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今月の主題　白血球

話題

慢性肉芽腫症の遺伝子治療への取り組み

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慢性肉芽腫症の遺伝子治療への取り組み

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〔KEYWORDS〕慢性肉芽腫症(CGD)，造血幹細胞移植(HSCT)，遺伝子治療

1. 慢性肉芽腫症(chronic granulomatous disease；CGD)の病因

好中球は，体内に微生物が侵入した際に，局所に遊走し，活性酸素を産生し殺菌を行う．CGDは，好中球の活性酸素産生能が欠損している先天性免疫不全症である．活性酸素産生に主要な役割を果たす酵素NADPHオキシダーゼは，細胞膜上のgp91^{phox}，p22^{phox}のヘテロ二量体と細胞質内に存在するp67^{phox}，p47^{phox}，p40^{phox}，Racp21から構成されるが，CGDではそのうちgp91^{phox}，p22^{phox}，p67^{phox}，p47^{phox}のいずれかを欠損する．患者は，乳児期から重症な細菌および真菌感染症に反復罹患し，諸臓器に肉芽腫を形成するのが特徴である．遺伝形式は，gp91^{phox}欠損型は，X連鎖性遺伝(このためX-CGDという)，他の3病型は常染色体劣性遺伝である．X-CGDは，X連鎖性遺伝のため患者のほとんどは男性で，母親が保因者であることが多い．保因者の末梢血好中球は，正常な細胞と異常な細胞とが混在するモザイクを呈し，その割合は，Lyon効果の程度によって決まる．保因者であっても，正常な好中球の割合が5%あれば，感染症に罹患しても重症にはなりにくい．

2. CGDに対する治療—遺伝子治療以外の治療

CGDの治療は，まず，感染症治療および予防を目的として抗生物質，抗真菌剤の投与が行われる．また，2つめの治療として，重症感染症の予防に約3分の1の症例に有効なインターフェロンγ(IFN-γ)が投与される．国内でも約4割の患者が投与を受けている．抗生剤の予防投与やIFN-γ投与によって，重症感染症の発症頻度は減少するが，いったん感染症に罹患すると，重症化・遷延化し，難治な場合が多く，平均寿命は，25～30歳である．死因は，敗血症やアスペルギルス感染症が多い．

CGDは，造血幹細胞に由来する骨髄系細胞の欠陥であり，その欠陥の根源は造血幹細胞にある．造血幹細胞移植(hematopoietic stem cell transplantation；HSCT)は，欠陥造血幹細胞を正常ドナーの造血幹細胞に置換し，治癒をめざす治療法である．1968年に重症複合型免疫不全症(severe combined immunodeficiency；SCID)に対する骨髄移植が成功して以来，CGDを含む先天性免疫不全症に対する根治療法としてHSCTが行われ，多くの知見が得られている．CGDの骨髄移植の成績は，1990年半ばまで，生存率が6割程度と，他の免疫不全症の成績と比較しても決してよくなかった．しかし，ここ10年は移植前の感染症治療や骨髄非破壊的前処置を用いた移植(reduced intensity stem cell transplantation；RIST)の進歩，移植片対宿主病(graft-versus-host disease；GVHD)に対する治療など移植後の管理技術の進歩によって，生存率が8割以上になった．

CGDの移植は，ドナーが，主要組織適合抗原(human leukocyte antigen；HLA)6/6座一致の

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表1　これまで行われたCGDに対する遺伝子治療臨床研究のまとめ

開始年	国	対象病型	症例数	ベクター	好中球機能の再構築(%)	持続期間または観察期間
1995	米　国	p47-CGD	5	MFGS-p47	0.004～0.051	6か月
1999	米　国	X-CGD	3	MFGS-gp91	0.055	1年
2003	ドイツ	X-CGD	2	SF71gp91	10～57	症例1：3年後死亡*注
	スイス	X-CGD	1	SF71gp91	未発表	未発表
	英　国	X-CGD	3	SF71gp91	未発表	未発表
2006	米　国	X-CGD	1	MFGS-gp91	24(2週間後) 1.12(6か月後)	6か月 (2007年5月末)
2007	韓　国	X-CGD	1	MT-gp91	10(2週間後) 1.0(3か月後)	3か月 (2007年5月末)

*注：死因は遺伝子治療によるものではないとされている．

場合は，同胞に限らず骨髄バンクの非血縁ドナーでも成績がよい．また，日本では，5/6座一致の移植の成功例もある．欧米では，ほぼ単一民族である日本と事情が違い，ドナーの条件がHLA 10/10座または9/10座一致であるため，ドナーの獲得が日本より困難である．また，HSCTは，アロ(同種)移植であるため，ある程度の前処置は必須である．CGDの場合，難治性感染症のため，前処置に耐えられない患者もいるし，感染病変の残存があると移植後急速に悪化する患者もいる．したがって，HSCTは，唯一確立された根治療法だが，すべての患者が適応ではない．

