Expression analysis showed that NFBD1 is regulated at mRNA level in response to ADR. To explore the molecular mechanisms regulating the p53-mediated DNA damage signaling pathways, it is necessary to identify the promoter region as well as the transcription factor(s) responsible for the regulation of NFBD1 upon treatment with ADR. Although the transcriptional regulatory mechanism of NFBD1 has remained elusive, the extensive search of the human genomic sequence information revealed that there exists a putative NF-κB-binding site within the intron 3 of the *NFBD1* gene (data not shown). Accumulating evidence suggests that NF-κB plays an important role in cellular protection against a wide variety of apoptotic stresses, including DNA damage (42–45). For example, camptothecin-mediated activation of NF-κB was transient, and the impaired activation of NF-κB resulted in an enhanced sensitivity to camptothecin (46). Similar results were also obtained in cells exposed to ADR (47). Furthermore, the recent study indicated that ATM is required for NF-κB activation in response to DNA damage (48, 49), and NF-κB activation decreases the stability of p53, which might be due to the up-regulation of MDM2 (50). Collectively, it is possible that the transient activation of NF-κB might maintain and/or induce the expression levels of NFBD1 and MDM2, which is required for cell survival following DNA damage. However, it remains to be clarified whether NF-κB could regulate the expression of NFBD1 in response to DNA damage. Recently, Townsend *et al.* (51) described that *NFBD1* might be a direct transcriptional target of STAT-1. According to their results, the expression levels of NFBD1 were reduced in STAT-1-deficient cells but were restored by the exogenous expression of STAT-1. Under our experimental conditions, however, we could not detect the STAT-1-mediated transcriptional up-regulation of *NFBD1* (data not shown). This discrepancy might be due to the cell type-specific effects. Thus, it is likely that there could exist a separate and distinct transcription factor(s) required for the transcriptional regulation of *NFBD1*. Studies to elucidate the molecular mechanisms of the transcriptional regulation of *NFBD1* in response to DNA damage are underway.

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Implications of *MYCN* amplification in patients with stage 4 neuroblastoma who undergo intensive chemotherapy☆

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Neuroblastoma;
MYCN amplification;
Intensive chemotherapy;
Stem cell transplantation

Abstract

Background/Purpose: This study aims to clarify the implications of *MYCN* amplification in patients with high-risk neuroblastomas treated with 2 different regimens of induction chemotherapy established by the Japan Study Group for Advanced Neuroblastoma.

Methods: Between 1985 and 2003 in Japan, 392 patients with stage 4 neuroblastomas who were older than 12 months were treated with 2 regimens of induction chemotherapy (the combination of cyclophosphamide [CTX], cisplatin [CDDP], pirarubicin, and vincristine or etoposide). Regimen 91A3 or 98A3 (A3) (CTX 2400 mg/m^2, CDDP 125 mg/m^2) was a higher dose combination of CTX and CDDP than regimen 85A1 or 91A1 (A1) (CTX 1200 mg/m^2, CDDP 90 mg/m^2). The 392 cases were classified into 3 groups (A, 1 copy; B, 2-9 copies; C, more than 10 copies) based on the *MYCN* amplification status by a Southern blot analysis.

Results: The 5-year overall survival rate (5-YS) was 41.1% for all 392 cases. Regarding the *MYCN* amplification status, the 5-YS was 46.6% for A group (n = 227), 22.7% for B group (n = 26), and 36.0% for C group (n = 139). A flouresence in situ hybridization analysis showed the presence of the cells with more than 10 copies in cases with 2 to 9 copies based on the Southern blot findings. Of the 227 patients in a group, the 5-YS was 46.7% for the 70 cases treated by A3 and 47.0% for 154 cases treated by A1 (nonsignificant). The 5-YS of the 210 patients with stem cell transplantation (SCT) (51.%) was significantly better than that of the 127 patients without SCT (41.1%) ($P < .05$).

Conclusions: Regarding the *MYCN* amplification status, the tumor aggressiveness might thus be different between 2 and 9 copies and a single copy of *MYCN*. In neuroblastomas with 2 and 9 copies of *MYCN* based on a Southern blot analysis, the *MYCN* amplification status should be analyzed using the flouresence in situ hybridization method. Induction chemotherapy followed by SCT according to the Japan Study Group for Advanced Neuroblastoma protocol improved the outcome of

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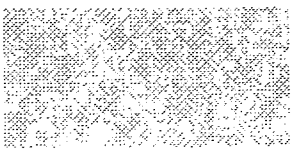

neuroblastomas with *MYCN* amplification; however, obtaining a further improvement in the long-term survival of stage 4 neuroblastomas may therefore require the development of an even more effective treatment modality.

Since 1985, advanced neuroblastomas in Japan have been treated according to the nationwide standard protocol established by the Japan Study Group for Advanced Neuroblastoma (JANB) [1-3]. After 6 courses of induction chemotherapy and a radical operation, the patients were further treated by high-dose chemotherapy with stem cell transplantation (SCT) or by consolidation chemotherapy without SCT. Table 1 shows the JANB induction chemotherapy regimens for stage 4 neuroblastoma patients older than 1 year from 1985 to 2003 [3]. Four different induction regimens were administered. The 91A3 and 98A3 regimens had higher doses of cyclophosphamide and cisplatin (CDDP) than did the 85A1 and 91A1 regimens. In the 91A1 and 91A3 regimens, etoposide was used instead of vincristine. Between 1985 and 1990, the 85A1 regimen was used in all patients. Between 1991 and 1997, the 91A1 regimen was used for patients with less than 10 copies of *MYCN*, whereas the 91A3 regimen was used for those with more than 10 copies of *MYCN*.

We previously reported the following results based on an analysis of the short-term survival curves by JANB [1,2]: (1) a significant improvement in the survival for stage 4 NB with more than 10 copies of *MYCN* using the 91A3 regimen was observed in comparison to the 85A1 regimen; (2) however, no significant difference in the survival between 85A1 and 91A1 for stage 4NB with less than 10 copies of *MYCN* was observed. Based on these results, we decided to administer the 98A3 regimen to all patients who were older than 1 year of age with or without *MYCN* amplification since 1998.

In the present study, we attempted to clarify the implications of *MYCN* amplification in patients with stage 4 neuroblastoma treated by 2 different systemic regimens of induction chemotherapy followed by SCT from the analysis of long-term survival curves in JANB.

1. Materials and methods

A total of 392 neuroblastomas older than 1 year with stage 4 disease were newly treated from 1985 to 2003 according to the JANB protocol [1-4].

Throughout the study, radical surgery was performed after the third but before the sixth cycle of chemotherapy. After completing 6 cycles of induction chemotherapy with these regimens, the patients received either continuation chemotherapy or myeloablative preconditioning regimens and, thereafter, underwent either autologous bone marrow transplantation or peripheral blood cell transplantation (SCT), as previously reported [1]. The preconditioning regimen most frequently used included melphalan 140 mg/m^2 and 90 mg/m^2 on successive days, CDDP 90 mg/m^2, and tetrahydropyranyl adriamycin (pirarubicin) 45 mg/m^2, with or without etoposide 200 mg/m^2 for 4 days. The next most frequently used preconditioning regimen consisted of melphalan 180 mg/m^2 with or without etoposide 200 mg/m^2 for 4 days. Total body irradiation up to a total of 10 Gy was administered at institutional discretion. Researchers were encouraged to perform SCT on patients with *MYCN* amplification when in complete remission.

