original article

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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

E. Nitta[1†], K. Izutsu[1†], T. Sato[1], Y. Ota[3], K. Takeuchi[4], A. Kamijo[2], K. Takahashi[2], K. Oshima[1], Y. Kanda[1], S. Chiba[1], T. Motokura[1] & M. Kurokawa[1*]

[1]*Department of Hematology and Oncology,* [2]*Department of Transfusion Medicine and Immunohematology, Graduate School of Medicine, University of Tokyo;* [3]*Department of Pathology, Toranomon Hospital;* [4]*Department of Pathology, Cancer Institute of Japanese Foundation for Cancer Research, Tokyo, Japan*

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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

original article

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

Correspondence to: Prof M. Kurokawa, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Tel: +81-3-5800-6527; Fax: +81-3-3815-8350; E-mail: kurokawa-tky@umin.ac.jp

†These authors contributed equally to this work.

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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

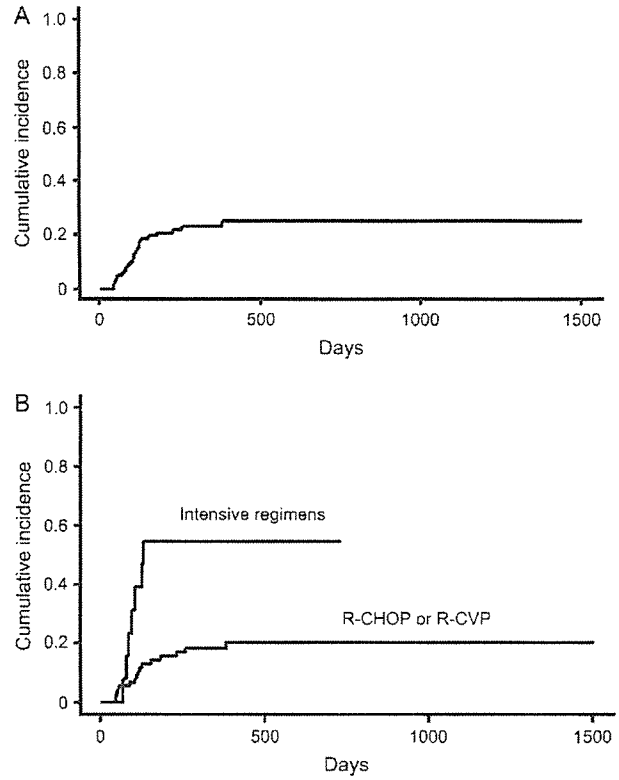

Figure 1. (A) The cumulative incidence of late-onset neutropenia (LON) after the last chemotherapy cycle. (B) The cumulative incidence of LON according to chemotherapy regimens [rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone (R-CHOP) or rituximab, cyclophosphamide, vincristine, prednisolone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

SHIGERU CHIBA

Department of Cell Therapy and Transplantation Medicine, University of Tokyo Hospital, Tokyo, Japan

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

Correspondence: Shigeru Chiba, M.D., Ph.D., Department of Cell Therapy and Transplantation Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Telephone: 81-3-5800-6421; Fax: 81-3-5689-7286; e-mail: schiba-tky@umin.ac.jp Received December 30, 2005; accepted for publication July 11, 2006; first published online in STEM CELLS *EXPRESS* August 3, 2006; available online without subscription through the open access option. ©AlphaMed Press 1066-5099/2006/$20.00/0 doi: 10.1634/stemcells.2005-0661

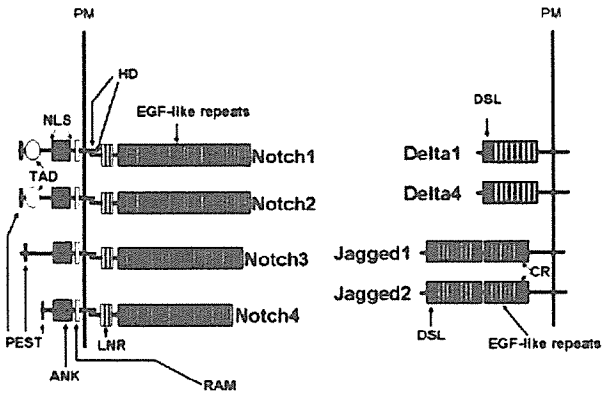

Figure 1. Protein structure of Notch receptors and their ligands. There are 36 EGF-like repeats in Notch1 and Notch2, 34 repeats in Notch3, and 29 repeats in Notch4. Some of the EGF-like repeats serve as a ligand-binding site. LNR has inhibitory function against the cleavage. HDs in the extracellular subunit and transmembrane subunit consist of 103 and 65 amino acids, respectively. RAM associates with CSL protein. ANK associates with proteins to form a complex. PEST negatively regulates the half-life of Notch proteins. DSL is a binding site for Notch. Abbreviations: ANK, ankyrin repeat; CR, cysteine-rich repeat; DSL, Delta-Serrate-Lag2 domain; EGF, epidermal growth factor; HD, heterodimerization domain; LNR, Lin-Notch repeat; NLS, nuclear localization signal; PEST, PEST domain; PM, plasma membrane; RAM, ram domain; TAD, transactivation domain.

