免疫学的分類・診断

国立成育医療センター研究所発生・分化研究部　清河信敬

I　はじめに

フローサイトメトリー(FCM)を用いた免疫学的検査は血液腫瘍の診断・分類に重要であり，特に急性リンパ性白血病(ALL)では初期治療の層別化に必須である．本稿では，小児の白血病マーカー検査に用いられるおもな抗原の特性や免疫学的分類・診断の基本的な考え方について概説し，現状における問題点や今後の課題について触れる．

II　小児がんに対するFCM検査

A　検査の対象

FCMは浮遊細胞を様々な抗体で蛍光染色し，個々について反応性を解析する検査である[1]．白血病患児の骨髄液や末梢血液がおもな対象であるが，固形腫瘍でも胸水，腹水，髄液等のほか，リンパ節や腫瘍組織もほぐして浮遊細胞を調整できれば検査可能である．

B　検査法の進歩

1970年代の免疫学的診断法はごく限られており，ALLについて①細胞膜表面免疫グロブリン(Ig)陽性のB-ALL，②羊赤血球とのE-ロゼット形成能陽性のT-ALL，③上記以外のnon-T，non-B ALL，という分類が行われていた．その後，単クローン性抗体作製法とFCMが開発されて普及し，免疫学的診断法が急速に進歩した．現在では細胞質内抗原染色を含めた3 color以上の多重染色法が一般化し，より詳細なマーカー解析が可能となった．

C　CD分類

CDは単クローン性抗体を認識抗原によって整理する統一分類であり，1980年代初頭に多数の抗体が一気に流通した際，名称に統一性がなかったため生じた混乱を解消する目的で開始された．1982年以来，数年ごとに開かれる国際ワークショップで順次追加され，当初の45番までから現在では339番まで増え，細胞表面抗原(以下，特に区別する場合はs-で表示)に加えて細胞質内抗原(以下cy-で表示)や白血球以外の抗原も含まれている．白血病診断に重要なIgやT細胞抗原受容体(TCR)，主要組織適合性抗原(MHC)などの可変性(多型性)のある分子や，TdT，MPOなどの酵素は含まれない．

III　白血病の免疫学的検査の実際

A　検査の意義・重要性

血液腫瘍の分類は，「腫瘍化しても正常発生母体の性格が保たれている」ことを前提に確立されてきた．体系的分類としては2001年に提唱された新WHO分類[2]が標準的であるが，ALLについては必ずしも十分ではない．免疫学的分類としてはEGIL分類[3]が代表的であるが，ほかにも様々な分類

がある.

　免疫学的検査が特に重要なのはALLの分類である．ALLは正常リンパ球の分化系統に準じて①precursor B，②B，③precursor T，④NKなどに分類されるが(図1)，免疫学的タイプの違いにより治療法が大きく異なる．臨床的には細胞形態を中心としたFAB分類が普及しているが，形態的特徴に乏しいALLでは治療層別化に必要な情報は得られず，免疫学的診断が必須である．

　急性骨髄性白血病(AML)の場合は，基本的にFAB分類のみで正確に診断可能であるが，一部の亜群ではALLとの鑑別が困難で，免疫学的診断が必須あるいは有用である．

B ALL診断の実際

　FCM検査に先立ち光顕的ペルオキシダーゼ(POX)陰性，非特異的エステラーゼ陰性であることを確認し，FAB分類にしたがったAMLとALLの鑑別が前提となる．小児ALLの約80%はprecursor B-ALLで最も頻度が高い．これに対してB-ALLが数%，precursor T-ALLが15%程度である．実際の鑑別は，①B細胞系か，T細胞系か；②B細胞系であればprecursor Bか，成熟型のB-ALLか，③上記いずれにもあてはまらない場合にはNK-ALLか，未分化ALLか，あるいはAMLか，といった手順がわかりやすい．

1 Precursor B-ALL

　Precursor B-ALLは，B細胞系であるがIg発現以前の前駆細胞由来の腫瘍である．B細胞系を示す基本的なマーカー(cy-)CD79a，CD22，CD19，HLA-DR陽性に加え，cy-TdTが陽性，s-Ig陰性である．

　CD79a(Igα)はIgとB細胞抗原受容体(BCR)複合体を形成しその刺激伝達に関与するB細胞の本質的マーカーでIg(陰性)の前駆細胞でも発現している．CD22，CD19はBCR刺激伝達の補助分子である．TdTは遺伝子再構成に必須の酵素で，TおよびBリンパ球前駆細胞のマーカーであり，B-ALLとの鑑別に有用である．

　Precursor B-ALLも，マーカー発現からみると多様である．約9割はCD10陽性のcommon-ALLとよばれてきた亜群で，CD24陽性で形態的にはFAB L1が多い．幹細胞マーカーとして知られるCD34や，成熟B細胞で発現するCD20が陽性の場合もある．

　Common-ALLのマーカーに加えて細胞質内にμ鎖を発現する亜群があり特にpre-B-ALLとよばれる．まれに細胞表面にもμ鎖を検出する例があるが，この場合は軽鎖(κあるいはλ鎖)ではなく代替軽鎖(CD179a/b)と複合体を形成していることからB-ALLと鑑別される．

　Precursor B-ALLの約1割はCD10陰性(大部分CD24も陰性)を示し，形態的にはFAB L2が多い．MLL遺伝子再構成を認める症例(MLL＋ALL)に多いので，1歳未満のいわゆる乳児白血病の大部分がこのタイプである．頻度は低いが1歳以上のMLL＋ALL症例も存在する．
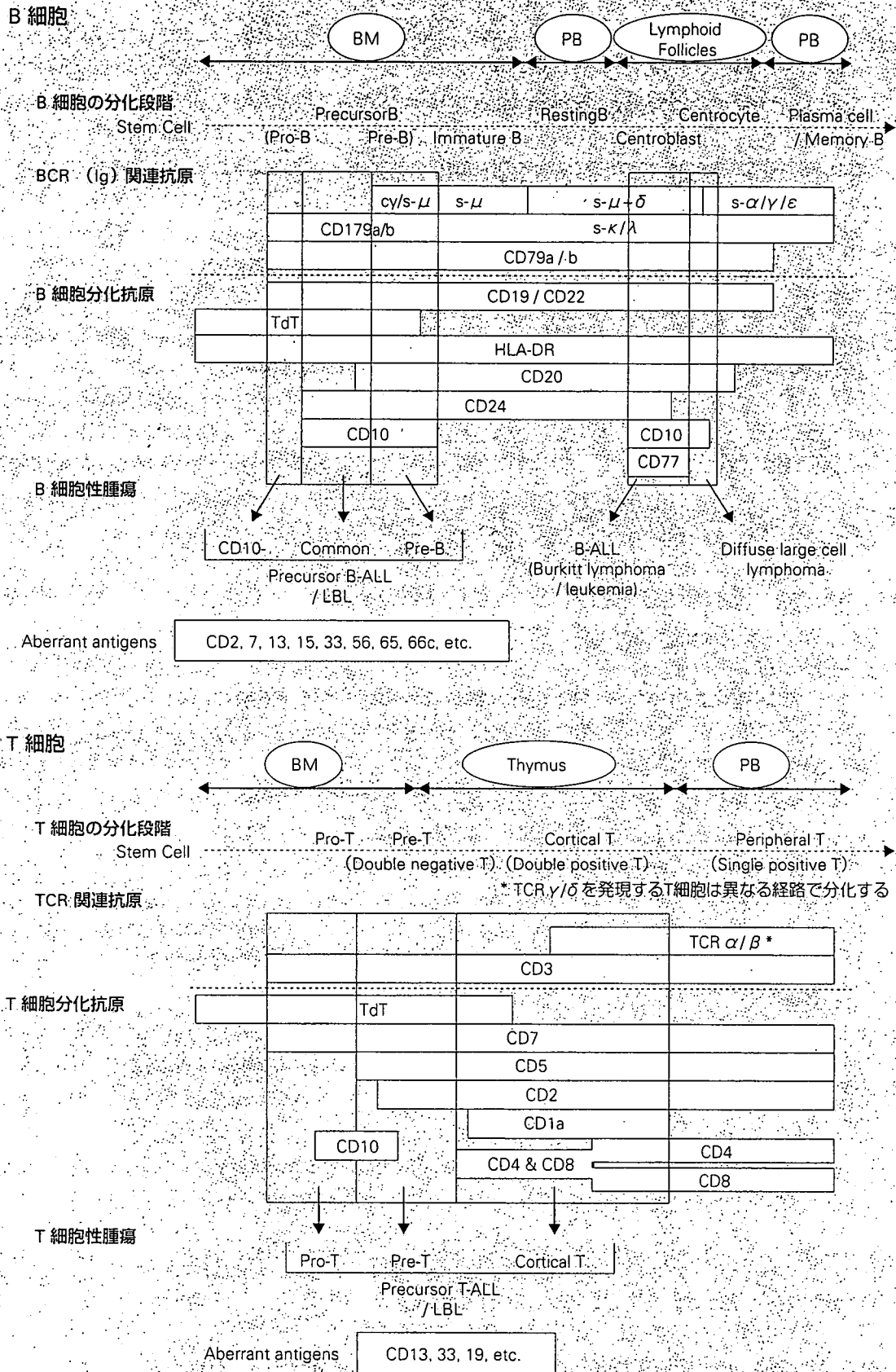
2 B-ALL

