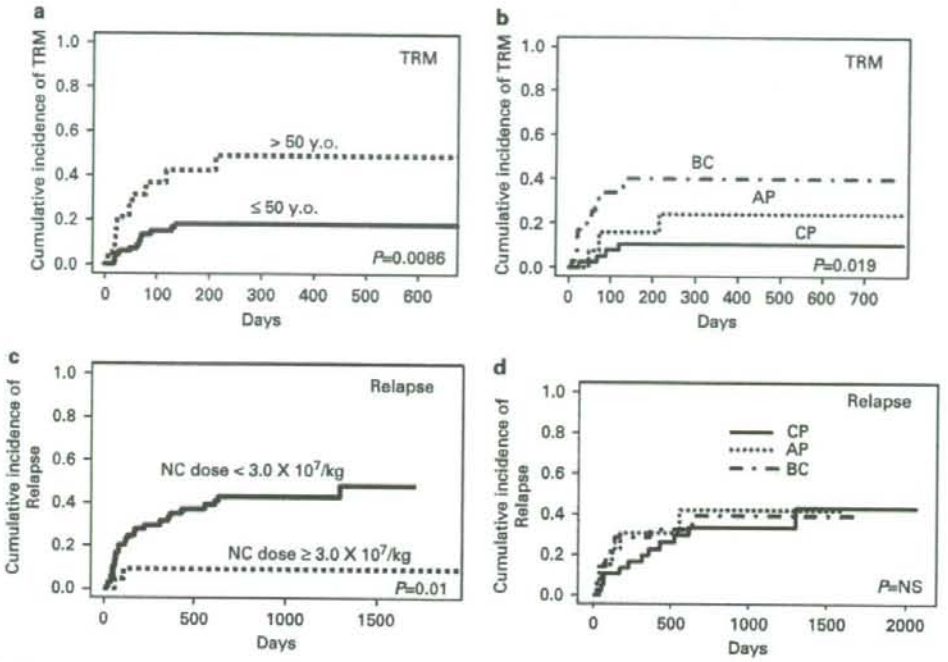

Figure 3 The cumulative incidence of transplant-related mortality (TRM) and relapse. Cumulative incidences of TRM are shown by age (**a**) and by the stage at unrelated cord blood transplantation (UCBT) (**b**). Cumulative incidences of relapse are shown by the dose of nucleated cells (NCs) before cryopreservation (**c**) and by disease stage at UCBT (**d**).

Table 4 Cause of death

Causes	n = *38*
Transplantation-related mortality	26
GVHD	5
VOD	2
Bleeding	2
Infection (5 bacterial, 1 bacteria plus fungus, 1 fungus, 1 tuberculosis, 1 viral encephalitis)	9
Tacrolimus-induced encephalopathy	1
Graft failure-related complications	7
Relapse or induction failure	10
Others	2
Post-second UCBT for relapse, TRM	1
Other malignancy	1

Abbreviations: TRM = transplantation-related mortality; UCBT = unrelated cord blood transplantation; VOD = veno-occlusive disease.

CI, 17–84%) for those in AP and 22% (95% CI, 10–48%) for those in BC ($P = 0.0004$; Figure 4b). The probability of 2-year OS for patients in CP, AP and BC was 71% (95% CI, 56–90%), 59% (95% CI, 37–94%) and 32% (95% CI, 20–55%), respectively ($P = 0.0004$; Figure 4e). The probability of survival remained higher for the patients in CP to AP at UCBT compared to LFS because the molecular or cytogenetic relapse in CP or AP might not be fatal. Note that NC-dose before cryopreservation also significantly affected the LFS and EFS (Table 3). Two-year EFS was 20% (95% CI, 12–35%) for the patients who received less than 3.0×10^7 per kg NCs before cryopreservation, whereas it was 68% (95% CI, 48–96%) for those who received more than 3.0×10^7 per kg NCs ($P = 0.0005$; Figure 4c). A marginally significant influence on 2-year EFS was observed if a patient was in the youngest age group (74; 95% CI, 48–100%) compared to those of young adults (33; 95% CI, 22–49%) and older adults (15; 95% CI, 3–72%; $P = 0.049$; Figure 4d). Hence, no significant influence by age was found in multivariate analysis for LFS, EFS and overall survival.

Pre-transplant risk scoring system of EBMT and TRM, LFS and OS

The pre-transplant risk scoring system was established by the EBMT to evaluate patients with CML who are candidates for HSCT.[28,29] We adjusted the data of the patients enrolled in the JCBBN to conform to the EBMT scoring system. The scores of all patients were raised by one point because of unrelated donors. When the EBMT scoring system was applied, 1 had a score of 1, 10 had scores of 3, 18 had scores of 4, 25 had scores of 5, 21 had scores of 6 and 10 had scores of 7 (Table 1). The outcome of TRM and survival had a tendency to be correlated with the pre-transplant scores. We compared the patients with scores of 0–4 ($n = 30$) vs the patients with scores of 5–7 ($n = 56$). The patients with a score of 5–7 had a significantly higher incidence of TRM and an unfavourable survival rate. The 1-year cumulative incidences of TRM were 7%

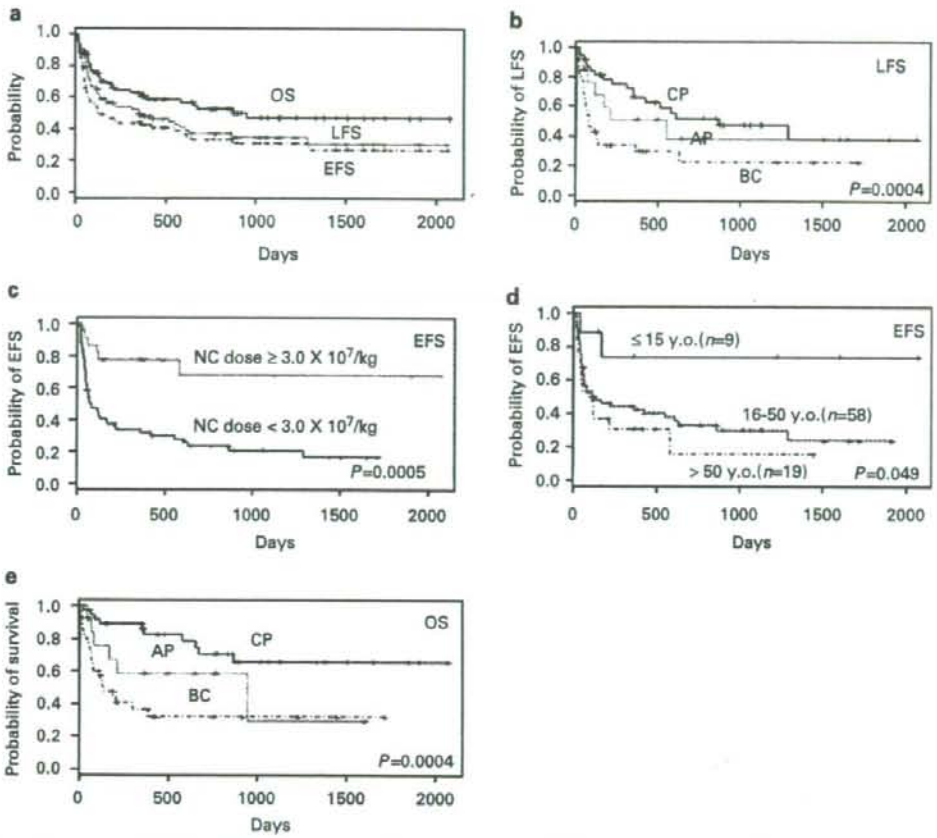

Figure 4 Kaplan–Meier estimate of leukaemia-free survival (LFS), event-free survival (EFS) and overall survival (OS). Overall LFS, EFS and OS are shown in (**a**). (**b**) LFS is shown by disease stage (chronic phase, CP; accelerated phase, AP and blast crisis, BC) at unrelated cord blood transplantation (UCBT). (**c**) EFS is shown by the dose of nucleated cells (NCs), NC dose of 3×10^7 per kg or more vs a lower dose. (**d**) Probability of EFS by age showed a marginally significant difference between children and adults. (**e**) OS is indicated by disease stage at UCBT.

(95% CI, 0–16%) for the patients with a score of 0–4 and 34% (95% CI, 21–47%) for those with a score of 5–7 ($P = 0.005$; Figure 5a). The 2-year survival rate was 66% (95% CI, 48–89%) for patients with a score of 0–4 and 47% (95% CI, 35–63%) for those with a score of 5–7 (Figure 5b).

Discussion

Reports of UCBT in patients with CML are limited. Sanz *et al.*[24] reported on nine cases from a single institute, but the remaining cases were reported only as part of a heterogeneous series of patients with different diseases[3] or as anecdotal case studies. To our knowledge, this retrospective registry-based analysis is the first that was specifically designed to describe the results of UCBT in patients with CML.

As expected, administering higher doses of NC and GM colonies before cryopreservation produced significantly quicker and more effective myeloid and platelet engraftment; however, the dose of GM colonies was not statistically significant because of missing data. Patients (14 adults and 8 children) who received more than 3.0×10^7 per kg NCs achieved the most successful myeloid and platelet engraftments. On the basis of these data, adult patients and their physicians are encouraged to obtain units containing a higher NC count. In contrast to previous studies,[30,31] $CD34^+$ cells did not have a significant influence on the incidence of myeloid engraftment. As with the dose of GM colonies, $CD34^+$ cell data were missing for several patients and different assay systems for $CD34^+$ analysis were used in each bank. Engraftment was influenced neither by duration from diagnosis to UCBT nor by prior therapy for CML. The patient with the longest duration from diagnosis to UCBT (17.6 years) achieved successful engraftment. Although the disease stage of CML at UCBT tended to influence the engraftment rate, no significant difference was detected (data not shown; $P = 0.08$). These results are encouraging for patients

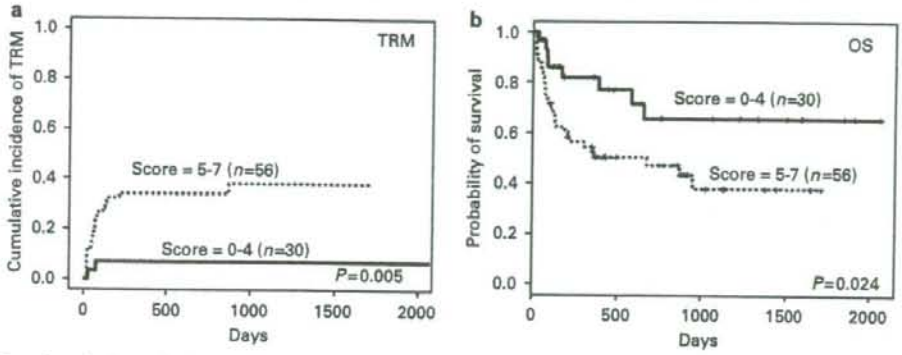

Figure 5 Transplant-related mortality (TRM) and overall survival (OS) according to the pre-transplant risk score of the European Group for Blood and Marrow Transplantation (EBMT). **(a)** TRM by pre-transplant risk score of the EBMT (0–4 vs 5–7). **(b)** Survival by pre-transplant risk score of the EBMT (0–4 vs 5–7).

with CML who are treated with imatinib or IFN before UCBT.

As expected, we found that an advanced disease stage at UCBT was significantly associated with lower LFS, EFS and OS. Moreover, although the disease stage of CML at UCBT did not have a significant influence on the cumulative incidence of relapse, it seemed to influence TRM (Figure 3b). However, in the multivariate analysis, the only significant factors that influenced higher TRM were age of 50 years or older and dose of GM colonies at less than 25×10^3 per kg (Table 3). The collinearity between older age and advanced stage of the disease may have had a large influence on this part of the analysis. In comparison to previous studies on unrelated BMT,[32–34] OS in UCBT seemed comparable to that in unrelated BMT. Two-year survival rates varied approximately 44–77% for patients in CP with unrelated BMT, whereas a 71% survival rate was observed for those in CP with UCBT. The finding that young age (1–15 years) indicated high EFS (74% at 2 years) with only marginal significance by univariate, but not by multivariate, analysis may have occurred because children included a relatively low percentage of the advanced disease cohort at CBT and a relatively high dose of NCs compared to the adults. Note that an NC dose higher than 3.0×10^7 per kg before cryopreservation improved the outcome of myeloid and platelet engraftment, the relapse rate and LFS. Dini *et al.*[35] reported that a NC dose higher than 3×10^8 per kg was associated with LFS in unrelated BMT, although the NC dose in UCBT was one log less than in UBMT; however, the dose of GM colonies did not influence the relapse and DFS. This discrepancy in the influence of the dose of GM colonies on engraftment and relapse suggests that non-myeloid stem cells in NC, such as T cells, are important in the immune system after HSCT.[36]

The most likely causes of death in UCBT for patients with CML were related to transplantation rather than relapse. The high incidence of rejection-related complications, such as infections and bleeding, remains to be resolved; however, unexpectedly, the incidence of GVHD as the cause of death was relatively higher in UCBT. CML is the only haematopoietic malignancy shown to respond effectively to immunotherapy with donor lymphocyte infusion after allogeneic HSCT.[22,23] Some unintended factors by physicians might promote the development of GVHD, although we could not assess this concern in detail in this retrospective registry-based analysis. To clarify this important issue, carefully designed prospective trials are needed.

