A robust, stromal cell-free culture procedure that is based on human factors would have many advantages over existing methods. For example, drugs or biologicals could be screened for direct effects on hematopoietic cells, identifying molecules with therapeutic potential. We have now systematically manipulated culture conditions, finding that hMSC produce soluble, heat-labile factors that support human B lymphopoiesis. The same is true for undefined substances in selected lots of fetal calf serum (FCS), and the active principal was not IL-7, thymic stromal lymphopoietin (TSLP), CXCL12 or hemokinin-1. High cell densities and addition of recombinant granulocyte colony stimulating factor (G-CSF), Stem cell factor (SCF) and flt-3 ligand (FL) were all critical for efficient B lymphopoiesis. While lymphocyte production from adult BM progenitors in culture is much more difficult than umbilical CB cells, both are responsive to G-CSF. This information should now facilitate basic studies of immune system replenishment, improving differentiation schemes useful for classifying malignancies as well as the discovery of cytokines that can counter immunodeficiency resulting from mutations or chemotherapy.

2. Materials and methods

2.1. Origin and isolation of cells

CB cells were collected from healthy, full-term neonates immediately after Caesarean section or normal delivery. BM cells were collected from normal donors. All samples were collected after informed consent, using protocols approved by the Investigational Review Boards at Osaka University and O.M.R.F. Mononuclear cells were separated by Ficoll-Paque PLUS (GE Healthcare Bio-Science AB, Uppsala, Sweden) or Lymphocyte Separation Medium (Mediatech, Inc., Manassas, VA) and centrifugation. Purification of CB and BM $CD34^+$ cells was performed using Direct human CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Auburn, CA). Human mesenchymal stem cells were purchased from Lonza (Walkersville, MD), and maintained in Mesenchymal Stem Cell Growth Medium (MSCGM, Lonza). Flow cytometric analysis confirmed that the cultured hMSC expressed CD105, CD166, CD29, and CD44, but not CD14, CD34, or CD45.

2.2. Co-cultures for human B lymphocytes

Co-cultures of CB $CD34^+$ cells on hMSC were performed as previously described (Ichii et al., 2008). hMSC were seeded in 12-well tissue plates 1 or 2 days before setting up the co-cultures. Isolated $CD34^+$ cells (2×10^3 cells/well) were plated on sub-confluent hMSC layers in MSCGM in the presence of 10 ng/ml SCF and 5 ng/ml FL. Recombinant human SCF and FL proteins were purchased from R&D systems (Minneapolis, NY). Half of the culture medium was replaced with fresh medium containing the same cytokines twice a week. In some experiments, direct contact between hMSC and cultured cells was prevented with

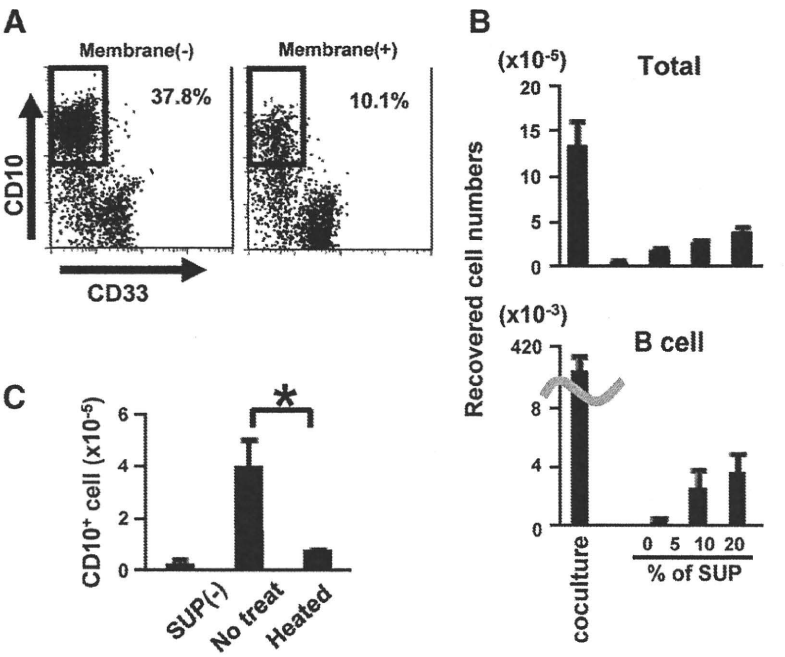

Fig. 1. Human mesenchymal stem cells (hMSC) produce unknown, heat-labile factors that promote human B lymphopoiesis. (A) CB $CD34^+$ cells (1×10^3 cells/ml) were cultured on hMSC in combinations of SCF and FL with or without membrane filters for 4 weeks. The generated cells were stained with PE-CD10 and FITC-CD33, and were analyzed by flow cytometry. Similar results were obtained in three independent experiments. (B) The indicated concentrations of hMSC supernatant (SUP) were added to the cultures of CB $CD34^+$ cells (1×10^3 cells/ml) in the presence of SCF and FL without stromal layers. The numbers of total and $CD10^+$ cells after 4 weeks of cultures are shown. Similar results were obtained in three independent experiments. (C) hMSC supernatants were heated at 56 °C for 1 h. CB $CD34^+$ cells (5×10^3 cells/ml) were cultured with or without hMSC supernatant (10%) in the presence of SCF and FL without stromal layers. The numbers of $CD10^+$ cells after 4 weeks of cultures are shown. Statistical differences from results with untreated supernatant are indicated with asterisks ($p<0.01$). Similar results were obtained in three independent experiments.

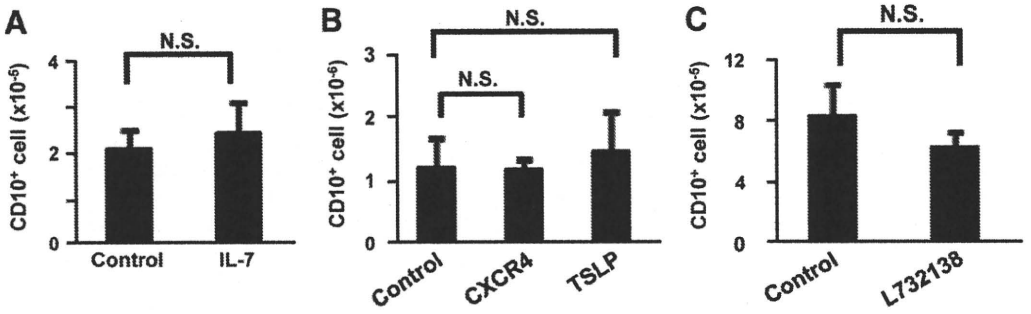

Fig. 2. IL-7, CXCR4, TSLP and Hemokinin-1 are not essential for human B lymphopoiesis. (A) CB CD34$^+$ cells (1×10^4 cells/ml) were cultured with a control (10 μg/ml Isotype-matched Ab) or a neutralizing antibody to IL-7(10 μg/ml) in QBSF®60 medium in the presence of hMSC supernatant (10%), SCF and FL for 4 weeks. Numbers of recovered CD10$^+$ cells determined by flow cytometry are shown. (B) CB CD34$^+$ cells (1×10^4 cells/ml) were cultured with control (10 μg/ml Isotype-matched Ab) or neutralizing antibodies (10 μg/ml) to CXCR4 or TSLP in QBSF®60 medium in the presence of hMSC supernatant (10%), SCF, FL and IL-7 for 4 weeks. (C) CB CD34$^+$ cells (1×10^4 cells/ml) were cultured with control (DMSO) or an inhibitor to Hemokinin-1 (20 μM L732138) in QBSF®60 medium in the presence of hMSC supernatant (10%), SCF, FL and IL-7 for 4 weeks. Similar results were obtained in three independent experiments. N.S.; differences were not significant.

0.45 μm polyethylene terephthalate membranes (FalconTM Cell Culture Inserts; Becton Dickinson Labware, Franklin Lakes, NJ).

