding exons of the *Ela2* gene. Five of 6 cases were missense mutations (P13L, R52P, S97L, V72P, and P110L) and 1 was a nonsense mutation (W212stop). Adult family members who also had histories of frequent bacterial infections had the same mutation of *Ela2* found in each proband. All missense mutations found in this study have been reported previously, whereas the nonsense mutation W212stop found in CN-1 was a novel *Ela2* mutation and has not been reported elsewhere.

All cases analyzed in this study had preventive medications or intravenous administration of antibiotics in case of bacterial infections, except for SCN-6 treated with cord blood transplantation. Prophylaxis included regular G-CSF injection and TMP-SMX administration in 3 SCN and 4 CN cases, TMP-SMX administration in 1 SCN and 1 CN, erythromycin administration in 1 SCN, and no prophylaxis in 3 SCN and 1 CN. The efficacy of these methods was analyzed by comparing the frequencies of various symptoms caused by infection before and after the establishment of strategies against bacterial infections. Manifestations of infection were divided into 4 groups: (1) stomatitis, tonsillitis, or lymphadenitis; (2) skin infections, including pyoderma or cellulitis; (3) pneumonia; and (4) sepsis or other life-threatening infections. Each episode was scored according to its frequency: 4, always present; 3, 2 to 4 times/mo; 2, 2 to 4 times/y; 1, once every 2 to 4 years; and 0, no episode. Each score was expressed as a number of boxes, as shown in Figure 1. Left and right pillars show the frequencies of infectious episodes before and after prophylaxis or early management against bacterial infection, respectively. The frequencies of infectious episodes decreased after prophylactic or early management in all cases except SCN-1. Next, the frequency of infectious episodes was compared at present time between adults (more than 20 y of age) and children (< 20 y of age) with familial SCN, who had the same background of gene mutations. The right pillars in each column of SCN-1, SCN-2, SCN-3, CN-1, CN-2, and CN-3 represent infection frequencies in adults and children in each family (Fig. 1). Adults were likely to have less frequent infectious episodes than children.

Next, the expression of neutrophil AMPs, including CAMP and NGAL, which play important roles in oxygen-independent bactericidal activities of neutrophils and in innate immune responses, were analyzed in SCN patients by flow cytometry.[13] Whole PB cells were stained with PerCP-conjugated CD45 antibodies and fluorescein isothiocyanate-conjugated CAMP or NGAL antibodies. Stained cells developed according to SSC characteristics and fluorescence intensities of PerCP, and the cell population of neutrophils was gated and analyzed for expression of each peptide. These cell populations were positive for CD16, which is a type III low-affinity Fc γ receptor expressed in neutrophils (data not shown). Figure 2A shows typical results of this analysis. As shown in Figure 2B, CAMP was positive in 85.4% of neutrophils in an average of 26 healthy controls. In contrast, 45.9% of neutrophils of 8 SCN patients were positive for CAMP ($P = 0.0021$). NGAL was positive for 93.2% and 46.0% of neutrophils in an average of 26 controls and 8 SCN, respectively ($P = 0.0018$). These results indicated that the contents of neutrophil secondary granule peptides, both CAMP and NGAL, were significantly lower in SCN than in normal controls.

DISCUSSION

Germline mutations were found in the *Ela2* gene in 3 (3/3, 100%) cases of familial SCN, 1 (1/3, 33%) of sporadic

TABLE 3. Results of Mutation Analysis in SCN and CN

	Ela2					
Patient	**Intron/Exon**	**Base Change**	**Predicted Protein Change**	**Mutation in Family (Father/Brother)**	***Gfi-1***	***Hax-1***
SCN-1	Exon 2	CCC→CTC	P13L	P13L (B)	—	ND
SCN-2	Exon 3	CGG→CCG	R52P	R52P (F)	—	ND
SCN-3	Exon 4	TCG→TTG	S97L	S97L (F)	—	ND
SCN-4	Exon 3	GTG→ATG	V72M	ND	—	ND
CN-1	Exon 5	TGG→TGA	W212stop	W212stop (F)	—	ND
CN-2	Exon 4	CCG→CTG	P110L	P110L (F)	ND	ND
SCN-5		No mutation	—	ND	—	—
SCN-6		No mutation	—	ND	—	—
CN-3		No mutation	—	—	—	—

B indicates brother; CN, cyclic neutropenia; F, father; ND indicates not determined; SCN, severe congenital neutropenia.