We also demonstrated that the pre-transplant risk scoring system established by the EBMT for CML[28] can be used to predict overall risk for UCBT. This scoring system is based on previously reported pre-transplant risk factors including histocompatibility, disease stage at the time of transplantation, age and sex of the donor and recipient and time from diagnosis to transplantation. According to this scoring system, we classified the patients using a 7-level risk score. Although a greater number of patients are needed to properly evaluate this scoring system, the group that scored 0–4 showed significantly better LFS, OS and TRM compared to the group that scored 5–7, but the TRM of the higher scoring group was much lower (40%) compared to a previous report (75%).[28] The lower TRM we observed may explain why advanced GVHD was limited and why imatinib, not intensive chemotherapy, was initiated prior to CBT.[37] This scoring system might provide a rational basis for counselling before UCBT as well as other HSCT; however, it does not address donor-side haematopoietic unit data, which is absolutely essential in the selection of CB.

The finding that the duration from diagnosis to UCBT did not affect the main outcome encouraged us to use targeted therapy, such as imatinib, even for patients with advanced-stage CML before transplantation. Furthermore, the patients with cytogenetic/molecular relapse after CBT could be started on imatinib therapy as we describe in this study. Recent evidence suggests that kinase inhibitors, such as imatinib, suppress T-cell functions and immunoglobulin production by B cells.[38,39] At present, how long patients will need to take imatinib is unclear, but the combination of imatinib and HSCT clearly results in improved survival in patients with CML.

We conclude that UCBT is an encouraging alternative treatment for patients with CML who do not have an identical sibling and/or those who cannot be expected to obtain a favourable cytogenetic response with imatinib treatment.

Acknowledgements

We thank Masato Takeuchi of Kitasato University Graduate School for the statistical discussion. We thank all of the physicians and staff at the hospitals and the 11 cord blood banks in Japan who collaborated on this study. We also thank all the data managers and officers of the Japan Cord Blood Bank Network and the Transplantation Data Management Committee. This work was supported in part by a Health and Labor Science Research Grant from the Ministry of Health, Labor and Welfare of Japan.

References

1 Gluckman E, Broxmeyer HA, Auerbach AD, Friedman HS, Douglas GW, Devergie A *et al.* Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. *N Engl J Med* 1989; **321**: 1174–1178.

2 Broxmeyer HE, Douglas GW, Hangoc G, Cooper S, Bard J, English D *et al.* Human umbilical cord blood as a potential source of transplantable hematopoietic stem/progenitor cells. *Proc Natl Acad Sci USA* 1989; **86**: 3828–3832.

3 Rubinstein P, Carrier C, Scaradavou A, Kurtzberg J, Adamson J, Migliaccio AR *et al.* Outcomes among 562 recipients of placental-blood transplants from unrelated donors. *N Engl J Med* 1998; **339**: 1565–1577.

4 Gluckman E, Rocha V, Arcese W, Michel G, Sanz G, Chan KW *et al.* Factors associated with outcomes of unrelated cord blood transplant: guidelines for donor choice. *Exp Hematol* 2004; **32**: 397–407.

5 Rocha V, Labopin M, Sanz G, Arcese W, Schwerdtfeger R, Bosi A *et al.* Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. *N Engl J Med* 2004; **351**: 2276–2285.

6 Talpaz M, Kantarjian H, Kurzrock R, Trujillo JM, Gutterman JU. Interferon-alpha produces sustained cytogenetic responses in chronic myelogenous leukemia. Philadelphia chromosome-positive patients. *Ann Intern Med* 1991; **114**: 532–538.

7 Lee MS, Kantarjian H, Talpaz M, Freireich EJ, Deisseroth A, Trujillo JM *et al.* Detection of minimal residual disease by polymerase chain reaction in Philadelphia chromosome-positive chronic myelogenous leukemia following interferon therapy. *Blood* 1992; **79**: 1920–1923.

8 Kantarjian HM, Cortes JE, O'Brien S, Giles F, Garcia-Manero G, Faderl S *et al.* Imatinib mesylate therapy in newly diagnosed patients with Philadelphia chromosome-positive chronic myelogenous leukemia: high incidence of early complete and major cytogenetic responses. *Blood* 2003; **101**: 97–100.

9 Deininger M, Buchdunger E, Druker BJ. The development of imatinib as a therapeutic agent for chronic myeloid leukemia. *Blood* 2005; **105**: 2640–2653.

10 Cortes J, Talpaz M, O'Brien S, Jones D, Luthra R, Shan J *et al.* Molecular responses in patients with chronic myelogenous leukemia in chronic phase treated with imatinib mesylate. *Clin Cancer Res* 2005; **11**: 3425–3432.

11 Kantarjian H, Talpaz M, O'Brien S, Giles F, Faderl S, Verstovsek S *et al.* Survival benefit with imatinib mesylate therapy in patients with accelerated-phase chronic myelogenous leukemia—comparison with historic experience. *Cancer* 2005; **103**: 2099–2108.

12 Druker BJ, Guilhot F, O'Brien SG, Gathmann I, Kantarjian H, Gattermann N *et al.* Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med* 2006; **355**: 2408–2417.

13 Cortes J, O'Brien S, Kantarjian H. Discontinuation of imatinib therapy after achieving a molecular response. *Blood* 2004; **104**: 2204–2205.

14 Merante S, Orlandi E, Bernasconi P, Calatroni S, Boni M, Lazzarino M. Outcome of four patients with chronic myeloid leukemia after imatinib mesylate discontinuation. *Haematologica* 2005; **90**: 979–981.

15 Bartholomeusz GA, Talpaz M, Kapuria V, Kong LY, Wang S, Estrov Z *et al.* Activation of a novel Bcr/Abl destruction pathway by WP1130 induces apoptosis of chronic myelogenous leukemia cells. *Blood* 2007; **109**: 3470–3478.

16 Ray A, Cowan-Jacob SW, Manley PW, Mestan J, Griffin JD. Identification of Bcr/Abl point mutations conferring resistance to the Abl kinase inhibitor AMN107 (Nilotinib) by a random mutagenesis study. *Blood* 2007; **109**: 5011–5015.

17 Hochhaus A, Erben P, Ernst T, Mueller MC. Resistance to targeted therapy in chronic myelogenous leukemia. *Semin Hematol* 2007; **44** (1 Suppl 1): 15–24.

18 Kimura S, Ashihara E, Maekawa T. New tyrosine kinase inhibitors in the treatment of chronic myeloid leukemia. *Curr Pharm Biotechnol* 2006; **7**: 371–379.

19 Weisberg E, Catley L, Wright RD, Moreno D, Banerji L, Ray A *et al.* Beneficial effects of combining nilotinib and imatinib in preclinical models of BCR-ABL+ leukemias. *Blood* 2007; **109**: 2112–2120.

20 Weisser M, Schleuning M, Haferlach C, Schwerdtfeger R, Kolb HJ. Allogeneic stem-cell transplantation provides excellent results in advanced stage chronic myeloid leukemia with major cytogenetic response to pre-transplant imatinib therapy. *Leuk Lymphoma* 2007; **48**: 295–301.

21 Koh LP, Hwang WY, Chuah CT, Linn YC, Goh YT, Tan CH *et al.* Imatinib mesylate (STI-571) given concurrently with nonmyeloablative stem cell transplantation did not compromise engraftment and resulted in cytogenetic remission in a patient with chronic myeloid leukemia in blast crisis. *Bone Marrow Transplant* 2003; **31**: 305–308.

22 Simula MP, Marktel S, Fozza C, Kaeda J, Szydlo RM, Nadal E *et al.* Response to donor lymphocyte infusions for chronic myeloid leukemia is dose-dependent: the importance of escalating the cell dose to maximize therapeutic efficacy. *Leukemia* 2007; **21**: 943–948.

23 de Weger RA, Tilanus MG, Scheidel KC, van den Tweel JG, Verdonck LF. Monitoring of residual disease and guided donor leucocyte infusion after allogeneic bone marrow transplantation by chimaerism analysis with short tandem repeats. *Br J Haematol* 2000; **110**: 647–653.

24 Sanz GF, Saavedra S, Jimenez C, Senent L, Cervera J, Planelles D *et al.* Unrelated donor cord blood transplantation in adults with chronic myelogenous leukemia: results in nine patients from a single institution. *Bone Marrow Transplant* 2001; **27**: 693–701.

25 Rubinstein P, Dobrila L, Rosenfield RE, Adamson JW, Migliaccio G, Migliaccio AR *et al.* Processing and cryopreservation of placental/umbilical cord blood for unrelated bone marrow reconstitution. *Proc Natl Acad Sci USA* 1995; **92**: 10119–10122.

26 Glucksberg H, Storb R, Fefer A, Buckner CD, Neiman PE, Clift RA *et al.* Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. *Transplantation* 1974; **18**: 295–304.

27 Michel G, Rocha V, Chevret S, Arcese W, Chan KW, Filipovich A *et al.* Unrelated cord blood transplantation for childhood acute myeloid leukemia: a Eurocord Group analysis. *Blood* 2003; **102**: 4290–4297.

28 Gratwohl A, Hermans J, Goldman JM, Arcese W, Carreras E, Devergie A *et al.* Risk assessment for patients with chronic myeloid leukaemia before allogeneic blood or marrow transplantation. Chronic Leukemia Working Party of the European Group for Blood and Marrow Transplantation. *Lancet* 1998; **352**: 1087–1092.

29 Gratwohl A, Brand R, Apperley J, Crawley C, Ruutu T, Corradini P *et al.* Allogeneic hematopoietic stem cell transplantation for chronic myeloid leukemia in Europe 2006: transplant activity, long-term data and current results. An analysis by the chronic leukemia working party of the European Group for Blood and Marrow Transplantation (EBMT). *Haematologica* 2006; **91**: 513–521.

30 Nagamura-Inoue T, Shioya M, Sugo M, Cui Y, Takahashi A, Tomita S *et al.* Wash-out of DMSO does not improve the speed of engraftment of cord blood transplantation: follow-up of 46 adult patients with units shipped from a single cord blood bank. *Transfusion* 2003; **43**: 1285–1295.

31 Wagner JE, Barker JN, DeFor TE, Baker KS, Blazar BR, Eide C *et al.* Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival. *Blood* 2002; **100**: 1611–1618.

32 Reiter E, Greinix HT, Keil F, Brugger S, Rabitsch W, Schulenburg A *et al.* Long-term follow-up of patients after related- and unrelated-donor bone marrow transplantation for chronic myelogenous leukemia. *Ann Hematol* 1999; **78**: 507–513.

33 Barker JN, Davies SM, DeFor TE, Burns LJ, McGlave PB, Miller JS *et al.* Determinants of survival after human leucocyte antigen-matched unrelated donor bone marrow transplantation in adults. *Br J Haematol* 2002; **118**: 101–107.

34 Morishima Y, Yabe T, Matsuo K, Kashiwase K, Inoko H, Saji H *et al.* Effects of HLA allele and killer immunoglobulin-like receptor ligand matching on clinical outcome in leukemia patients undergoing transplantation with T-cell-replete marrow from an unrelated donor. *Biol Blood Marrow Transplant* 2007; **13**: 315–328.

35 Dini G, Lamparelli T, Rondelli R, Lanino E, Barbanti M, Costa C *et al.* Unrelated donor marrow transplantation for chronic myelogenous leukaemia. *Br J Haematol* 1998; **102**: 544–552.

36 Molldrem JJ, Lee PP, Wang C, Felio K, Kantarjian HM, Champlin RE *et al.* Evidence that specific T lymphocytes may participate in the elimination of chronic myelogenous leukemia. *Nat Med* 2000; **6**: 1018–1023.

37 Deininger M, Schleuning M, Greinix H, Sayer HG, Fischer T, Martinez J *et al.* The effect of prior exposure to imatinib on transplant-related mortality. *Haematologica* 2006; **91**: 452–459.

38 Seggewiss R, Lore K, Greiner E, Magnusson MK, Price DA, Douek DC *et al.* Imatinib inhibits T-cell receptor-mediated T-cell proliferation and activation in a dose-dependent manner. *Blood* 2005; **105**: 2473–2479.

39 Appel S, Balabanov S, Brummendorf TH, Brossart P. Effects of imatinib on normal hematopoiesis and immune activation. *Stem Cells* 2005; **23**: 1082–1088.

Biology of Blood and Marrow Transplantation 14:1057-1063 (2008)

1083-8791/08/1409-0001$32.00/0
doi:10.1016/j.bbmt.2008.07.003

Unrelated Cord Blood Transplantation for Severe Aplastic Anemia

Ayami Yoshimi,[1] Seiji Kojima,[2] Shuichi Taniguchi,[3] Junichi Hara,[4] Toshimitsu Matsui,[5] Yoshiyuki Takahashi,[2] Hiroshi Azuma,[6] Koji Kato,[7] Tokiko Nagamura-Inoue,[8] Shunro Kai,[9] Shunichi Kato[10]

[1]Department of HSCT Data Management, Nagoya University, School of Medicine, Nagoya, Japan; [2]Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Japan; [3]Department of Hematology, Toranomon Hospital, Tokyo, Japan; [4]Hematology/Oncology Department of Pediatrics, Osaka General Medical Center, Osaka, Japan; [5]Hematology/Oncology, Department of Medicine, Kobe University Graduate School of Medicine, Kobe, Japan; [6]Hokkaido Cord Blood Bank, Sapporo, Japan; [7]Tokai Cord Blood Bank, Nagoya, Japan; [8]Tokyo Cord Blood Bank, Tokyo, Japan; [9]Hyogo Cord Blood Bank, Nishinomiya, Japan; and [10]Tokai University Cord Blood Bank, Isehara, Japan; on behalf of the Japan Cord Blood Bank Network (JCBBN)

Correspondence and reprint requests to: Seiji Kojima, MD, Department of Pediatrics, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, 466-8550, Japan (e-mail: kojimas@med.nagoya-u.ac.jp).

