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Ⅳ　研究成果の刊行物・別刷

先天性造血不全―Shwachman-Diamond症候群

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専攻
小児血液・腫瘍学

1．はじめに

Shwachman-Diamond症候群(SDS；MIM260400)は、1960年代に初めて記載された、膵外分泌不全、骨髄不全、骨格異常を主徴とする稀な先天性骨髄不全症候群である。常染色体劣性遺伝の遺伝形式をとり、原因遺伝子として*SBDS*が同定されている。SDS患者は、骨髄異形成症候群(MDS)、急性骨髄性白血病(AML)を発症するリスクが高いことが知られている。欧米では、Fanconi貧血、Diamond-Blackfan貧血に次いで多い先天性骨髄不全症候群であるが、本邦では20例程度が報告されているに過ぎず、実態は明らかになっていない。

2．臨床症候

典型例では、乳児期に、発育障害、脂肪性下痢、感染を繰り返すことを契機に、好中球減少に気づかれ、診断に至ることが多い。骨の異常も高率に認められる。但し、SDSでは、脂肪性下痢は年齢が高くなるにつれ軽快することが多く、好中球減少もしばしば間欠的であり、症例により臨床像は多様であるため、診断は必ずしも容易でない場合がある。

1）血液学的異常

大部分の症例(77%～100%)で好中球減少が認められる。絶対数500/μl未満の好中球減少は平均61%(23%-67%)の症例でみられる。好中球減少は、約2／3の症例で間欠的であるため、一度の検査では好中球減少がみられないことがあり、経時的に複数回検査することが重要である。

貧血は約半数で認められ、通常網状赤血球数は低値である。正球性貧血であることが多いが、大球性の場合もある。血小板減少も約半数で認められる。汎血球減少は、10%から44%の患者で認められる。重症再生不良性貧血の基準を満たす場合もある。

骨髄所見は多様であり、本症候群に特異的な所見が認められる訳ではない。細胞密度も低形成から過形成まで様々な場合があり、軽度の異形成はしばしば認められる。

好中球遊走能異常、Bリンパ球、Tリンパ球の機能異常があることが報告されている。

2）MDS、AMLへの転化

他の先天性骨髄不全症候群と同様、SDSではMDS、AMLの発症リスクが高く、13%から33%の症例でMDS/AMLへの転化がおこると報告されている。SDS患者で1年毎に骨髄をフォローしていくと、5年間で約30%にクローナルな染色体異常が認められるとする報告がある。このような染色体異常では、monosomy 7、isochromosome 7q、7q欠失と7番染色体の異常が最もよくみられる。しかし、中には臨床的意義が不明なものもあり、特にisochromosome 7qはMDS/AMLへの転化のリスクは低く、自然に消失することもある。原因不明であるが、SDSでは、MDS/AML転化をおこすのはほとんどが男性である。白血化した細胞は、しばしば7番染色体の異常を含む複雑な核型異常を示す。MDS/AMLの予後は不良で、SDSの主たる致死的合併症である。

3）膵外分泌不全

SDSは、欧米では嚢胞線維症に次いで多い先天性膵外分泌不全の原因である。典型例では、乳児期早期から、脂肪性下痢、発育不全がみられる。病理学的には、膵管、膵島は比較的保たれているが、腺房が広範に脂肪に置換されている。膵外分泌不全は、血清トリプシン、膵型アミラーゼ測定、膵刺激試験、便中脂肪定量で評価することができる。脂溶性ビタミンの欠乏がみられる。さらに、CTやMRIといった画像診断によって、膵が脂肪に置き換わっている像が得られる場合があり、しばしばSDSを疑うきっかけとなる。原因ははっきりしていないが、ほとんどの場合、脂肪性下痢は年齢が上がるにつれて改善する。

4）骨異常

SDSでは、胸郭異常、metaphyseal dystosis（骨幹端異形成）、骨低形成などの、骨異常が非常に高率に認められる。約半数で3パーセンタイル以下の低身長が認められる。適切な膵酵素補充を行えば、ほとんどの患者で成長速度は正常化するが、約半数で身長は3パーセンタイル以下に留まったままとなる。

5）その他の異常

肝トランスアミナーゼ軽度上昇がしばしばみられる。精神発達遅滞、歯牙の異常が認められることが多い。糖尿病、成長ホルモン分泌不全、腎尿細管性アシドーシス、口蓋裂、心筋症が報告されている。

3．診断

1）臨床診断

表にSDSの診断基準を示す。膵外分泌不全と血液学的異常の両方を認める症例がSDSと臨床診断される。但し両者は必ずしも同時に認められなくてもよい。症状や検査所見が経時的に変化していく可能性があるからである。

2）遺伝子診断

SDSの責任遺伝子は連鎖解析により同定され、*SBDS*と命名された。*SBDS*は7番染色体上に局在し、約90％の患者で同遺伝子の両アリル変異が認められる。exon 2にある183_184delinsCT、258+2 T>Cの2つの変異が最も頻度が高く、これらの複合ヘテロ接合変異が最も多くみられるため、まずこの2つの変異が存在するかどうかが解析される。

3）鑑別診断

他の原因による膵外分泌不全を除外する。代表的なのは嚢胞線維症であるが、sweat testで鑑別が可能である。他の先天性骨髄不全とも鑑別を要する。Pearson病は、膵外分泌不全、鉄芽球性貧血、骨髄で血液細胞に空胞が認められる疾患であり、ミトコンドリアDNAの欠失が原因である。Cartilage-hair hypoplasia（CHH）は、低身長、metaphyseal dystosis、血球減少、毛髪低形成を主徴とする疾患である。CHHはRMRP複合体のRNAサブユニットをコードするRMRP（RNase mitochondrial RNA processing）遺伝子の変異によっておこる。

表　SDS臨床診断基準

以下のIかつIIを認める。

I．膵外分泌不全
以下の一つ以上を満たす
1．膵外分泌酵素低値
3歳未満でトリプシノーゲン低値
かつ／または
3歳以上で膵型アミラーゼ低値
2．画像で小型あるいは脂肪の多い膵を認める
3．便中脂質の増加（72時間収集）