2.3. Stromal cell-free cultures for human B lymphocytes

Isolated CD34$^+$ cells were seeded with cytokines as indicated in the figures. The cultures were usually maintained in QBSF®60 (Quality Biological, Inc., Gaithersburg, MD) containing10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin). Recombinant human IL-7 and G-CSF proteins were purchased from R&D Systems. Half of the culture medium was replaced with fresh medium containing the same cytokines once a week. Supernatants of one week hMSC cultures were collected, filtered and added to lymphocyte cultures. This conditioned medium was incubated for one hour at 56 °C to assess lability and also used in experiments with neutralizing antibodies to IL-7, CXCR4 or TSLP (R&D Systems). A hemokinin-1 inhibitor (L732138) was obtained from Sigma-Aldrich (St. Louis, MO).

2.4. Flow cytometry and cell sorting

Flow cytometric analysis was performed with a FACSCalibur or FACS LSRII (BD Biosciences Immunocytometry Systems, San Jose, CA) using standard multicolor immunofluorescent staining protocols. Mouse monoclonal Abs against the following human cell surface molecules were purchased: PE-CD3, PE-CD10, APC-CD10, PE-CD19, PE-CD20, FITC-CD33, PE-CD34, APC-CD34, FITC-CD45, PE-CD56, and PE-glycophorin A (GPA) from BD Biosciences/BD Pharmingen (Franklin Lakes, NJ); phycoerythrin 5-succinimidylester (PC5)-CD19 from Beckman Coulter (Marseilles, France); and FITC-IgM from Southern Biotechnology Associates (Birmingham, AL).

2.5. Transplantation of cultured cells into immunodeficient mice

Xenotransplantation of cultured cells from CB CD34$^+$ cells were performed using a previously reported method (Hiramatsu et al., 2003). Briefly, 8- to 12-week old NOD/SCID/common γ^{null} (NOG) mice received 240 cGy radiation divided in two fractions. Isolated CB CD34$^+$ cells or cells generated in stromal cell-free cultures were injected via the tail vein. At intervals after transplantation, peripheral blood cells were taken and assessed for human lineage specific marker expression by flow cytometry. The same mice were sacrificed more than 2 months after transplantation, and phenotypes of BM and spleen cells were analyzed using flow cytometry.

2.6. Statistical analyses

Student's *t*-test was performed to assess statistical differences. All results are shown as mean values ± SD.

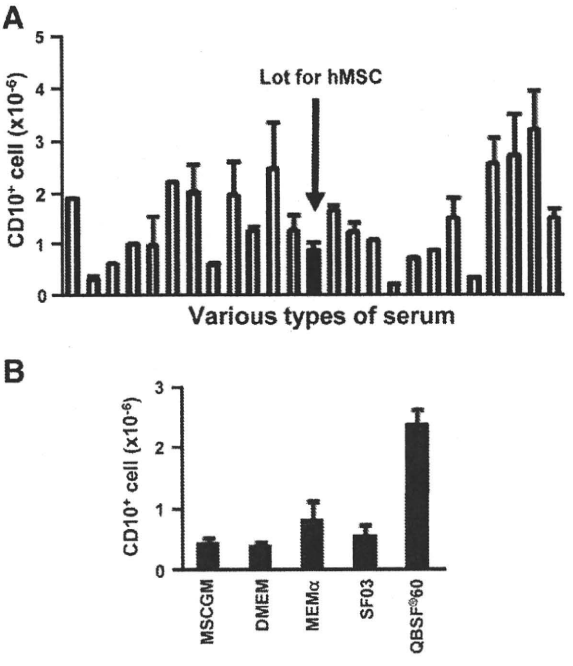

Fig. 3. FCS and medium selection were important variables for stromal cell-free cultures that support human B lymphopoiesis. (A) CB CD34$^+$ cells (1×10^4 cells/ml) were cultured in QBSF®60 medium supplemented with 10% of various lots of fetal calf sera in the presence of hMSC supernatant (10%), SCF, FL and IL-7 for 4 weeks. The solid bar indicates CD10$^+$ cell numbers in cultures with the serum batch selected for maintaining hMSC. Similar results were obtained in three independent experiments. (B) CB CD34$^+$ cells (1×10^4 cells/ml) were cultured in various types of media with 10% FCS in the presence of hMSC supernatant (10%), SCF and FL for 4 weeks. Numbers of recovered CD10$^+$ cells are shown. Similar results were obtained in three independent experiments. Data are shown as means ± SD numbers of CD10$^+$ cells generated in triplicate samples.

3. Results

3.1. Mesenchymal stem cells produce unknown, heat-labile factors that promote human B lymphopoiesis

Membrane inserts have been extensively used to probe requirements for physical contact in hematopoietic cell cultures (Miller et al., 1998; Nishihara et al., 1998; Verfaillie, 1993). We used this approach to determine that hMSC or conditioned medium from them supported formation of $CD10^+$ $CD33^-$ lymphoid cells in cultures of $CD34^+$ CB (Fig. 1A, B). The yield of lymphocytes was much less than when hematopoietic cells were in direct contact with hMSC, suggesting that the diffusible factors might be short-acting, and they were completely inactivated by heating for 1 h at 56 °C (Fig. 1C).

Stromal cell derived IL-7 is essential for murine adult B lymphopoiesis (Peschon et al., 1994;Von Freeden-Jeffry et al., 1995) and parts of the IL-7 receptor are used to recognize the cytokine TSLP (Levin et al., 1999; Vosshenrich et al., 2003). Chemokines recognized by the CXCR4 receptor and the hemokinin-1 peptide also influence lymphopoiesis in the mouse (Milne et al., 2004; Tokoyoda et al., 2004; Zhang et al., 2000; Zhu et al., 2007). Addition of neutralizing antibody to IL-7 had no effect on human cord blood cell cultures (Fig. 2A). The same was true for antibodies to the CXCR4 receptor or TSLP, or addition of an inhibitor for hemokinin-1 (Fig. 2B, C). The experiments described above were conducted with a batch of FCS that is optimal for growth of hMSC, and screening revealed no correlation with efficiency of lymphopoiesis (Fig. 3A). All subsequent studies were done with a batch that was optimal in