Received April 30, 2008; accepted July 2, 2008

ABSTRACT

In the present study we evaluated the feasibility of unrelated cord blood transplantation (UCBT) in patients with severe aplastic anemia (SAA). The outcome of 31 SAA patients (median age 28; range: 0.9-72.3 years old) who received UCBT was analyzed. The cumulative incidences of the neutrophil and platelet recovery after UCBT were 54.8 and 72.2%, respectively (95% confidence interval [CI] = 36.0%-70.3% and 51.3%-85.3%, respectively). The cumulative incidences of grade ≥II acute and chronic graft-versus-host disease (aGVHD, cGVHD) were 17.1% (95% CI = 6.2%-32.8%) and 19.7% (95% CI = 6.2%-38.8%), respectively. Currently, 13 patients are alive, having survived for 33.7 months (median; range: 6-77 months) after UCBT. The probability of overall survival (OS) at 2 years was 41.1% (95% CI = 23.8%-57.7%). A conditioning regimen that included low-dose total body irradiation (TBI) (2-5 Gy), fludarabine, and cyclophosphamide resulted in a favorable OS (80%; 95% CI = 20.4%-96.9%). This result suggests that UCBT using the optimal conditioning regimen can be a salvage treatment for patients without a suitable bone marrow donor and warrants evaluation in further prospective studies.

KEY WORDS

Unrelated cord blood transplantation • Severe aplastic anemia

INTRODUCTION

Over the last 2 decades, the outcome of patients with severe aplastic anemia (SAA) has dramatically improved regardless of whether patients received immunosuppressive therapy (IST) or bone marrow transplantation (BMT) [1-3]. BMT from an HLA-matched sibling is curative in the majority of younger patients with SAA, and is currently recommended as first-line treatment [4]. IST, with a combination of antithymocyte globulin (ATG) and cyclosporine (CSA), has been an alternative therapy for patients without an HLA-matched sibling. BMT from an unrelated donor (UD) is used as a salvage therapy for patients who fail to respond to IST or who experience a relapse of the disease. However, in general, the results of UD-BMT have been inferior to those achieved with an HLA-matched sibling.

The report the Center for International Blood and Marrow Transplant Research (CIBMTR) on UD-BMT (n = 231), for the period 1988-1998, showed that the overall survival (OS) rates for matched and mismatched UD-BMT in patients with SAA were 39% and 36%, respectively [5]. The Japan Marrow Donor Program (JMDP) reported a favorable outcome with 56% survival rate in 154 patients with SAA who received UD-BMT between 1993 and 2000 [6]. In

the recent 2 reports from the European Group for Blood and Marrow Transplantation (EBMT) and the French Society of Bone Marrow Transplantation and Cellular Therapy (SFGM-TC), the outcomes of UD-BMT for SAA before and after 1998 were compared. The results demonstrated improved OS rates of UD-BMT since year 1998 (32% versus 57% for EBMT and 29% versus 50% for SFGM-TC) [7,8]. The authors speculated that the better HLA matching because of the introduction of high-resolution HLA typing may have contributed to the improved outcomes. In pediatric series, 90% OS rates have been recently reported for UD-BMT patients, which is comparable to that observed for BMT from a matched sibling [9,10].

Treatment approaches for patients who lack a suitable unrelated bone marrow donor remain a great challenge. Cord blood has been used as an alternative source of HSCT, and it has the advantages of rapid availability on demand and a low incidence of graft-versus-host disease (GVHD). There were only a few reports on unrelated cord blood transplantation (UCBT), which included patients with SAA. The results showed poor outcome and high incidence of graft failure [11,12]. However, a few small series and case reports of successful UCBT for SAA have recently been reported [13-17]. Because of the possible reporting bias, the general efficacy of UCBT is still unknown. Therefore, we decided to further examine this procedure by using the database of the Japan Cord Blood Bank Network (JCBBN). We identified 31 patients with acquired SAA who received UCBT and analyzed the outcome.

PATIENTS AND METHODS

Patients

From September 1998 until February 2006, 53 patients with acquired SAA received UCBT through JCBBN. Twenty-two patients who received UCBT as a salvage therapy for the engraftment failure after previous HSCT were excluded, and the remaining 31 patients were included in this study. Patient characteristics and the cord blood units are summarized in Table 1. Patients were eligible for UCBT if they had no HLA-identical related or unrelated bone marrow donor. Patients who could not wait for UD-BMT because of unstable diseases were also considered to be eligible for UCBT. Cord blood units with 0 to 2 HLA locus mismatches by serology in HLA-A, HLA-B, and HLA-DRB1 were searched and then the unit with the largest cell dose was selected. At least 2.0×10^7/kg mononuclear cells (MNCs) were given in all patients.

The age of the patients ranged 0.9 to 72.7 years (median 27.9 years), and there were 8 patients older than 50 years of age. There were 25 patients who had been previously treated with IST, including ATG + CSA (n = 13), ATG only (n = 4), or CSA only (n = 8). In 4 patients, androgen had been given. The remaining 2 patients were given only supportive therapy. All patients or their guardians gave informed consent for transplantation and submission of the data to the JCBBN.

Table 1. *Patient and Donor Characteristics (n = 31)*

Characteristic	
Median patient age, years (range)	27.9 (0.8-72.7)
Sex (male/female)	
Patient (n)	11/20
Donor (n)	14/17
Etiology of aplastic anemia	
Idiopathic/hepatitis associated (n)	30/1
Disease duration before UCBT: median, days (range)	337 (31-5063)
1 year or less/ 1-3 year/3 year or more/unknown (n)	13/4/8/5
Red blood cell transfusions before UCBT	
Less than 20 times/20 or more times/unknown (n)	8/21/2
Platelet transfusions before UCBT	
Less than 20 times/20 or more times/unknown (n)	7/22/2
HLA mismatches (serologic): GVHD direction (n = 31)	
0/1/2 (n)	4/18/9
HLA mismatches (serologic): rejection direction (n = 31)	
0/1/2 (n)	6/17/8
HLA mismatches (DNA typing): GVHD direction (n = 22)	
0/1/2/3/4 (n)	2/6/6/6/2
HLA mismatches (DNA typing): rejection direction (n = 22)	
0/1/2/3/4 (n)	1/5/12/3/1

UCBT indicates unrelated cord blood transplantation; GVHD, graft-versus-host disease.

Recipient-Donor HLA Matching

Data were available for 31 patients with serology-based recipient-donor HLA matching and for 22 patients who underwent high-resolution DNA typing for class I-HLA-A, HLA-B, and DRB1 (Table 1). The HLA disparities for both GVHD and rejection directions are shown in Table 1.

Transplantation Procedure

Characteristics of the transplantation procedures are listed in Table 2. The conditioning regimens varied according to the individual centers used. The 3 most commonly used regimens were: TBI (4-5 Gy) + fludarabine (FLU; 120-175 mg/m^2) + Melphalan (MEL) (80-120/mg/m^2) (n = 12), TBI (2-4 Gy) + FLU (90-250/mg/m^2) and cyclophosphamide (CY; 50-100 mg/kg or 2250/mg/m^2) (n = 5), and TBI (10-12 Gy) + CY (120-200 mg/kg) + ATG (n = 3). Of the 25 patients given irradiation, 24 received TBI

Table 2. *Transplant Procedures (n = 31)*

	No. of Patients
Conditioning Regimen	
TBI (4-5 Gy) + MEL+ FLU	12
TBI (2-4 Gy) + CY + FLU	5
TBI (10-12 Gy) + CY + ATG	3
Others	11
Radiation	
TBI/TAI	25/1
No radiation	7
ATG	
Yes/No	7/24
GVHD prophylaxis	
CSA	6
CSA + others (MTX/steroid/MMF)	10
Tacrolimus	7
Tacrolimus + others (MTX/steroid)	8
MNC cell dose	
$\geq 2.0 \times 10^7$/kg, $< 3.0 \times 10^7$/kg	15
$\geq 3.0 \times 10^7$/kg	16
CFU-GM cell dose	
$< 2.0 \times 10^4$/kg	14
$\geq 2.0 \times 10^4$/kg	15
Unknown	2
CD34 cell dose	
$< 1.0 \times 10^5$/kg	10
$\geq 1.0 \times 10^5$/kg	15
Unknown	6

TBI indicates total body irradiation; TAI, thoracoabdominal irradiation; MEL, melphalan; FLU, fludarabine; CY, cyclophosphamide; ATG, antithymocyte globulin; CSA, cyclosporine; MTX, methotrexate; MMF, mycophenolate mofetil; MNC, mononuclear cell; CFU-GM, colony-forming unit granulocyte-macrophage.

and 1 underwent thoracoabdominal irradiation. A total of 7 patients were administered with ATG, either horse ATG (Lymphoglobulin 30-75 mg/kg in 5 patients) or rabbit ATG (Thymoglobulin 10 mg/kg in 2 patients). GVHD prophylaxis also varied according to the individual centers (Table 2). To facilitate the recovery of neutrophils, all patients received recombinant human granulocyte colony-stimulating factor. The number of mononucleated cells, colony-forming units of granulocyte-macrophage (CFU-GM), and CD34-positive cells of the cord blood units at the time of freezing are shown in Table 2.

Definitions and Statistical Analysis

The status of all patients was evaluated based on the last follow-up report, which was performed using the standardized forms provided by the JCBBN. All results were analyzed as of June 2008.

Date of engraftment was defined as the first of the 3 consecutive days where the neutrophil recovery was $>0.5 \times 10^9$/L. Platelet recovery was defined as the first of the 3 consecutive days where the unsupported platelet count was $>50 \times 10^9$/L. Chimerism was evaluated in 12 patients, with fluorescent in situ hybridization for the Y chromosome performed in 6 sex-mismatched grafts and quantitative polymerase chain reaction analysis for microsatellite DNA markers performed in 6 sex-matched transplantations. Acute and chronic GVHD (aGVHD, cGVHD) were diagnosed and graded according to standard clinical criteria [18,19].

Probability of OS was estimated according to the Kaplan-Meier method. GVHD and engraftment were assessed using the cumulative incidence procedure, and death was the competing event. Univariate comparisons among various groups were made using the log-rank test. The variables evaluated included age of the patient, donor sex, sex mismatch, disease duration before UCBT, the number of pre-UCBT transfusions for red cells and platelets, IST before UCBT, HLA matching by serology and high-resolution DNA typing for both GVHD and rejection directions, the number of mononuclear cells, CFU-GM, CD34-positive cells of the cord blood units at the time of freezing, conditioning regimens, and the administration of ATG and GVHD prophylaxis (single agent versus ≥ 2 agents, MTX versus no MTX, or CSA versus tacrolimus). All statistical analyses were carried out with version 10 of the STATA software (StataCorp, College Station, TX).

RESULTS

Engraftment

Sustained engraftment was observed in 17 patients. The cumulative incidences of the neutrophil and platelet recovery after UCBT were 54.8 and 72.2%, respectively (95% confidence interval [CI] = 36.0%-70.3% and 51.3%-85.3%, respectively; Figure 1). The median times to achieve a neutrophil count $\geq 0.5 \times 10^9$/l and a platelet count $\geq 50 \times 10^9$/l were 19 days (range: 12-35 days) and 59 days (range: 39-145 days), respectively. Chimerism analysis results were available in 8 patients with sustained neutrophil engraftment. All of these patients showed complete donor chimerism with more than 99% donor cells. No mixed chimerism was observed. There were 7 patients who failed to achieve sustained engraftment among patients who survived more than 28 days after UCBT. Five patients did not achieve a primary engraftment. Although 3 of them underwent a second UCBT, all died of infections, with (n = 1) or without (n = 2) engraftment of the second graft. Autologous recovery was noted in 1 patient, which was proven by the chimerism analysis that demonstrated 100% recipient cells. One patient had achieved engraftment on day 19, but she suffered from late graft failure at day 176 and received second HSCT at day 203. The patient was still alive at the time of the last follow-up.

Results of the univariate analysis for engraftment are shown in Table 3. The GVHD prophylaxis with a single agent (CSA or tacrolimus) exhibited a significantly better engraftment rate than that seen for the other methods (75.0% versus 33.3%, $P = 0.02$).

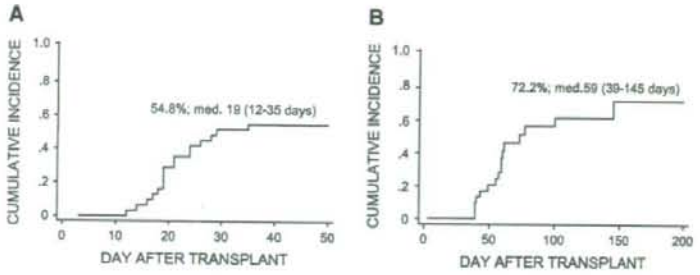

Figure 1. (A) Cumulative incidence of sustained donor neutrophil engraftment ($>0.5 \times 10^9$/L) and (B) platelet engraftment ($>50 \times 10^9$/L) after unrelated cord blood transplantation in patients with aplastic anemia.

When there was a lower number of transfusions (<20 times) of red cells and platelets prior to the HSCT, there was a trend for a better chance of successful engraftment compared to cases where there were higher number of transfusions (≥20 times), although this was not statistically significant. The number of infused MNCs, CFU-GM, and CD34 had no impact on the engraftment.

