

various combinations of cytokines or other reagents, as indicated in Figures 1,3,5,6,8. After cultivation for the periods indicated, the cells were harvested with 0.25% trypsin plus 0.02% ethylenediamine tetraacetic acid (IBL Co. Ltd., Gunma, Japan), the number of cells per well was counted, and cells were analyzed by flow cytometry.

For the colony assay, CD34⁺ BM cells were cultured for 1 week on MS-5 cells in the presence or absence of goat anti-IL-7 Ab and the floating cell fraction was first collected with culture medium. The remaining adherent cell fraction with MS-5 cells were treated with trypsin, harvested, and plated in 6-well tissue culture plate (Asahi Techno Glass). After removing MS-5 cells by letting them attach to the bottom of the plate by 15-minute incubation, subsequent suspension cells were collected as adherent cell fraction. After counting the cell number by flow cytometry using Flow-Count (Beckman/Coulter), cells from each fraction were passaged into methylcellulose cultures containing the cocktail of cytokines (Methocult™ GF+H4435; Stem Cell Technologies Inc, Northampton, UK). Morphology and number of colonies comprising more than 50 cells was scored at 14 days. All experiments were performed in triplicate and the mean + SD of the values were shown in Figures 1,3,5,6,8.

Immunofluorescence study

Cells were stained with fluorescence-labeled monoclonal antibodies and analyzed by flow cytometry (EPICS-XL, Beckman/Coulter) as described previously [20]. Two-color immunofluorescence study was performed with a combination of PE and PC-5. Experiments were performed in triplicate, and the mean + SD of the cell counts were indicated in the Figures 1,3,5,6,8.

For cell sorting, human BM CD34⁺ cells cocultured with MS-5 cells for 4 weeks were harvested and stained with PE-conjugated anti-CD33 monoclonal Ab and PC-5-conjugated anti-CD19 monoclonal Ab. CD33⁻CD19⁻, CD33⁺ and CD19⁺ cells were sorted in an EPICS-ALTRA cell sorter (Beckman/Coulter). Total RNA was extracted and used for reverse transcription polymerase chain reaction (RT-PCR).

RT-PCR

Total RNA was extracted from cultured cells, and cDNA was generated with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and a FirstStrand cDNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden). cDNA synthesized from 150 ng total RNA was used as a template for one amplification. The sets of primers used in this study were listed in Table 1.

PCR was repeated for 30 to 35 cycles of heating at 94°C for 60 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 2 minutes; the products were then separated on a 1.5% agarose gel.

Results

MS-5 cells secrete IL-7

Murine stromal cell line MS-5 has been reported to possess the ability to support the differentiation of B-lineage cells and myeloid cells from human cord blood CD34⁺ cells [21–25]. Consistent with previous observations, the human BM CD34⁺ cells in our study generated CD19⁺CD33⁻ B cells and CD19⁻CD33⁺ myeloid cells after 4 weeks of

Table 1. List of primers used in this study

Name of gene	Primer sequence	Product size (bp)
Murine IL-7		
Forward	5'-TAAATCGTGCTGCTCGCAAAGT-3'	
Reverse	5'-AGCAGTCAGCTGCAITTTCTGTG-3'	392
Human IL-7R α		
Forward	5'-GTCACCTCCAGAAAGCTTTGG-3'	
Reverse	5'-AGGAACCTAGACTTCCCTTT-3'	
Human CD19		
Forward	5'-GTTCCGGTGGAAATGTTTCGG-3'	386
Reverse	5'-AGATGAAGAATGCCACAAGG-3'	576
Human TdT		
Forward	5'-ACACGAATGCAGAAAGCAGGA-3'	
Reverse	5'-AGGCAACCTGAGCTTTTCAA-3'	315
Human PAX5		
Forward	5'-CCATCAAGTCTGAAAAATC-3'	
Reverse	5'-CCCAAAGTGGTGGAAAAAT-3'	319
Human Ig α		
Forward	5'-TAGTCGACATGCTGGGGTCCAGGATCCTC-3'	
Reverse	5'-GATGTCCAGCTGGAGAAGCCGTGA-3'	681
Human GAPDH		
Forward	5'-CCACCATGGCAAATTCATGGCA-3'	598
Reverse	5'-TCTAGACGGCAGGTCAGGTCCACC-3'	
Murine actin		
Forward	5'-TGACGGGGTCACCCACACTGTGCCATCTA-3'	
Reverse	5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3'	661

GAPDH = glyceraldehyde phosphate dehydrogenase; IL = interleukin.

cocultivation with MS-5 cells (Fig. 1). Immunocytological analysis showed that the CD19⁺ B cells in our culture system were surrogate light chain⁺ μ^- pro-B cells [25]. Consistent with these observations, the human BM CD34⁺ cells in our study generated CD19⁺ B cells and CD33⁺ myeloid cells after 4 weeks of cocultivation with MS-5 cells (Fig. 1). The detailed characterization of our culture system has been reported previously [25]. Starting with 4×10^4 CD34⁺ cells, that containing <8% of CD19⁺CD34⁺, 0.4 to 1.3×10^6 mononuclear cells, 30.1% to 68.2% of which were CD19⁺CD34⁻ cells, were obtained (data not shown). Immunocytological analysis showed that most of these CD19⁺ B cells expressed cytoplasmic-CD179a, a component of surrogate light chain known to be most specific molecular marker of precursor-B cells, whereas only a few percent of the CD19⁺ cells were positive for surface and/or cytoplasmic- μ^- heavy chain. Considering the additional observations that CD10, CD24, and CD43 were expressed but CD20 were not in the CD19⁺ cells, we concluded that most of the CD19⁺ B cells obtained in our culture system were pro-B cells [25].

We investigated the expression of IL-7 by the MS-5 cells and IL-7R α by cultured CD34⁺ BM cells. RT-PCR analysis showed expression of murine IL-7 by MS-5 cells (Fig. 2A). In addition, expression of human IL-7R α mRNA by the cultured human BM CD34⁺ cells was observed (Fig. 2B).

Elimination of IL-7 reduced pro-B-cell development

Because murine IL-7 is known to react with human IL-7R [26], the IL-7 secreted by MS-5 cells possibly affects cultured CD34⁺ BM cells. We therefore investigated the effect of anti-mouse IL-7 antibodies, which neutralizes the effect of IL-7 on cultured CD34⁺ BM cells. As shown in Figure 1, when anti-mouse IL-7 Ab was added, the CD19⁺CD33⁻ B-cell development was significantly reduced. In contrast, when goat immunoglobulin (Ig) G was similarly added, as a control experiment for Figure 1A, the CD19⁺CD33⁻ B-cell development was not reduced (Fig. 1B), indicating that the effect of anti-mouse IL-7 Ab is specific. The inhibitory effect of anti-mouse IL-7 Ab on pro-B-cell differentiation was found to be dose-dependent and time-dependent (Figs. 1 and 3). It is noteworthy that no significant change in CD19⁺CD33⁺ myeloid cell development was observed, whereas the subsequent cell number of CD19⁺CD33⁻ was also suppressed by addition of anti-mouse IL-7 Ab (Fig. 1).

Because we observed the inhibitory effect of anti-mouse IL-7 Ab on CD19⁺CD33⁻ cell fraction, we next investigated the expression of B-lineage marker genes to evaluate more detail characterization of these cells. As shown in Figure 4A, in addition to CD19⁺CD33⁻ pro-B cell, CD19⁺CD33⁻ cells but not CD19⁺CD33⁺ cells also expressed IL-7R α , after cultivation for 4 weeks. Expression of TdT was also detected in CD19⁺CD33⁻ cells. Although 30 cycles amplification failed in detection of PAX5 and Igz

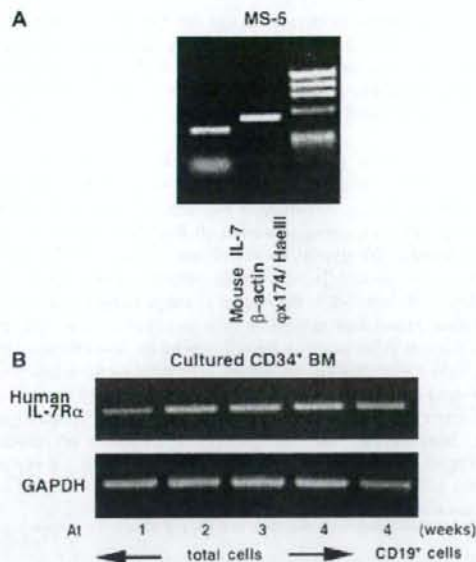


Figure 2. Expression of interleukin (IL)-7 by murine stromal MS-5 cells and of IL-7 receptor by cultured human bone marrow CD34⁺ cells. (A) Expression of IL-7 by MS-5 cells was investigated by reverse transcription polymerase chain reaction (RT-PCR). Expression of mouse β -actin was also investigated as an internal control. The ϕ x174/HaeIII molecular weight marker is shown on the right side. (B) Human bone marrow CD34⁺ cells cultured on MS-5 cells for 1, 2, 3, and 4 weeks. At the end of each culture period, cultured human bone marrow cells was collected by gentry pipetting, and the expression of IL-7 receptor (R) α was investigated by RT-PCR. CD19⁺ cells were sorted from 4-week cultured human bone marrow CD34⁺ cells and similarly examined. Expression of human glyceraldehyde phosphate dehydrogenase was investigated as an internal control.

genes, 35 cycles amplification revealed the expression of these genes in CD19⁺CD33⁻ cells (Fig. 4B).