II．骨髄不全
以下の一つ以上を満たす
1．絶対数1500未満の好中球減少（間欠的あるいは慢性的；3ヶ月以上に渡って少なくとも3回）
2．年齢別正常値と比較したヘモグロビン低値あるいはMCV高値
3．血小板数15万以下の血小板減少
4．汎血球減少
5．骨髄検査で白血病、骨髄異形成症候群、染色体異常のうち一つ以上に該当する。

＊ 膵外分泌不全、骨髄不全の原因となる他疾患を除外する。
＊
（Nathan and Oski's Hematology of Infancy and Childhood, 7th edition. p344より一部改変）

4．病態

*SBDS*遺伝子変異のほとんどはSDSの近傍に存在するpseudogene（SBDSP）とのconversionの結果おこったものである。変異により正常より短い蛋白（truncated protein）が産生され、ほとんどの場合SBDS蛋白の発現は著明に低下している。しかし、183_184delinsCTのホモ接合変異をもった患者は存在せず、ヌル対立遺伝子のホモ接合体で、機能を持つSBDS蛋白を全く産生できない場合には生存不可能と考えられる。さらにSBDSノックアウトマウスも胎生致死になることから、SBDSは生命維持に必須の蛋白であることが示唆されている。実際、*SBDS*遺伝子のコードする蛋白は、進化の過程で高度に保存されている。

SBDS蛋白は既知のドメイン構造を持っておらず、その機能は不明であったが、様々な解析からSBDS蛋

白の主な機能はリボソーム生成の制御であることが明らかとなってきている。SBDS蛋白は、細胞周期依存的に核小体に局在し、リボソームの28Sサブユニット、リボソーム生成や白血病発症に関与するヌクレオフォスミンと結合する。酵母では、SBDSの相同遺伝子であるSdo1を欠失させると、リボソーム生成が阻害される。Sdo1はpre-60Sリボソームサブユニットからのpre-60Sリボソームサブユニットからの Tif 6の放出を促進させる働きがあり、その結果60Sサブユニットは他のリボソームサブユニットとの結合が可能となり、リボソームは成熟し翻訳が活性化される。最近Diamond-Blackfan貧血等いくつかの骨髄不全がリボソーム生成の異常によることがわかってきているが、以上のような知見から、SDSもこのような疾患群の一つであると考えられる。

SDS患者のCD34陽性骨髄細胞では、Fasを介したアポトーシスが亢進していることが報告され、造血前駆細胞の細胞死が血球減少の原因であることが示唆されている(Dror Y et al.Blood,2001)。Hela細胞でsiRNAを用いSBDSをノックダウンしても、細胞死が亢進することが報告されている(Watanabe K at al. Haemaotologica, 2008, Apoptosis,2008)。

SDS患者においてMDS/AMLが発症しやすい原因は解明されていない。SDS患者の骨髄細胞で染色体異常がよくみられることは、造血前駆細胞でゲノムの不安定性が増していることを示唆している。SDS患者の白血球でテロメア長が短縮していることを示した論文が一つだけあるが、これがどの程度ゲノム不安定性に関与するかは不明である。最近、SBDS蛋白が細胞分裂の際の紡錘糸に局在することが報告された。SBDSの欠失により、細胞分裂の異常や異数性がおこることが示され、紡錘糸の安定性の喪失が、SDS患者における骨髄不全や白血病発症の機構に寄与していることが示唆されている。尚、SDSにおいては、未だ固形癌の発症リスクが上がるという報告はない。

Calado等は91例の再生不良性貧血患者で4例の*SBDS*ヘテロ接合変異がみつかったと報告し、*SBDS*のハプロ不全があると、再生不良性貧血が発症しやすくなると主張した。しかし、本邦での96例の再生不良性貧血患者の解析では1人もSBDS変異をもつものは見つからなかった(Wang Y, et al.2007)。SBDSのハプロ不全と再生不良性貧血発症の関係については、統計学的に十分な症例数が解析されているとは言えず、未だ確定的ではないと考えられている。

5．治療

膵外分泌不全に対しては、膵酵素補充、脂溶性ビタミンの投与が行われる。約50%の患者では、脂肪性下痢は年齢が高くなるにつれ改善するが、膵酵素は低値にとどまる可能性があり、定期的な膵外分泌機能検査が推奨される。

血球数について定期的に検査を行う。これに加え、年1回骨髄検査を行い、形態や染色体異常について評価することが推奨される。重度の好中球減少や重症感染症を繰り返す場合には、G-CSFの使用や予防的抗生剤投与を考慮する。G-CSF投与と白血化の関連は明らかではないが、G-CSFはできるだけ少量を用いた方がよいとする考えがある。

造血幹細胞移植は、SDSに伴う血液学的異常に対する唯一の根治的治療法であり、重度の血球減少、MDS/AML転化を伴う患者に行われている。サイクロファスファミドにブスルファンあるいは全身放射線照射を加えた骨髄破壊的前処置が使用されていたが、SDS患者に対する造血幹細胞移植では、治療関連毒性、特に心臓、肺に対する毒性が高率に認められた。French Severe Chronic Neutropenia Registryでは、10例のSDS患者が同種移植を受け、5年無イベント生存率は60%であった。European Group for blood and marrow transplantation(EBMT)から報告されたSDS患者25例の同種移植後粗生存率は中央値1.1年のフォローアップ期間で64%であった。MDS/AMLに移行してから移植が行われた場合の方が、重度の骨髄不全に対して行われた場合より予後が不良であった。最近、前処置による毒性を軽減するため、フルダラビンやCampath-1Hを含む骨髄非破壊的前処置を用いた移植の報告がされている。SDSは稀で移植を受ける例も少数であるため、十分な症例数のまとまった報告は未だない。至適な移植法の確立は今後の課題と言えよう。

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Journal of Cellular Physiology

