the highest number of ex vivo expanded HSCs. Based on this condition, some additional explorations should be considered. Increase of differentiated cells surpasses that of immature cells, which might interfere with the SRC expansion because the differentiated cells might secrete various substances that inhibit SRC expansion. Indeed, removal of differentiated cells during culture of CB Lin^- cells has been shown to have strongly positive effect on the efficient SRC expansion in a serum-free culture with 3GFs [51]. Therefore, a much higher level of SRC expansion might be possible if we apply similar differentiated cell-removal protocols in our culture condition. Culture under the hypoxic condition may also improve the expansion efficiency. In this study, we expanded HSCs by a 3-week culture system. In general, shorter ex vivo culture periods are preferable in clinical settings from the viewpoint of safety or costs. Further studies based on our results and above ideas may provide improved methods with shorter culture periods and higher expansion efficiency, which could be the most efficient ex vivo HSC expansion system for clinical applications in the future.

ACKNOWLEDGMENTS

This work was supported by Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Research on Pharmaceutical and Medical Safety, Health and Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan and Grant-in-Aid for Scientific Research, KAKENHI (17014023) from the Japan Society for the Promotion of Science, and a grant from the Takeda Science Foundation. We thank Dr. A. Kikuchi for the supply of CB samples and Kyokuto Pharmaceutical Industrial Co., Ltd. for generously providing us with the serum-free medium. We also thank Y. Sato for taking care of the animals.

DISCLOSURES

M.N. owns stock in and has received financial support from Kirin Brewery Co., Ltd. S.S. owns stock in and has received financial support from Asahi Kasei Corporation.

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AML1/Runx1 rescues Notch1-null mutation-induced deficiency of para-aortic splanchnopleural hematopoiesis

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The Notch1-RBP-Jκ and the transcription factor Runx1 pathways have been independently shown to be indispensable for the establishment of definitive hematopoiesis. Importantly, expression of Runx1 is down-regulated in the para-aortic splanchnopleural (P-Sp) region of *Notch1*- and *Rbpsuh*-null mice. Here we demonstrate that Notch1 up-regulates Runx1 expression and that the defective hematopoietic potential of *Notch1*-null P-Sp cells is successfully rescued in the OP9 culture system by retroviral transfer of Runx1. We also show that Hes1, a known effector of Notch signaling, potentiates Runx1-mediated transactivation. Together with the recent findings in zebrafish, Runx1 is postulated to be a cardinal downstream mediator of Notch signaling in hematopoietic development throughout vertebrates. Our findings also suggest that Notch signaling may modulate both expression and transcriptional activity of Runx1. (Blood. 2006;108:3329-3334)

Introduction

Mammalian hematopoietic development is believed to arise from 2 distinct cellular origins. In mice, primitive hematopoiesis arises in the yolk sac (YS) blood island at embryonic day (E) 7.5, while definitive hematopoiesis starts at the ventral region of the aorta-gonad-mesonephros (AGM) around E10.5, which shifts to the liver, spleen, and bone marrow, in this order. Progenitors for definitive hematopoiesis are first detected in the para-aortic splanchnopleural (P-Sp) region at E7.5 to E9.5,[1,2] where the *Notch1* gene has a nonredundant role in hematopoietic stem cell (HSC) development.[3] *Notch1* encodes a 300-kDa heterodimeric single-span transmembrane receptor consisting of a 180-kDa extracellular and a 120-kDa transmembrane subunit. Together with 3 other paralogs, it belongs to the evolutionarily conserved Notch family receptors that mediate cell-fate determination in multiple species. The Notch signaling is initiated by the binding of the Jagged and Delta families of ligands expressed on the neighboring cells, which induces the cleavage of the Notch transmembrane subunit and the release of the Notch intracellular domain. The latter in turn translocates to the nucleus and forms a transactivation complex by interacting with the DNA-binding protein RBP-Jκ and induces the expression of their target genes, such as those for the hairy/enhancer of split (*Hes*) family of basic helix-loop-helix transcription factors.[4] Molecular channels downstream of these, however, are largely unknown.

Mice deficient in *Runx1* (also known as *AML1*, *CBFA2*, or *PEBP2αB*), *Scl*, and *Gata2* genes are lethal during the embryonic stage and show failure in the establishment of definitive hematopoiesis.[5-7] A connection between Notch signaling and these transcription factors has been shown by the analyses of *Notch1*- and RBP-Jκ–encoding *Rbpsuh*-null mice. In the E9.5 P-Sp cells from *Notch1*-null mice, expression levels of SCL, GATA2, and Runx1 mRNA are significantly reduced.[3] *Rbpsuh*-null mice also show markedly reduced levels of SCL, GATA2, and Runx1 mRNA in the endothelial-cell layer of the E9.5 P-Sp region,[8] supporting the notion that the Notch1-RBP-Jκ pathway up-regulates the expression of these key transcription factors. Among these, Runx1, which has close homology to a *Drosophila* protein, Runt, functions as a transcriptional activator or repressor for its target genes in concert with several specific coactivators or corepressors, depending on the context.[9] Importantly, presence of the Notch-Runx pathway has been proposed in *Drosophila* embryonic hemocytogenesis[10] and zebrafish hematopoiesis during both developmental and postnatal periods.[11] Similarly reported has been transcriptional regulation by Notch of the *Gata2* gene in mouse AGM hematopoiesis[8] and of the *Gata* homolog *Serpent* gene in *Drosophila* embryonic hemocytogenesis.[12] In mammals, the existence of Notch-Runx pathway has been unclear.

In this study, we show that Notch1 up-regulates Runx1 mRNA expression in NIH3T3 cells. When introduced to the defective prehematopoietic precursor cells derived from the P-Sp region of *Notch1*-null embryos using retroviruses, Runx1, but neither SCL nor GATA2, restores the definitive hematopoiesis. We also demonstrate that Hes1, one of the Notch signal effectors, augments the transcriptional activity of Runx1 protein. These findings indicate that Runx1 is a key molecule in Notch1-RBP-Jκ–mediated mammalian hematopoiesis.

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Submitted April 25, 2006; accepted July 3, 2006. Prepublished online as *Blood* First Edition Paper, August 3, 2006; DOI 10.1182/blood-2006-04-019570.

Supported in part by Grant-in-Aid for Scientific Research (KAKENHI no. 17390274) and Grant-in-Aid for Japan Society for the Promotion of Science (JSPS), Fellows from JSPS, Research on Pharmaceutical and Medical Safety, Health and Labour Sciences Research Grants, Ministry of Health, Labour and Welfare of Japan.

The authors declare no competing financial interests.

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Materials and methods

Mice and embryos

C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan) and *Notch1* mutant mice[13] were from Jackson Laboratory (Bar Harbor, ME). To generate embryos, timed matings were set up between *Notch1*$^{+/-}$ mice. The time at midday (12 PM) was taken to be E0.5 for the plugged mice.

In vitro P-Sp culture

P-Sp culture was performed as described previously.[14] In brief, isolated P-Sp regions of E9.5 embryos were dissociated by incubation with 250 protease units (PU)/mL dispase (Godo Shusei, Tokyo, Japan) for 20 minutes and cell-dissociation buffer (Gibco BRL, Carlsbad, CA) for 20 minutes at 37°C, followed by vigorous pipetting. Approximately 5×10^4 P-Sp–derived cells were suspended in 300 μL of serum-free StemPro media (Life Technologies, Gaithersburg, MD) supplemented with 50 ng/mL stem-cell factor (SCF), 5 ng/mL interleukin-3 (IL3; gifts from Kirin Brewery, Takasaki, Japan), and 10 ng/mL mouse oncostatin M (R&D Systems, Minneapolis, MN). Single-cell suspensions were seeded on preplated OP9 stromal cells[15] in the 24-well plate, followed by incubation at 37°C in a 5% CO_2 incubator. Images were visualized with a Nikon Eclipse TE2000-U microscope equipped with 40×/0.60 and 10×/0.30 NA objective lenses (Nikon, Tokyo, Japan), and were captured with a C5810 camera (Hamamatsu Photonics, Hamamatsu, Japan).

Plasmid construction

The cDNA of human Runx1 was subcloned into the *Eco*RI restriction site of the retrovirus vector pMYs/internal ribosomal entry site–enhanced green fluorescent protein (IRESEGFP; pMYs/IG).[16] The cDNAs for FLAG-tagged murine SCL and FLAG-tagged murine GATA2 were inserted into the *Eco*RI and *Not*I restriction sites of pMYs/IG. The cDNA for murine Notch1 intracellular domain (NICD)[3] was subcloned into the *Bam*HI restriction site of pMYs/IG. To assess the domain functions of Runx1, we used mutants and wild-type Runx1 constructed in pMY/IG.[14] The pME18S-HA-Runx1 and pME18S-PEBP2β were described previously.[17] The cDNA for FLAG-tagged murine Hes1 was inserted into the *Eco*RI and *Not*I restriction sites of the pME18S-expression vector and in-frame into the *Eco*RI and *Xba*I restriction sites of the p3xFLAG-myc-CMV-25–expression vector (Sigma, St Louis, MO).

Retroviral transduction

Plat-E packaging cells (2×10^6)[16] were transiently transfected with 3 μg of retrovirus vectors, mixed with 9 μL of FuGENE6 (Roche Applied Science, Indianapolis, IN), and incubated at 37°C. Supernatant containing retrovirus was collected 48 hours after transfection and used immediately for infection. Retroviral transduction of the cells derived from *Notch1*-null P-Sp regions was performed as described previously.[14] In brief, the viral supernatant was added to the P-Sp cells seeded on the OP9 stromal-cell layer together with 10 μg/mL polybrene (Sigma). After 72 hours of incubation, virus-containing medium was replaced by the original culture medium. The cells were incubated for another 10 days and processed for analysis. To confirm the expression of proteins, NIH3T3 cells were also infected with the same viral supernatants. The efficiency of infection was evaluated by the positivity of GFP. The proteins were detected by Western blot using anti-Runx1 antibody (PC284L; Oncogene, Cambridge, MA), anti-FLAG monoclonal antibody (M2; Sigma), and anti-FLAG polyclonal antibody (F7425; Sigma) to detect Runx1, GATA2, and SCL, respectively. F7425 antibody was used to exclude the overlap of SCL and nonspecific band by M2 antibody.