Effect of elimination of IL-7 on colony formation of CD34⁺ BM cells

We also examined the effect of IL-7 elimination on colony formation ability of CD34⁺ BM cells. The CD34⁺ cells were cultured on MS-5 cells with and without anti-mouse IL-7 Ab for 1 week and examined by colony formation assay. As we reported previously [25], cultured CD34⁺ cells on MS-5 cells can be classified into two subpopulations, namely, floating and adherent cell fraction. Interestingly, treatment with anti-mouse IL-7 Ab distinctively affected each cell fraction and the number of adherent cells was slightly decreased, whereas the floating cells were not reduced (Fig. 5A). Moreover, after treatment with anti-mouse IL-7 Ab, granulocyte-erythrocyte-macrophage-megakaryocyte (GEMM) colony formation from floating cells was slightly reduced and burst-forming unit erythroid (BFU-E) colony formation

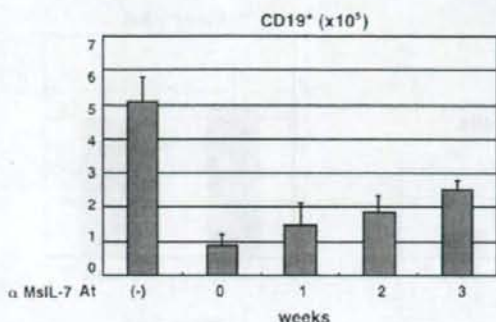


Figure 3. Time-dependency of anti-mouse interleukin (IL-7) antibody-mediated inhibition of pro-B-cell development. Human bone marrow CD34⁺ cells were cultured on MS-5 cells for 4 weeks. Goat polyclonal anti-mouse IL-7 antibody (2.5 µg/mL) was added at the start of culture (0), and after 1, 2, and 3 weeks of culture, and the number of CD19⁺ cells was estimated by flow cytometry.

from adherent cells was significantly increased (Fig. 5B). Especially, subsequent BFU-E colony formation from total cells was also increased by anti-mouse IL-7 Ab treatment.

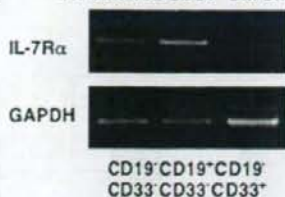
Effect of cytokines on anti-IL-7

Ab-mediated reduction in B-cell development

Since the reduction in CD19⁺ B-cell development induced by anti-mouse IL-7 Ab was reversed by addition of recombinant human IL-7 to the coculture of CD34⁺ BM cells and MS-5 cells (Fig. 6A), the effect of anti-mouse IL-7 Ab was concluded to be IL-7-specific. However, when we investigated the effect of exogenous recombinant human IL-7 alone, no significant increase in CD19⁺ B-cell development was observed (Fig. 6A). Also, the proportion of different lineages cells was not affected by exogenous recombinant human IL-7 (data not shown). It is noteworthy that although exogenous recombinant human IL-7 did not change the number of pro-B cells, it increased the intensity of CD19 expression on CD34⁺ BM cells (Fig. 7), while further differentiation of pro-B to pre-B cell was not observed (data not shown).

Next, we investigated the effect of exogenous recombinant human IL-2, IL-4, IL-9, IL-11, IL-15, and IL-21, which mediates signal transduction via common γ chain on the reduction in pro-B-cell development induced by anti-mouse IL-7 Ab, and no significant recovery in pro-B-cell development was observed (Fig. 6B). TSLP has been reported to mediate signal transduction via IL-7R and TSLPR heterodimer and have overlapping function with IL-7 [27,28]. Thus, we also investigated the effect of exogenous recombinant murine and human TSLP on reduction in pro-B-cell development induced by anti-mouse IL-7 Ab, whereas no significant recovery in pro-B-cell development was observed (Fig. 6C).

A 4W cultured CD34⁺ BM on MS-5



B



Figure 4. Expression of B-cell differentiation marker mRNAs by cultured human bone marrow CD34⁺ cells. (A) Human bone marrow CD34⁺ cells cultured on MS-5 cells for 4 weeks, CD33⁺CD19⁺, CD33⁺CD19⁻, and CD33⁻CD19⁺ cells were sorted, and expression of IL-7R α was investigated by reverse transcription polymerase chain reaction with 30 cycles amplification. Expression of human glyceraldehyde phosphate dehydrogenase (GAPDH) was also investigated as an internal control. (B) CD33⁺CD19⁺ and CD33⁺CD19⁻ cells were sorted from 4-week cultured human bone marrow CD34⁺ cells and expression of B-cell-differentiation marker genes as indicated were similarly examined as in (A) with either 30 or 35 cycles amplification. Expression of human GAPDH was investigated as an internal control.

Inhibition of IL-7 signaling

reduced pro-B-cell development

Next, we investigated whether anti-human IL-7R α Ab inhibits pro-B-cell development. As shown in Figure 8, addition of human IL-7R α Ab that block the effect of IL-7 reduced the number of pro-B-cell development. Because IL-7R signaling transduces to JAK3, we investigated the effect of a JAK3 kinase inhibitor. As shown in Figure 8, the JAK3 kinase inhibitor significantly reduced pro-B-cell development.

Discussion

In this article, we demonstrated that IL-7 plays a certain role in development of human pro-B cells from

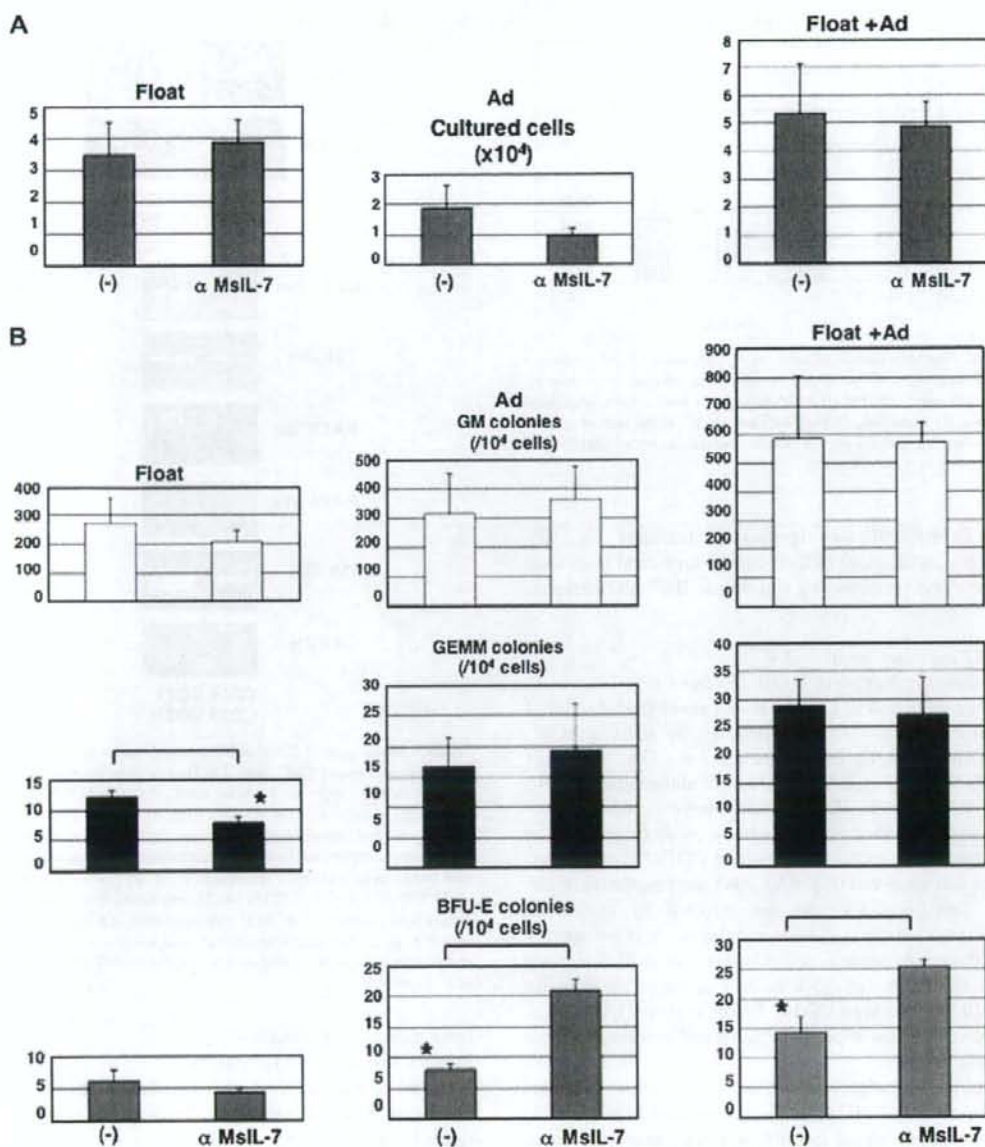


Figure 5. Effect of anti-mouse interleukin (IL)-7 antibody on colony formation ability of human bone marrow CD34⁺ cells. Human bone marrow CD34⁺ cells were cultured on MS-5 cells for 1 week in the presence or absence ((-)) of goat polyclonal anti-mouse IL-7 antibody (2.5 μ g/mL), and colony assay was performed with floating and adhesion cells separately as described in Materials and Methods. The number of granulocyte macrophage (GM), granulocyte-erythrocyte-macrophage-megakaryocyte (GEMM), and burst-forming unit erythroid (BFU-E) colonies per 10⁴ cultured cells (A), and 1 weed-cultured cell number (B) was counted. *Statistically significant differences ($p < 0.05$).

hematopoietic stem cells in vitro. Results of the present study showed that MS-5 murine stromal cells produce IL-7 and that neutralization of the IL-7 they secrete with anti-mouse IL-7 Ab markedly reduced pro-B-cell develop-

ment. As mentioned above, murine IL-7 is known to be capable of binding to the human IL-7R [26,29]. Although previous study of structure evaluation and enthalpy calculation performed on computer predicted that murine IL-7

