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Regulation of human B lymphopoiesis by the transforming growth factor-β superfamily in a newly established coculture system using human mesenchymal stem cells as a supportive microenvironment

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Objectives. **To characterize and evaluate the validity of a novel coculture system for studying human B-lymphocyte developmental biology.**

Materials and Methods. **We developed a long-term culture system to produce B lymphocytes from human CD34$^+$ cells purified from umbilical cord blood using human mesenchymal stem cells (hMSC) as stroma. We evaluated the effects of several low molecular weight inhibitors, recombinant proteins, and neutralizing antibodies (Abs) as potential regulators of B-lymphocyte development.**

Results. **Our cocultures of 2000 CD34$^+$ cells in the presence of stem cell factor and Flt3-ligand produced 1–5 × 10^5 CD10$^+$ cells after 4 weeks of culture. Surface IgM$^+$ immature B cells began to appear after 4 weeks. We evaluated the negative-regulatory effects of the transforming growth factor (TGF)-β superfamily on human B lymphopoiesis, and found that adding an anti–activin A antibody enhanced generation of CD10$^+$ cells two- to three-fold. As well, the proportion of CD10$^+$ cells in the generated cells increased markedly, indicating that activin A downregulated B lymphopoiesis more efficiently than myelopoiesis. Addition of TGF-β1 suppressed B-lymphocyte production by 20% to 30%, while addition of an anti–bone morphogenetic protein (BMP)-4 antibody or recombinant BMP-4 had no effect. Therefore, the strength of ability to suppress human B lymphopoiesis seemed to be activin A > TGF-β1 > BMP-4. None of these three factors influenced the emergence of IgM$^+$ cells.**

Conclusions. **hMSC coculture supported human B lymphopoiesis. Activin A selectively suppressed B lymphocyte production.** © **2008 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

B lymphocytes develop from hematopoietic stem cells within bone marrow (BM), and play an essential role in immune system function. B-lymphocyte production is regulated by an elaborate scheme involving many different soluble or adhesion molecules; failure to control production adequately can promote the development of diseases with quantitative and/or qualitative B-lymphocyte abnormalities [1]. A variety of murine assay systems are used to evaluate the mechanisms of B-lymphocyte regulation, such as Whitlock-Witte–type long-term BM cultures, cocultures of murine BM cells on stromal cell lines, and colony assays. Studies utilizing these culture systems have elucidated many regulatory mechanisms of B-lymphocyte development in mice. For example, CD44 [2], vascular cell adhesion molecule-1, and very-late activation antigen-4 [3] are adhesion molecules essential for B lymphopoiesis. We have also reported that a novel interferon-ζ/limitin suppressed colony formation of B-lymphocyte progenitors [4,5] and that an adipocyte-specific protein, adiponectin,

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inhibited stroma-dependent B-lymphocyte growth through induction of prostaglandins (PGs) [6]. In addition, members of the TGF superfamily [7], the Wnt family [8], and the Notch family [9] are known to regulate proliferation, differentiation, and survival, depending on the developmental stage of the B lymphocytes. Although early B-lymphocyte development was believed to require direct interactions with supportive stromal layers, in previous work we generated $CD45RA^+CD19^+$ B lymphocytes from murine $Lin^-c\text{-}kit^{high}$ and $Lin^-c\text{-}kit^{low}$ cells in the presence of stem cell factor (SCF), Flt3-ligand (FL), and interleukin (IL)-7, in the absence of stromal cells [10,11].

In contrast to mice, systems to evaluate human B-lymphocyte developmental biology are not fully established because of a lack of appropriate human stromal materials. Several culture systems for human hematopoietic cells have utilized murine stromal cell lines as supportive microenvironment [12–15]. Although murine stromal cells produce cytokines that could potentially affect human B-lymphocyte development, some of these molecules have no interspecies cross-reactivity [16]. As well, there are some mechanistic differences between murine and human B-lymphocyte development. For example, signaling through the IL-7 receptor (IL-7R) is critical for adult murine B-lymphocyte development [17,18]. However, in humans, disrupting IL-7R does not induce arrest of B lymphopoiesis [19,20]. In addition, human B-lymphocyte progenitor cells cannot expand without stroma [12], whereas murine progenitor cells can [10,11]. Therefore, assay systems more relevant to human biology are needed. A previous study reported the establishment of a serum-free human BM stromal cell culture; however, the investigators isolated cellular components from fetal BM, which is currently unavailable for research purposes [21,22].

In this study, we show that human mesenchymal stem cells (hMSC) can support the commitment and differentiation of human $CD34^+$ cells into B lymphocytes. Our cocultures of 2000 human $CD34^+$ cells on hMSC in the presence of SCF and FL produced $1–5 \times 10^5$ $CD10^+$ B lymphocytes after 4 weeks. Using this coculture system, we determined that members of the transforming growth factor-β (TGF-β) superfamily, activin A and TGF-β1, were negative regulators for early onset of human B lymphopoiesis. The TGF-β superfamily has more than 20 members, including three TGF-βs, two inhibins, three activins, seven bone morphogenetic proteins (BMPs), and nodal [23]. There are two types of receptors for the TGF-β superfamily, type I (activin receptor-like kinase [ALK]-1-7) and type II receptors [24]. Their specific ligand–receptor interactions induce critical effects on a wide range of physiological and pathological processes, such as immune responses, angiogenesis, tumor development, and wound healing [25]. In addition, TGF-βs, activin A, and BMPs have been reported to influence lymphohematopoiesis [7,26,27]. Here, we explore the similarities and/or differences of functions of the TGF-β superfamily members between humans and mice by comparing our results obtained from the human B-lymphocyte coculture with data from several previous reports.

Materials and methods

Origin and isolation of cells

Cord blood (CB) cells were collected from healthy, full-term neonates immediately after delivery by Cesarean section. All participants provided prior informed consent. Mononuclear cells were separated by Ficoll-Paque PLUS (GE Healthcare Bio-Science AB, Uppsala, Sweden) centrifugation. CB $CD34^+$ cells were purified using the Direct CD34 Progenitor Cell Isolation Kit (human; Miltenyi Biotec, Auburn, CA, USA). BM-derived hMSC were purchased from Cambrex Bio Science Walkersville (Walkersville, MD, USA) and maintained in MSC Growth Medium (Cambrex Bio Science Walkersville). Human umbilical vein endothelial cells (HUVEC) were purchased from Cascade Biologics (Portland, OR, USA), and maintained in Humedia-EG2 (Kurabo, Osaka, Japan). The murine stromal cell line MS-5, kindly provided by Dr. Mori (Niigata University), was maintained in α-minimum essential medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS).

Recombinant proteins and reagents

Recombinant human SCF, FL, IL-7, TGF-β1, activin A, and BMP-4 proteins were purchased from R&D Systems (Minneapolis, NY, USA). Granulocyte-colony stimulating factor (G-CSF) was gifted from Kirin Brewery (Tokyo, Japan). N-acetylcysteine was purchased from Sigma (St. Louis, MO, USA), DUP697 from Cayman Chemicals (Ann Arbor, MI, USA), BIO from Calbiochem (Darmstadt, Germany), and SB431542 from TOCRIS Bioscience (Ellisville, MO, USA). Neutralizing antibodies (Abs) against human TGF-β1, activin A, and BMP-4 were purchased from R&D Systems. Follistatin was purchased from Calbiochem.

Cocultures for human B lymphocytes

hMSC were seeded in 12-well tissue culture plates (Iwaki, Tokyo, Japan) 1 or 2 days before coculture. Isolated CB $CD34^+$ cells (2000 cells/well) were plated on subconfluent hMSC layers in MSC growth medium in the presence of 10 ng/mL SCF and 5 ng/mL FL. Half of culture medium was replaced with fresh medium containing the same cytokines twice per week. When appropriate, the cultured cells on hMSC were collected and their phenotypes were analyzed with flow cytometry. In some experiments, cultures were performed in medium containing low molecular weight inhibitors or neutralizing Abs, as indicated. In other experiments, HUVEC or MS-5 cells were used as stroma. In cocultures containing HUVEC or MS-5 cells, the culture media were Iscove's modified Eagle's medium (Gibco) supplemented with 20% FCS and 2 mM glutamine or α-minimum essential medium supplemented with 10% FCS, respectively.