CFC assay

The nonadherent or semiadherent cells that emerged from wild-type and *Notch1*-null P-Sp regions were used for colony-forming–cell (CFC) assays. Cells (6×10^4) were plated into MethoCult GF M3434 medium (StemCell Technologies, Vancouver, BC, Canada) and cultured in a 5% CO_2 incubator at 37°C. Colony types were determined at day 7 by morphologic appearance and by Wright-Giemsa staining of each colony. Images were taken with a Nikon Eclipse TE2000-U.

Flow cytometric analysis

Flow cytometric analysis was performed with a BD LSRII (BD Biosciences, San Jose, CA) after addition of 7-amino-actinomycin D (7-AAD) (Via-Probe; BD PharMingen, San Diego, CA) to exclude dead cells. For surface staining, cell suspensions collected from the P-Sp cultures were incubated on ice for 30 minutes in the presence of various mixtures of labeled monoclonal antibodies. The following monoclonal antibodies were purchased from BD PharMingen: phycoerythrin (PE)–conjugated anti–granulocyte 1 (anti–Gr-1), anti–macrophage antigen 1 (anti–Mac-1), anti–stem-cell antigen 1 (anti–Sca-1), anti–Ter-119, allophycocyanin (APC)–conjugated anti-CD45, anti–c-Kit, and biotin-conjugated anti-CD34. Biotinylated antibodies were labeled with PE- or APC-conjugated streptavidin.

Immunoprecipitation and Western blotting

COS7 cells were transfected with expression plasmids (pME-HA-Runx1 and p3xFLAG-myc-CMV-25-Hes1) using the FuGENE6 according to the manufacturer's instruction. The cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) for 48 hours after transfection and were lysed in radioimmunoprecipitation assay (RIPA) buffer.[14] These cell lysates were precleared with protein G–sepharose (Amersham Bioscience, Little Chalfont, United Kingdom) and mixed with anti-FLAG antibody (M2; Sigma) or anti-HA antibody (HA.11; Covance Research Products, Berkeley, CA) for 2 hours. The antibody-associated proteins were then recovered on protein G–sepharose beads. The beads were washed 4 times with the RIPA buffer. Whole-cell lysates containing 100 μg of proteins and immunoprecipitates were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA). The membranes were blocked with 5% skim milk treated with either peroxidase-conjugated anti-FLAG monoclonal antibody (M2; Sigma) or peroxidase-conjugated anti-HA monoclonal antibody (12CA5; Roche Applied Science). The blots were visualized using the enhanced chemiluminescence (ECL) system (Amersham Bioscience).

Transcriptional response assays

Luciferase assays were performed as described previously[18] with minor modifications. Briefly, HeLa cells were transfected with 300 ng of reporter (pM-CSF-R-luc),[19] and expression plasmids (combinations of 200 ng of pME18S-HA-Runx1 and 160 ng of pME18S-PEBP2β and 60, 200, or 600 ng of pME18S-FLAG-Hes1 or control) using FuGENE6 according to the manufacturer's instructions. As a control of transfection efficiency, a plasmid expressing β-galactosidase was cotransfected. The cells were harvested 48 hours after transfection and assayed for luciferase activity. The data were normalized to β-galactosidase activity.

Quantitative PCR analysis

NIH3T3 cells were infected with NICD or mock retrovirus. The cells were cultured in DMEM medium supplemented with 10% FCS for 48 hours after infection and were selected by the expression of GFP with the FACSAria (BD Biosciences). Total cellular RNA was extracted with RNeasy (QIAGEN, Hilden, Germany) and converted into cDNAs by reverse transcriptase (Superscript III; Invitrogen, Carisbad, CA). Real-time polymerase chain reaction (PCR) was performed using TaqMan Gene Expression Assays Mm00486762_m1 (Applied Biosystems, Foster City, CA) with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. Amplification of 18S ribosomal RNA cDNA was used as the endogenous normalization standard.

Results

Retroviral expression of Runx1 rescues hematopoietic defects of *Notch1*-null P-Sp regions

It has been reported that expression of Runx1 or its homolog, *Lozenge*, is up-regulated by positive Notch signaling in zebrafish and *Drosophila* systems, respectively.[10,11] We thus first evaluated whether Notch activation results in up-regulation of Runx1 also in the mammalian system. When NIH3T3 cells were transiently transfected with Notch1 intracellular domain (NICD), which represents the constitutive active form of Notch1, the mRNA level of Runx1 increased (Table 1).

We then examined whether forced expression of Runx1 could rescue the hematopoietic defect of *Notch1*-null mice. Wild-type P-Sp cells gave rise to round-shaped nonadherent cells when overlayed on the OP9 stromal cells. Flow cytometric analysis of these cells revealed that they were viable (7-AAD negative) CD45-positive cells (top panels in Figure 1A), representing hematopoietic cells. No such cells were generated from *Notch1*-null P-Sp cells and only background OP9 cells were observed (bottom panels in Figure 1A).[3] We retrovirally infected *Notch1*-null P-Sp cells that were seeded on the OP9 layer with Runx1, SCL, or GATA2, and assessed whether *Notch1*-null P-Sp cells could generate hematopoietic cells. Titers of the retroviruses containing Runx1, SCL, and GATA2 were similar to each other as evaluated by infecting NIH3T3 cells with these viruses (Figure 2A). Expression of individual proteins was confirmed by a Western blot analysis (Figure 2B). Mock, SCL, and GATA2 transduction did not generate round-shaped nonadherent cells morphologically or viable CD45-positive cells detectable by flow cytometric analysis. In contrast, Runx1-transduced P-Sp cells gave rise to round-shaped nonadherent cells. These cells were shown to be viable CD45-positive cells by flow cytometric analysis (Figure 1B). This pattern was identical to the positive control ($Notch1^{+/+}$ P-Sp cells; top panels in Figure 1A).

To confirm that the cells developed from Runx1-infected *Notch1*-null P-Sp cells (hereafter referred to as Runx1-rescued cells) retain the features of hematopoietic cells, we evaluated these cells for surface markers and CFC activities. The flow cytometric analysis of the Runx1-rescued cells at day 12 revealed that they express hematopoietic cell-surface markers such as a panleukocyte marker (CD45), stem-cell markers (c-Kit, CD34, and Sca1), myeloid-cell markers (Gr-1 or Mac-1), and an erythroid-cell marker (Ter-119) (Figure 3A). Their expression profiles were reminiscent of those of hematopoietic cells generated from the wild-type P-Sp cells (Figure 3B). The P-Sp culture system faithfully reproduced the generation of hematopoietic cells, and there were no consistent differences between Runx1-rescued and wild-type P-Sp–derived cells in the surface-marker expression levels, although we observed variable minor differences in individual experiments partly because of the variation in the time required for hematopoietic development (Figure 3A-B).

When the Runx1-rescued cells were seeded into semisolid medium at day 12 and cultured for an additional 7 days, they generated mixed, granulocyte/macrophage, and erythroid colonies containing enucleated erythrocytes (Figure 4A) at a frequency comparable to that of wild-type P-Sp–derived cells (Figure 4B-C). There were no statistical differences in the numbers of total ($P = .11$) and individual (erythroid, $P = .20$; granulocyte/macrophage, $P = .11$; mixed, $P = .07$) colonies generated from $Notch1^{+/+}$

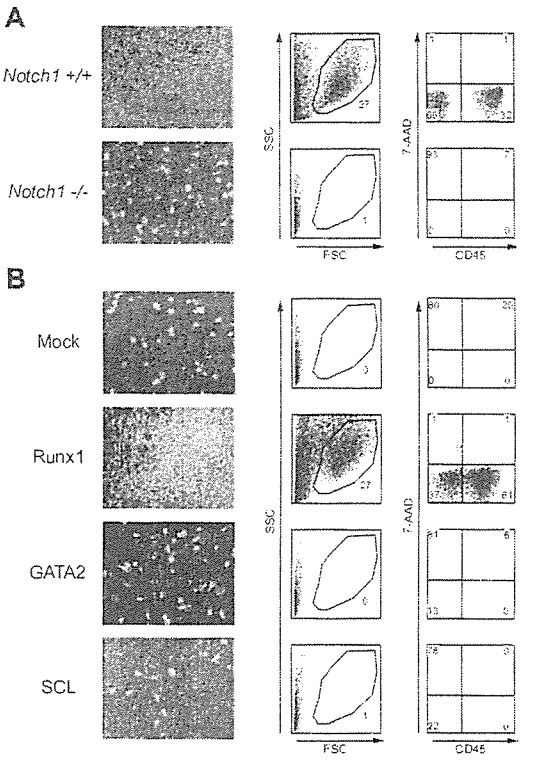

Figure 1. Retroviral expression of Runx1 rescues hematopoietic defect of *Notch1*-null P-Sp region. (A) P-Sp cells from wild-type ($Notch1^{+/+}$) and *Notch1*-null ($Notch1^{-/-}$) embryos at E9.5 were cultured for 5 days on OP9 cells. (B) P-Sp cells from *Notch1*-null embryos at E9.5 were infected with mock retrovirus or retrovirus containing Runx1, SCL, or GATA2, and cultured for 12 days on OP9 cells. Microscopic representation (left column; original magnification, × 100). Only cocultured OP9 cells are shown if hematopoietic cells are not produced. Flow cytometric analyses (center and right columns) of cells generated in the culture. Percentages of cells gated (center columns) and cells in each quadrant (right columns) are indicated.

Table 1. Notch activation up-regulates the expression of Runx1

	RAU	Mean	Notch-mock
Experiment 1			2.78*
NIH3T3-Mock	0.112634; 0.077514; 0.093663	0.094604	
NIH3T3-Notch	0.263982; 0.241864; 0.282636	0.262827	
Experiment 2			4.12*
NIH3T3-Mock	0.038500; 0.045755; 0.044123	0.042792	
NIH3T3-Notch	0.186016; 0.148638; 0.194443	0.176366	

Data are from 2 independent experiments in triplicate. RAU, relative arbitrary units; Notch-mock, the ratio of RAU by constitutive active Notch 1 infection and RAU by mock infection.