HSCTの適応にならない患者に対する新しい治療戦略として，欠損遺伝子を患者細胞に導入し，機能回復をめざす遺伝子治療の研究が精力的に行われてきた．

CGDは，①原因遺伝子が明らかな単一遺伝病である，②造血幹細胞移植で完治可能，③遺伝子導入の標的細胞(造血幹細胞)の採取技術が確立されている，④標的細胞に遺伝子を発現させ，維持可能であることなど，遺伝子治療の前提条件を満たしており，研究の初期の段階から，遺伝子治療の候補疾患の1つに挙げられてきた．また，保因者が正常の5%程度の活性酸素産生能しか持たなくても，感染症を起こさず健康に生活していることから，機能回復の目標値が比較的低いこともあり，遺伝子治療の効果が大いに期待される疾患である．

3. 遺伝子治療の方法

遺伝子治療法には，生体外遺伝子治療法と生体内遺伝子治療法とがあり，どちらを適用するかは，対象疾患の病変と遺伝子導入に用いるベクター(細胞に遺伝子を導入する際の運び屋)の特性により決められる．遺伝子導入法には，ウイルスが元来持つ細胞侵入機構を利用したウイルスベクター法と，化学的物理的な操作で細胞に遺伝子を取り込ませる非ウイルスベクター法とがある．これまでの遺伝子治療臨床研究では，ウイルスベクター(レトロウイルスベクター，アデノウイルスベクター，アデノ随伴ウイルスベクター，レンチウイルスベクター)の使用が主流である．またCGDの遺伝子治療臨床研究では，すべてレトロウイルスベクターが使用されている．

4. CGDに対する遺伝子治療(表1参照)

最初のCGDの遺伝子治療臨床研究は，米国国立衛生研究所(National Institutes of Health；NIH)のMalech博士らによって，5名の$p47^{phox}$欠損型CGD患者を対象に1995年に開始された．彼らは，遺伝子導入標的細胞として患者の末梢血CD34陽性細胞を使用した．標的細胞を閉鎖式ガス透過性プラスチックバック内で無血清下［ウシ胎仔血清(fetal bovine serum；FBS)は用いず，ヒトアルブミンを用いた］にて前培養したのち，MFGS-$p47^{phox}$レトロウイルスベクターを用いて，遺伝子導入を行った(**図1**)．細胞増殖因子(サイトカイン)として，前培養と遺伝子導入中にPIXY321［インターロイキン3：IL-3と顆粒球マクロファージコロニー刺激因子(granulocyte macrophage colony-stimulating factor；GM-CSF)の合成蛋白質］と顆粒球コロニー刺激因子(granulocyte colony-stimulating factor；G-CSF)を使用した．2日間の遺伝子導入後，遺伝子導入

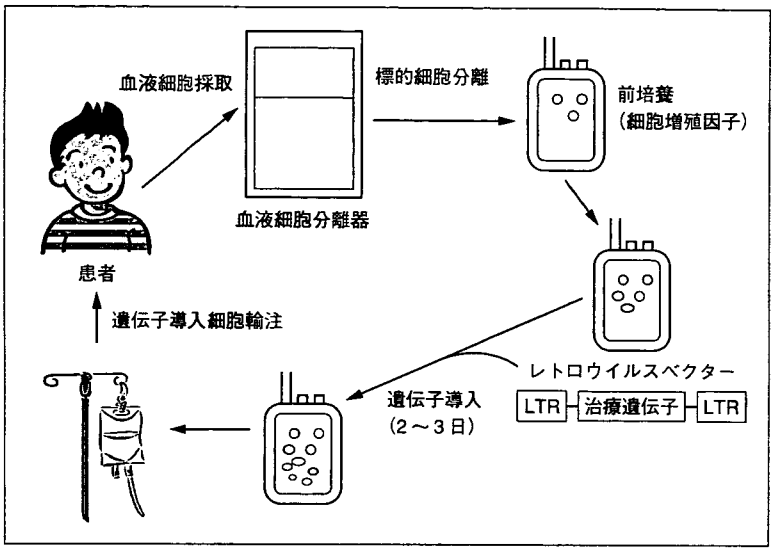

図1　造血幹細胞を用いた生体外遺伝子治療の概要

細胞を患者に投与した．患者に投与した残りの遺伝子導入細胞を引き続きサイトカインを添加した培地で培養し，17日目にDHR123法で活性酸素産生能を測定したところ，正常HSTを同じ条件下で培養して得られた値の21～90%を示した．メチルセルロースコロニーアッセイで得られたコロニーのNBTテストの結果は，9～28%のコロニーがNBT陽性であった．また，遺伝子導入細胞のゲノムDNAの組み込まれたウイルスベクターのコピー数は，1細胞あたり0.05～0.19コピーであった．遺伝子導入効率は高かったが，投与後の患者の体内での遺伝子導入細胞の動態をみてみると，治療後24～34日に末梢血中に導入遺伝子を持った細胞が検出され，40～147日まで確認できた．しかし，活性酸素産生能をもった好中球の比率は，最高で0.004～0.051%と低かった．臨床的に感染症を治療するレベルまでは達しなかったが，治療後に肺炎に罹患した症例で，気道分泌物中に活性酸素産生好中球が検出され，感染部位への遊走は確認できたと報告されている[1]．