The status of *MYCN* amplification was analyzed by a Southern blot analysis. The assessment of *MYCN* amplification for several samples using the flouresence in situ hybridization (FISH) method was done as reported previously [5]. The category of the status of *MYCN* amplification was classified into the following 3 groups: A group had 1 copy, B group had 2 to 9 copies, and C group had more than 10 copies. The category of the induction chemotherapy regimen was classified into 2 groups. The A1 system consisted of the 85A1 and 91A1 regimens, whereas the A3 system consisted of the 91A3 and 98A3 regimens. Regarding the categories of SCT, the patients who were

Table 1 JANB induction chemotherapy regimens for stage 4 neuroblastoma (≧1 year old) (mg/m^2)

MYCN status	1985-1990	1991-1997		1998-2003
	All patients	*MYCN*		All patients
		<10 copies	≧10 copies	
Regimen	85A1	91A1	91A3	98A3
Cyclophosphamide	1200 × 1	1200 × 1	1200 × 2	1200 × 2
Vincristine	1.5 × 1			1.5 × 1
Etoposide		100 × 5	100 × 5	
Pirarubicin	40 × 1	40 × 1	40 × 1	40 × 1
Cisplatin	90 × 1	90 × 1	25 × 5	25 × 5

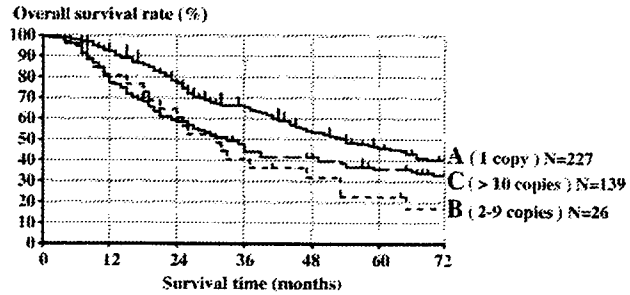

Fig. 1 Association between the *MYCN* amplification status and prognosis. A group vs B group: $P < .01$. A group vs C group: $P < .01$. B group vs C group: NS.

event-free for 9 months from the initial treatment were classified into 2 groups. The patients undergoing SCT(+) received high dose chemotherapy with SCT, whereas the patients with SCT(−) received consolidation chemotherapy without SCT.

The survival curves for each category were constructed using the Kaplan-Meier method and then were statistically evaluated by the log-rank test.

2. Results

2.1. Association between the *MYCN* amplification status and prognosis

The 5-year overall survival rate (5-YS) was 41.1% for all 392 cases. Fig. 1 shows the relationship between the *MYCN* amplification and prognosis. The 5-YS was 46.6% for the single copy *MYCN* group (group A) (n = 227), 22.7% for the *MYCN* group with 2 to 9 copies (group B) (n = 26), and 36.0% for the *MYCN* group with more than 10 copies (group C) (n = 139), respectively. Group A had a significantly better prognosis than did either group B or group C ($P < .01$). No significant difference in the 5-year survival rate between groups B and C was observed.

Table 2 *MYCN* amplification and induction chemotherapy

Status of *MYCN* amplification	5-YS		*P*
	A1 system	A3 system	
All cases	44.5% (n = 210)	39.9% (n = 182)	NS
A group (1 copy) (n = 227)	47.0% (n = 157)	46.7% (n = 70)	NS
B group (2-9 copies) (n = 26)	13.3% (n = 15)	40.9% (n = 11)	NS
C group (>10 copies) (n = 139)	34.3% (n = 38)	36.3% (n = 101)	NS

In order to assess the reason for the high tumor aggressiveness in the *MYCN* group with 2 to 9 copies, we analyzed the status of *MYCN* amplification using FISH for 5 samples in the *MYCN* group with 2 to 9 copies. As a result, all 5 samples by analyzed by FISH showed most nuclei to display more than 10 copies of *MYCN*, as shown in Fig. 2.

Table 2 summarizes the effect of induction chemotherapy based on the status of *MYCN* amplification. Of the 227 patients in A group, the 5-YS was 46.7% for the 70 cases treated by A3 and 47.0% for 154 cases treated by A1 (nonsignificant [NS]). Of the 26 patients in B group, the 5-YS was 40.9% for the 11 cases treated by A3 and 13.3% for the 15 cases treated by A1 (NS). Of the 139 patients in C group, the 5-YS was 36.3% for the 101 cases treated by A3 and 34.7% for the 38 cases treated by A1 (NS).

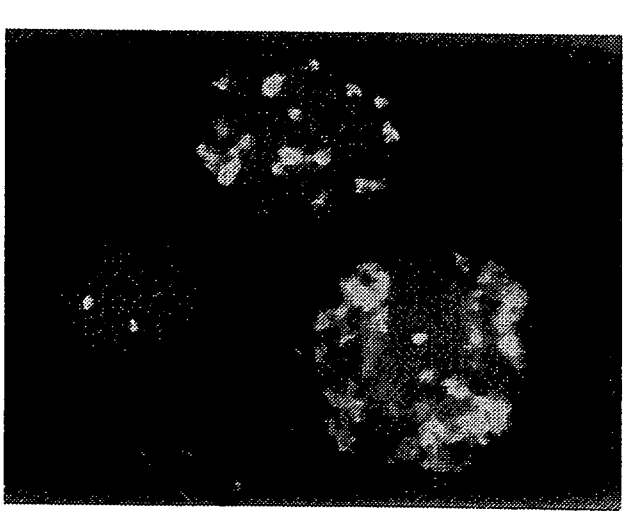

Fig. 2 The status of *MYCN* amplification using FISH for a case with 6 copies of *MYCN* based on the Southern Blotting findings. Cells with more than 10 copies of *MYCN* were found at 65% of the nuclei in the tumor sample.

2.2. The association between the high dose chemotherapy with SCT and the prognosis

Of the 392 patients, 337 were event-free for 9 months after the initial treatment. Among the 337 patients who were event-free for 9 months from initial treatment, 210 received high

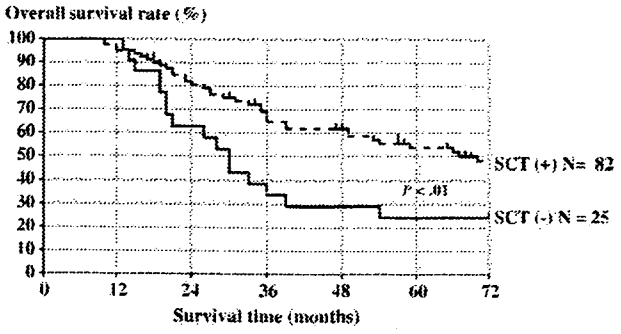

Fig. 3 The association between the SCT and prognosis in the patients with stage 4 neuroblastomas demonstrating more than 10 copies of *MYCN*.

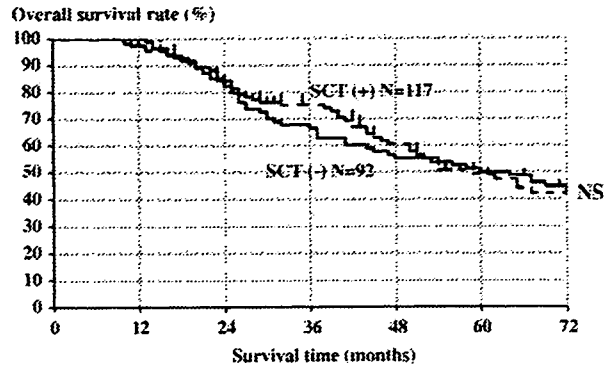

Fig. 4 The association between the SCT and prognosis in the patients with stage 4 neuroblastomas demonstrating a single copy of *MYCN*.

dose chemotherapy with SCT, whereas 127 patients received consolidation chemotherapy without SCT. The 5-YS of the 210 patients with SCT (51.%) was significantly better than that of the 127 patients without SCT (41.1%) ($P < .05$).

Among the 107 patients with more than 10 copies of *MYCN*, the outcome of 85 patients with SCT improved significantly better than in the 25 patients without SCT ($P < .01$) (Fig. 3). In contrast, in the 209 patients with a single copy of *MYCN*, no significant difference in the outcome was observed between the patients with SCT (n = 117) and those without SCT (n = 92) (Fig. 4).

3. Discussion

An amplification of the *MYCN* gene is the strongest unfavorable prognostic factor in neuroblastoma [6-8]. In particular, the outcome of stage 4 neuroblatomas demonstrating *MYCN* with more than 10 copies had been extremely poor. The neuroblastomas from patients in the clinical trials in both the United States and Europe have been routinely assessed for the presence of *MYCN* amplification [9,10]. In Japan, 2 chemotherapeutic regimens for stage 4 neuroblastomas have been designed based on the *MYCN* amplification status (*MYCN* with either more than 10 copies or less than 10 copies) from 1991 to 1997 [1]. The clinical significance *MYCN* with from 2 to 9 copies in stage 4 neuroblastomas, however, remains unclear [11,12]. In the present study, regarding the association between the *MYCN* amplification status and prognosis, the *MYCN* group with 2 to 9 copies had a significantly worse prognosis than did the *MYCN* group with a single copy. Furthermore, no significant difference in the outcome between the *MYCN* group with 2 to 9 copies and the *MYCN* group with more than 10 copies was observed. Although not all of the neuroblastomas demonstrating *MYCN* with 2 to 9 copies were analyzed using FISH, all 5 samples analyzed by FISH showed most of the nuclei to display more than 10 copies of *MYCN*. These findings might be associated with the contents of the tumor cells in the samples analyzed [5]. The confirmation of tumor cells in the sample using a microdissection system may therefore be necessary to precisely assess the status of *MYCN* amplification as analyzed by a Southern blot analysis [13,14]. We therefore recommend that *MYCN* amplification status be analyzed using the FISH method for neuroblastomas demonstrating *MYCN* with 2 to 9 copies by a Southern blot analysis. In fact, FISH method is the standard technique for evaluating *MYCN* copy in COG. Furthermore, in international neuroblastoma risk group committee of 2006 advance neuroblastoma research, the standard criteria of *MYCN* amplification is defined as 4-fold over of *MYCN* signal using 2-color FISH method.