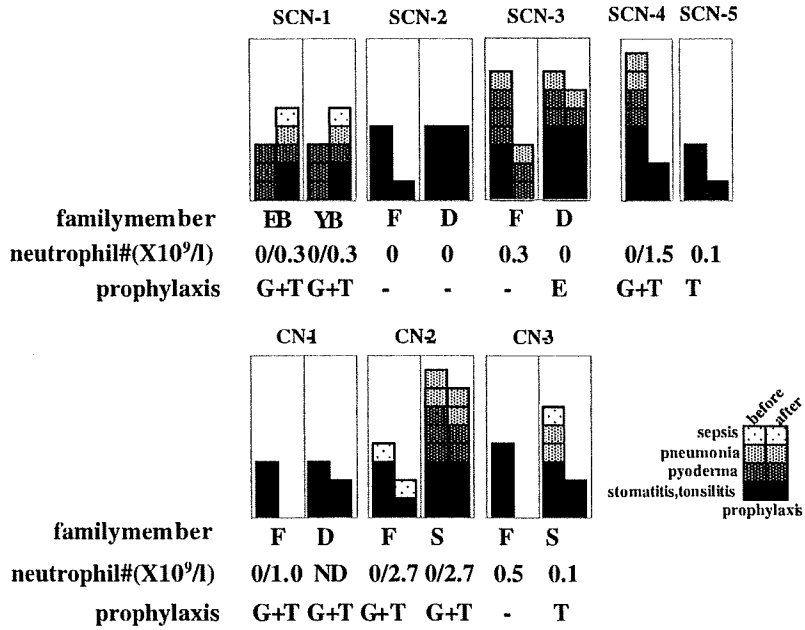


FIGURE 1. Changes of clinical manifestations by prophylaxis or early intervention against bacterial infection. Diagnoses are indicated as follows: ■, stomatitis, tonsillitis, or lymphadenitis; ▩, pyoderma; ▨, pneumonia; and ⊡, sepsis or other life-threatening infections. The frequencies of each manifestation are presented by the number of boxes: 4 boxes, always present; 3 boxes, 2 to 4 times/mo; 2 boxes, 2 to 4 times/y; and 1 box, once every 2 to 4 years or less. Categories and frequencies before and after prophylaxis or early intervention are presented in the left and right pillars in each column, respectively. Affected family member, the neutrophil count without G-CSF/with G-CSF, and prophylactic methods of each patient are described below the schema. The dose of G-CSF was 2 μg/kg, 2 to 3 times/wk. D indicates daughter; E, erythromycin; EB indicates elder brother; F, father; G+T, G-CSF plus TMP-SMX; S, son; T, TMP-SMX; YB, younger brother; −, no prophylaxis. G-CSF indicates granulocyte colony-stimulating factor; TMP-SMX, trimethoprim-sulfamethoxazole.

SCN, and in 2 (2/3, 66%) familial CN cases. Five of 6 mutations have already been characterized, and 1 found in CN-1 (W212stop) was novel.[7] W212stop was localized to the final exon of the *Ela2* gene, and was expected to yield a transcript and a shortened protein that may act via a dominant negative or loss-of-function mechanism on the genes. The nonsense mutation of *Ela2* in CN detected in the present study is the first such mutation reported to date. Inheritance was suggested to be autosomal dominant, as reported previously.[5] Mutation analysis of the *Ela2* gene is useful in differential diagnosis of SCN and CN with other chronic neutropenias, especially in infancy.

Gfi-1[9] and *Hax-1*[10,11] had also been reported to be mutated in a limited number of SCN patients. *Gfi-1* mutations were not found in the 13 cases examined, except CN-2 that was not analyzed. *Hax-1* mutations were not found in 4 cases without either *Ela2* or *Gfi-1* mutations.

The data reported here demonstrated the efficacy of treatment by G-CSF and prophylaxis against bacterial infection. SCN-1, with increased frequency of infectious episodes despite prophylaxis with TMP-SMX and G-CSF treatment, was refractory to G-CSF in neutrophil number. Pediatric patients SCN-2 (R52P) and SCN-3 (S97L) tolerated the prophylactic antibiotics well. In particular, the usefulness and safety of TMP-SMX in prophylactic use in autoimmune neutropenia in infancy was reported.[14] No side effects, including anemia, thrombocytopenia, or liver dysfunction, were observed in the present study.

Adult SCN or CN patients had a tendency to show less frequent bacterial infection than those < 20 years old in each family. The severity of each infectious episode was also lower in adults than in younger family members (data not shown). Four out of 7 adult patients had G-CSF treatment compared with patients under 20 years old all of whom received prophylaxis or early management against bacterial infection. There were few differences in the neutrophil or monocyte count in the PB of each family member (data not shown). The reason for the improvement of symptoms with transition from childhood to adulthood is unknown. The adult patients had experienced similar infection frequencies during childhood as their counterparts, and received similar prophylaxis or treatment between the present and in childhood. Intrafamilial variations observed in diseases inherited in an autosomal dominant fashion, such as Marfan syndrome[15] or long QT syndrome,[16] were not obvious in this study. An alternative phagocytic function, including the monocyte/macrophage system, may develop. This study was not designed as a prospective randomized control analysis, and criteria for prophylaxis or treatment were not followed as a protocol. Efficacy of treatment or prophylaxis or improvement of symptoms by transition may not be significant, but merely represent a tendency. Therefore, these results must be interpreted carefully.

AMPs in neutrophils have a broad spectrum of antimicrobial activities against fungi and viruses and Gram-positive and Gram-negative bacteria.[17] Neutrophil AMPs are classified into primary, secondary (specific), and tertiary granular proteins. Both CAMP and NGAL are classified as secondary granule proteins. Neutrophil-specific