*$P < .01$ (2-tailed, unequal variance *t* test).

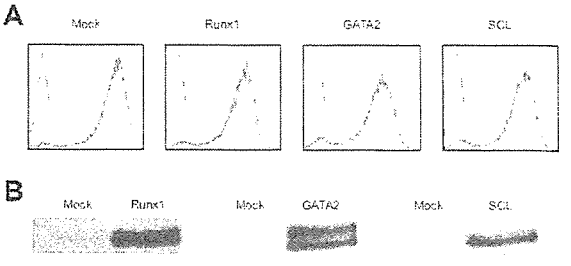

Figure 2. Retroviruses properly create Runx1, GATA2, and SCL proteins. (A) The efficiency of retrovirus-mediated gene transfer of Runx1, GATA2, or SCL was estimated by infecting NIH3T3 cells. Retrovirus-infected cells were evaluated by the expression of GFP (shaded histograms). Uninfected NIH3T3 cells are also shown as a control (open histograms). (B) Expression of individual proteins was confirmed by a Western blot analysis.

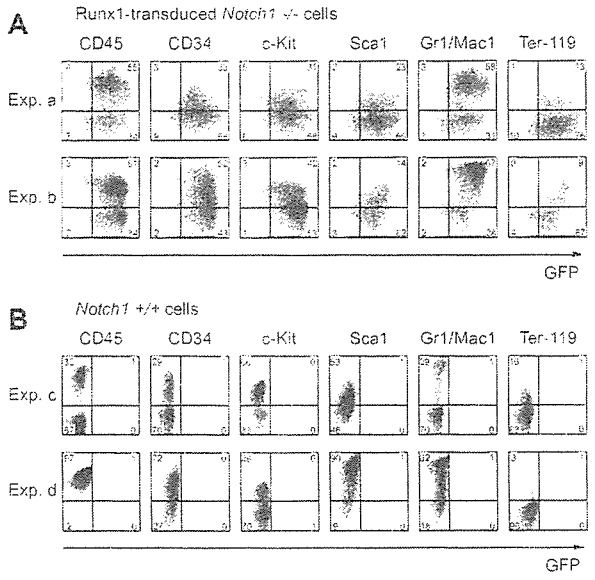

Figure 3. Runx1-rescued cells express hematopoietic surface markers. Expression of hematopoietic surface markers of cultured cells at day 12 from Runx1-transduced *Notch1*-null (*Notch1*$^{-/-}$) embryos (A) or wild-type (*Notch1*$^{+/+}$) embryos (B) was evaluated by flow cytometric analyses. GFP intensity (marking retrovirus-transduced cells) is plotted on the x-axis and intensity of counterstaining of hematopoietic surface markers is plotted on the y-axis. The results show representative results of independent replicates from 5 experiments. Percentages of cells in each quadrant are indicated.

and Runx1-transduced *Notch1*$^{-/-}$ P-Sp–derived cells. These observations indicate that the hematopoietic characteristics of Runx1-rescued cells were similar to those of wild-type P-Sp–derived cells.

Functional implication of Runx1 at the downstream of Notch-RBP-Jκ pathway

Runx1 has several distinct domains with defined biochemical functions. The Runt domain mediates both binding to DNA and dimerization with a partner protein, CBFβ/PEBP2β, whereas the transactivation domain interacts with transcriptional coactivators. Inhibitory domain counteracts the effect of the transactivation domain. The VWRPY motif located near the C-terminus mediates the interaction with a corepressor, TLE. A domain that interacts with mSin3A corepressor is also identified.[9] To assess whether Runx1 functions as an activator or a repressor[20] to restore the hematopoietic defect of *Notch1*-null embryo, we examined a series of Runx1 mutants (Figure 5)[14] for hematopoietic rescue.

Infection of retroviruses containing wild-type and several mutants, Δ444, Δ397, and Δ205-332 of Runx1 (Figure 5) resulted in the rescue of the *Notch1*-null phenotype, giving the same pattern with the culture of wild-type P-Sp cells (Figure 1A, top panels). In contrast, other mutants, Δ335, Δ288, AML1a, ΔRD, Δ205-332, and R139G (Figure 5) could not rescue the *Notch1*-null phenotype, giving the same pattern with the negative control (Figure 1A, bottom panels). Therefore, wild-type of Runx1 and the mutants that lack the VWRPY domain (Δ444, Δ397) or the mSin3A-binding region (Δ181-210) could restore the production of hematopoietic cells in the *Notch1*-null P-Sp culture, whereas those mutants that lack transactivation domain (Δ335, Δ288, AML1a, and Δ205-332) or Runt domain (ΔRD) could not rescue hematopoiesis from the *Notch1*-null P-Sp cells. Since changes in the tertiary structure of the protein could influence the function independent of the role of each domain, we also examined R139G, a mutant isolated from a patient with myelodysplastic syndrome (MDS) that harbors a point mutation causing substitution of Arg139 in the Runt domain with Gly. The DNA-binding ability is severely impaired in R139G, although the ability to heterodimerize with CBFβ/PEBP2β is spared.[21] This mutant could not restore hematopoiesis. These results suggest that, in the presence of an intact Runt domain, the transcriptional activating function is necessary and sufficient for Runx1 to rescue the hematopoietic defect of *Notch1*-null mice in the P-Sp culture system, while the transcriptional repressing function is dispensable.

Notch signaling also regulates transactivating function of Runx1

Hes1 is known to be a canonical Notch-RBP-Jκ target gene in mammals. It is also evident, however, by a number of studies that Hes1 mediates a part of, but not the whole, Notch-RBP-Jκ signaling.[22] In adult hematopoiesis, Hes1 maintains HSCs in vitro and expands them in vivo when retrovirally introduced to a highly HSC-enriched population.[23] Because Hes1 is expressed in the hematopoietic clusters budding from the dorsal aorta,[8] this transcription factor is a candidate as a physiologic target of the Notch-RBP-Jκ pathway in the embryonic hematopoietic development. Hes1 has also been known to mediate cross-talk between Notch and

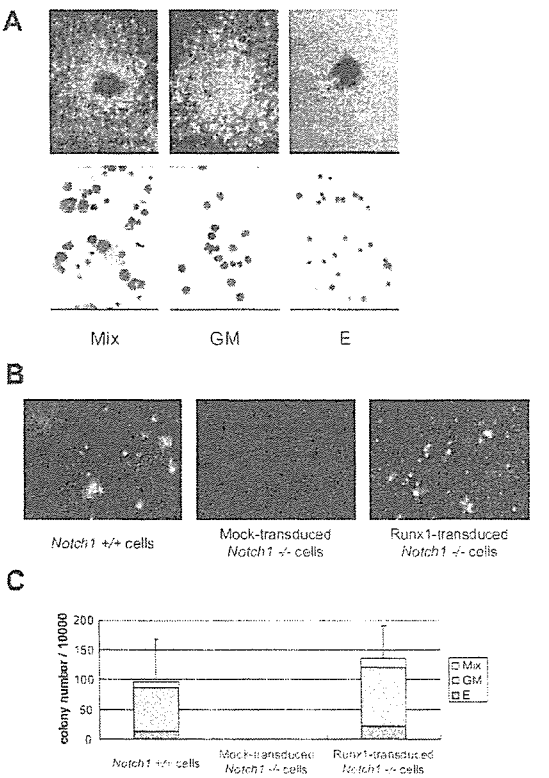

Figure 4. Runx1-rescued cells generate hematopoietic colonies. Colony formation of the Runx1-rescued cells from *Notch1*-null embryos. The rescued cells were harvested at day 12 and plated into MethoCult GF M3434 medium. (A) Representative hematopoietic colonies at day 7 are shown. Mix indicates mixed colony; GM, granulocyte/macrophage colony; and E, erythroid colony. Morphology of the colonies (top panels); original magnification, × 100. Wright-Giemsa–stained cytospin preparation of corresponding cell populations (bottom panels); original magnification, × 600. (B) Photographs of representative colonies. Original magnification, × 3. (C) The total number of colonies and the frequencies of different kinds of colonies. The results show the mean values of 5 independent experiments, each in duplicate, with standard deviations for the total colony numbers. Data were statistically analyzed by 2-tailed, unequal-variance *t* test.

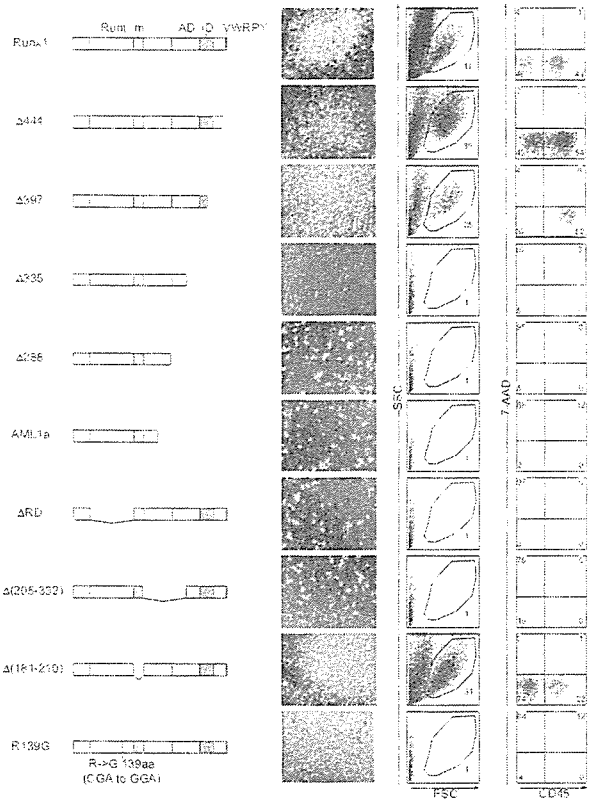

Figure 5. The transcriptionally active form of Runx1 is required for hematopoietic rescue. P-Sp cells from *Notch1*-null embryos at E 9.5 were infected with retroviruses containing *Runx1* mutants and cultured on OP9 cells for 12 days. Structures of Runx1 mutants are depicted (left column). Runt indicates the Runt domain; m, a binding region for mSin3A; AD, transactivation domain; ID, inhibitory domain; and VWRPY, VWRPY motif. Microscopic representations (center column; original magnification, × 100) and flow cytometric analyses (right 2 columns) of cells produced in the culture. Percentages of cells gated (center columns) and cells in each quadrant (right columns) are indicated.