that are in direct contact. Binding triggers the cleavage of the extracellular region of the Notch transmembrane subunit (S2 cleavage) [26]. This cleavage facilitates the next cleavage, which occurs within the transmembrane domain (S3 cleavage) [26, 27]. In a simplified scheme, the consequence of S3 cleavage is nuclear translocation of the cleaved intracellular domain of Notch (NICD) and its association with the constitutive DNA-binding protein CSL (after CBF1, Suppressor of hairless, Lag-1; CBF1 is also called RBP-Jκ). This turns the CSL complex from a transcriptional repressor to a transcriptional activator [28], for which the mastermind adaptor protein is an essential component [29, 30]. The most well-defined targets of the NICD-CSL complex are the HES family [31] and their homologs, the Hey (also called HERP) family [9] of basic helix-loop-helix transcription factors. The regulated intramembrane proteolysis (RIP) of transmembrane proteins was first recognized to have biologic significance in the amyloid precursor protein (APP). In APP, RIP occurs at more than one site, which is believed to be linked to the pathogenesis of Alzheimer disease. Interestingly, a second RIP (designated S4 cleavage) has also been detected in Notch1 [32, 33]; this RIP might be related to the Notch signaling pathway. A number of Notch signal-modifying proteins have been identified, and the presence of noncanonical, CSL-independent pathways has been advocated, which are not included in this review.

Notch Signaling in Stem Cells During Embryonic Development

During embryogenesis, it is not always easy to distinguish between primordial cells and tissue-specific stem cells and between stem cells and progenitors. Therefore, stem cells are most broadly considered in this section. Studies using gene-modified animals, which have demonstrated roles of Notch signaling in stem and early progenitor cells, are summarized in Table 1.

Phenotypes of Mice with Mutated *Notch* and Ligand Genes and Their Roles in Embryogenesis

Mutations have been introduced in mice for each of the four *Notch* genes (*Notch1–Notch4*) and four Notch ligand genes (*Delta-like1*, *Delta-like4*, *Jagged1*, and *Jagged2*). Mice, homozygously disrupted with either *Notch1* [34, 35] or *Notch2* [36] are fatal at approximately embryonic day (E) 11. *Notch3*-null [37] and *Notch4*-null mice [38] survive without any apparent phenotypic abnormalities, but the contribution of *Notch3* and *Notch4* to embryonic vascular development has been clarified [38, 39]. Homozygous inactivation of *Delta-like1*, *Delta-like4*, or *Jagged1* causes embryonic lethality during E9.5–E12.5 [40–43], and *Jagged2*-null mice die perinatally [44]. These findings indicate that most of the individual *Notch* genes and ligand genes have nonredundant roles in mouse embryogenesis. Somitogenesis, abnormal vasculature formation, increased cellular apoptosis, excessive neuronal differentiation, etc., are observed in these mutant mice. There are, however, both similar and dissimilar phenotypes in these mice, and the causes of fatality in early to midgestation stages are not likely to be uniform in the knockout mice for each gene.

On the other hand, successful progression to midgestation stages implies that Notch signaling is unnecessary for the very early stage of embryogenesis, including the fertilized egg stage [45]. This would be consistent with the fact that activation of Notch signaling in ESCs, which are derived from the inner cell mass, does not block differentiation of ESCs [46].

Roles in the Central Nervous System During Embryogenesis

Among undifferentiated neuroectodermal cells with the same potential during *Drosophila* embryogenesis, some cells will eventually express Delta at higher levels, which sends a signal to surrounding cells that uniformly express the Notch receptor. Cells receiving the signal are blocked from differentiating to neuroglioblasts (NGBs) and eventually assume another differentiation fate. On the other hand, cells that express Delta differentiate to NGBs and subsequently to neurons and glial cells [47]. Accordingly, insufficient levels of Notch signals result in the "neurogenic phenotype," in which all cells with neuronal potential differentiate into neurons.

Precocious neuronal differentiation observed in Notch pathway-deficient neurocompetent cells is also detected in mammals, such as in mice with inactivated *Notch1* [48]. A conditional *Notch1* knockout study [49] provides further support that Notch signaling inhibits the premature onset of neurogenesis. These studies, as well as those of *rbpsuh* (gene for CSL) knockout mice [50], suggest that, in addition to the differentiation blockade, this pathway is required for the maintenance and expansion of the neural stem/progenitor cell pool. Inactivation of *HES1* [51]; *HES1* and *HES5* [52]; and *HES1*, *HES5*, and *HES3* [53] in mice variably induces precocious neuronal differentiation accompanied by a decrease in neural progenitors. Together, these studies and HES protein overexpression studies [54] indicate that *HES* genes are the conserved targets of Notch-CSL signaling for regulating the expansion and differentiation of neural progenitors [6, 31, 54].