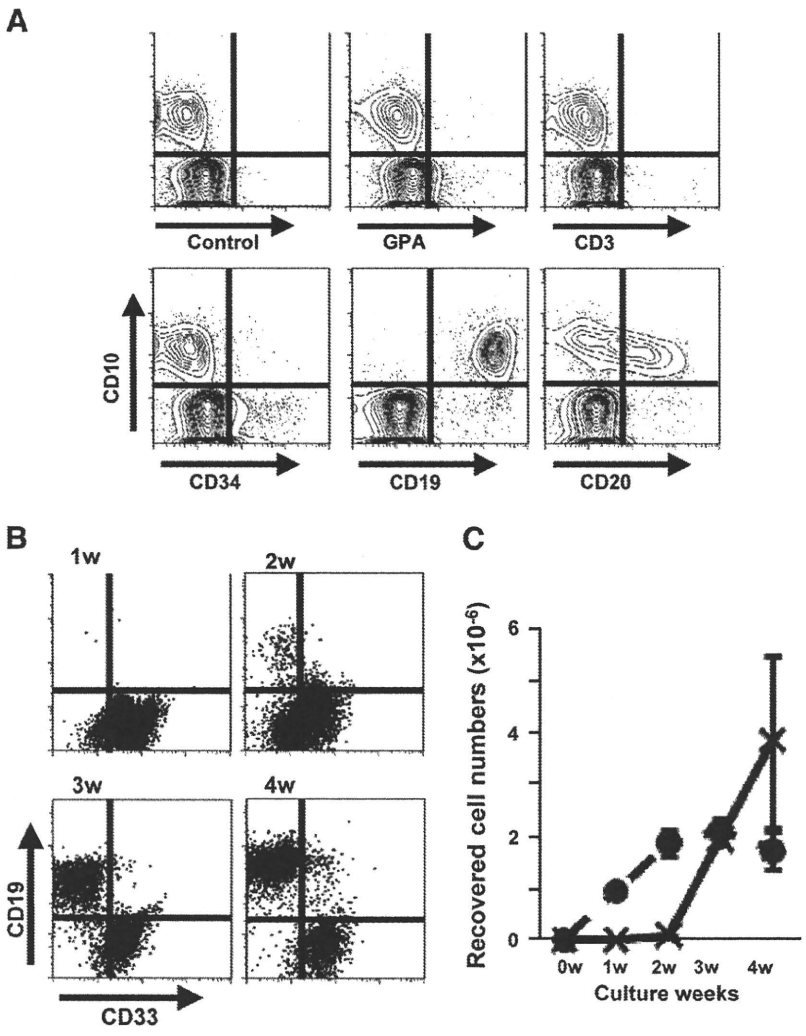

Fig. 4. Characteristics of cells generated in stromal cell-free cultures. (A) CB $CD34^+$ cells (1×10^4 cells/ml) were cultured for 5 weeks in the presence of hMSC supernatant (10%), SCF, FL and IL-7. The generated cells were stained with FITC-CD33 and APC-CD10 as well as the indicated PE-conjugated Abs. All of the flow cytometry results shown in panel A were gated for $CD33^-$ cells. Isotype-matched Abs were used as negative controls. Similar results were obtained in three independent experiments. (B, C) CB $CD34^+$ cells (1×10^4 cells/ml) were cultured with hMSC supernatant (10%), SCF, FL and IL-7 for 4 weeks. The cultured cells were collected, and surface expression of CD10, CD19 and CD33 was determined weekly by flow cytometry. Numbers of $CD33^+$ myeloid cells (dotted line) and $CD10^+$ $CD19^+$ lymphoid cells (solid line) were calculated, and similar results were obtained in three independent experiments.

this respect. Medium selection was another important variable, and QBSF®60 was selected for all of our studies (Fig. 3B). We conclude that while direct contact with stromal cells is not essential for human B lymphopoiesis, unknown stromal cell-derived factors do facilitate this process.

3.2. Characteristics of cells generated in cultures with hMSC conditioned medium

We found that cultures initiated with 1×10^4 highly enriched CB $CD34^+$ cells together with 10% hMSC conditioned medium, SCF, FL, and IL-7 generated approximately 2×10^6 $CD10^+$ lymphoid cells within 4 weeks. Fig. 4A shows representative flow cytometry results for cultures held one week longer. Approximately 5% of the cultured cells still expressed CD34, but neither GPA^+ erythroid cells nor $CD3^+$ T lineage cells were ever detected. Most of the generated $CD10^+$ cells also expressed CD19, while more than half of them were $CD20^+$. $CD33^+$ myeloid cells peaked after 2 weeks of culture and then $CD10^+$ $CD19^+$ B lineage cells appeared (Fig. 4B, C).

We then assessed the differentiation potential of cells expanded in three week cultures by transplanting 1×10^7 of them into sub-lethally irradiated, immunodeficient NOG mice. Control animals received 5×10^4 freshly isolated CB $CD34^+$ cells. $CD45^+$ human cells were detected in peripheral blood of all transplanted mice six weeks later and B lineage lymphoid cells predominated (Fig. 5A). The animals were then sacrificed 3 months after transplantation and more detailed flow cytometry was performed with BM and spleen (Fig. 5B). Human $CD19^+$, $CD20^+$ and IgM^+ lymphocytes, but not NK or

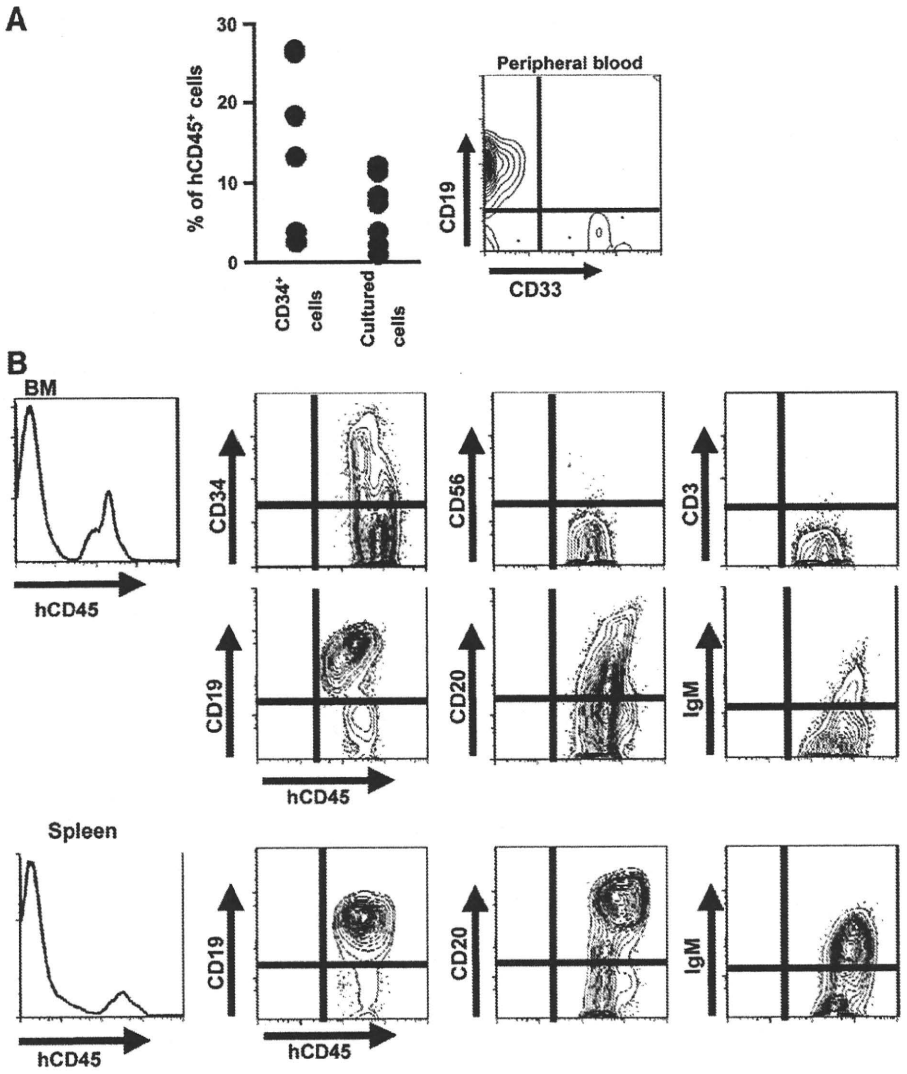

Fig. 5. Differentiation of cultured human hematopoietic cells in immunodeficient mice. (A) NOG mice (n = 5 for each group) were each transplanted with 5×10^4 freshly isolated CB $CD34^+$ cells or 1×10^7 cells recovered from 3 week stromal cell-free cultures. Six weeks later, peripheral blood samples were analyzed by flow cytometry. Percentages of human $CD45^+$ cells are shown. Similar results were obtained in three independent experiments. Representative flow cytometry data for CD19 and CD33 expression by gated human $CD45^+$ cells is also shown. (B) CB $CD34^+$ cells were held 3 weeks in stromal cell-free cultures in the presence of hMSC supernatant (10%), SCF, FL and IL-7. Then, 1×10^7 recovered cells were transplanted into NOG mice. After 12 weeks, their BM and spleen cells were collected and subjected to flow cytometry analysis. Cells of culture origin were gated with APC-anti human CD45 and staining is shown with the indicated Abs. Results obtained in 5 independent experiments were very similar.