other signaling pathways such as Janus-activating kinase/signal transducer and activator of transcription (JAK/STAT), Wnt, and Ras/mitogen-activated protein kinase (MAPK) pathways.[24-26] Furthermore, the transactivating function of Runx2, another Runx family member, is modified by Hes proteins and their relatives Hey proteins. When overexpressed, Hes1 potentiates Runx2-mediated transactivation in the transfected cells,[27] while Hey represses Runx2-mediated transactivation.[28,29]

Based on these pieces of information, we assessed whether Hes1 also modulates Runx1-mediated transactivation. Consistent with a previous report in which Hes1 was shown to bind to Runx1 in glutathione S-tranferase (GST) pull-down assays,[27] we detected HA-tagged Runx1 protein in the anti-FLAG immunoprecipitant, and reversely, FLAG-tagged Hes1 protein in the anti-HA immunoprecipitant, indicating physical interaction of Hes1 with Runx1 (Figure 6A). Moreover, Hes1 potentiated Runx1-mediated transactivation when expressed in HeLa cells, depending on the expression levels of Hes1 (Figure 6B).

Discussion

In this study, we showed that Runx1 rescues the defective hematopoiesis of *Notch1*-null mice in the OP9 culture system. The functional relationship between Notch and Runx families during hematopoietic development was first indicated in *Drosophila*, in which Notch up-regulates the expression of a *Runt* family gene, *Lozenge*.[16] More recently, it was shown that a zebrafish Notch-signaling mutant *mind bomb* fails in the specification of definitive HSCs during embryogenesis, and that Runx1 is required for expansion of HSCs in the zebrafish AGM region sufficient to restore the HSC specification in the *mind bomb* mutant.[11] The data shown in the present study strongly indicate that the Notch-Runx pathway is conserved from invertebrates to mammals and that Runx1 locates at a very proximal position in the Notch1 signaling pathway during establishment of definitive hematopoiesis.

GATA2 is also reported to have an important role downstream of Notch signaling in the establishment of definitive hematopoiesis. It was reported that NICD directly binds to the *Gata2* promoter and increases its expression level in mouse AGM cells.[8] Similarly in *Drosophila*, Notch up-regulates *Serpent* and induces emergence of hemocyte progenitors in lymph glands.[12] In our retroviral expression system, however, GATA2 could not rescue the hematopoietic defect of *Notch1*-null P-Sp cells (Figure 1B). It remains unknown whether GATA2 expression in more regulated levels and/or timings could rescue the hematopoietic deficient phenotype of *Notch1*-knockout P-Sp cells.

We clearly demonstrated that definitive hematopoiesis is rescued by forced expression of Runx1 in the *Notch1*-null P-Sp cells, but it should be directly shown whether transplantable HSCs are generated from the *Notch1*-null Runx1-introduced P-Sp cells. Fresh P-Sp cells obtained from wild-type embryos can be engrafted

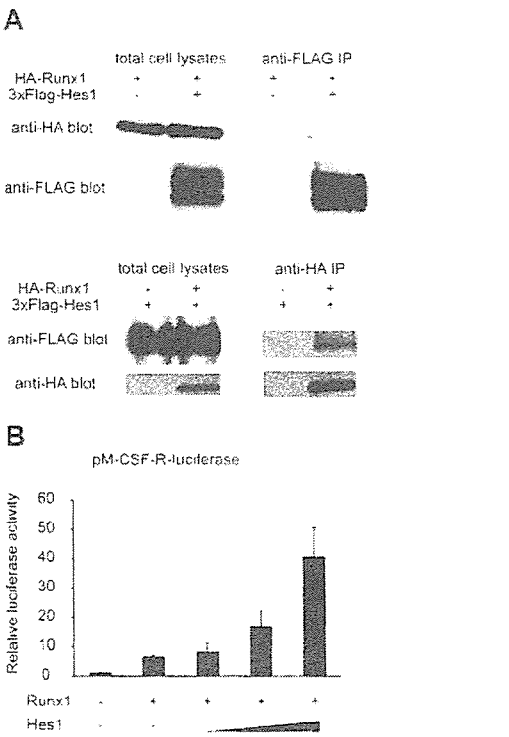

Figure 6. Notch signaling regulates transcriptional level of Runx1 and modulates the function of Runx1 protein through the effector protein, Hes1. (A) COS7 cells were transfected with HA-tagged Runx1 and 3xFLAG-tagged Hes1. Whole-cell extracts were immunoprecipitated (IP) with anti-FLAG antibody or anti-HA antibody followed by immunoblotting (blot) using anti-HA antibody or anti-FLAG antibody. (B) Relative luciferase activity in HeLa cells transfected with Runx1 (200 ng) and Runx1-dependent macrophage colony-stimulating factor receptor (pM-CSF-R) luciferase reporter (300 ng) with or without cotransfection of Hes1 (60, 200, or 600 ng). Data are means ± standard errors of duplicate wells in a representative experiment. Reproducible results were obtained in 3 independent experiments.

to mouse bone marrow if injected in the preconditioned newborn mice, as described.[3,30] It is unknown, however, whether the cultured P-Sp cells are also engraftable with the same method. We were unable to observe engraftment of the cultured P-Sp cells unlike fresh P-Sp cells, when injected to busulfan-pretreated newborn mice (data not shown). Culturing the cells, even for just a short time, is prerequisite for the retroviral gene transfer, which stands as a major technical obstacle to assess the engraftability of the *Notch1*-null Runx1-introduced P-Sp cells. Transgenic expression of Runx1, under an appropriate promoter, in the *Notch1*-null background may reveal further that the Notch1-Runx1 pathway represents an essential physiologic channel for the mammalian HSC generation from the P-Sp cells.

We also showed that Hes1, a known mediator of Notch signaling, cooperatively activates the Runx1-responsive pM-CSF-R luciferase reporter. This observation suggests that the Notch1 pathway modulates expression of Runx1 target genes through multiple mechanisms. There is a possibility that Notch1 directly augments the expression of Runx1 target genes. Although overexpression of Runx1 is sufficient to restore hematopoietic potential in *Notch1*-null P-Sp cells, both of these mechanisms might cooperatively contribute to HSC generation during normal development.

Acknowledgments

We thank M. Ohki for the gift of the human Runx1 cDNA, Y. Ito for the PEBP2β cDNA, D.-E. Zhang for the pM-CSF-R-luc vector, T. Kitamura for the Plat-E packaging cells and the pMYs/IRES-EGFP retrovirus vector, T. Nakano for the OP9 stromal cells, R. Kageyama for the Hes1 cDNA, and Kirin Brewery Pharmaceutical Research Laboratory for the cytokines. We dedicate this paper for the late Prof Hisamaru Hirai, who passed away during the progress of this study.

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American Journal of Hematology 81:875–879 (2006)

Pharmacokinetics of Alemtuzumab after Haploidentical HLA-Mismatched Hematopoietic Stem Cell Transplantation Using in Vivo Alemtuzumab With or Without CD52-Positive Malignancies

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We recently reported that the addition of in vivo alemtuzumab to the conditioning regimen enables 2- or 3-locus-mismatched hematopoietic stem cell transplantation without an excessive risk of graft rejection or graft-versus-host disease. In a later series of patients, however, one patient with refractory chronic lymphocytic leukemia with large residual tumors at transplantation developed graft rejection. While the peak alemtuzumab concentration in the previous patients without graft rejection was higher than 5 μg/ml, the peak alemtuzumab concentration in this patient was only 1.44 μg/ml. We considered that alemtuzumab was bound to the large residual tumors, which resulted in a low blood concentration of alemtuzumab. Therefore, it is important to debulk tumors before the conditioning regimen for patients with refractory CD52-positive hematological malignancies, or the dose of alemtuzumab should be adjusted by monitoring the blood concentration, when alemtuzumab is used for in vivo T-cell depletion in 2- or 3-locus-mismatched transplantation. Am. J. Hematol. 81:875–879, 2006. © 2006 Wiley-Liss, Inc.

Key words: chronic lymphocytic leukemia; hematopoietic stem cell transplantation; alemtuzumab; serum concentration; rejection

INTRODUCTION

Alemtuzumab (Campath-1H) is a humanized monoclonal antibody directed against human CD52 that is expressed at a high density on B- and T-cells and dendritic cells, but not on hematopoietic stem cells [1]. Although alemtuzumab was approved for the treatment of fludarabine-refractory chronic lymphocytic leukemia (CLL) [2], it has also been used for in vivo T-cell depletion to prevent graft rejection and graft-versus-host disease (GVHD) in allogeneic hematopoietic stem cell transplantation (HSCT) [3,4]. The addition of alemtuzumab to a conditioning regimen decreases graft rejection by depleting host T-cells. In addition, it has a long terminal half-life (15–21 days) and the blood concentration is maintained at a lympholytic level for about 2 months after transplantation, which contributes to the prevention of GVHD [5]. We extended the use of in vivo alemtuzumab to 2- or 3-locus-mismatched transplantation and successfully reduced the incidence of grade III-IV acute GHVD to only 9% without graft rejection in the first 12 patients in a prospective study approved by the ethics commitee [6]. However, in a later series of patients, a patient with fludarabine-refractory CLL with large residual tumors at transplantation developed graft rejection after an initial neutrophil recovery. We describe here the clinical course and discuss the pharmacokinetics of alemtuzumab in patients with or without CD52-positive hematological malignancies.