　B-ALLは成熟B細胞に由来し，小児ではほとんどがBurkitt leukemiaで，形態的にFAB L3を示す．s-Ig(ほとんどがIgM＝μ＋κ or λ鎖)陽性である点でprecursor B-ALLと鑑別される．cy-TdTは陰性で，CD20が強陽性である．CD10も陽性の場合が多い．

3 Precursor T-ALL

　Precursor T-ALLは，骨髄でT細胞への分化を運命づけられた段階から胸腺で成熟途上のT前駆細胞を発生母体とし，マーカー上多様である．T細胞の最も本質的なマーカーは特異的抗原認識を担うTCR複合体だが，未分化な段階では発現していない．CD3はTCRと複合体を形成し刺激伝達を担う分子で，未熟T細胞でも細胞質内に発現し最も信頼性が高いマーカーである．cy-CD3陽性の場合，通常CD7も陽性であるが，CD7自体はT細胞系以外の未分化な細胞にも発現する．cy-CD3とCD7のみ陽性の亜群はpro-T-ALLとよばれ，最も未分化な段階のT細胞由来と考えられる．この二者に加えてCD2やCD5を発現する亜群はpre-T-ALLとよばれる．骨髄原発のprecursor T-ALLは以上の2つの亜群のいずれかである場合が多く，CD34，CD10等の発現を伴う場合がある．胸腺皮質T細胞の特徴であるCD1aやCD4とCD8の双方を同時に発現する亜群はcorticalT-ALLとよばれ，α/β-TCRの発現も認

図 1 リンパ球の分化と小児のリンパ性腫瘍

B 細胞

BM　　　PB　　Lymphoid Follicles　　PB

B 細胞の分化段階

| Stem Cell | PrecursorB (Pro-B)　Pre-B)　Immature B | RestingB | Centrocyte Centroblast | Plasma cell / Memory B |

BCR （Ig）関連抗原

	cy/s-μ	s-μ	s-μ + δ		s-α/γ/ε
CD179a/b		s-κ/λ			
	CD79a / b				

B 細胞分化抗原

| CD19 / CD22 |
| TdT | HLA-DR |
| CD20 |
| CD24 |
| CD10 | CD10 |
| | CD77 |

B 細胞性腫瘍

| CD10- Common Pre-B | B-ALL (Burkitt lymphoma / leukemia) | Diffuse large cell lymphoma |
| Precursor B-ALL / LBL | | |

Aberrant antigens　　CD2, 7, 13, 15, 33, 56, 65, 66c, etc.

T 細胞

BM　　Thymus　　PB

T 細胞の分化段階

| Stem Cell | Pro-T　Pre-T (Double negative T) | Cortical T (Double positive T) | Peripheral T (Single positive T) |

* TCR γ/δ を発現するT細胞は異なる経路で分化する

TCR 関連抗原

| | | TCR α/β * |
| CD3 | | |

T 細胞分化抗原

| TdT |
| CD7 |
| CD5 |
| CD2 |
| CD1a |
CD10		
	CD4 & CD8	CD4
		CD8

T 細胞性腫瘍

| Pro-T Pre-T Cortical T |
| Precursor T-ALL / LBL |

Aberrant antigens　　CD13, 33, 19, etc.

リンパ球の分化段階やリンパ性腫瘍の分類，呼称については様々な意見があり必ずしも統一されていない．

める場合があり，胸腺原発のALL/リンパ腫に多い．以上のほかにγ/δ-TCRの発現を認める亜群も存在する．

4┃ その他のALL

TあるいはB細胞系以外では，CD56陽性のNK-ALL[4]や，CD7，CD34，CD38，HLA-DRなどのみ陽性で系統特異的抗原を発現しない未分化ALLが存在するが，頻度は低い．

Ⓖ AMLの免疫学的診断

AMLは光顕的POX陽性であることで診断される．免疫学的マーカーではcy-MPO，CD13，CD14，CD15，CD33，CD64，CD65，CD117などの骨髄単球系抗原が様々な様式で発現するが，FAB M1〜M5の亜群との明確な相関は認めない．リンパ球抗原は原則的に陰性であるが，一部(特にM1，M2)でB細胞抗原であるCD19，CD79a陽性である．CD7，CD4も時に陽性となるが，この場合はそれぞれ未分化な血球，単球の性格を示すと考えられる．また単球系などではHLA-DRも陽性となる．M6(赤白血病)の場合はCD235a(グリコホリンA)の発現が特徴的である．

FAB分類に1985年に追加されたM7(巨核芽球性)は形態的にALLと鑑別困難な場合があり，巨核球/血小板に特異的なCD41 and/or CD42の発現の免疫学的な確認が必須である．特異性では劣るがCD36やCD61も診断に有用である．

1991年に追加されたM0は光顕的POX陰性のAMLであり，リンパ球抗原(CD79a or CD22，CD3)陰性かつ免疫学的なMPO陽性，あるいはCD13 and/or CD33陽性であることが診断基準である．

Ⅳ 白血病の免疫学的検査の注意点

Ⓐ Gating

白血病細胞，特にprecursor B-ALLでは白血球共通抗原であるCD45が弱陽性〜陰性の場合が多く，この特性を利用して白血病細胞を選択的に解析するCD45-blast gatingが行われる[1]．白血病細胞のマーカー発現をより正確に評価することが可能であり，特に芽球の割合が低い検体の場合に非常に有用である．しかし，この特性はB前駆細胞の性格を反映したものであり，非白血病検体でも白血病の場合と同様の結果になる場合があるので注意が必要である．

Ⓑ Aberrant antigen

白血病細胞では時に細胞系統の特異性に反する抗原発現を認める(aberrant antigen)．極端な場合，複数の細胞系統の本質的な性格を同時に示すこともあり，mixed lineage(biphenotypic or biclonal) leukemiaとよばれるが，診断基準については様々である[3]．Precursor B-ALLでは2割程度の症例でCD13，CD15，CD33，CD65，CD66cなどの骨髄単球抗原やCD2，CD7等のT細胞抗原のいずれかの発現を認める．特に1歳以上のMLL＋ALL症例では複数のaberrant antigenの発現を認める場合がある．特定の遺伝子異常を認める場合を除き，aberrant antigenと予後との関係は明らかではない．

Ⓒ 診断上の問題点

診断に用いられる抗原のなかで，絶対的系統特異性を示すものはB細胞系のIgやCD22，T細胞系のTCRやCD3，骨髄球系のMPOなどに限られ，他の抗原は単独では系統特異的とはいいきれない．マーカー診断では複数の抗原の発現のパターン認識が必要であり，「陰性所見」も時に重要である．

まれではあるが症例のなかには診断基準から逸脱するものが必ず存在する．その場合も抗原の発現パターンが重要であり，他の検査所見も参考に総合的な判断が必要である．

腫瘍細胞特異的抗原というものは存在しない．白血病のマーカー診断は形態学的な診断が確定していることが前提に成り立つ検査である．特に芽球の割合が低い場合には結果の判定に十分な注意が必要である．例外的に，正常では絶対に存在しえないマーカー発現，すなわちaberrant antigenを有する

症例や，末梢血におけるCD10陽性細胞やCorticalT形質を示す細胞の存在などは，マーカー所見のみでも腫瘍細胞の存在を強く示唆する．

同じCD番号で分類される抗体でも用いる抗体のクローン（認識する抗原決定基）の違いにより反応性が大きく異なる場合がある．また標識蛍光色素の種類によって抗体の反応性が阻害される場合もあるので，注意が必要である．

V 今後の課題

血液腫瘍の免疫学的診断は初期治療の層別化に重要であるばかりでなく，近年は微小残存白血病（MRD）検出にも応用されている[5]．検査法に関してはすでに確立された感もあるが，MLL＋ALLに対する7.1抗原や，precursor B-ALLに対するCD179a/bなど新たなマーカーの有用性の検討が必要である．また，aberrant antigenの発現などのマーカーの多様性と治療予後との関係についても検討が必要である．

現在小児血液腫瘍の治療研究は国内で統一される方向にある．これに際し，免疫学的診断法についても検査方法や診断基準について統一されることが望ましく，実際に標準化をめざした活動が行われている．今後FCMを用いた検査法がさらに進歩し，小児血液腫瘍の診療に一層貢献することが期待される．

文献

1) 日本臨床検査標準協議会血液検査標準化検討委員会フローサイトメトリーワーキンググループ：フローサイトメトリーによる造血器腫瘍細胞表面抗原検査に関するガイドライン（JCCLS H2-P V1.0）．日本臨床検査標準協議会会誌 2003；18：69-107

2) Brunning RD, Borowitz M, Matutes E, *et al.* : Precursor B-cell and T-cell neoplasms. In : Jaffe ES, Harris NL, Stein H, *et al.*(eds), *World Health Organization Classification of Tumorsse──Pathology and genetics of tumours of haematopoietic and lymphoid tissues.* IARC press, Lyon, 2001；109-117

3) Bene MC, Castoldi G, Knapp W, *et al.* : Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias(EGIL). *Leukemia* 1995；9：1783-1786

4) Oshimi K : Leukemia and lymphoma of natural killer lineage cells. *Int J Hematol* 2003；78：18-23