Flow cytometry and cell sorting

Flow cytometry analysis was performed with a FACSCalibur (BD Biosciences Immunocytometry Systems, San Jose, CA, USA) using standard multicolor immunofluorescent staining protocols [28]. Murine monoclonal Abs against the following human cell surface molecules were purchased: phycoerythrin (PE)-CD3,

PE-CD10, allophycocyanin (APC)-CD10, PE-CD19, PE-CD20, fluorescein isothiocyanate (FITC)-CD33, APC-CD33, PE-CD34, APC-CD34, FITC-CD38, FITC-CD45, and PE-glycophorin A from BD Biosciences/BD Pharmingen; PC5-CD19 from Beckman Coulter (Marseilles, France), FITC-IgM from Southern Biotechnology Associates (Birmingham, AL, USA). Cultured cells were categorized as myeloid lineage cells ($CD33^+$ and $CD10^-$), B lymphoid lineage cells ($CD33^-$ and $CD10^+/CD19^+$), or immature B cell ($CD33^-$, $CD10^+$, $CD19^+$, and IgM^+). In some experiments, $CD34^+CD38^-$, $CD34^+CD38^+CD10^-$, and $CD34^+CD38^+CD10^+$ cells were sorted using a FACS Aria (BD Biosciences Immunocytometry Systems).

Limiting dilution assays

Limiting dilution assays were performed in 96-well plates (Iwaki) preseeded with hMSC. CB $CD34^+$ cells were plated at various concentrations from 1 to 100 cells/well. Each well contained 200 μL MSC Growth Medium with 10 ng/mL SCF and 5 ng/mL FL, with or without 10 μM SB431542. Half of culture medium was replaced with fresh medium containing the same cytokines twice per week. After 28 days of coculture, wells with cell expansion were scored. Individual expanded cells were analyzed by flow cytometry, and the number of culture wells containing $CD10^+$ cells was determined.

Reverse transcription polymerase chain reaction

Total RNA was extracted from CB cells and hMSCs using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. RNAs were reverse transcribed and target cDNAs were amplified by polymerase chain reaction using 0.5 U Taq DNA polymerase (Applied Biosystems, Branchburg, NJ, USA; primers sequences are available upon request) [29].

Enzyme-linked immunosorbent assay

TGF-β1 was detected using Immunoassay Kit (Biosource International, Camarillo, CA, USA; sensitivity 15.6 pg/mL). Activin A was detected using DuoSet ELISA Development System (R&D Systems; sensitivity 117.2 pg/mL). Each step of the reactions was performed according to manufacturer's instructions.

Statistical analysis

Student's *t*-test was used to analyze statistically significant differences between data sets. All results are reported as mean values ± standard deviation.

Results

hMSC support B-lymphocyte development from human CB $CD34^+$ cells

The murine stromal cell line MS-5 is used widely to support murine and human lymphohematopoietic cells in culture [12,13]. HUVECs express several adhesion molecules, such as vascular cell adhesion molecule-1 [30], that interact with B lymphocytes. hMSC have the ability to support human hematopoietic stem cells in culture [31], and can enhance engraftment of human hematopoietic stem cell transplantation [32]. Therefore, we compared the ability of these three cell types to support human B lymphopoiesis in coculture. When purified human CB $CD34^+$ cells were cultured on each different monolayer, $CD19^+$ cells were generated within 4 weeks. As shown in Figure 1, cocultures on hMSCs generated many more $CD19^+$ cells than those on MS-5 or HUVEC. In addition, similar supporting activity for human B lymphopoiesis was observed for two different lots of hMSC (data not shown). Finally, HUVEC started to detach from the culture wells within 3 weeks of culture, while hMSC did not.

Therefore, hMSC appear to better support human B-lymphocyte progenitor cell development than HUVEC or MS-5.

SCF and FL enhance human B lymphopoiesis in coculture

In mice, SCF, FL, and IL-7 are critical for early B-lymphocyte development [10,11]. Therefore, coculture of human CB $CD34^+$ cells on hMSC included various combinations of SCF, FL, and IL-7. Although the addition of SCF, FL, or IL-7 individually to the cocultures enhanced production of B lymphocytes slightly (data not shown), many more B lymphocytes were recovered when these factors were added in combination (Fig. 2A). Anti-CD33 Ab recognizes a 67-kD type I transmembrane glycoprotein expressed mainly on monocytes, granulocytes, and myeloid progenitors, but not on lymphocytes and hematopoietic stem cells. Anti-

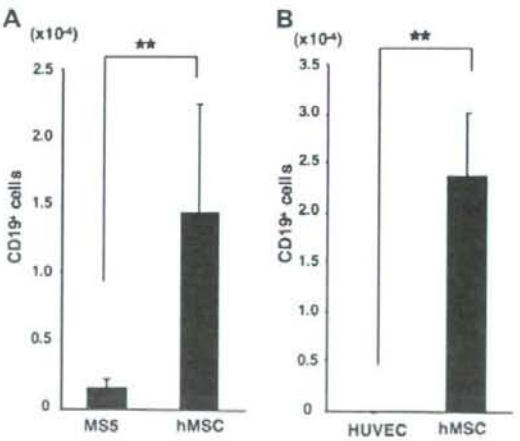


Figure 1. Human mesenchymal stem cells (hMSC) have high supporting activity of human B lymphocytes. Purified cord blood (CB) $CD34^+$ cells (2000 cells/well) were cultured on subconfluent of hMSC (**A**, **B**), MS-5 (**A**), or human umbilical vein endothelial cells (**B**) in the absence of any cytokines for 4 weeks. Total numbers of the generated cells were calculated, and surface phenotypes of the cells were analyzed with flow cytometry. Data are shown as mean ± standard deviation of the generated $CD19^+$ cell numbers in triplicated samples. Statistically differences from control values are shown with two asterisks ($p < 0.01$). Similar results were obtained in three independent experiments.