Table 1. Gene-modified animal studies demonstrating roles of Notch signaling in stem and early progenitor cells

Stem/early progenitor cell system	Observations	Genes and modifications	Specific references	Review article
Embryogenesis				
CNS development	Precocious neuronal differentiation	Notch1-null, Notch1-cond null, rbpsuh-null, Hes1-null	48, 49, 50, 51	6, 31, 47
	Decrease in neural stem/progenitor cell pool	Hes1-null/Hes5-null, Hes1-null/Hes5-null/Hes3-null	52, 53	6, 31, 47
	Expansion of neural stem cells	Hes1:Tg, Hes5:Tg	54	6, 31, 47
HSC generation	Deficient HSC generation at P-Sp	Notch1-null, rbpsuh-null	70, 72	5
	Failure in contribution to hematopoiesis	Notch1-null ES cell chimera	71	5
Melanocyte generation	Severe coat color dilution in the initial hair	rbpsuh-null	73	
Vasculature system development	Abnormalities in blood vessel formation and remodeling	Notch1-null, Notch1-null/Notch4-null, rbpsuh-null	38, 74	2
		Jagged1-null, Hey1-null/Hey2-null	43, 76	2
	Failure in arterial patterning of endothelial cells	Dll4-hetero, rbpsuh-null	41, 42, 74	2
	Failure in arterial specification of smooth muscle cells	Notch3-null	39	2
Organogenesis	Hypoplastic gromeruli and heart	Notch2-ΔEGFrep	82	
	Eye dysmorphology	Jagged1-null	43	
	Abnormalities of bile duct, heart, eyes, and vertebrae[a]	Jagged1-hetero (Aligille syn)[a]	83, 84	
	Excess endocrine cells in pancreas	Dll1-null, rbpsuh-null, HES1-null	86, 87	
	Insulin-deficient diabetes with both endocrine and exocrine pancreatic hypoplasia	rbpsuh-cond null	88	
	Gall bladder agenesis, hypoplastic bile duct	HES1-null	89	
	Differentiation to pancreatic cells from bile duct epithelium	HES1-null		
	Excess differentiation to secretary cells in small intestine	HES1-null	87	
	Amplification of intestinal progenitor pool			
	Inhibition of intestinal cell differentiation	aNotch1-cond Tg	90	
Stem cell systems in adults				
Skin and hair systems	Hair loss; epidermal hyperkeratinization and epidermal cyst formation	Notch1-cond null, Notch1-cond null/Notch2-cond null	92, 93, 95	
		Notch1-cond null/Notch2-cond null/Notch3-null	92, 93, 95	
		PS1-cond null/PS2-null, rubsuh-cond null; Notch1-Tg	92, 93, 95	
	Generation of squamous carcinoma of skin	Notch1-cond null	97	
Hematopoietic and immune systems				
HSC maintenance in bone marrow	Increase in bone marrow HSCs	CaPTHER-Tg (Jagged 1 overexpression in trabecular bone)	100	
Early T cell development	Impaired thymocyte development, increased B lineage cell development in the thymus	Notch1-cond null, 1-Fringe-Tag	123, 125, 126	
Intestinal mucosal system	Conversion of proliferative crypt cells to postmitotic Goblet cells	rbpsuh-null	146	
Skeletal muscle system	Restoration of regenerative potential in old mouse skeletal muscle by Notch activation using Notch-stimulatory antibody[b]		140, 149	
	Impairment of regenerative potential in young mouse skeletal muscle by Notch inhibition using Jagged1-Fc			

[a] Alagille syndrome (human disease).

[b] Gene-modified animals are not used.

Abbreviations: aNotch1, constitutive active form of Notch1; caPTH, constitutively active form of parathyroid hormone receptor; CNS, central nervous system; cond, conditional; ΔEGFrep, deletion of EGF-like repeat(s); hetero, heterozygous null mutation; HSC, hematopoietic stem cell; l-Fringe, lunatic Fringe; null, homozygous null mutation; P-Sp, paraaorta-splanchnopleura; Tg, transgenic.

Importantly, Notch signaling either promotes or, by default, facilitates glial cell fate, perhaps as a consequence of inhibiting neuronal cell fate [55–59]. It further induces astrocyte differentiation [60, 61]. Therefore, Notch signaling might act on neural stem cells in two steps: (a) initially inhibiting neuronal fate while allowing for glial cell fate; and (b) then promoting differentiation to astrocytes while inhibiting differentiation to both neurons and oligodendrocytes [61]. These concepts fit the conventional view of the fundamental function of Notch signaling to prevent cells from taking the first pathway while guiding them into a secondary pathway.

To add to the complexity, neural stem cells have been identified in glial cell populations, particularly in astrocytes [47]. It remains to be clarified how Notch signaling is involved specifically in the maintenance of stem cell characteristics and the promotion of glial differentiation.