1999年に，Malech博士らは，MFGS-gp91^{phox}を用い，X-CGDの遺伝子治療を試みた．前回の遺伝子治療から改良した点は，①ウイルスベクターと標的細胞の接着効率を上げるためファイブロネクチンを使用したこと，②遺伝子導入効率改善のためウイルスベクター産生細胞を変更したこと，③培養時添加するサイトカインにFlt3リガンドを加えたことである．この改良が功を奏して，遺伝子導入効率は60%以上を超え，末梢血中に約1年間にわたり遺伝子導入細胞を検出することができた．しかし，治療後の患者末梢血の活性酸素産生好中球の割合は，最高で0.055%と，目標の5%には届かなかった[2]．遺伝子導入効率は高くても，機能未修復の好中球の分化・増殖能が正常なため，遺伝子導入細胞由来の好中球の増殖優位性がないことが原因と考えられた．これを克服する方策として，遺伝子治療は基本的には自己HSCTだが，①移植された遺伝子導入細胞が増殖できる空間を作る目的で軽い前処置を行うこと，②生着率を上げるため，末梢血HSCではなく骨髄血HSCを使用することが考えられる．

造血幹細胞を標的とした他の免疫不全症［アデノシンデアミナーゼ(ADA)欠損症とX-SCID］に対する遺伝子治療研究から得られた知見でも，骨髄HSCを用いた遺伝子治療研究が有効であることが示されている[3]．また，X-SCIDの場合，遺伝子導入細胞自体が生存選択性と増殖優位性を持っているため，前処置は不要だが，CGDと同様増殖優位性を持たないADA欠損症では，前処置による骨髄抑制を行って良好な結果を得ている[4,5]．

2003年から，ドイツ，スイス，イギリスにおいて，前処置を加えたX-CGDに対する遺伝子治療が開始された．この研究では，標的細胞は末梢

血 HSC が，レトロウイルスベクターは，SF71gp91 が使用された．サイトカインは，IL-3，SCF，Flt3 リガンド，トロンボポエチンが用いられた．骨髄抑制前処置として，ドイツ，スイスでは抗がん剤のブスルファンを 4 mg/kg×2 日，イギリスでは，メルファラン 140 mg/m^{2}が投与された．ドイツの 2 症例について論文報告されており[6]，前処置による骨髄抑制で好中球数が 500 個/μl 以下となった期間は，症例 1 で 10 日間，症例 2 で 6 日間であった．末梢血白血球における遺伝子導入細胞の比率を定量 PCR 法で評価したところ，症例 1 は，day21～day157 に 13～21%，症例 2 は，day21～day149 に 12～31% であったが，その後，症例 1 は，day180～day381 に 40～46% と増加し，day542 に 27% に減少した．症例 2 では，day413 に 53% まで増加し，day491 に 30% であった．さらに，末梢血白血球を CD15 陽性細胞と CD3 陽性細胞に分けて，同じ解析を行うと，導入された遺伝子は CD15 陽性細胞に特異的に導入されていることが判明した．

レトロウイルスの性質として，標的細胞の DNA に導入遺伝子がランダムに組み込まれる(インテグレーションという)が，フランスの X-SCID の遺伝子治療では，T 細胞の転座遺伝子 11p13 の下流に位置する LMO-2 癌原性遺伝子の翻訳開始領域近傍にインテグレーションが起こり転写が亢進したため，T 細胞が単クローン性に増殖し，白血病になった症例がある[7]．ドイツの CGD の症例でも，インテグレーションの部位が詳細に検討され，MDS1-EVI1，PRDM16，SETBP1 遺伝子の近傍へのインテグレーションが確認されたが[6]，現在までのところ，造血系細胞の単クローン性の増殖は見られず，白血病は発症していない．

末梢血好中球における gp91^{phox} の発現と活性酸素産生能は，症例 1，2 ともに導入遺伝子の定量 PCR の結果と平行して動いており，導入遺伝子発現の増減とともに gp91^{phox} の発現も機能も増減し，推移している．症例 1 は，ブドウ球菌による肝膿瘍，症例 2 は，肺のアスペルギルス症が改善しており，臨床上の利益は十分あったと考えられる．しかし，治療後 2 年を過ぎた頃から，PCR で遺伝子の存在は確認されても，活性酸素の産生能が次第に低下しており，導入遺伝子のサイレンシングが起きている可能性が懸念されている．症例 1 は，治療後約 3 年で歯科治療中に起こした感染症のため死亡したが，遺伝子治療が原因ではないとされている．論文報告はされていないが，スイスの症例は，対麻痺を伴った脊椎炎が改善し，歩行可能になった(私信)．