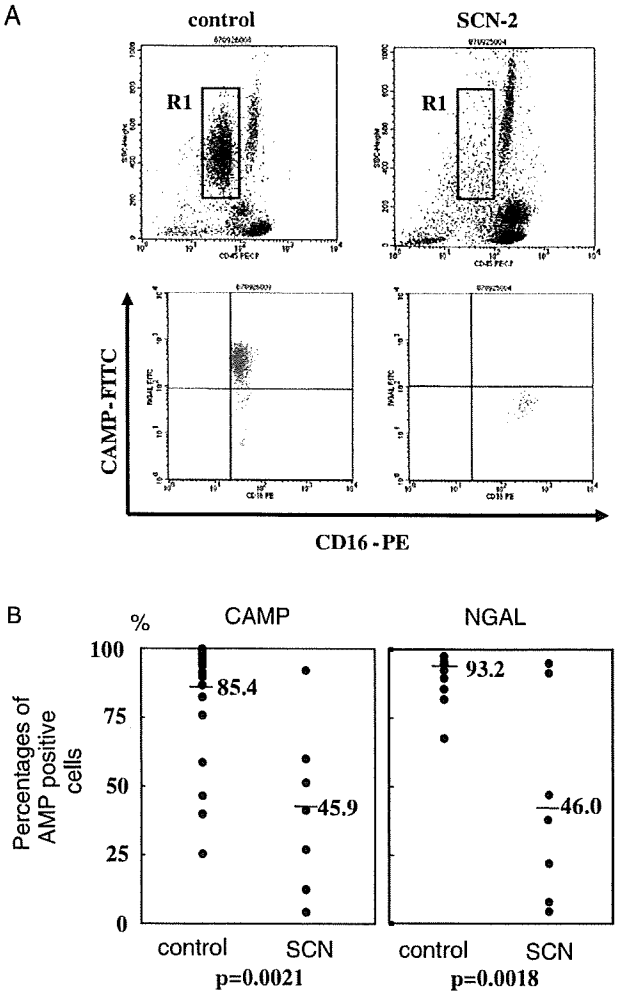


FIGURE 2. Flow cytometric analysis of CAMP expression in neutrophils derived from peripheral blood (PB) of control (left) or SCN patients (right). A, PB cells were stained with CD45-PerCP, CD16-PE, and FITC-conjugated CAMP Ab, followed by flow cytometric analysis. The upper panels show dot blots of total PB cells developed with intensities of CD45-PerCP and SSC. Neutrophil population was gated in rectangles indicated as R1. The lower panels show dot blot analyses of cells gated as R1 stained with CD16 and CAMP Ab, respectively. Figures show representative results of flow cytometric analyses. B, Percentages of CAMP or NGAL expression in neutrophils derived from 26 healthy controls and 7 SCN revealed by flow cytometric analysis are shown as dot blots, and the mean percentage of each sample was calculated. Statistical analysis was performed and *P* values are indicated at the bottom of each figure. Ab indicates antibody; CAMP, cathelicidin antimicrobial peptide; FITC, fluorescein isothiocyanate; NGAL, neutrophil gelatinase-associated lipocalin; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; SCN, severe congenital neutropenia; SSC, side-scattering characteristics.

granule deficiency, which is associated with defects in the expression of several AMPs, shows frequent and severe bacterial infection from infancy.[18,19] CAMP and NGAL contents were significantly lower in SCN than in healthy controls, indicating that bactericidal defects of neutrophils were another cause of frequent infection in SCN in addition to the decrease in number. Plasma levels of pro-LL-37 have also been reported to be useful in diagnosis of SCN, in which pro-LL-37 was deficient.[20]

The production of specific granule proteins started at the myelocyte stage of myeloid differentiation.[21] Maturation arrest occurred at a similar differentiation stage of myeloid cells in SCN. Pütsep et al[22] reported that primary granules, α-defensins, and CAMP contents in neutrophils were also reduced in morbus Kostmann, which is inherited in an autosomal recessive manner. SCN-5, who did not have *Ela2* mutation, showed normal CAMP and NGAL contents as revealed by flow cytometry (Fig. 2B). It has been reported that G-CSF treatment of SCN did not correct abnormal expression of AMPs despite improvement of neutrophil number.[23] In contrast, vitamin D3 induced pro-LL-37 expression in neutrophil progenitors in SCN.[24] These findings indicated that defects in AMPs were not a direct effect of a mutated gene, but were the result of an impaired myeloid differentiation pathway. Recently, we found that the expression of neutrophil primary granule protein, bactericidal/permeability-increasing protein, was inducible by all–trans-retinoic acid in cord blood cells in vitro.[12] Furthermore, vitamin D3 was reported to induce CAMP expression in myeloid progenitors in SCN and normal human cells.[24,25] These agents may have effects in increasing the content of neutrophil AMPs and may be useful in prophylactic or therapeutic approaches against bacterial infection in SCN.

REFERENCES

1. Ancliff PJ. Congenital neutropenia. *Blood Rev.* 2003;17: 209–216.
2. Welte K, Zeidler C, Dale DC. Severe congenital neutropenia. *Semin Hematol.* 2006;43:189–195.
3. Boxer LA, Newburger PE. A molecular classification of congenital neutropenia syndromes. *Pediatr Blood Cancer.* 2007; 49:609–614.
4. Horwitz M, Benson KF, Person RE, et al. Mutations in ELA2, encoding neutrophil elastase, define a 21-day biological clock in cyclic haematopoiesis. *Nat Genet.* 1999;23:433–436.
5. Dale DC, Person RE, Bolyard AA, et al. Mutations in the gene encoding neutrophil elastase in congenital and cyclic neutropenia. *Blood.* 2000;96:2317–2322.
6. Ancliff PA, Gale RE, Liesner R, et al. Mutations in the ELA2 gene encoding neutrophil elastase are present in most patients with sporadic severe congenital neutropenia but only in some patients with the familial form of the disease. *Blood.* 2001; 98:2645–2650.
7. Horwitz MS, Duan Z, Korkmaz B, et al. Neutrophil elastase in cyclic and severe congenital neutropenia. *Blood.* 2007;109: 1817–1824.
8. Bohn G, Welte K, Klein C. Severe congenital neutropenia: new genes explain an old disease. *Curr Opin Rheumatol.* 2007;19: 644–650.
9. Person RE, Li FQ, Duan Z, et al. Mutations in proto-oncogene GFI1 cause human neutropenia and target ELA2. *Nat Genet.* 2003;34:308–312.
10. Klein C, Grudzien M, Appaswamy G, et al. HAX1 deficiency causes autosomal recessive severe congenital neutropenia (Kostmann disease). *Nat Genet.* 2007;39:86–92.
11. Ishikawa N, Okada S, Miki M, et al. Neurodevelopmental abnormalities associated with severe congenital neutropenia due to the B86X mutation in the HAX1 gene. *J Med Genet.* 2008;45:802–807.
12. Tanaka M, Gombart AF, Koeffler HP, et al. Expression of bactericidal/permeability-increasing protein requires C/EBPε. *Int J Hematol.* 2007;85:304–311.