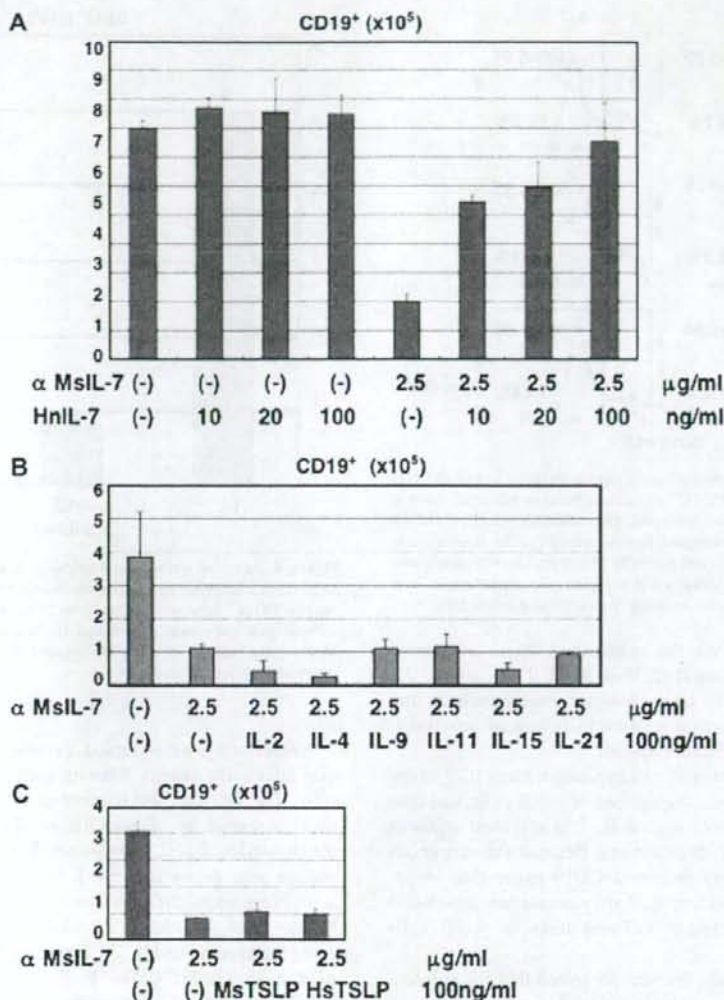


Figure 6. Effect of recombinant human interleukin (IL)-7 on anti-mouse IL-7 antibody-mediated inhibition of human pro-B-cell development. (A) Human bone marrow CD34⁺ cells were cultured on MS-5 cells for 4 weeks with or without the indicated combinations of goat anti-mouse IL-7 antibody (2.5 μg/mL) and recombinant human IL-7 (10 to 100 ng/mL, as indicated). The number of CD19⁺ cells was counted, the same as described in Figure 5. (B) Human bone marrow CD34⁺ cells were cultured on MS-5 cells for 4 weeks with or without the indicated combinations of goat anti-mouse IL-7 antibody (2.5 μg/mL) and recombinant human IL (100 ng/mL), as indicated. The number of CD19⁺ cells was counted and presented as in Figure 5. (C) Human bone marrow CD34⁺ cells were cultured on MS-5 cells for 4 weeks with or without the indicated combinations of goat anti-mouse IL-7 antibody (2.5 μg/mL) and recombinant murine or human thymic stromal lymphopoietin (TSLP) (100 ng/mL), as indicated. The number of CD19⁺ cells was counted and presented as in Figure 5.

may display weaker binding with human IL-7R than human IL-7 [30], however, it has been reported that murine IL-7 still affect human CD19⁺ cells and can induce downstream signaling of IL-7R [31]. Indeed, the anti-mouse IL-7 Ab-induced reduction in pro-B-cell development was reversed by the addition of recombinant human IL-7, suggesting specific inhibition of IL-7 function by anti-IL-7 Ab.

Inhibition of IL-7 binding to human IL-7R by anti-human IL-7Rα Ab also reduced pro-B-cell development, and a JAK3 kinase inhibitor that blocks signaling downstream of IL-7R showed a similar reduction in pro-B-cell development. As we presented, more significant inhibition of B lymphopoiesis was induced by addition of the JAK3 inhibitor. It may be because the reason of that JAK3 mediates

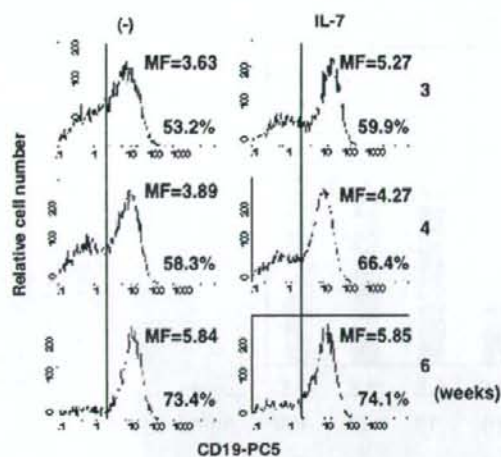


Figure 7. Effect of recombinant human interleukin (IL)-7 on human CD19 expression in pro-B cells. CD34⁺ cells were cultured on MS-5 cells for 3, 4, and 6 weeks with or without 100 ng/mL recombinant human IL-7, and expression of CD19 was investigated by flow cytometry. The value of mean fluorescence intensity (MF) and positivity (%) of each histogram are indicated. Experiments were performed in triplicate, and similar results were obtained. X-axis, fluorescence intensity; Y-axis, relative cell number.

signal transduction via the common γ chain of several lymphokines, including IL-2, IL-4, IL-9, IL-15, and IL-21, beside IL-7. All of the above findings clearly indicate that eliminating IL-7 function resulted in failure of pro-B-cell development in our culture system.

By contrast, addition of recombinant human IL-7 to the culture did not increase the number of pro-B cells, and thus the MS-5 cells possibly secrete IL-7 in sufficient amounts to support pro-B-cell development. Because the exogenous human IL-7 relatively increased CD19 expression on induced pro-B cells, excess IL-7 may accelerate pro-B-cell maturation, while further differentiation to pre-B cells was not occurred.

In the present study, we also presented that the elimination of IL-7 function results in the inhibition of cell growth in CD19⁺CD33⁻ cell fraction. Because we detected the gene expression of IL-7 R α in the cell fraction of CD19⁺CD33⁻, but not CD19⁺CD33⁺, it is reasonable to consider that IL-7 can directly affect CD19⁺CD33⁻ cell fraction. The fact of the expression of B-lineage marker genes, such as PAX5 and Ig α , should indicate that CD19⁺CD33⁻ cell fraction contain the B cell progenitors in which CD19 gene is not yet expressing. Consistently, Reynaud et al. reported that IL-7R α ⁺Ig α ⁺CD19⁻ cells that produced by CD34⁺CD19⁻CD10⁻ cord blood cells cultured in the presence of MS-5 with IL-2, IL-15, and stem cell factor cytokines, transcribed the B-lymphoid-specific genes E2A, EBF, TdT, Rag-1, had initiated DJH rearrangement [32]. Alternatively, IL-7 may affect not only lymphoid progenitor but also other lineage cells.

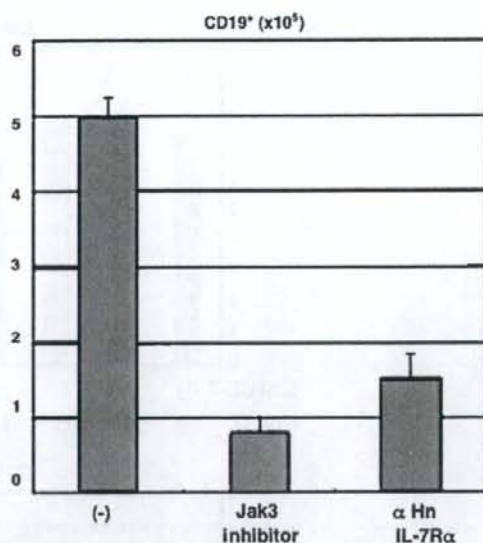


Figure 8. Effect of anti-human interleukin (IL)-7 receptor antibody and JAK3 kinase inhibitor on human pro-B-cell development. Human bone marrow CD34⁺ cells were cultured on MS-5 cells for 4 weeks with or without goat polyclonal anti-human IL-7R α antibody (2.5 μ g/mL) or JAK3 kinase inhibitor (5 μ M). The number of CD19⁺ cells was counted the same as described in Figure 5.

Interestingly, we observed that elimination of IL-7 function affects the colony-forming ability of cultured CD34⁺ cells. The fact that the number of BFU-E colony remarkably increased by elimination of IL-7 function suggests the possibility that IL-7 promotes differentiation of hematopoietic progenitors into the B-lineage cells and thus lead a suppression of their differentiation into the erythroblast besides. The observation of Adolfsson et al. [33] that indicated the upregulated IL-7R gene expression in a population of Lin-Scal⁺c-kit⁺CD34⁺Flt3⁺ lymphoid-myeloid stem cells in which the ability to adopt erythroid and megakaryocyte lineage fates have lost [33] should support our hypothesis. Moreover, decrease in the number of colony-forming unit-GEMM colony in adhesion cell fraction might suggest that IL-7 is taking part in the amplification of multipotent progenitor cells, though a more detailed investigation is necessary. It is also notable that floating and adhesion cell fractions seem to have different receptivity for the effect of IL-7 in our observation. Alternatively, IL-7 may influence the ability of adhesion of CD34⁺ cell.

As shown above, IL-7 is required for human pro-B-cell development, at least in our culture system. In contrast to our observation, however, Pribyl et al. [11] showed that IL-7 is not necessary for human B-cell development in an *in vitro* study. They cocultured human CD34⁺ hematopoietic stem cells and BM stromal cells from fetal BM for 3

weeks without exogenous cytokines and induced immature B cells expressing μ/λ or μ/κ surface Ig receptors. In their study enzyme-linked immunosorbent assay revealed secretion of about 1 to 2 pg/mL IL-7 by BM stromal cells, and addition of recombinant human IL-7 or anti-human IL-7 neutralizing Ab had no effect on the CD19⁺ cell number. Consistent with this, congenital immunodeficiency patients who have mutations in common γ chain, IL-7R α chain or JAK3 tyrosine kinase, have normal numbers of peripheral B cells [12–15].

Although the exact reason for the discrepancy is unknown, several explanations are possible. In contrast to our study, for example, they used human BM stromal cells, and the difference between the microenvironments produced by the human and murine stromal cells may have contributed to the difference in effect of IL-7 on human B-cell development. Another possibility is that, stimulation by another cytokine or a growth factor may compensate for the lack of IL-7 function in human B-cell development. In the mouse microenvironment, however, the factor may be absent or not have an IL-7 function-compensating effect. In this study, we have tried to identify the substitutional factor for IL-7, whereas IL-2, IL-4, IL-9, IL-11, IL-15, IL-21, and TSLP failed to compensate for the lack of IL-7 function. Therefore, another candidate(s) having substitutional effect for IL-7 need to be identified in the future experiments.