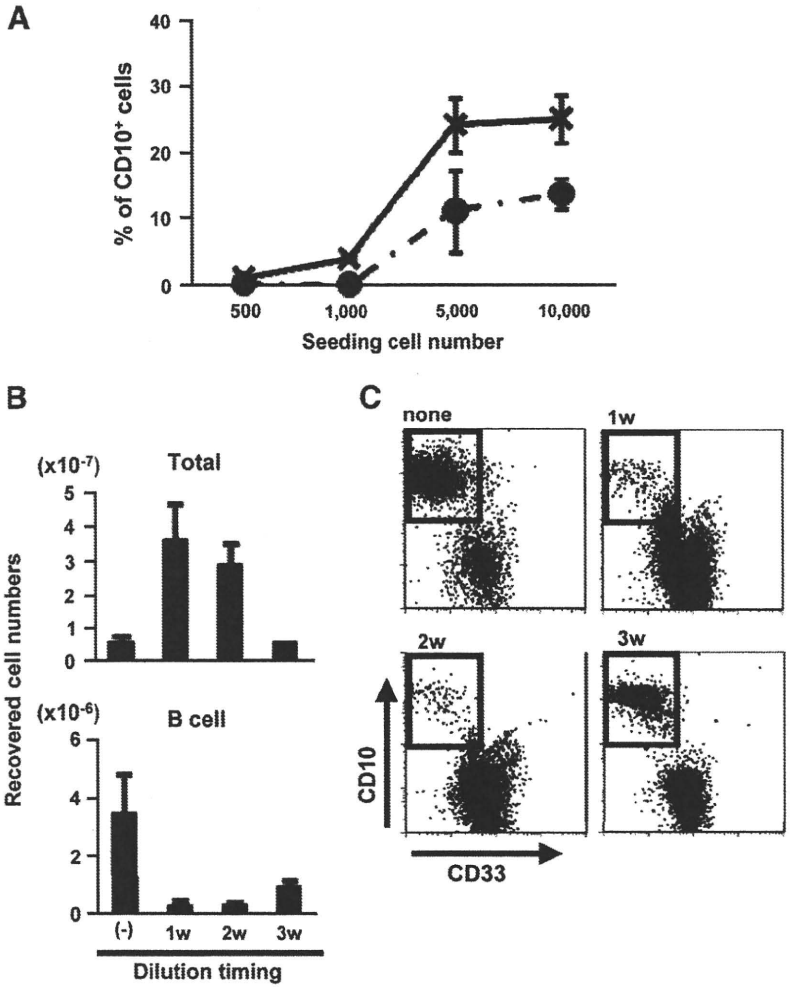

Fig. 6. High cell density favors lymphopoiesis in stromal cell-free cultures. (A) The indicated numbers of CB $CD34^+$ cells were cultured in the presence of SCF and FL with (solid line) or without (dotted line) 10% hMSC supernatant for 4 weeks. Percentages of $CD10^+$ cells recovered were determined by flow cytometry. (B,C) CB $CD34^+$ cells (1×10^4 cells/ml) were cultured in the presence of SCF, FL and IL-7. The harvested cells were then adjusted back to 1×10^4 cells/ml on days 7, 14, or 21 of culture, respectively. (B) Numbers of total and CD10 cells generated cells are also shown. (C) Representative flow cytometry results were shown for analyses done after a total of 4 weeks of culture. Similar results were obtained in three independent experiments.

T cells were found in both sites. Similar results were obtained when transplanted mice were examined six months after transplantation. Thus, these culture conditions support the generation of human B lymphoid cells and cultured progenitors retain substantial potential for B lymphopoiesis in chimeric mice.

3.3. Lymphopoiesis can be observed in stromal cell-free, hMSC supernatant-free cultures if cell density is high

Some studies have stressed the importance of high cell density for lymphocyte viability in culture (Milne et al., 2004; Zhang et al., 2000), and we found this was also the case for human lymphoid progenitors (Fig. 6A). In fact, significant numbers of lymphocytes were made even in the absence of hMSC or their products when cultures were initiated with greater than 5×10^3 CB $CD34^+$ cells/ml. These generated cells had similar surface phenotypes to the cultured cells with 10% hMSC conditioned medium shown in Fig. 4A (data not shown). A series of cultures were then set up with a starting density of 1×10^4 cells/ml and subgroups were then diluted to this same density at intervals of 1, 2 or 3 weeks of culture. Total nucleated cells expanded approximately 500 fold in the control, unmanipulated group and this was the most efficient condition for generating lymphocytes (Fig. 6B). Adjustment of the density at any subsequent time, and especially during the first two weeks, compromised lymphocyte recovery. Note that non-lymphoid cells represented in the total nucleated cell counts expanded well when culture cell numbers were readjusted on weeks two and three. Also, crowding of cells in round bottom rather than flat bottom 96 well trays improved cloning efficiencies for human progenitors (data not shown). Therefore, contact between maturing hematopoietic progenitors favors their survival, proliferation and/or differentiation.

3.4. Addition of recombinant G-CSF promotes lymphopoiesis even with adult marrow progenitors

The developmental age of hematopoietic cell donors has a strong influence over their potential to generate lymphocytes

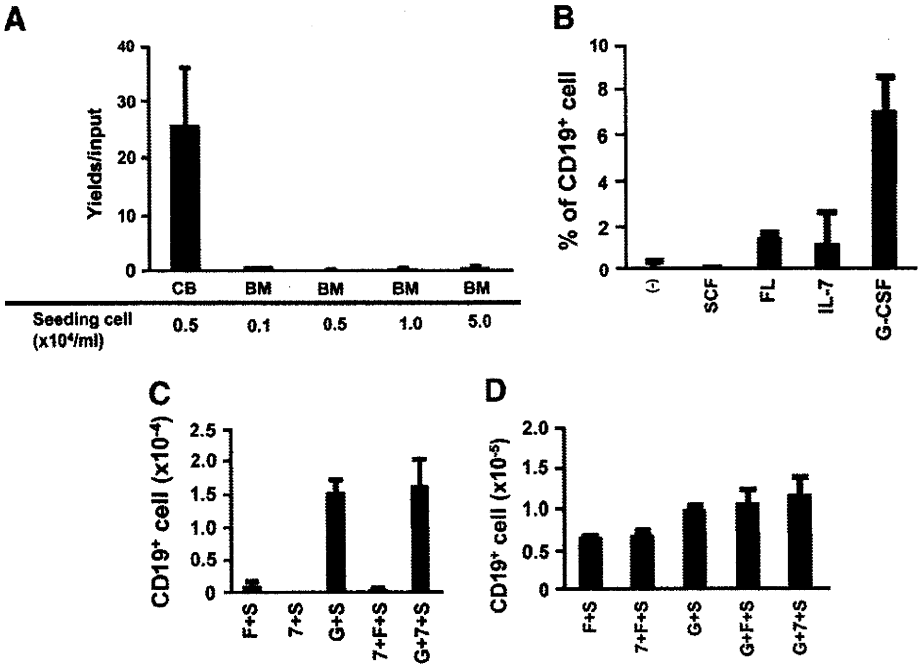

Fig. 7. Human BM $CD34^+$ cells require G-CSF to generate B lineage cells in stromal cell-free cultures. (A) 1×10^4 cells/ml CB $CD34^+$ cells or the indicated concentrations of BM $CD34^+$ cells were cultured in the presence of SCF, FL and IL-7 for 4 weeks. Lymphoid cells were identified by flow cytometry and the results are given as numbers of $CD19^+$ cells generated/input progenitor cell. (B) BM $CD34^+$ cells (5×10^4 cells/ml) were cultured alone or with the indicated cytokines. After 4 weeks of culture, CD19 expression of the recovered cells was assessed by flow cytometry. (C,D) BM $CD34^+$ cells (5×10^4 cells/ml) (C) or CB $CD34^+$ cells (1×10^4 cells/ml) (D) were cultured for 4 weeks in the indicated combinations of SCF (S), FL (F), IL-7 (7) and/or G-CSF (G). The results are given as mean total numbers of $CD19^+$ cells recovered ± S.D. Similar results were obtained in three independent experiments.

in culture (Rossi et al., 2003). That is, conditions that support vigorous lymphopoiesis from CB $CD34^+$ cells yield few lymphocytes with progenitors harvested from adult BM. We observed this phenomenon when cells of the two types were cultured on hMSC or in conditioned medium from hMSC, regardless of the initial cell density (Fig. 7A and data not shown). It was reported that G-CSF facilitated lymphopoiesis in co-cultures with murine stromal cells (Nishihara et al., 1998), and we found the same was true in stromal cell-free cultures (Fig. 7B and C). That is, even $CD34^+$ cells from adult BM generated lymphocytes when this recombinant cytokine was present. Yields of lymphocytes were best when recombinant SCF and G-CSF were added together and refreshed with each weekly medium change. Progenitors from umbilical CB were less fastidious than those from adult marrow (Fig. 7A). However, even CB 34^+ cells generated more lymphocytes in culture when G-CSF was present (Fig. 7D).