2006 年末から 2007 年にかけて，米国 Malech 博士らと韓国の Kim 博士らのグループも，ブスルファンによる骨髄非破壊的前処置を行う遺伝子治療臨床研究を開始している．米国の研究では，1998 年と同じレトロウイルスベクターを用い，ブスルファン 10 mg/kg を使用した．治療 2 週間後には，24% の末梢血好中球が活性酸素を産生していたが，治療後 6 か月後(2007 年 5 月末現在)で 1.12% まで減少した．臨床的には，ブドウ球菌による肝膿瘍は縮小している(学会報告)．韓国の研究では，新たに開発した MT レトロウイルスベクターを用い，前処置にブスルファン 4 mg/kg を使用した．活性酸素を産生する好中球は，2 週間後に 10%，3 か月後(2007 年 5 月末現在)には 1% まで減少している(私信)．

5. 日本での CGD 遺伝子治療への取り組み

日本でも，当施設において X-CGD に対する造血幹細胞を標的とした遺伝子治療臨床研究の実施計画が進行中である．これまでの CGD に対する遺伝子治療と違う点として，遺伝子導入標的細胞に患者自己骨髄細胞を使用し，細胞増殖因子に，SCF，TPO，Flt3-L，IL-6，IL-6 レセプターを使用する予定である．候補となる患者は，当施設で診療している患者に限らず，全国の施設から登録をしていただき，専門家の合意のもと，遺伝子治療の適否の検討を行ったうえ，選定をする予定である．現在，実施体制を整備しているところである．

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お知らせ

平成19年度第1回肺がん検診従事者講習会（東京都健康診査従事者講習会）

実施日：2007年10月23日(火)　14：30～16：30
会　場：東京都健康プラザ「ハイジア」3階研修室
対　象：都内の施設で，肺がん検診に従事している医師，放射線技師，臨床検査技師等
受講定員：先着50名程度
受講料：無料
申込方法：必要事項を御記入のうえ，FAX・電子メール・郵送にてお申込みください．必要事項(講習会名・氏名・職種・勤務先の名称，所在地，電話番号，メールアドレス)
申込締切：2007年10月16日(火)　必着(受講できない場合のみ，御連絡いたします．)
テーマ及び講師：科学的根拠に基づく肺がん検診のすすめ
地方独立行政法人大阪府立病院機構大阪府立成人病センター調査部疫学課・課長　中山富雄　先生
〈講習概要〉
肺がん検診は，他のがん検診に比べて，その有効性に疑問が投げかけられてきました．こうした背景のもと，中山先生をはじめとする，厚労省がん研究助成金「がん検診の適切な方法とその評価法の確立に関する研究」班(主任研究者　祖父江友孝氏)によって昨年作成された「有効性評価に基づく肺がん検診ガイドライン」をレビューするとともに，肺がん検診に対する批判の問題点および今後の展開をご紹介いただきます．
お申込み・お問合せ先：
財団法人東京都保健医療公社東京都多摩がん検診センター研修担当　丸山
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※東京都健康診査従事者講習会の「お知らせ」及び「申込書」は，次のホームページからダウンロードできます．
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ELSEVIER

Experimental Hematology

Experimental Hematology 35 (2007) 1398–1407

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.**

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-7Rα 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-7Rα Ab were obtained from R&D Systems (Abingdon, UK) and used in the cultures at concentrations of 1 to 5 μg/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 μM) was obtained from Calbiochem-Novabiochem Co. (San Diego, CA, USA) and used in the cultures at a concentration of 5 μM. 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 32Dcl1-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% CO_2 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