Notch signaling is also involved in the regulation of apoptosis in mammals. It remains controversial, however, whether it is pro- or antiapoptotic in developing neural stem/progenitor cells. Conditional deletion of *Notch1* [49] and both *Notch1* and *Notch3* [62] genes causes apoptosis and massive loss of developing neural progenitors as well as newly differentiating neurons. These studies suggest an antiapoptotic activity of Notch signaling. The results of other studies that analyzed conditional transgenic and knockout mice, however, led to the opposite conclusion. Conditional expression of a constitutively active form of Notch1 leads to p53-dependent apoptosis of early neural progenitors. Conversely, disruption of *presenilin-1*, the gene for a component of γ-secretase that is essential for Notch signaling, and conditional deletion of *Notch1* result in reduced apoptosis of early neural progenitor cells [63]. Proapoptotic activity of Notch signaling occurs in keratinocytic [3, 64] and endothelial [65, 66] cells, partly through inactivation of the phosphatidylinositol 3-kinase (PI3K) pathway, whereas activation of the PI3K pathway and antiapoptotic effects have also been demonstrated in other cells, including tumor cells [67, 68]. Thus, the regulation of apoptosis and survival by Notch signaling must be highly context-dependent, and reciprocal directions should be considered on this axis for understanding the development of neural stem/progenitor cells.

Roles in Generating Hematopoietic Stem Cells

In mice, phenotypically defined endothelial cells that possess hematopoietic potential, called "hematogenic endothelial cells," are generated by E9.5 at the paraaorta-splanchnopleura (P-Sp) region. On E10.5, when the P-Sp develops to become the aorta-gonad-mesonephros (AGM), hematogenic endothelial cells develop into hematopoietic stem cells that have the potential to engraft in adult mouse bone marrow [69]. In the *Notch1*-null mutant mouse embryo, apparent hematogenic endothelial cells are recognizable, but they do not develop into hematopoietic stem cells [70]. Using different approaches, either *Notch1*-null ESC-chimeric mice [71] or *rbpsuh*-null mutant mice [72] produced similar results, indicating that Notch1 has an indispensable role at the point right before hematopoietic stem cells are generated.

On the other hand, Notch1 is dispensable for primitive hematopoiesis and secondary hematopoiesis that is derived from progenitors, not from HSCs, in the yolk sac. Thus the requirement of Notch signaling during hematogenesis is most prominent in adult-type HSC generation [70, 71].

Role in Melanoblast Survival

In embryonic skin, melanoblasts (Mbs) develop in the suprabasal and basal layers of the epidermis. The cleaved form of Notch1 is detected and the HES1 promoter is activated in the embryonic Mbs. A γ-secretase inhibitor induces apoptosis of embryonic Mbs, and *tyrosinase* promoter-dependent deletion of the *rbpsuh* gene results in severe coat color dilution in the initial hair [73]. These results indicate that the embryonic Mbs use the Notch1-CSL-Hes1 pathway to protect themselves from apoptosis.

Roles in Vasculature Formation

Mice with disrupted *Notch*-related molecules display various abnormalities in blood vessel formation, such as proliferation and migration of endothelial cells, smooth muscle differentiation, vascular remodeling processes [2], and arterial-venous identification [41, 42, 74, 75]. These studies describe the involvement of Notch1 [38], Notch3 [39], and Notch4 [38] receptors and Delta-like4 [41, 42, 74] and Jagged1 [43] ligands in vasculature formation.

In the vascular system, Hey1, Hey2, and HeyL are considered candidate Notch-CSL targets [2]. In fact, mice lacking both *Hey1* and *Hey2* are embryonic lethal due to abnormalities in the heart and vasculature [76]. The phenotypes of these mice, however, do not precisely recapitulate those of *Notch1/Notch4* double mutant mice or *Delta-like4* heterozygous mice. Thus, further studies are required before definitive conclusions can be drawn as to whether the Hey family alone comprises the transcription factors functioning downstream of the Notch signals in vasculature development. Endothelial cells of the artery and vein have distinct gene expression patterns at the early developmental stage. Many Notch-related molecules are preferentially expressed in the arterial endothelial cells [77, 78]. Strikingly, signals from Delta-like4 to the Notch receptors confers the identity of the arterial endothelium to the precursor cells, which is implicated by the analysis of *Delta-like4* heterozygous mice [41, 42, 74]. *rbpsuh*-null mice have a similar arterio-venous misidentification [74]. In addition to the endothelium, Notch signaling is also involved in the differentiation and maturation of vascular smooth muscle cells, particularly their specification to arterial smooth muscle cells [39].

It has been proposed that the default pathway of the endothelial cells is "venous fate" and that Notch signaling instructively modulates endothelial cell fate to the "arterial fate" [79, 80]. It was recently reported, however, that the venous fate of endothelial cells is induced by a transcription factor, COUP-TFII, that inhibits Notch signaling [75]. In any case, it is clear that the Notch pathway is a critical determinant for arterial-venous endothelial cell fate.

Gain-of-function and loss-of-function experiments in vascular development sometimes yield similar results [81]. These findings often appear to contradict those observed in neural progenitors in which gain- and loss-of-function studies exhibit mirroring phenomena, that is, progenitor expansion with blocked differentiation by the gain-of-function studies and precocious differentiation with decreased progenitor pool by the loss-of-function studies. This might indicate that appropriate

Notch signaling levels are most important for survival of the endothelial cells, given that Notch signals often affect endothelial cells in a proapoptotic manner [65, 66].