Neutrophil Differentiation From Human-Induced Pluripotent Stem Cells

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Induced pluripotent stem (iPS) cells are of potential value not only for regenerative medicine, but also for disease investigation. The present study describes the development of a neutrophil differentiation system from human iPS cells (hiPSCs) and the analysis of neutrophil function and differentiation. The culture system used consisted of the transfer of hiPSCs onto OP9 cells and their culture with vascular endothelial growth factor (VEGF). After 10 days, TRA 1-85^{+}CD34^{+}VEGF receptor-2 (VEGFR-2)^{high} cells were sorted and co-cultured with OP9 cells in the presence of hematopoietic cytokines for 30 days. Floating cells were collected and subjected to morphological and functional analysis. These hiPSC-derived neutrophils were similar to peripheral blood mature neutrophils in morphology, contained functional neutrophil specific granules, and were equipped with the basic functions such as phagocytosis, superoxide production, and chemotaxis. In the process of differentiation, myeloid cells appeared sequentially from immature myeloblasts to mature segmented neutrophils. Expression patterns of surface antigen, transcription factors, and granule proteins during differentiation were also similar to those of granulopoiesis in normal bone marrow. In conclusion, differentiation of mature neutrophils from hiPSCs was successfully induced in a similar process to normal granulopoiesis using an OP9 co-culture system. This system may be applied to elucidate the pathogenesis of various hematological diseases that affect neutrophils.

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Neutrophils and/or myeloid differentiation are most commonly affected in various hematological diseases including inherited bone marrow failure syndromes and neutrophil function disorders. Responsible genes have been identified in most of these syndromes or diseases, but the association between the gene mutation and the specific phenotype is not always clear. Moreover, often patients who present with a specific syndrome lack mutations in the known genes (Alter, 2007). Understanding the pathophysiology of these syndromes has been challenging despite the information provided by recent molecular findings, and in many of these syndromes, experimental models have not yet been generated.

Murine models of human congenital and acquired diseases are invaluable for disease investigation, but they provide a limited representation of human pathophysiology because they often do not faithfully mimic human diseases. The differences between murine and human physiologies make human cell culture an essential complement to research with animal models of disease.

Induced pluripotent stem (iPS) cells are reprogrammed somatic cells with embryonic stem (ES) cell-like characteristics generated by the introduction of combinations of specific transcription factors (Takahashi and Yamanaka, 2006; Meissner et al., 2007; Okita et al., 2007; Takahashi et al., 2007; Yu et al., 2007; Park et al., 2008b). Given the robustness of the approach, direct reprogramming promises to be a facile source of patient-derived cell lines. Such lines would be immediately valuable not only for regenerative medicine, but for disease investigation and drug screening as well.

The pluripotency and self-renewal potential of ES cells contributes to their value in various fields of science (Evans and Kaufman, 1981). Previous studies using normal or gene-manipulated ES cells have helped elucidate the process of normal embryogenesis and the genetic mechanisms of certain diseases (Lensch and Daley, 2006; Tulpule et al., 2010). Use of human embryos, however, faces ethical controversies that hinder the applications of human ES cells (hESCs). In addition, it is difficult to generate patient- or disease-specific ES cells, which are required for their effective application. The use of iPS cells would avoid the controversies surrounding human embryonic stem cell research.

Patient-specific iPS cells can be used for the generation of disease-corrected, patient-specific cells for cell therapy applications. Disease-specific pluripotent cells capable of differentiation into the various tissues affected in each condition can also provide new insights into disease pathophysiology by permitting analysis in a human system, under controlled conditions in vitro. Recent studies reported the generation of disease-specific iPS cell lines from patients with a variety of diseases (Park et al., 2008a; Raya et al., 2009; Agarwal et al., 2010). Therefore, disease-specific iPS cells are expected to be good models for the investigation of different diseases, and

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effective neutrophil differentiation systems are required to investigate the pathogenesis of various hematological conditions that affect neutrophils using human iPS cells (hiPSCs).

Recent reports describe in vitro culture systems for neutrophil differentiation from hESCs (Choi et al., 2009; Saeki et al., 2009; Yokoyama et al., 2009); however, neutrophil differentiation from hiPSCs has not yet been reported in detail. One of these studies demonstrated that myeloid differentiation could be induced from hiPSCs using the same methodology employed for their differentiation from hESCs (Choi et al., 2009), but the differentiation process and the functions of hiPSC-derived neutrophils were not shown in detail. A system for erythroid differentiation from primate ES and murine iPS cells by co-culture with OP9 stromal cells was developed in previous studies (Umeda et al., 2004; Umeda et al., 2006; Shinoda et al., 2007; Niwa et al., 2009). In the present study, a neutrophil differentiation system from hiPSCs was established by modifying the erythroid differentiation system, and the functions of the hiPSC-derived neutrophils and their differentiation process were analyzed in detail. This system may contribute to the elucidation of the pathogenesis of various blood diseases and the development of novel therapeutic approaches.

Materials and Methods

Maintenance of cells

The human iPS cell lines 201B6, 253G1 and 253G4 were a kind gift from Dr. Yamanaka (Kyoto University, Kyoto), and were generated from human dermal fibroblasts by retrovirus-mediated transfection of four (201B6) or three (253G1 and 253G4) transcription factors (Oct3/4, Sox2, and Klf4, with or without c-Myc) (Takahashi et al., 2007; Nakagawa et al., 2008). The human iPS cell lines and the human ES cell line KhES3-EGFPneo (KhES-3G) were maintained on mitomycin-C (Kyowa Hakko Kirin, Tokyo, Japan) -treated mouse embryonic fibroblasts (MEFs) in DMEM/F12 (Sigma-Aldrich, St. Louis, MO) supplemented with 20% Knockout™ Serum Replacement (Invitrogen, Carlsbad, CA), 5 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN), 1% non-essential amino acids solution (Invitrogen), 5 mM sodium hydroxide solution, 100 μM 2-mercaptethanol, and 2 mM L-glutamine. The culture medium was replaced daily with fresh medium. Colonies were passaged onto new MEFs every 3 or 4 days. The human ES cell line was used in conformity with The Guidelines for Derivation and Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science, and Technology, Japan. OP9 stromal cells, which were a kind gift from Dr. Kodama (Osaka University, Osaka), were maintained in α-MEM (Invitrogen) supplemented with 20% fetal calf serum (FCS; Biological Industries, Bet Haemek, Israel).