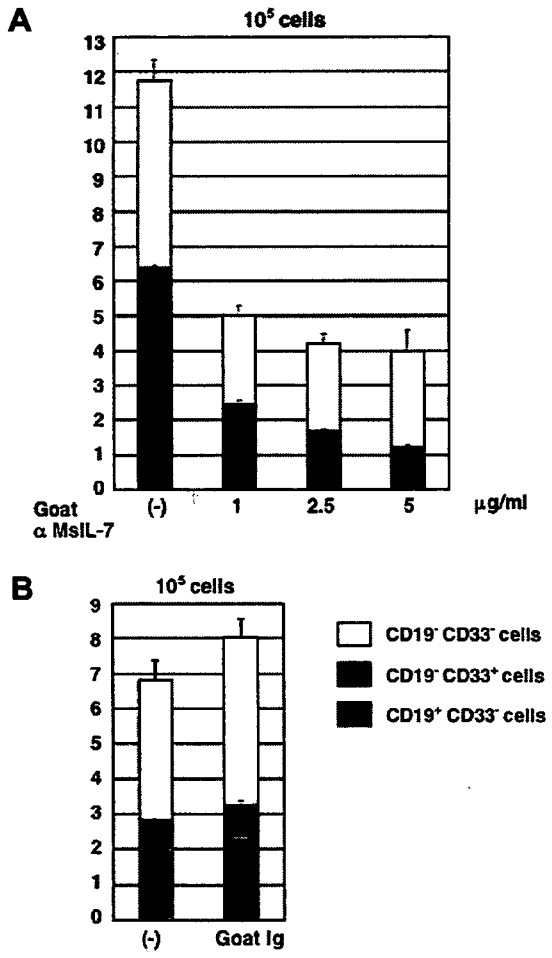

Figure 1. Effect of anti-mouse interleukin (IL)-7 antibody on human pro–B-cell development. (**A**) Human bone marrow $CD34^+$ cells were cultured on MS-5 cells for 4 weeks in the presence or absence {(–)} of different concentrations of goat polyclonal anti-mouse IL-7 antibody. The subsequent $CD19^+CD33^-$ cell number (lower light gray column), $CD19^-CD33^+$ cell number (middle dark gray column), and $CD33^-CD19^-$ 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′-AGCAGTCAGCTGCATTTCTGTG-3′	392
Human IL-7R α		
Forward	5′-GTCACTCCAGAAAGCTTTGG-3′	
Reverse	5′- AGGAACTCTAGACTTCCCTTT-3′	
Human CD19		
Forward	5′-GTTCCGGTGGAATGTTTCGG-3′	386
Reverse	5′-AGATGAAGAATGCCCACAAGG-3′	576
Human TdT		
Forward	5′-ACACGAATGCAGAAAGCAGGA-3′	
Reverse	5′-AGGCAACCTGAGCTTTTCAAA-3′	315
Human PAX5		
Forward	5′-CCATCAAGTCCTGAAAAATC-3′	
Reverse	5′-CCCAAAGTGGTGGAAAAAAT-3′	319
Human Igα		
Forward	5′-TAGTCGACATGCCTGGGGGTCCAGGAGTCCTC-3′	
Reverse	5′-GATGTCCAGCTGGAGAAGCCGTGA-3′	681
Human GAPDH		598
Forward	5′-CCACCCATGGCAAATTCCATGGCA-3′	
Reverse	5′-TCTAGACGGCAGGTCAGGTCCACC-3′	
Murine actin		
Forward	5′-TGACGGGGTCACCCACACTGTGCCCATCTA-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-7Rα 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-7Rα mRNA by the cultured human BM $CD34^+$ cells was observed (Fig. 2B).

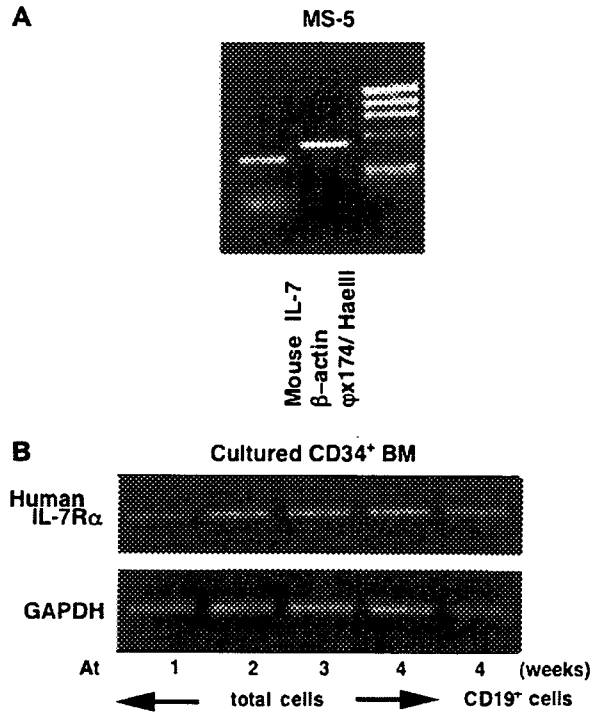

Figure 2. Expression of interleukin (IL)-7 by murine stromal MS-5 cells and of IL-7 receptor by cultured human bone marrow $CD34^+$ cells. (**A**) Expression of IL-7 by MS-5 cells was investigated by reverse transcription polymerase chain reaction (RT-PCR). Expression of mouse β-actin was also investigated as an internal control. The $\phi\chi174$/*Hae*III 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.

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-7Rα, 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 Igα 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

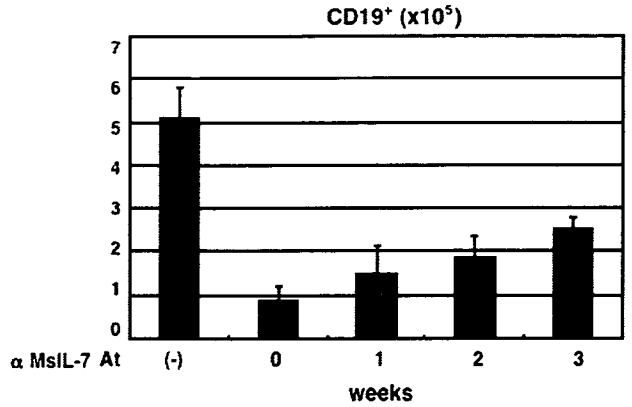

Figure 3. Time-dependency of anti-mouse interleukin (IL)-7 antibody–mediated inhibition of pro–B-cell development. Human bone marrow $CD34^+$ cells were cultured on MS-5 cells for 4 weeks. Goat polyclonal anti-mouse IL-7 antibody (2.5 μg/mL) was added at the start of culture (0), and after 1, 2, and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