Roles in Organogenesis

Analyses of *Notch* gene knockout mice and identification of *Jagged1* as a responsible gene for a hereditary disease that accompanies defects in organ morphogenesis provide evidence that Notch signaling has important roles in the development of kidney, liver, pancreas, heart, etc.

Notch2-null mouse embryos die before mesonephros generation [36] (described above). *Notch2*-hypomorphic mice, in which Notch2 lacks one or two epidermal growth factor-like domains in the extracellular region, survive until birth. In these mice, the glomeruli are severely hypoplastic, and the heart is also variably hypoplastic [82].

Alagille syndrome is an autosomal-dominant human disorder characterized by intrahepatic cholestasis; abnormalities of heart, eyes, and vertebrae; and a peculiar facial appearance. Haploinsufficiency of *Jagged1* is responsible for this disorder [83, 84], suggesting that reduced Jagged1-Notch signaling results in organogenesis failure. Both hepatocytes and biliary epithelial cells are derived from common liver progenitor cells or liver stem cells [85]. Whether adequate Jagged1 function is required for the biliary epithelial cell induction from liver stem cells is not known. The phenotypes of *Jagged1*-disrupted mice [43] and *Notch2*-hypomorphic mice [82] are similar to that of Alagille syndrome.

In the pancreas of *Delta-like1* or *rbpsuh* gene knockout mice, there is an excess of endocrine cells [86], suggesting that Notch signaling inhibits endocrine-lineage differentiation from the common exocrine and endocrine progenitors during pancreatic development. Analysis of *HES1*-null mice leads to a similar conclusion about the role of Notch signaling in pancreatic development [87]. Recently, somewhat different results were obtained from conditional deletion of the *rbpsuh* gene; the mice exhibited insulin-deficient diabetes with both endocrine and exocrine pancreatic hypoplasia [88]. *HES1*-null mice show agenesis of the gall bladder and hypoplastic bile ducts. Furthermore, bile epithelial cells differentiate to pancreatic cells in these mice [89], suggesting that Notch signaling physiologically inhibits pancreatic differentiation from bile epithelial cells, which have the potential to take the pancreatic cell fate in the absence of Notch signaling.

The same mice exhibit excess differentiation to secretory cells, such as Goblet cells, in the intestinal mucosa at the expense of enterocytes [87]. A mirroring phenotype has been reported in *Villin* promoter-controlled Cre and Rosa-Notch transgenic mice. These mice, in which constitutively active Notch1 is expressed in the intestinal epithelium, survive until birth at Mendelian ratios but die of malnutrition within 3 days after birth. The architecture of the intestinal epithelium in neonatal mice is markedly altered by amplification of the intestinal progenitor pool and inhibition of cell differentiation [90]. These findings together suggest a role of Notch signaling in the developing intestinal epithelium to regulate specification of the intestinal progenitor cells.

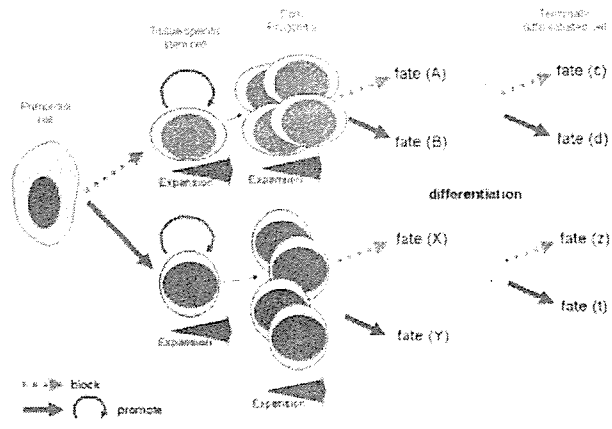

Figure 2. Influence of Notch signaling on the fate of stem and progenitor cells. The biologic effects of Notch signaling can be generalized as depicted in this figure. Notch signaling guides cells to differentiate or not and to differentiate to A instead of B or to B instead of A. Thus, Notch signaling has a role in increasing the number of stem or early progenitor cells.

Cell Fate Determination by Notch Signaling During Embryogenesis

The above observations indicate that from the viewpoint of cell differentiation, Notch signaling has three major roles during embryonic development. First, it affects differentiation from primordial cells to tissue-specific stem cells in the early- to midstage embryo. In the traditional view of *Drosophila* nervous system development, differentiation from undifferentiated neuroectodermal cells to NGBs is inhibited. In mammals, however, induction rather than inhibition is postulated for HSC generation from upstream progenitors. The effect of Notch signaling on differentiation fate from primordial cells to tissue-specific stem cells might be context-dependent. Second, it inhibits tissue- or organ-specific stem cells or immature progenitors from further differentiation and presumably helps them expand while maintaining the immature state. Third, it blocks the default pathway and promotes the alternative pathway, which is typically observed during mid- to late-stage embryo development, such as during organ formation (Fig. 2).

Roles of Notch Signaling in Stem Cells Postdevelopment

Adult stem cells are considered to maintain homeostasis of cells and tissues throughout life. The adult stem cells maintain the number of stem cells, as well as terminally differentiated cells, during normal turnover and repair damage after injury. Involvement of Notch signaling occurs during both normal status and injury in various stem cell systems. Studies along this line using gene-modified animals are summarized in Table 1.