13. Borregaard N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood.* 1997;89: 3503–3521.
14. Kobayashi M, Sato T, Kawaguchi H, et al. Efficacy of prophylactic use of trimethoprim-sulfamethoxazole in autoimmune neutropenia in infancy. *J Pediatr Hematol Oncol.* 2003;25:553–557.
15. De Backer J, Loeys B, Leroy B, et al. Utility of molecular analyses in the exploration of extreme intrafamilial variability in the Marfan syndrome. *Clin Genet.* 2007;72: 188–198.
16. Schulze-Bahr E, Wedekind H, Haverkamp W, et al. The LQT syndromes-current status of molecular mechanisms. *Z Kardiol.* 1999;88:245–254.
17. Jenssen H, Hamill P, Hanock REW. Peptide antimicrobial agents. *Clin Microbiol Rev.* 2006;19:491–511.
18. Gombart AF, Shiohara M, Kwok SH, et al. Neutrophil-specific granule deficiency: homozygous recessive inheritance of a frameshift mutation in the gene encoding transcription factor CCAAT/enhancer binding protein-ε. *Blood.* 2001;97: 2561–2567.
19. Shiohara M, Gombart AF, Sekiguchi Y, et al. Phenotypic and functional alterations of peripheral blood monocytes in neutrophil-specific granule deficiency. *J Leukoc Biol.* 2004;75: 190–197.
20. Karlsson J, Carlsson G, Ramme KG, et al. Low plasma levels of the protein pro-LL-37 as an early indication of severe disease in patients with chronic neutropenia. *Br J Haematol.* 2007;137:166–169.
21. Borregaard N, Theilgaard-Monch K, Sorensen OE, et al. Regulation of human neutrophil granule protein expression. *Curr Opin Hematol.* 2001;8:23–27.
22. Pütsep K, Carlsson G, Boman HG, et al. Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. *Lancet.* 2002;360:1144–1149.
23. Donini M, Fontana S, Savoldi G, et al. G-CSF treatment of severe congenital neutropenia reverses neutropenia but does not correct the underlying functional deficiency of the neutrophil in defending against microorganisms. *Blood.* 2007; 109:4716–4723.
24. Karlsson J, Carlsson G, Larne O, et al. Vitamin D3 induces pro-LL-37 expression in myeloid precursors from patients with severe congenital neutropenia. *J Leukoc Biol.* 2008;84:1279–1286.
25. Gombart AF, O'Kelly J, Saito T, et al. Regulation of the CAMP gene by 1,25(OH)2D3 in various tissues. *J Steroid Biochem Mol Biol.* 2007;103:552–557.

Transfusion Medicine, 2009, **19**, 105–108 doi: 10.1111/j.1365-3148.2009.00909.x

SHORT COMMUNICATION

Respiratory complications after haematopoietic stem cell transplantation in a patient with chronic granulomatous disease

K. Hara,* T. Kajiume,* T. Kondo,† Y. Sera,* H. Kawaguchi* & M. Kobayashi* **Department of Pediatrics, and †Department of Molecular and Internal Medicine, Hiroshima University, Hiroshima, Japan*

Received 3 July 2008; accepted for publication 29 October 2008

SUMMARY. Chronic granulomatous disease (CGD) is an inherited immunodeficiency disorder caused by defects in NADPH oxidase and characterized by recurrent life-threatening bacterial and fungal infections. Although CGD has been considered to be a target for gene therapy, bone marrow transplantation (BMT) is now selected as the radical treatment in most cases. We performed BMT in a patient with CGD with severe infections and experienced respiratory complications of diffuse alveolar haemorrhage and/or infection-associated alveolar haemorrhage. We suggest that attention be paid to signs of onset of alveolar haemorrhage during BMT in CGD patients.

Key words: chronic granulomatous disease, diffuse alveolar haemorrhage, haematopoietic stem cell transplantation.

Most of the chronic granulomatous disease (CGD) patients experience recurrent infections since infancy. The most frequent infections for CGD cases include pneumonia (*Aspergillus* sp.), purulent lymphadenitis (*Staphylococcus* sp.), subcutaneous abscess (*Staphylococcus* sp.), liver abscess (*Staphylococcus* sp.), osteomyelitis (*Serratia* sp.) and sepsis (*Salmonella* sp.); these infections often prove fatal to the patients (Winkelstein *et al.*, 2000). It is reported that in one third of the CGD patients who were administered interferon-γ as a treatment, the effectiveness of prevention against severe infection was demonstrated (International Chronic Granulomatous Disease Cooperative Study Group, 1991). Following the recent advances in post-transplantation management and the prevalent use of transplantation with a non-myeloablative conditioning regimen, haematopoietic stem cell transplantation (HSCT) is becoming the standard radical treatment for CGD (Horwitz *et al.*, 2001). In this study, we present a case of a 17-year-old male CGD patient whom we treated. As he was suffering from a severe infection, the only treatment option for him was HSCT. Generally, most CGD patients are still infectious when they undergo HSCT. In our case too, HSCT was performed in the patient while he was still infectious. Our patient died of an associated complication such as diffuse alveolar haemorrhage (DAH) and/or infection-associated alveolar haemorrhage (IAH). Therefore, we suggest that it is necessary to consider the complication of alveolar haemorrhage for CGD patients undergoing bone marrow transplantation (BMT).