During revision, a similar observation to that presently reported has been published by Johnson et al. [31]. Using coculture system of CD34⁺ cord blood cells and MS-5 cells supplemented with granulocyte-colony stimulating factor and stem cell factor to develop CD19⁺ pro-B cells, they presented that murine and human IL-7 affect human pro-B cells and activate STAT5, resulting in proliferation. They also presented that neutralizing anti-murine IL-7 inhibited development of CD19⁺ cells on their culture system. Our study further extends their observation and indicated that IL-7 is involved in the development of human pro-B cells from hematopoietic stem cells in vitro and affect CD19⁺CD33⁺IL7R⁺ B-cell precursor fraction and hence influence on their colony-formation ability.

In view of the above findings, we concluded that the IL-7 is required for human pro-B-cell development from CD34⁺ BM cells in our culture system and that IL-7 appears to play a certain role in early human B lymphopoiesis. Although further investigation needed to be done, our observations should contribute to a better understanding of the functional roles of IL-7 in the regulation of B lymphopoiesis.

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Interleukin-7 contributes to human pro-B-cell development in a mouse stromal cell-dependent culture system

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Objective. The role of interleukin (IL)-7 in human B lymphopoiesis is still controversial. We used an in vitro culture system to verify involvement of IL-7 in development of human pro-B cells from hematopoietic stem cells.

Materials and Methods. Human CD34⁺ bone marrow cells were cultured for 4 weeks on MS-5 mouse stromal cells to induce pro-B cells. Expression of IL-7 receptor α or other B-cell differentiation marker genes on cultured human CD34⁺ bone marrow cells was investigated by reverse transcription polymerase chain reaction (RT-PCR). Colony assay of human CD34⁺ bone marrow cells was also performed to determine the effect of IL-7 on colony-forming ability. Neutralizing antibody or reagent that eliminates the effect of IL-7 was added to the culture system, and the number of pro-B cells induced was estimated by flow cytometry.

Results. RT-PCR analysis revealed mRNA expression of IL-7 receptor α as well as B-cell differentiation marker genes in not only CD19⁺ pro-B cells but also CD19⁻ CD33⁻ cells induced from CD34⁺ bone marrow cells after cultivation for 4 weeks on MS-5 cells. Addition of anti-mouse IL-7 antibody, anti-human IL-7 receptor α antibody, or JAK3 kinase inhibitor reduced the number of pro-B cells induced, demonstrating that elimination of IL-7 reduces pro-B-cell development. Addition of anti-mouse IL-7 antibody emphasized the colony-forming ability of burst-forming unit erythroid cells.

Conclusions. IL-7 produced by MS-5 cells is required for human pro-B-cell development from CD34⁺ bone marrow cells in our culture system, and IL-7 appears to play a certain role in early human B lymphopoiesis. © 2007 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

Interleukin (IL)-7 is a cytokine that was first cloned from a murine bone marrow (BM) stromal cell line and is involved in the regulation of lymphopoiesis [1]. Several studies have shown that IL-7 is crucial to proliferation and development of murine B cells. For example, injection of mice with recombinant IL-7 has been shown to greatly increase the number of B cells [2], whereas injection of anti-IL-7 antibodies severely represses B-cell development [3,4]. Study of the effect of IL-7 on fractionated B-lineage cells from normal mouse BM in a stromal-cell-dependent

culture system revealed that IL-7 is required for effective differentiation of pro-B cells into pre-B cells [5]. IL-7 is sufficient to induce differentiation of murine common lymphoid progenitors into pro-B cells in cultures under stromal-cell-free conditions [6].

The requirement for IL-7 in B-lymphocyte development in mice was further demonstrated by experiments in which components of the IL-7 signal transduction pathways were deleted by gene targeting [7–10]. Results showed that B-cell development is severely arrested at the common lymphoid progenitor stage in the BM of adult IL-7 receptor (R) α and common γ -chain-deficient mice, leading to a striking paucity of peripheral B cells.

In contrast to murine B-cell development, however, human B-cell development does not appear to require IL-7

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[11]. Unlike the mouse common γ knockouts, patients with human X-linked severe combined immunodeficiency, who lack a functional common γ chain, produce normal numbers of B cells [12]. Immunodeficiency patients with autosomal recessive mutations in either IL-7R α chain or JAK3 tyrosine kinase, a downstream signaling molecule of IL-7R, also have normal numbers of peripheral B cells [13–15]. All of this evidence indicates that IL-7 is not always required for B-cell development in humans.

Nevertheless, some studies found that IL-7 affects human B-cell development in some way. For example, it was found that IL-7 transduces signals that lead to specific changes in gene expression during human B-cell development. IL-7 stimulation induces a specific increase in CD19 on the surface of human pro-B cells and decrease in RAG-1, RAG-2, and TdT messenger RNA levels [16]. Proliferation of CD19⁺CD34⁺ pro-B cells on human BM stromal cells is enhanced by inclusion of exogenous IL-7 in the culture [17]. Therefore, if not essential, IL-7 may play an integral role in some aspects of human B-cell development.

In an attempt to clarify the effect of IL-7 on human B-cell development, we used an in vitro culture system in which human hematopoietic stem cells are cocultured with murine BM stromal cells that induce pro-B-cell differentiation. In this article, we report finding that IL-7 is essential for the differentiation of human CD34⁺ BM cells into pro-B cells in our culture system, and we discuss the possible role of IL-7 in early human B-cell development.

Materials and methods

Reagents

Monoclonal antibodies used were phycoerythrin (PE)-conjugated anti-CD33, from Becton Dickinson Biosciences (San Diego, CA, USA), and PE-cyanine (PC)-5-conjugated anti-CD19, from Beckman/Coulter Inc. (Westbrook, MA, USA). Goat polyclonal anti-mouse IL-7 antibody (Ab) and goat anti-human IL-7R α Ab were obtained from R&D Systems (Abingdon, UK) and used in the cultures at concentrations of 1 to 5 μ g/mL, as indicated. Recombinant human IL-2, -4, -7, -9, and -11 were obtained from PeproTech EC Ltd. (London, UK) and recombinant human IL-15, -21, and both human and mouse thymic stromal lymphopoietin (TSLP) were obtained from R&D Systems.

4-[(3'-Bromo-4'-hydroxyphenyl) amino]-6,7-dimethoxyquinazoline, a potent specific inhibitor of JAK3 kinase (IC_{50} = 5.6 μ M) was obtained from Calbiochem-Novabiochem Co. (San Diego, CA, USA) and used in the cultures at a concentration of 5 μ M. The specificity of this chemical compound as a JAK3 kinase inhibitor has been examined by Goodman et al. [18] and Sudbeck et al. [19]. They demonstrated that this compound exhibited detectable inhibitory activity only against recombinant JAK3, but not JAK1 or JAK2, in immune complex kinase assays and also inhibited IL-2-induced JAK3-dependent signal transducers and activators of transcription (STAT) activation, but not inhibited IL-3-induced JAK1/JAK2-dependent STAT activation in 32Dc11-IL2R cells. Unless otherwise indicated, all chemical reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cells, cultures, and colony assay

Human BM CD34⁺ cells used were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD, USA). BM cells were isolated from human tissue after obtaining informed consent. A cloned murine BM stromal cell line, MS-5, was kindly provided by Dr. A. Manabe (St. Luke's International Hospital, Tokyo, Japan) and Dr. K. Mori (Nigata University, Nigata, Japan), and maintained in RPMI-1640 medium (Sigma-Aldrich Fine Chemical Co., St. Louis, MO, USA) supplemented with 10% (v/v) fetal calf serum (Sigma-Aldrich) at 37°C under a humidified 5% CO₂ atmosphere.

To induce pro-B cells, MS-5 cells were plated at a concentration of 1×10^5 cells in 12-well tissue plate (Asahi Techno Glass Co., Chiba, Japan) 1 day prior to seeding human BM CD34⁺ cells. CD34⁺ cells were plated 4×10^4 cells/well/2 mL onto the MS-5 cells in RPMI-1640 supplemented with 10% fetal calf serum and

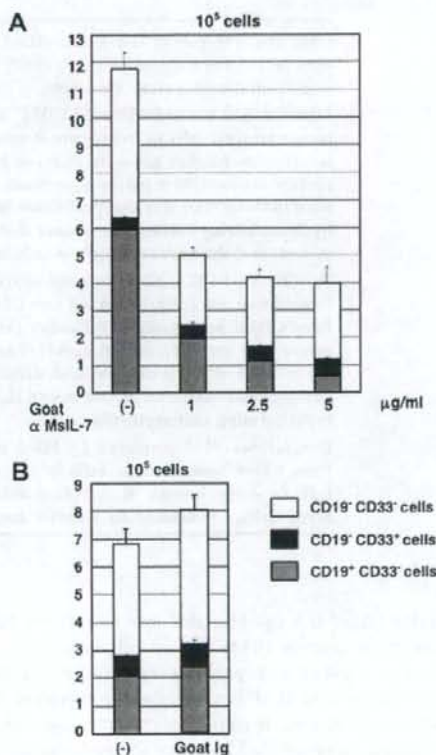


Figure 1. Effect of anti-mouse interleukin (IL-7) antibody on human pro-B-cell development. (A) Human bone marrow CD34⁺ cells were cultured on MS-5 cells for 4 weeks in the presence or absence [(-)] of different concentrations of goat polyclonal anti-mouse IL-7 antibody. The subsequent CD19⁺CD33⁺ cell number (lower light gray column), CD19⁺CD33⁻ cell number (middle dark gray column), and CD19⁻CD33⁺ cell number (upper white column) of cultured CD34⁺ cells were calculated by flow cytometry. (B) Human bone marrow CD34⁺ cells were cultured on MS-5 cells for 4 weeks in the presence or absence of goat immunoglobulin (goat Ig) as a negative control.

various combinations of cytokines or other reagents, as indicated in Figures 1,3,5,6,8. After cultivation for the periods indicated, the cells were harvested with 0.25% trypsin plus 0.02% ethylenediamine tetraacetic acid (IBL Co. Ltd., Gunma, Japan), the number of cells per well was counted, and cells were analyzed by flow cytometry.