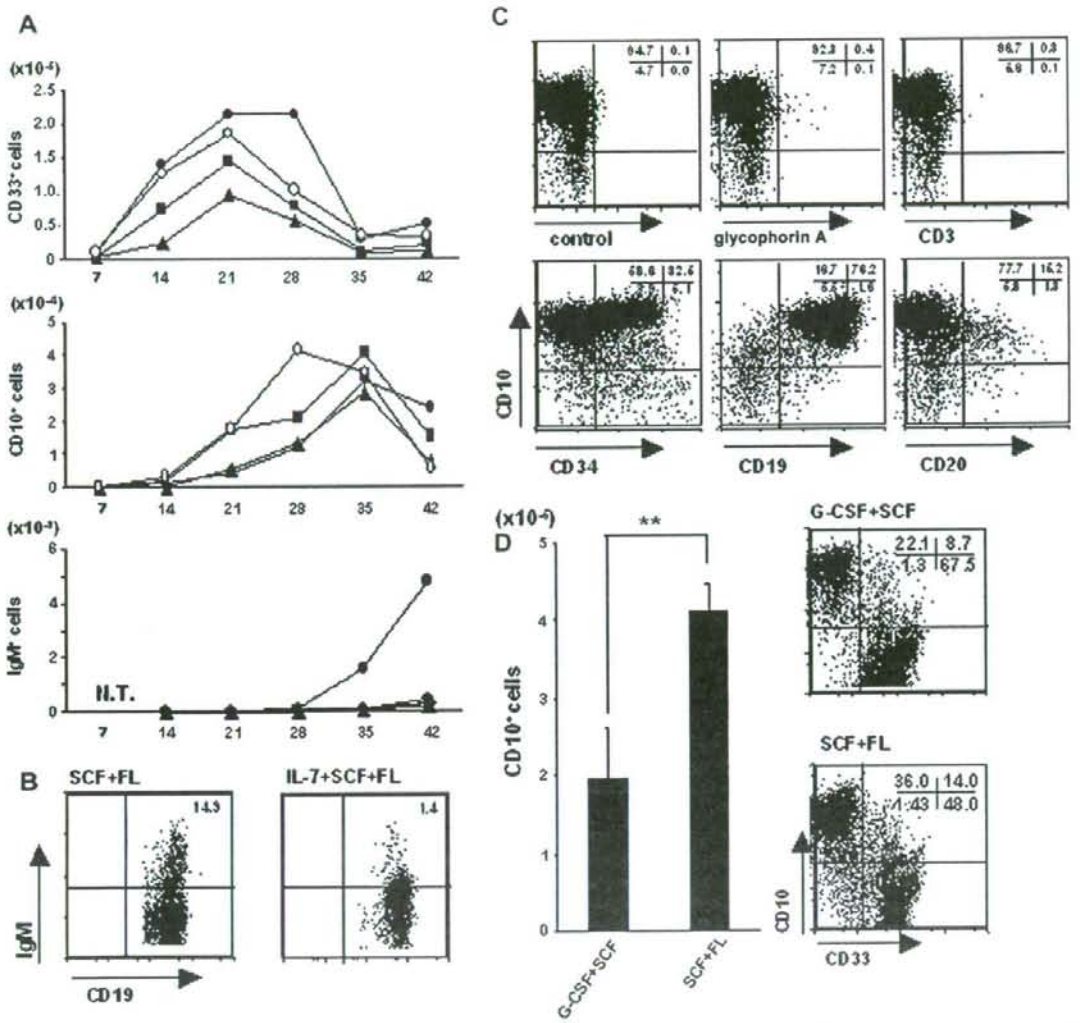


Figure 2. Combination of cytokines enhances human B-lymphocyte production in cocultures of cord blood (CB) $CD34^+$ cells on human mesenchymal stem cells (hMSC). (**A**) Purified CB $CD34^+$ cells (2000 cells/well) were cultured on hMSC in combinations of 10 ng/mL stem cell factor (SCF) + 5 ng/mL, Flt3-ligand (FL) (closed circle), 5 ng/mL FL + 5 ng/mL interleukin (IL)-7 (closed triangle), 5 ng/mL IL-7 + 10 ng/mL SCF (closed square), 10 ng/mL SCF + 5 ng/mL FL + 5 ng/mL IL-7 (open circle) for 6 weeks. The cultured floating cells were collected, and numbers of the generated $CD33^+$ cells (upper panel), $CD10^+$ cells (middle panel), and IgM^+ cells (lower panel) were estimated weekly. Similar results were obtained in three independent experiments. (**B**) Purified CB $CD34^+$ cells (2000 cells/well) were cultured on hMSC in the presence of 10 ng/mL SCF + 5 ng/mL FL or 10 ng/mL SCF + 5 ng/mL FL + 5 ng/mL IL-7, respectively. At day 42, the generated cells were stained with fluorescein isothiocyanate (FITC)-IgM and phycoerythrin (PE)-CD19, and analyzed with flow cytometry. Similar results were obtained in three independent experiments. (**C**) Purified CB $CD34^+$ cells (2000 cells/well) were cultured on hMSC in the presence of 10 ng/mL SCF and 5 ng/mL FL. The generated cells were stained with FITC-CD33 and allophycocyanin (APC)-CD10 as well as the indicated PE-conjugated antibody (Ab), and analyzed with flow cytometry at day 40. Isotype-matched Abs were used as negative controls. Similar results were obtained in three independent experiments. (**D**) Purified CB $CD34^+$ cells (2000 cells/well) were cultured on hMSC in the presence of 10 ng/mL SCF + 5 ng/mL FL or 10 ng/mL SCF + 10 ng/mL G-CSF. Numbers of the generated $CD10^+$ cells were analyzed at 4 weeks of the cocultures. Data are shown as mean ± standard deviation in triplicated samples. Statistically differences from control values are shown with two ($p < 0.01$) asterisks. One representative flow cytometry data for CD10 and CD33 expression was also shown. Similar results were obtained in two independent experiments. NT = not tested.

CD10 Ab recognizes neutral endopeptidase, a 100-kD type II transmembrane glycoprotein, which is referred to as the common acute lymphoblastic leukemia antigen. CD10 antigen is expressed on some subsets of B- and T-lymphoid progenitors and germinal center cells. Short-term expansion of $CD33^+$ cells was observed within 2 weeks of culture (Fig. 2A, upper panel). $CD10^+$ cells started to appear at 2 weeks, and their numbers increased rapidly until 4 or 5 weeks of culture (Fig. 2A, middle panel). Cocultures on hMSC in the presence of SCF and FL led to generation of $1–5 \times 10^5$ $CD10^+$ cells from 2000 CB $CD34^+$ cells after 4 weeks of culture. Further addition of IL-7 to these cocultures had limited effect on $CD10^+$ cell production.

With regard to the transition into immature B cells, part of the generated $CD19^+$ cells began to express surface IgM after 4 weeks in coculture that included SCF and FL (Fig. 2A, lower panel; Fig. 2B). Surface phenotypes of the generated cells were analyzed after 6 weeks of culture (Fig. 2C), and most of the generated $CD10^+$ cells expressed CD19. Approximately 16.4% of $CD10^+$ cells expressed CD20. At that time, 32.5% of $CD10^+$ cells still showed surface expression of CD34. We did not detect any $CD3^+$ T-lineage cells or glycophorin A^+ erythroid cells.

Coculture of human $CD34^+$ cells on MS-5 stromal cells in the presence of SCF and G-CSF was reported to support human B lymphopoiesis [12]. Therefore, we compared B-lymphocyte production in hMSC coculture with added SCF plus FL vs SCF plus G-CSF. As shown in Figure 2D, the cocultures with SCF and FL generated B lymphocytes more efficiently than cocultures with SCF and G-CSF.

Therefore, we concluded that the coculture of CB $CD34^+$ cells on hMSC in the presence of SCF and FL was a suitable system for analyzing human B-lymphocyte development.

Screening for positive and negative regulators of human B lymphopoiesis

Because our coculture system was composed only of human-derived materials, except for FCS, we thought that it might be a suitable system for screening regulators of human B lymphopoiesis, and added several different low molecular weight inhibitors to the cocultures. Reactive oxygen species generation is related to induction of apoptosis in hematopoietic stem cells [33]. We confirmed that 100 μM N-acetylcysteine reduced apoptosis in murine hematopoietic stem cells by inhibiting reactive oxygen species generation. DUP697 is an inhibitor of Cox-2, which induces PGE_2 production. Endogenous PGE_2 was found in the supernatants of the cocultures and addition of 0.1 μM DUP697 blocked its production ($[PGE_2] = 0.74 \pm 0.40 \times 10^{-9}$ M without DUP697 and undetectable with DUP697). However, these inhibitors had no effect on human B-lymphocyte production (Fig. 3A). BIO is an inhibitor of a glycogen synthase kinase-3, which induces degradation of β-catenin [34]. Addition of BIO diminished the emergence of $CD10^+$ cells by approximately 30% (Fig. 3A), indicating that signals mediated by β-catenin inhibit human B-lymphocyte development. SB431542 is an inhibitor of ALK4/5/7, which are receptors for the TGF-β superfamily. Addition of SB431542 enhanced expansion of $CD10^+$ cells in a dose-dependent manner (Figs. 3A and B). Importantly, the influence of SB431542 on B-lymphocyte progenitors was greater than its influence on myeloid progenitors, because the percentage of $CD10^+$ cells increased significantly in the cocultures with added SB431542 (Fig. 3C).