Table 3. *Outcome following Unrelated Cord Blood Transplantations for Aplastic Anemia: Univariate Analysis*

Covariates	2-Year-OS (%) (95% CI)	P	Engraftment (%) (95% CI)	P
Recipient age				
<20 year (n = 9)	44.4 (13.6-71.9)	.18	44.4 (13.6-71.9)	.76
20- 40 year (n = 12)	56.3 (24.4-79.1)		66.7 (33.7-86.0)	
>40 year (n = 10)	20.0 (3.0-47.5)		50.0 (18.4-75.3)	
Disease duration before UCBT				
<1 year (n = 13)	35.7 (13.0-59.4)	.34	57.1 (28.4-78.0)	.67
≥1 year (n = 12)	47.6 (18.2-72.4)		58.3 (27.0-80.1)	
RBC transfusions before UCBT				
<20 (n = 8)	62.5 (22.9-86.1)	.26	75.0 (31.5-93.1)	.08
≥20 (n = 21)	31.4 (13.1-51.7)		47.6 (25.7-66.7)	
Platelet transfusions before UCBT				
<20 (n = 7)	57.1 (17.2-83.7)	.28	85.7 (33.4-97.9)	.05
≥20 (n = 22)	35.0 (16.1-54.7)		45.4 (24.4-64.3)	
HLA matching by serologic typing (GVHD direction)				
0-1 mismatched (n = 22)	49.2 (27.3-68.0)	.34	63.6 (40.3-79.9)	.10
2 mismatched (n = 9)	22.2 (3.4-51.3)		33.3 (78.3-62.3)	
HLA matching by serologic typing (Rejection direction)				
0-1 mismatched (n = 23)	43.5 (23.3-62.1)	.64	52.2 (30.5-70.0)	.59
2 mismatched (n = 8)	37.5 (8.7-67.4)		62.5 (22.9-86.1)	
Conditioning regimen				
TBI + CY + FLU (n = 5)	80.0 (20.4-96.9)	.02	75.0 (40.8-91.2)	.17
TBI + MEL + FLU (n = 12)	46.9 (17.6-71.9)		80.0 (20.4-96.9)	
Others (n = 14)	21.4 (5.2-44.8)		28.6 (08.8-52.4)	
ATG				
No (n = 24)	48.9 (27.8-67.0)	.007	66.7 (44.3-81.7)	.19
Yes (n = 7)	14.3 (0.7-46.5)		14.3 (0.7-46.5)	
GVHD prophylaxis				
CSA or tacrolimus only (n = 13)	54.6 (27.4-75.3)	.07	75.0 (46.3-89.8)	.02
CSA or tacrolimus+others (n = 18)	26.7 (8.3-49.6)		33.3 (12.2-56.4)	
MTX				
No (n = 20)	38.5 (17.7-59.1)	.93	60.0 (35.7-77.6)	.24
Yes (n = 11)	45.5 (16.7-70.7)		45.5 (16.7-70.7)	
MNC				
2×10^7/kg-3×10^7/kg (n = 15)	45.0 (19.4-67.8)	.61	60.0 (31.8-79.7)	.70
$\geq 3 \times 10^7$/kg (n = 15)	37.5 (15.4-59.8)		50.0 (24.-71.0)	
CD34				
$<1 \times 10^5$/kg (n = 15)	45.7 (14.3-73.0)	.32	70.0 (32.9-89.2)	.52
$\geq 1 \times 10^5$/kg (n = 15)	33.3 (12.2-56.4)		53.3 (26.3-74.4)	

GVHD indicates graft-versus-host disease; TBI, total-body irradiation; CY, cyclophosphamide; Mel, melpharan; Flu, fludarabine; ATG, antithymocyte globulin; CSA, cyclosporine; MTX, methotrexate; MNC, mononuclear cell; CFU-GM, colony-forming unit-granulocyte macrophage; UCBT, unrelated cord blood transplantation.

GVHD and Viral Infections

Acute GVHD (≥grade II) was observed in 5 patients (grade II; n = 4, grade III; n = 1) on days 8 through 56, and was lethal in the 1 patient with grade III aGVHD. Chronic GVHD was observed in 4 patients (extensive: n = 1, limited: n = 3; de novo n = 2, progression from aGVHD n = 2) on days 124 through 213. Figure 2 depicts the cumulative incidence of grade II-IV aGVHD (17.1%; 95% CI = 6.2%-32.8%) and cGVHD (19.7%; 95% CI = 6.2%-38.8%). Viral reactivations were commonly observed in this study. CMV reactivation was noted in 9 patients, and 1 of them developed CMV disease. Epstein-Barr virus (EBV) reactivation was noted in 1 patient, having developed cerebral infarction, which was considered to be related with EBV. Adenovirus induced cystitis occurred in 1 patient.

Survival

Of the 31 total patients, 13 are presently alive, with survival durations of 6 to 77 months (median 33.7 months) after the transplantations. The probability of OS at 2 years was 41.1% (95% CI = 23.8%-57.7%). The results of univariate analysis of the factors influencing survival are shown in Table 3. The conditioning regimen and the administration of ATG were the only factors that were significantly related to the survival. The conditioning regimen, which included low-dose TBI, FLU, and CY, resulted in better outcomes than were seen for the other regimens (Table 3 and Figure 3). The administration of ATG was associated with poor outcome (Table 3 and Figure 3). There were 5 out of 7 patients given ATG that died before engraftment because of infections (n = 3) or hepatic veno-occlusive disease (VOD) (n = 2). In the 2 other patients, 1 demonstrated autologous recovery, whereas the other patient has had sustained engraftment and is currently still alive. There tended to be a better outcome noted for GVHD prophylaxis with a single agent (either CSA or tacrolimus) compared to prophylaxis with 2 or more agents. The outcome for the patients aged 40 years and older was inferior to that seen for the younger patients, although this was not statistically significant.

In the 18 patients who died, the causes of death were graft failure (n = 7), bacterial/fungal infections (n = 3), EBV-related cerebral infarction (n = 1), VOD (n = 3), aGVHD (n = 1), acute respiratory distress syndrome (n = 1), encephalopathy (n = 1), and cardiac toxicity (n = 1).

DISCUSSION

The outcome of 31 patients with SAA who received UCBT was analyzed in this study. This is the first report on a nationwide multicentral study that focused on UCBT for SAA as far as we know. The overall survival rate was 41%, which is comparative to the results of the large registry-based analysis of UD-BMT for SAA by CIBMTR [5], but inferior to the results of some recent reports of UD-BMT [6,20]. The incidence and the severity of aGVHD and cGVHD were considerably lower in this study, which is advantageous for UCBT. The major problem encountered, however, was still the high incidence of engraftment failure after UCBT. In the present study the conditioning regimen with the low-dose TBI, FLU, and CY resulted in better outcome (80% survival rate) compared to other regimens. This regimen and the selection of optimal donor with better HLA match and higher cell dose may improve the outcome of UCBT for SAA.

Previous reports on the conditioning regimen of UCBT for SAA are limited. Mao et al. [13] reported on 9 patients with SAA who were conditioned with ATG and CY (60 mg/kg) prior to undergoing UCBT. A total of 7 out of 9 of these patients survived with hematologic recovery. However, a donor-recipient mixed chimerism was present in all patients. There are a few case reports of UCBT for SAA using more intensified regiments, which resulted in successful engraftment along with complete chimerism [14-16,21].

Radiation-containing regimens are efficient in achieving better engraftments and widely used within the UD-BMT settings for patients with SAA, although these regimens are associated with significant early and late toxicities, including secondary malignancies [22]. Recent study by Deeg et al. [20] to define the optimal TBI dose in combination with CY (200 mg/kg) and

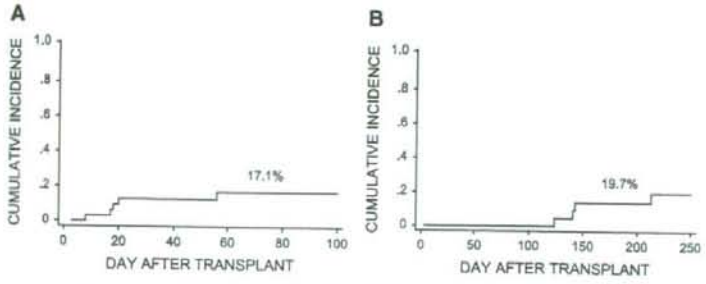

Figure 2. Cumulative incidence of ≥ grade II aGVHD (A) and cGVHD (B) in patients with aplastic anemia who received UCBT.

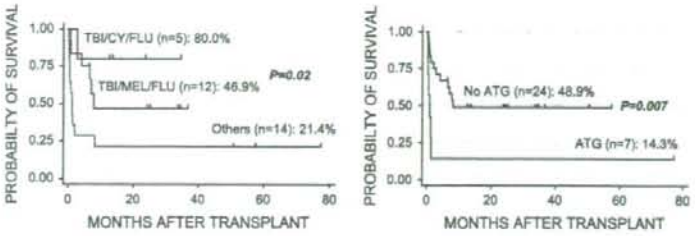

Figure 3. Probability of survival after conditioning regimens in patients with aplastic anemia, who received unrelated cord blood transplantation. TBI: total body irradiation, CY: cyclophosphamide, MEL: melphalan, FLU: fludarabine, ATG: antithymocyte globulin.

ATG for use with UD-BMT in patients with SAA showed that 2 Gy was sufficient to allow engraftment without increasing toxicities. This finding was also supported by a Japanese study on UD-BMT in patients with SAA, which reported that in a small group given a conditioning regimen of low-dose TBI (2-5 Gy), CY (200 mg/kg), and ATG, there was a 90% survival rate [7].

Fludarabine is currently widely used for nonmyeloablative transplants for a variety of diseases including SAA [23-26]. In the recent study on UD-SCT from the Severe Aplastic Anemia Working Party of the EBMT (SAA WP-EBMT), they designed a non-TBI regimen that used FLU (120 mg/m^2), CY (1200 mg/m^2), and ATG [27]. In this study, a total of 38 both pediatric and adults patients with SAA were included (36 BMT and 2 PBSCT patients) and the 2-year survival rate was 73%, with a low incidence of aGVHD and cGVHD. Therefore, this result suggests that a FLU containing regimen might be effective for use with UD-HSCT in SAA. The authors suggested that the conditioning regimen might need to be modified for adults through the addition of a low dose of TBI, as there was a significantly lower engraftment rate seen in the adult patients (82% overall, 68% in adults). Overall, these findings in previous reports and in this study suggest that the conditioning regimen that included the low-dose TBI and FLU resulted in favorable outcomes. In present study, the 7 patients given ATG were poor. Only 1 of them achieved engraftment and is alive. However, the number of patients given ATG was too small to reach any definitive conclusions and the benefit of ATG in UCBT for SAA should be evaluated in a large prospective study.

The GVHD prophylaxis using a single agent (CSA or tacrolimus) exhibited a better engraftment rate and a marginally better survival rate compared to that seen when 2 or more immunosuppressive agents were used. In the latter group, steroid, MTX, or mycophenolate mofetil (MMF) were given in addition to CSA or tacrolimus. Because of the limited number of patients and the highly heterogeneous regimen of the GVHD prophylaxis in this study, it is difficult to define the optimal GVHD prophylaxis based on the current results. However, the low incidence and severity of GVHD that we noted in our study suggests that a single agent, regardless of whether it is tacrolimus or CSA, may be effective enough to prevent GVHD in UCBT for SAA.

One of the most important factors that determine the success of UCBT is the cell dose in the CB [11,28-30]. In the present study, a minimum of 2×10^7/kg MNCs were infused in all patients. In this condition, the dose of MNCs, CFU-GM, and CD34 had no impact on engraftment and survival. One of the benefits of UCBT is that it can overcome the HLA barrier. Despite the HLA disparity in the majority of the patients, the incidence of GVHD was quite low in this study. There was a tendency for better HLA matching to result in a better outcome, although this was not statistically significant. Selection of the CB units with higher cell dose and better HLA match may be essential to improve the outcome of UCBT for SAA.

In our study there were also 8 patients who were older than 50 years of age, which is generally considered to be over than the cutoff age for transplantation. Because of the poor outcome of UCBT in older patients (OS = 20% in group with age >40 years old), UCBT cannot be recommended for older patients at present, and repeated IST should be considered in these patients [31,32].

In summary, this first multicentral study focused on the UCBT for SAA suggests that UCBT can be an alternative treatment for SAA patients who failed to IST and have no suitable bone marrow donor. The results may be improved by using the optimal conditioning regimen such as low-dose TBI, FLU, and CY and by donor selection of better HLA match and higher cell dose.

ACKNOWLEDGMENTS

This work was supported by a Research Grant for Tissue Engineering (H17-014) from the Japanese Ministry of Health, Labor, and Welfare. The authors would like to thank all of the staff members of the collaborating institutes of the Japan Cord Blood Bank Network for taking care of and reporting information on the patients. We would also like to thank Dr. Yoshiko Atsuta for the statistical analysis support.