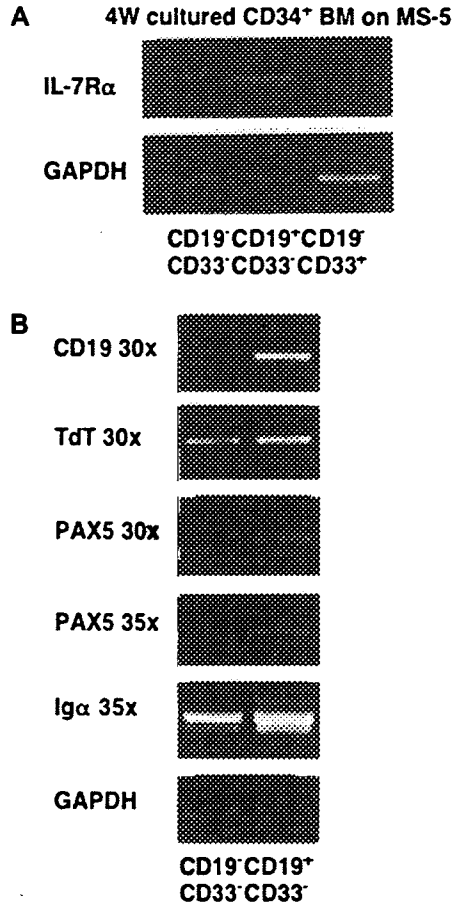

Figure 4. Expression of B-cell differentiation marker mRNAs by cultured human bone marrow $CD34^+$ cells. (**A**) Human bone marrow $CD34^+$ cells cultured on MS-5 cells for 4 weeks, $CD33^-CD19^-$, $CD33^+CD19^-$, and $CD33^-CD19^+$ cells were sorted, and expression of IL-7Rα was investigated by reverse 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-7Rα Ab inhibits pro–B-cell development. As shown in Figure 8, addition of human IL-7Rα 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