Since 1998, we have administered the A3 system to all patients who were older than 1 year either with or without *MYCN* amplification. In the present study, the A3 system established by JANB was as effective as the A1 system for stage 4 neuroblastomas without *MYCN* amplification. These results suggest that some unknown prognostic factors besides the *MYCN* amplification may thus be associated with the tumor aggressiveness for stage 4 neuroblastomas over 1 year of age [15-17].

The benefit of high-dose chemotherapy with SCT for high risk neuroblastoma has been reported by the Children's Cancer Group or other groups since a few years ago [10,18,19]. In this study, the induction chemotherapy followed by high-dose chemotherapy with SCT, as established by JANB, was thus found to improve the outcome of stage 4 neuroblastoma with *MYCN* amplification. However, for stage 4 neuroblastoma without *MYCN* amplification, induction chemotherapy followed by SCT was found to be not superior to induction chemotherapy without SCT. One possible problem with the SCT is that it was not a randomized clinical study because the choice of using high-dose chemotherapy with SCT or consolidation chemotherapy without SCT was an institutional choice.

In conclusion, the tumor aggressiveness in cases demonstrating *MYCN* with 2 to 9 copies based on a Southern blot analysis was as worse as in cases demonstrating *MYCN* with more than 10 copies. It is therefore necessary for neuroblastomas demonstrating *MYCN* with 2 to 9 copies based on a Southern blot to have their *MYCN* amplification status analyzed using the FISH method. The induction chemotherapy followed by SCT based on the JANB protocol improved the outcome of neuroblastoma with *MYCN* amplification. However, to obtain any further improvement in the long-term survival of stage 4 neuroblastoma cases, an even more effective treatment modality will thus have to be developed.

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原　著

Granulocyte colony stimulating factor（G-CSF）動員ヒドロキシエチルでんぷん（HES）非使用採取法による同種顆粒球輸血の安全性と有効性について

The efficacy and safeness of granulocyte colony stimulating factor (G-CSF) mobilized granulocyte transfusion collected without hydroxyethylstarch (HES) in five neutropenic children.

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要　旨

【目的】ヒドロキシエチルでんぷん(HES)非使用採取法によるG-CSF動員顆粒球輸血の安全性と臨床的有効性とを後方視的に確認する．【方法】赤血球輸血可能な血液型の血縁ドナーにG-CSF　75～100 μgを皮下注し，約12時間後にapheresis(HES非使用)法または遠心＋用手分離によってbuffy coatを採取，15Gyの放射線照射後に患者に輸注する手法を用いた．【結果】全例で速やかな解熱が得られた．ドナー，患者いずれにも特記すべき副作用を認めなかった．用手法による顆粒球採取および輸注は簡便かつ安全であり，自己顆粒球造血が回復するまでの間の支持療法として有効である可能性が示唆された．

Key words：**顆粒球輸血，ヒドロキシエチルでんぷん，顆粒球コロニー刺激因子**(G-CSF)
granulocyte transfusion, hydroxyethylstarch(HES), granulocyte colony stimulating factor(G-CSF)

I　はじめに

がん化学療法や造血幹細胞移植において見られる無顆粒球症に合併する重症感染症に対し，従来行われているapheresisによる顆粒球採取法では採取効率や，ドナーにヒドロキシエチルでんぷん(hydroxyethylstarch；HES)やG-CSFを使用することについての安全性が課題とされている．今回我々は，当院で施行したG-CSF動員HES非使用採取法による顆粒球輸血のドナーならびに患者

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に対する安全性と臨床的有効性とについて後方視的に検討した.

II 目　的

G-CSF 動員 HES 非使用採取法による顆粒球輸血のドナーに対する安全性ならびに患者に対する安全性と臨床的有効性について検討する.

III 対　象（表 1）

対象は，当院で化学療法を受けた 1 か月から 16 歳の患児 5 名（男児が 4 名，女児が 1 名）．体表面積 0.18 ～ 1.75 m^2．原疾患は，家族性血球貪食性リンパ組織球症が 2 名，神経芽腫が 2 名，急性リンパ性白血病が 1 名であった．5 例とも化学療法後の顆粒球減少時における，抗生剤治療に不応の発熱に際して顆粒球輸血が行われた．顆粒球輸血の適応については，小池ら，大坂ら，小原の適応基準を参考にした[1)~3)]．5 例のうち 3 例（症例 2：*Psuedomonas aeruginosa*，症例 3：*Staphylococcus aureus*（MSSA），症例 5：*Enterobacter cloacae, Staphylococcus hominis*）が検出されたが，他の 2 例は各種検索から起炎菌は検出されなかった.

IV 方　法（表 2, 3）

顆粒球採取法（表 2）は，まず，インフォームドコンセント[1), 2)]を得た血液型一致血縁ドナーに対し，G-CSF 製剤を 75 ～ 100 μg 皮下注射した．その約 12 時間後に用手法，もしくは血球分離装置を用いて顆粒球を採取した．血球分離装置は Spectra（COBE 社）を使用した．採取した顆粒球に 15Gy の放射線を照射した後，患者に輸注した（表 2）.

用手法について表 3 に示す（根本らの手法[4)]に準拠した）．ドナーより全血 200 ～ 400ml を自己血採血バッグに採血した．採血バッグを 2,000 回転，15 分間，20℃ で遠心分離した．遠心後の採血バッグを分離スタンド（テルモ）にかけて buffy coat を 10 ～ 50ml 分離採取した．Buffy coat を採取した残りの血液はドナーに返血した．以上の過程を 1 サイクルとし，これを 1 ～ 3 サイクル反復して顆粒球を採取した.

顆粒球輸血の有効性は，治療後の解熱，CRP 値の低下，その他臨床症状の改善の有無から評価した．また，患者およびドナーに対する安全性は，過去に報告のあった副反応出現の有無，その他不

表 1　顆粒球輸血の対象症例

症例 No.	年齢，性別	原疾患	診　断	体表面積（m^2）
1.	男児，1 か月	血球貪食症候群	発熱を伴う顆粒球減少	0.18
2.	男児，1 歳	血球貪食症候群	菌血症※	0.4
3.	女児，4 歳	神経芽腫	菌血症※	0.53
4.	男児，5 歳	神経芽腫	発熱を伴う顆粒球減少	0.74
5.	男児，16 歳	急性リンパ性白血病	菌血症※	1.85

※：症例 2 は血液培養から *Pseudomonas aeruginosa*，症例 3 は血液培養から *Staphylococcus aureus*（*MSSA*），症例 5 は血液培養から *Enterobacter cloacae* と *Staphylococcus hominis* が検出された.

表 2　顆粒球採取法・1（輸注法）

1．血縁ドナーに対し，G - CSF を 75～100 μ g 皮下注射した.

2．G - CSF 皮下注射の約 12 時間後に用手法，もしくは血球分離装置（COBE spectra）を用いて顆粒球を採取した.

3．用手法，もしくは血球分離装置を用いて採取した顆粒球に 15Gy の放射線照射後，患者に輸注した.

利益を生ずる臨床症状の出現の有無から評価した[1)~3),5)].