REFERENCES

1. Young NS, Calado RT, Scheinberg P. Current concepts in the pathophysiology and treatment of aplastic anemia. *Blood.* 2006; 108:2509-2519.
2. Kojima S, Frickhofen N, Deeg HJ, et al. Aplastic anemia. *Int J Hematol.* 2005;82:408-411.
3. Marsh JC. Management of acquired aplastic anaemia. *Blood Rev.* 2005;19:143-151.
4. Storb R, Blume KG, O'Donnell MR, et al. Cyclophosphamide and antithymocyte globulin to condition patients with aplastic anemia for allogeneic marrow transplantations: the experience in four centers. *Biol Blood Marrow Transplant.* 2001;7:39-44.
5. Passweg JR, Perez WS, Eapen M, et al. Bone marrow transplants from mismatched related and unrelated donors for severe aplastic anemia. *Bone Marrow Transplant.* 2006;37:641-649.
6. Kojima S, Matsuyama T, Kato S, et al. Outcome of 154 patients with severe aplastic anemia who received transplants from unrelated donors: the Japan Marrow Donor Program. *Blood.* 2002; 100:799-803.
7. Viollier R, Socie G, Tichelli A, et al. Recent improvement in outcome of unrelated donor transplantation for aplastic anemia. *Bone Marrow Transplant.* 2008;41:45-50.
8. Maury S, Balere-Appert ML, Chir Z, et al. Unrelated stem cell transplantation for severe acquired aplastic anemia: improved outcome in the era of high-resolution HLA matching between donor and recipient. *Haematologica.* 2007;92:589-596.
9. Kennedy-Nasser AA, Leung KS, Mahajan A, et al. Comparable outcomes of matched-related and alternative donor stem cell transplantation for pediatric severe aplastic anemia. *Biol Blood Marrow Transplant.* 2006;12:1277-1284.
10. Kojima S, Inaba J, Kondo M, et al. Unrelated donor marrow transplantation for severe acquired aplastic anemia using cyclophosphamide, antithymocyte globulin, and total body irradiation. *Blood.* 1995;85:291-292.
11. Rubinstein P, Carrier C, Scaradavou A, et al. Outcomes among 562 recipients of placental-blood transplants from unrelated donors. *N Engl J Med.* 1998;339:1565-1577.
12. Neudorf SM, Blatt J, Corey S, et al. Graft failure after an umbilical cord blood transplant in a patient with severe aplastic anemia. *Blood.* 1995;85:2991-2992.
13. Mao P, Zhu Z, Wang H, et al. Sustained and stable hematopoietic donor-recipient mixed chimerism after unrelated cord blood transplantation for adult patients with severe aplastic anemia. *Eur J Haematol.* 2005;75:430-435.
14. Ohga S, Ichino K, Goto K, et al. Unrelated donor cord blood transplantation for childhood severe aplastic anemia after a modified conditioning. *Pediatr Transplant.* 2006;10:497-500.
15. Lim SH, Zhang Y, Wang Z, et al. Umbilical cord blood transplant in hepatitis C-associated severe aplastic anemia. *Bone Marrow Transplant.* 2004;33:565-567.
16. Kusumi E, Miyakoshi S, Murashige N, et al. Successful reduced-intensity stem cell transplantation (RIST) with mismatched cord blood in a 70-year-old patient with severe aplastic anemia (SAA). *Bone Marrow Transplant.* 2003;32:1111-1112.
17. Lau FY, Wong R, Chui CH, Cheng G. Successful engraftment in two adult patients with severe aplastic anemia using nonmyeloablative conditioning followed by unrelated HLA-mismatched cord blood transplantation. *J Hematother Stem Cell Res.* 2001;10:309-311.
18. Glucksberg H, Storb R, Fefer A, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. *Transplantation.* 1974;18: 295-304.
19. Shulman HM, Sullivan KM, Weiden PL, et al. Chronic graft-versus-host syndrome in man. A long-term clinicopathologic study of 20 Seattle patients. *Am J Med.* 1980;69:204-217.
20. Deeg HJ, O'Donnell M, Tolar J, et al. Optimization of conditioning for marrow transplantation from unrelated donors for patients with aplastic anemia after failure of immunosuppressive therapy. *Blood.* 2006;108:1485-1491.
21. Shaw PH, Haut PR, Olszewski M, Kletzel M. Hematopoietic stem-cell transplantation using unrelated cord-blood versus matched sibling marrow in pediatric bone marrow failure syndrome: one center's experience. *Pediatr Transplant.* 1999;3: 315-321.
22. Champlin RE, Horowitz MM, van Bekkum DW, et al. Graft failure following bone marrow transplantation for severe aplastic anemia: risk factors and treatment results. *Blood.* 1989;73: 606-613.
23. Giralt S, Logan B, Rizzo D, et al. Reduced-intensity conditioning for unrelated donor progenitor cell transplantation: long-term follow-up of the first 285 reported to the national marrow donor program. *Biol Blood Marrow Transplant.* 2007;13: 844-852.
24. Giralt S, Thall PF, Khouri I, et al. Melphalan and purine analog-containing preparative regimens: reduced-intensity conditioning for patients with hematologic malignancies undergoing allogeneic progenitor cell transplantation. *Blood.* 2001;97: 631-637.
25. Kang HJ, Shin HY, Choi HS, Ahn HS. Fludarabine, cyclophosphamide plus thymoglobulin conditioning regimen for unrelated bone marrow transplantation in severe aplastic anemia. *Bone Marrow Transplant.* 2004;34:939-943.
26. George B, Mathews V, Shaji RV, et al. Fludarabine-based conditioning for allogeneic stem cell transplantation for multiply transfused patients with Fanconi's anemia. *Bone Marrow Transplant.* 2005;35:341-343.
27. Bacigalupo A, Locatelli F, Lanino E, et al. Fludarabine, cyclophosphamide and anti-thymocyte globulin for alternative donor transplants in acquired severe aplastic anemia: a report from the EBMT-SAA Working Party. *Bone Marrow Transplant.* 2005;36: 947-950.
28. Wagner JE, Barker JN, Defor TE, et al. Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival. *Blood.* 2002;100:1611-1618.
29. Kurtzberg J, Laughlin M, Graham ML, et al. Placental blood as a source of hematopoietic stem cells for transplantation into unrelated recipients. *N Engl J Med.* 1996;335:157-166.
30. Gluckman E, Rocha V, Boyer-Chammard A, et al. Outcome of cord-blood transplantation from related and unrelated donors. Eurocord Transplant Group and the European Blood and Marrow Transplantation Group. *N Engl J Med.* 1997;337:373-381.
31. Tichelli A, Passweg J, Nissen C, et al. Repeated treatment with horse antilymphocyte globulin for severe aplastic anaemia. *Br J Haematol.* 1998;100:393-400.
32. Di Bona E, Rodeghiero F, Bruno B, et al. Rabbit antithymocyte globulin (r-ATG) plus cyclosporine and granulocyte colony stimulating factor is an effective treatment for aplastic anaemia patients unresponsive to a first course of intensive immunosuppressive therapy. Gruppo Italiano Trapianto di Midollo Osseo (GITMO). *Br J Haematol.* 1999;107:330-334.

STEM CELLS

TISSUE-SPECIFIC STEM CELLS

Quiescent Human Hematopoietic Stem Cells in the Bone Marrow Niches Organize the Hierarchical Structure of Hematopoiesis

TAKASHI YAHATA,[a,b,c] YUKARI MUGURUMA,[a] SHIZU YUMINO,[a] YIN SHENG,[a] TOMOKO UNO,[a] HIDEYUKI MATSUZAWA,[a] MAMORU ITO,[d] SHUNICHI KATO,[a,c] TOMOMITSU HOTTA,[a,b] KIYOSHI ANDO[a,b]

[a]Division of Hematopoiesis, Research Center for Regenerative Medicine; [b]Department of Hematology; [c]Department of Cell Transplantation and Regenerative Medicine; Tokai University School of Medicine, Kanagawa, Japan; [d]Central Institute for Experimental Animals, Kawasaki, Japan

Key Words. Cell cycle • Clonal assays • Hematopoietic stem cell transplantation • Long-term repopulation • Mesenchymal stem cells • Stem cell-microenvironment interactions • Human hematopoietic stem cells • Severe combined immunodeficient repopulating cell

ABSTRACT

Hematopoiesis is a dynamic and strictly regulated process orchestrated by self-renewing hematopoietic stem cells (HSCs) and the supporting microenvironment. However, the exact mechanisms by which individual human HSCs sustain hematopoietic homeostasis remain to be clarified. To understand how the long-term repopulating cell (LTRC) activity of individual human HSCs and the hematopoietic hierarchy are maintained in the bone marrow (BM) microenvironment, we traced the repopulating dynamics of individual human HSC clones using viral integration site analysis. Our study presents several lines of evidence regarding the in vivo dynamics of human hematopoiesis. First, human LTRCs existed in a rare population of $CD34^+CD38^-$ cells that localized to the stem cell niches and maintained their stem cell activities while being in a quiescent state. Second, clonally distinct LTRCs controlled hematopoietic homeostasis and created a stem cell pool hierarchy by asymmetric self-renewal division that produced lineage-restricted short-term repopulating cells and long-lasting LTRCs. Third, we demonstrated that quiescent LTRC clones expanded remarkably to reconstitute the hematopoiesis of the secondary recipient. Finally, we further demonstrated that human mesenchymal stem cells differentiated into key components of the niche and maintained LTRC activity by closely interacting with quiescent human LTRCs, resulting in more LTRCs. Taken together, this study provides a novel insight into repopulation dynamics, turnover, hierarchical structure, and the cell cycle status of human HSCs in the recipient BM microenvironment. STEM CELLS *2008;26:3228–3236*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

One of the essential features of hematopoietic stem cells (HSCs) is their ability to remain in a quiescent state to maintain long-term repopulating activity [1]. Regulatory mechanisms that govern this quiescent state are crucial for organizing the hierarchical structure of hematopoiesis and are also of critical biological importance in preventing premature HSC exhaustion under conditions of hematopoietic stress. Several murine studies have demonstrated that interactions between HSCs and stem cell niches, specialized bone marrow (BM) microenvironments created by supporting cells, via receptor-ligand interactions and cell-adhesion molecules expressed in both cell types play central roles in regulating stem cell properties [2]. At present, at least two distinct niches have been identified in the endosteal areas of BM: the osteoblastic niche and the vascular niche [3–5]. However, it remains unclear whether these principles of the niche-HSC regulatory system, extrapolated from murine studies, could apply to human situations.

The severe combined immunodeficient (SCID) mouse-repopulating cell (SRC) assay is considered to be the most reliable research tool for in vivo analysis of the biological processes of human hematopoiesis [6]. In this assay system, SRCs, defined by their ability to reconstitute human hematopoiesis in immunodeficient mice, can be classified into several subtypes on the basis of the lineage restriction of their progenies and the timing of their appearance after transplantation. Although long-term repopulating cell (LTRC) activities with lymphomyeloid potential are mostly restricted to the $CD34^+CD38^{neg}$ population, the $CD34^+CD38^+$ population exhibits only short-term repopulating cell (STRC) activities [7–9]. STRCs are further subdivided into myeloid-restricted STRCs (STRC-Ms) and lymphomyeloid STRCs (STRC-MLs) [10]. The hierarchical relationships of each SRC pop-

Author contributions: T.Y.: conception and design, collection and assembly of data, data analysis and interpretation, and manuscript writing; Y.M.: collection and assembly of data, data analysis and interpretation, and manuscript writing; S.Y., Y.S., T.U., and H.M.: collection of data; M.I., S.K.: provision of study material; T.H. and K.A.: conception and design, financial support, and final approval of manuscript.

Correspondence: Kiyoshi Ando, M.D., Ph.D., Division of Hematopoiesis, Research Center for Regenerative Medicine and Department of Hematology, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan. Telephone: 81-463-93-1121; Fax: 81-463-92-4511; e-mail: andok@keyaki.cc.u-tokai.ac.jp Received June 11, 2008; accepted for publication September 3, 2008; first published online in STEM CELLS *EXPRESS* September 11, 2008. 1066-5099/2008/$30.00/0 doi: 10.1634/stemcells.2008-0552

ulation and the precise characteristics regarding functions of individual human HSCs remain elusive.

Clonal analysis of the unique proviral integration sites is a powerful approach to identify and trace the activity of individual SRCs for comprehensive understanding of the properties of individual HSCs [11]. Random and permanent integration of virus vectors into the host cell genome makes the vector-genomic DNA junction a unique marker by which to identify the originally transduced cells and their progenies. By combining lineage-cell sorting and a linear amplification-mediated (LAM)-polymerase chain reaction (PCR) technique that verifies individual proviral integration into the human genome by direct sequence, we have developed a strategy by which to examine the multipotency of a single human HSC from two angles [12]. First, clonal analysis of each lineage cell proves the presence of the repopulating cell, which is the ancestor of the currently analyzed lineage cell (retrospective identification). Second, the stem cell phenotype and the capacity of individual SRC clones at the time of analysis distinguish self-renewing clones and differentiating clones (current status identification). Using this approach, we have recently documented heterogeneity among individual SRC clones regarding their stem cell activity, differentiation potential, and clonal longevity within the stem cell pool [12].

In this study, we investigated the in vivo repopulating dynamics of 228 sequence-verified individual SRC clones with respect to differentiation potential, self-renewal capacity, and cell cycle status in the niche. We successfully identified human LTRCs that were responsible for lifelong hematopoiesis in the BM microenvironment. Those human LTRCs localized to and interacted with the key components of the stem cell niche in a quiescent state. Once activated, they divided into LTRCs and STRCs by asymmetric self-renewal division, ultimately creating a hierarchical hematopoietic structure.

MATERIALS AND METHODS

Collection and Fractionation of Cord Blood CD34$^+$ Cells

Cord blood (CB) samples were obtained from full-term deliveries according to the institutional guidelines approved by the Tokai University Committee on Clinical Investigation. CD34$^+$ cell fractions were prepared using the CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Sunnyvale, CA, http://www.miltenyibiotec.com). Pooled CD34$^+$-enriched cells from multiple donors were stained with allophycocyanin (APC)-conjugated anti-lineage-specific antigens CD3 (UCHT1), CD41 (P2), glycophorin A (11E4B-7-6) (all from Coulter/Immunotech, Marseille, France), CD14 (MfP9), CD19 (SJ25C1), and CD56 (NCAM16.2) (all from BD Biosciences, San Jose, CA, http://www.bdbiosciences.com) and with phycoerythrin (PE)-conjugated anti-CD38 (HB7; BD Biosciences) and fluorescein isothiocyanate (FITC)-conjugated anti-CD34 (581; Beckman Coulter, Fullerton, CA, http://www.beckmancoulter.com) monoclonal antibodies. Cells were gated on lineage marker-negative and/or low-expression region, and Lin$^{-/low}$CD34$^+$ cells were fractionated according to their CD38 expression levels using the FACSVantage flow cytometer (BD Biosciences). To eliminate the contamination of each subpopulation, we performed two consecutive rounds of cell sorting to ensure more than 99% cell purity.