For the colony assay, CD34⁺ BM cells were cultured for 1 week on MS-5 cells in the presence or absence of goat anti-IL-7 Ab and the floating cell fraction was first collected with culture medium. The remaining adherent cell fraction with MS-5 cells were treated with trypsin, harvested, and plated in 6-well tissue culture plate (Asahi Techno Glass). After removing MS-5 cells by letting them attach to the bottom of the plate by 15-minute incubation, subsequent suspension cells were collected as adherent cell fraction. After counting the cell number by flow cytometry using Flow-Count (Beckman/Coulter), cells from each fraction were passaged into methylcellulose cultures containing the cocktail of cytokines (MethocultTM GF+H4435; Stem Cell Technologies Inc, Northampton, UK). Morphology and number of colonies comprising more than 50 cells was scored at 14 days. All experiments were performed in triplicate and the mean + SD of the values were shown in Figures 1,3,5,6,8.

Immunofluorescence study

Cells were stained with fluorescence-labeled monoclonal antibodies and analyzed by flow cytometry (EPICS-XL, Beckman/Coulter) as described previously [20]. Two-color immunofluorescence study was performed with a combination of PE and PC-5. Experiments were performed in triplicate, and the mean + SD of the cell counts were indicated in the Figures 1,3,5,6,8.

For cell sorting, human BM CD34⁺ cells cocultured with MS-5 cells for 4 weeks were harvested and stained with PE-conjugated anti-CD33 monoclonal Ab and PC-5-conjugated anti-CD19 monoclonal Ab. CD33⁻CD19⁻, CD33⁺ and CD19⁺ cells were sorted in an EPICS-ALTRA cell sorter (Beckman/Coulter). Total RNA was extracted and used for reverse transcription polymerase chain reaction (RT-PCR).

RT-PCR

Total RNA was extracted from cultured cells, and cDNA was generated with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and a FirstStrand cDNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden). cDNA synthesized from 150 ng total RNA was used as a template for one amplification. The sets of primers used in this study were listed in Table 1.

PCR was repeated for 30 to 35 cycles of heating at 94°C for 60 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 2 minutes; the products were then separated on a 1.5% agarose gel.

Results

MS-5 cells secrete IL-7

Murine stromal cell line MS-5 has been reported to possess the ability to support the differentiation of B-lineage cells and myeloid cells from human cord blood CD34⁺ cells [21–25]. Consistent with previous observations, the human BM CD34⁺ cells in our study generated CD19⁺CD33⁻ B cells and CD19⁺CD33⁺ myeloid cells after 4 weeks of

Table 1. List of primers used in this study

Name of gene	Primer sequence	Product size (bp)
Murine IL-7		
Forward	5'-TAAATCGTGCTGCTCGCAAGT-3'	
Reverse	5'-AGCAGTCAGCTGCAITTCGTG-3'	392
Human IL-7R α		
Forward	5'-GTCACCTCCAGAAAGCTTTGG-3'	
Reverse	5'-AGGAACTCTAGACTTCCCTTT-3'	
Human CD19		
Forward	5'-GTTCCGGTGAATGTTTCGG-3'	386
Reverse	5'-AGATGAAGAATGCCCAAGG-3'	576
Human TdT		
Forward	5'-ACACGAATGCAGAAAGCAGGA-3'	
Reverse	5'-AGGCAACCTGAGCTTTTCAA-3'	315
Human PAX5		
Forward	5'-CCATCAAGTCCTGAAAAATC-3'	
Reverse	5'-CCCAAAGTGGTGAAAAATC-3'	319
Human Ig α		
Forward	5'-TAGTCGACATGCCTGGGGTCCAGGAGTCCCTC-3'	
Reverse	5'-GATGTCCAGCTGGAGAAGCCGTGA-3'	681
Human GAPDH		
Forward	5'-CCACCATGGCAAAATCCATGGCA-3'	598
Reverse	5'-TCTAGACGGCAGGTCAGGTCCACC-3'	
Murine actin		
Forward	5'-TGACGGGTACCCACACTGTGCCCATCTA-3'	
Reverse	5'-CTAGAAGCAATTCGGGTGGACGATGGAGGG-3'	661

GAPDH = glyceraldehyde phosphate dehydrogenase; IL = interleukin.

cocultivation with MS-5 cells (Fig. 1). Immunocytological analysis showed that the CD19⁺ B cells in our culture system were surrogate light chain⁺ μ^- pro-B cells [25]. Consistent with these observations, the human BM CD34⁺ cells in our study generated CD19⁺ B cells and CD33⁺ myeloid cells after 4 weeks of cocultivation with MS-5 cells (Fig. 1). The detailed characterization of our culture system has been reported previously [25]. Starting with 4×10^4 CD34⁺ cells, that containing <8% of CD19⁺CD34⁺, 0.4 to 1.3×10^6 mononuclear cells, 30.1% to 68.2% of which were CD19⁺CD34⁻ cells, were obtained (data not shown). Immunocytological analysis showed that most of these CD19⁺ B cells expressed cytoplasmic-CD179a, a component of surrogate light chain known to be most specific molecular marker of precursor-B cells, whereas only a few percent of the CD19⁺ cells were positive for surface and/or cytoplasmic- μ^- heavy chain. Considering the additional observations that CD10, CD24, and CD43 were expressed but CD20 were not in the CD19⁺ cells, we concluded that most of the CD19⁺ B cells obtained in our culture system were pro-B cells [25].

We investigated the expression of IL-7 by the MS-5 cells and IL-7R α by cultured CD34⁺ BM cells. RT-PCR analysis showed expression of murine IL-7 by MS-5 cells (Fig. 2A). In addition, expression of human IL-7R α mRNA by the cultured human BM CD34⁺ cells was observed (Fig. 2B).

Elimination of IL-7 reduced pro-B-cell development

Because murine IL-7 is known to react with human IL-7R [26], the IL-7 secreted by MS-5 cells possibly affects cultured CD34⁺ BM cells. We therefore investigated the effect of anti-mouse IL-7 antibodies, which neutralizes the effect of IL-7 on cultured CD34⁺ BM cells. As shown in Figure 1, when anti-mouse IL-7 Ab was added, the CD19⁺CD33⁻ B-cell development was significantly reduced. In contrast, when goat immunoglobulin (Ig) G was similarly added, as a control experiment for Figure 1A, the CD19⁺CD33⁻ B-cell development was not reduced (Fig. 1B), indicating that the effect of anti-mouse IL-7 Ab is specific. The inhibitory effect of anti-mouse IL-7 Ab on pro-B-cell differentiation was found to be dose-dependent and time-dependent (Figs. 1 and 3). It is noteworthy that no significant change in CD19⁺CD33⁺ myeloid cell development was observed, whereas the subsequent cell number of CD19⁺CD33⁻ was also suppressed by addition of anti-mouse IL-7 Ab (Fig. 1).

Because we observed the inhibitory effect of anti-mouse IL-7 Ab on CD19⁺CD33⁻ cell fraction, we next investigated the expression of B-lineage marker genes to evaluate more detail characterization of these cells. As shown in Figure 4A, in addition to CD19⁺CD33⁻ pro-B cell, CD19⁺CD33⁻ cells but not CD19⁺CD33⁺ cells also expressed IL-7R α , after cultivation for 4 weeks. Expression of TdT was also detected in CD19⁺CD33⁻ cells. Although 30 cycles amplification failed in detection of PAX5 and Ig α

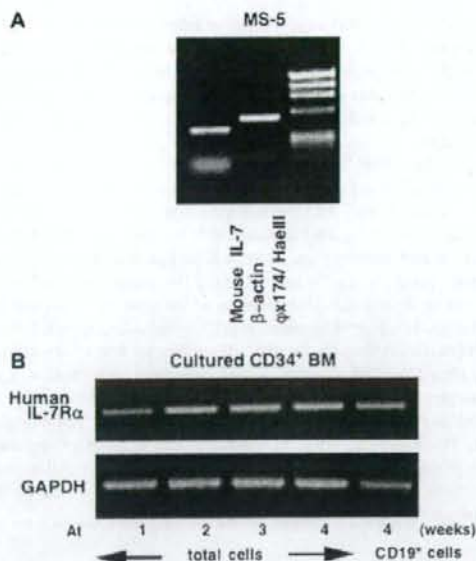


Figure 2. Expression of interleukin (IL)-7 by murine stromal MS-5 cells and of IL-7 receptor by cultured human bone marrow CD34⁺ cells. (A) Expression of IL-7 by MS-5 cells was investigated by reverse transcription polymerase chain reaction (RT-PCR). Expression of mouse β -actin was also investigated as an internal control. The ϕ X174/HaeIII molecular weight marker is shown on the right side. (B) Human bone marrow CD34⁺ cells cultured on MS-5 cells for 1, 2, 3, and 4 weeks. At the end of each culture period, cultured human bone marrow cells were collected by gentry pipetting, and the expression of IL-7 receptor (R) α was investigated by RT-PCR. CD19⁺ cells were sorted from 4-week cultured human bone marrow CD34⁺ cells and similarly examined. Expression of human glyceraldehyde phosphate dehydrogenase was investigated as an internal control.

genes, 35 cycles amplification revealed the expression of these genes in CD19⁺CD33⁻ cells (Fig. 4B).

Effect of elimination of IL-7 on colony formation of CD34⁺ BM cells

We also examined the effect of IL-7 elimination on colony formation ability of CD34⁺ BM cells. The CD34⁺ cells were cultured on MS-5 cells with and without anti-mouse IL-7 Ab for 1 week and examined by colony formation assay. As we reported previously [25], cultured CD34⁺ cells on MS-5 cells can be classified into two subpopulations, namely, floating and adherent cell fraction. Interestingly, treatment with anti-mouse IL-7 Ab distinctively affected each cell fraction and the number of adherent cells was slightly decreased, whereas the floating cells were not reduced (Fig. 5A). Moreover, after treatment with anti-mouse IL-7 Ab, granulocyte-erythrocyte-macrophage-megakaryocyte (GEMM) colony formation from floating cells was slightly reduced and burst-forming unit erythroid (BFU-E) colony formation

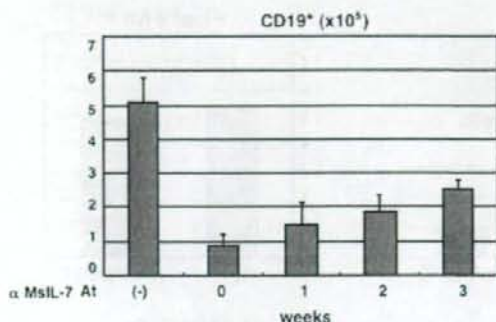


Figure 3. Time-dependency of anti-mouse interleukin (IL)-7 antibody-mediated inhibition of pro-B-cell development. Human bone marrow CD34⁺ cells were cultured on MS-5 cells for 4 weeks. Goat polyclonal anti-mouse IL-7 antibody (2.5 μ g/mL) was added at the start of culture (0), and after 1, 2, and 3 weeks of culture, and the number of CD19⁺ cells was estimated by flow cytometry.

from adherent cells was significantly increased (Fig. 5B). Especially, subsequent BFU-E colony formation from total cells was also increased by anti-mouse IL-7 Ab treatment.