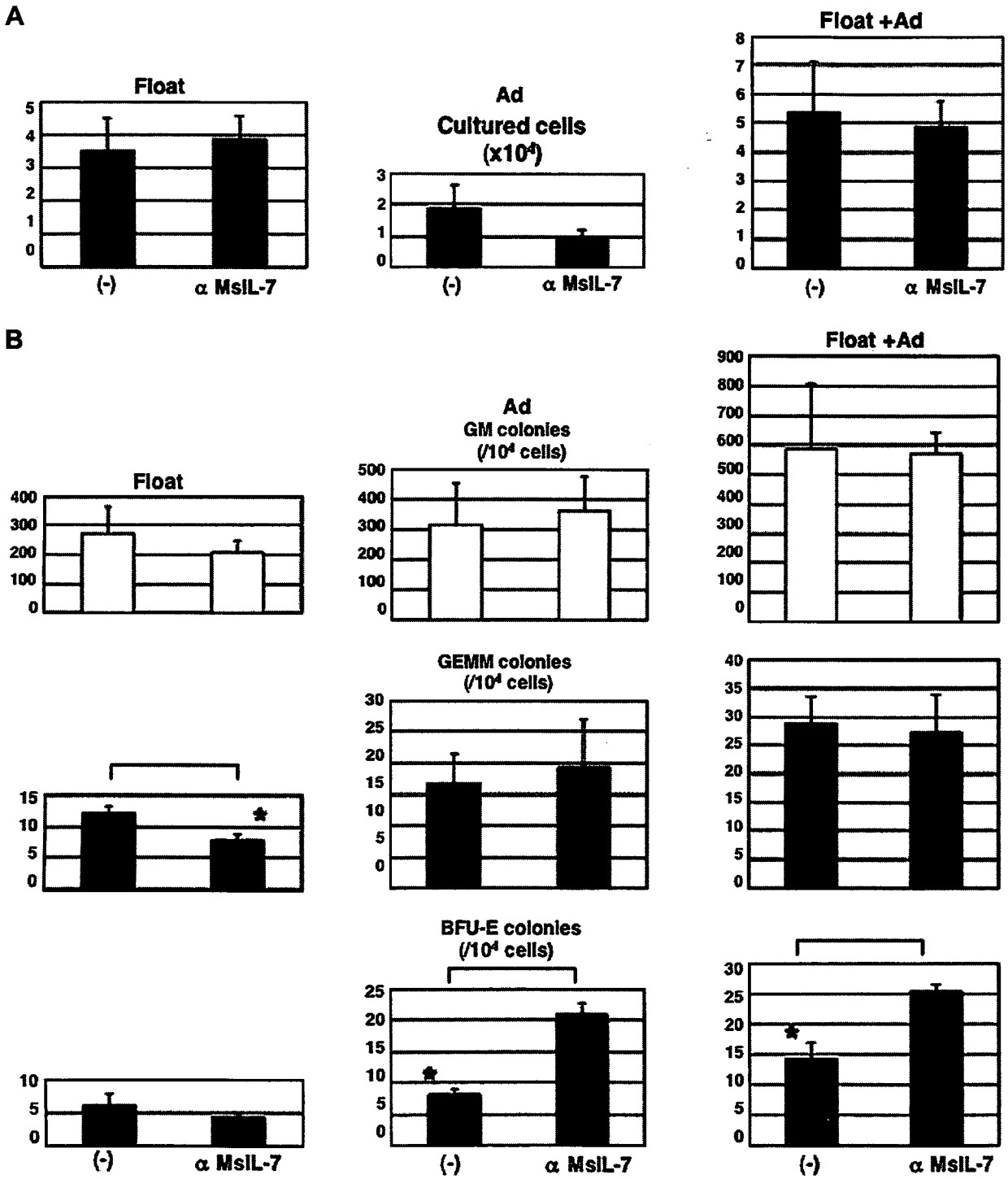

Figure 5. Effect of anti-mouse interleukin (IL)-7 antibody on colony formation ability of human bone marrow $CD34^+$ cells. Human bone marrow $CD34^+$ cells were cultured on MS-5 cells for 1 week in the presence or absence {(–)} of goat polyclonal anti-mouse IL-7 antibody (2.5 μg/mL), and colony assay 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 10^4 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 development. 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