4. Discussion

It is believed that bone marrow contains specialized niches where B lineage lymphocytes are generated (Tang et al., 1993; Tokoyoda et al., 2004; Zhu et al., 2007). However, the molecules that comprise these lymphopoietic micro-environments are poorly understood. To our knowledge, this represents the first successful generation of human B lineage lymphocytes in stromal cell-free cultures. Important variables include selection of fetal calf serum lots, appropriate medium, high cell density and addition of G-CSF. Cultures initiated with 1×10^4 CB $CD34^+$ cells/ml or 5×10^4 BM $CD34^+$ cells/ml in QBSF®60 contained with 10% selected FCS, SCF, FL, and G-CSF generated $CD10^+$ $CD19^+$ lymphoid cells within 4 weeks. Although far from being an efficient system, it provides an opportunity to systematically screen candidate substances and learn how diffusible factors regulate replenishment of the humoral immune system. Assessment of survival and growth of lymphoid leukemia may represent another application of this new technology.

G-CSF was discovered for its support of myelopoiesis, and while it is not made by hMSC under normal steady-state conditions, the factor is produced by osteoblasts (Eaves et al., 1991; Majumdar et al., 2000; Taichman and Emerson, 1994; Zhang et al., 2004). B lineage progenitors were also mobilized in patients whose stem cells were mobilized by injection of this cytokine (Imamura et al., 2005). In addition, the factor augmented human lymphopoiesis when added to co-cultures supported by murine MS5 stromal cells (Nishihara et al., 1998; Ohkawara et al., 1998). The same investigators reported that lymphoid and non-lymphoid cells have G-CSF receptors. We have now found that G-CSF directly stimulates human $CD34^+$ cells and supports their ability to generate B lymphopoiesis. The effect was most pronounced when stem and progenitors from adult BM was used to initiate cultures. It may be that G-CSF is functioning as a multipoietin, improving the retention of $CD34^+$ cells that subsequently generate lymphocytes. However, we have also found that G-CSF augments lymphocyte formation in cultures initiated with Lin^- $CD34^+$ $CD38^+$ $CD123^-$ $CD45RA^+$ $CD10^-$ lymphoid progenitors (M.I. unpublished observations).

Consistent with many previous studies (Pribyl and LeBien, 1996; Puel et al., 1998), we found no influence of IL-7 on B lymphopoiesis in human cultures and active heat-labile substances released by stromal cells remain undefined. TSLP is a cytokine that preferentially expands fetal lymphopoiesis in mice (Levin et al., 1999; Vosshenrich et al., 2003). Consistent with that, addition of the factor augmented lymphocyte formation in cultures initiated with human cord blood, but not adult marrow progenitors in preliminary experiments (data not shown). However, addition of neutralizing TSLP specific antibodies to our cultures did not identify it as an activity in hMSC supernatants.

High cell densities reportedly improve murine pre-B cell survival in part because of their release of hemokinin-1 (Milne et al., 2004; Zhang et al., 2000). Autocrine lymphocyte-derived factors may also be important for human progenitors, but we never observed changes in cultures treated with Hemokinin-1 or an inhibitor specific for it. Chemokines such as CXCL12 may be recognized by CXCR4 on progenitors and deliver pro-adhesive signals important within bone marrow (Tokoyoda et al., 2004; Zhu et al., 2007), but there was no obvious role for this receptor in our stromal cell-free cultures.

It is noteworthy that B lineage lymphocytes always appeared after the expansion of myeloid cells in all culture conditions. When limiting numbers of $CD34^+$ cells were used to initiate cultures, some wells contained myeloid cells but no lymphocytes. Even highly enriched murine stem cell populations are heterogeneous, and it is believed that subsets differ with respect to lymphopoietic potential. The methods we describe here may suggest experimental approaches to such questions with human stem cells.

Fetal and neonatal $CD34^+$ cells differ from their adult counterparts in multiple ways (Ng et al., 2004; Wang et al., 1997; Weekx et al., 1998). For example, cord blood cells slowly reconstitute adult transplant recipients but smaller numbers are effective (Laughlin et al., 2004; Schmitz and Barrett, 2002). A better understanding of these differences may lead to improved and accelerated transplant recovery as well as reduced incidences of disease relapse. Neonatal cells have also been more robust and less fastidious in previous lymphoid culture studies (Ichii et al., 2008; Rossi et al., 2003). As noted above, G-CSF preferentially influences adult progenitors but lymphopoiesis is still less efficient than when cord blood $CD34^+$ cells are used. Again, experimental approaches involving cultures might be used to seek a molecular basis for neonatal/adult differences.

These findings represent an increment in techniques for observing human B lymphopoiesis in culture. While there is considerable room for further improvement, it is already clear that complex questions about species specific and lymphoid leukemia regulators as well as stem and progenitor cell heterogeneity can now be addressed.

Acknowledgments

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Original Article

Diffuse large B-cell lymphoma in the spinal epidural space: A study of the Osaka Lymphoma Study Group ☆

Naoki Wada [a], Masaharu Kohara [a], Junichiro Ikeda [a], Yumiko Hori [a], Shigeki Fujita [a], Masaya Okada [b], Hiroyasu Ogawa [b], Haruo Sugiyama [c], Shirou Fukuhara [d], Akihisa Kanamaru [e], Masayuki Hino [f], Yuzuru Kanakura [g], Eiichi Morii [a], Katsuyuki Aozasa [a,*]

[a] Departments of Pathology, Osaka University Graduate School of Medicine, Suita, 2-2 Yamadaoka, Suita, Osaka 565-0871, Osaka, Japan
[b] Department of Internal Medicine, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan
[c] Functional Diagnostic Science, Osaka University Graduate School of Medicine, Suita, Osaka, Japan
[d] First Department of Internal Medicine, Kansai Medical University, Moriguchi, Osaka, Japan
[e] Department of Hematology, Kinki University School of Medicine, Sayama, Osaka, Japan
[f] Department of Clinical Haematology and Diagnostics, Osaka City University Graduate School of Medicine, Osaka, Japan
[g] Hematology and Oncology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan

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ABSTRACT

Diffuse large B-cell lymphoma (DLBCL) involving spinal epidural space (SEDLBCL) is relatively rare, constituting 1.8% of DLBCLs in Osaka, Japan. The aim of this study was to analyze SEDLBCL cases for their clinical and histopathologic findings, including an association with Epstein-Barr virus (EBV) and immunohistochemical characteristics.

We analyzed the clinicopathologic findings of 27 SEDLBCL cases. They consisted of 16 males and 11 females, their age ranging from 37–86 years (median 64 years). Eight patients had stage I disease, 3 had stage II, 5 had stage III, and 11 had stage IV. Based on the staining pattern for anti-CD10, bcl-6, and MUM-1, the cases were categorized into 17 cases of the germinal center B-cell (GCB) type and nine of the non-GCB type. There was a 4%-positive rate for EBV in the tumor cells. When compared to nodal DLBCL, the frequency of patients with a high performance status (PS) is higher in SEDLBCL. Compared to the DLBCL of the central nervous system (CNS), the frequency of cases with high stage, 2 or more extranodal lesions, high international prognostic index (IPI), and GCB-type is higher in SEDLBCL. There were no significant differences in the histologic features between SEDLBCL and nodal/CNS DLBCL. Univariate analysis revealed that advanced stage was an unfavorable factor for overall survival (P=0.060).

SEDLBCL is different from nodal and CNS DLBCL, but an association with EBV is unlikely in every group.