Roles in the Skin

Involvement of Notch signaling in postdevelopmental stem cell systems is best understood in the skin, particularly in the hair follicles. Notch1, Notch2, and Notch3 are expressed and differentially localized to various layers of the hair follicle [91, 92]. Notch signals do not affect the pattern of skin formation during embryogenesis. Thin, short, and wavy hair grows when *Notch1*

is inactivated specifically in the hair follicles during embryogenesis, where Notch1 activity in the cortical progenitors affects the neighboring inner root sheath and medulla cells from their progenitors in a nonautonomous manner [92].

After birth, the first hair cycle of *Notch1*-null mice shows a shortened anagen phase and premature entry into the catagen phase, and inactivation of *Notch1* in adult mice results in almost complete hair loss followed by cyst formation. These observations indicate that Notch1 is essential for postnatal hair follicle development and homeostasis [92, 93]. Compared with the phenotypes of *Notch1*-null mice, *Notch1* and *Notch2* double-null mice display stronger hair follicle phenotypes. The phenotypes of triple-null (i.e., *Notch1*-, *Notch2*-, and *Notch3*-null) mice appear to be even stronger than those of double-null mice. Presenilins are necessary for hair follicle maintenance in mice, and the hair and skin phenotypes of the *Notch1*-, *Notch2*-, and *Notch3*-null mice are very similar to those of *presenilin-1* and *presenilin-2* double-null mice [92].

Notch overactivity in hair follicle cells also leads to abnormal hair formation [94, 95]. Notch1 overactivity in the cortex affects neighboring cells, indicating a nonautonomous role for Notch-regulated transcripts [94]. Transgenic mice expressing constitutively active Notch1 in the suprabasal epidermal keratinocytes and inner root sheath of the hair follicle have epidermal hyperplasia and delayed inner root sheath differentiation, which leads to hair shaft abnormalities and alopecia associated with defects in the anagen phase of the hair cycle [95]. Thus, it is noteworthy that both loss and gain of Notch signaling similarly result in hyperkeratosis and hair loss due to hair cycle disturbances.

Analysis of mice with a specific deletion of *rbpsuh* in the skin using a *nestin* promoter-controlled Cre-lox system reveals that Notch signaling inhibits stem cells in the bulge, the stem cell niche in the hair follicle, from differentiating into epidermal cells and promotes hair formation [96]. This conclusion is consistent with observations that defects in Notch signaling result in markedly impaired hair formation and excess differentiation to the epidermal cells during the catagen phase, which eventually causes epidermal hyperplasia and epidermal cyst formation [92, 93]. Moreover, a lack of *Notch1* caused by a tamoxifen-inducible *K5* promoter-controlled Cre-lox system leads to skin tumor generation [97], indicating that Notch signaling is sometimes tumor-suppressive [98].

In summary, Notch signals are likely to promote the selection of hair formation in bulge stem cells. Thus, ex vivo expansion of hair stem cells with the use of an artificial stem cell niche could revolutionize the dermatology/cosmetology field. Notch signaling should be an important component of such an artificial niche for hair stem cells. Moreover, the regulation of Notch signaling might be considered as a means for skin surface management.

Roles in Hematopoietic and Immune Systems

In the hematopoietic and immune systems, Notch1, Notch2, and Notch3 are expressed in immature, as well as mature, blood cells and lymphocytes. Notch ligands are mainly expressed in the stromal cells and antigen-presenting cells, yet subsets of hematolymphopoietic cells also express some Notch ligands [4]. A number of studies demonstrate that Notch signaling inhibits myeloid differentiation from progenitor cells [5]. Whether this signaling pathway is involved in HSC maintenance in the bone marrow niche and whether ex vivo HSC expansion is feasible using Notch signaling activators are issues that remain to be examined.

HSC Maintenance in Adult Bone Marrow. Osteoblasts on the surfaces of trabecular bone have been identified as one of the bone marrow HSC niches [99–101]. Sinusoidal endothelial cells in the bone marrow and spleen also provide a niche for HSCs [102]. Two groups have proposed that Notch signaling is actively involved in HSC maintenance/growth in the osteoblastic niche using different experimental animals. One group, using transgenic mice that express constitutive active parathyroid hormone receptor under the control of the collagen type-IV promoter, reported that the trabecular bone mass is increased and the Notch ligand, Jagged1, is overexpressed in the osteoblasts. The authors of this report argue that the increase in bone marrow HSCs is a direct consequence of the increased osteoblastic niche area and overexpression of Jagged1 in the niche cells [100]. This finding does not necessarily imply that physiologic levels of Notch signaling contribute to HSC maintenance, particularly as the long-term culture-initiating cells decreased only to basal levels, but not below basal levels, by a γ-secretase inhibitor in the coculture with bone marrow stromal cells derived from the transgenic mice. The other group used mice harboring green fluorescence protein as a reporter under the CSL binding sequence and explored Notch activation at the cellular level in situ. In these mice, green fluorescence protein is expressed in the c-Kit-positive cells near the trabecular bone area, indicating Notch activation in the HSCs, which contributes to inhibiting HSC differentiation [103].