顆粒球採取，および輸注施行後の観察期間は，3～61か月（中央値27か月）であった．

V 結　果（表4）

以上の2通りの方法で顆粒球輸血をのべ12回（用手法10回，apheresis 2回，1人のドナーからの採取回数は，1～4回，中央値1.5回，1症例に対する顆粒球輸注回数は1～6回，中央値1回）施行した．ドナーは血液型一致血縁者6名（患者の父親が5名，母親が1名），採取顆粒球数は処理したドナー全血400mlあたり2.3～18.4×10^9個，輸注顆粒球数は患者体表面積1 m^2あたり0.97～4.4×10^{10}個であった（表4）．症例5は，同一ドナーに対し，用手法と血球分離装置を用いた

表3　顆粒球採取法・2（用手法）

1．血縁ドナーに対し，G-CSFを75～100μg皮下注射した．

2．G-CSF皮下注射の約12時間後にドナーより全血を200mlまたは400mlを採血バッグに採血した．

3．2000rpm，15分，20℃で遠心分離した．

4．遠心後の採血バッグを分離スタンドにかけ，buffy coatを分離採取した．

5．残りの血液をドナーに返血した．

6．以上，1.～5.の過程を1サイクルとして，1～3サイクル反復した．

表4　当院で行われた12回の顆粒球採取・顆粒球輸血のまとめ

症例No.	ドナー（年齢）	採取法	総処理血液量（ml）	輸注量（ml）	採取顆粒球数（×10^9/400ml）	輸注顆粒球数（×10^{10}/患児体表面積m^2）	ドナーの副作用	解熱／CRP陰性化までの期間(日)※
1	父(40)	用手法	400	10	7.95	4.4	なし	5／9
		用手法	200	20	4.4	1.2	なし	2／9
		用手法	400	20	3.78	2.1	なし	1／－*
		用手法	400	20	3.56	1.98	なし	1／－
	母(33)	用手法	200	20	6.6	1.83	なし	1／－
		用手法	200	10	6.3	1.75	なし	2／－
2	父(38)	用手法	800	100	2.3	1.2	なし	2／5
3	父(39)	用手法	1200	165	4.9	2.77	軽度倦怠感	4／27
4	父(39)	用手法	800	45	4.0	1.08	なし	1／24
5	父(39)	用手法	1200	84	18.4	0.99	なし	6／18
		血球分離装置	4500	278	5.3	1.06	なし	1／－
		血球分離装置	4500	190	4.9	0.97	なし	2／30

※：（解熱までの期間（日））／（CRP陰性化までの期間（日））を表す．＊：－は，CRPが陰性化しなかったことを示す．全例で速やかな解熱が見られた．症例1はのべ6回施行した顆粒球輸血のうち4回でCRPの陰性化を認めなかったが，原病の血球貪食性リンパ組織球症によるものと考えられた．

方法の2方法で顆粒球を採取したが，用手法はapheresisの約3分の1の全血処理量で同等の顆粒球数が採取可能であり，3倍の効率で顆粒球を採取できた．

ドナーの副作用は症例3で軽度倦怠感を訴えたのみで，ほかに特記すべき副作用は見られなかった．

のべ12回の顆粒球輸血において，患者には特記すべき副作用を認めなかった．

いずれの顆粒球採取においても，顆粒球輸血に必要な十分量の顆粒球が採取可能であった．また，全例において，顆粒球輸血により解熱や炎症反応の低下を得られた．症例1はのべ6回の顆粒球輸血を施行し，いずれの場合でも速やかな解熱を得た．6回の顆粒球輸血のうち4回でCRPの陰性化を認めなかったが，原病の家族性血球貪食性リンパ組織球症による影響と考えられた．

以上より，造血能の自力回復までの間，感染症の悪化を防止できたと考えられた．

VI 考　察

広域抗生物質や抗真菌剤，G-CSFなどを用いた感染対策が進歩したにもかかわらず，高度の好中球減少症に合併する細菌感染症や真菌感染症は，依然として造血幹細胞移植やがん化学療法などの際には，しばしば患者の生命を脅かし，治療成績向上の妨げとなっている．こうした場合，一部の症例では顆粒球輸血を行い，以前からその有効性が報告されていた[6)~8)]．G-CSFが普及した後，健常人にG-CSFを投与して大量の顆粒球を採取し，難治性感染症を合併した好中球減少症患者に顆粒球輸血を行うことの有効性は，既に複数の報告がある[9)~16)]．

一方で，G-CSF投与量に数十倍の開きがあるにせよ，同種末梢血幹細胞ドナーの2次性発がんやMDSなどが少数ながら報告されており[17)]，健常人に対する使用には安全性の面で検討が必要であろう．また，HESについては，腎尿細管への沈着蓄積があり，同一ドナーへの反復使用はできないとされている[18)]．今回，我々が顆粒球採取を行ったドナーにおいて，副作用は軽度倦怠感を1例に認めたのみで，他に特記すべき副作用は見られなかったが，G-CSFによる2次性発がんなどについては，今後も慎重な長期的追跡が必要である．

G-CSF動員顆粒球輸血を受けた患者の副反応について，Wrightらは，amphotericin-B投与とG-CSF動員顆粒球輸血を受けた57例のうち，12例でARDSを発症したと報告し，G-CSFにより活性化した顆粒球から分泌されたサイトカインが臓器障害を引き起こしたと考えた[19)]．一方で，Adkinsらは，悪寒，発熱，蕁麻疹の他に，呼吸促迫（低酸素症）が副作用として見られたが，重篤な副反応は認めなかったと報告している[16)]．また，Priceらは，悪寒，発熱，発疹，蕁麻疹，掻痒などの副反応を認めたものの，いずれも一過性のもので，のべ165回の顆粒球輸血で重篤な副反応は見られなかったと報告している[15)]．今回我々の経験した顆粒球輸血を受けた患者においては，特記すべき副作用を認めず，また，全例において解熱・炎症反応の低下が見られ，臨床的に有効と考えられた．

国内では医薬品として認可された高分子HESの入手が困難で，臍帯血の赤血球除去用試薬であるHES40（ニプロ社，分子量40万）が販売されているのみである．今回のわれわれの経験からも，HESを使用しない顆粒球採取が有用である可能性が示唆された．

血球分離装置を用いた方法は実施条件が限られるなどの問題点も挙げられる．近年，用手法を用いて採取した顆粒球輸血が有効であったとの報告が見られる[1), 4), 20)]．用手法による顆粒球採取は採取効率もよく簡便であり，機材などによる制約を受けないという利点があり，また，HESを使用せず，同一ドナーからの反復採取も可能であった．今回我々が経験した5例の症例のうち，1例で同一ドナーに対し，用手法と血球分離装置を用いた方法の2方法で顆粒球を採取したが，用手法は装置を用いた方法の約3分の1の全血処理量で同等の顆粒球数が採取可能であり，3倍の効率で顆粒球を採取できたことから，用手法による顆粒球採取は簡便かつ効率の高い採取法である可能性が期待できると考えられた．

VII 結　語

5症例に対してG-CSF動員HES非使用採取法による顆粒球輸血をのべ12回施行した．用手法による顆粒球採取および輸注は簡便かつ安全であり，自己顆粒球造血が回復するまでの間の支持療法として有効である可能性が示唆された．

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ORIGINAL ARTICLE

Quantification of progenitors capable of generating T cells in human cord blood

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Abstract

Objective: For transplantation of cord blood (CB) cells, it is important to select a CB sample that can reconstitute not only myelo-erythropoiesis but also lymphopoiesis in recipients. However, until now the reconstitution ability of CB samples has been assessed by colony forming unit-culture (CFU-C) assay or by simply counting $CD34^+$ cells. The present study aims at establishing a method capable of assessing the potential of T lymphopoieses of CB samples. *Methods:* $CD34^+CD38^-$ cells sorted from CB were cultured on a monolayer of murine stromal cell line TSt-4, transduced with the human Delta-like 1 gene. *Results:* Immature T cells expressing CD5 and/or CD7 were generated in the culture. As these immature T cells can easily be discriminated from mature T cells that are included in the mononuclear cell population (MNCs), we can use the MNCs as starting material for quantification of progenitors capable of generating T cells (TGP). By applying a limiting dilution analysis, we succeeded in determining the frequency of TGP in MNCs. It was found that the ratios for the number of TGP vs. that of CFU-C differ among CB samples maximally by 3.5 times. *Conclusion:* The present assay system provides a novel tool for the evaluation of CB samples, especially for their T-cell-generating potential.