Correspondence: Teruyuki Kajiume, MD, PhD, Department of Pediatrics, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan.
Tel.: +81-82-257-5212; fax: +81-82-257-5214;
e-mail: kajiume@hiroshima-u.ac.jp

CASE REPORT

Our patient was a 17-year-old male with a positive family history; his older brother had been diagnosed with CGD and had died of severe pneumonia 2 years before the patient was referred to our hospital. Therefore, our patient was promptly diagnosed with CGD (deficiency of gp91; a subunit of NADPH oxidase) at birth itself. He has been repeatedly hospitalized for a skin abscess, severe pneumonia and aspergillosis since infancy. We selected HSCT as the radical treatment for CGD. He demonstrated certain infectious symptoms when he was admitted to our hospital. In particular, severe damage to his right lung because of aspergillosis was identified (Fig. 1a). Therefore, a variety of antibiotics along with amphotericin B were used for treating the patient before the transplantation to reduce the number and intensity of infectious foci.

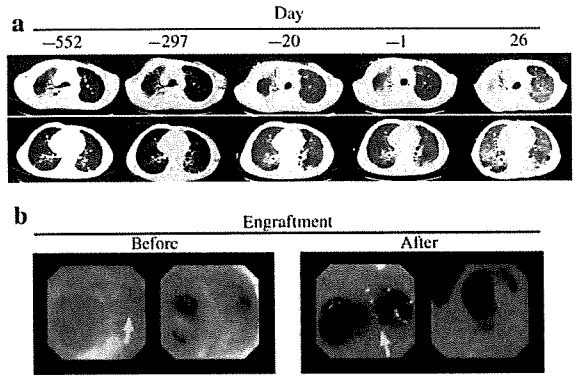


Fig. 1. Chest computed tomography (CT) changes over time and bronchoscopy findings. (a) High-density areas were found all over the lungs more than a year before BMT. A cavity was also identified in the right upper lobe. The lungs appeared to worsen with time; however, a day before the transplantation, a temporary improvement was observed. On day 26, ground-glass opacity was observed in both lungs covering each lung. (b) A small amount of bronchial haemorrhage was observed before engraftment. Although bleeding from the right bronchial tube was also identified, this haemorrhage was arrested within a few days (left panel). Bronchial haemorrhage started again after engraftment. At this time, a blood clot (indicated by arrow) was deposited in the bifurcation of the trachea, and active bleeding was identified in the right S6 area (right panel).

As he did not have an human leukocyto antigen (HLA) full-match sibling donor, he received reduced-intensity stem cell transplantation from an unrelated one-locus HLA-mismatched donor. Briefly, the patient received conditioning therapy (3 Gy of total body irradiation, 750 mg m^{-2} day^{-1} cyclophosphamide for 4 days, 25 mg m^{-2} day^{-1} fludarabine for 5 days, 70 mg m^{-2} melphalan for 1 day and 15 mg kg^{-1} day^{-1} antithymocyte globulin for 4 days) before transplantation.

Although the patient did not show any symptoms for the first few days after HSCT, his haematopoietic recovery was gradually delayed and the signs of infection became worse, particularly his respiratory status progressively deteriorated. Blood gas analysis showed that the CO_2 levels were extremely high on day 26. When the respiratory status became worse, 3–8 L min^{-1} of oxygen was given to patient with oxygen mask. Therefore, mechanical ventilation was used to alleviate the respiratory failure in the intensive care unit. As shown in Fig. 1a, worsened pneumonia was identified on day 26. On day 33, the white blood cell count from peripheral blood was 70 μL^{-1}, and his haematopoietic recovery was considered to have begun. Around the same time, bloody secretions were suctioned through the intubation tube, and we identified the cause as a small tracheal haemorrhage. Meanwhile, the FiO_2 was observed to be 0·4, and the SpO_2 was 100%. On day 44, although the white blood cell count from peripheral blood was above 500 μL^{-1} and engraftment was identified, he had extensive tracheal haemorrhage with progressive worsening of his respiratory status. Bronchoscopy demonstrated haemorrhage from the right upper and lower lobes (Fig. 1b). His respiratory status further worsened; it was not possible to maintain 90% of SpO_2 with 1·0 of FiO_2. Furthermore, CO_2 accumulation occurred, and acidosis was observed. On day 54, his blood pressure decreased and ventricular tachycardia appeared. His heart rate rapidly slowed down, and death occurred soon after. Figure 2 shows the summary of the process. Any bacteria and fungi had been not detected by blood cultures or through bronchoscope.

The autopsy showed traces of haemorrhage all over the lungs; there was no air infiltration area (Fig. 3a–c). Based on these findings, we diagnosed it as a case of DAH and/or IAH. Figure 3d shows the cavity in the right upper lobe from which *Aspergillus* was isolated (Fig. 3e–g).