Effect of cytokines on anti-IL-7

Ab-mediated reduction in B-cell development

Since the reduction in CD19⁺ B-cell development induced by anti-mouse IL-7 Ab was reversed by addition of recombinant human IL-7 to the coculture of CD34⁺ BM cells and MS-5 cells (Fig. 6A), the effect of anti-mouse IL-7 Ab was concluded to be IL-7-specific. However, when we investigated the effect of exogenous recombinant human IL-7 alone, no significant increase in CD19⁺ B-cell development was observed (Fig. 6A). Also, the proportion of different lineages cells was not affected by exogenous recombinant human IL-7 (data not shown). It is noteworthy that although exogenous recombinant human IL-7 did not change the number of pro-B cells, it increased the intensity of CD19 expression on CD34⁺ BM cells (Fig. 7), while further differentiation of pro-B to pre-B cell was not observed (data not shown).

Next, we investigated the effect of exogenous recombinant human IL-2, IL-4, IL-9, IL-11, IL-15, and IL-21, which mediates signal transduction via common γ chain on the reduction in pro-B-cell development induced by anti-mouse IL-7 Ab, and no significant recovery in pro-B-cell development was observed (Fig. 6B). TSLP has been reported to mediate signal transduction via IL-7R and TSLPR heterodimer and have overlapping function with IL-7 [27,28]. Thus, we also investigated the effect of exogenous recombinant murine and human TSLP on reduction in pro-B-cell development induced by anti-mouse IL-7 Ab, whereas no significant recovery in pro-B-cell development was observed (Fig. 6C).

A 4W cultured CD34⁺ BM on MS-5

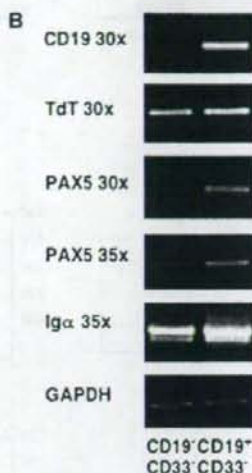


Figure 4. Expression of B-cell differentiation marker mRNAs by cultured human bone marrow CD34⁺ cells. (A) Human bone marrow CD34⁺ cells cultured on MS-5 cells for 4 weeks, CD33⁻CD19⁺, CD33⁺CD19⁺, and CD33⁻CD19⁺ cells were sorted, and expression of IL-7R α was investigated by reverse transcription polymerase chain reaction with 30 cycles amplification. Expression of human glyceraldehyde phosphate dehydrogenase (GAPDH) was also investigated as an internal control. (B) CD33⁻CD19⁺ and CD33⁺CD19⁺ cells were sorted from 4-week cultured human bone marrow CD34⁺ cells and expression of B-cell-differentiation marker genes as indicated were similarly examined as in (A) with either 30 or 35 cycles amplification. Expression of human GAPDH was investigated as an internal control.

Inhibition of IL-7 signaling reduced pro-B-cell development

Next, we investigated whether anti-human IL-7R α Ab inhibits pro-B-cell development. As shown in Figure 8, addition of human IL-7R α Ab that block the effect of IL-7 reduced the number of pro-B-cell development. Because IL-7R signaling transduces to JAK3, we investigated the effect of a JAK3 kinase inhibitor. As shown in Figure 8, the JAK3 kinase inhibitor significantly reduced pro-B-cell development.

Discussion

In this article, we demonstrated that IL-7 plays a certain role in development of human pro-B cells from

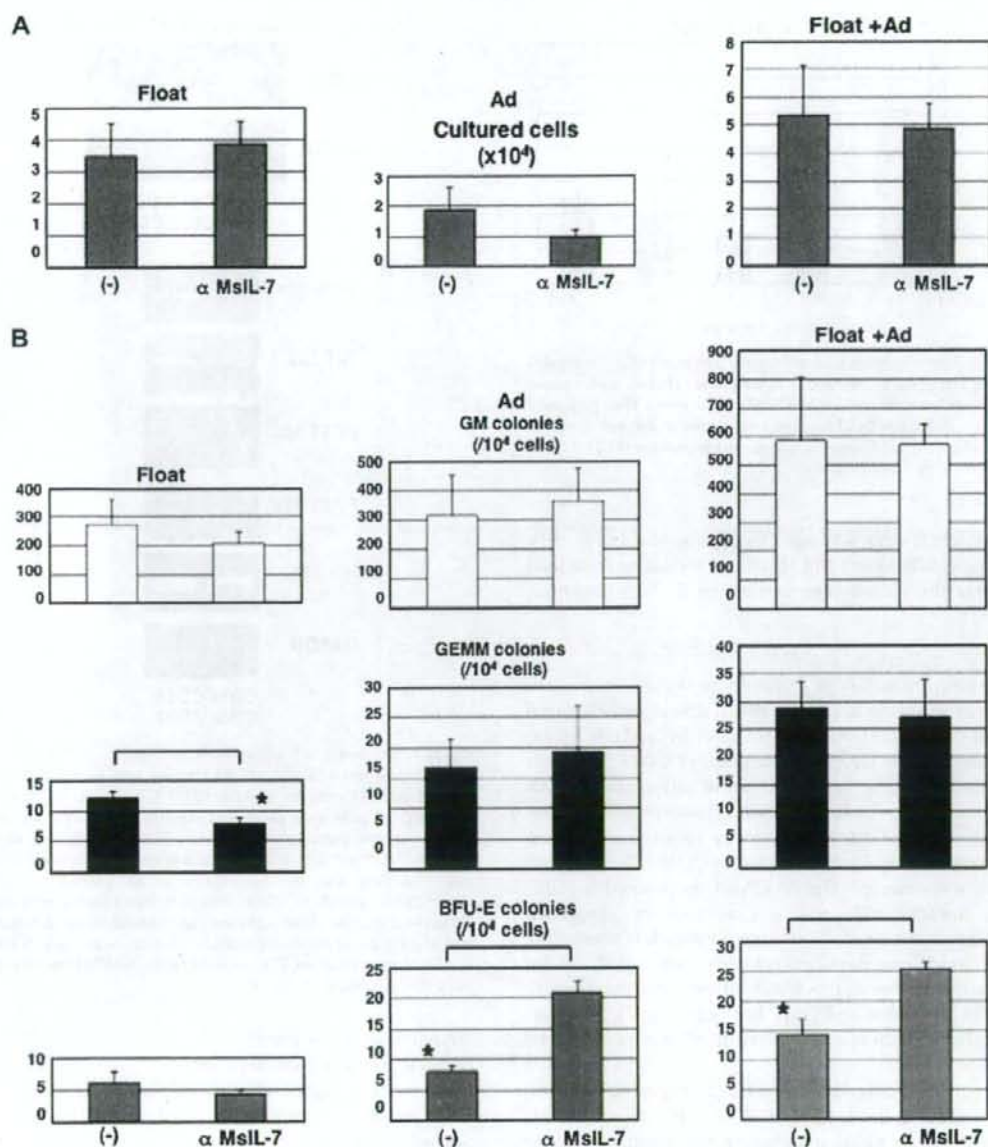


Figure 5. Effect of anti-mouse interleukin (IL)-7 antibody on colony formation ability of human bone marrow CD34⁺ cells. Human bone marrow CD34⁺ cells were cultured on MS-5 cells for 1 week in the presence or absence [(-)] of goat polyclonal anti-mouse IL-7 antibody (2.5 μ g/mL), and colony assay was performed with floating and adhesion cells separately as described in Materials and Methods. The number of granulocyte macrophage (GM), granulocyte-erythrocyte-macrophage-megakaryocyte (GEMM), and burst-forming unit erythroid (BFU-E) colonies per 10⁴ cultured cells (A), and 1 weed-cultured cell number (B) was counted. *Statistically significant differences ($p < 0.05$).

hematopoietic stem cells in vitro. Results of the present study showed that MS-5 murine stromal cells produce IL-7 and that neutralization of the IL-7 they secrete with anti-mouse IL-7 Ab markedly reduced pro-B-cell develop-

ment. As mentioned above, murine IL-7 is known to be capable of binding to the human IL-7R [26,29]. Although previous study of structure evaluation and enthalpy calculation performed on computer predicted that murine IL-7