Key words cord blood; human; progenitor; T cell; limiting dilution analysis

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Transplantation of hematopoietic stem/progenitor cells has become a standard treatment for high-risk acute leukemia, chronic myeloid leukemia, severe aplastic anemia and congenital immunodeficiency (1–3). Stem/progenitor cell transplantation is also applied to patients with malignant tumors and sarcomas and those with metabolic diseases such as Hurler syndrome (4–8). Currently, umbilical cord blood (CB) is becoming the major source of stem/progenitor cells even for adult patients, because there is no risk to the donors as seen in the cases of transplantation of bone marrow or granulocyte-colony stimulating factor (G-CSF)-mobilized peripheral blood cells (9). Especially in adults, however, the limited number of hematopoietic stem cells (HSCs) available in most CB samples has been the major obstacle in hematopoietic reconstitution therapy. To insure reconstitution of lympho-hematopoiesis in recipients, it is important to use CB samples that contain sufficient numbers of stem/progenitor cells capable of reconstituting all components of the hematopoietic system including T cells. For selecting such CB samples, however, only the colony forming unit-culture (CFU-C) assay is employed that has the limitation in detecting the potential for just the erythroid and myeloid lineages (10, 11). The lymphoid potential of the samples cannot be determined, because no method is available to quantitatively evaluate the progenitors for the T- or B-cell lineages. The present study aims at establishing a method for quantification of stem/progenitor cells that are able to generate T cells [T-cell-generating progenitors (TGP)], which represent the key players in acquired immunity.

It is possible to detect the human TGP, for example by culturing with a murine fetal thymus, or more effectively by culturing with the fetal thymus of severe combined immunodeficiency mice (12–14). The fetal thymus organ culture, however, is not amenable to use in clinical laboratories. Recently, a simpler method to detect human T-cell progenitors has been reported by La Motte-Mohs *et al.* (15), where it was shown that OP9 stromal cells expressing the human *Delta-like 1* (*DLL1*) gene supported the development of $CD4^+CD8^+$ T cells from $CD34^+CD38^-$ cells of human CB. However, no trial has been made to determine the frequency of TGP using this culture system.

For an assay system to be adapted in clinical laboratories, simplicity is important. It is desirable for the assay to be carried out avoiding isolation of stem/progenitor cells, such as $CD34^+CD38^-$ cells, with a cell sorter. In the present study, we developed a culture system in which stem/progenitor cells from human CB are induced to differentiate into immature T cells. As no mature T cells are generated in this culture, the T lineage cells, namely the immature T cells, can easily be discriminated from mature T cells included in a crude fraction of CB, which survive the culture. Thus, we were able to use a crude fraction, specifically the mononuclear cells (MNCs) in CB as the starting material for the T-cell progenitor assay. By applying a limiting dilution analysis, we succeeded in quantifying TGP in CB. The present assay system will contribute to the evaluation of CB samples for their immune reconstitution potential.

Materials and methods

Preparation of CB cells

Human CB samples were obtained for research from the Tokyo Cord Blood Bank (Tokyo, Japan). Experiments were performed under the institutional guidelines approved by the Institutional Review Board of Nihon University. Within 24 h of collection, MNCs were isolated from CB by density gradient separation at 350 *g* for 30 min, using Lymphoprep (density: 1.077 g/cm^3) (AXIS-SHIELD PoC AS, Oslo, Norway). The separated MNCs were washed twice with phosphate-buffered saline (PBS), and then frozen in Cellbanker (Juji Field Inc., Tokyo, Japan). $CD34^+$ cells were isolated from a portion of MNCs using a CD34 Progenitor Cell Isolation Kit and a MS Column (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. The $CD34^+$ cells were frozen in Cellbanker.

For cultivation, frozen MNCs and $CD34^+$ cells were thawed, passed through 40-μm nylon mesh, washed, resuspended in medium, and counted using an automated hematology analyzer (Sysmex, Kobe, Japan). The proportion of viable cells was determined by fluorescence microscopy after staining with ethidium-acridine orange.

Culture medium

The culture medium was RPMI1640, supplemented with 10% fetal calf serum (Lot.511042; BioSource International, Inc. Camarillo, CA, USA), sodium pyruvate (1 mM), nonessential amino acid solution (0.1 mM), 2-mercaptoethanol (ME) (5×10^{-5} M), streptomycin (100 μg/mL) and penicillin (100 U/mL).

Antibodies

For staining human cells, anti-CD3 (HIT3a), anti-CD4 (RPA-T4), anti-CD5 (UCHT2), anti-CD7 (M-T701), anti-CD8 (HIT8a), anti-CD14 (M5E2), anti-CD19 (HIB19), anti-CD34 (581), anti-CD38 (HIT2), anti-CD56 (B159), anti-glycophorin A [Ga-R2(HIR2)], which were purchased from BD Pharmingen (San Diego, CA, USA), and anti-CD11b (Bear1) and anti-CD45 (J33), which were purchased from IMMUNOTECH (Marseille, France), were used. Anti-CD3, anti-CD4, anti-CD5, anti-CD7, anti-CD8, anti-CD14, anti-CD19, anti-CD56 and anti-glycophorin A were used as lineage markers (Lin). For staining mouse cells, anti-c-kit (2B8), anti-erythroid lineage cells (TER119), anti-Gr-1 (RB6-8C5), anti-B220 (RA3-6B2), anti-Thy1.2 (53-2.1), anti-CD4 (H129.19), anti-CD8 (53-6.7), anti-NK1.1 (PK136) and anti-CD19 (1D3), which were purchased from BD Pharmingen, were used. TER119, anti-Gr-1, anti-B220, anti-CD19, anti-NK1.1 and anti-Thy-1.2 were used as Lin.

Stromal cells

TSt-4 stromal cells (16) were transduced with the MSCV retroviral vector engineered to express the *DLL1* gene and green fluorescent protein (GFP) in the same way as transduction of murine *delta-like 1* gene in TSt-4 (17). The transduced stromal cells were sorted on the basis of GFP expression, and established as TSt-4/hDLL1.

Preparation of murine fetal cells and FT organs

C57BL/6 (B6) mice were purchased from Charles River Japan (Kanagawa, Japan). Fetal thymus (FT) lobes from fetuses of day 15 post-coitum (dpc) were obtained, and the lobes were treated with deoxyguanosine (dGuo) before use in organ culture experiments (18). Fetal liver (FL) cells and fetal blood (FB) cells used as the progenitor source were prepared from fetuses at 12 dpc as described previously (19).

Sorting of hematopoietic stem/progenitors cells and T cells

Basic methods for surface staining of cells, flow cytometric analysis and sorting of stained cells are as previously described (20). Thawed human $CD34^+$ cells were three-color stained with fluorescein isothiocyanate (FITC)-anti-Lin, phycoerythrin (PE)-anti-CD34 and allophycocyanin (APC)-anti-CD38, and thawed MNCs were three-color stained with FITC-anti-CD19, PE-anti-CD3 and APC-anti-CD45. Murine FL and FB cells were two-color stained with anti-Lin and anti-c-kit. Cells were sorted using a FACSVantage (BD-Biosciences, San Jose, CA, USA). Nonviable cells were excluded by forward and side scatter profiles. Sorted cells were reanalyzed to check their purity and were found to be >98% pure.

Coculture of stem/progenitor cells with stromal cells and flow cytometric analysis

TSt-4/hDLL1 stromal cells were used to determine the T-cell potential of human CB progenitors. TSt-4 stromal cells were used as control. $CD34^+CD38^-Lin^-$ cells (50 cells per well) sorted from thawed CB $CD34^+$ cells, $CD3^+$ cells (1000 cells per well) sorted from thawed CB MNCs, and CB MNCs (5000 cells per well) were cultured on monolayers of these stromal cell lines in 48-well plates. Cells grown in these cultures were divided into four groups and stained with various combinations of monoclonal antibodies for three-color fluorescence-activated cell sorter (FACS) analysis. One group was stained with FITC-anti-CD19, PE-anti-CD11b and APC-anti-CD45, the second group was stained with FITC-anti-CD3, PE-anti-CD5 and APC-anti-CD45, the third group was stained with FITC-anti-CD7, PE-anti-CD5 and APC-anti-CD45, and the fourth group was stained with FITC-anti-CD4, PE-anti-CD8 and APC-CD45. Anti-CD45 was used to screen for the presence of human hematopoietic cells. To examine the T-cell development from mouse progenitor cells, $Lin^-c\text{-}kit^+$ cells from mouse FL and FB were cultured (100 cells per well) for 14 d on monolayers of these stromal cell lines in 12-well plates, and the generated cells were examined by FACS for expression of Thy-1.2, CD19, CD4, and CD8.