DISCUSSION

During the 1980s and 1990s, the incidence of pulmonary complications after BMT was 30–60% (Cordonnier *et al.*, 1986; Jules-Elysee *et al.*, 1992). Although most of the complications involved infectious diseases such as pneumonia, this incidence decreased considerably

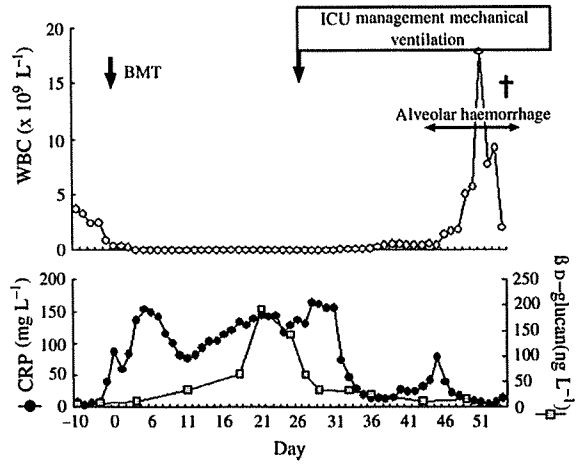


Fig. 2. Significant changes in laboratory results following HSCT. The delay in the haematopoietic recovery is shown. Because of the delay, the mycotic infection appears to have worsened. Alveolar hemorrhage appeared following the increase in the white blood cell (WBC) count. CRP, C-reactive-protein; ICU, intensive care unit. †, death of patient.

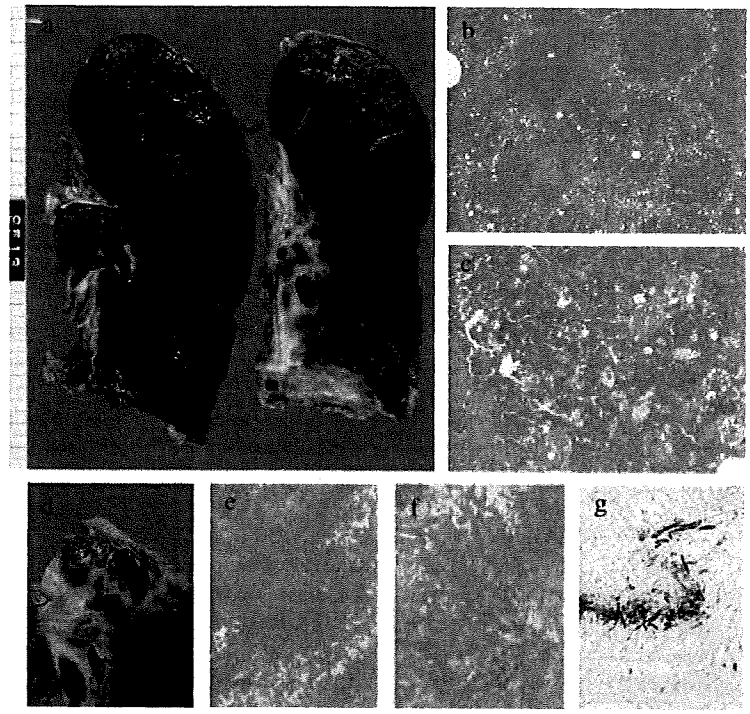


Fig. 3. Autopsy findings of the lungs. (a) The entire lungs are observed to be filled with blood with a complete absence of air-infiltrated areas. (b) All alveoli are also filled with blood. (c) A number of red blood cells are identified in the alveoli under high magnification. (d) There is a cavity in the right upper lobe. (e) Under high magnification, stick-like *Aspergillus* can be observed with haematoxylin and eosin staining. (f) *Aspergillus* observed with periodic acid-Schiff (PAS) staining. (g) *Aspergillus* observed with Grocott staining.

because of the development of new medicines and facilities. DAH is an associated complication in 5% of BMT patients (Afessa *et al.*, 2002b), and there is no significant difference in incidence between adults and children (Heggen *et al.*, 2002). The frequency of alveolar haemorrhage in our facility is 2·78%.

Non-infectious inflammation at the time of engraftment following HSCT is known as engraftment syndrome (ES) (Cahill *et al.*, 1996; Moreb *et al.*, 1997; Marin *et al.*, 1998; Ravenel *et al.*, 2002), and DAH is one of the typical findings of ES. Although the causes of DAH are not completely known, angiopathy because of conditioning therapy or radiation and immunogenicity caused by graft-vs.-host disease have been considered as causes. However, when symptoms caused by infectious diseases are observed, the condition is called IAH. Although it is difficult to differentiate between DAH and IAH, a case in which no infection is identified within 7 days of the occurrence of alveolar haemorrhage is diagnosed as DAH, and a case in which a pathogen is identified by either a blood test or a bronchoalveolar lavage (BAL) is diagnosed as IAH (Majhail *et al.*, 2006). A previous report has stated that high levels of certain types of cytokines – such as IL-1β, IL-6, IL-8, TNFα, MIP1α and G-CSF – identified by BAL after BMT are characteristic of DAH (Kharbanda *et al.*, 2006). Our patient already had a respiratory tract infection by aspergillosis when he underwent BMT. However, the mycotic infection had improved a week before the bronchial bleeding began (day 33), and when bronchial haemorrhage was observed (day 44), all other infections also showed improvement. For these reasons, although we diagnosed the patient as a case of DAH, it may be described as IAH because *Aspergilli* were observed by the autopsy.