Therefore, we determined that β-catenin and the TGF-β superfamily members act as negative regulators of human B-lymphocyte development in our coculture system.

Activin A and TGF-β1 negatively regulate human B lymphopoiesis

Among members of the TGF-β superfamily, TGF-β1 recognizes ALK-1 and -5, activin A binds to ALK-4, and BMP-4 uses ALK-2, -3, and -6 as receptors [24]. We added recombinant proteins as well as neutralizing Abs of these molecules to the cocultures. Production of human B lymphocytes decreased in a dose-dependent manner with the addition of TGF-β1, but not with activin A or BMP-4 (Fig. 4). A neutralizing Ab for activin A enhanced B-lymphocyte production approximately threefold, but neutralizing Abs for TGF-β1 and BMP4 had no effect (Fig. 5A). The physiological antagonist of activin A, follistatin, enhanced human B-lymphocyte production in a dose-dependent manner (Fig. 5B), and the percentage of $CD10^+$ cells in the generated cells increased markedly with the addition of follistatin (Fig. 5C), indicating that activin A downregulates human B lymphopoiesis more efficiently than myelopoiesis.

Therefore, both activin A and TGF-β1 inhibit human B-lymphocyte development, while BMP-4 has no apparent regulatory effect on human B-lymphocyte progenitor cells in our cocultures.

Both activin A and TGF-β1 inhibit early onset of human B lymphopoiesis

We next examined the effect of SB431542 on the frequency of B-lymphocyte progenitor cells. In limiting dilution culture with SB431542, the frequency of progenitor cells capable of generating $CD10^+$ cells increased significantly (Fig. 6A). Moreover, when a neutralizing Ab for activin A was added to cocultures derived from subpopulations of CB $CD34^+$ cells, the production of B lymphocytes, but not myeloid cells, was significantly enhanced (Fig. 6B and C). The enhancing effect on B-lymphocyte production was particularly great when the cocultures were started from $CD34^+CD38^-$ and $CD34^+CD38^+CD10^-$ subpopulations.

Therefore, both activin A and TGF-β1 inhibit early onset of human B lymphopoiesis.

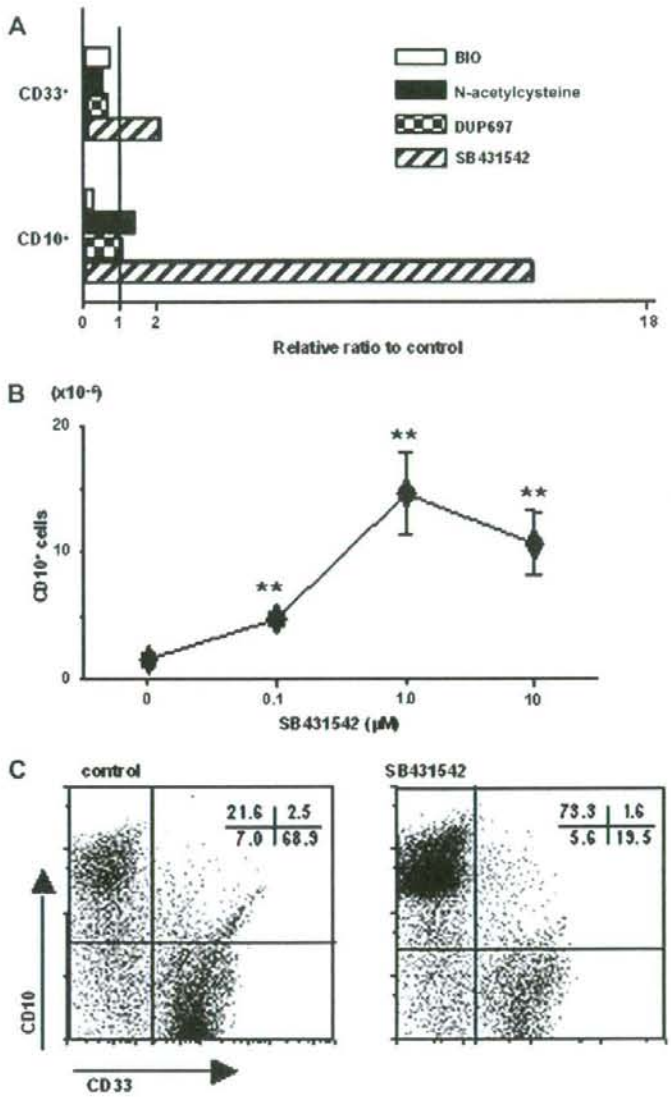


Figure 3. Addition of an inhibitor for activin receptor-like kinase [ALK]-4/5/7 to the cocultures selectively enhances human B-lymphocyte production. (**A**) 5 nM BIO (glycogen synthase kinase-3 inhibitor), 100 μM N-acetylcysteine (reactive oxygen species inhibitor), 0.1 μM DUP697 (Cox-2 inhibitor) or 10 μM SB431542 (ALK-4/5/7 inhibitor) was added to the cocultures of CB $CD34^+$ cells (2000 cells/well) on human mesenchymal stem cells (hMSC) in the presence of 10 ng/mL stem cell factor (SCF) and 5 ng/mL Flt3-ligand (FL). At day 28, the generated $CD33^+$ or $CD10^+$ cells were calculated from the recovered total cell numbers and the percentages of the positive cells confirmed by flow cytometry. Data are shown as mean in duplicated samples. Similar results were obtained in three independent experiments. (**B**) The indicated concentrations of SB431542 were added to the cocultures of CB $CD34^+$ cells (2000 cells/well) on hMSC in the presence of 10 ng/mL SCF and 5 ng/mL FL. Data are shown as mean ± standard deviation of numbers of the generated $CD10^+$ cells in triplicated samples. Statistically differences from control values (without SB431542) are shown with two ($p < 0.01$) asterisks. Similar results were obtained in two independent experiments. (**C**) Purified CB $CD34^+$ cells (2000 cells/well) were cultured on hMSC in the presence of 10 ng/mL SCF and 5 ng/mL FL with or without 1.0 μM SB431542. At day 28, the generated cells were stained with phycoerythrin (PE)-CD10 and fluorescein isothiocyanate (FITC)-CD33, and analyzed with flow cytometry. Similar results were obtained in four independent experiments.

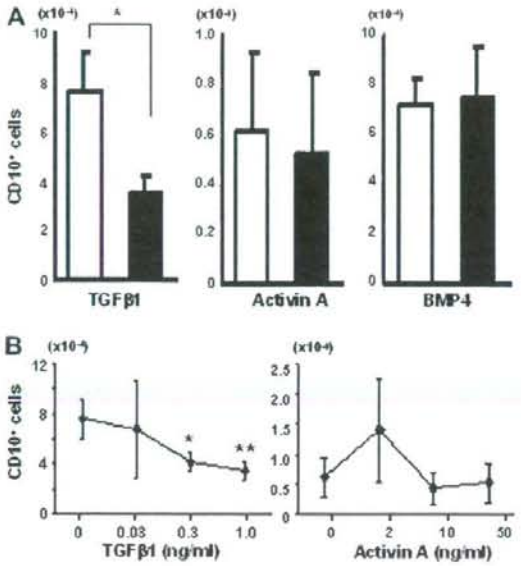


Figure 4. Transforming growth factor (TGF)-β1 negatively regulates human B lymphopoiesis in the cocultures. (**A**, **B**) Purified cord blood (CB) $CD34^+$ cells (2000 cells/well) were cultured on human mesenchymal stem cells (hMSC) in the presence of 10 ng/mL stem cell factor (SCF) and 5 ng/mL Flt3-ligand (FL) with control (open column), 1 ng/mL TGF-β1, 50 ng/mL activin A, or 50 ng/mL bone morphogenetic protein (BMP)-4 (closed column) (**A**), with the indicated concentrations of TGF-β1 or activin A (**B**). At day 28, the generated $CD10^+$ cells were calculated from total cell numbers and percentages of the positive cells confirmed by flow cytometry. Data are shown as mean ± standard deviation in triplicated samples. Statistically differences from control values are shown with one ($p < 0.05$) or two ($p < 0.01$) asterisks. Similar results were obtained in three independent experiments.