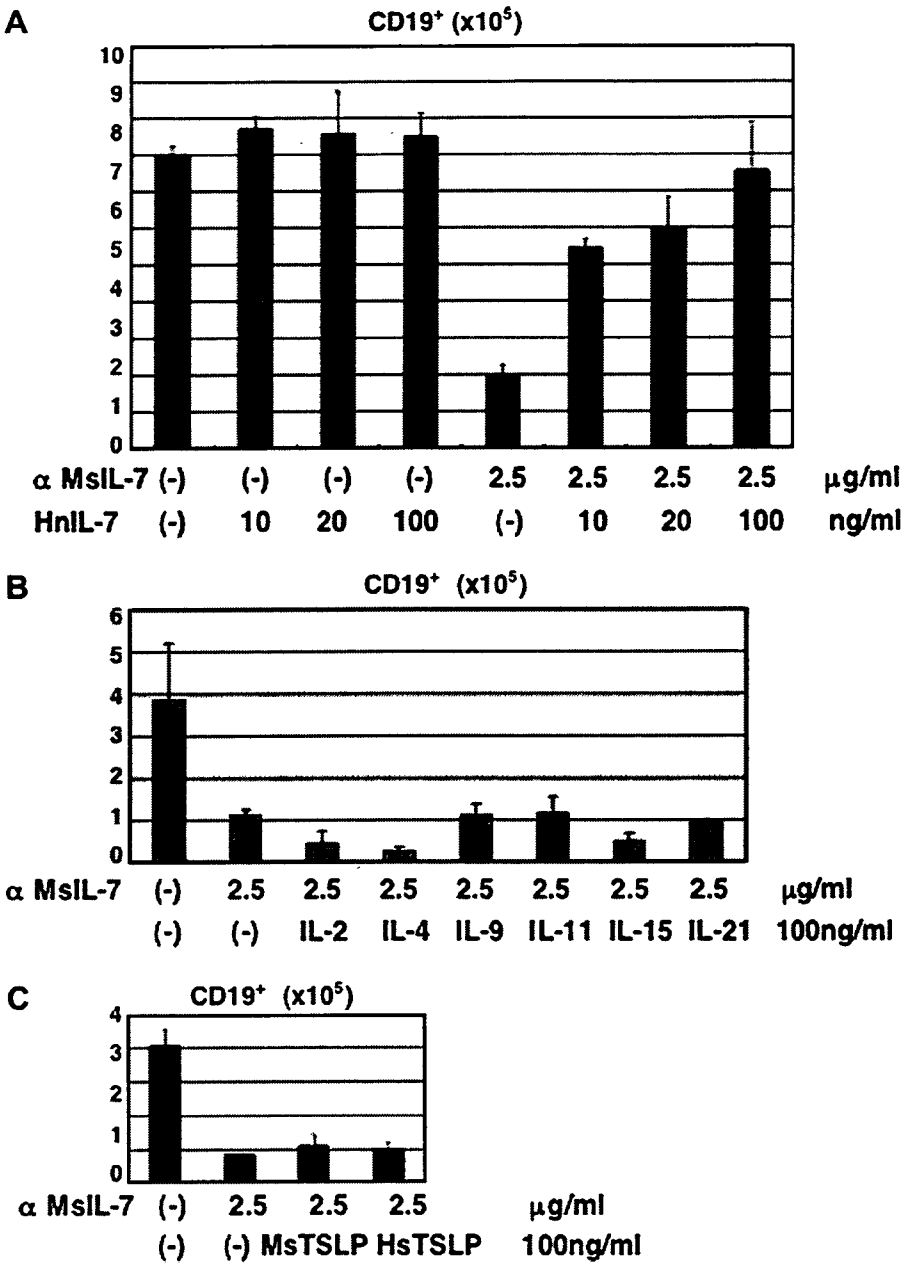

Figure 6. Effect of recombinant human interleukin (IL)-7 on anti-mouse IL-7 antibody–mediated inhibition of human pro–B-cell development. (**A**) Human bone marrow $CD34^+$ cells were cultured on MS-5 cells for 4 weeks with or without the indicated combinations of goat anti-mouse IL-7 antibody (2.5 μg/mL) 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 because the reason of that JAK3 mediates

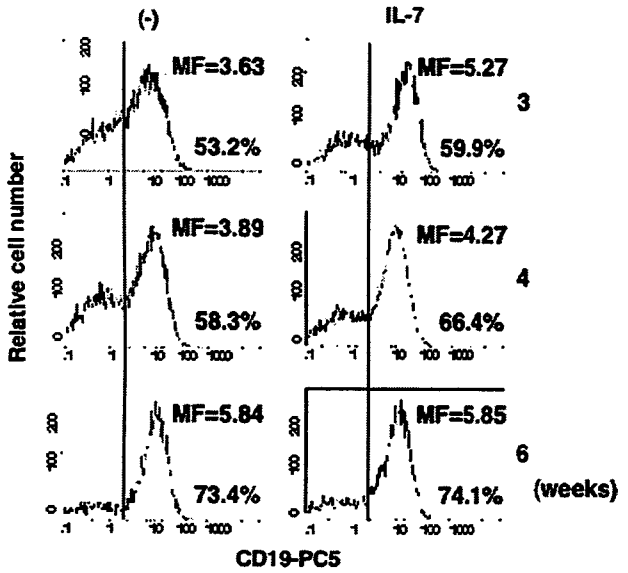

Figure 7. Effect of recombinant human interleukin (IL)-7 on human CD19 expression in pro-B cells. $CD34^+$ cells were cultured on MS-5 cells for 3, 4, and 6 weeks with or without 100 ng/mL recombinant human IL-7, and expression of CD19 was investigated by flow cytometry. The value of mean fluorescence 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.

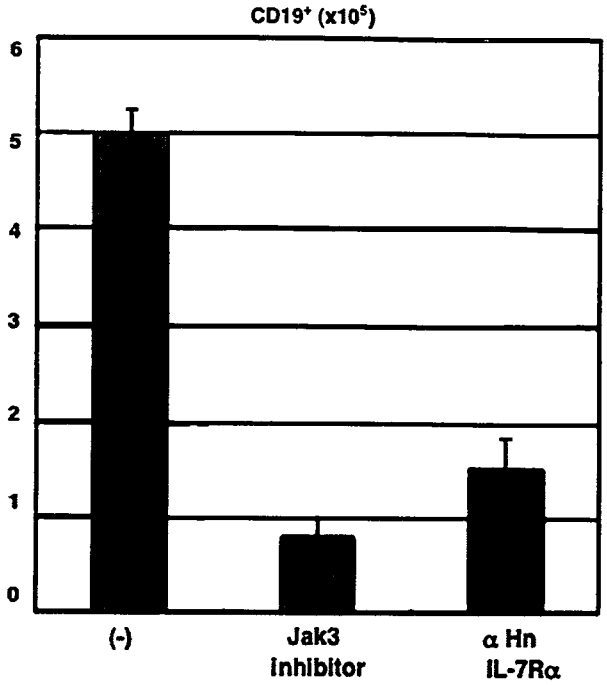

Figure 8. Effect of anti-human interleukin (IL)-7 receptor antibody and JAK3 kinase inhibitor on human pro–B-cell development. Human bone marrow $CD34^+$ cells were cultured on MS-5 cells for 4 weeks with or without goat polyclonal anti-human IL-7Rα antibody (2.5 μg/mL) or JAK3 kinase inhibitor (5 μM).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

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 Rα 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 Igα, 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 $IL\text{-}7R\alpha^+Ig\alpha^+CD19^-$ 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.

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 $Lin\text{-}Sca1^+c\text{-}kit^+CD34^+Flt3^+$ 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-7Rα 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.

Acknowledgments

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Preferential localization of SSEA-4 in interfaces between blastomeres of mouse preimplantaion 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.

Keywords: SialylGb5; SSEA-4; Embryonic stem cells; Embryonal carcinoma cells; Preimplantation embryo; Immunostaining

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% CO_2 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 IgG_3, 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.

BDF_1 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 Hepes 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 CO_2 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 40× 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