Antibodies

The antibodies used for flow cytometric analysis included fluorescein isothiocyanate (FITC)-conjugated anti-human TRA 1-85 (R&D Systems), CD45 (Becton-Dickinson, Franklin Lakes, NJ) antibodies, phycoerythrin (PE)-conjugated anti-human CD11b, CD34 (Beckman Coulter, Fullerton, CA), CD13, CD16, CD33 (Becton-Dickinson) antibodies, and allophycocyanin (APC)-conjugated anti-human vascular endothelial growth factor receptor-2 (VEGFR-2) (eBioscience, San Diego, CA) antibody. The primary antibodies used for immunocytochemical analysis included goat anti-human lactoferrin (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-human MMP9 (Abcam, Cambridge, UK). Biotinylated horse anti-goat or anti-rabbit antibodies (Vector Laboratories, Burlingame, CA) were used as secondary antibodies.

Differentiation of iPS cells

Methods used for the initial differentiation of iPS cells and cell sorting were based on earlier reports (Umeda et al., 2004, 2006). Briefly, trypsin-treated undifferentiated iPS cells were transferred onto OP9 cells and cultured with 20 ng/ml vascular endothelial growth factor (VEGF) (R&D Systems). After 10 days, the induced cells were harvested with cell dissociation buffer (Invitrogen), and sorted TRA 1-85^{+}CD34^{+}VEGFR-2^{high} cells were transferred onto fresh OP9 cells in six-well plates at a concentration of 3×10^3 cells per well. Sorted cells were cultured in α-MEM (Invitrogen) containing 10% FCS (Sigma, St Louis, MO), 50 μM 2-mercaptoethanol, 20 ng/ml interleukin (IL)-3, 100 ng/ml stem cell factor (SCF) (R&D Systems), and 10 ng/ml thrombopoietin (TPO) for 20 days. On day 20 after cell sorting, cytokines were changed into 20 ng/ml IL-3 and 10 ng/ml granulocyte colony-stimulating factor (G-CSF). IL-3, TPO and G-CSF were kindly provided by Kyowa Hakko Kirin.

Flow cytometric analysis and cell sorting

Cells were trypsinized and stained with antibodies. Dead cells were excluded by 4',6-diamidino-2-phenylindole (DAPI) staining. Samples were analyzed using an LSR flow cytometer and Cell Quest software (Becton Dickinson). Cell sorting was performed using a FACSVantage SE flow cytometer (Becton Dickinson).

Cytostaining

Floating cells were centrifuged onto glass slides using a Shandon Cytospin® 4 Cytocentrifuge (Thermo, Pittsburgh, PA), and analyzed by microscopy after May–Giemsa, myeloperoxidase (MPO), or alkaline-phosphatase staining. Sequential morphological analysis was performed as follows: all adherent cells including OP9 cells were trypsinized, harvested, and incubated in a new tissue-culture dish (Becton-Dickinson) for 1 h to eliminate adherent OP9 cells (Suwabe et al., 1998). Floating cells were then collected, centrifuged onto glass slides, and analyzed by microscopy after May–Giemsa staining. For immunocytochemical analysis, cells were fixed with 4% paraformaldehyde (PFA), immersed in citrate buffer, and autoclaved for 5 min at 121°C for antigen retrieval (Toda et al., 1999). The slides were then incubated with primary antibodies followed by application of the streptavidinbiotin complex immunoperoxidase technique with diaminobenzidine as chromogen, and nuclei were counterstained with hematoxylin.

Electron microscopy

Cells were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (PB) for at least 2 h, and then postfixed in 1% osmium tetroxide in 0.1 M PB for 1.5 h. After fixation, samples were dehydrated in a graded ethanol series, cleared with propylene oxide, and embedded in Epon. Thin sections of cured samples were stained with uranyl acetate and Reynolds lead citrate. The sections were inspected using a transmission electron microscope, H7650 (Hitachi, Tokyo, Japan).

Chemotaxis assay

Chemotactic ability was determined using a modified Boyden chamber method (Boyden, 1962; Harvath et al., 1980). Briefly, 500 μl of the reaction medium (Hank's Balanced Salt Solution (HBSS) containing 2.5% FCS) with or without 10 nM formyl-Met-Leu-Phe (fMLP; Sigma-Aldrich) was placed into each well of a 24-well plate, and the cell culture insert (3.0-μm pores; Becton Dickinson) was gently placed into each well to divide the well into upper and lower sections. Floating cells were suspended in the reaction medium at 7.0×10^4/ml, and a 500-μl cell suspension was added to the upper well, allowing the cells to migrate from the upper to the lower side of the membrane for 4 h at 37°C. After incubation, cells in the lower chamber were collected and counted using an LSR flow cytometer. Cells were counted by flow cytometry as follows:

equivalent amounts of counting beads were added to each sample and counted until the bead count reached 10,000.

MPO activity assay

The EnzChek Myeloperoxidase (MPO) Activity Assay Kit (Molecular Probes, Leiden, The Netherlands) was used for rapid and sensitive determination of MPO chlorination activity in cell lysates. The procedure was performed following the manufacturer's instructions. Cell lysate samples were prepared from 1×10^4 floating cells by freeze-thaw cycles. Fluorescence was measured with a fluorescence microplate reader (Wallac 1420 ARVO sx; PerkinElmer, Waltham, MA) using fluorescence excitation and emission at 485 and 530 nm, respectively. The background fluorescence measured for each zero-MPO control reaction was subtracted from each fluorescence measurement before plotting.

DHR assay

Neutrophil production of reactive oxygen species was detected by flow cytometry using dihydrorhodamine123 (DHR) as described previously (Vowells et al., 1995). Briefly, 3.5×10^4 floating cells were suspended in 100 µl of the reaction buffer (HBSS containing 0.1% FCS and 5 mM glucose) per tube, and two tubes were prepared for each sample. Catalase (Sigma-Aldrich) at a final concentration of 1000 U/ml and DHR at a final concentration of 1.0×10^5 nM were added and incubated for 5 min in a 37°C shaking water bath. After incubation, phorbol myristate acetate (PMA; Sigma-Aldrich) at a final concentration of 400 ng/ml was added to one of the two tubes and tubes were returned to the water bath for an additional 15 min. Following incubation, rhodamine fluorescence from the oxidized DHR was detected using an LSR flow cytometer.