Lentivirus Infection

Fractionated CD34$^+$ cells were plated on fibronectin CH-296 fragment (Takara Shuzo, Shiga, Japan, http://www.takara-bio.co.jp) and incubated with highly concentrated viral supernatant at a multiplicity of infection of 50 in serum-free StemPro-34 medium (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) containing the following cytokines for 16 hours: recombinant human thrombopoietin (50 ng/ml; kindly donated by Kirin Brewery Co., Tokyo, http://www.kirin.co.jp/english), stem cell factor (50 ng/ml; donated by Kirin Brewery), and Flk-2/Flt-3 ligand (50 ng/ml; R&D Systems Inc., Minneapolis, http://www.rndsystems.com). The infection efficiency was 72.4% ± 8.5%.

Human Hematopoietic Repopulation

NOD/Shi-scid, IL-2Rγc^{null} (NOG) mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan) and maintained in the animal facility of the Tokai University School of Medicine in microisolator cages; the animals were fed with autoclaved food and water. This strain exhibits a high engraftment rate of human hematopoietic cells than other existing strains such as NOD/SCID and NOD/SCID/$\beta 2m^{null}$, allowing the reconstitution capacity and lymphomyeloid differentiation of human repopulating stem cells to be assessed [13, 14]. Nine- to 20-week-old NOG mice were irradiated with 250 cGy of x-rays. The following day, transduced cells were injected into the retro-orbital plexus of the NOG mice. At the indicated times after transplantation, the mice were humanely killed, and BM cells, splenocytes, and thymocytes were harvested. Human hematopoietic cells were distinguished from mouse cells by the expression of human CD45. Enhanced green fluorescent protein (EGFP)-expressing CD45$^+$ human hematopoietic cells were further classified into human stem/progenitor (CD34$^+$), myeloid (CD33$^+$), B-lymphoid (CD19$^+$), and T-lymphoid (CD3$^+$ or CD4$^+$/CD8$^+$) subpopulations and were sorted using a FACSVantage Diva option (BD Biosciences). Sorted populations had purities of more than 99%. All experiments were approved by the animal care committee of Tokai University.

Secondary Transplantation

BM cells were obtained from mice transplanted with CD34$^+$CD38^{neg} cells at 18 weeks after transplantation, and CD34$^+$ cells were isolated by cell sorting. Purified CD34$^+$ cells of the primary recipients were divided in half and injected intravenously into two sublethally irradiated secondary NOG recipients (1.5×10^6 cells per recipient). One recipient was sacrificed at 3 weeks and the other at 18 weeks after transplantation. BM cells and thymocytes were collected from each secondary recipient and used for flow cytometric analysis and lineage cell sorting as described earlier.

Integration Site Analysis of Lentivirally Marked SRCs

Genomic DNA isolation and LAM-PCR were carried out as described previously [12]. The proviral integration sites of CD33$^+$ cells were sequenced, and the sequences were examined for alignment to the human genome using NCBI BlastN (http://www.ncbi.nlm.nih.gov/blast). The verified genomic sequence information of these CD33$^+$ cell integration sites was used to design new primers (all primer sequences used in this study are listed in supplemental online Tables 1 and 2). PCR was performed on each LAM-PCR product using the unique genomic flanking primers.

Estimation of Clone Size by Real-Time Quantitative PCR

The relative clone size of individual LTRC clones that was detected in the CD34$^+$ cells of primary and secondary recipients was examined as described previously [12]. For real-time quantitative PCR, each target DNA was amplified on the same plate, with β-globin as the reference, using the QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany, http://www1.qiagen.com) and the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com). The relative clone amounts and range were determined in reference to β-globin. A comparative threshold cycle (C_T) was used to determine the proportion of CD34$^+$ clones in paired recipients. For each sample, the clone C_T value was normalized using the formula $\Delta C_T = \Delta C_{T\ clone} - \Delta C_{T\ \beta\text{-globin}}$. To determine relative clone size, the following formula was used: $\Delta\Delta C_T = \Delta C_{T\ clone}$ in CD34$^+$ cells of the primary recipient $- \Delta C_{T\ clone}$ in CD34$^+$ cells of the secondary

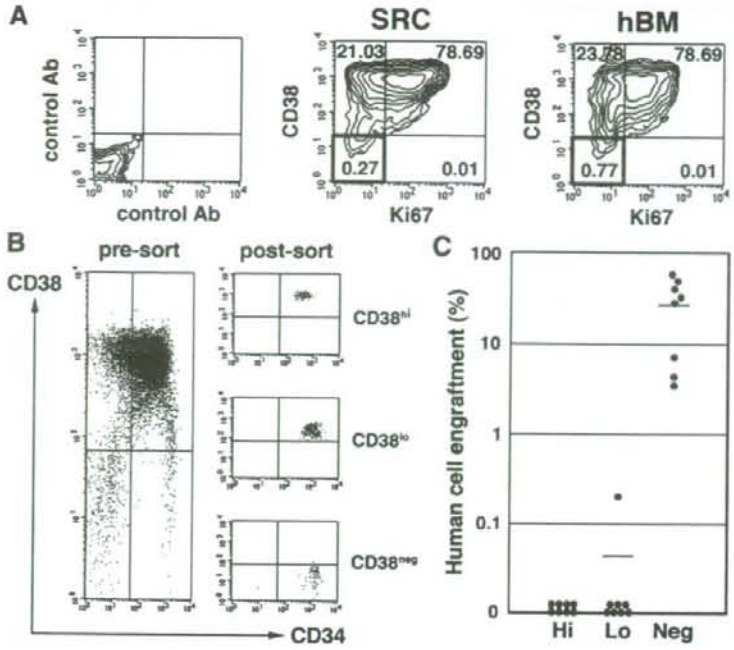

Figure 1. Cell cycle status and stem cell activity of $CD34^+$ cells. **(A):** Bone marrow (BM) $CD34^+$ cells were analyzed for their expression of CD38 and Ki-67 by flow cytometry. Representative fluorescence-activated cell sorting profiles of isotype control; $CD34^+$ cells in the NOD/Shi-scid, IL-$2R\gamma c^{null}$ (NOG) recipient BM; and freshly isolated hBM $CD34^+$ cells from five independent experiments are shown. The relative frequencies of each population are indicated. **(B):** Representative sorting profiles of $CD34^+$ cells obtained from primary recipient BM. Fractionated $CD34^+$ cells were transplanted into secondary NOG hosts ($CD34^+CD38^{neg}$, $1–7 \times 10^4$ cells; $CD34^+CD38^{lo}$, $1–3.5 \times 10^5$ cells; $CD34^+CD38^{hi}$, $5–8 \times 10^5$ cells). **(C):** Secondary recipient BM cells were analyzed for the expression of human CD45. Each symbol represents one mouse, and horizontal bars indicate the average engraftment level in three independent experiments. Abbreviations: Ab, antibody; hBM, human bone marrow; Hi, high; Lo, low; Neg, negative; SRC, severe combined immunodeficient mouse-repopulating cell.

recipient. The value was calculated by the expression $2^{-\Delta\Delta C_T}$. Each reaction was performed at least in triplicate.

Cell Cycle Analysis

$CD34^+$ BM cells were stained with PE-conjugated anti-CD38 and APC-conjugated anti-CD34 antibodies. The cells were then fixed and permeabilized using a Cell Permeabilization Kit (BD Biosciences) and stained with FITC-conjugated anti-Ki-67 antibody (BD Biosciences). Fluorescence-activated cell sorting analysis was performed on a FACSCalibur using CELLQuest software (BD Biosciences).

Histological Analysis of BM Microenvironment

Tissue processing and immunofluorescent staining were performed as described previously [15]. For in situ examination of transplanted nonhematopoietic human cells, genetically EGFP-marked human mesenchymal stem cells (MSCs) were transplanted directly into the right tibias of NOG mice. The following antibodies were used for tissue immunostaining: anti-human CD34 (My10; BD Biosciences), anti-human CD38 (HI12; BD Biosciences), anti-human CD31 (TECHNE, Minneapolis, http://www.techne-corp.com), anti-human SDF-1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com), and anti-proliferating cell nuclear antigen (anti-PCNA; Abcam, Cambridge, U.K., http://www.abcam.com). Immunofluorescent-stained slides were examined, and images were captured using an LSM510 META confocal microscope (Carl Zeiss, Jena, Germany, http://www.zeiss.com). Images were processed by Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA, http://www.adobe.com).

Statistical Analysis

Data are represented as mean ± SD. p values $<.05$ were considered to be significant.

Results

Only Quiescent $CD34^+CD38^{neg}$ Cells in the Recipient Sustain Long-Term Hematopoiesis

To investigate the characteristics of human LTRCs in the recipient BM, we transplanted CB $CD34^+CD38^{neg}$ cells into sublethally irradiated NOG mice. Eighteen weeks after transplantation, we examined the cell cycle status and repopulating ability of engrafted $CD34^+$ cells in recipient BM. $CD38^{neg}$ cells accounted for a very small population of $CD34^+$ cells in the BM of transplanted mice ($1.33\% \pm 1.18\%$; $n = 7$). The expression of Ki-67, a nuclear protein expressed during all active parts of the cell cycle in proliferating cells, was examined to evaluate the cell cycle status of $CD34^+$ cells in the BM of transplanted mice. Although a majority of $CD34^+CD38^+$ cells expressed Ki-67, less than 0.1% of $CD34^+CD38^{neg}$ cells were positive for Ki-67 (Fig. 1A), confirming that $CD34^+CD38^{neg}$ cells in the recipient BM were indeed dormant. Interestingly, the cell surface phenotype, high levels of CD38 expression in the majority of $CD34^+$ cells, and the cell cycle status of $CD34^+$ cells in the NOG mice closely resembled those of human BM-derived $CD34^+$ cells (Fig. 1A), suggesting that the human BM microenvironment of NOG mouse can substitute, at least in part, for the function of a human BM microenvironment.

To examine the LTRC activity of $CD34^+$ cells in recipient BM, we separated $CD34^+$ cells into $CD38^{neg}$, $CD38^{lo}$, and $CD38^{hi}$ fractions and transplanted these fractions into secondary NOG hosts (Fig. 1B). Secondary transplantable LTRCs were found almost exclusively in the extremely rare fraction of $CD34^+CD38^{neg}$ cells (Fig. 1C). These cells were able to display multilineage engraftment in secondary recipients (supplemental online Fig. 1). Our results revealed that $CD34^+CD38^{neg}$ cells in recipient BM stayed in the quiescent state and that only these

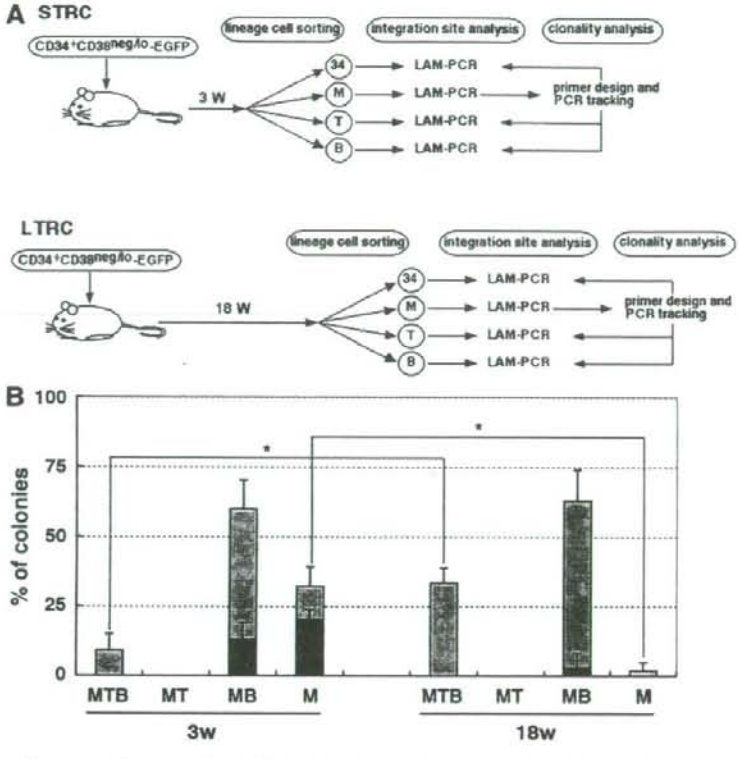

Figure 2. Differentiation ability and self-renewal capacity of individual severe combined immunodeficient mouse-repopulating cell clones. (**A**): Study design for clonal analysis of STRC and LTRC activity. (**B**): Relative frequencies of each clone type. Gray areas in each bar represent the clones detected in $CD34^+$ cells, and black areas represent the clones not detected in $CD34^+$ cells. A total of 116 clones were analyzed (supplemental online Table 1). Mean ± SD of four independent experiments are shown. *, $p < .01$. Abbreviations: 34, $CD34^+$ stem/progenitor cells; B, $CD19^+$ B-lymphoid lineage cells; EGFP, enhanced green fluorescent protein; LAM, linear amplification-mediated; lo, low; LTRC, long-term repopulating cell; M, $CD33^+$ myeloid lineage cells; neg, negative; PCR, polymerase chain reaction; STRC, short-term repopulating cell; T, $CD3^+$ (spleen) or CD4/CD8 DP (thymus) T-lymphoid lineage cells; W, weeks.

cells were able to continuously reconstitute hematopoiesis in vivo.