It has been reported that certain underlying diseases are strongly associated with DAH; e.g. breast cancer patients are categorized as a high-risk group for DAH (Robbins *et al.*, 1989), and myeloma patients are in a higher risk group for DAH than are patients of malignant lymphoma (Capizzi *et al.*, 2001). HSCT has not been reported for many immunodeficient patients such as CGD patients, and few reports discuss this treatment. It has been reported that a delay in haematopoietic recovery and the administration of amphotericin B are considered as risk factors for ES, manifesting as DAH (Afessa *et al.*, 2002b; Gorak *et al.*, 2005). In this case, our patient had several risk factors. Most CGD patients are expected to undergo HSCT while already having certain risk factors. Therefore, caution should be exercised in patients who are considered to be at risk for ES. Although the administration of steroid hormones, particularly high doses of methylprednisolone, is effective for treating DAH, the patient outcome is not promising; the mortality rate is approximately 80% (Afessa *et al.*, 2002a,b; Robbins *et al.*, 1989; Lewis *et al.*, 2000; Huaringa *et al.*, 2000). In

particular, 61% of the patients who died had an onset of DAH 30 days or more after BMT (Afessa *et al.*, 2002a); the onset appears to be related to the delay in haematopoietic recovery. Other than steroid hormones, it has been reported that recombinant factor VIIa (Pastores *et al.*, 2003) and aminocaproic acid (Wanko *et al.*, 2006) are also effective as treatments for DAH. There are bound to be some cases where HSCT is needed as an emergency procedure such as in immunocompromised patients with CGD. However, in the case of non-emergency patients, we strongly suggest that the infectious disease that could delay the haematopoietic recovery be cured or adequately treated before HSCT.

REFERENCES

Afessa, B., Tefferi, A., Litzow, M.R. & Peters, S.G. (2002a) Outcome of diffuse alveolar hemorrhage in hematopoietic stem cell transplant recipients. *American Journal of Respiratory and Critical Care Medicine*, **166,** 1364–1368.

Afessa, B., Tefferi, A., Litzow, M.R., Krowka, M.J., Wylam, M.E. & Peters, S.G. (2002b) Diffuse alveolar hemorrhage in hematopoietic stem cell transplant recipients. *American Journal of Respiratory and Critical Care Medicine*, **166,** 641–645.

Cahill, R.A., Spitzer, T.R. & Mazumder, A. (1996) Marrow engraftment and clinical manifestations of capillary leak syndrome. *Bone Marrow Transplant*, **18,** 177–184.

Capizzi, S.A., Kumar, S., Huneke, N.E. *et al.* (2001) Peri-engraftment respiratory distress syndrome during autologous hematopoietic stem cell transplantation. *Bone Marrow Transplant*, **27,** 1299–1303.

Cordonnier, C., Bernaudin, J.F., Bierling, P., Huet, Y. & Vernant, J.P. (1986) Pulmonary complications occurring after allogeneic bone marrow transplantation. A study of 130 consecutive transplanted patients. *Cancer*, **58,** 1047–1054.

Gorak, E., Geller, N., Srinivasan, R., Espinoza-Delgado, I., Donohue, T., Barrett, A.J., Suffredini, A. & Childs, R. (2005) Engraftment syndrome after nonmyeloablative allogeneic hematopoietic stem cell transplantation: incidence and effects on survival. *Biology of Blood and Marrow Transplantion*, **11,** 542–550.

Heggen, J., West, C., Olson, E., Olson, T., Teague, G., Fortenberry, J. & Yeager, A.M. (2002) Diffuse alveolar hemorrhage in pediatric hematopoietic cell transplant patients. *Pediatrics*, **109,** 965–971.

Horwitz, M.E., Barrett, A.J., Brown, M.R. *et al.* (2001) Treatment of chronic granulomatous disease with nonmyeloablative conditioning and a T-cell-depleted hematopoietic allograft. *New England Journal of Medicine*, **22,** 881–888.

Huaringa, A.J., Leyva, F.J., Giralt, S.A., Blanco, J., Signes-Costa, J., Velarde, H. & Champlin, R.E. (2000) Outcome of bone marrow transplantation patients requiring mechanical ventilation. *Critical Care Medicine*, **28,** 1014–1017.

International Chronic Granulomatous Disease Cooperative Study Group. (1991) A controlled trial of interferon gamma to prevent infection in chronic granulomatous disease. *New England Journal of Medicine*, **324,** 509–516.

Jules-Elysee, K., Stover, D.E., Yahalom, J., White, D.A. & Gulati, S.C. (1992) Pulmonary complications in lymphoma patients treated with high-dose therapy autologous bone marrow transplantation. *American Review of Respiratory Disease*, **146,** 485–491.

Kharbanda, S., Panoskaltsis-Mortari, A., Haddad, I.Y. *et al.* (2006) Inflammatory cytokines and the development of pulmonary complications after allogeneic hematopoietic cell transplantation in patients with inherited metabolic storage disorders. *Biology of Blood and Marrow Transplantion*, **12,** 430–437.

Lewis, I.D., DeFor, T. & Weisdorf, D.J. (2000) Increasing incidence of diffuse alveolar hemorrhage following allogeneic bone marrow transplantation: cryptic etiology and uncertain therapy. *Bone Marrow Transplant*, **26,** 539–543.

Majhail, N.S., Parks, K., Defor, T.E. & Weisdorf, D.J. (2006) Diffuse alveolar hemorrhage and infection-associated alveolar hemorrhage following hematopoietic stem cell transplantation: related and high-risk clinical syndromes. *Biology of Blood and Marrow Transplantion*, **12,** 1038–1046.