5) Coustan-Smith E, Sancho J, Hancock ML, *et al.* : Clinical importance of minimal residual disease in childhood acute lymphoblastic leukemia. *Blood* 2000；96：2691-2696

15 合併症と支持療法
Ⅳ）臓器合併症対策

慶應義塾大学医学部小児科学教室　嶋　晴子
国立成育医療センター特殊診療部小児腫瘍科　森　鉄也

Ⅰ　はじめに

　小児がんの治療成績は向上し，最も頻度の高い小児がんである急性リンパ性白血病では80%以上の長期生存率が期待される．一方，治療早期死亡例が2%程度存在することが知られている．小児がん診療において遭遇し得る合併症を理解し，適切な支持療法を行うことは，安全で効率的な医療の提供のみならず，治療成績の向上にも寄与すると考えられる．本稿では小児がん診療における一般的な臓器合併症の病態，症状・所見，および対応について，「腫瘍そのものの作用による臓器合併症」，「治療毒性による臓器合併症」に大別し概説する．感染症，oncologic emergencyに関連する臓器合併症については該当項を参照されたい．

Ⅱ　腫瘍そのものの作用による臓器合併症とその対策

Ａ　神経合併症

1｜ 中枢神経浸潤

1）病態

　クモ膜の血管壁や脈絡叢に直接浸潤，神経根に沿ってクモ膜下腔まで到達し脳脊髄液に浸潤すると考えられている．急性白血病，横紋筋肉腫，悪性リンパ腫などに合併する．

2）症状・所見

　早期には症状を呈さないことが多い．頭痛，嘔吐，項部硬直，けいれんなど頭蓋内圧上昇に関連した臨床症状を示す．髄液細胞数増加，CT・MRIで髄膜の肥厚・造影効果を認める．診断には髄液細胞診など病理学的手法が用いられる．

3）対応

　頭蓋照射，抗腫瘍剤の髄腔内投与（髄注）．

2｜ 脊髄圧迫

1）病態

　血行性転移，頭蓋内腫瘍の浸潤，傍脊椎腫瘍の増大などにより脊髄が圧迫される．脊髄血管障害による脊髄浮腫，腫瘍の椎体浸潤，治療に関連した骨粗鬆症による脊椎破壊も原因となり得る．小児では胸髄レベルでの脊髄圧迫が多い．

2）症状

　圧迫所見のある部位レベルの疼痛が初発症状であることが多い．運動麻痺で発症する例もある．深部反射亢進，感覚神経麻痺は約半数でみられる．自律神経障害は遅れて出現するが，馬尾圧迫がある場合は神経因性膀胱が初発症状となる．造影MRIが診断に有効である．

3）対応

　非可逆性神経障害を避けるために早期診断・治療が必要である．感受性が期待される場合には化学療法，放射線照射を開始する．ステロイド大量療法が脊髄浮腫軽減に有効とされる．組織診断が必要

な場合，頸髄圧迫による呼吸障害のリスクがある場合，放射線照射や化学療法に早期効果が期待できない場合，神経症状が急速に進行する場合には，椎弓切除による減圧術の適応である．

Ｂ 縦隔に関連する合併症

1 上大静脈症候群

1）病態

上大静脈が部分的または完全に閉塞し静脈還流量が減少した状態．腫瘍による上大静脈の外部からの圧迫，血管内腫瘍の増殖，血栓により生じる．上大静脈の血管壁は繊細で内圧も低いため圧迫・閉塞されやすい．非Hodgkinリンパ腫，T前駆細胞性急性リンパ性白血病，胚細胞腫，神経芽腫などに合併する．中心静脈カテーテル長期留置により生じることもある．

2）症状・所見

顔面・眼瞼・上肢浮腫，頸胸部静脈怒張などの症状を認める．頭蓋内圧上昇に伴って頭痛，嗜眠，けいれんを生じ得る．胸水や心囊液の貯留を伴うこともある．

3）対応

重症の場合は診断を待たずに早期に抗腫瘍治療を開始する．リンパ系腫瘍に対してはステロイド剤の投与が有効である．200〜400 cGyの緊急放射線照射も有効である．頭部挙上（セミFowler位）により呼吸状態の改善をはかる．過剰輸液に注意する．静脈血栓が原因の場合は抗凝固剤の投与を行う．

2 気道狭窄

1）病態

縦隔腫瘍によって気管および気管支が圧迫された状態．縦隔腫瘍のある小児の約50%にみられ，そのうち10%では重度の気道狭窄症状を伴う．気道閉塞は鎮静，気管内挿管・抜管，腰椎穿刺などでの体位変換がリスクとなる．

2）症状・所見

乾性咳嗽，努力性呼吸，胸痛，胸部不快感，チアノーゼ，喘鳴，呼吸音減弱を呈する．仰臥位がとれない場合，急激に呼吸困難に陥る可能性がある．胸部X線では気管偏位，無気肺，胸水を認めることがある．

3）対応

鎮静による気道閉塞を回避するため，可能な限り局所麻酔で侵襲の少ない検査で診断を行い早期に治療を開始する．鎮静を要する場合には気道閉塞の危険性を十分に説明し同意を得て，気道確保，呼吸管理の準備を麻酔科医などに依頼して対応する．

Ｃ leukostasis syndrome

1）病態

著しい血球数増加による過粘稠症候群と類似した病態．末梢血白血球数100,000/mm³以上でleukostasis syndromeのリスクが高まる．血球数増加による血液粘稠度亢進に加え，血球の大きさ，変形能，容積指数（leukocrit）が関連すると推測されている．急性骨髄性白血病では，白血病細胞の容積が大きく，変形能が低く，臓器浸潤性が高いためにleukostasis syndromeのリスクが高いと考えられている．詳細な発症機序は不明であるが，白血病細胞の増殖により微小血管の血流が減少し末梢循環不全を起こすとともに，集簇した白血病細胞により微小血管が閉塞し組織の虚血および出血をもたらすと考えられている．

2）症状

出血，眼障害（乳頭浮腫，網膜剥離，網膜静脈血栓症），神経障害，心血管障害（うっ血性心不全，狭心症，不整脈），呼吸障害を呈する．

3）対応

末梢血白血球数200,000/mm³以上の症例では白血球フェレーシス（白血球数が20〜60%減少すると

いわれている）・交換輸血が勧める向きもあるが，短期的・長期的な有効性が証明されているわけではない．ヘモグロビン値は8 g/dl以下に，血小板数2万以上に維持する．中枢神経症状を認める場合，頭蓋照射が行われていたが効果には議論があり最近は積極的には行なわれていない．

Ⓓ 高カルシウム血症

1）病態

血清カルシウム値>11.5 mg/dlと定義され，local osteolytic hypercalcemia(LOH)とhumoral hypercalcemia of malignancy(HHM)に分類される．LOHでは腫瘍の骨浸潤により骨吸収が促進される．HHMは骨浸潤を伴わず，腫瘍細胞がカルシウム制御ホルモン関連レセプターに作用する物質(PTHrP: parathyroid hormone-related protein, Prostaglandin E, IL-1, TNFなど)を分泌することにより，破骨細胞の活性化に伴う骨吸収，腎尿細管でのカルシウム再吸収，小腸でのカルシウム再吸収が促進される．成人の腫瘍でみられることが多く小児ではまれである．

2）症状・所見

易疲労感，食欲不振，多飲，多尿，嘔吐，腹痛，腰痛，便秘，脱水など非特異的症状を呈する．重度の場合，けいれん，昏睡などの神経症状をもたらす．急激な血清カルシウムの上昇では不整脈による突然死も免れない．血液腫瘍ではPTHrPが関連することが最も多く，原発性副甲状腺機能亢進症と異なりPTH，尿中cAMPは正常であり，PTHrPの上昇を認める．

3）対応

Henle上行脚からの尿中カルシウム排泄を促すためフロセミドを併用しながら生理食塩水による脱水の補正，近位尿細管からのカルシウムの再吸収を阻害する．症状を伴う場合，上記に加え，カルシトニンを投与する．通常，投与数時間後に血清カルシウムの低下を認めるが効果は一時的である．ビスホスホネートは効果発現までに時間がかかるが，カルシトニンよりも有効性は高い．

Ⅱ　治療による臓器合併症

Ⓐ 神経合併症

1〕 白質脳症

1）病態

大脳白質，大脳の神経線維が障害されるために，情報伝達が行われず意識障害，精神症状，運動障害を中心とした様々な神経障害を呈する病態．メトトレキサート静注，髄注，頭蓋放射線照射が原因となる．Sytarabine静注で合併することもある．

2）症状

初発症状として頭痛，けいれん，悪心，嘔吐がみられることが多い．ほかに性格変化，言語障害，健忘，視力障害，片麻痺，意識障害，痴呆などの症状を認める．MRI検査で大脳白質にT2延長像を認める．早期診断には拡散強調画像(DWI)が有用である．