Effect of TGF-β superfamily members on the transition into immature B cells

As shown in Figure 2, part of $CD19^+$ cells develop into IgM^+ immature B cells after 4 weeks in culture. Therefore, we evaluated the effects of TGF-β superfamily members on the transition into immature B cells in coculture. Blocking ALK-4/5/7 by SB431542 failed to influence the percentage of the IgM^+ population in the cultured $CD19^+$ cells at 5 weeks of culture (Fig. 7A). Similarly, we did not detect any difference in the percentage of IgM^+ cells by the addition of recombinant TGF-β1, a neutralizing Ab for activin A, or follistatin (Fig. 7B, C, and D).

Therefore, we did not observe any influence by TGF-β superfamily members on the transition into immature B cells in our human B-lymphocyte coculture system.

Coculture production of activin A and TGF-β1

Using reverse transcription PCR, we confirmed that RNAs of activin A type I and type II receptors were expressed by both CB $CD34^+$ cells and $CD34^-$ cells (Fig. 8A). hMSC expressed RNAs of the TGF-β superfamily members (Fig. 8B). Supernatants from the cocultures contained 1700 ± 410 pg/mL activin A and 40.8 ± 19.4 pg/mL TGF-β1 at day 3 of culture, and 3200 ± 130 pg/mL activin A and 114.7 ± 16.1 pg/mL TGF-β1 at day 10 (Fig. 8C). When we examined BM sections from normal healthy individuals, we detected activin A- and TGF-β1–positive cells (data not shown).

Therefore, both activin A and TGF-β1 are produced in our cocultures and in human BM.

Discussion

We established a novel coculture system to analyze human B lymphopoiesis. In our system, hMSC could support the commitment and differentiation of CB $CD34^+$ cells into $CD10^+$ cells, followed by transition into IgM^+ immature B cells. hMSC retained their capacity for cell-to-cell contact inhibition; therefore, we could continue the cocultures for up to 6 weeks without passage of hMSC. Moreover, our coculture system is a biologically relevant model for human B-lymphocyte development in that it excludes the effects of xenograft materials. When IL-7 was added to the SCF and FL-containing cocultures, only a few IgM^+ cells appeared. Thus, adding the combination of SCF and FL enhanced the B-lymphocyte–supporting capacity of hMSC.

We found that the addition of SB431542, an inhibitor for ALK-4/5/7, enhanced the output of $CD10^+$ cells markedly. Follistatin, a physiological inhibitor of activin A, and a neutralizing Ab for activin A enhanced B-lymphocyte production, while a neutralizing Ab for TGF-β1 had no discernable effect. TGF-β1, but not activin A, suppressed B-lymphocyte production in a dose-dependent manner. The different effects of inhibitors and factors on human B-lymphocyte production seemed to be related to the fact that the culture supernatant contained a much higher concentration of activin A than of TGF-β1. Adding a neutralizing Ab for BMP-4 or recombinant BMP-4 protein itself had no effect on B-lymphocyte production. Therefore, the strength of the ability to suppress human B lymphopoiesis seemed to be activin A > TGF-β1 > BMP-4.

Members of the TGF-β superfamily are implicated in control of many biological processes, such as cell cycle, cell growth and differentiation, and lymphocyte development and function [25]. The importance of these regulatory cytokines on immune homeostasis is reflected by the phenotypes of TGF-β1–deficient mice that develop autoimmune diseases with production of autoantibodies [35], although suppression of self-reactive lymphocyte clones involves actions of TGF-β1 on both B and T lymphocytes [36]. There are several reports suggesting that the TGF-β superfamily can modulate B-lymphocyte proliferation, expression of surface antigen receptors, and Ab secretion [37–40]. In murine B lymphopoiesis, TGF-β [7] and activin

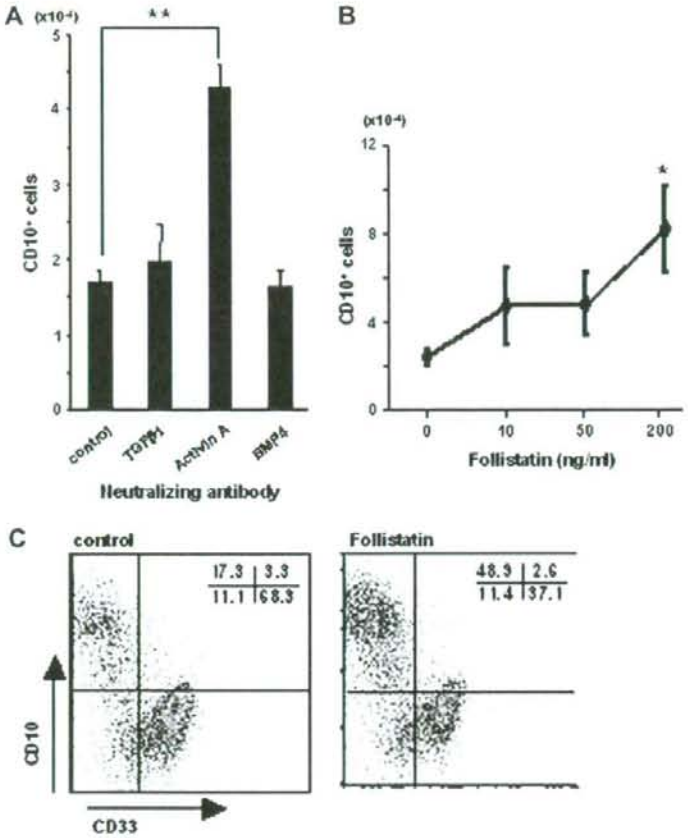


Figure 5. Inhibition of activin A enhances human B lymphopoiesis in the cocultures. (**A**, **B**) Purified cord blood (CB) $CD34^+$ cells (2000 cells/well) were cultured on human mesenchymal stem cells (hMSC) in the presence of 10 ng/mL stem cell factor (SCF) and 5 ng/mL Flt3-ligand (FL) with 10 μg/mL antibodies (Abs) for TGF-β1, activin A, or bone morphogenetic protein (BMP)-4 (**A**), or with the indicated concentrations of follistatin (**B**). At day 28, the generated $CD10^+$ cells were calculated from total cell numbers and percentages of the positive cells confirmed by flow cytometry. Data are shown as mean ± standard deviation in triplicated samples. Statistically differences from control values are shown with one ($p < 0.05$) or two ($p < 0.01$) asterisks. Similar results were obtained in three independent experiments. (**C**) Purified CB $CD34^+$ cells (2000 cells/well) were cultured on hMSC in the presence of 10 ng/mL SCF and 5 ng/mL FL with or without 200 ng/mL follistatin for 4 weeks. The generated cells were stained with phycoerythrin (PE)-CD10 and fluorescein isothiocyanate-CD33, and analyzed with flow cytometry. Similar results were obtained in three independent experiments.