Marin, D., Berrade, J., Ferra, C. *et al.* (1998) Engraftment syndrome and survival after respiratory failure post-bone marrow transplantation. *Intensive Care Medicine*, **24,** 732–735.

Moreb, J.S., Kubilis, P.S., Mullins, D.L., Myers, L., Youngblood, M. & Hutcheson, C. (1997) Increased frequency of autoaggression syndrome associated with autologous stem cell transplantation in breast cancer patients. *Bone Marrow Transplant*, **19,** 101–106.

Pastores, S.M., Papadopoulos, E., Voigt, L. & Halpern, N.A. (2003) Diffuse alveolar hemorrhage after allogeneic hematopoietic stem-cell transplantation: treatment with recombinant factor VIIa. *Chest*, **124,** 2400–2403.

Ravenel, J.G., Scalzetti, E.M. & Zamkoff, K.W. (2002) Chest radiographic features of engraftment syndrome. *Journal of Thoracic Imaging*, **15,** 56–60.

Robbins, R.A., Linder, J., Stahl, M.G. *et al.* (1989) Diffuse alveolar hemorrhage in autologous bone marrow transplant recipients. *American Journal of Medicine*, **87,** 511–518.

Wanko, S.O., Broadwater, G., Folz, R.J. & Chao, N.J. (2006) Diffuse alveolar hemorrhage: retrospective review of clinical outcome in allogeneic transplant recipients treated with aminocaproic acid. *Biology of Blood and Marrow Transplantion*, **12,** 949–953.

Winkelstein, J.A., Marino, M.C., Johnston, R.B. Jr. *et al.* (2000) Chronic granulomatous disease. Report on a national registry of 368 patients. *Medicine*, **79,** 155–169.

Int J Hematol
DOI 10.1007/s12185-009-0482-3

ORIGINAL ARTICLE

Significance of immature platelet fraction and CD41-positive cells at birth in early onset neonatal thrombocytopenia

Hirotaka Kihara · Norioki Ohno · Syuhei Karakawa · Yoko Mizoguchi · Rie Fukuhara · Michiko Hayashidani · Shinji Nomura · Kazuhiro Nakamura · Masao Kobayashi

Received: 13 September 2009 / Revised: 25 November 2009 / Accepted: 21 December 2009

Abstract Early thrombocytopenia is a common hematological abnormality in sick neonates. Here, we examined the relationship between early thrombocytopenia in neonates and parameters associated with thrombopoiesis to identify predictive factors at birth. Two hundred and forty-four neonates admitted to the neonatal intensive care unit were divided into thrombocytopenic ($n = 55$, 23%) and non-thrombocytopenic ($n = 189$, 77%) groups based on platelet counts, which were monitored within 72 h of birth. Immature platelet fraction (IPF) and platelet count at birth were determined simultaneously soon after phlebotomy with an automated hematology analyzer. Megakaryocytes and their precursors positive for CD41 in peripheral blood were examined at birth by flow cytometry. The thrombocytopenic group showed significantly higher IPF percentage and lower percentage of $CD41^+$ mononuclear cells (MNCs) than did the non-thrombocytopenic group ($P < 0.01$). Moreover, the percentage of $CD41^+$ MNCs significantly differentiated neonates with platelet counts $>150 \times 10^3/\mu L$ at birth and nadir platelet count $<150 \times 10^3/\mu L$ over the clinical course from neonates without thrombocytopenia. These observations suggest that the percentage of $CD41^+$ MNCs at birth and IPF percentage are useful predictors of early thrombocytopenia in the majority of sick neonates.

Keywords Thrombocytopenia · Neonate · Immature platelet fraction · Megakaryocyte

1 Introduction

Thrombocytopenia is one of the most common hematological abnormalities in newborn infants, affecting 22–35% of neonates admitted to neonatal intensive care units (NICUs) [1, 2]. Multiple disease processes can cause neonatal thrombocytopenia, and these can be classified as those inducing early thrombocytopenia (<72 h after birth) and those inducing late-onset thrombocytopenia (>72 h). The causes of early thrombocytopenia are well understood: neonatal alloimmune thrombocytopenia [3], maternal immune thrombocytopenic purpura (ITP) or lupus [4], neonatal giant hemangioma [5], perinatal asphyxia, perinatal infection, congenital infection [6], inherited thrombocytopenia caused by reduced platelet production, and congenital errors of metabolism [7]. However, the most frequent cause of early thrombocytopenia is chronic fetal hypoxia, which occurs in infants born to mothers with pregnancy-induced hypertension (PIH) [8] or diabetes [9] and in those who are small for gestational age (SGA) [10]. In the majority of such cases, thrombocytopenia occurs within 3 days and resolves spontaneously within 10 days [1]. The mechanism of transient thrombocytopenia in neonates with these conditions has not yet been elucidated. The method for quantifying reticulated platelets known as the immature platelet fraction (IPF) has been utilized in a variety of clinical conditions such as thrombocytopenic disorders [11–13]. The percentage value and the absolute

H. Kihara · N. Ohno · S. Karakawa · Y. Mizoguchi · R. Fukuhara · M. Hayashidani · S. Nomura · K. Nakamura · M. Kobayashi (✉)
Department of Pediatrics, Hiroshima University Graduate School of Biomedical Sciences, 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8551, Japan
e-mail: masak@hiroshima-u.ac.jp

H. Kihara
e-mail: kihah@mpd.biglobe.ne.jp

Published online: 16 January 2010