3）対応

メトトレキサート髄注に伴う白質脳症は一過性のことが多く，臨床症状および画像上の改善がみられれば治療を再開する．

2〕 末梢神経障害

1）病態

Vincristinの神経毒性としてみられることが多い．

2）症状・所見

手指のしびれ，下顎のしびれ，眼瞼下垂で気づかれることが多い．自律神経障害の症状として腹部膨満，頑固な便秘もみられる．深部腱反射は減弱または消失する．

3）対応

一過性であることが多い．高ビリルビン血症を伴うときはビンクリスチンの排泄が遷延し，神経症状が増悪するため減量または投与を延期する．

B 呼吸器合併症

1 薬剤性肺炎

1）病態

抗がん剤，抗生剤，消炎鎮痛剤，顆粒球コロニー刺激因子（G-CSF）など複数の薬剤の副作用としてみられる．頻度は0.1％未満である．レチノイン酸（all-trans retinoic acid：ATRA）やG-CSF関連性では急激な白血球増加によるスーパーオキサイドやエラスターゼが過剰に産生され，肺障害や肺血管の透過性亢進と関連すると考えられている．

2）症状・所見

間質性肺炎，肺浸潤，肺うっ血などが現われることがある．薬剤によるリンパ球刺激試験（DLST）陽性率は67％程度といわれており，投与薬剤との関連が不明である場合も多い．

3）対応

発症時は速やかに疑われる薬剤の投与を中止し，中等症以上の症例にはプレドニゾロン1 mg/kgを，無効例や急速進行重症例にはステロイドパルス療法を行う．

2 放射線肺炎

1）病態，症状・所見

胸部放射線照射後2〜3か月に発症し，急性期肺炎とそれに続く慢性肺線維症を呈する．

2）対応

急性期の中等症，散発性にはプレドニゾロン0.5〜1 mg/kgを，重症例にはパルス療法を行う．

C 循環器合併症

1 不整脈

アントラサイクリン系抗がん剤，cyclophosphamide，ifosfamideなどのアルキル化剤，cytarabine，フルオロウラシルなどが不整脈誘発性抗がん剤として報告されている．通常，一過性で致命的となることはまれである．造血幹細胞移植時に細胞凍結保存液であるDMSO（dimethyl sulfoxide）が原因と考えられる不整脈が出現することがある．冷凍保存した自家骨髄例の9.7％に2度以上の房室ブロックを認めたという報告もあり，心電図モニター管理は必須である．

2 心筋障害

1）アントラサイクリン系抗がん剤

アントラサイクリン系抗がん剤は総投与量依存性に心筋障害をもたらす．化学療法継続中，あるいはアントラサイクリン系抗がん剤の最終投与から1年以内に発症する心筋症を早発型（early form of cardiomyopathy），それ以降に発症するものを遅発型（late form of cardiomyopathy）と分類する．早発型心筋症の頻度はアントラサイクリン系抗がん剤の総投与量がdoxorubicinで450 mg/m²，daunorubicinで700 mg/m²を超えると増加する．心筋症発症の危険因子としてこの他に，doxorubicinの急速な投与，縦隔への放射線照射，心疾患の既往，5歳未満，女児が報告されている．遅発型心筋症は小児においてより高頻度である．doxorubicinの総投与が300mg/m²以下での発症も報告されている．アントラサイクリン系抗がん剤による治療歴のある患者が，ウイルス感染などを契機に急速な心機能低下を生じることがある．最近の晩期障害などに関する研究では，小児期に安全量と考えられたアントラサイクリン系抗がん剤治療歴がある例において，症状に現れない（subclinical）心機能障害は高頻度であることが報告されている．長期経過観察が必要である．

2）アルキル化剤大量療法

Cyclophosphamide100〜200 mg/kg，ifosfamide10〜18 g/m²の大量投与により心筋症，不整脈が生じ

ることが報告されている.

3）放射線照射

縦隔への放射線照射はアントラサイクリン系抗がん剤による心筋障害を増加させるという報告がある.

D 肝機能障害

1 血清肝逸脱酵素(transaminase)値，ビリルビン値の上昇

血清肝逸脱酵素値，ビリルビン値の上昇は化学療法中にしばしばみられる副作用である．その多くは化学療法の中止や肝庇護剤投与により改善する．輸血歴がある場合，肝炎ウイルス感染の鑑別が必要である.

2 肝中心静脈閉塞症(veno-occlusive disease : VOD)

1）病態

肝の微小静脈の血栓性閉塞とその周囲の肝細胞の壊死をきたす病態で，重症例では致命的な合併症である．薬剤(actinomycin D，dacarbazine，busulfan など)，放射線照射などにより肝中心静脈，小葉下静脈の血管内皮障害，血管透過性の亢進，凝固因子の活性化，内皮下層の浮腫などを生じ静脈閉塞をきたす．薬剤による直接作用，および中心静脈閉塞による類洞血の循環障害による肝細胞障害を伴う．造血細胞移植後VODは移植後20日以内に発症することが多いが，通常の化学療法のみでもみられる．GVHD，感染症・薬剤による肝障害との鑑別が問題となる例もある.

2）症状・所見

急激な肝腫大による右季肋部痛，および腹水などの水分貯留による体重増加で発症する．黄疸は肝腫大にやや遅れて出現する．生検による病理組織診断が確定診断であるが出血傾向などにより困難な場合が多い．シアトルグループによる診断基準では，「総ビリルビン値>2 mg/dL 以上の黄疸」，「肝腫大と右季肋部痛」，「原因不明の2%以上の体重増加あるいは腹水」の3項目のうち2項目を認める場合VODと診断する．PAI-1の増加，C蛋白，AT-III など様々な凝固・線溶因子の変化が報告されている．超音波Doppler検査で門脈血流の逆流，減少を認めるが特異性は低い.

3）治療

有効性が確立された治療は存在しない．軽症例は体液貯留・血管内脱水に対する支持療法のみで軽快することがあるが，重症例では致死率が高い．recombinant human tissue plasminogen(rh-tPA)の有効性が示唆されているが，副作用として重篤な出血が示され，進行例に対する効果は否定的とする報告も存在する．日本で未発売のdefibrotideは抗血栓作用，抗虚血作用，血栓融解作用を有すが，全身的な抗凝固作用が乏しく，VODに対する有効性が報告されている.

4）予防

ウルソデオキシコール酸，低分子ヘパリン，プロスタグランジンE$_1$による予防効果を示す報告が存在する.

E L-アスパラギナーゼによる急性膵炎

1）病態

L-アスパラギナーゼによる蛋白合成抑制作用が膵炎発症に関連すると考えられている．L-アスパラギナーゼに伴う膵炎発症の頻度は約2%とされるが，1回投与量，投与回数，投与開始または投与中止から膵炎発症までの期間に一定の傾向は報告されていない.

2）症状・所見

L-アスパラギナーゼ投与後に腹痛を認めた場合は，膵炎を念頭に対応しなければならない．背部に放散する上腹部痛，嘔吐を認める．小児では不機嫌など非特異的症状で発症する場合や，ショックなどで急激に発症することが多い．血清膵アミラーゼ，リパーゼの上昇を伴うが，初期には目立たないことも多い．CT，腹部エコーによる膵腫大，腹水の貯留などが診断に有用である.

3）対応

初期対応として循環の維持，禁食・除圧などによる消化管の安静をはかる．蛋白分解酵素阻害薬の

早期投与が有効であるとの報告がある．

🅵 耐糖能異常

L-アスパラギナーゼ，ステロイド剤の副作用としてみられることが多い．治療継続のためインスリン投与を要することがある．

🅶 腫瘍崩壊症候群

1）病態

腫瘍細胞が急激に崩壊することにより血中に放出された尿酸，リン，カリウムなどの崩壊産物が腎排泄能を上回り臓器障害をもたらす状態．治療開始前の発症，治療誘発性のいずれもある．白血球数（巨大腫瘍），LDH高値，縦隔腫瘍，増殖能の高い腫瘍（Burkittリンパ腫，T前駆細胞性急性リンパ性白血病など）がリスク因子である．

2）症状・所見

高尿酸血症，高リン血症では腎尿細管，集合管に尿酸塩，リン酸塩が析出し腎不全を生じる．乏尿・肺水腫・呼吸不全・低酸素血症・脳浮腫により致命的なこともある．高リン血症では二次性の低カルシウム血症を生じる．高カリウム血症，低カルシウム血症はテタニー，不整脈，心停止の原因となる．

3）対応

大量輸液により利尿を促進し，アルカリ化輸液により尿pHを7.0〜7.5に維持し尿酸塩，リン酸塩の析出を予防する．高尿酸血症に対しアロプリノール投与，高カリウム血症（6 mEq/l以上）に対しカリウム摂取制限，イオン交換樹脂製剤投与，グルコース-インスリン療法を行う．高リン血症に対しては水酸化アルミニウム内服により消化管からのリン吸収を抑制する．高リン血症に伴う低カルシウム血症に対するカルシウム補充は禁忌である．乏尿・無尿を認めたら早期に血液透析を行う．

🅷 出血性膀胱炎

1）病態

Cyclophosphamideやifosfamide等のアルキル化剤の代謝産物であるアクロレインが膀胱上皮を傷害して生じる．投与量，接触時間が発症に関係する．膀胱炎発症のピークは投与後5〜8日頃である．

2）症状・所見

血尿，排尿痛，腹痛．

3）対応

発症予防として，大量輸液により利尿を促進，膀胱カテーテル留置によりアクロレインと膀胱上皮の接触時間を短縮，メスナ投与によりアクロレインの毒性中和を行う．Cyclophosphamideに関連する乏尿に対しては利尿剤の投与を行う．