1. Introduction

In the World Health Organization (WHO) classification for lymphoid neoplasias, malignant lymphomas are largely divided into B-cell neoplasias, T/NK-cell neoplasias, and Hodgkin's lymphomas (HL). Diffuse large B-cell lymphoma (DLBCL), the most common category, representing approximately 30% of all non-Hodgkin's lymphomas (NHL) worldwide, is defined as diffuse proliferation of large neoplastic lymphoid B-cells [19,21]. Patients with DLBCL present with a predominantly nodal disease, but approximately 40% of the cases initially present with extranodal lesions. The gastrointestinal tract, especially the stomach, is most commonly involved [19]. Several subtypes of DLBCL are listed in the classification according to the specific sites of involvement, for example primary DLBCL of the central nervous system, primary cutaneous DLBCL, leg type, primary mediastinal (thymic) large B-cell lymphoma, and primary effusion lymphoma [19].

Spinal epidural NHL is a rare disease accounting for 10% to 30% of all epidural malignancies [3], and for 0.1% to 3.3% of all lymphomas [11]. Since the introduction of lymphoid neoplasm as a new modality for lymphoma classification (1994) into the Revised European–American Classification of Lymphoid Neoplasms (REAL), clinicopathologic findings of approximately 200 cases of spinal epidural malignant lymphomas have been reported to date. Information for immunophenotypes is available in 94 cases, in which B-cell lymphomas account for 84 cases and T-cell

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* Corresponding author: Tel.: 81 6 6879 3710; fax: 81 6 6879 3713.
E-mail address: aozasa@molpath.med.osaka-u.ac.jp (K. Aozasa).

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lymphomas for 10. A diagnosis of DLBCL was rendered in 30 cases: the items evaluated in these cases varied from report to report [2,5,7–9,12,13,17]. There is no information or only limited information available regarding the frequency of the germinal center B-cell (GCB) type and the non-GCB type of DLBCL, the accurate frequency of Epstein-Barr virus (EBV)-positive cases, and factors affecting prognosis.

Reviewing the cases registered by the Osaka Lymphoma Study Group (OLSG) between 1999 and 2009, we found that 33 cases had undergone histologic examination for spinal epidural malignant lymphoma (SEML). They consisted of 27 DLBCL cases, 2 HLs, 2 plasmacytomas, 1 marginal zone B-cell lymphoma, and 1 hematopoietic neoplasia not further specified. In this study, twenty-seven cases of spinal epidural DLBCL (SEDLBCL) were analyzed for their clinical and histopathologic findings, including the association with EBV and immunohistochemical characteristics.

2. Material and methods

2.1. Patients

Between November 1999 and February 2009, a total of 4162 cases were registered by OLSG, Japan. The histologic specimens obtained by biopsy were fixed in 10% formalin and routinely processed for paraffin-embedding. Histologic sections (4 μm) were stained with hematoxylin/eosin and immunoperoxidase (ABC method). All of the histologic sections were reviewed by one of the authors (KA) and classified according to the WHO classification. The diagnosis of malignant lymphoma was confirmed in 3307 (79.5%) of 4162 cases. Of these 3307 cases, 3031 (91.7%) were NHL and 276 (8.3%) HL. There were 1471 DLBCL cases, constituting 48.5% of all NHLs. In 33 cases, spinal epidural space was the site mainly involved, and was subject to surgery: DLBCL was diagnosed in 27 cases, HL in 2, plasmacytoma in 2, marginal zone B-cell lymphoma in 1, and hematopoietic neoplasia, not further specified in 1. Twenty-seven cases of DLBCL involving the spinal epidural space (SEDLBCL) were selected for the present study. Thirty-nine cases of nodal DLBCL and 31 cases of central nervous system (CNS) DLBCL with adequate clinical data and unstained sections for additional immunohistochemical analyses and in situ hybridization were included as controls: the nodal DLBCLs were registered by OLSG between April 2000 and February 2005, and the CNS cases at the Department of Neurosurgery, Osaka University, between December 1999 and July 2009.

Table 1 summarizes the clinicopathologic findings of SEDLBCL and the control groups. The SEDLBCL group consisted of 16 males and 11 females, their age ranging from 37–86 (median 64) years. Every patient had its main lesion in the spinal epidural space. Based on the records of physical examinations, surgical notes, and pathologic examinations of the specimens, the Ann Arbor staging system was applied. Eight cases had stage I of disease, 3 had stage II, 5 had stage III, and 11 had stage IV. The IPI score was calculated using five adverse factors (age > 60 years; Ann Arbor stages III and IV; Eastern Cooperative Oncology Group performance score 2–4; elevation of serum lactate dehydrogenase (LDH); and 2 or more extranodal lesions) present at the time of diagnosis [20]. For cases under 60 years, an age-adjusted IPI score was applied, in which advanced stage, high performance score (PS), and elevation of LDH were considered as adverse factors [20]. The IPI score 0/1 or age-adjusted IPI score 0 was categorized as low risk group, IPI score 2 or age-adjusted IPI score 1 as low-intermediate, IPI score 3 or age-adjusted IPI score 2 as high-intermediate, IPI score 4/5 or age-adjusted IPI score 3 as high. Twelve patients with SEDLBCL received a combination of radiotherapy and chemotherapy, and 15 patients received chemotherapy only. The clinical outcome was evaluated according to the guidelines of the International Workshop to standardize response criteria for NHL [4].

2.2. Immunohistochemistry

For immunophenotyping the following monoclonal antibodies were used: CD20, CD3, Bcl-6, MUM1, MIB-1 (Dakocytomation, Glostrup, Denmark, dilution at 1:400, 1:50, 1:50, 1:100, 1:1, respectively), and CD10 (NICHIREI BIOSCIENCES, Tokyo, Japan, used as prediluted antibody). Tonsils with reactive lymphoid hyperplasia served as external control tissues. In MIB-1 staining, the number of positive cells among 300–1000 large lymphoid cells was counted: MIB-1 index was calculated as positive cells/100 cells.

2.3. In situ hybridization

RNA in situ hybridization using the EBER-1 (Epstein-Barr encoded RNAs) probe was performed to examine the presence of EBV genome on the formalin-fixed, paraffin-embedded sections

Table 1
Brief clinicopathologic findings of diffuse large B-cell lymphoma in spinal epidural space and others.

Characteristic	Site			*P*
		Control		vs. Lymph node / CNS
	Spinal epidural space	Lymph node (*n*=39)	CNS (*n*=31)	
Age (years): range (mean/median)	37–86 (64.9/64)	38–79 (61.3/61)	31–82 (64.3/67)	NS/NS
Age > 60 years, *n* (%)	19/27 (70.4%)	20/39 (51.3%)	22/31 (71.0%)	NS/NS
Sex, male:female	16:11	25:14	15:16	NS/NS
Serum LDH level > normal, *n* (%)	16/26 (61.5%)	24/39 (61.5%)	14/27 (51.9%)	NS/NS
Performance status 2–4, *n* (%)	21/27 (77.8%)	8/39 (20.5%)	19/31 (61.3%)	< 0.01/NS
Stage 3/4, *n* (%)	16/27 (59.3%)	22/39 (56.4%)	1/31 (3.2%)	NS/ < 0.01
Involved extranodal organ > 1, *n* (%)	13/27 (48.1%)	10/39 (25.6%)	0/31 (0%)	0.059/ < 0.01
IPI, HI/H, *n* (%)	19/26 (73.1%)	20/39 (51.3%)	10/27 (37.0%)	0.079/ < 0.01
Fibrosis, present:absent	16:11	16:23	0:28	NS/ < 0.01
Mitotic count (/high-power field) mean (range)	2.9 (0–7)	3.1 (0–10)	2.8 (0–10)	NS/NS
MIB-1, %, mean (range)	58.8 (30–90)	61.8 (30–80)	63.1 (20–90)	NS/NS
GCB:non-GCB	17:9	18:20	5:16	NS/ < 0.01
EBV-positive, *n* (%)	1/25 (4%)	1/14 (7.1%)	1/20 (5%)	NS/NS

IPI: International prognostic index; HI/H, high-intermediate/high; GCB, germinal center B-cell type; CNS, central nervous system; NS, not significant.

according to the previously described method with some modifications [23]. Briefly, we synthesized a 30-base oligonucleotide probe; 5′-AGACACCGTCCTCACCACCCGGGACTTGTA-3′, which was the sense and antisense for a portion of the EBER-1 gene, a region of the EBV genome that is actively transcribed in latently infected cells. The Raji cell line was used as a positive control. The hybridizing mixture containing sense or antisense probe after RNase treatment was used as a negative control. The presence of EBV genomes was evaluated in 25 SEDLBCL cases and in 14 nodal and 20 CNS DLBCL cases as controls. When the in situ hybridization yielded positive signals in the nuclei of more than 1% of the proliferating cells, such cases were defined as EBV-positive.