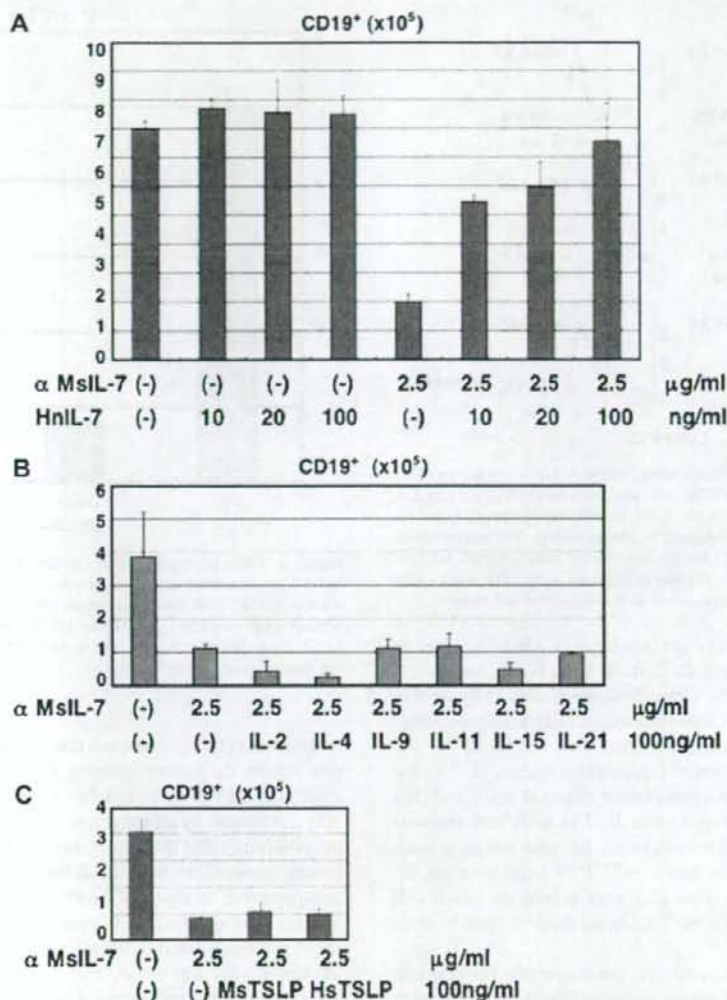


Figure 6. Effect of recombinant human interleukin (IL)-7 on anti-mouse IL-7 antibody-mediated inhibition of human pro-B-cell development. (A) Human bone marrow CD34⁺ cells were cultured on MS-5 cells for 4 weeks with or without the indicated combinations of goat anti-mouse IL-7 antibody (2.5 μg/mL) and recombinant human IL-7 (10 to 100 ng/mL, as indicated). The number of CD19⁺ cells was counted, the same as described in Figure 5. (B) Human bone marrow CD34⁺ cells were cultured on MS-5 cells for 4 weeks with or without the indicated combinations of goat anti-mouse IL-7 antibody (2.5 μg/mL) and recombinant human IL (100 ng/mL), as indicated. The number of CD19⁺ cells was counted and presented as in Figure 5. (C) Human bone marrow CD34⁺ cells were cultured on MS-5 cells for 4 weeks with or without the indicated combinations of goat anti-mouse IL-7 antibody (2.5 μg/mL) and recombinant murine or human thymic stromal lymphopoietin (TSLP) (100 ng/mL), as indicated. The number of CD19⁺ cells was counted and presented as in Figure 5.

may display weaker binding with human IL-7R than human IL-7 [30], however, it has been reported that murine IL-7 still affect human CD19⁺ cells and can induce downstream signaling of IL-7R [31]. Indeed, the anti-mouse IL-7 Ab-induced reduction in pro-B-cell development was reversed by the addition of recombinant human IL-7, suggesting specific inhibition of IL-7 function by anti-IL-7 Ab.

Inhibition of IL-7 binding to human IL-7R by anti-human IL-7Rα Ab also reduced pro-B-cell development, and a JAK3 kinase inhibitor that blocks signaling downstream of IL-7R showed a similar reduction in pro-B-cell development. As we presented, more significant inhibition of B lymphopoiesis was induced by addition of the JAK3 inhibitor. It may be because the reason of that JAK3 mediates

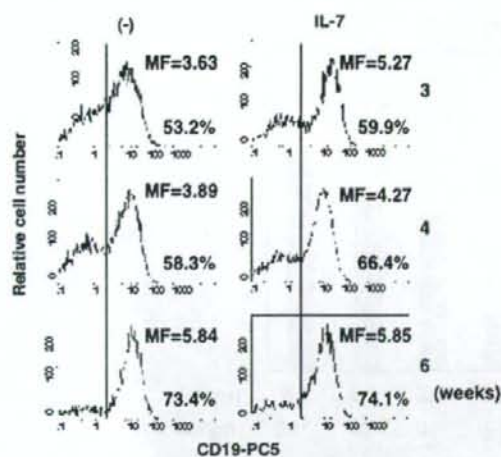


Figure 7. Effect of recombinant human interleukin (IL)-7 on human CD19 expression in pro-B cells. CD34⁺ cells were cultured on MS-5 cells for 3, 4, and 6 weeks with or without 100 ng/mL recombinant human IL-7, and expression of CD19 was investigated by flow cytometry. The value of mean fluorescence intensity (MF) and positivity (%) of each histogram are indicated. Experiments were performed in triplicate, and similar results were obtained. X-axis, fluorescence intensity; Y-axis, relative cell number.

signal transduction via the common γ chain of several lymphokines, including IL-2, IL-4, IL-9, IL-15, and IL-21, beside IL-7. All of the above findings clearly indicate that eliminating IL-7 function resulted in failure of pro-B-cell development in our culture system.

By contrast, addition of recombinant human IL-7 to the culture did not increase the number of pro-B cells, and thus the MS-5 cells possibly secrete IL-7 in sufficient amounts to support pro-B-cell development. Because the exogenous human IL-7 relatively increased CD19 expression on induced pro-B cells, excess IL-7 may accelerate pro-B-cell maturation, while further differentiation to pre-B cells was not occurred.

In the present study, we also presented that the elimination of IL-7 function results in the inhibition of cell growth in CD19⁺CD33⁻ cell fraction. Because we detected the gene expression of IL-7 R α in the cell fraction of CD19⁺CD33⁻, but not CD19⁺CD33⁺, it is reasonable to consider that IL-7 can directly affect CD19⁺CD33⁻ cell fraction. The fact of the expression of B-lineage marker genes, such as PAX5 and Ig α , should indicate that CD19⁺CD33⁻ cell fraction contain the B cell progenitors in which CD19 gene is not yet expressing. Consistently, Reynaud et al. reported that IL-7R α ⁺Ig α ⁺CD19⁻ cells that produced by CD34⁺CD19⁻CD10⁻ cord blood cells cultured in the presence of MS-5 with IL-2, IL-15, and stem cell factor cytokines, transcribed the B-lymphoid-specific genes E2A, EBF, Tdt, Rag-1, had initiated DJH rearrangement [32]. Alternatively, IL-7 may affect not only lymphoid progenitor but also other lineage cells.

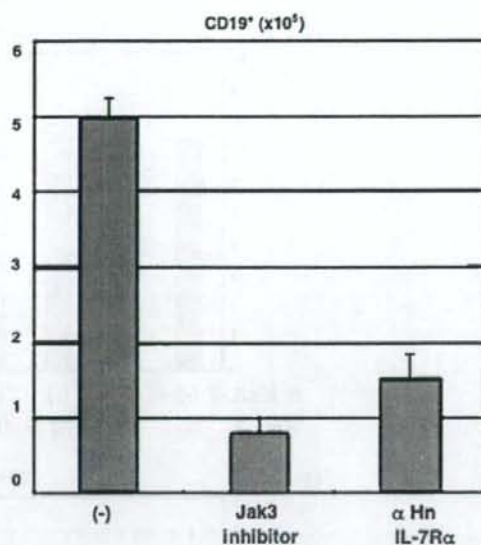


Figure 8. Effect of anti-human interleukin (IL)-7 receptor antibody and JAK3 kinase inhibitor on human pro-B-cell development. Human bone marrow CD34⁺ cells were cultured on MS-5 cells for 4 weeks with or without goat polyclonal anti-human IL-7R α antibody (2.5 μ g/mL) or JAK3 kinase inhibitor (5 μ M). The number of CD19⁺ cells was counted the same as described in Figure 5.

Interestingly, we observed that elimination of IL-7 function affects the colony-forming ability of cultured CD34⁺ cells. The fact that the number of BFU-E colony remarkably increased by elimination of IL-7 function suggests the possibility that IL-7 promotes differentiation of hematopoietic progenitors into the B-lineage cells and thus lead a suppression of their differentiation into the erythroblast besides. The observation of Adolfsson et al. [33] that indicated the upregulated IL-7R gene expression in a population of Lin-Sca1⁺c-kit⁺CD34⁺Flt3⁺ lymphoid-myeloid stem cells in which the ability to adopt erythroid and megakaryocyte lineage fates have lost [33] should support our hypothesis. Moreover, decrease in the number of colony-forming unit-GEMM colony in adhesion cell fraction might suggest that IL-7 is taking part in the amplification of multipotent progenitor cells, though a more detailed investigation is necessary. It is also notable that floating and adhesion cell fractions seem to have different receptivity for the effect of IL-7 in our observation. Alternatively, IL-7 may influence the ability of adhesion of CD34⁺ cell.

As shown above, IL-7 is required for human pro-B-cell development, at least in our culture system. In contrast to our observation, however, Pribyl et al. [11] showed that IL-7 is not necessary for human B-cell development in an *in vitro* study. They cocultured human CD34⁺ hematopoietic stem cells and BM stromal cells from fetal BM for 3

weeks without exogenous cytokines and induced immature B cells expressing μ/λ or μ/κ surface Ig receptors. In their study enzyme-linked immunosorbent assay revealed secretion of about 1 to 2 pg/mL IL-7 by BM stromal cells, and addition of recombinant human IL-7 or anti-human IL-7 neutralizing Ab had no effect on the CD19⁺ cell number. Consistent with this, congenital immunodeficiency patients who have mutations in common γ chain, IL-7R α chain or JAK3 tyrosine kinase, have normal numbers of peripheral B cells [12–15].

Although the exact reason for the discrepancy is unknown, several explanations are possible. In contrast to our study, for example, they used human BM stromal cells, and the difference between the microenvironments produced by the human and murine stromal cells may have contributed to the difference in effect of IL-7 on human B-cell development. Another possibility is that, stimulation by another cytokine or a growth factor may compensate for the lack of IL-7 function in human B-cell development. In the mouse microenvironment, however, the factor may be absent or not have an IL-7 function-compensating effect. In this study, we have tried to identify the substitutional factor for IL-7, whereas IL-2, IL-4, IL-9, IL-11, IL-15, IL-21, and TSLP failed to compensate for the lack of IL-7 function. Therefore, another candidate(s) having substitutional effect for IL-7 need to be identified in the future experiments.