Limiting dilution analysis of progenitors that have T-cell potential

Graded numbers of MNCs from human CB were cultured on a monolayer of TSt-4/hDLL1 in 48-well plates for 33 d, and determination of positive and negative wells for T-cell generation was made by FACS analysis of the recovered cells after staining with anti-CD3, anti-CD4, anti-CD5, anti-CD7, anti-CD8 and anti-CD45.The frequency of TGP was estimated by using the Poisson distribution formula (21). Thirteen to 19 wells per point were used.

CFU-C assay

Mononuclear cells (2×10^4 cells per dish) of human CB were cultured in MethoCult GF H4434V (Lot. 3H07906; Stem Cell Technologies, Vancouver, Canada). Numbers of colonies were counted on day 14 for burst-forming unit-erythrocyte, CFU-granulocyte/macrophage (CFU-GM), and colony-forming unit-granulocyte/erythroid/macrophage/megakaryocyte. Only CFU-GM-type colonies are mentioned in this paper, because only the number of CFU-GM-type colonies is used as an index for safety in CB transplantation.

PCR analysis for TCRβ chain gene rearrangement

Cells (1×10^5) recovered from the coculture of human $CD34^+CD38^-Lin^-$ CB cells and TSt-4/hDLL1 stromal cells (on day 33 of culture) were resuspended in 20 μL of 1x polymerase chain reaction (PCR) buffer [10 mM Tris-HCl (pH 9.0), 50 mM KCl and 1.5 mM $MgCl_2$] including 0.45% Nonidet P-40, 0.45% Tween 20, and 1.2 μg of Proteinase K (Sigma, St Louis, MO, USA), and incubated at 55°C for 1 h, then 95°C for 10 min. Samples of these disrupted cells were used as templates for PCR amplification. The primers were 5′-TGGTGGTCTCTCCCAGGCTCT-3′ (Dβ1.1) and 5′-CCAGCTGTCCAGCCTTGACTT-3′ (Jβ1.3), and the reaction volume was 20 μL, containing 5 μL of the cell extract, 1.5 μL of 10x PCR buffer, 0.16 μL of 25 mM dNTPs, 4 pmol of each primer, and 0.6 U of *Taq* polymerase (Invitrogen Corporation, Carlsbad, CA, USA). Thermocycling conditions were as follows: 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, 2 min at 72°C, and a final extension step of 10 min at 72°C. Amplified DNA products were loaded on a 1.2% agarose gel, electrophoresed and stained with ethidium bromide.

Results

T-cell-inducing capability of DLL1-transduced stromal cell line

We first investigated whether TSt-4/hDLL1 stromal cells are able to support T-cell development from murine progenitor cells. $Lin^-c\text{-}kit^+$ cells (100 cells per well) from murine FL or FB were cultured on a monolayer of this stromal cell line. As negative and positive controls, a coculture with TSt-4 and a culture with a dGuo-treated FT lobe (FTOC), respectively, were set up in parallel. After 14 d of culture, grown cells were

harvested and stained with antibodies to various lineage markers for FACS analysis. Profiles for Thy-1.2 vs. CD19 and CD4 vs. CD8 expression are shown in Fig. 1A (FL) and Fig. 1B (FB). The results indicate that TSt-4/hDLL1 is able to support the development of $CD4^+CD8^+$ [double positive (DP)] cells from $c\text{-}kit^+$ prethymic progenitors of the mouse, although differentiation to $CD4^+$ or $CD8^+$ mature, single positive cells is marginal, if any.

Development of immature T cells from human $CD34^+CD38^-Lin^-$ CB cells by culturing with TSt-4/hDLL1

$CD34^+CD38^-Lin^-$ cells were sorted from thawed $CD34^+$ CB cells, and were cultured at 50 cells per well on a monolayer of TSt-4/hDLL1 (Fig. 2A). TSt-4 was used as a control. Cell recovery reaches the maximum level around day 30 and the levels decrease thereafter (Fig. 2B). Representative flow cytometric profiles of cells

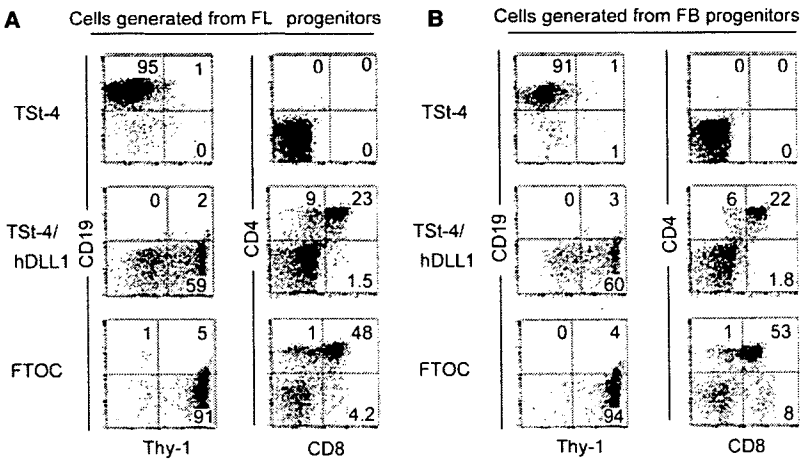

Figure 1 Capability of TSt-4/hDLL1 to induce T-cell generation from murine FL and FB progenitors. Representative flow cytometric profiles of cells generated from FL $Lin^-c\text{-}kit^+$ cells (A) and FB $Lin^-c\text{-}kit^+$ cells (B) are shown. These cells (100 cells per well) were cultured on a stromal cell line TSt-4/hDLL1 in a 12-well plate for 14 d. As negative and positive controls, the cells generated from these progenitors by culturing with TSt-4 and dGuo-FT (FTOC), respectively, are shown.

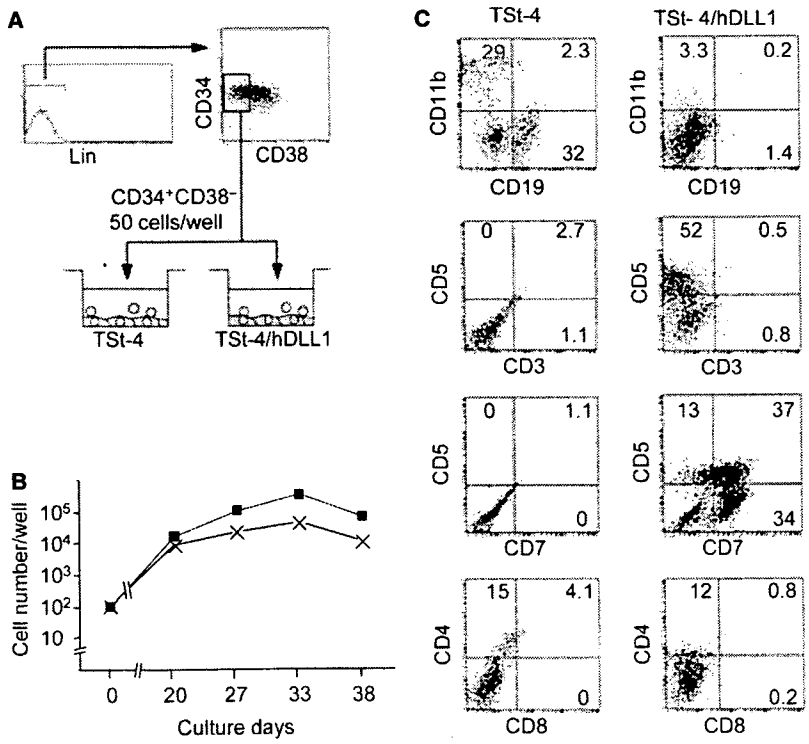

Figure 2 Generation of $CD5^+CD7^+CD3^-$ immature T cells from human hematopoietic stem/progenitor cells by culturing with TSt-4/hDLL1. (A) Experimental procedures for cultivation of human hematopoietic stem/progenitor cells. $CD34^+$ cells from a CB sample were stained in three colors with anti-Lin, anti-CD34, and anti-CD38, and the $CD34^+CD38^-Lin^-$ cells were sorted and cultured at 50 cells per well on a monolayer of TSt-4 or TSt-4/hDLL1 in a 48-well plate. (B) Time course of cell recovery in cultures of $CD34^+CD38^-$ cells on a monolayer of TSt-4 (x) or TSt-4/hDLL1 (■). (C) Representative flow cytometric profiles of recovered cells in cultures with TSt-4 (left panels) or TSt-4/hDLL1 (right panels). The cells were stained in three colors with CD45 and various combinations of monoclonal antibodies. CD19 vs. CD11b, CD3 vs. CD5 and CD5 vs. CD7 profiles, and CD8 vs. CD4 profiles of $CD45^+$ cells are shown. The mean numbers of recovered cells per well in cultures on TSt-4 and TSt-4/hDLL1 were 2.0×10^4 and 1.8×10^5, respectively. Results are representative of three independent experiments.