A [26,41] inhibit early onset of B-lymphocyte development. Because production of $CD10^+$ cells from CB $CD34^+$ cells was suppressed by both activin A and TGF-β1, our results from the coculture experiments coincide well with previous reports about the negative regulatory effects of these molecules on murine B-lymphocyte development. Notably, production of $CD10^+$ cells was influenced by the inhibition of TGF-β superfamily members even when the $CD34^+CD38^-$ stem cell population was used to initiate cocultures, and the influence was greater in the cocultures derived from $CD34^+CD38^-$ and $CD34^+CD38^+CD10^-$ cells than in cocultures derived from $CD34^+CD38^+CD10^+$ cells. These data suggest that the TGF-β superfamily inhibits early onset of human B lymphopoiesis. Furthermore, production of $CD10^+$ cells was also influenced when the inhibitor was added after 2 weeks of coculture (data not shown), indicating that the TGF-β superfamily might suppress the proliferation of relatively differentiated B-lymphocyte progenitors. Thus, members of the TGF-β superfamily are likely to suppress human B lymphopoiesis at a wide range of differentiation stages. This hypothesis is supported in part by our reverse transcription PCR data showing that both $CD34^+$ cells and $CD34^-$ cells express receptors for the TGF-β superfamily. Although a number of investigators have reported regulatory effects of the TGF-β superfamily on class switching

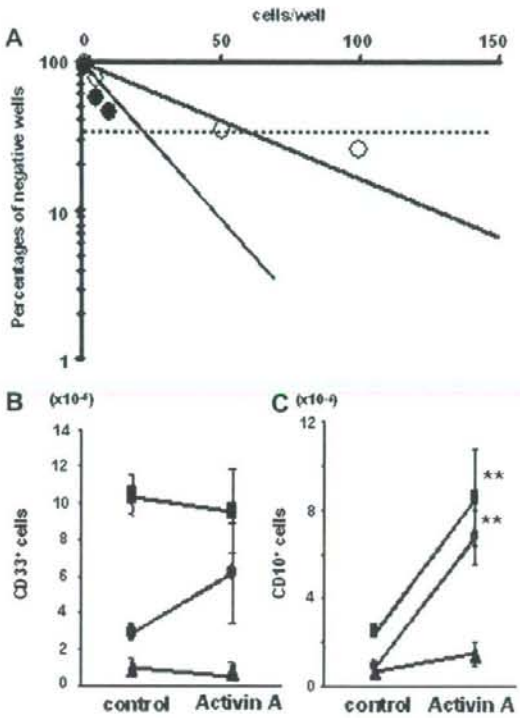


Figure 6. Transforming growth factor (TGF)-β superfamily inhibit early onset of human B lymphopoiesis. (**A**) Limiting dilution assays were performed in 96-well plates. Cord blood (CB) $CD34^+$ cells were cultured on human mesenchymal stem cells (hMSC) at indicated concentrations in the presence of 10 ng/mL stem cell factor (SCF) and 5 ng/mL Flt3-ligand (FL) with (closed circle) or without 10 μM SB431542 (open circle). After 28 days of coculture, wells with cell expansion were scored. The expanded cells were analyzed by flow cytometry and calculated the well number of cultures generated $CD10^+$ cells. Similar results were obtained in three independent experiments. (**B**, **C**) Purified CB $CD34^+$ cells were stained with allophycocyanin (APC)-CD34, fluorescein isothiocyanate (FITC)-CD38, and phycoerythrin (PE)-CD10. $CD38^-$ cells (square), $CD38^+$ $CD10^-$ cells (circle), and $CD38^+$ $CD10^+$ cells (triangle) were then sorted with FACSAria. The sorted cells (2000 cells/well) were cultured on hMSC in the presence of 10 ng/mL SCF and 5 ng/mL FL with or without 10 μg/mL antibodies for activin A for 4 weeks. Numbers of the generated $CD33^+$ cells (**B**) or $CD10^+$ cells (**C**) were evaluated. Data are shown as mean ± standard deviation in triplicated samples. Statistically differences from control values are shown with two ($p < 0.01$) asterisks. Similar results were obtained in two independent experiments.

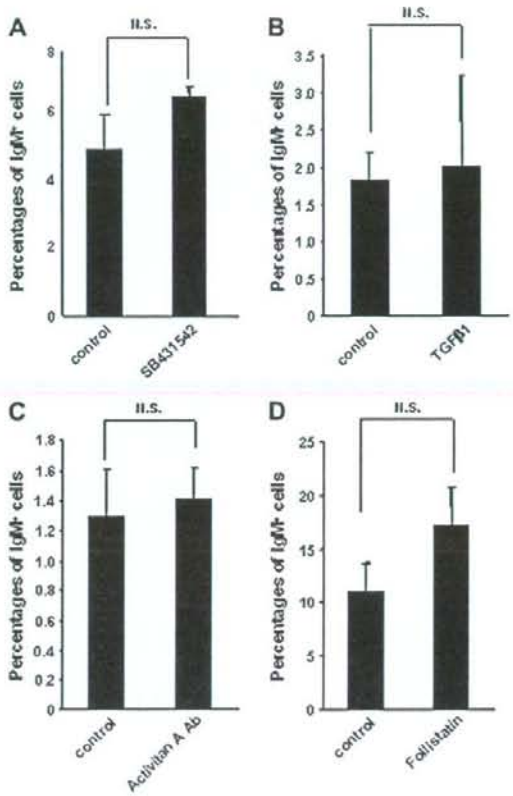
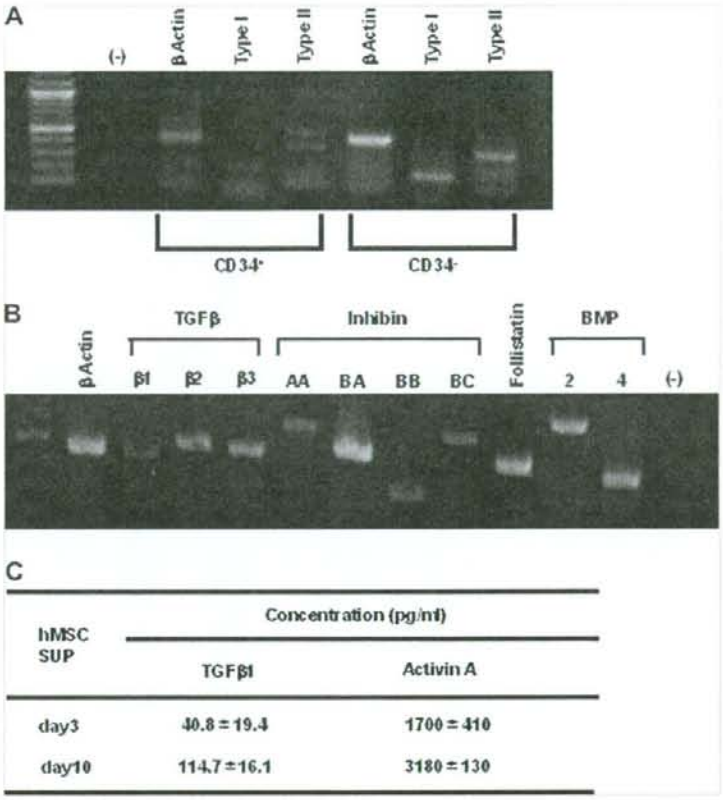


Figure 7. Transition from pre-B cells into immature B cells is not influenced by manipulating the transforming growth factor (TGF)-β superfamily in the cocultures. (**A**–**D**) Purified cord blood (CB) $CD34^+$ cells (2000 cells/well) were cultured on human mesenchymal stem cells (hMSC) in the presence of 10 ng/mL stem cell factor (SCF) and 5 ng/mL Flt3-ligand (FL) with 1 μM SB431542 (**A**), with 0.3 ng/mL TGF-β1 (**B**), with 10 μg/mL an anti-activin A (**C**), or with 200 ng/mL follistatin (**D**). At day 42, the generated cells were stained with fluorescein isothiocyanate (FITC)-IgM and phycoerythrin (PE)-CD19, and analyzed with flow cytometry. Similar results were obtained in two independent experiments. NS = not significant.

and immunoglobulin secretion, little information about the influence on the transition from pre-B to immature B cells is available. We found that the percentage of surface IgM^+ cells in $CD19^+$ cells was not influenced by manipulating the TGF-β superfamily, while the production of $CD19^+$ cells was affected. Therefore, the transition into immature B cells is unlikely to be influenced by the TGF-β superfamily.