2.4. Clonality analysis with use of Ig gene rearrangement (Gene Scan analysis) and BCL2-IgH gene rearrangement

One to five sections (4–10 μm) were cut from the paraffin-embedded samples, deparaffinized with xylene, washed with 70% and absolute ethanol, and subsequently digested in lysis buffer (50 mM Tris–HCl, 10 mM EDTA, 150 mM NaCl, 0.5% SDS and 0.4 mg/l proteinase K) at 55 °C overnight. DNA was extracted according to phenol–chloroform extraction-based protocol, followed by ethanol precipitation, and was redissolved in TE buffer. Immunoglobulin (Ig) gene rearrangement was assessed by 8 PCRs with 41 primers according to BIOMED-2 protocols (4 multiplex PCRs and 1 single PCR with 28 primers for Ig heavy chain (IgH) gene; 2 multiplex PCRs with 10 primers for Ig kappa light chain (Igκ) gene; 1 multiplex PCR with 3 primers for Ig lambda light chain (Igλ) gene) [22]. For this, fluorescence-labeled (6-FAM, VIC, NED, PET), custom-made primers were purchased from Applied Biosystems (Tokyo, Japan). When the PCR amplification was not sufficient because of the small amount of extracted DNA or fragmentation of DNA, we modified the PCR condition of BIOMED-2 protocols (Table 2). The amplified PCR products were mixed and diluted to ×16 or ×32 volumes with distilled water. Diluted samples (0.5 μl) were added to 11.5 μl Hi-Di formamide containing 0.5 μl internal size standard (Gene Scan-600 LIZ; Applied Biosystems). After denaturation at 95 °C for 3 min, the samples were cooled with ice-cold water, and analyzed using ABI PRISM 310 genetic analyzer with DS-33 dye-set and software v. 3.1. (Applied Biosystems). For detection of BCL2-IgH chimera gene generated by t(14;18)(q32;q21), BIOMED-2 PCR protocol for MBR, 3'MBR and mcr was applied as described previously [22]. The amplified PCR products were electrophoresed in 5.0% or 6.6% polyacrylamide gel based on fragment sizes. Samples for clonality analysis were not available in 10 SEDLBCL cases.

Table 2
Summary of the PCR protocols for the original and modified BIOMED-2 PCR reactions.

	Original BIOMED-2	Modified BIOMED-2
PCR reaction mix		
Template DNA	40 ng/50 μL	20 ng/50 μL
dNTP	200 μM	No change
Buffer ($MgCl_2$)	1.5 mM	No change
Primer	0.2 μM	0.5 μM
AmpliTaq gold	1 U/50 μL	2.5 U/50 μL
PCR run parameters		
Denature	30 s	No change
Annealing	30 s	No change
Extention	30 s	No change
Cycles	35	40

2.5. Follow-up

SEDLBCL patients were followed until July 2009. The follow-up period for survivors ranged from 4.6–49 (average 26.1) months. Sixteen of 27 patients were alive at the end of the observation period. The Kaplan–Meier estimated survival rate for the SEDLBCL at 4 years was 58.0%.

2.6. Statistical analysis

Differences in the frequencies of various clinical and pathologic factors between SEDLBCL and control DLBCL cases were compared using the Chi-square test or the Fisher's exact probability test. Differences in mean values were compared with the *t* test or the Mann–Whitney test. Survival curves and overall survival rates were calculated using the Kaplan–Meyer method and were compared by the log-rank test. Multivariate analysis was performed with the Cox proportional hazard regression model.

3. Results

3.1. Clinical findings

The clinical and pathologic findings of SEDLBCL are summarized in Table 1. One patient suffering from psoriasis was treated with cyclosporine A for six years, and SEDLBCL developed. One patient suffered from uterus cancer and another from large bowel cancer. In the remaining patients, there were no findings that suggested the presence of immunodeficient conditions. Three patients had a history as carrier of hepatitis B virus. Five patients suffered from chronic hepatitis caused by hepatitis C virus, with liver cirrhosis in one. There were no significant differences in age, sex ratio, and serum LDH level between SEDLBCL and control DLBCL cases. When compared to nodal DLBCL, the frequency of patients with high performance status (PS 2–4) is higher in SEDLBCL. When compared to CNS DLBCL, the frequency of patients with advanced stage (stage 3/4), 2 or more extranodal lesions, high IPI score (high-intermediate and high risk groups), and ratio of GCB-type to non-GCB type is higher in SEDLBCL. Back pain was the commonest symptom (70.4% of patients), followed by weakness of the lower limbs (66.7%), sensory disturbance (59.3%), dysfunction of bladder (29.6%) and bowel (22.2%), and weakness of the upper limbs (11.1%). Roentgenographic examination revealed the involvement of thoracic, lumbar, and other areas in 48.1%, 18.5%, and 7.4% of cases, respectively. Multiple lesions involving the thoraco-lumbar, cervico-thoracico, lumbo-sacral, and thoraco-lumbo-sacral areas were found in three, two, one, and one case. Bone infiltration was found in 7 cases.

3.2. Histologic and immunohistochemical findings

There were no significant differences in histologic features between the SEDLBCL and control groups. Minute to small necrotic foci were occasional in three SEDLBCL cases. Finely fibrous tissue in the background of the lesions was found in 16 (59.3%) of 27 SEDLBCL cases and in 16 (41.0%) of 39 nodal DLBCL cases. The difference was not statistically significant. The mean mitotic count in one high-power field and MIB-1 labeling index among large lymphoid cells in SEDLBCL, nodal and CNS DLBCL were 2.9, 3.1 and 2.8, and 58.8, 61.8 and 63.1, respectively.

Immunohistochemically, the large lymphoid cells were $CD20^+$ and $CD3^-$. According to the criteria proposed by Hans et al. [6], SEDLBCL could be categorized into GCB ($CD10^+$ or $CD10^-$/bcl-6^+/

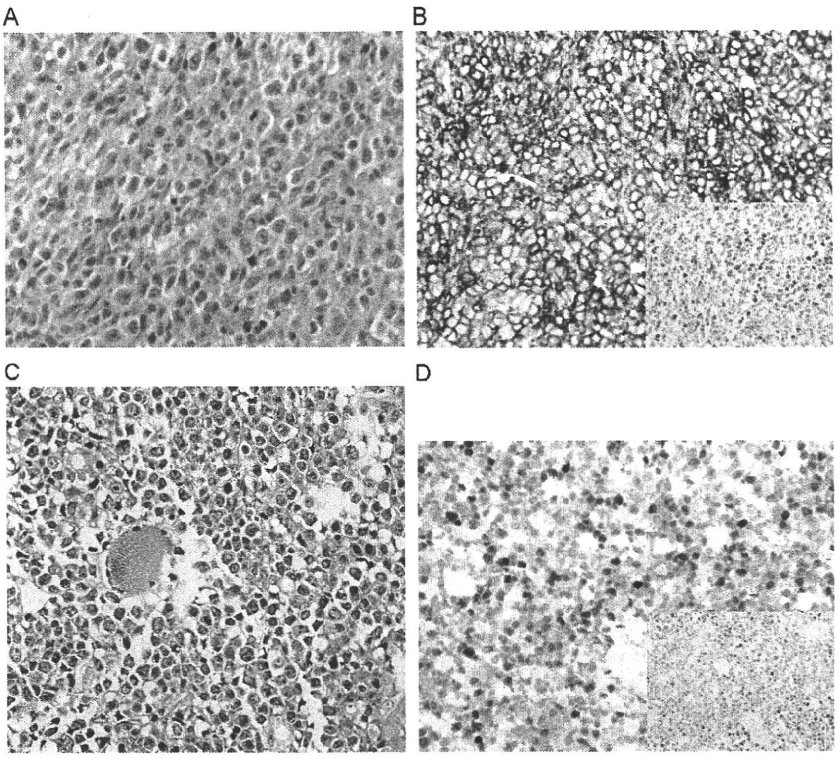

Fig. 1. (A) Diffuse large B-cell lymphoma of germinal center B-cell (GCB) type. Proliferating cells have an oval to round nucleus with occasional slight indentation, in which one to several rather small nucleoli are discernible. H&E. (B) Proliferating cells show strong immunoreactivity for CD10. Inset: they also show immunoreactivity for bcl-6. (C) Non-GCB type. There was a diffuse proliferation of large lymphoid cells with rather rich, basophilic cytoplasm and occasional prominent nucleoli mimicking immunoblasts. H&E. (D) Proliferating cells show strong nuclear staining for MUM-1. Inset: they also show immunoreactivity for bcl-6. All ×400.