Phagocytosis and detection of reactive oxygen species

Phagocytosis and neutrophil production of reactive oxygen species was detected by chemiluminescent microspheres (luminol-binding carboxyl hydrophilic microspheres; TORAY, Tokyo, Japan) as described previously (Uchida et al., 1985). Briefly, 2×10^4 floating cells were suspended in 50 µl of the reaction buffer (HBSS containing 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES)) per tube. To activate the system, 5 µl of chemiluminescent microspheres was added, and light emission was recorded continuously. During the measurement, samples were kept at 37°C. To inhibit the phagocytosis, 1.75 µg of cytochalasin B (Sigma-Aldrich) was added to the sample. Chemiluminescence from the microspheres was detected using a luminometer (TD-20/20; Turner Designs, Sunnyvale, CA).

RNA extraction and RT-PCR analysis

RNA samples were prepared using silica gel membrane-based spin-columns (RNeasy Mini-Kit™, Qiagen, Valencia, CA) and subjected to reverse transcription (RT) with the Ominscript-RT Kit™ (Qiagen). All procedures were performed following the manufacturer's instructions. For reverse transcriptase-polymerase chain reaction (RT-PCR), yields were adjusted by dilution to produce equal amounts of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplicon. The complementary DNA (cDNA) templates were initially denatured at 94°C for 5 min, followed by 30–40 amplification reactions consisting of 94°C for 15–30 sec (denaturing), 55–63°C for 15–30 sec (annealing), and 72°C for 30–60 sec (extension), with a final extension at 72°C for 7 min. The oligonucleotide primers were as follows: NANOG, 5′-CAG CCC TGA TTC TTC CAC CAG TCC C-3′ and 5′-TGG AAG GTT CCC AGT CGG GTT CAC C-3′ (Takahashi et al., 2007); human GAPDH, 5′-CAC CAG GGC TGC TTT TAA CTC TG-3′ and 5′-ATG GTT CAC ACC CAT GAC GAA C-3′ (Umeda et al., 2006); PU.1, 5′-CTG CAT TGG CCC CCA CCG AG-3′ and 5′-AGG TCT TCT GAT GGC TGA GGG GG-3′; C/EBPα, 5′-TAA CCT TGT GCC TTG GAA ATG CAA AC-3′ and 5′- ATG TTT CCA CCC CTT TCT AAG GAC A-3′ (Duan and Horwitz, 2003); C/EBPε, 5′- AGT CTG GGG AAG AGC AGC TTC-3′ and 5′- ACA GTG TGC CAC TTG GTA CTG-3′ (Mori et al., 2009); MPO, 5′-TGA GGA CGG CTT CTC TCT TC-3′ and 5′-CCC GGT AAG TGA TGA TCT GG-3′; Lactoferrin, 5′-AGC TGG CAG ACT TTG CGC T-3′ and 5′-TTC AGA TTA GTA ATG CCT GCG ACA TAC-3′ (Kholodnyuk et al., 2006); Gelatinase (MMP-9), 5′-GCC TCC AAC CAC CAC CAC AC-3′ and 5′-GCC CAG CCC ACC TCC ACT C-3′ (Sugimoto et al., 2001); mouse GAPDH, 5′-ACG GCC GCA TCT TCT TGT GCA-3′ and 5′-CAC CCT TCA AGT GGG CCC CG-3′. PCR amplification reaction cycles were performed in the linear range for each primer by carrying out primer titrations. The number of reaction cycles per sample were: NANOG, 35 cycles; human GAPDH, 30 cycles; PU.1, 40 cycles; C/EBPα, 40 cycles; C/EBPε, 40 cycles; MPO, 35 cycles; Lactoferrin, 35 cycles; Gelatinase (MMP-9), 40 cycles; mouse GAPDH, 30 cycles.

Statistics

Statistical analyses were conducted using the Student's *t*-test. Statistical significance was defined as $P < 0.05$.

Results

Neutrophil differentiation from hiPSCs in co-culture with OP9 stromal cells

A culture system for the induction of erythroid cell differentiation from primate ES and murine iPS cells by co-culture with OP9 stromal cells (Umeda et al., 2004; Umeda et al., 2006; Shinoda et al., 2007; Niwa et al., 2009) was established, and this system was applied for neutrophil differentiation from hiPSCs. Prior data in primate ES cells suggested that the VEGFR-2^{high} fraction of differentiated cells contained hemangioblasts and VEGFR-2^{high}CD34$^+$ cells had more hematopoietic potential (Umeda et al., 2006). Therefore, the expression of VEGFR-2 and CD34 was examined using three human iPS cell lines (201B6, 253G1, 253G4) and one ES cell line (KhES-3G). After 10 days of co-culture with OP9 in the presence of 20 ng/ml VEGF, VEGFR-2^{high}CD34$^+$ cells appeared from all hiPSC lines in a similar manner to the ES cell line (Fig. 1A). Among these three human iPS cell lines, the highest percentage of VEGFR-2^{high}CD34$^+$ cells was detected in 253G4 (Fig. 1B), and the data on this cell line is therefore presented below.

The VEGFR-2^{high}CD34$^+$ cell fraction was sorted (Fig. 1C) and 1.1×10^4 (range; $0.6–2.2 \times 10^4$ in 14 independent cultures) VEGFR-2^{high}CD34$^+$ cells were grown in one 10-cm dish containing hiPSCs. They were then transferred onto fresh OP9 cells and cultured in the presence of hematopoietic cytokines. Around 10 days after cell sorting (day10 + 10), small, round cell colonies appeared (Fig. 1D), and these colonies gradually grew in both size and number (Fig. 1E). At the same time, floating cells also appeared, and the average number of floating cells from 1×10^4 sorted VEGFR-2^{high}CD34$^+$ cells at 30 days after cell sorting (day10 + 30) was 4.1×10^4 (range; $0.2–9.9 \times 10^4$ in 11 independent cultures).