参考文献

1）Raul C. Ribeiro, Ching-Hon Pui : Acute complications. In : Ching-Hon Pui (eds), *Childhood Leukemias*. Cambridge University Press. 1999 ; 443-457

2）Arthur R. Ablin, Richard S.Pieters, Larry Singher : The Management of Oncologic Emergencies. In Arthur R. Ablin (eds), *Supportive Care of Children with Cancer*. The Johns Hopkins University Press, 1993;85-96

3）Shari Derengowski, Eileen O'Brien : Critical Care of Pediatric Oncology Patient. *Pediatric Oncology Patient* 1996;7:109-119

4）John J. Iacuone, Laurel J.Steinherz, Arthur R. Ablin : Modifications for Toxicity. In Arthur R. Ablin (eds), *Supportive Care of Children with Cancer*. The Johns Hopkins University Press, 1993;37-49

5）Hudson M : Late complications after leukemia therapy. In : Ching-Hon Pui (eds), *Childhood Leukemias*. Cambridge University Press. 1999 ; 463-475

6）Pizzo PA, Poplack DG(eds) : *Principles and Practice of Pediatric Oncology*, 4th ed. Lippincott Williams & Wilkins, Philadelphia, 2002

Characterization of Monocyte-Macrophage–Lineage Cells Induced from CD34+ Bone Marrow Cells In Vitro

Kyoko Suzuki,[a,b] Nobutaka Kiyokawa,[a] Tomoko Taguchi,[a] Hisami Takenouchi,[a] Masahiro Saito,[b] Toshiaki Shimizu,[b] Hajime Okita,[a] Junichiro Fujimoto[a]

aDepartment of Developmental Biology, National Research Institute for Child Health and Development, Tokyo, Japan;
bDepartment of Pediatrics, Juntendo University, School of Medicine, Tokyo, Japan

Abstract

We characterized the expression of cell surface antigens and cytokine-secreting ability of monocyte-macrophage–lineage cells induced in vitro from CD34+ bone marrow cells. After cultivation for 3 weeks, we observed 2 distinct cell fractions: a floating small, round cell fraction and an adherent large, protruding cell fraction. Both cell fractions expressed myelocyte-monocyte–lineage antigens, but mature-macrophage markers such as CD206 were expressed only by the adherent cells. An assessment of cells cultured for 5 weeks revealed spontaneous secretion of interleukin 8 (IL-8) and IL-6, and lipopolysaccharide (LPS)-induced tumor necrosis factor α (TNF-α) secretion in both fractions, but only the adherent cell fraction secreted IL-10 after LPS stimulation. In contrast, both fractions of cells cultured for 3 weeks spontaneously secreted low levels of IL-8, but none of the other cytokines. Upon LPS stimulation, the cells secreted IL-6 and TNF-α, but not IL-10. We also assessed the effect of granulocyte colony-stimulating factor (G-CSF) pretreatment on TNF-α secretion by each cell fraction and found that G-CSF reduced TNF-α secretion only in the adherent fraction of cells cultured for 3 weeks. Monocyte-macrophage–lineage cells induced in vitro should provide an ideal model for functional analysis of monocyte-macrophage cells.
Int J Hematol. 2007;85:384-389. doi: 10.1532/IJH97.06213
© 2007 The Japanese Society of Hematology

Key words: Monocyte-macrophage lineage; Antigen; Cytokine; Expression

1. Introduction

The mononuclear phagocyte system includes a widely distributed family of related cells (such as peripheral blood monocytes, macrophages, Kupffer cells, dendritic cells, osteoclasts, and microglia) that exhibit highly specialized functions. Macrophages resident in a number of tissues act as professional phagocytes and remove pathogens or apoptotic cells [1]. Dendritic cells are specialized to capture and present antigens, initiating the immune response through naive T-cell activation [2]; dendritic cells are also implicated in maintaining tolerance to self antigens [3]. Osteoclasts, multinucleated bone-resorbing cells found in the vicinity of bone, play an

essential role in bone remodeling as well as in regulating calcium homeostasis [4]. Microglia represent a unique category of mononuclear phagocytes distributed throughout the central nervous system [5], and in addition to their role as the immune effectors of the central nervous system, they perform nonimmunologic functions, including the production of neurotrophic factors and glutamate uptake [6,7].

The mononuclear phagocytic cells are believed to originate from hematopoietic stem cells in the bone marrow (BM). In the conventional view, monocytes that develop in the BM are released into the circulation and then enter the tissues to become resident macrophages and other mononuclear phagocytic cells [8,9]. Consistent with this view, Kennedy and Abkowitz demonstrated in a mouse transplantation system that more mature monocytes give rise to tissue macrophages, including alveolar macrophages in the lung and Kupffer cells in the liver [10]. The results of a number of studies have suggested, however, that the mechanism of mononuclear phagocytic cell development is more complicated. One intriguing possibility is that less mature marrow-derived cells, such as macrophage colony-forming units, enter

Correspondence and reprints requests: Nobutaka Kiyokawa, MD, PhD, Department of Developmental Biology, National Research Institute for Child Health and Development, 2-10-1, Okura, Setagaya-ku, Tokyo 154-8535, Japan; 81-3-3417-2496; fax: 81-3-3417-2496 (e-mail: nkiyokawa@nch.go.jp).

the tissues and differentiate into macrophages [11]. Prior studies have shown that tissue macrophages divide in situ, indicating that these cells are responsible for the renewal and expansion of this population [12-14].

The functional and phenotypic heterogeneity within the phagocyte system may be evidence of the differentiation plasticity of a common progenitor, but the details of the developmental pathways leading to the maturation of mononuclear phagocytic cells are still unclear. In vitro culture systems in which mature mononuclear phagocytic cells are induced from hematopoietic stem cells or monocyte precursors have been employed in a number of studies to clarify the molecular mechanism of phagocytic cell development. For example, monocyte-macrophage–lineage cells can be induced from CD34⁺ cord blood hematopoietic stem cells by liquid culture with cytokines [15] and by cocultivation with BM stromal cell lines in the presence of cytokines [16]. BM progenitors have recently been identified by their ability to differentiate into dendritic cells or osteoclasts, depending on whether they are exposed to RANKL in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) or M-CSF [17].

To evaluate the usefulness of monocyte-macrophage–lineage cells as a source for an in vitro model for the functional analysis of a monocytic phagocyte system, we analyzed the immunophenotype of and cytokine production by monocyte-macrophage–lineage cells induced from CD34⁺ BM cells in vitro. In this study, we showed that several distinct developmental stage–related subpopulations are present in monocyte-macrophage–lineage cells induced from CD34⁺ BM cells in vitro.

2. Materials and Methods

2.1. Cells and Reagents

Human BM CD34⁺ cells from Cambrex Bio Science Walkersville (Walkersville, MD, USA) were used. The cells had been isolated from human tissue after informed consent had been obtained. Recombinant human cytokines were purchased from PeproTech (London, UK). Fluorescently conjugated monoclonal antibodies were purchased from Beckman Coulter (Westbrook, MA, USA). Unless otherwise indicated, all other chemical reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Cultures

Monocyte-macrophage–lineage cells were induced by incubating human BM CD34⁺ cells (1×10^5 cells/well of a 6-well plate) at 37°C under 5% carbon dioxide in 5 mL of 10% (vol/vol) fetal calf serum (Sigma-Aldrich, St. Louis, MO, USA) containing RPMI 1640 medium (Sigma-Aldrich) supplemented with a cytokine mixture consisting of interleukin 3 (IL-3) (20 ng/mL), IL-6 (20 ng/mL), M-CSF (100 ng/mL), GM-CSF (20 ng/mL), and Flt-3 ligand (100 ng/mL) [15,18]. Every week, half of the medium was replaced with fetal calf serum–containing medium supplemented with M-CSF alone. After 3 weeks of cultivation, the medium was completely replaced with the medium supplemented with M-CSF alone, and the cells were cultured for another 2 weeks. At the end

of 5 weeks of cultivation, the floating cells in the medium were collected, and the adherent cells were harvested with 0.25% trypsin plus 0.02% EDTA (Immuno-Biological Laboratories Co, Gunma, Japan). These 2 cell fractions were used for further examination.

For the histology studies, cells were harvested and immobilized on glass slides with Cytospin 2 (Shandon, Pittsburgh, PA, USA). After Giemsa staining, cell morphology was assessed by light microscopy (BX-61; Olympus, Tokyo, Japan). Cells were tested for cytokine secretion by exposing the cells to G-CSF and stimulating them with lipopolysaccharide (LPS) (Sigma-Aldrich) for 24 hours, as described previously [19].

2.3. Reverse Transcriptase–Polymerase Chain Reaction Analysis

Total RNA was extracted from cultured human BM cells, and complementary DNA (cDNA) was generated with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and a First-Strand cDNA Synthesis Kit (Pfizer, Uppsala, Sweden). cDNA synthesized from 150 ng of total RNA was used as a template for one amplification reaction. The following sets of primers were used: 5′-ttattaccccctccttcagacac-3′ (sense) and 5′-aagtctggaaacatctggagagag-3′ (antisense), for amplification of a 347-bp fragment of human tumor necrosis factor α (TNF-α) cDNA; 5′-aagtggtgttctccatgtcc-3′ (sense) and 5′-gagcgaatgacagagggttt-3′ (antisense), for amplification of a 664-bp fragment of human IL-1β cDNA; and 5′-gctggag-gactttaagggtt-3′ (sense) and 5′-cccagatccgattttggaga-3′ (antisense), for amplification of a 394-bp fragment of human IL-10 cDNA. The set of primers for amplification of human glyceraldehyde-3-phosphate dehydrogenase was obtained from Stratagene (La Jolla, CA, USA). The polymerase chain reaction (PCR) was repeated for 30 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 PCR products were then separated on a 1.5% agarose gel.