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Received for publication 3 October 2005; Accepted 18 March 2006

Published online 21 July 2006 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/ajh.20694

Clinical Course of a Patient Who Developed Graft Rejection

A 56-year-old woman with CLL, which was refractory to 10 courses of fludarabine (30 mg/m^2 × 5 days), 2 courses of rituximab (375 mg/m^2), and 4 courses of CVP therapy (cyclophosphamide 750 mg/m^2 × 1 day, vincristine 1.4 mg/m^2 × 1 day, prednisolone 60 mg/m^2 × 5 days), chose to participate in a clinical study of 2- or 3-locus-mismatched HSCT using in vivo alemtuzumab, since she did not have an available HLA-matched or 1-locus-mismatched donor among her family members and her disease status precluded a time-consuming donor coordination to identify an HLA-matched unrelated donor. Just before starting the conditioning regimen, she still had large residual tumors in the abdomen, although the peripheral blood lymphocyte count was decreased to 2.66 × 10^9/L. The conditioning regimen consisted of alemtuzumab (0.2 mg/kg/day from day −8 to −3), fludarabine (30 mg/m^2 from day −8 to −3), busulfan (4 mg/kg/day on days −5 and −4) and total body irradiation (TBI; 2 Gy twice daily on day −1). Peripheral blood mononuclear cells were collected from her 3-locus-mismatched son following a mobilization with filgrastim, cryopreserved without ex vivo manipulation, and infused on day 0. The number of infused CD34- and CD3-positive cells was 4.75 × 10^6 cells/kg and 0.86 × 10^8 cells/kg of recipient body weight, respectively. Posttransplantation prophylaxis against GVHD was performed with the continuous infusion of cyclosporine A (3 mg/kg) and short-term methotrexate (15 mg/m^2 on day 1 and 10 mg/m^2 on days 3, 6, and 11). Regimen-related toxicities were mild. Neutrophil engraftment, defined as the first of 3 consecutive days with an absolute neutrophil count of at least 0.5 × 10^9/L, was documented on day 15. On day 18, however, the granulocytic count began to rapidly decrease, associated with a high fever up to 104°F, disseminated intravascular coagulation, and a high lactate dehydrogenase level. We restarted filgrastim but the neutrophil count decreased to below 0.10 × 10^9/L. Eighty-two percent of the bone marrow cells were of donor origin on day 22, but donor cells became undetectable in both the bone marrow and peripheral blood on day 28. The abdominal CT scan on day 22 showed decreased but residual tumors. Flow cytometry analysis of the peripheral blood on day 26 showed that more than 90% of lymphocytes were CD8-positive T-cells. Although we waited for autologous hematopoietic recovery, the neutrophil count remained below 0.10 × 10^9/L. Therefore, we performed the second peripheral blood stem cell transplantation from the same donor on day 51 after the first transplantation following a conditioning regimen consisting of alemtuzumab (0.2 mg/kg/day from day −10 to −5), cyclophosphamide (30 mg/kg/day on days −7 and −6), and fludarabine (25 mg/m^2/day from day −5 to −1). The number of CD34- and CD3-positive cells infused at the second transplantation was 2.86 × 10^6 cells/kg and 0.53 × 10^8 cells/kg of recipient body weight, respectively. We started the continuous infusion of CsA on day −1. However, she developed acute renal failure and thus we replaced CsA with prednisolone at 1 mg/kg/day from day 2. At the same time, high fever, skin rash, fluid retention, weight gain, and noncardiogenic pulmonary edema rapidly progressed, which required mechanical ventilation from day 2 and continuous hemodiafiltration from day 9. Twice, we administered high-dose methyl-prednisolone at 1000 mg/day for 3 days (from days 9 and 15). Despite these treatments, capillary leak syndrome did not improve and she died on day 18 due to severe hypotension, although donor cell engraftment was confirmed on day 11. The clinical course of the CLL patient is summarized in Figure 1. Autopsy revealed no residual CLL cells and no microbiologically documented infections. Pathological finding of the skin showed the degeneration of epidermal cells and sweat gland cells with little lymphocyte infiltration, which were compatible with acute GVHD.

Blood Concentration of Alemtuzumab

The serum concentration of alemtuzumab was determined by indirect immunofluorescence using frozen sera as described in detail elsewhere [7,8]. The serial serum concentrations of alemtuzumab of the present patient in the first and second transplantations and those of three control patients who underwent haploidentical HSCT using alemtuzumab from a 3-locus-mismatched related donor and whose serum samples before and after transplantation were available are shown in Figure 2. The current patient and the three control patients participated in the same study to evaluate the safety of unmanipulated peripheral blood stem cell transplantation from 2- or 3-locus-mismatched related donors using alemtuzumab in vivo and received exactly the same alemtuzumab dosage schedule (0.2 mg/kg/day from day −8 to −3) and the same supportive care [6]. Of the three control patients, two (C1 and C2) had myeloid malignancies and the other (C3) had diffuse large B-cell lymphoma in partial remission. Patients C2 and C3 received the same conditioning regimen as the CLL patient did, whereas patient C1 received TBI at 2 Gy twice daily on days −7, −6, and −5, followed by cyclophosphamide at 60 mg/kg on days

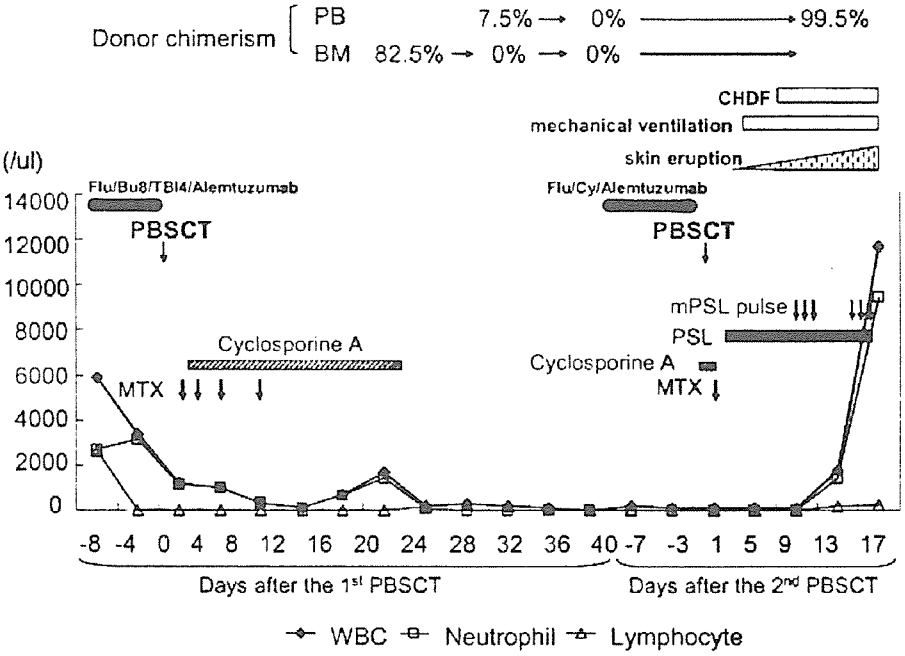

Fig. 1. Clinical course of the first and second haploidentical transplantation using in vivo alemtuzumab. PB, peripheral blood; BM, bone marrow; CHDF, continuous hemodiafiltration; Flu, fludarabine; Bu, busulfan; TBI, total body irradiation; Cy, cyclophosphamide; PBSCT, peripheral blood stem cell transplantation; MTX, methotrexate; PSL, prednisolone; WBC, white blood cell.

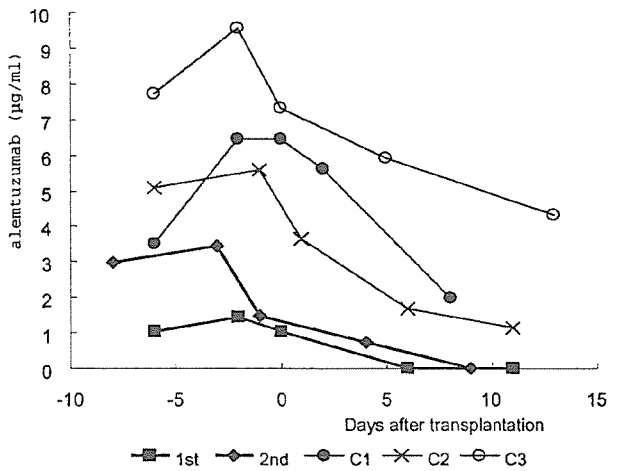

Fig. 2. Serial serum concentrations of alemtuzumab. Alemtuzumab activity was measured by indirect immunofluorescence. The lower limit of this assay for serum samples is 0.50 μg/ml and the upper limit is 20.00 μg/mL.

−3 and −2. Although the serum alemtuzumab concentrations in the three control patients were comparable to those in previous studies where alemtuzumab was used in a conditioning regimen [9,10], the serum alemtuzumab concentration in the CLL patient was persistently lower than 2.0 and 4.0 μg/ml in the first and second transplantations, respectively, and it quickly decreased to an undetectable level (<0.5 μg/ml) after transplantation. Therefore, the serum concentration of alemtuzumab before transplantation was too low to suppress host T-cells, which may have resulted in graft rejection after the first transplantation. In the second transplantation, host T-cells might have been sufficiently suppressed by the repeated conditioning regimen, but a strong reaction compatible with hyperacute GVHD occurred a few days after the infusion of donor graft, probably due to the insufficient alemtuzumab concentration on day 0 and thereafter.

DISCUSSION

The use of in vivo alemtuzumab in an HSCT setting enables durable engraftment and a significant reduction of GVHD, even in 2- or 3-locus-mismatched HSCT [6,10]. Pharmacokinetic studies of alemtuzumab at a dose of 20 mg/day for 5 days before transplantation with a reduced-intensity conditioning regimen have demonstrated that the serum alemtuzumab concentration was higher than the level that was required to kill infused donor T-cells at the time of transplantation and remained at

TABLE I. The Incidence of Graft Failure after Allogeneic Transplantation Using Fludarabine, Melphalan, and Alemtuzumab

Underlying disease	*n*	Donor R/U	Dose of alemtuzumab	Primary graft failure	Secondary graft failure
CLL	41	24/17	100 mg in 27 60 mg in 6 50 mg in 7 40 mg in 1	3	5
AML/MDS	76	35/41	100 mg	2	0
MM	25	0/25	100 mg	0	0
NHL	88	65/23	100 mg	1	3
HL	49	31/18	100 mg	0	0

Note. CLL, chronic lymphocytic leukemia; AML, acute myeloblastic leukemia; MDS, myelodysplastic syndrome; MM, multiple myeloma; NHL, non-Hodgkin's lymphoma; HL, Hodgkin's lymphoma; R, related donor; U, unrelated donor.

a potentially lympholytic level for approximately 2 months after transplantation [5].