May–Giemsa staining of the floating cells on day 10 + 30 revealed that 38.0 ± 1.6% of the cells were stab and segmented neutrophils (Fig. 1F), which were positive for MPO (Fig. 1G) and neutrophil alkaline-phosphatase (Fig. 1H). The rest were mainly immature myeloid cells and a small number of macrophages, and cells of other lineages, such as erythroid or lymphoid cells, were not observed. The frequency of MPO- and neutrophil alkaline-phosphatase-positive cells is shown in Table 1. The results were consistent with the morphological features revealed by May–Giemsa staining.

Surface marker analysis revealed that these floating cells were positive for CD45 and CD11b, and partially positive for CD13, CD33, and CD16 (Fig. 1I). The expression pattern of these surface markers was similar to that of neutrophils or immature myeloid cells in healthy bone marrow (van Lochem et al., 2004), although the CD16 expression level was lower.

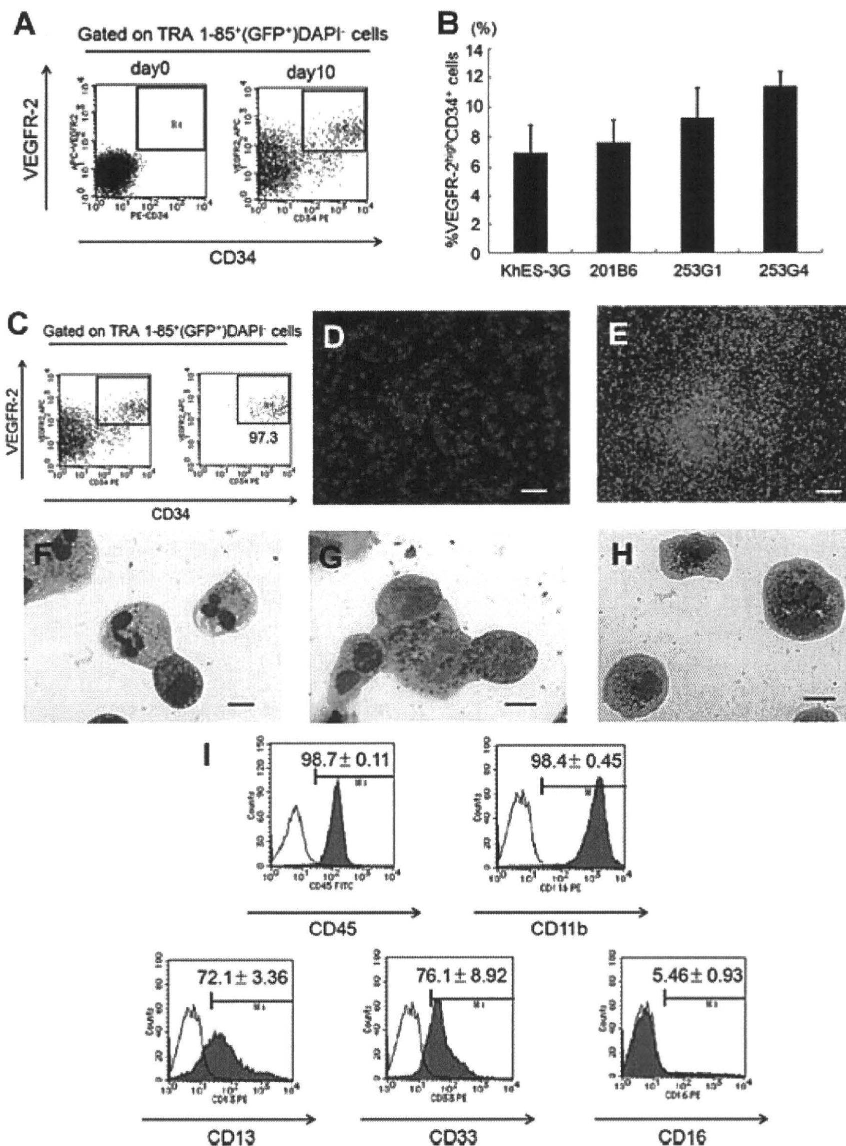

Fig. 1. Neutrophil differentiation from hiPSCs in co-culture with OP9 stromal cells. (A–B) Flow cytometric analysis of VEGFR-2 and CD34 during differentiation induction. TRA 1-85$^+$ (GFP$^+$) DAPI$^-$ cells were gated as human iPS (ES) cell-derived viable cells. Undifferentiated iPS (ES) cells and 10-day culture cells were stained with antibodies specific for VEGFR-2 and CD34. Representative results from one of three independent experiments (A) and percentages of VEGFR-2^{high}CD34$^+$ cells on day 10 (B) are shown (n = 3; bars represent SDs). (C) VEGFR-2^{high}CD34$^+$ cells were sorted on day 10. Representative dot plots and percentages of gated cells are shown. Purities of viable VEGFR-2^{high}CD34$^+$ cells were calculated at 95.5 ± 1.9% from 14 independent experiments. (D–E) Micrographs of adherent hematopoietic cell clusters generated on day 10 (D) and day 30 (E) after cell sorting. Scale bars: 200 μm. (F–H) May–Giemsa staining (F), myeloperoxidase staining (G), and neutrophil alkaline-phosphatase staining (H) of floating cells on day 10 + 30. Scale bars: 10 μm. (I) Flow cytometric analysis of floating cells on day 10 + 30 were stained with antibodies specific for CD45, CD11b, CD13, CD33, or CD16. Plots show the negative control profile (open bars) versus the specific antibody staining profiles (shaded bars). Representative results from one of three independent experiments are shown. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 1. Frequency of staining-positive cells for neutrophil specific granules

Staining	Frequency of positive cells (%)
Myeloperoxidase	93.7 ± 1.7
Neutrophil alkaline-phosphatase	39.0 ± 2.2
Lactoferrin	79.0 ± 1.4
Gelatinase	59.0 ± 3.7

Data are shown as mean ± SD (n = 3 independent experiments).