2.4. Immunofluorescence Study and Cytokine Measurement

A multicolor immunofluorescence study was performed with a combination of fluorescein isothiocyanate, phycoerythrin, and phycoerythrin–Cyanine 5 (PC-5). Cells were stained with fluorescently labeled monoclonal antibodies and analyzed by flow cytometry (Epics XL; Beckman Coulter), as described previously [19]. The concentrations of cytokines and chemokines in culture supernatants were determined with a Cytometric Bead Array (CBA) (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions.

3. Results

3.1. Differentiation into Monocyte-Macrophage–Lineage Cells of Human BM CD34⁺ Cells Cultured with a Combination of Cytokines

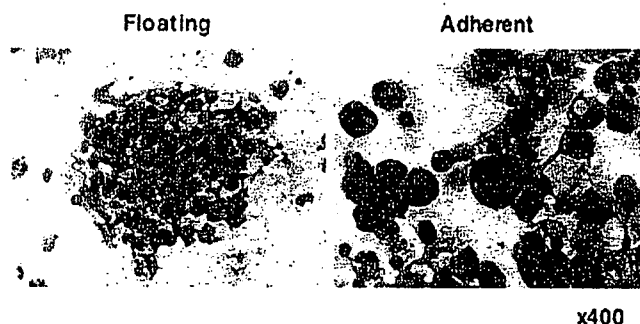We first characterized the morphology and surface-antigen expression of human BM CD34⁺ cells cultured with the

Floating   **Adherent**

x400

Figure 1. Morphology of monocyte-macrophage cells induced from CD34+ bone marrow (BM) cells in vitro. Human BM CD34+ cells were cultured for 5 weeks in the presence of a cytokine mixture, as described in "Materials and Methods." Floating and adherent cell fractions were subsequently collected separately and cytocentrifuged on a glass slide. Morphology was assessed after Giemsa staining. The experiments were repeated 3 times, and reproducible results were obtained. Representative data are shown (original magnification ×400).

combination of cytokines indicated in "Materials and Methods." During the second week of culture, microscopical observation revealed that a portion of the cells had started to adhere to the bottom of the culture dish. After 3 weeks of cultivation, the cells were clearly divided into floating and adherent fractions. When 1×10^5 CD34+ cells were cultured, approximately 7×10^6 adherent cells and 3×10^6 floating cells were obtained after 5 weeks of cultivation. At the end of the 5-week cultivation period, the floating and adherent fractions were collected separately, and their morphologies were assessed after May-Giemsa staining. As shown in Figure 1, the cells in the floating fraction were small and round and contained little cytoplasm. In contrast, the cells in the adherent fraction were large and contained abundant foamy cytoplasm with protrusions.

We also used flow cytometry to examine the cells for expression of monocyte-macrophage–lineage markers (Figure 2). Most floating cells expressed CD11b, CD31, CD33, and CD97, but no other mature-macrophage markers. The adherent cells, on the other hand, expressed markers of the myelocyte-monocyte lineage, such as CD13, CD14, CD36, CD54, CD64, CD85k, and CD105. It is noteworthy that the adherent cells expressed the mature-macrophage marker CD206, which was not expressed by the peripheral blood monocytes examined as a control (Figure 3).

3.2. Cytokine Secretion by Monocyte-Macrophage–Lineage Cells Induced from Human BM CD34+ Cells

Next, we assessed the cytokine-secreting ability of human BM CD34+ cells cultured with the cytokine combination. At the end of 5 weeks of cultivation, the floating and adherent fractions were collected separately, and cytokine secretion was assessed with the CBA system with and without LPS stimulation. Figure 4 shows that both the floating and adherent cell fractions spontaneously secreted IL-8 and IL-6 without LPS stimulation. After 24 hours of LPS stimulation, IL-6 secretion was enhanced in both fractions, but IL-8 secretion by adherent cells was decreased. TNF-α secretion, on the

other hand, was induced in the 2 fractions only after LPS stimulation. It is noteworthy that LPS stimulation induced IL-10 secretion only in the adherent cell fraction and not in the floating cell fraction. Neither IL-1β nor IL-12 secretion was induced by LPS stimulation in either fraction.

We also used reverse transcriptase–polymerase chain reaction analysis to assess the effect of LPS stimulation on the expression levels of cytokine messenger RNA (mRNA). Consistent with the results of the CBA analysis, LPS stimulation enhanced the expression of TNF-α mRNA in both the adherent and floating cell fractions (Figure 5). IL-10 mRNA expression, however, was already detectable in both cell fractions in the unstimulated state, and LPS stimulation did not enhance expression. In addition, LPS stimulation reduced IL-10 mRNA expression in the floating cell fraction. It is interesting that although no secretion of IL-1β protein was detected by the CBA assay, LPS stimulation significantly increased IL-1β mRNA expression in both cell fractions.
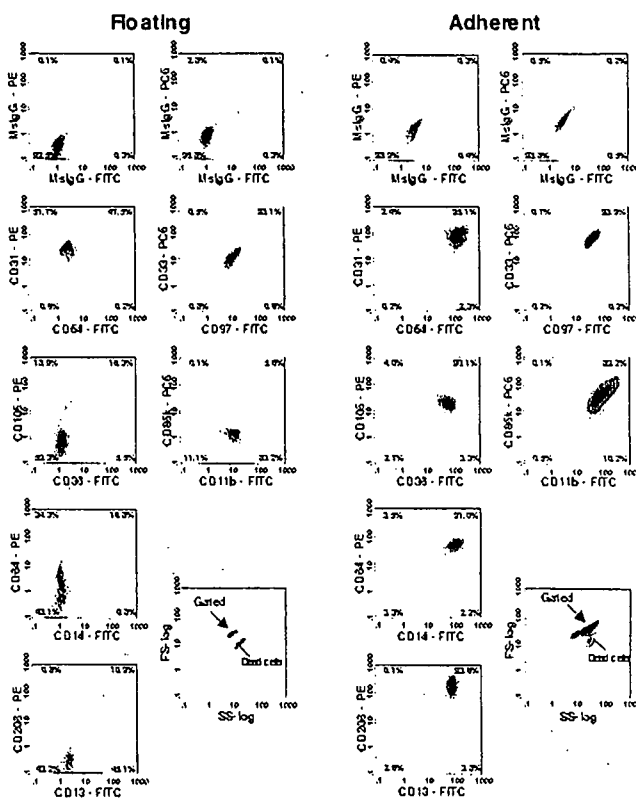

Floating   **Adherent**

Figure 2. Immunophenotypic analysis of monocyte-macrophage cells induced from CD34+ bone marrow (BM) cells in vitro. Human BM CD34+ cells were cultured for 5 weeks (as in Figure 1). At the end of the culture period, the floating and adherent cell fractions were collected separately, stained with combinations of fluorescently labeled antibodies as indicated, and examined by flow cytometry. The experiments were repeated 3 times, and reproducible results were obtained. Representative histogram data are shown. MsIgG, mouse immunoglobulin G; PE, phycoerythrin; PC-5, PE-Cyanine 5; FITC, fluorescein isothiocyanate; FS, forward light scatter; SS, side light scatter.
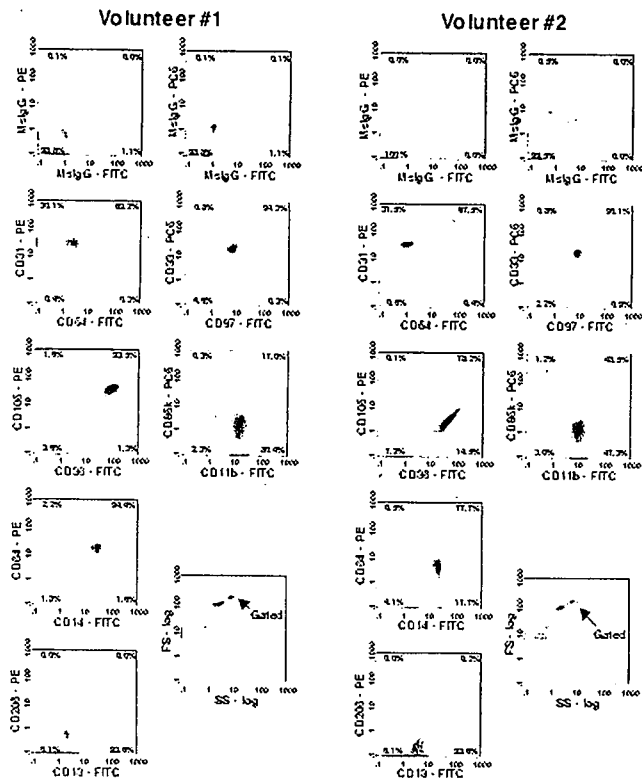
Figure 3. Immunophenotypic analysis of peripheral blood monocytes. Mononuclear cells were obtained from the peripheral blood of healthy volunteers via Ficoll-Paque centrifugation and stained with the indicated combinations of fluorescently labeled antibodies. Monocytes were gated, and the expression of each antigen was analyzed as in Figure 2.
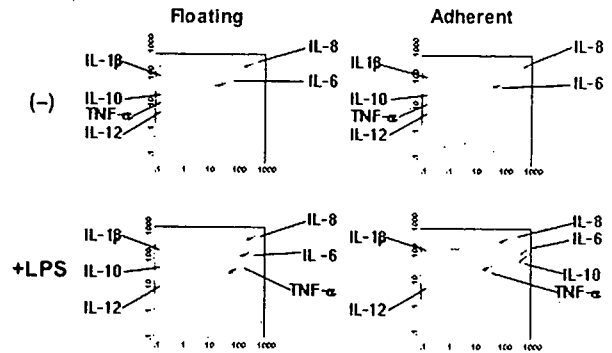
4. Discussion

This study has shown that monocyte-macrophage–lineage cells were efficiently induced from CD34⁺ BM cells in liquid culture in the presence of a cocktail of cytokines and that the monocyte-macrophage–lineage cells induced in vitro were capable of cytokine secretion upon stimulation with LPS. Several different subsets of monocyte-macrophage–lineage cells were induced during the course of culture.