Repopulation Dynamics of Individual SRC Clones During Human Hematopoietic Reconstitution Originated from $CD34^+CD38^{neg}$ Cells

Next, we dissected out the repopulation dynamics of SRCs derived from the quiescent subpopulations of $CD34^+CD38^{neg}$ cells. Following transplantation, NOG recipient mice were sacrificed at several time points, and the engraftment and development of human hematopoietic cells were analyzed. Mice that received $CD34^+CD38^{neg}$ cells exhibited high levels of $CD45^+$ human hematopoietic cell engraftment at 3 weeks after transplantation (the early phase of repopulation) (supplemental online Fig. 2A). The recipients continued to display high levels of human hematopoietic cell ($CD45^+$) and stem/progenitor cell ($CD34^+$) repopulation between 9 and 18 weeks (the later phase of repopulation). As expected, the human hematopoietic graft in NOG mice consisted mainly of $CD33^+$ myeloid lineage cells at 3 weeks after transplantation, but $CD19^+$ B-lymphoid cells outgrew after 9 weeks (supplemental online Fig. 2B).

To clarify the specific stem cell activity of individual SRCs originating from quiescent $CD34^+CD38^{neg}$ cells that contribute to various stages of hematopoietic reconstitution, we examined the functional aspects of individual SRC clones using LAM-PCR-based viral integration site analysis (Fig. 2A). Each NOG mouse that received EGFP-transduced $CD34^+CD38^{neg}$ population was analyzed at two time points. At each time point, EGFP-expressing human hematopoietic lineage cells were sorted, and the fate of individual SRC clones was examined by clone-tracking analysis. Using primers that were designed on the basis of the genomic sequence information of the $CD33^+$ myeloid cell integration site, we traced distribution of each clone among phenotypically distinct populations: $CD34^+$ stem/progenitor, T-lymphoid, and B-lymphoid cells (all primer sequences are listed in supplemental online Table 1). Three different clone types were observed at each time point: a multipotent type (MTB), in which insertion sites originally detected in $CD33^+$ myeloid cells were also detected in T- and B-lymphoid cell populations; a unipotent progenitor containing exclusively myeloid cells; and a bipotent M/B progenitor. We found that the early phase of hematopoietic reconstitution was mostly attributed to myeloid-restricted clones (Fig. 2B; $p < .01$ compared with later phase of reconstitution). On the other hand, the majority of SRC clones found in the later phase of hematopoiesis were of the multilineage cell-producing type (Fig. 2B; $p < .01$).

We previously reported that the presence or the absence of a common integration site in $CD34^+$ stem cell populations indicated the current status of individual SRC clones in terms of their stem cell function [12]. The three clone types were further examined to determine whether they stayed in the $CD34^+$ stem cell pool (Fig. 2B). Consistent with our previous observations, the proportion of clones that stayed in the $CD34^+$ cell population decreased as the differentiation potential of clones restricted to bipotency or unipotency at 3 weeks, suggesting that initial myeloid-producing STRCs were rapidly exhausted from the $CD34^+$ stem cell pool. At 18 weeks, the vast majority of clones (>95%) stayed in the $CD34^+$ stem cell pool, suggesting the self-replication of virally transduced SRC clones that have the ability to produce both lymphoid and myeloid lineages within the stem cell pool.

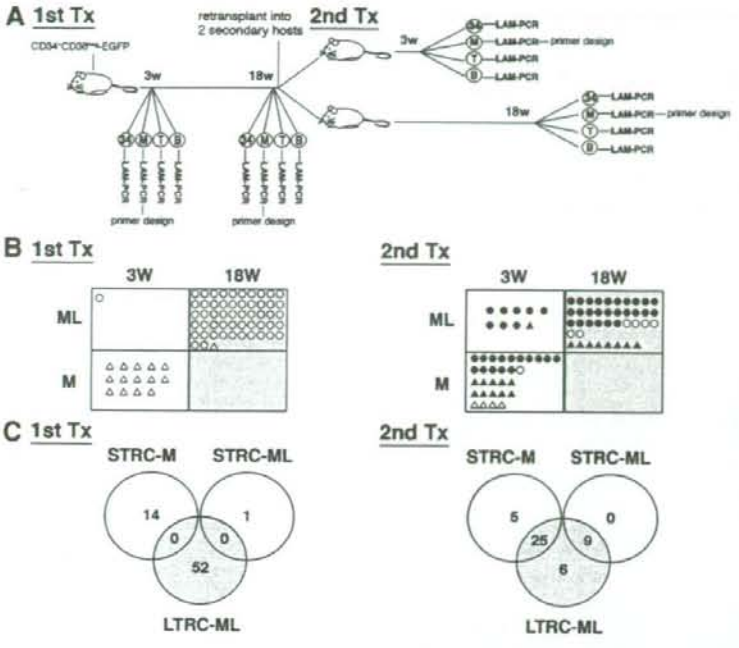

Figure 3. Sequential analysis of individual severe combined immunodeficient mouse-repopulating cell (SRC) clones derived from $CD34^+CD38^{neg}$ population. **(A):** Study design for sequential analysis of individual SRC clones. Lineage-specified cells were separated at the indicated time points, and clone-tracking analysis was performed to examine the presence and persistence of specific clones. **(B):** Summary of the sequential clone-tracking analysis from three independent experiments. A total of 112 clones were analyzed (supplemental online Table 2). Open symbols: clones detected at one time point only. Filled symbols: clones simultaneously detected at two time points. Circles: clones detected in the $CD34^+$ stem cell pool. Triangles: clones not detected in the $CD34^+$ stem cell pool. **(C):** Cumulative number of different types of clones. Numbers in overlapping circles represent clones present at both time points. Abbreviations: B, CD19+ B-lymphoid lineage cells; LAM, linear amplification-mediated; LTRC, long-term repopulating cell; M, CD33+ myeloid lineage cells; ML, multilineage; PCR, polymerase chain reaction; STRC, short-term repopulating cell; T, CD3+ (spleen) or CD4/CD8 DP (thymus) T-lymphoid lineage cells; TX, transplantation; w, weeks.

Sequential SRC Clone-Tracking Analysis Revealed that the Quiescent $CD34^+CD38^{neg}$ Cells Constitute the Hierarchical Organization of Human Hematopoiesis

In the recipients of $CD34^+CD38^{neg}$ cells, the early phase of repopulation was dominated by myeloid-restricted clones (M-clones), as opposed to the later phase, in which the majority of repopulating clones were multilineage type clones (ML-clones) (Fig. 2B). At this point, it is not clear whether the identical clones produced different types of cells depending on the reconstitution phase or independent clones were responsible for either the early or later phase of repopulation. To find out the origin of repopulating clones in the early and later phases, we performed integration site analysis on individual clones recovered from both phases of the same $CD34^+CD38^{neg}$ cell recipient (Fig. 3A, left part; all primer sequences are listed in supplemental online Table 2). At 3 weeks after transplantation, BM cells were aspirated from the tibia of each recipient, and at 18 weeks recipients were sacrificed and BM cells were collected from four long bones. At each time point, EGFP-expressing human hematopoietic lineage cells were sorted for integration site analysis using LAM-PCR. Each proviral integration site serves as a unique marker by which to identify individual clones. When the identical proviral integration site was recognized at different time points, it was regarded as the persistence of the same clone. In that way, we were able to examine the fate of individual SRC clones in the same recipient at different time points. As reported earlier in this study (Fig. 2B), transient M-clones were responsible for the early phase of hematopoietic reconstitution, and self-replicating ML-clones were responsible for the late phase. Interestingly, the transient M-clones detected at the early phase and self-replicating ML-clones were distinctly different (Fig. 3B, 3C, left), indicating that the $CD34^+CD38^{neg}$ population, which is highly enriched for human LTRCs, is composed of distinct clonal subsets that are heterogeneous in repopulating kinetics, lineage cell-producing ability, and self-renewal capacity.

To further examine clonal differences in the repopulating cells, we performed clone-tracking analysis on paired secondary transplanted mice. $CD34^+$ cells recovered from primary recipients at 18 weeks of transplantation were subsequently transplanted into two secondary recipients, one of which was sacrificed at 3 weeks and the other at 18 weeks after secondary transplantation (Fig. 3A, right). Most of the clones (26 of 38; 68.4%) found in the primary recipients did not engraft in the secondary recipient, suggesting that the repopulating potential of most clones deteriorates during long-term reconstitution in the primary recipient. In contrast, all clones detected in paired secondary recipients had originated from the primary LTRC clones. To our interest, in all the clones found in the secondary recipient pair (a total of 45), there was a significant overlap between STRC and LTRC (34 of 45; 75.6%) (Fig. 3B, 3C, right), which is in contrast to the earlier observation in the primary recipient. This indicates that LTRCs produce both STRCs and LTRCs by asymmetric self-renewal division. It is important to note that although all the STRC-ML clones (9 of 9) demonstrated LTRC activity, some of the STRC-M clones (5 of 30; 16.7%) showed only a transient engraftment, indicating that STRC-M tends to have a more restricted potential than STRC-ML (Fig. 3B, 3C, right). We also identified a small proportion of clones (6 of 45; 13.3%) that were uniquely found in the later phase of secondary repopulation, suggesting the existence of quiescent LTRCs that are reactivated only at the later phase. Taken together, our findings constitute clonal evidence that self-replicating LTRCs produce widely heterogeneous SRC compartments, ultimately constituting hierarchical organization of the human HSC pool (supplemental online Fig. 3).

LTRCs with Higher Self-Renewal Capacity Are Constituted by Relatively Dormant Clones and Expanded Clonally upon Secondary Transplantation

Finally, we quantitatively compared the relative clone size of each LTRC clone that was found in the $CD34^+$ stem cell pool of both primary and secondary recipients. Interestingly, all

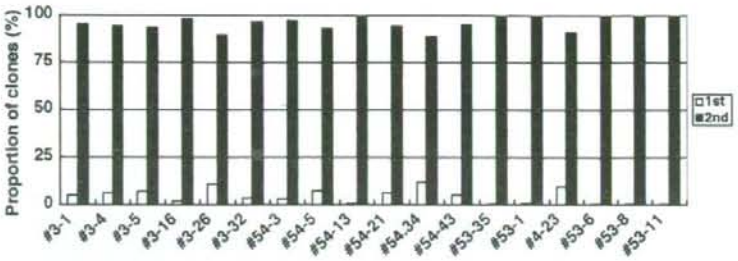

Figure 4. Relative clone size of individual long-term repopulating cell (LTRC) clones in primary and secondary recipients. The clone size of individual LTRC clones detected in $CD34^+$ cells was examined by real-time quantitative polymerase chain reaction. The relative clone sizes of each LTRC clone in primary (open bars) and secondary (filled bars) recipients are shown.

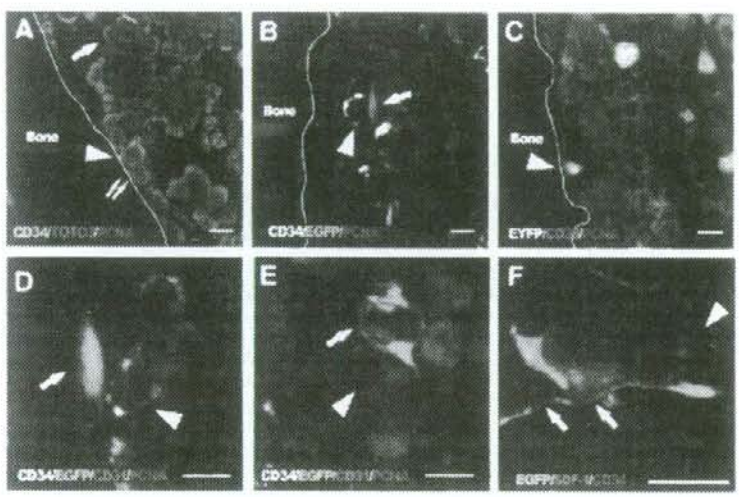

Figure 5. Quiescent human long-term repopulating cells interacting with niche components in the endosteal region. **(A):** Two human $CD34^+$ cells, a PCNA-negative (arrowhead) and a PCNA-positive (double arrows), were adjacent to each other and are attached to the endosteum. A large $CD34^+$ PCNA-positive cell was found away from endosteum (arrow). **(B):** A PCNA-negative $CD34^+$ cell (arrowhead) interacted with CD31-expressing murine endothelial cells (arrow) in the endosteal region. **(C):** The majority of human cells were positive for CD38. A $CD38^{neg}$PCNA-negative EYFP-transduced human cell was attached to the endosteum (arrowhead). **(D, E):** EGFP-marked HMRCs differentiated into fibroblastic reticular cells that associated with $CD31^+$ vascular cells. PCNA-negative quiescent $CD34^+$ cells (arrowheads) interacted with human reticular cells (arrow). **(F):** HMRCs in the vascular niche expressed SDF-1 (arrows) and interacted with a $CD34^+$ cell (arrowhead). Scale bars = 10 μm. Abbreviations: EGFP, enhanced green fluorescent protein; PCNA, proliferating cell nuclear antigen.

LTRC clones in the $CD34^+$ stem cell pool of the secondary recipient were much larger than they had been in the primary recipient (Fig. 4). This suggests that LTRC clones with a higher self-renewal ability are relatively inactive or proliferate slowly, but they could become activated upon secondary transplantation. We also discovered that all quiescent LTRCs were indeed capable of producing multilineage cells in the primary recipients, suggesting that stem cells are not always quiescent but can actively contribute to hematopoiesis when necessary. These results provide evidence for the clonal dynamics of repopulating clones by showing that LTRCs are quiescent at one point and are active at another point.