Taken together, interactions between osteoblast-expressed Notch ligands such as Jagged1 and signal transmission to the Notch receptor-expressing HSCs might be one of the molecular mechanisms underlying the regulation of HSC in the osteoblastic niche in the bone marrow. The conditional deletion of both *Notch1* and *Jagged1*, however, fails to show a clear-cut role for the Jagged1-Notch1 pathway in HSC maintenance [104]. Therefore, truly convincing evidence must be demonstrated before we can draw conclusions about the physiologic role of Notch signaling for HSC maintenance in the bone marrow niche, including the osteoblastic and sinusoidal endothelial niches.

Ex Vivo Expansion of HSCs Using Notch Signaling. Ex vivo expansion of HSCs maintained in the immature state has long attracted interest because of the potential utilization of expanded HSCs for transplantation, gene therapy, etc. [105]. Forced expression of a constitutively active form of Notch1 [106, 107] or wild-type HES1 [108] inhibits murine HSC differentiation and potentially expands the HSCs. There are no clear-cut data, however, showing ex vivo expansion of transplantable murine HSCs that retain full hematopoietic activity by stimulating with Notch ligands, although a number of studies have demonstrated the influence of Notch signaling on murine hematopoietic progenitor cells (Table 2) [109–121]. In contrast, investigations using human cord blood-derived cells demonstrated ex vivo expansion of severe combine immunodeficiency (SCID) mouse-repopulating cells (SRCs), which are established surrogates for HSCs (Table 2). For the latter, soluble forms of Delta-like1,

Table 2. Effects of Notch ligands on murine and human hematopoietic stem/progenitor cells in culture

Notch ligands[a]	Cytokines	Increased (maintained) progenitor cells and the source of culture	References
Murine hematopoietic cells			
Jagged1 (human) on 3T3 cells; Jagged1 (human) immobilized to beads	SCF, FL, IL6, IL11	Total CFC and CFU-Mix from BM KSL cells	109
Jagged1 (human) on S17 cells	Serum alone	Total CFC from $CD34^+$c-Kit^+ cells in AGM and fetal liver	110
Jagged2 (murine) on Rab2 fibroblasts	Serum alone	CFU-Mix, CFU-GM, CFU-G, and CFU-M from BM Lin^-c-Kit^+ cells	111
GST-Dll1 (human); Dll1 (human) immobilized to beads	SCF, FL, IL6, IL11, IL1, G-CSF, GM-CSF, EPO (no serum)[c]	Total CFC, CFU-Mix, CFU-GM, BFU-E, and CFU-S from BM Lin^- cells	112
Dll1-Fc (human) immobilized to flask[a]	FL, IL6, IL11	Short-term repopulating cell from BM KSL cells	113
Jagged1 (rat) immobilized to beads[a]	WEHI-3B-conditioned medium or SCF, TPO, FL	CFU-Mix, CFU-GM, BFU-E, and cobblestone area-forming cells from BM mononuclear cells and Lin^- cells	114
Human hematopoietic cells			
Jagged2 on 3T3 cells	FL, IL3/GM-CSF fusion protein	Total CFC, CFU-Mix, and CFU-GM from CB $CD3^+$ cells	115
Jagged1-Fc	SCF, FL, IL6, IL3, G-CSF (no serum)	SRC and total CFC from CB $CD34^+CD38^-Lin^-$ cells	116
Dll1-Fc (Dll4-Fc)[b]	SCF, FL, IL6, IL3, G-CSF (no serum)	SRC and total CFC from CB $CD34^+CD38^-Lin^-$ cells	117
Dll1-myc immobilized to flask	SCF, TPO, FL, IL6, IL3 (no serum)	SRC and total CFC from CB $CD34^+CD38^-$ cells	118
Dll4 on S17 cells (Dll4-Fc)	SCF, PEG-MGDF, FL, IL3	LTC-IC from CB $CD34^+CD38^-$ cells	119
Dll4 on S17 cells	SCF, TPO, FL, IL3	BFU-E and LTC-IC from FL $CD34^+CD38^-$ cells	120
Dll1-Fc immobilized to flask	SCF, TPO, FL, IL3, IL6	SRC from CB $CD34^+CD38^-$ cells[d]	121
	SCF, TPO, FL, IL3, FP6	SRC from CB $CD133^+$ cells	122

[a] Immobilization of proteins was required for the effects noted; free proteins were ineffective or antagonistic.
[b] SRC expansion was demonstrated only by Dll1-Fc.
[c] Fetal calf serum or horse serum is used unless indicated.
[d] Effective only in the low dose of Dll1-Fc.
Abbreviations: AGM, aorta-gonad-mesonephros; BFU-E, burst-forming units-erythroid; BM, bone marrow; CAFC, cobblestone area-forming cells; CB, cord blood; CFC, colony-forming cells; CFU-GM, colony-forming units-granulocytes/macrophages; CFU-Mix, colony-forming units-mixed; CFU-S, colony-forming units-spleen; Dll1, Delta-like1; Dll4, Delta-like4 (all the soluble proteins are consisted of extracellular domain of each ligand); EPO, erythropoietin; Fc, Fc portion of human immunoglobulin G; FL, flt-3 ligand; FP6, fusion protein 6; GST, glutathione *S*-transferase; KSL, c-Kit(+) Sca-1(+) Lin(-); Lin, lineage marker; PEG-MGDF, polyethylene glycol-megakaryocyte growth and development factor; SCF, stem cell factor; SRC, SCID-repopulating cells; TPO, thrombopoietin.