For example, 2 distinct fractions, adherent cells and floating cells, were observed at the end of 5 weeks of culture. These 2 fractions were distinctive in both morphology and immunophenotype. Adherent cells were large, had a macrophage-like appearance, and expressed the mature-macrophage markers CD14, CD105, and CD206. In contrast, the floating cells were relatively small and contained little cytoplasm. Only some of them expressed mature-macrophage markers, whereas most

3.3. Effect of G-CSF on TNF-α Secretion by Monocyte-Macrophage–Lineage Cells Induced from Human BM CD34⁺ Cells

As we reported previously, G-CSF directly affects peripheral blood monocytes and reduces LPS-induced TNF-α secretion in a time-dependent manner [20]. We therefore tested the effect of G-CSF on TNF-α secretion by monocyte-macrophage–lineage cells induced from human BM CD34⁺ cells. Because our testing of monocyte-macrophage–lineage cells that had been induced with cytokines and harvested after 5 weeks of cultivation showed that G-CSF did not affect LPS-induced TNF-α secretion (data not shown), we tested cells harvested at different time points. Our assessment of LPS-stimulated cytokine secretion by adherent cells (Figure 6) (but not floating cells; data not shown) collected after 3 weeks of cultivation revealed cytokine-secretion patterns different from those of cells collected after 5 weeks of cultivation. Figure 6 shows that cells cultured for 3 weeks spontaneously secreted low levels of IL-8, but not other cytokines. LPS stimulated the cells to secrete IL-6 and TNF-α and an increased level of IL-8. Pretreatment with G-CSF reduced LPS-induced TNF-α secretion in a time-dependent manner.

		Floating, ng/mL	Adherent, ng/mL
IL-8	(-)	9.24	26.13
	LPS	12.03	3.90
IL-1 β	(-)	0.07	0.07
	LPS	0.07	0.10
IL-6	(-)	0.87	2.03
	LPS	7.35	20.30
IL-10	(-)	0.02	0.02
	LPS	0.02	34.62
TNF-α	(-)	<0.00	<0.00
	LPS	2.55	0.87
IL12	(-)	<0.00	<0.00
	LPS	<0.00	<0.00

Figure 4. Cytometric Bead Array (CBA) analysis of lipopolysaccharide (LPS)-stimulated cytokine secretion by monocyte-macrophage cells induced from CD34⁺ bone marrow (BM) cells in vitro. Monocyte-macrophage–lineage cells were induced from human BM CD34⁺ cells by cultivation for 5 weeks, as described for Figure 1. At the end of the culture period, the floating and adherent cells were stimulated with and without LPS for 24 hours. Subsequent cytokine secretion was assessed with the CBA system. The histograms obtained (upper panels) and calculated concentrations of each cytokine (table at bottom; <0.00 indicates undetectable) are shown. The experiments were repeated 3 times, and reproducible results were obtained. Representative data are shown. IL-1β indicates interleukin 1β; TNF-α, tumor necrosis factor α.

RT-PCR

AD   FL
──────────
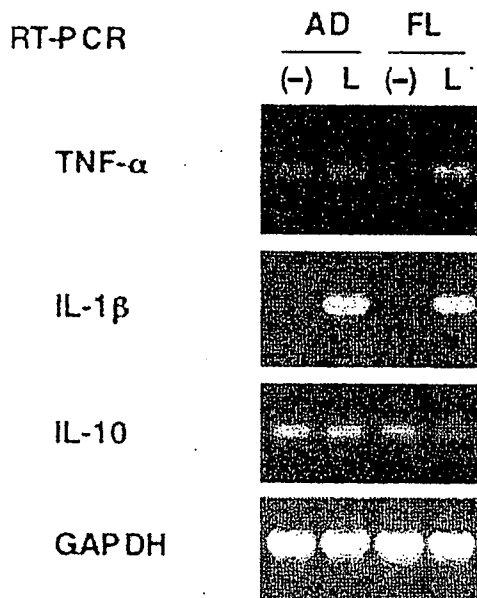(-) L (-) L

TNF-α

IL-1β

IL-10

GAPDH

Figure 5. Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of lipopolysaccharide (LPS)-stimulated cytokine production by monocyte-macrophage cells induced from CD34⁺ bone marrow (BM) cells in vitro. Monocyte-macrophage–lineage cells were induced from human BM CD34⁺ cells as described for Figure 1, and then floating (FL) and adherent (AD) cell fractions were collected separately. After stimulation with (L) or without (-) LPS for 24 hours (as described for Figure 4), total RNA was extracted, and the indicated messenger RNA molecules were analyzed by the RT-PCR after complementary DNA synthesis. The experiments were repeated 3 times, and reproducible results were obtained. Representative data are shown. TNF-α indicates tumor necrosis factor α; IL-1β, interleukin 1β; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cells expressed myelomonocytic antigens, including CD31, CD33, and CD97. It is interesting that these 2 fractions exhibited different cytokine-secretion abilities. Figure 4 shows that both fractions spontaneously secreted IL-8 and IL-6, and they both secreted TNF-α upon stimulation with LPS. Only the adherent cell fraction secreted IL-10 after LPS stimulation, however. These characteristics suggest that the adherent cell fraction represents mature macrophages, whereas the floating cell fraction may be related to immature monocytes. Evidence that further cultivation of the floating cell fraction induced an adherent cell fraction (data not shown) supports this idea.

It is noteworthy that both cell fractions contained more IL-1β mRNA after LPS stimulation but that no IL-1β secretion at the protein level was detected in either fraction. The data indicate that monocyte-macrophage–lineage cells induced in vitro are capable of producing IL-1β upon stimulation with LPS but that the stimulation is insufficient to induce secretion of IL-1β.

On the other hand, the adherent cells exhibited a profile of cytokine secretion after 3 weeks of cultivation that was distinct from that obtained after 5 weeks. At 3 weeks, the adherent cell fractions displayed almost the same immunophenotype as monocyte-macrophage–lineage cells cultured

for 5 weeks; however, the cells spontaneously secreted only low levels of IL-8, and not other cytokines. Although the cells secreted IL-6 and TNF-α after LPS stimulation, they did not secrete IL-10. Thus, our data indicate that different culture conditions induce different monocyte-macrophage–lineage subsets or monocyte-macrophage–lineage cells with different degrees of maturity.

Several studies have shown the induction of monocyte-macrophage–lineage cells by in vitro culture of cells from different cell sources. For example, Akagawa reported that M-CSF–induced monocyte-derived macrophages (M-Mphi) and GM-CSF–induced Mphi (GM-Mphi) differ in morphology, cell surface antigen expression, and function, including Fcγ receptor–mediated phagocytosis, hydrogen peroxide production and sensitivity, catalase activity, susceptibilities to human immunodeficiency virus type 1 and *Mycobacterium tuberculosis*, and suppressor activity [21]. She therefore concluded that the characteristics of GM-Mphi resemble those of human alveolar macrophages. Servet-Delprat et al also
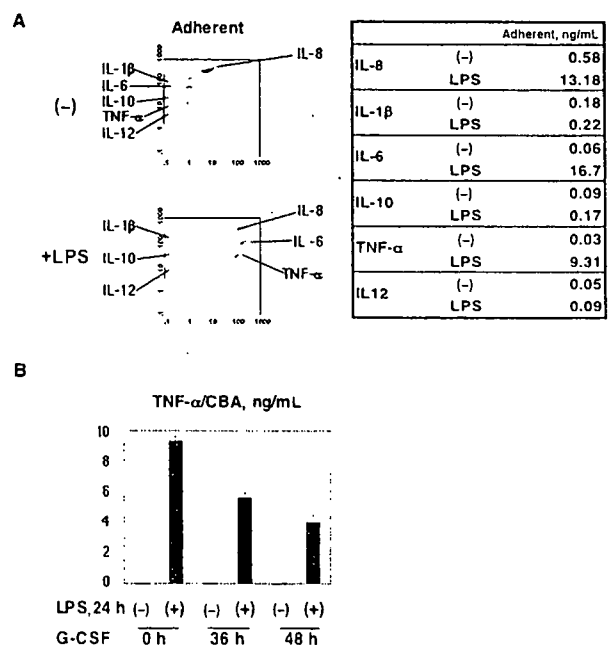