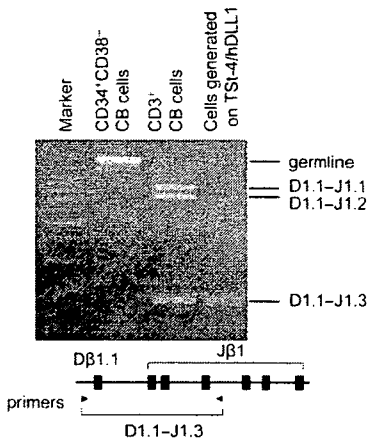

Figure 3 D-J rearrangement of the TCRβ chain gene in immature T cells generated from human hematopoietic stem/progenitor cells *in vitro*. $CD34^+CD38^-Lin^-$ cells from a CB sample were cultured on a monolayer of TSt-4/hDLL1 (50 cells per well) for 33 d, and genomic DNA was prepared from the recovered cells. As negative and positive controls, $CD34^+$ cells and $CD3^+$ cells purified from CB were used. DNA samples (equivalent to 2.0×10^4 cells) were PCR-amplified using primers for Dβ1.1-Jβ1.3-1.4, with their locations schematically shown under the figure. Three independent experiments showed a similar expression pattern.

recovered on day 33 of the culture are shown in Fig. 2C. $CD11b^+$ myeloid cells and $CD19^+$ B cells were generated by culturing with TSt-4, but no T-cell generation was seen in this culture (Fig. 2C, left panels). In contrast, in the group cultured with TSt-4/hDLL1, virtually all cells generated express either or both CD5 and CD7 (Fig. 2C, right panels), which are T-lineage cell markers. These cells lack expression of CD4, CD8 or CD3, indicating that the TSt-4/hDLL1 supports the differentiation until a certain immature T-cell stage. On the other hand, TSt-4/hDLL1 does not induce the generation of myeloid or B cells.

Cells generated on TSt-4/hDLL1 monolayers were analyzed by PCR for their Dβ-Jβ rearrangement status (Fig. 3). As positive and negative controls, $CD3^+$ cells and $CD34^+$ cells in CB, respectively, were examined. The cells generated on TSt-4/hDLL1 monolayers exhibited a clear D1.1-J1.3 band and a faint D1.1-J1.2 band, indicating that the cells expressing CD5 and/or CD7 have rearranged their TCRβ-chain gene and therefore are surely immature T cells.

Generation of immature T cells from MNCs

The experiments in the preceding section showed that TSt-4/hDLL1 is able to support the generation of immature T cells from $CD34^+CD38^-$ CB cells. The major purpose of the present study, however, is to quantify TGP in an MNC fraction without further fractionations with a cell sorter. The critical point to be clarified for this purpose is whether the T cells generated from progenitors *in vitro* can be discriminated from the T cells that are present in the MNC fraction and survived the culture. As shown in Fig. 4A, $CD5^+$ cells are present in the MNCs, but they are all $CD3^+$ and are therefore of the mature type. No immature type $CD5^+CD3^-$ cells were seen. When these $CD3^+$ T cells were cultured with TSt-4/hDLL1 for 33 d (Fig. 4B), their phenotype remained $CD5^+CD3^+$, although the CD3 expression levels were slightly reduced. Flow cytometric profiles of cells recovered from cultures of MNCs with TSt-4/hDLL1 are shown in Fig. 4C. A large majority of the cells are $CD5^+CD3^-$ immature T cells, while a small number of $CD5^+CD3^+$ cells also exist. As these latter cells are virtually not generated in the culture of $CD34^+CD38^-$ cells (see Fig. 2), the $CD5^+CD3^+$ cells were regarded as mature T cells that survived the culture. Thus, mature T cells included in the MNCs do not interfere with the detection of immature T cells generated *de novo* from progenitors.

Frequency determination of TGP in MNCs

Serially reduced numbers of MNCs were cultured with TSt-4/hDLL1 for 33 d, and the cells in all wells were analyzed by FACS. Proportions of negative wells, where T-cell growth was not seen, were plotted against the number of cultured cells (Fig. 5). From the titration curve, the frequency of progenitors generating T cells determined by this figure was 1/1174. From this value, the number of TGP in 100 mL of CB was calculated as 35.0×10^4.

Frequency analysis was repeated three times with different CB samples, and the number of TGP per unit volume was calculated from the frequency. To make comparison of data among samples easier, TGP numbers were normalized to values per 100 mL CB, and the mean ± SD are shown in Table 1. The fluctuation of the determination observed in this experiment is at the same level usually experienced in the CFU-C assay. Therefore, we believe that the limiting dilution analysis for TGP can be carried out stably.

Further characterization of TGP in reference to CFU-GM

To investigate whether the TGP of a CB sample is related to the progenitors determined by the CFU-C assay, which is the only method currently used to evaluate the developmental potential of CB samples, we compared the number of TGP and that of CFU-GM in 10 different neonate samples. Data for TGP and CFU-GM are normalized to express the values per 100 mL CB,

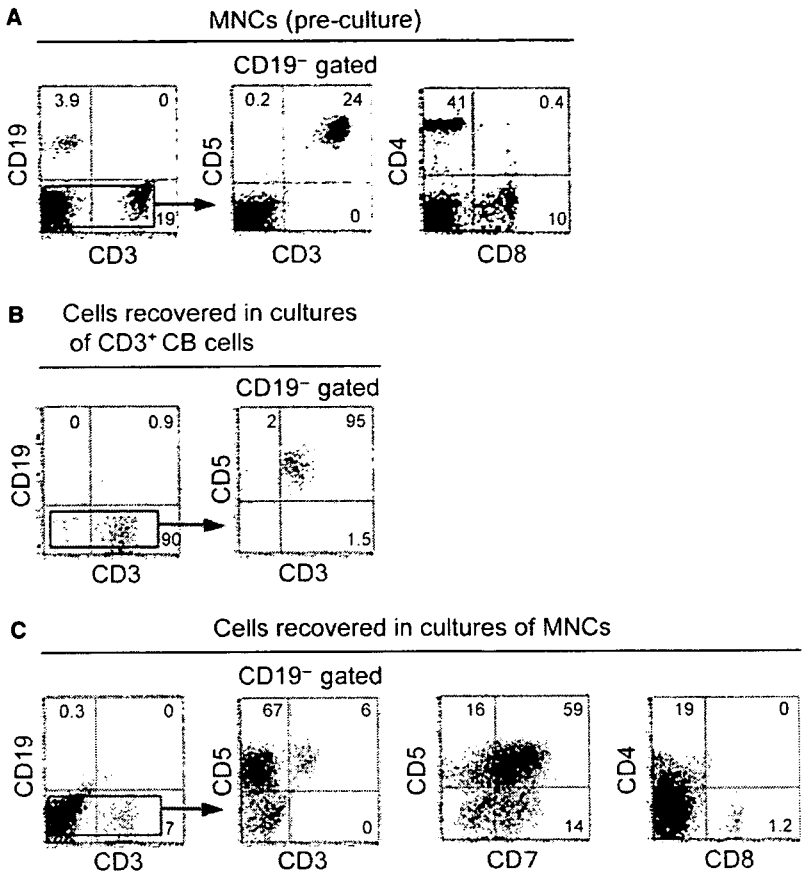

Figure 4 Detection of immature T cells generated *in vitro* from CB progenitors. (A) Representative flow cytometric profiles of MNCs in CB. MNCs from a CB sample were stained in three colors with anti-CD19, anti-CD3 and anti-CD5, or in two colors with anti-CD4 and anti-CD8, and the profiles for CD3 vs. CD19, CD3 vs. CD5 of CD19⁻ cells, and CD4 vs. CD8 of whole MNCs are shown. (B, C) CD3⁺ cells (1000 cells per well) or MNCs (5000 cells per well) were cultured on a monolayer of TSt-4/hDLL1 for 33 d, and the cells recovered from these cultures were stained as in (A). In (B), profiles for CD3 vs. CD19 and CD3 vs. CD5 of CD19⁻ cells are shown. In (C), profiles for CD3 vs. CD19, CD3 vs. CD5 of CD19⁻ cells, and CD4 vs. CD8 are shown. CD5 vs. CD7 profiles are also shown in this figure. The mean cell recovery in cultures of CD3⁺ cells and MNCs in each triplicate culture were 3.3×10^3 and 5.7×10^4, respectively. The results are representative of three independent experiments.