In conclusion, our coculture system of CB $CD34^+$ cells on hMSC in the presence of SCF and FL is suitable for analyzing the regulatory mechanisms of human B-lymphocyte development. With this system, we showed that members of TGF-β superfamily, activin A and TGF-β1, are negative regulators of human B-lymphocyte development at a range of differentiation stages. We expect that our coculture system will be applicable to a variety of research and development processes, such as screening for regulatory molecules or drugs that influence human B-lymphocyte development, evaluating B-lymphocyte progenitors in patients with B-cell malignancies, and cloning human B-lymphocyte–supportive molecules.



C

hMSC SUP	Concentration (pg/ml)	
	TGFβ1	Activin A
day3	40.8 ± 19.4	1700 ± 410
day10	114.7 ± 16.1	3180 ± 130

Figure 8. Production of activin A and transforming growth factor-β1. (**A**, **B**) Expression of RNAs of type I and type II receptor of activin A in cord blood (CB) $CD34^+$ and $CD34^-$ cells (**A**) and of the various TGF-β superfamily members in human mesenchymal stem cells (hMSC) (**B**) was analyzed with reverse transcription polymerase chain reaction. (**C**) Purified CB $CD34^+$ cells were cultured on hMSC in the presence of 10 ng/mL stem cell factor (SCF) and 5 ng/mL Flt3-ligand (FL). Culture supernatant was collected just before medium change at day 3 and day 10, and subjected to enzyme-linked immunosorbent assay for activin A or TGF-β1, respectively. Data are shown as mean ± standard deviation (n = 3 each).

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付録

急性白血病患者に対する
同種臍帯血由来 ex vivo 増幅 CD34 陽性細胞移植に関する
臨床第Ⅰ相／前期第Ⅱ相試験
実施計画書

急性白血病等患者に対する
同種臍帯血由来 *ex vivo* 増幅 CD34 陽性細胞移植に関する
臨床第 I 相／前期第 II 相試験

実施計画書

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免疫アレルギー疾患等予防・治療研究事業

「新たな移植細胞療法に向けた造血幹細胞の ex vivo 増幅技術の開発と応用」

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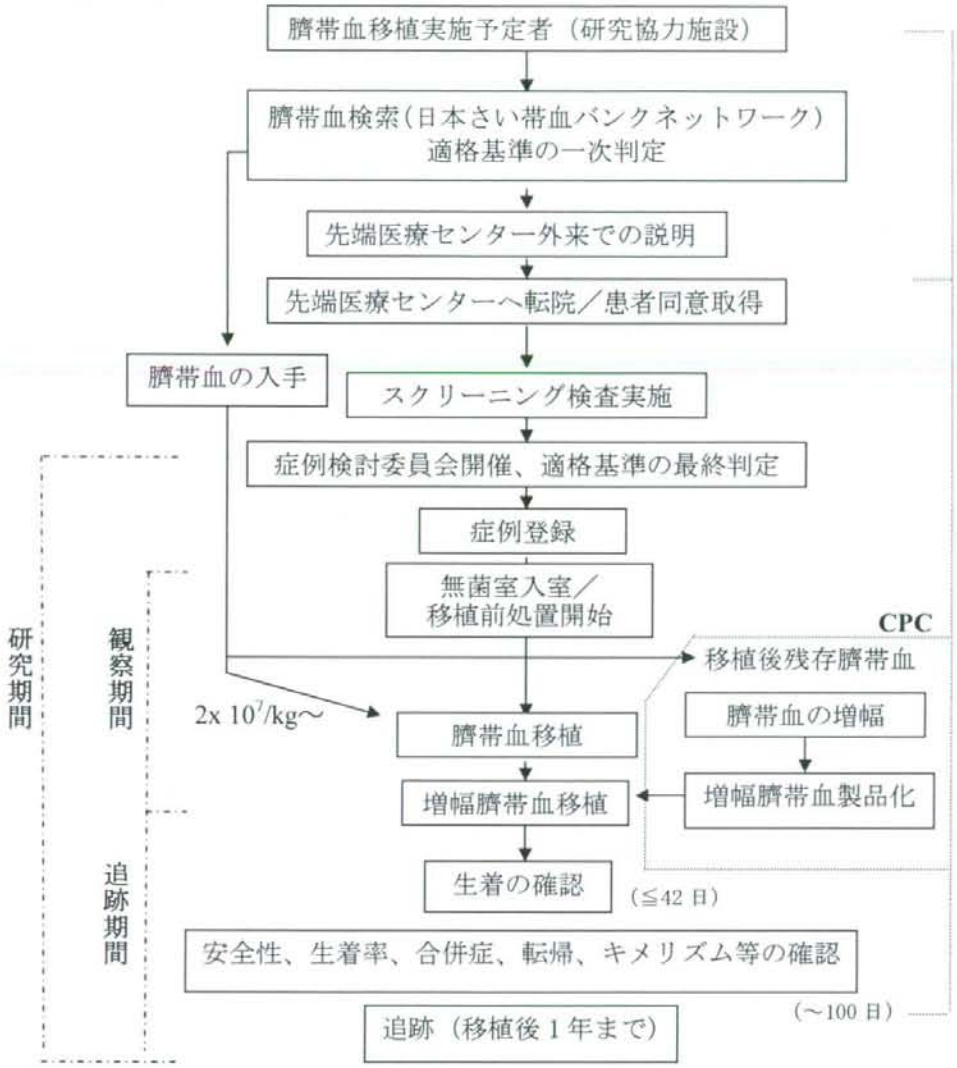
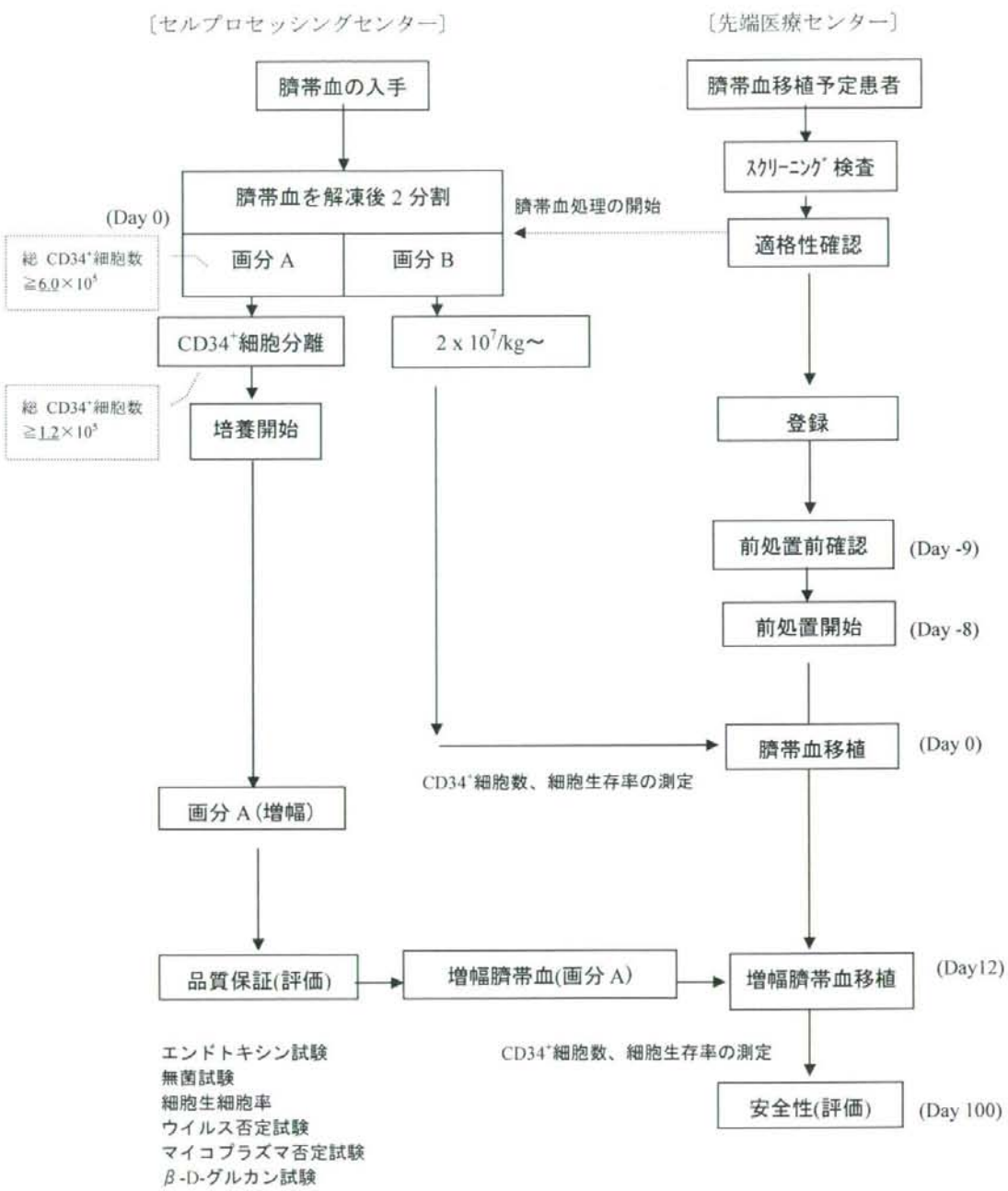
付録 14. MDS の国際予後スコアリングシステム(International prognostic scoring system, IPSS)