Quiescent LTRCs Localizes to BM Niches

Maintenance and regulation of LTRCs in vivo depends on the specific microenvironment, known as the niche. We examined the localization of quiescent human $CD34^+$ cells in the BM microenvironment of recipients. The quiescent $CD34^+$ cells were identified by the lack of PCNA expression. Most of the $PCNA^-CD34^+$ cells (77.8%; n = 198 $PCNA^-CD34^+$ cells) were located in the endosteal region (arbitrarily defined as within 12 cells of the endosteum, as described previously [16]) and were found to be associated with bone-lining osteoblasts or endothelial cells (Fig. 5A, 5B). These niche-interacting quiescent human $CD34^+$ cells were confirmed as CD38-negative (Fig. 5C), the only cell population transplantable in secondary recipients. In line with previous mouse studies [3–5], these results revealed that human HSCs stayed in a quiescent state to maintain their LTRC activity by actively interacting with the osteoblastic and vascular niche components in the endosteal region of BM.

Mesenchymal Stem Cells Serve As Stem Cells for the Hematopoietic Microenvironment

These experiments had so far demonstrated behavior of human hematopoietic cells in the murine microenvironment. To inspect whether the principles of niche regulatory system extrapolated from murine studies could apply to the human situation, human hematopoietic reconstitution was examined in the "humanized" hematopoietic microenvironment. Previously, we established an experimental system in which a functional human microenvironment was reproduced by the intramedullary injection of human BM MSC-derived HMRCs [15]. The presence of HMRCs was shown to be important in human hematopoietic reconstitution in immunodeficient mice. We examined whether the HMRCs play roles in the maintenance of the LTRC activity in BM. EGFP-transduced human MSCs were intramedullary injected into the right tibia of irradiated NOG mice, and then $CD34^+$ cells were transplanted intravenously into each mouse. Consistent with our previous report [15], a majority of HMRCs differentiated into vasculature-associated fibroblastic reticular cells, which are known to play a pivotal role in HSC regulation [17]. In line with this notion, HMRCs expressed one of the important hematopoietic regulatory molecules, SDF-1 (Fig. 5F). Interestingly, the quiescent human $CD34^+$ cells often interacted with these $EGFP^+$ human reticular cells in the endosteal region, strongly suggesting the hematopoiesis-supporting function of these cells (Fig. 5D, 5E). Much to our interest, the number of

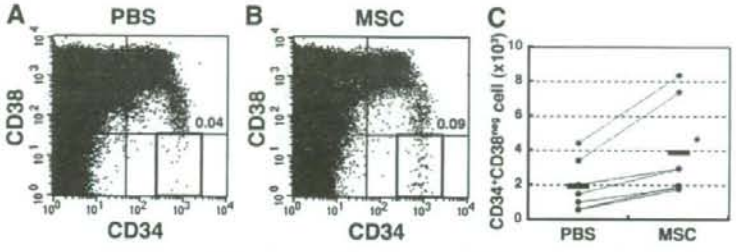

Figure 6. Effect of HMRCs on the engraftment of long-term repopulating cells. **(A):** BM cells obtained from the MSC-injected (right) or PBS-injected (left) tibia from the same mouse were examined by flow cytometry at 12 weeks after transplantation. Representative flow cytometric profiles are shown. The relative frequencies of the $CD34^+CD38^{neg}$ population are indicated. **(B):** Absolute numbers of $CD34^+CD38^{neg}$ cells in the MSC-injected (right) and the PBS-injected (left) tibias of mice. Each dot represents one mouse. Values obtained from the same mouse are connected with lines. *, $p < .01$ relative to the PBS-injected tibia. Abbreviations: MSC, mesenchymal stem cell; neg, negative; PBS, phosphate-buffered saline.

$CD34^+CD38^{neg}$ LTRCs in the HMRC-injected tibia was higher than in the saline-injected tibia in the same recipient (Fig. 6). These results confirmed the previous mouse study and demonstrated for the first time that human reticular cells in the endosteal niches were one of the critical components of human LTRC niches.

DISCUSSION

In this study, we demonstrated that the hierarchical organization of human HSCs originated from quiescent $CD34^+CD38^{neg}$ cells that resided in the endosteal region of the BM microenvironment, particularly in osteoblastic and vascular niches. Our study presented several lines of evidence that are important in understanding the in vivo dynamics of human hematopoiesis. First, human hematopoiesis was supported by functionally heterogeneous and distinct clonal subsets. Human repopulating cells with predetermined STRC activity contributed to the initial myeloid production, and those clones were rapidly exhausted from the $CD34^+$ stem cell pool and lost repopulating ability. In contrast, multilineage-producing LTRC clones remained in the $CD34^+$ stem cell pool and sustained hematopoiesis. Asymmetric division of LTRC clones produced clones with different potentials, resulting in the construction of the hierarchical organization of the human HSC pool in the BM microenvironment. Second, repopulating activity of long-lasting LTRCs fluctuated. We quantitatively demonstrated that the self-renewing LTRCs normally stayed in a quiescent state in the primary recipient and expanded clonally upon secondary transplantation. Finally, the hematopoietic microenvironment activity influenced LTRC maintenance. We demonstrated that MSCs served as stem cells for the key components of the hematopoietic microenvironment and that the number of LTRCs could be increased by manipulating microenvironments.

Stem cell quiescence is an indispensable property for the maintenance of hematopoiesis. Interaction of HSCs with their particular microenvironments, known as the stem cell niches, is critical in regulating stem cell quiescence [2]. Although it has been reported that the most primitive human hematopoietic cells, when freshly isolated [18–22], stayed predominantly in the quiescent phase, the cell cycle status and potential of transplanted human HSCs in recipient BM has not been fully examined. In this study, we successfully demonstrated that human LTRCs existed in a rare population of $CD34^+CD38^-$ cells that localized to the stem cell niches and maintained their stem cell activities while remaining in a quiescent state. Furthermore, we successfully visualized interaction between BM niche cells and a rare population of dormant LTRCs that fulfilled the functional criteria of HSCs: cell cycle quiescence and multilineage engraftment in a secondary host. Combined with our previous finding that showed preferential localization of transplanted $CD34^+CD38^{neg}$ cells in the endosteal area [15], the present study indicates that LTRCs protect themselves from extinguishing their stem cell activity by firmly attaching to the endosteal niches in cases of various hematopoietic stresses, as has been demonstrated in mouse studies [23, 24].

Several studies, including the present one, showed that LTRC activity was highly enriched in the $CD34^+CD38^{neg}$ fraction, and the self-renewal capacity and differentiation potential of SRCs became restricted, coinciding with the appearance and increasing expression of the CD38 antigen [7–10]. Recently, unexpectedly high repopulating activity was discovered within a $CD34^+CD38^{lo}$ subpopulation in direct intrafemoral transplantation experiments [25]. However, the present study demonstrated that $CD34^+CD38^{lo}$ cells appeared to be more restricted in their self-renewal ability. Although we were unable to determine the reason for this discrepancy, it is possible that the high self-renewal activity of the $CD34^+CD38^{lo}$ subpopulation could be grasped only by direct intrafemoral transplantation. Nevertheless, our experiment has presented the idea that the self-renewal potentials of the $CD34^+CD38^{lo}$ and $CD34^+CD38^{neg}$ fractions are distinctly different. Furthermore, we demonstrated for the first time that a rare population of $CD34^+CD38^{neg}$ cells in the primary recipient BM niches was the only group of SRCs that could successfully reconstitute hematopoiesis in the secondary recipient.

A previous study has reported that in an event that enforces active proliferation of HSCs, such as transplantation, initial active proliferation of SRC population is markedly downregulated after a while [26]. Another study demonstrated an initial upsurge and rapid decline of STRCs in human subjects [27]. Our study showed a similar decline of STRCs. Importantly, our clone-tracking lineage analysis revealed that transient unipotent STRC repopulation was followed by the emergence of multipotent LTRCs. Those LTRCs became activated and produced self-renewing LTRCs and STRCs with limited self-renewal activity. These STRCs in the later phase served as a functional element of the hematopoietic hierarchy. Together with earlier findings, the results of our study provided experimental evidence that quiescent human LTRCs asymmetrically divide in the BM niches, producing one daughter cell that remains in a quiescent state as a "reservoir" of the stem cell pool and another daughter cell that proliferates and differentiates into $CD38^+$ cells as a "contributor" for sustaining hematopoiesis.

Several studies in mice and larger animals have concluded that long-term hematopoiesis is sustained by a limited number of self-renewing stem cells that maintain their stem cell activities by staying in a quiescent state and are activated as the occasion demands [28–32]. However, the quantitative dynamics

of individual HSC clones during a quiescent state and an active state remain unclear. In the present study, we quantitatively unveiled that self-renewing LTRC clones normally occupied very small clone size in the primary recipient but expanded remarkably (10–1,000-fold) upon secondary transplantation. Importantly, these quiescent LTRCs were capable of producing multilineage cells even in a steady state in the primary recipients, suggesting that stem cells are not always remained in a quiescent state but can actively contribute to hematopoiesis, depending on the situation.

The exact nature of the HSC niche and the mechanism of HSC regulation have remained largely unknown because of the complex structure of the niche itself and the technical difficulties of examining it. Over the past few years, however, a number of important papers have been published [3–5, 17], and we began to understand how the niche regulates HSCs, at least in mice. Unfortunately, experimental examination of human hematopoietic niches had been hampered by the lack of an appropriate model system until now. We recently established a mouse model that recapitulates a functional human hematopoietic microenvironment [15]. Intramedullary transplanted human MSCs, termed HMRCs, reconstituted functional components of hematopoietic niches. The majority of HMRCs became fibroblastic reticular cells that were often associated with the vasculature, and the rest differentiated into osteoblasts, osteocytes, and endothelial cells. These HMRCs actively interacted with primitive human HSCs to maintain secondary transplantable repopulating activity. Importantly, the HMRCs preferentially distributed to the endosteal region and expressed several niche factors, such as N-cadherin [4], SDF-1 [33–35], and fibronectin [35–37] (T.Y., Y.M., and K.A., unpublished observation). In another published study [35], we demonstrated the important in vivo roles of these niche factors, especially SDF-1, for the engraftment of human HSCs in the BM microenvironment by directly injecting antibody-treated human HSCs into the BM cavity. In the present study, quiescent HSCs interacted with HMRC-derived human reticular cells, and cotransplantation with MSCs resulted in increased engraftment of the $CD34^{+}CD38^{neg}$ population (i.e., the LTRC compartment), emphasizing the importance of the presence of a human microenvironment in an experimental animal. Consistent with our past [15] and present findings, a recent study by Sugiyama et al. elegantly demonstrated that SDF-1-expressing fibroblastic reticular cells play a pivotal role in both osteoblastic and vascular niches at the endosteal region of the murine microenvironment [17]. Taken together, those findings and our own indicated that HMRC-derived fibroblastic reticular cells in the osteoblastic and vascular niches participate in the maintenance of LTRC activity by closely interacting with quiescent human HSCs and secreting hematopoietic-regulatory factors.

Detailed examinations of the innate properties of individual human HSCs that contribute to various phases of reconstitution is important not only to understand human hematopoietic development but also to exploit the therapeutic potential of HSCs. Clinically speaking, rapid recovery of hematopoiesis and sustainable long-term hematopoiesis in patients are primary factors for successful HSC transplantation. In other words, appropriate control of initial hematopoietic recovery and prevention of premature HSC exhaustion could remarkably improve the therapeutic outcome of clinical transplantation. The clonal analysis presented in this study enabled us to accurately evaluate in vivo properties of individual human HSCs. Our strategies may serve as indicators to assess the therapeutic effects of emerging stem cell therapies and help advance clinical transplantation medicine.

Conclusion

Through this study, we successfully identified human LTRCs that were responsible for lifelong hematopoiesis in the BM microenvironment. In a steady state, those quiescent human LTRCs localized to and interacted with the key components of the stem cell niche. Once activated, they divided asymmetrically into LTRCs and STRCs, ultimately creating a hierarchical hematopoietic structure. Further identification and characterization of LTRC and STRC subsets will be particularly useful in optimizing protocols for stem cell therapies. Our study also confirmed that MSCs serve as stem cells for the key components of the hematopoietic microenvironment. Therefore, it should be possible to develop MSCs into useful therapeutic tools for conditioning the hematopoietic niches.

Acknowledgments

We thank members of the animal facility of Tokai University for meticulous care of the experimental animals and members of the Tokai Cord Blood Bank for assistance. We also thank members of the Research Center for Regenerative Medicine of Tokai University for helpful discussion and assistance. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and by a Research Grant on Human Genome, Tissue Engineering from The Ministry of Health, Labor, and Welfare of Japan.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

References

1 Cheng T, Rodrigues N, Shen H et al. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. Science 2000;287:1804–1808.
2 Wilson A, Trumpp A. Bone-marrow haematopoietic-stem-cell niches. Nat Rev Immunol 2006;6:93–106.
3 Calvi LM, Adams GB, Weibrecht KW et al. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 2003;425:841–846.
4 Zhang J, Niu C, Ye L et al. Identification of the haematopoietic stem cell niche and control of the niche size. Nature 2003;425:836–841.
5 Kiel MJ, Yilmaz OH, Iwashita T et al. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 2005;121:1109–1121.
6 Shultz LD, Ishikawa F, Greiner DL. Humanized mice in translational biomedical research. Nat Rev Immunol 2007;7:118–130.
7 Bhatia M, Wang JC, Kapp U et al. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc Natl Acad Sci U S A 1997;94:5320–5325.
8 Hogan CJ, Shpall EJ, Keller G. Differential long-term and multilineage engraftment potential from subfractions of human CD34+ cord blood cells transplanted into NOD/SCID mice. Proc Natl Acad Sci U S A 2002;99:413–418.
9 Kerre TC, De Smet G, De Smedt M et al. Both CD34+38+ and CD34+38- cells home specifically to the bone marrow of NOD/LtSZ scid/scid mice but show different kinetics in expansion. J Immunol 2001;167:3692–3698.
10 Glimm H, Eisterer W, Lee K et al. Previously undetected human hematopoietic cell populations with short-term repopulating activity selec-