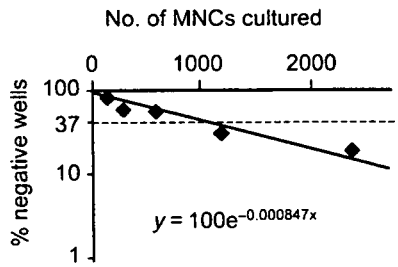

Figure 5 Quantification of TGP in MNCs. Graded numbers of MNCs were cultured on a monolayer of TST-4/hDLL1 for 33 d. T-cell progenitor activity of cells seeded in each well was determined by flow cytometric analysis of cells recovered from individual wells for expression of CD5 and CD3. Progenitor frequency was estimated from the proportions of negative wells by using the Poisson distribution formula. Thirteen to 19 wells per point were used. The frequency was determined as 1/1174.

and the results are summarized in Table 2. Quite a large difference is seen among samples in the levels of TGP as well as CFU-GM. Samples showing high values in both assays, e.g. samples g, j and o, may be the most useful ones in transplantation. However, it was found that the

Table 1 Reproducibility test for TGP determination

Sample	TGP number per 100 mL of CB (mean ± SD)[1]
a	13.3 ± 2.0
b	3.0 ± 0.4
c	9.1 ± 2.8
d	4.5 ± 0.4
e	19.9 ± 3.7

Frequency of T-cell-generating progenitors (TGP) was determined as in Fig. 5.
[1]Mean ± SD of three determinations are shown.

TGP and CFU-GM do not necessarily run parallel. The largest CFU-GM/TGP ratio is 6.67 (sample f), whereas the smallest is 1.92 (sample o), indicating that an evaluation for reconstitution ability of CB fluctuates maximally 3.5 times when only the CFU-C assay or only the TGP assay is adopted.

Discussion

The present study succeeded in establishing a method for detection of stem/progenitor cells that can generate T

Table 2 Determination of TGP and CFU-GM in 10 CB samples

Sample	Volume of sample (mL)	No. per 100 mL of CB			
		MNCs	TGP	CFU-GM	CFU-GM/TGP[1]
f	48	41.9 ($\times 10^7$)	14.0 ($\times 10^4$)	93.6 ($\times 10^4$)	6.6
g	57	38.4	22.9	94.2	4.12
h	62	9.5	2.0	7.9	4.00
i	66	18.5	6.1	20.0	3.25
j	58	20.3	15.8	44.9	2.84
k	40	22.5	12.1	29.5	2.43
l	77	14.4	4.1	9.4	2.29
m	20	9.0	10.3	23.2	2.27
n	83	9.2	5.0	9.9	1.96
o	32	22.2	17.3	33.2	1.92

The frequency of T-cell-generating progenitors (TGP) was estimated as in Figure 5. The numbers per 100 mL CB are shown.
[1]The ratio for the numbers of CFU-GM and TGP.

cells, which we termed TGP, in human CB. TGP were detected by culturing MNCs with a murine stromal cell line TSt-4 expressing human *DLL1* (TSt-4/hDLL1). By combining this culture system with limiting dilution analysis, we succeeded for the first time in quantifying TGP in CB samples. The number of TGP in CB samples does not necessarily correlate with that of CFU-GM, and thus the enumeration of TGP provides different information than the CFU-C assay in the evaluation of CB samples used for reconstitution of lympho-hematopoiesis of recipients.

For transplantation of hematopoietic stem/progenitor cells, reconstitution of erythrocytes, platelets and myeloid cells in recipients is clinically a requirement. Reconstitution of lymphocytes is also important, as life-time resistance to infectious microorganisms is mediated mainly by lymphocytes. Serious opportunistic infections are the main complication and reason for the death of patients after stem cell transplantation because of lack of T-cell reconstitution (22). It takes usually around 1 yr for T cells to be fully reconstituted (23, 24). It is still unknown why such a long period is required for T-cell reconstitution, nor is it understood whether such a delay in T-cell reconstitution can be attributed to an insufficient number of TGP transplanted or to the recipients' conditions. Our success in quantification of TGP will contribute towards clarifying whether administering a larger number of TGP can improve T-cell reconstitution in recipients. The present assay system will further contribute, after some modifications, to evaluate the expansion or reconstitution levels of TGP in recipients.

Several methods for detection of human T-cell progenitors have already been described. For example, T-cell development can be induced by coculturing human hematopoietic stem/progenitor cells with a dGuo-treated murine FT lobe (12–14), or more efficiently with a dGuo-treated FT lobe of mice with severe combined immunodeficiency (14, 25). Human FT fragments have also been used as the culture environment (26, 27). These culture systems, however, cannot easily be applied for clinical use, because of their technical difficulty and complicatedness for introducing thymic organ culture systems in clinical laboratories. After the discovery that Notch signaling is a key factor for T-cell induction (28), a murine stromal cell line OP9 transduced with the mouse delta-like 1 gene (OP9-DL1) was reported to support T-cell development from human hematopoietic stem/progenitor cells (15). The OP9-DL1 cell line, however, has not been applied to quantify human hematopoietic stem/progenitor cells. As TSt-4/hDLL1 stably functions as a stromal support over a very long period (> 5 weeks) without passage, this cell line is effective in examining T-cell progenitor activity of limiting numbers of cells. Although the ability of TSt-4/hDLL1 is restricted to support differentiation up to an immature stage of T lineage cells, we were able to use this characteristic to our advantage, because the newly generated $CD5^+$ or $CD7^+$ immature T cells were easily discriminated in flow cytometric analysis from mature T cells surviving in the culture of MNCs. By a limiting dilution analysis using the TSt-4/hDLL1 cell line, it became possible to enumerate the TGP in CB.

Using 10 CB samples, the CFU-C assay was undertaken in parallel with TGP determination (Table 2). Samples showing quite high CFU-GM values (samples f and g) also show high TGP values, and those showing low CFU-GM values were also low in TGP levels (samples h, l and n). However, TGP levels are not necessarily proportional to CFU-GM levels. Sample f shows quite a high CFU-GM, but the TGP value is at an average level. The reverse is true in sample o. These findings strongly suggested that the capability for T-cell reconstitution and also myeloid reconstitution differs depending upon the CB samples used. To find out whether the TGP levels really represent the potential of CB samples in reconstituting T cells, however, it is necessary to perform clinical studies after CB transplantation to compare the effectiveness of samples showing different TGP levels.

The TGP detected in the present study do not necessarily represent T cell lineage committed progenitors alone, but is probably a mixture of hematopoietic stem cells, common myelo-lymphoid progenitors, myeloid-T bipotent progenitors, and T cell lineage-committed progenitors, which have been determined with the multilineage progenitor assay in mice (20, 29–31). It is still unclear, however, whether such progenitor types seen in mice also exist in human or human CB, as no detailed processes of lympho-hematopoiesis have been elucidated in humans. The search for surface markers specific for these progenitors, like the paired immunoglobulin-like

receptors (PIR) on T-cell lineage-committed progenitors in mice (17), is also an important approach. Identification and isolation of different types of progenitors that give rise to T cells with surface markers will further facilitate our understanding of human T-cell development, and may contribute to the promotion of clinical success in the reconstitution of patients' immune responsiveness.

Development of an assay system to quantify B-cell progenitors will allow an additional effective evaluation of samples used in transplantation. Murine stromal cell lines have been used for B-cell induction from human hematopoietic progenitor/stem cells. It was shown that $CD19^+CD10^+CD20^-$ and surface Ig^- immature B cells were generated by culturing with murine stromal cell lines (32, 33). More quantitative evaluation of B-cell progenitors will be attained by modifying these culture systems. In the present study we show that the TSt-4 cell line is able to induce myeloid and immature B cells from human CB progenitors (see Fig. 2C). This ability of the mother TSt-4 cell line is virtually completely lost after the transduction of the *DLL1* gene . Our future aim is to produce a culture environment that supports the development of all hematopoietic cells including myeloid, T and B lineage cells. Studies are in progress to establish a cell line that is able to induce cells of all these lineages, which may provide the opportunity to elucidate the detailed process of human lympho-hematopoiesis that has been obtained with the multilineage progenitor assay in mice (20, 29–31).

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