0. シェーマ及び概要

0.1. シェーマ



0.2. 臍帯血の移植および増幅 CD34 陽性細胞の製造・移植



1. 目的

本研究では、日本造血細胞移植学会の「造血幹細胞移植のガイドライン」に合致する患者で、骨髄移植および末梢血幹細胞移植において適切なドナーを得ることができない急性白血病、骨髄異形成症候群、及び一部悪性リンパ腫を対象として、臍帯血内の CD34 陽性細胞の一部を *ex vivo* 増幅して臍帯血移植を実施し、その安全性・効果を検討する。さらに、臍帯血内の CD34 陽性細胞および増幅培養した CD34 陽性細胞における輸注細胞数と生着率の相関を検討する。

2. 背景と根拠

2.1. 白血病の治療成績の現状

白血病の治療の基本は化学療法であるが、白血病の病型ごとに標準的化学療法による予後因子が明らかになってきている。白血病における造血幹細胞移植と化学療法の優劣に関する治療成績は我が国においても海外においてもまだ病型、病期、危険因子毎に層別化された前方視的比較試験が行われておらず、十分にエビデンスが得られているとはいえない状況ではある。ただし、いくつかの試験は行われている。急性骨髄性白血病に対しては、Zittoun らが、同種骨髄移植と自家骨髄移植と化学療法の治療成績を前方視的に比較した。その結果、同種骨髄移植が 4 年無病生存率、再発率とも他の治療群より有意に優れていた[1]。急性リンパ性白血病においては、未だに前方視的臨床比較試験は実施されていないが、IBMTR(The International Bone Marrow Transplant Registry)の同種移植成績と JALSG(Japan Adult Leukemia Study Group)の化学療法を比較した検討においては、30 歳以下では同種移植の成績がよく、30 歳以上では同等であったと報告されている[2]。しかし、成人の急性リンパ性白血病の化学療法による長期寛解率は約 30%しかなく、造血幹細胞移植が必要と考えられている[3]。慢性骨髄性白血病においては、IFN-α の登場により、化学療法の成績が格段に向上したが、IFN-α の反応性による予後との関連も明らかになってきており、反応が悪い例では造血細胞移植は有用な手段と考えられている[4]。このような現状をふまえ、造血細胞移植学会では白血病の病型ごとに移植適応規準を設けて 2002 年に移植適応ガイドラインを作成した。本邦では 1980 年代より骨髄移植が施行されるようになったが、1994 年に末梢血幹細胞移植が保険適応となったのに引き続き、1998 年には臍帯血移植も保険適応となったことで造血幹細胞移植の選択肢が広がっている。現在では各々の移植の特性を考慮して患者に適した移植が実施されている。

どの移植幹細胞ソースを用いるかについては、現時点において治療成績を評価するための十分臨床データが蓄積されているわけではないが、最適な時期に移植できる点や移植片対宿主病(Graft Versus Host Disease: GVHD)や他の移植関連合併症が少ない点で、ヒト白血球抗原(Human Leukocyte Antigen: HLA)一致血縁者間移植が全ての病型、病期において第一適応になると考えられる。HLA 一致血縁者によるドナーがいない場合には、骨髄バンク登録非血縁者ドナーからの骨髄移植が行われることが一般的である。これらのドナーが見つからないとき、あるいはドナーコーディネートに時間がかかり、最適な移植時期である寛解維持が困難である場合などは、臍帯血移植が行われている。

2.2. 臍帯血移植について

臍帯血中には未分化で増殖能力の高い造血幹細胞が多数含まれていることが 1980 年代前半に明らかにされていたが、1988 年にフランスの Gluckman らのグループにより世界最初の臍帯血移植が Fanconi 貧血患者に対し実施された[5]。一方、本邦においては 1994 年に東海大学の Hattori らのグループにより第 1 例目の臍帯血移植が急性骨髄性白血病患者に対して実施された[6]。

2.3. 臍帯血移植の現状

骨髄移植では、血縁者にドナーを見出せない場合は公的機関である骨髄バンクにて非血縁者ドナーを検索する。ただし、骨髄バンクにおいても必ずしもドナー候補が見つかるわけではないのが現状で、いまだ十分なドナー登録者数が確保されているとはいえない。さらに、末梢血幹細胞移植は現時点においてドナーは血縁者のみに限られていることから、ドナーの負担が全くない臍帯血移植への期待は大きい。

臍帯血移植は、日本さい帯血バンクネットワークを通じ、予めさい帯血バンクに保存されている臍帯血を検索し、患者の HLA と一致または 1 ないし 2 抗原不一致までの臍帯血が選択され移植が実施されている。日本さい帯血バンクネットワークによると、平成 16 年 3 月 30 日現在、全国 11 バンクに保存され検索対象となっている臍帯血数は 18,419 個であり、平成 16 年度中には 2 万個を超えることが予想されている[7]。また、平成 14 年度の臍帯血移植数は 294 例であり骨髄移植及び末梢血幹細胞移植が平成 13 年度をピークに減少に転じたのに反して年々増加傾向にあり、平成 15 年には累計で 1,000 例を超え、近年では臍帯血移植の約 8 割が 16 歳以上の成人を対象に実施されている (表 1)。

表 1. 本邦における移植の種類別件数の年次推移[7,8]

平成(年度)	10	11	12	13	14	15*
同種移植(総数)	1094	1181	1364	1560	1288	—
骨髄	940	989	972	943	713	—
末梢血	81	109	289	520	461	—
骨髄＋末梢血	26	20	9	13	3	—
臍帯血移植	77	114	169	221	294	105
(累計)	(97)	(211)	(380)	(601)	(895)	(1000)

同種移植：平成 15 年度 日本造血細胞移植学会 全国調査報告書より，臍帯血移植：日本さい帯血バンクネットワーク HP より
*6 月 30 日現在の集計結果(臍帯血移植のみ)

2.4. 臍帯血移植の特徴

臍帯血移植の長所としては、ドナーの直接的負担がないことは言うまでもないが、ドナー検索から移植までの期間が短いことも大きな特徴の一つとしてあげられる。これは、臍帯血が凍結保存されている各臍帯血バンクがすでにネットワーク化されており、HLA の適合度について随時検索できるシステムが構築されていることによるものが大きい。さらに移植後