During revision, a similar observation to that presently reported has been published by Johnson et al. [31]. Using coculture system of CD34⁺ cord blood cells and MS-5 cells supplemented with granulocyte-colony stimulating factor and stem cell factor to develop CD19⁺ pro-B cells, they presented that murine and human IL-7 affect human pro-B cells and activate STAT5, resulting in proliferation. They also presented that neutralizing anti-murine IL-7 inhibited development of CD19⁺ cells on their culture system. Our study further extends their observation and indicated that IL-7 is involved in the development of human pro-B cells from hematopoietic stem cells in vitro and affect CD19⁺CD33⁺IL7R⁺ B-cell precursor fraction and hence influence on their colony-formation ability.

In view of the above findings, we concluded that the IL-7 is required for human pro-B-cell development from CD34⁺ BM cells in our culture system and that IL-7 appears to play a certain role in early human B lymphopoiesis. Although further investigation needed to be done, our observations should contribute to a better understanding of the functional roles of IL-7 in the regulation of B lymphopoiesis.

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Preferential localization of SSEA-4 in interfaces between blastomeres of mouse preimplantation embryos

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Abstract

The monoclonal antibody 6E2 raised against the embryonal carcinoma cell line NCR-G3 had been shown to also react with human germ cells. Thin-layer chromatography (TLC) immunostaining revealed that 6E2 specifically reacts with sialosylglobopentaosylceramide (sialylGb5), which carries an epitope of stage-specific embryonic antigen-4 (SSEA-4), known as an important cell surface marker of embryogenesis. The immunostaining of mouse preimplantation embryos without fixation showed that the binding of 6E2 caused the clustering and consequent accumulation of sialylGb5 at the interface between blastomeres. These results suggest that SSEA-4 actively moves on the cell surface and readily accumulates between blastomeres after binding of 6E2.

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Embryonal carcinoma (EC) cells isolated from teratocarcinomas have been shown to possess pluri- or multipotency in both mouse and human systems [1–3]. In mice, certain EC cells as well as embryonic stem (ES) cells have been considered to be developmentally equivalent to the inner cell mass of blastocysts [1]. These EC cells are useful for clarifying the molecular characteristics of early embryonic cells and thus many efforts have been made to establish EC cell lines and monoclonal antibodies (Mabs) that

detect differentiation-related molecules on EC cells. As a consequence, a number of stage-specific markers for embryogenesis have been identified. Notably, it is important that this molecular information is adapted to research on ES cells or mouse preimplantation embryos. Stage-specific embryonic antigen (SSEA) -1, -3, and -4, as well as tumor rejection antigen (TRA) -1-60 and -1-81 [4], have been used as stage-specific markers for embryogenesis, though their functional significance in early development remains unclear. Interestingly, however, most of these antigens are carbohydrates themselves or closely related to the carbohydrates carried on glycosphingolipids (GSLs) and glycoproteins [5].

6E2 is a Mab established by immunizing with NCR-G3 cells, a previously established multipotent human EC cell

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line capable of differentiating into trophoblastic cell lineages other than somatic cells [3]. It has been revealed that 6E2 reacts with not only human ECs, including NCR-G2 and 3 cells, but also other germ cell tumors, as well as normal human germ cells such as spermatogonia and oocytes [6]. Although a previous study reported that 6E2 immunoprecipitates a cell surface protein having a molecular weight of approximately 80 kDa from 125 I-labeled NCR-G3 cells, the specific antigen recognized by 6E2 still remains unknown. To characterize the antigen specificity of 6E2, we examined the reactivity of the Mab with other cell lines using several distinct methods. In this paper, we present evidence that 6E2 recognizes SSEA-4 carried by sialylGb5. Using 6E2, we determined the localization of SSEA-4 in "living" mouse preimplantation embryos and observed its preferential localization in interface between blastomeres.

Materials and methods

Cells, antibodies, and animals. The human renal carcinoma cell line ACHN was purchased from American Type Culture Collection. The African green monkey kidney cell line Vero was a gift from Dr. T. Takeda of Department of Infectious Diseases Research, National Children's Medical Research Center, Tokyo, Japan. Cells were maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM) (Sigma Chem., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS). The human EC cell line NCR-G2 [3] was cultured in a 1:1 mixture of DMEM and Ham's F12 medium (DMEM/F12) (Invitrogen Gibco, Carlsbad, CA) supplemented with 10% FBS/JRH Bioscience, non-essential amino acid solution (NEAA) (Invitrogen Gibco), and Insulin-Transferin-Sodium Selenite media (Invitrogen Gibco). The cynomolgus monkey ES cell line CMK-6 [7] were provided by Dr. Yasushi Kondo of Mitsubishi Tanabe Pharma Corporation. ES cells were grown on mouse embryonic fibroblast feeder cells that were inactivated by gamma-irradiation in DMEM/F12 supplemented with 20% Knockout™ Serum Replacement, 2 mM Glutamax-I, 1% NEAA, 50 units/ml penicillin, 50 µg/ml streptomycin, 0.1 mM 2-mercaptoethanol, 1% sodium pyruvate, and 5 ng/ml bFGF (all from Invitrogen GIBCO). The cultures were performed at 37°C in a 5% CO₂ incubator. The human venous blood from a healthy consenting volunteer was drawn in a heparin-coated syringe. The blood was spun at 3000 rpm for 15 min and human red blood cells (hRBCs) were washed three times in phosphate buffered saline (PBS).

The conjugation of affinity-purified 6E2 (mouse IgG₃, κ) [6] to the fluorescence reagent was performed with an Alexa Fluor® 488 monoclonal antibody labeling kit (Molecular Probes, Eugene, OR.) according to the manufacturer's instructions. The anti-SSEA-4 Mabs used in this study were Raft.2 [8] and MC813-70 (R&D Systems, Inc Minneapolis, MN). Alexa Fluor® 488 goat anti-mouse IgG and Streptavidin Alexa Fluor® 568 were purchased from Molecular probes.

BDF₁ mice were purchased from Clea Japan (Tokyo, Japan).

TLC immunostaining of GSLs. TLC immunostaining of GSLs from cultured cells and hRBCs was performed as previously described [9]. Reference GSLs were purchased from Matlayer, Inc. (Pleasant Gap, PA). SialylGb5 was purified from ACHN cells by preparative TLC. Purified GM1 b was kindly provided by Dr. Nakamura of RIKEN, Saitama, Japan [10].

Flow cytometry. Cells were harvested and incubated with a primary antibody (1 µg/ml) for 1 h on ice, followed by treatment with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a dilution of 1:50 and analyzed with an EPICS-XL flow cytometer (Beckman Coulter, Inc, Miami, FL).

Dot blot analysis. Purified sialylGb5 was serially diluted (0.1–60 ng) and vacuum blotted onto a PVDF membrane by using a 96-well format

dot blot apparatus (Bio-Rad Laboratories, Richmond, CA). The membrane was immunostained with the Mab 6E2 or MC813-70 (0.5 µg/ml) according to a previously described procedure [9]. The antibodies that bound to the membranes were visualized with ECL-plus Western Blotting Detection Reagents (GE Healthcare UK Ltd, Buckinghamshire, UK) and scanned with a LAS-1000 luminescent imaging analyzer (Fujifilm, Tokyo, Japan). Scanned images were analyzed using the software Image Gauge with which the LAS-1000 was equipped.

Indirect immunostaining of cynomolgus monkey ES cells. Cells were grown on a glass-bottomed dish (IWAKI) for 3 days and then these cells were fixed for 30 min with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-200 in PBS for 20 min. Subsequently, the cells were washed three times with PBS for 5 min and blocked with 5% normal goat serum in PBS for 30 min. The fixed cells were incubated with anti-SSEA-4 antibodies or isotype-matched mouse IgG at a dilution of 1:300 for 2 h, followed by incubation with Alexa Fluor® 488-conjugated goat anti-mouse IgG at a dilution of 1:300 for 30 min. DAPI was used for counter staining of nuclei.

Immunostaining of mouse preimplantation embryos. Mouse preimplantation embryos were collected from superovulated mice. Seven-week-old BDF₁ female mice were induced to superovulate with intraperitoneal injections of pregnant mare's serum gonadotropin (ASKA Pharmaceutical co., Ltd., Tokyo, Japan) (5 IU) and human chorionic gonadotropin (hCG) (ASKA Pharmaceutical co) (5 IU) 48 h apart and mated with individual BDF₁ male mice after the hCG injection. The 2-cell, the 8-cell, and the morula stage embryos were flushed out from oviducts at 36, 60, and 72 h after the hCG injection, respectively. Animals were treated according to the institutional animal care and use guidelines of National Research Institute for Child Health and Development.

Embryos immediately after being collected and those prefixed with 2% paraformaldehyde in Heps buffered saline were incubated in 30 µl drops of M16 medium containing 0.45 µg of Alexa Fluor® 488-conjugated 6E2 for 1 h or biotinylated MC813-70 for 1 h, treated with streptavidin Alexa Fluor® 568 diluted 1:300, and then they were washed three times in 30 µl drops of M16 medium. All staining steps were carried out at 37°C in a CO₂ incubator for fresh embryos and at 4°C for fixed embryos. The stained embryos were placed in drop of a M16 medium on glass-bottomed dishes (IWAKI, Tokyo, Japan), and were observed with a LSM510 Zeiss Confocal laser-scanning microscope (Carl Zeiss, Thornwood, NY) to obtain a field of view of the embryo only with a 40x objective lens.

Results and discussion

6E2 specifically binds to sialylGb5

In order to examine whether the 80 kDa membrane protein is recognized by 6E2, we performed a Western analysis of the cell lysates or their immunoprecipitates with 6E2. Since no significant signal was detected on the blot (data not shown), we examined TLC immunostaining of GSLs extracted from several 6E2-positive cell lines. ACHN cells showed the expression of comparable amounts of Gb3, Gb4, Gb5, and sialylGb5, whereas Vero cells and NCR-G2 cells expressed predominantly Gb3 (Fig. 1A). TLC immunostaining analysis revealed that 6E2 binds to a major slow-migrating GSL extracted from these three cell lines. The slow-migrating GSL was identified as sialylGb5, defined by the Mab Raft.2. We observed that 6E2 bound to sialylGb5 (LKE-antigen) of hRBCs [13] (Fig. 1B). Finally, we examined the reactivity of 6E2 with purified GSLs and found that the Mab reacts with purified sialylGb5, but not purified GM1 b (Fig. 1C). These results indicate that 6E2 specifically binds to sialylGb5 and thus is an anti-SSEA-4