		Adherent, ng/mL
IL-8	(-)	0.58
	LPS	13.18
IL-1β	(-)	0.18
	LPS	0.22
IL-6	(-)	0.06
	LPS	16.7
IL-10	(-)	0.09
	LPS	0.17
TNF-α	(-)	0.03
	LPS	9.31
IL12	(-)	0.05
	LPS	0.09

Figure 6. Effect of granulocyte colony-stimulating factor (G-CSF) on lipopolysaccharide (LPS)-stimulated tumor necrosis factor α (TNF-α) secretion by monocyte-macrophage cells induced from CD34⁺ bone marrow (BM) cells in vitro. A, Monocyte-macrophage–lineage cells were induced from human BM CD34⁺ cells after 3 weeks of cultivation in the presence of the mixture of cytokines described in "Materials and Methods." At the end of the culture period, cells were stimulated with LPS for 24 hours. Subsequent cytokine secretion was assessed as in Figure 4. B, Induced monocyte-macrophage cells pretreated and not pretreated with G-CSF were stimulated with (+) and without (-) LPS for 24 hours, and subsequent cytokine secretion was assessed as in (A). The experiment was performed in triplicate, and the data are presented as the mean + SD. The experiments were repeated 3 times, and reproducible results were obtained. Representative data are shown. IL-1β indicates interleukin 1β.

reported that a variety of monocyte-macrophage–lineage cells, including macrophages, osteoclasts, dendritic cells, and microglia, can be induced from murine BM cells by ex vivo culture with different combinations of cytokines [22]. These reports further support our hypothesis that different culture conditions can induce different subsets of monocyte-macrophage–lineage cells.

In conclusion, the results of this study indicate that monocyte-macrophage–lineage cells induced from CD34+ BM cells in vitro can be used for functional assays, at least in terms of cytokine secretion. Further investigation is clearly necessary, however; the establishment of culture conditions that enable the induction of different subsets of monocyte-macrophage–lineage cells should provide an ideal experimental model for the analysis of monocyte-macrophage–lineage cell function.

Acknowledgments

References

1. Gordon S. The macrophage. *Bioessays*. 1995;17:977-986.
2. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature*. 1998;392:245-252.
3. Steinman RM, Turley S, Mellman I, Inaba K. The induction of tolerance by dendritic cells that have captured apoptotic cells. *J Exp Med*. 2000;191:411-416.
4. Teitelbaum SL. Bone resorption by osteoclasts. *Science*. 2000;289: 1504-1508.
5. Perry VH. A revised view of the central nervous system microenvironment and major histocompatibility complex class II antigen presentation. *J Neuroimmunol*. 1998;90:113-121.
6. Elkabes S, DiCicco-Bloom EM, Black IB. Brain microglia/ macrophages express neurotrophins that selectively regulate microglial proliferation and function. *J Neurosci*. 1996;16:2508-2521.
7. Noda M, Nakanishi H, Nabekura J, Akaike N. AMPA-kainate subtypes of glutamate receptor in rat cerebral microglia. *J Neurosci*. 2000;20:251-258.
8. Crofton RW, Diesselhoff-den Dulk MM, van Furth R. The origin, kinetics, and characteristics of the Kupffer cells in the normal steady state. *J Exp Med*. 1978;148:1-17.
9. Thomas ED, Ramberg RE, Sale GE, Sparkes RS, Golde DW. Direct evidence for a bone marrow origin of the alveolar macrophage in man. *Science*. 1976;192:1016-1018.
10. Kennedy DW, Abkowitz JL. Mature monocytic cells enter tissues and engraft. *Proc Natl Acad Sci USA*. 1998;95:14944-14949.
11. Takahashi K, Naito M, Takeya M. Development and heterogeneity of macrophages and their related cells through their differentiation pathways. *Pathol Int*. 1996;46:473-485.
12. Yamamoto T, Naito M, Moriyama H, et al. Repopulation of murine Kupffer cells after intravenous administration of liposome-encapsulated dichloromethylene diphosphonate. *Am J Pathol*. 1996;149: 1271-1286.
13. Wijffels JF, Hendrickx RJ, Steenbergen JJ, Eestermans IL, Beelen RH. Milky spots in the mouse omentum may play an important role in the origin of peritoneal macrophages. *Res Immunol*. 1992; 143:401-409.
14. Lawson LJ, Perry VH, Gordon S. Turnover of resident microglia in the normal adult mouse brain. *Neuroscience*. 1992;48:405-415.
15. Sawano A, Iwai S, Sakurai Y, et al. Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans. *Blood*. 2001;97:785-791.
16. Taghon T, Stolz F, De Smedt M, et al. HOX-A10 regulates hematopoietic lineage commitment: evidence for a monocyte-specific transcription factor. *Blood*. 2002;99:1197-1204.
17. Miyamoto T, Ohneda O, Arai F, et al. Bifurcation of osteoclasts and dendritic cells from common progenitors. *Blood*. 2001;98:2544-2554.
18. Xu MJ, Tsuji K, Ueda T, et al. Stimulation of mouse and human primitive hematopoiesis by murine embryonic aorta-gonad-mesonephros-derived stromal cell lines. *Blood*. 1998;92: 2032-2040.
19. Kiyokawa N, Kokai Y, Ishimoto K, Fujita H, Fujimoto J, Hata JI. Characterization of the common acute lymphoblastic leukaemia antigen (CD10) as an activation molecule on mature human B cells. *Clin Exp Immunol*. 1990;79:322-327.
20. Saito M, Kiyokawa N, Taguchi T, et al. Granulocyte colony-stimulating factor directly affects human monocytes and modulates cytokine secretion. *Exp Hematol*. 2002;30:1115-1123.
21. Akagawa KS. Functional heterogeneity of colony-stimulating factor-induced human monocyte-derived macrophages. *Int J Hematol*. 2002;76:27-34.
22. Servet-Delprat C, Arnaud S, Jurdic P, et al. Flt3+ macrophage precursors commit sequentially to osteoclasts, dendritic cells and microglia. *BMC Immunol*. 2002;3:15.

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

Tomoko Taguchi[a,b], Hisami Takenouchi[a], Yusuke Shiozawa[a],
Jun Matsui[a], Noriko Kitamura[a], Yoshitaka Miyagawa[a], Yoko U. Katagiri[a],
Takao Takahashi[b], Hajime Okita[a], Junichiro Fujimoto[a], and Nobutaka Kiyokawa[a]

[a]*Department of Developmental Biology, National Research Institute for Child Health and Development,*
Setagaya-ku, Tokyo, Japan; [b]*Department of Pediatrics, Keio University, School of Medicine, Shinjuku-ku, Tokyo, Japan*

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

Offprint requests to: Nobutaka Kiyokawa, M.D., Ph.D., Department of Developmental Biology, National Research Institute for Child Health and Development, 2-10-1, Okura, Setagaya-ku, Tokyo 154-8535, Japan; E-mail: nkiyokawa@nch.go.jp

[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 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% 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
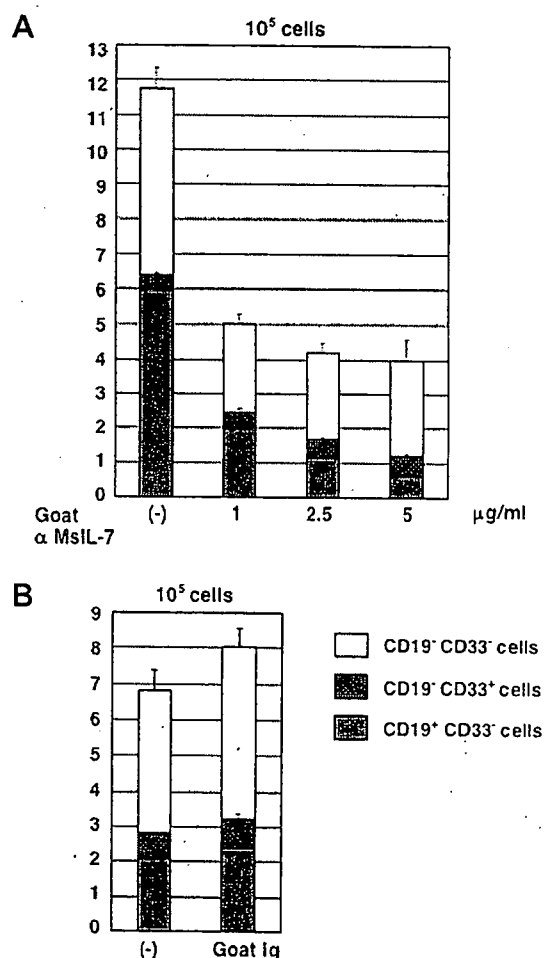

Figure 1. Effect of anti-mouse interleukin (IL)-7 antibody on human pro-B-cell development. (A) Human bone marrow CD34+ cells were cultured on MS-5 cells for 4 weeks in the presence or absence [(−)] of different concentrations of goat polyclonal anti-mouse IL-7 antibody. The subsequent CD19+CD33− cell 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.