The pharmacokinetics of alemtuzumab in the treatment of CLL are quite different. The peak blood concentration after the first infusion of alemtuzumab at 30 mg for refractory CLL patients showed a wide variation among patients [7]. In addition, the blood concentrations of alemtuzumab showed a modest negative correlation with the starting lymphocyte counts [7]. These results suggest that the pharmacokinetics of alemtuzumab in CLL patients were affected by the number of tumor cells. The alemtuzumab concentration tends to be lower when the patient has bulky tumor cells, since alemtuzumab may bind to tumor cells.

In a transplantation setting, only a cumulative dose of 100 mg or less, which is far lower than that in the treatment of CLL, is highly immunosuppressive and clinically effective for the prevention of graft rejection and GVHD [11]. However, the cumulative dose of 1.2 mg/kg (66 mg/body) might have been insufficient to prevent graft rejection in this patient with bulky CD52-positive residual tumor cells. On the other hand, previous transplantation studies have not pointed out any differences in the pharmacokinetics of alemtuzumab between patients with CD52-positive lymphoid malignancies and those with myeloid malignancies [5]. However, Delgado et al. recently reported the results of 41 consecutive allogeneic hematopoietic cell transplantation for CLL using fludarabine, melphalan, and alemtuzumab [12]. They showed a higher incidence of primary or secondary graft failure (8 of 41) than that in transplantation for the other hematological malignancies using the same regimen (Table I) [13–16]. The alemtuzumab concentrations in these patients were assumed to be lower than those in transplantation for the other hematological malignancies, because they had CLL and/or the dose of alemtuzumab was reduced in 14 of the 41 patients. These data further support our hypothesis that the low alemtuzumab concentration might have resulted in graft rejection in the current CLL patient.

In conclusion, a residual CD52-positive tumor may strongly affect the blood concentration of alemtuzumab. It may be worthwhile to decrease tumor cells before the conditioning regimen or to increase the dose of alemtuzumab in a conditioning to prevent graft rejection and GVHD. More data on the pharmacokinetics of alemtuzumab are needed to determine an optimal dose for HSCT, especially from a 2- or 3-locus-mismatched donor.

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original article

Annals of Oncology 18: 364–369, 2007
doi:10.1093/annonc/mdl393
Published online 1 November 2006

A high incidence of late-onset neutropenia following rituximab-containing chemotherapy as a primary treatment of CD20-positive B-cell lymphoma: a single-institution study

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Received 1 August 2006; revised 12 September 2006; accepted 14 September 2006

Background: Late-onset neutropenia (LON) has been reported following rituximab-containing chemotherapy. Its incidence and risk factors, however, have not been extensively studied.

Patients and methods: We retrospectively reviewed the medical records of 107 patients treated with rituximab-containing chemotherapy as a primary treatment of CD20-positive B-cell lymphomas and identified cases with LON as defined by the neutrophil count of $\leq 1.0 \times 10^9$/l without an apparent cause after the recovery of neutrophil count following completion of the intended chemotherapy.

Results: With a median follow-up of 411 days, 23 patients developed LON out of the 107 at a median of 106 days after the last chemotherapy. Cumulative incidence of LON among the total patients was 24.9%. The median neutrophil count nadir was 0.61×10^9/l. The LON episodes were generally self-limited, and filgrastim was administered in one patient. Including this patient, there were no serious infectious episodes in the cases with LON. In multivariate analysis, intensive chemotherapy regimens including high-dose therapy followed by autologous hematopoietic stem cell transplantation (ASCT) and high-dose methotrexate-containing regimens without ASCT were a risk factor for LON.

Conclusion: This study suggests that LON is a frequent complication of rituximab-containing intensive chemotherapy.

Key words: late-onset neutropenia, lymphoma, neutropenia, rituximab

introduction

Rituximab, a chimeric monoclonal antibody against human CD20, is widely used as a single agent or in combination with chemotherapy for various types of CD20-positive B-cell malignancies [1–5]. This agent adds little toxicity when combined with chemotherapy regimens aside from mild to moderate infusion-related reaction, which is frequently encountered with the first dose [1, 2]. Late-onset neutropenia (LON) is a newly recognized late complication of rituximab-combining chemotherapy. The cause of LON has been attributed to rituximab, but the mechanism for developing LON remains undetermined [6–9]. According to previous reports, LON is usually considered to be an uncommon event with standard-dose chemotherapy, while a higher incidence has been reported after high-dose therapy followed by stem cell transplantation [10, 11]. The incidence and the clinical course of LON, however, are unclear, especially among patients who receive rituximab-containing regimen as the first-line chemotherapy. For instance, the incidence of LON after the combination therapy of rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP), the most common first-line regimen for B-cell lymphomas, has not been reported. Such a late complication may be elusive unless it leads to infection in large-scale prospective studies. Thus, we investigated the incidence and the clinical course of LON in clinical practice at our department.

patients and methods

patient population

We retrospectively reviewed the medical charts of consecutive patients who had completed the intended primary treatment of CD20-positive lymphomas at our department from March 1996 through May 2006. Patients who had achieved complete response or complete response undetermined according to the International Workshop criteria and with

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a follow-up period of at least 1 month after the last chemotherapy cycle were analyzed. Two patients with indolent lymphomas who had previously received involved field radiotherapy as an initial treatment followed by the rituximab-containing regimen for the progressive disease were included. Characteristics of patients and primary treatments are shown in Table 1. After the primary treatment, the interval of follow-up visits and blood tests were at physicians' discretion. Generally, complete blood count with differential and reticulocyte counts was carried out every 2–8 weeks during first 2 years of follow-up.

One hundred and seven patients were treated with a rituximab-containing chemotherapy regimen. Ninety-four patients underwent rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone (R-CHOP), with patients followed by consolidative high-dose chemotherapy with autologous hematopoietic stem cell transplantation (ASCT) in five, and followed by radiotherapy in 23. Among them, 25 patients were treated with four cycles of chemotherapy or less (abbreviated chemotherapy) mainly because it was combined with involved field radiotherapy for localized diffuse large B-cell lymphoma (DLBCL) ($n = 20$). Three patients underwent rituximab combined with cyclophosphamide, vincristine, and prednisolone (R-CVP). In this study, we defined R-CHOP followed by consolidative ASCT ($n = 5$, as described above) and high-dose methotrexate-containing regimens: fractionated cyclophosphamide, doxorubicin, vincristine, dexamethasone alternating with high-dose methotrexate–cytarabine (R-hyperCVAD/MA) ($n = 5$), the Cancer and Leukemia Group B 9251 regimen for Burkitt's lymphoma (BL regimen) ($n = 3$), and other high-dose methotrexate-containing regimens ($n = 2$), as intensive regimens. Among them, three patients underwent ASCT as a consolidation after completing R-hyperCVAD/MA, and two patients with the BL regimen received radiotherapy. These intensive regimens were carried out according to the protocol in our department for high-risk DLBCL according to the age-adjusted international prognostic index, mantle cell lymphoma, DLBCL of intravascular variant, Burkitt's lymphoma, and DLBCL with involvement of the central nervous system or the testis. All of these patients received six cycles of chemotherapy or more according to the protocol. Thus, the chemotherapy duration as defined by the time from the start of chemotherapy through completion, including high-dose therapy, was longer in the patients who had intensive regimens than those with R-CHOP or R-CVP (median 166 days, range 96–206 days, versus 112 days, range 22–214 days, the Mann–Whitney U-test, $P < 0.0001$). For rituximab-containing chemotherapy, one dose of rituximab 375 mg/m^2 was administered with each chemotherapy cycle in general. There was no patient who had been treated with maintenance rituximab therapy.

Table 1. Patient characteristics

			Rituximab-containing chemotherapy	Therapy without rituximab	*P* value
Number			107	52	
Age					
Median			62	62	0.570
Range			24–91	22–88	
Sex					
Male (%)			66 (61.7%)	24 (46.1%)	0.064
BM involvement			38 (35.5%)	4 (7.7%)	0.0002
Stage III, IV			66 (61.7%)	19 (36.5%)	0.003
Histology					
Aggressive			71 (66.4%)	47 (90.4%)	0.001
DLBCL			63	44	
MCL			5	1	
BL			3	2	
Indolent			36 (33.6%)	5 (10.6%)	
FL			31	5	
MALT			4	0	
LPL			1	0	
Regimen	RT	ASCT			
R-CHOP or CHOP	–	–	66	32	
	+	–	23	15	
	–	+	5	1	
	+	+	–	2	
R-CVP	–	–	3	–	
R-hyperCVAD/MA	–	–	2	–	
	–	+	3	–	
BL regimen	–	–	1	–	
	+	–	2	2	
Others	–	–	2	–	

BM, bone marrow; DLBCL, diffuse large B-cell lymphoma; MCL, mantle cell lymphoma; BL, Burkitt's lymphoma; FL, follicular lymphoma; MALT, mucosa-associated lymphoid tissue lymphoma; LPL, lymphoplasmacytic lymphoma; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisolone; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone; R-CVP, rituximab, cyclophosphamide, vincristine, prednisolone; RT, radiotherapy; ASCT, consolidative high-dose therapy followed by autologous hematopoietic stem cell transplantation; R-hyperCVAD/MA, rituximab, fractionated cyclophosphamide, vincristine, doxorubicin, dexamethasone, alternating with high-dose methotrexate–cytarabine.
–, not applicable.

Twenty-five patients received involved field radiotherapy after the completion of chemotherapy.