MUM1$^-$) and non-GCB (CD10$^-$/bcl-6$^+$ or CD10$^-$/bcl-6$^-$/MUM1$^+$) type (Fig. 1). The ratio of the GCB to the non-GCB subgroup was significantly higher in SEDLBCL (17:9) than in CNS DLBCL (5:16) ($P < 0.01$).

3.3. *In situ hybridization*

In situ hybridization with EBER-1 probe revealed positive signals in the nucleus of large lymphoid cells in 1 (4%) of 25 SEDLBCL cases. The EBV-positive rate was similar between cases with SEDLBCL and the control groups.

3.4. *Molecular genetic study*

Genotypic study was performed in 17 cases with SEDLBCL. All cases showed monoclonal rearrangement of Ig genes with at least one primer. Monoclonal bands for both IgH and Ig light chain (IgL) genes were found in 11 cases, only for IgH gene in 3, and only for IgL gene in 3. Monoclonal rearrangement of BCL2-IgH gene was detected in 2 (11.8%) of 17 cases examined. Tumor cells in these two cases showed positive immunoreactivity for CD10.

3.5. *Prognostic factors*

The results of the univariate analysis are shown in Table 3. The stage of disease showed a marginal significance for prognosis (P=0.060). Multivariate analysis for age, sex, serum LDH level, PS, stage, extranodal involvement, IPI, GCB/non-GCB, and therapy (chemotherapy or the combination of chemotherapy and radiotherapy) revealed that none of them significantly affected the prognosis of the patients.

4. Discussion

In previous reports, spinal epidural involvement by malignant lymphoma has been described in 0.1% and 3.3% of cases [11]. The frequency was considerably lower in the present series, i.e., in 33 of 3307 cases (0.1%). Although it has been reported that T-cell lymphomas are occasionally found among NHL involving spinal epidural space [3,8,10,12,14,17], NHLs in the present series were composed exclusively of B-cell lymphomas, the vast majority being DLBCL. In the SEDLBCL presented here, age distribution and sex ratio were rather similar to those reported previously [2,5,7–9,12,17].

At diagnosis, approximately 60% of the SEDLBCL patients presented here had advanced stages of disease (stages III–IV). As clinicians regarded the spinal epidural space as the main lesion, it was extirpated for histologic examination. This might lead to the hypothesis that spinal epidural involvement is a manifestation of systemic DLBCL, an aggressive lymphoma, or local spread of DLBCL from the vertebral bones or paravertebral lesion. Another possibility would be a development of *de novo* DLBCL in the spinal epidural space. Russell and Rubinstein state that native lymphoid cells are derived from mesodermal cells in the spinal epidural space, and it is conceivable that they could follow a transformation cascade to produce primary SEML [16]. On the other hand, formation of lymphoid tissue in the chronically inflamed tissue might provide a basis for the development of malignant lymphoma consisting mostly of the B-cell type. Under such a

Table 3
Univariate analysis for overall survival in patients with diffuse large B-cell lymphoma of spinal epidural space.

Characteristic	Number of patients	OS (%)	P
Age, years		4-Year OS	
Age≦60	8	75.0	NS
Age > 60	19	50.1	
Sex		4-Year OS	
Male	16	68.2	NS
Female	11	50.5	
Serum LDH level		4-Year OS	
Normal or lower	10	75.0	NS
Higher than normal	16	50.9	
Performance status		3-Year OS	
Ambulatory (0–1)	6	50.0	NS
Not ambulatory (2–4)	21	58.5	
Ann Arbor stage		3-Year OS	
Stage ½	11	78.7	0.060
Stage ¾	16	35.8	
Extranodal involvement		3-Year OS	
0–1 Site	14	65.0	NS
> 1 Sites	13	55.9	
International prognostic index		4-Year OS	
L/LI	7	85.7	NS
HI/H	19	45.4	
GCB/non-GCB		2-Year OS	
GCB	17	75.1	NS
Non-GCB	9	50.0	
Chemo alone/Chemo+RT		4-Year OS	
Chemo alone	15	61.3	NS
Chemo+RT	12	55.6	

OS: overall survival; L/LI, low/low-intermediate; HI/H, high-intermediate/high; GCB, germinal center B-cell type; Chemo alone, chemotherapy alone; Chemo+RT, combined chemotherapy and radiotherapy; NS, not significant.

condition, the formed lymphoid tissue is referred to as mucosa-associated lymphoid tissue (MALT), and the lymphoma originating from it is a marginal zone B-cell lymphoma. Indeed, the present series of spinal epidural NHL includes a case of marginal zone B-cell lymphoma. Among the current SEDLBCL cases, the history included narrowing of the lumbar vertebral canal, tuberculosis, pleuritis, interstitial pneumonia, or psoriasis vulgaris in a total of five cases.

DLBCL is the most common lymphoma worldwide, and comprises heterogenous groups of diseases showing a wide range of response patterns to treatment. There were no significant differences in histologic features between SEDLBCL and nodal/CNS DLBCL, except for the ratio of the GCB to the non-GCB type as discussed later. Based on the gene expression profiles, Alizadeh et al. categorized the DLBCL cases into germinal center and activated B-cell signatures, with a more favorable prognosis for the former than for the latter group [1]. Hans et al. proposed to categorize DLBCL into GCB and non-GCB types based on the immunohistochemical findings [6]: $CD10^+$ or $CD10^-$/bcl-6^+/$MUM1^-$ as GCB and $CD10^-$/bcl-6^- or $CD10^-$/bcl-6^+/$MUM1^+$ as non-GCB type. There are no reports categorizing SEDLBCL as GCB or non-GCB type. The ratio of GCB and non-GCB cases in the present cases of SEDLBCL (17:9) was higher than that in a previous report on the ordinary type of DLBCL (about 1:1) [15]. The ratio in SEDLBCL was significantly higher than that in CNS DLBCL (5:16) ($P < 0.01$). It has been reported that DLBCL of the GCB type had a more favorable prognosis than that of the non-GCB type [1,6,18]. The SEDLBCL with the GCB type showed a rather favorable prognosis compared to those with the non-GCB type, although the difference was not statistically significant (log-rank, $P > 0.05$): 2-year survival rate was 75.1% and 50.0%, respectively (Table 3).

The EBV-positive rate in the ordinary DLBCL has been reported to account for approximately 10% worldwide [19], but it is really much lower in Osaka, Japan, i.e., less than 5% [24]. An EBV-positive rate in SEDLBCL in a large series of cases has not been reported to date. The EBV-positive rate in the present SEDLBCL cases (4%) was rather similar to that in ordinary DLBCL, indicating that a role of EBV for the development of SEDLBCL is unlikely.

Univariate analysis revealed that stage of disease showed a marginal significance for prognosis (P=0.060). Multivariate analysis revealed that none of the clinicopathologic factors was useful for predicting the overall survival of the SEDLBCL. This might be due to the relatively small number of cases analyzed in the present study.

In conclusion, SEDLBCL is different from nodal and CNS DLBCL, but an association with EBV is unlikely in every group.

Conflict of interest statement

We declare that we have no conflict of interest.

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