such as fusion with the Fc portion of human immunoglobulin G (Dll1-Fc), have been used to show the increase in the potential or the numbers of human SRCs when HSC-enriched cells are cultured in the presence of hematopoietic cytokines, such as stem cell factor and thrombopoietin [116, 118, 121, 122]. These findings raise the possibility of the clinical use of these proteins. To date, however, the degrees of SRC expansion have not been robust enough to establish clinically applicable ex vivo HSC expansion methods, although expansion of progenitors has been demonstrated to be more massive.

A recent work by Delaney et al. indicates that low doses of immobilized Dll1-Fc are required for SRC expansion, whereas higher doses induce apoptosis of the cord blood-derived immature cells [121]. In addition, it appears that there are differences in the biologic effects of diverse Notch ligands if they are used ex vivo. These issues raise another possibility that the bone marrow microenvironment can be better mimicked if we learn more about the biologic outcomes of diverse levels of Notch signaling or Notch signaling by different ligands.

Effects of Notch Signaling on Early Lymphopoietic Cells. Several important conclusions have been drawn from hematolymphopoietic cells differentiated from HSCs, such as that Notch signaling guides further differentiation of HSC-derived hematolymphopoietic cells, for example: (a) T and B lineage determination (in the progenitor stage between the HSCs and the most early thymocytes, Notch signaling blocks B lineage differentiation and promotes T-lineage differentiation) [123–126], (b) further acceleration of thymocyte differentiation and proliferation [127–130], and (c) splenic marginal zone B cell forma-

tion [131–134]. Sambandam et al. identified "early T lineage progenitors" in the thymus, which express Flt3 and preserve B lineage potential, as the cell subset that first receives Notch signals rather than multipotent hematopoietic progenitors circulated in the blood [135]. Notch signals are also necessary for the differentiation, proliferation, and rescue from apoptosis at multiple checkpoints during thymocyte development. Interestingly, the glucose metabolism induced by activation of phosphatidylinositol-3-OH kinase is proposed to be the major contributor to apoptosis prevention by Notch signaling at the β-selection checkpoint [13]. It is now known that either Delta-like1 or Delta-like4 is sufficient to induce T-lineage cells down to the $CD4^+CD8^+$ stage from upstream progenitors in in vitro culture [134–136].

Information regarding the involvement of Notch signaling in the immune system is accumulating rapidly, and a comprehensive discussion of this topic is beyond the scope of this review. A number of review papers summarize the influence of Notch signals on lymphocyte development, particularly on T-cell development in the thymus [4, 7, 137, 138].

Roles in Intestinal Mucosal Cells

Intestinal epithelial stem/progenitor cells are localized in the basal area of the crypts and continuously supply multiple types of mature cells [139, 140]. These cells express Notch receptors and molecules necessary for Notch signaling [141, 142].

The administration of γ-secretase inhibitors induces gross histologic changes in the intestinal epithelial layer of mice, such as an increased number of Goblet cells, endocrine cells, and abnormal crypts [143–146]. Conditional inactivation of *rbpsuh* in the epithelium of the small intestine and colon by an inducible *Cyp1A* promoter-expressed Cre enzyme induces rapid and massive conversion of proliferative crypt cells to postmitotic Goblet cells [146]. These findings are consistent with observations of *HES1*-null mice [87] and *Villin*-Cre *Rosa*-Notch transgenic mice [90] and suggest that Notch signaling in adults functions to maintain intestinal epithelial stem/progenitor cells.

Roles in Skeletal Muscle Regeneration

Satellite cells are stem cells of skeletal muscle fibers [147]. In aged mice, satellite cells have a markedly impaired propensity to proliferate and produce the myoblasts necessary for muscle regeneration. This is attributed to insufficient upregulation of Delta1, in contrast to the injured muscle in young mice in which Delta1 is sufficiently upregulated. Ultimately, Notch signaling is insufficient for the regeneration of injured muscle in aged mice. Notch inhibition impairs regeneration in young mouse muscle, and forced Notch activation restores the regenerative potential to aged mouse muscle [148, 149]. Thus, Notch signaling is a key determinant of the muscle regenerative potential that declines with age.

CONCLUSION

This report reviews the current knowledge of the Notch signaling pathway in various types of stem and early progenitor cells, but not germ cells. Ex vivo stem cell expansion is fundamental to the success of stem cell-based regeneration medicine, and it is likely that Notch signaling has a role in stem cell expansion. The effects of Notch signaling on progenitor cell survival have been demonstrated, and tumorigenic aspects must be considered.

DISCLOSURES

The author indicates no potential conflicts of interest.

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