As a control, we reviewed the charts of 52 consecutive patients who underwent chemotherapy without rituximab as a primary treatment of CD20-positive lymphomas at our department (Table 1). Proportions of patients who had intensive regimens (14.0% versus 9.6%, $P = 0.432$), abbreviated chemotherapy (23.4% versus 19.2%, $P = 0.555$), consolidative ASCT (7.5% versus 5.8%, $P = 0.691$), and radiotherapy (23.4% versus 36.5%, $P = 0.082$) were comparable between the rituximab-containing chemotherapy-treated group and the control group. Rituximab was included in the standard protocol for indolent lymphomas in 2001 and for aggressive lymphomas in 2003. Therefore, treatment periods of the control group predated those of the studied patients. Otherwise, patients in the both groups were followed in the same manner and there were no changes in supportive treatments.

definition of LON

Various definitions of LON have been used in previous reports [6–8, 12]. In this study, we defined LON as neutropenia of $\leq 1.0 \times 10^9$/l [grade 3 according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC)] without an apparent cause after the recovery of neutrophil count following completion of the intended chemotherapy and before progression of lymphoma and/or additional chemotherapy. Severe LON was defined as neutropenia of $\leq 0.5 \times 10^9$/l (grade 4 according to the NCI-CTC). Neutropenia that was observed after the recovery from the first episode of LON was defined as the second episode of LON as far as the patient had not had progressive lymphoma and/or been treated with additional chemotherapy. One patient had interferon and lamivudine because of exacerbation of chronic hepatitis B. This patient was censored at the start of interferon. For patients with LON, we had not defined a protocol for work-up or indication for the use of granulocyte colony-stimulating factor.

statistical analysis

Univariate and multivariate analyses for time-to-event covariates were carried out using the log-rank test and proportional-hazard modeling, respectively. Factors associated with at least borderline significance ($P < 0.10$) in univariate analyses were subjected to a multivariate analysis and deleted stepwise from the model. The cumulative incidence of LON was evaluated using Gray's method considering progression of lymphoma before LON as a competing risk [13].

results

incidence and risk factors of LON

With a median follow-up of 411 days, 23 (21.5%) patients developed LON out of the 107 patients who received rituximab-containing chemotherapy as a primary treatment of CD20-positive B-cell lymphomas. The cumulative incidence of LON was 24.9% (Figure 1A). Severe LON was observed in 10 (9.3%) patients, and its cumulative incidence was 15.4%. In contrast, no episodes of LON were observed in the control group (52 patients).

In univariate analysis, advanced stages (Ann Arbor stages III and IV), intensive primary treatment regimens, consolidative high-dose therapy followed by ASCT, and absence of radiotherapy as a primary treatment were associated with a higher incidence of LON (Table 2). There was a trend for a higher incidence of LON in patients who were 65 years old or younger. In this study, sex, histology, bone marrow involvement at diagnosis, or adopting abbreviated chemotherapy was not

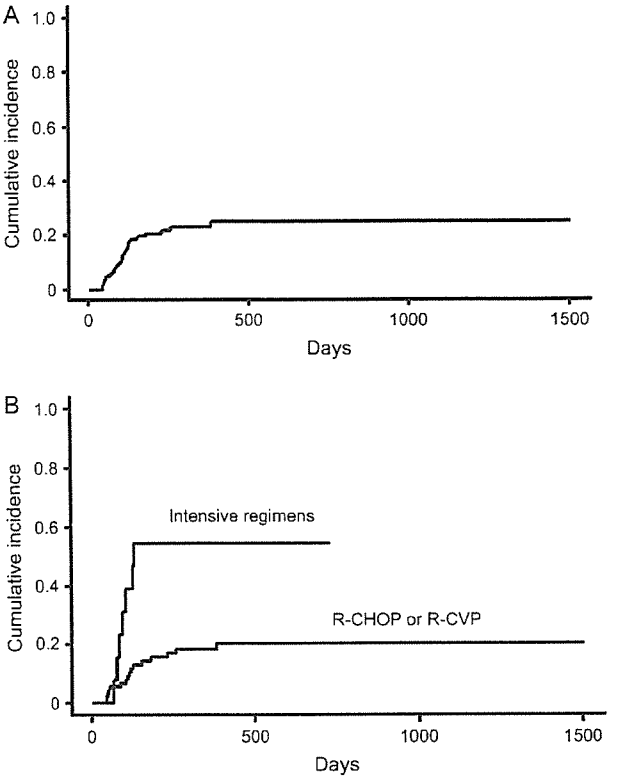

Figure 1. (**A**) The cumulative incidence of late-onset neutropenia (LON) after the last chemotherapy cycle. (**B**) The cumulative incidence of LON according to chemotherapy regimens [rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone (R-CHOP) or rituximab, cyclophosphamide, vincristine, prednisolone (R-CVP) versus intensive regimens] combined with rituximab.

a risk factor for LON. In the multivariate analysis, the use of primary treatment regimens with higher intensity than that of R-CHOP or R-CVP was an independent risk factor for LON (Figure 1B, Table 2). Although, by definition, patients who had consolidative ASCT were included in the group of intensive primary treatment regimens, a high incidence of LON (50.3%) was also observed when only the patients without ASCT were analyzed. Even for the patients who received R-CHOP or R-CVP without consolidative ASCT, the incidence of LON was 20.4%.

clinical courses of LON

Clinical courses of the patients with LON are summarized in Table 3. The first LON episode in each patient developed at a median of 106 (range 46–384) days after the last chemotherapy and at a median of 124 (range 46–384) days after the last administration of rituximab. Neutrophil count nadir during LON episodes in each patient ranged from 0.008×10^9 to 0.96×10^9/l (median 0.6×10^9/l). The recovery from neutropenia was observed at a median of 28 (range 5–84) days. In most cases, neutropenia was observed at only one visit. Sustained neutropenia lasting at least >3 weeks was, however, observed in six episodes (range 22–81 days). Including these cases, there was no concurrent drop in platelet or reticulocyte count in the

Table 2. Risk factors for late-onset neutropenia

Factors	Variables	N	Incidence (%)	P value
Univariate analyses				
Age	≤65	64	30.8	0.095
	>65	43	16.5	
Sex	Male	66	32.4	0.388
	Female	41	20.8	
Histology	Aggressive lymphomas	71	22.5	0.291
	Indolent lymphomas	36	30.1	
Stage	I, II	41	11.6	0.016
	III, IV	66	33.2	
Bone marrow involvement	Yes	38	32.5	0.215
	No	69	20.4	
Chemotherapy regimen	R-CHOP or R-CVP	92	20.4	0.005
	Intensive regimens	15	54.4	
Consolidative ASCT	Yes	8	57.1	0.019
	No	99	22.5	
Radiotherapy in primary treatment	Yes	25	8.00	0.031
	No	82	31.1	
Abbreviated chemotherapy	Yes	25	22.2	0.544
	No	82	25.7	
	Relative risk	95% CI	P value	
Multivariate analysis				
Intensive regimens versus R-CHOP or R-CVP	3.425	1.426–8.223	0.0059	

Aggressive lymphomas: diffuse large B-cell lymphoma, Burkitt's lymphoma, mantle cell lymphoma; indolent lymphomas: follicular lymphoma, mucosa-associated lymphoid tissue lymphoma, lymphoplasmacytic lymphoma. ASCT, autologous hematopoietic stem cell transplantation; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone; R-CVP, rituximab, cyclophosphamide, vincristine, prednisolone; CI, confidence interval.

patients with LON. Bone marrow examination was carried out in two patients. In one patient (case 11-1), the result was unremarkable, while in the other (case 23-1), maturation arrest of myeloid series was seen. Filgrastim was administered in one patient for LON, and neutrophil count recovered promptly after one dose. Otherwise, all LON episodes recovered spontaneously without administering hematopoietic factors. All LON cases were detected in blood tests at routine follow-up, and no serious complications with LON were observed aside from one patient with mild tonsillitis. Three patients (cases 11-2, 13-2, and 23-2) developed the second episode of LON at 49, 53, and 56 days after the recovery from the first episode of LON, respectively. Time to the recovery from the second episodes of LON was 28, 42, and 63 days, respectively. Progression of lymphoma was observed in five patients out of 23 patients who had LON. The median progression-free survival was 28.4 months after completion of primary therapy which was comparable to that of the patients without LON (data not shown).

One patient (case 11-1) developed neutropenia immediately after the stem cell mobilization with filgrastim alone 3 months after completion of R-hyperCVAD/MA. In this case, upon consecutive administration of 600 μg of filgrastim per day for 4 days, there was only a slight increase in white blood cell count (3.6×10^9 to 6.7×10^9/l) and we could collect only 0.084×10^6/kg CD34-positive cells, which were not enough for transplantation. The neutrophil count on the day of stem cell collection was 5.2×10^9/l, and after cessation of filgrastim, it decreased steadily to 0.55×10^9/l on the fifth day. LON in this case lasted for 1 week, and there was no sign of infection during this period.

discussion

Rituximab has changed the treatment paradigm of CD20-positive B-cell malignancies. This agent is used as a single agent or in combination with chemotherapy. Improvement in complete response rates and in long-term prognosis has been shown by adding rituximab to the original chemotherapy regimen in several B-cell malignancies including DLBCL and follicular lymphoma [1, 2, 4, 5]. The toxicity of rituximab is generally mild, if any, and a major concern is restricted to infusion-related toxicity at the first administration of rituximab [2]. With its increased use, however, uncommon adverse events attributed to rituximab have been recognized [6, 14]. LON is one of such events and we also attribute LON to the use of rituximab because it was never seen in the patients treated with chemotherapy alone as described in the literature [7, 8], although clinical characteristics of the studied group and the control group were not comparable because of the retrospective design of this study (Table 1).