This lower CD16 expression level was similar to that of neutrophils derived in vitro from bone marrow CD34$^+$ cells by stimulation with G-CSF (Kerst et al., 1993b) and to the effect in vivo when G-CSF is administered to healthy volunteers (Kerst et al., 1993a). These results indicated that the modified OP9 co-culture system could differentiate mature neutrophils from immature hiPSCs.

hiPSC-derived neutrophils contain neutrophil specific granules

Mature neutrophils in vivo have intracellular granules that are important for their bactericidal function. The granules can be

classified into three types based on their size, morphology, or electron density, or with reference to a given protein: primary (azurophilic) granules contain MPO, secondary granules contain lactoferrin, and tertiary granules contain gelatinase (Borregaard and Cowland, 1997).

To assess the presence of these granules in hiPSC-derived neutrophils, they were imaged using transmission electron microscopy, which showed that the hiPSC-derived mature neutrophils contained peroxidase-positive and negative granules, as was observed in peripheral blood neutrophils (Fig. 2A–B). Immunocytochemical analysis revealed that hiPSC-derived mature neutrophils were also positive for lactoferrin and gelatinase (Fig. 2C–D). The frequencies of cells that were positive for neutrophil specific granules, as observed by transmission electron microscopy (Table 2) and immunocytochemical analysis (Table 1), were more than 90% for primary granules, about 80% for secondary granules, and approximately 60% for tertiary granules. These results indicated that hiPSC-derived neutrophils contained neutrophils-specific granules.

hiPSC-derived neutrophils exhibit biological bactericidal activities

Because neutrophils patrol circulating blood and play a key role in early phase defense mechanisms, the chemotactic, phagocytotic, and bactericidal activities of hiPSC-derived neutrophils were analyzed.

Chemotactic activity was assessed using a modified Boyden chamber method (Boyden, 1962; Harvath et al., 1980). After incubation with or without fMLP in the lower well, neutrophils had migrated from the upper side to the lower side of the membrane. Incubation with fMLP caused an increase in the number of migrated cells of more than three times compared to cells without fMLP, suggesting that hiPSC-derived neutrophils had chemotactic activity in response to a chemoattractant similar to natural neutrophils derived from bone marrow (Fig. 3A).

The MPO-dependent chlorination activity and reactive oxygen production of hiPSC-derived neutrophils, which are

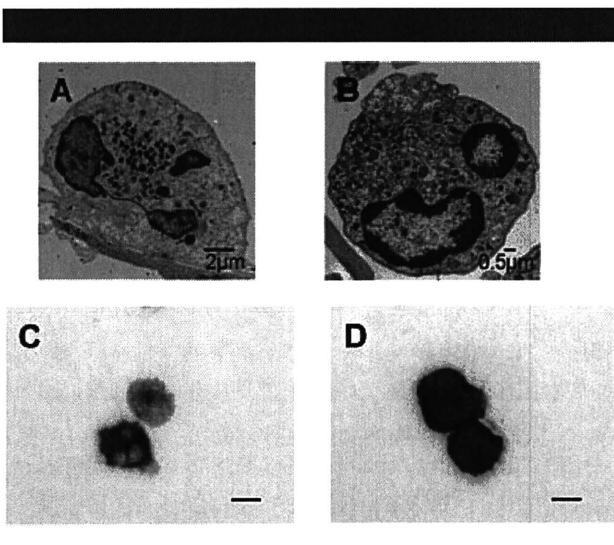

Fig. 2. Neutrophil-specific granules in hiPSC-derived neutrophils. (A–B) Floating cells on day10 + 30 (A) and peripheral blood neutrophils (B) were analyzed by transmission electron microscope. (C–D) Immunocytochemical analysis. Floating cells on day10 + 30 were stained for lactoferrin (C) and MMP9 (gelatinase) (D). Scale bars: 10 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 2. Frequency of positive cells for neutrophil specific granules under transmission electron microscopy

Granules	Frequency of positive cells (%)
Peroxidase-positive granules	95.1 (135/142)
Peroxidase-negative granules	86.6 (123/142)

both essential for their bactericidal function, were determined next. MPO reacts with hydrogen peroxide (H_2O_2) to form the active redox and enzyme intermediate compound MPO-I, which oxidizes chloride (Cl^-) to HOCl (Winterbourn, 2002). As shown in Figure 3B, hiPSC-derived neutrophils showed MPO-dependent chlorination activity. To evaluate reactive oxygen production, the ability to convert DHR to rhodamine was assessed using flow cytometry (Vowells et al., 1995) and the results revealed that hiPSC-derived neutrophils characteristically produced superoxide in response to PMA (Fig. 3C).

Finally, phagocytotic activity and phagosome-dependent reactive oxygen production were measured using luminol-bound microspheres (Uchida et al., 1985). As shown in Figure 3D, the captured data confirmed that hiPSC-derived neutrophils could produce reactive oxygen species in response to the phagocytosis of microspheres, which was completely abolished in the presence of the antiphagocytic agent cytochalasin B. Moreover, transmission electron microscopy successfully captured a screenshot of a neutrophil phagocytosing the microbeads (Fig. 3E). The above results clearly show that neutrophils derived from hiPSC using the present culture system maintain their functional status.

Step-wise neutrophil differentiation from hiPSCs is similar to normal granulopoiesis

Disorders of neutrophil differentiation are observed in various hematological diseases, among them the maturation arrest of neutrophil precursors in the bone marrow at the promyelocyte stage in severe congenital neutropenia. Thus, in clinical applications for disease investigation, the sequential analysis of the differentiation process from hiPSC to mature neutrophils in this culture system is required.

Observation of the sequential changes in cell morphology was done using May–Giemsa staining. Visualization of the morphology of day10 + 10 cells revealed that the cells were mainly myeloblasts and promyelocytes (Fig. 4A). On day10 + 20, myelocytes and metamyelocytes became predominant (Fig. 4B), and on day 10 + 30, stab and segmented neutrophils became predominant (Fig. 4C).