In this study, the cumulative incidence of LON was 24.9% with higher incidence (54.4%) in patients receiving intensive primary treatment compared with patients who received conventional treatment with R-CHOP or R-CVP (20.4%). A high incidence of LON has been reported in patients treated with

Table 3. Characteristics of patients with late-onset neutropenia

Case	Age/Sex	Diagnosis	Stage	Bone marrow involvement at diagnosis	Primary treatment	LON episodes			
						Onset (days after the last chemotherapy)	Neutrophil count nadir (/ml)	G-CSF	Infection
1	42/M	BL	IV	+	BL regimen + rituximab	130	960	–	Tonsillitis
2	47/M	DLBCL	II	–	R-CHOP	126	920	–	–
3	72/F	DLBCL	I	–	R-CHOP, IFRT	109	726	–	–
4	56/M	DLBCL	IV	–	R-CHOP	105	486	–	–
5	34/F	DLBCL	IV	–	R-CHOP, ASCT	78	909	–	–
6	63/M	DLBCL	III	–	R-CHOP, ASCT	106	196	–	–
7	59/F	DLBCL	II	–	R-CHOP, IFRT	119	638	–	–
8	55/M	DLBCL	IV	+	R-CHOP	115	160	1 day	–
9	48/M	DLBCL	IV	–	R-CHOP, ASCT	69	384	–	–
10	50/M	DLBCL	I	–	R-CHOP	259	863	–	–
11-1	60/F	DLBCL	IV	+	R-hyperCVAD/MA	92	552	–	–
11-2					–	155	595	–	–
12	77/F	DLBCL	IV	+	R-CHOP	384	920	–	–
13-1	62/M	FL	IV	+	R-CHOP	153	380	–	–
13-2					–	220	775	–	–
14	71/M	FL	IV	+	R-CHOP	46	806	–	–
15	64/F	FL	IV	+	R-CHOP	89	486	–	–
16	53/F	FL	IV	–	R-CHOP	46	466	–	–
17	58/F	FL	IV	+	R-CHOP	49	165	–	–
18	70/F	FL	IV	–	R-CHOP	56	800	–	–
19	66/M	FL	III	–	R-CHOP	231	960	–	–
20	72/F	FL	IV	+	R-CVP	52	322	–	–
21	58/F	FL	III	–	R-CHOP	182	875	–	–
22	39/M	MCL	IV	+	R-hyperCVAD/MA	143	624	–	–
23-1	53/M	MCL	IV	+	R-hyperCVAD/MA, ASCT	85	8	–	–
23-2					–	228	550	–	–

BL, Burkitt's lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; G-CSF, granulocyte colony-stimulating factor; LON, late-onset neutropenia; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone; R-CVP, rituximab, cyclophosphamide, vincristine, prednisolone; ASCT, autologous hematopoietic stem cell transplantation; R-hyperCVAD/MA, rituximab, fractionated cyclophosphamide, vincristine, doxorubicin, dexamethasone, alternating with high-dose methotrexate–cytarabine; IFRT, involved field radiotherapy.

rituximab before and/or after ASCT [10–12]. A French group reported six patients with LON (neutrophil count of $<0.5 \times 10^9$/l) out of 39 patients (15%) treated with the same protocol using rituximab and consolidative ASCT [10]. Consistent with this, we observed a high incidence of LON (57.1%) in patients who underwent consolidative ASCT. Furthermore, the current study revealed that patients who primarily received rituximab in combination with intensive regimens incorporating high-dose methotrexate without consolidative ASCT also had a high incidence (50.3%) of LON. It is possible that factors associated with the diseases on their own, which necessitated these intensive regimens, may have directly contributed to the development of LON, although all of these patients were free of progression for >1 year after the episode of LON.

In the present study, patients having radiotherapy as a primary treatment had lower incidence of LON (Table 2). Among these 25 patients, 20 were treated with a combination of abbreviated chemotherapy plus involved field radiation. The other five patients had radiotherapy after completing six cycles of chemotherapy for initial bulky lesion and so on. Thus, it would be very convincing that it is not absence of radiotherapy as a primary treatment, but the amount of chemotherapy that is a risk factor for LON, and this is in line with another finding that the use of intensive regimens was a risk factor for LON. In this study, however, the use of abbreviated chemotherapy itself was not associated with lower incidence of LON. Although there may be some unknown confounding factor, we have no account of this at the moment.

We also found a high incidence (20.4%) of LON even in patients treated with R-CHOP and R-CVP, which are the most commonly used regimens for CD20-positive lymphomas. We assume that this result could be applied widely to patients who are treated with these regimens. The manufacturer of rituximab reported the calculated post-marketing reporting rate of LON of <0.02% [15]. These facts suggest the possibility that many cases with rituximab-associated LON are unrecognized in clinical practice. The report from Australia described eight episodes of severe LON among 53 patients (15%) who were treated with rituximab [7]. That study, however, included mainly patients with relapsed follicular lymphoma, and patients treated with rituximab alone were analyzed together, which makes it difficult to estimate the incidence of LON in a primary treatment setting. The cumulative incidence of severe LON of 9.3% in the current study is consistent with the previous report from NCI of the USA in which the incidence was reported to be 8% out of 76 patients treated with dose-adjusted

EPOCH with rituximab as a frontline treatment of DLBCL, AIDS-related lymphomas, and mantle cell lymphoma [8]. We believe that less severe LON is more commonly seen than is generally thought. Because episodes of LON can be short and rarely complicated with severe infection, as shown in this study, it can elude recognition by physicians. As this retrospective study was carried out based on routine clinical practice, some LON episodes may have eluded recognition as they developed and subsided between blood tests. Thus, the actual incidence of LON might be even higher than revealed by this study.

The median time to LON in this study was similar to that observed in the previous studies [6, 7]. Although B-cell count was not followed serially in this study, the onset of LON coincided with or preceded the timing of normal B-cell recovery described in the literature [3], supporting the hypotheses that etiology of LON is related with the recovery of nonmalignant B-cell population after administration of rituximab. Production of anti-neutrophil autoantibody by repopulating B cells has been implicated [6]. Recently, another hypothesis has been reported in which perturbation of stromal-derived factor-1 during B-cell recovery inhibits the egression of neutrophil from the bone marrow [8]. B-cell recovery-associated mechanism may not be the only cause of LON, however, because it has rarely been reported in patients who were treated with rituximab alone [3], and the incidence of it might be associated with chemotherapy regimen used along with rituximab, as shown in this study.

In conclusion, we found a high incidence of LON in the series of patients who underwent rituximab-containing primary chemotherapy for CD20-positive B-cell lymphoma. The use of intensive primary chemotherapy regimen was a risk factor for LON. It was generally self-limited and not associated with severe infections. We should, however, be aware of it especially when applying intensive chemotherapy regimens along with rituximab.

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STEM CELLS
TISSUE-SPECIFIC STEM CELLS

Concise Review: Notch Signaling in Stem Cell Systems

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Key Words. Stem cell • Notch • Embryogenesis • Homeostasis

ABSTRACT

The Notch signaling pathway is among the most commonly used communication channels in animal cells. Recent studies have demonstrated that this pathway is indispensable for cells in various stages of maturation, including terminal differentiation. One main focus in mammalian studies is the role of Notch in embryonic and postembryonic stem cell systems. In this review, the roles of Notch signaling in various mammalian stem and early progenitor cells are summarized. STEM CELLS *2006;24: 2437–2447*

INTRODUCTION

In mammals, a wide variety of cells use the Notch signaling system for embryonic development and, in adults, maintenance of homeostasis. A number of review articles have focused on the developmental biology [1], cell biology [2–7], and molecular biology [8–10] of the Notch signaling cascade in individual cellular systems. The Notch signaling pathway has also been discussed in review papers summarizing the molecular mechanisms that regulate stem cell self-renewal, together with other signaling pathways, such as Wnt and hedgehog [11]. The present paper reviews the current knowledge of the roles of Notch signals in various stem and early progenitor cell systems in both the developmental and adult phases.

HISTORICAL BACKGROUND OF NOTCH

The *Notch* gene was named for the phenotype of a mutant *Drosophila* with an indentation in the wings [12]. In the 1930s, it was suggested that the genetic locus responsible for this phenotype has an important role in the cell fate decision during *Drosophila* embryogenesis and that the homozygous mutation of this locus results in excessive differentiation to neuronal tissue (thus, the term "neurogenic" began to be used) [13]. Molecular cloning studies in the 1980s revealed that the *Notch* gene encodes a single-pass transmembrane protein [14] that functions as a receptor for the ligand present on the cell surfaces of neighboring cells [15]. It was subsequently demonstrated that this ligand-receptor interaction redirects the fate of signal-receiving cells to non-neuronal cells by inhibiting neuronal differentiation [16] and that this process governs the "lateral specification" that is essential for normal embryonic development [17, 18].

Although such a concept was established in lower animals such as *Drosophila* and *Caenorhabditis elegans*, homologs in vertebrates were first found in *Xenopus* [19] and then in humans [20]. In humans, the gene located at the break point on chromosome 9 in the t(7;9)(q34;q34) translocation that is found in a subset of acute T lymphoblastic leukemias was identified as a *Notch* homolog and named translocation-associated *Notch* homolog 1 (*TAN-1*) [20]. This gene is now called *Notch1*, and its discovery revealed that the *Notch* genes are very well conserved from nematode to humans. Leukemia cells harboring the t(7;9) translocation express a Notch1 protein with a large part of the extracellular domain truncated. The TAN-1 protein is localized intracellularly and is constitutively activated. Because disproportionately enhanced Notch signals, such as the one transduced by TAN1, were considered tumorigenic, the mechanisms of signaling through the Notch receptors further attracted the attention of researchers [18, 21, 22]. It is now known that the Notch signaling pathway also influences cell fate decisions in mammals, such as cell differentiation, survival/apoptosis, and cell cycle in both physiologic and pathologic contexts, particularly in conjunction with stem cell behavior.

NOTCH SIGNALING PATHWAY AND ITS COMPONENTS

In mammals, four Notch receptors (Notch1–Notch4) and five structurally similar Notch ligands (Delta-like1 [also called Delta1], Delta-like3, Delta-like4, Jagged1, and Jagged2) have been identified, yet there is very little evidence that Delta-like3 physically binds to the Notch receptors or that it truly functions as a Notch ligand [23]. Notch ligands are also single-pass transmembrane proteins. Notch receptors undergo intramolecular cleavage of the precursor protein (S1 cleavage) to form heterodimers, composed of an extracellular subunit and a transmembrane subunit, on the plasma membrane [24–26] (Fig. 1).

Under physiologic conditions, the ligand expressed on one cell binds to a Notch receptor expressed on neighboring cells

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