Surface antigen expression at each differentiation stage of hiPSC-derived cells was analyzed by flow cytometry (Fig. 4D). CD34, cell surface marker on normal immature hematopoietic cells, was detected in about 20% of the cells on day 10 + 10, but disappeared gradually thereafter. From day 10 + 10 to 10 + 30, the common myeloid antigens CD11b and CD33 were expressed in almost all the cells. Interestingly, expression of CD13, also a common myeloid antigen, was observed in less than 20% of cells at day 10 + 10 and did not subsequently increase. The expression level of CD16, which is a representative marker of matured neutrophils (van de Winkel and Anderson, 1991), doubled from day 10 + 10 to day 10 + 20, although the increase in expression was not statistically significant. These expression patterns were consistent with the patterns observed during normal neutrophil differentiation in healthy bone marrow (van Lochem et al., 2004).

The gene expression patterns of the pluripotency marker, transcription factors and granule proteins during neutrophil

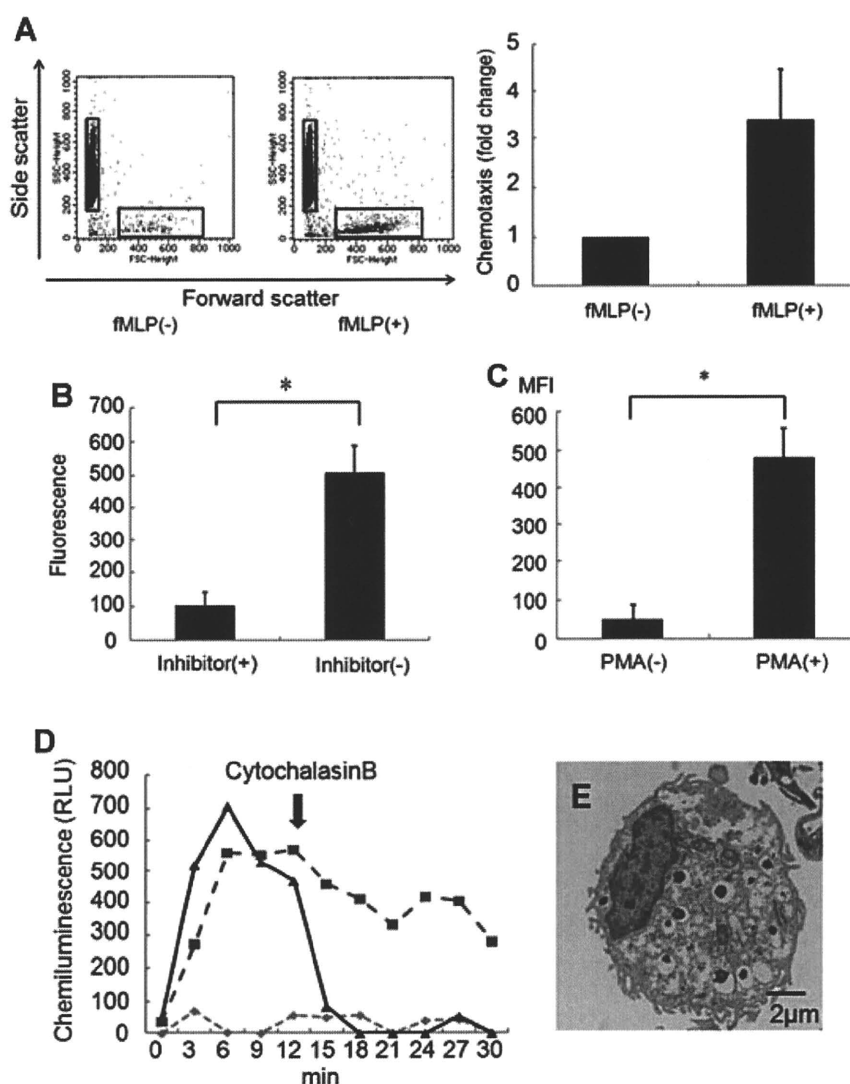

Fig. 3. Functional analysis of hiPSC-derived neutrophils. (A) Chemotactic activity of floating cells on day10 + 30 in response to fMLP was determined as described in Materials and Methods section. After a 4-h culture, the transwell inserts were removed, and the cells in the lower chamber were counted by an LSR flow cytometer (n = 3; bars represent SDs). (B) MPO chlorination activity in cell lysates from floating cells on day10 + 30 was analyzed by EnzChek Myeloperoxidase (MPO) Activity Assay Kit as described in the Materials and Methods section. The chlorination activity in neutrophil cell lysates was almost completely abolished by the addition of a chlorination inhibitor (n = 3; bars represent SDs; *$P < 0.05$.). (C) Floating cells on day10 + 30 were subjected to DHR assay. DHR was reacted with neutrophils with or without PMA, and the resultant rhodamine fluorescence was detected by flow cytometry. The addition of PMA increased the levels of fluorescence. Results are expressed as mean fluorescence intensity (MFI) (n = 3; bars represent SDs; *$P < 0.05$.). (D) Floating cells on day10 + 30 were subjected to the assay for phagocytosis-induced respiratory burst activity using chemiluminescent microspheres (luminol-binding microspheres). Gradual increase in chemiluminescence indicates the respiratory burst triggered by the phagocytosis of luminol-binding microspheres (squares). The increase in chemiluminescence was almost completely abolished by the addition of cytochalasin B (diamonds) and inhibited by its later addition (triangles). The figures are representative of three independent experiments. Abbreviation: RLU, relative light units. (E) hiPSC-derived neutrophils phagocytosing the microbeads were analyzed by transmission electron microscopy.

differentiation in this culture system were investigated by RT-PCR (Fig. 4E–F). NANOG, a pluripotency marker, was expressed in undifferentiated iPS cells but disappeared in sorted VEGFR2^{high}CD34^{+} cells after 10 days differentiation. PU.1 and C/EBPα, essential transcription factors for commitment and differentiation of the granulocytic lineage (Borregaard et al., 2001; Friedman, 2007) were first detected on day 10 + 10 and persisted thereafter. C/EBPε, which had a critical role for the later stages of neutrophil development and transcription of key granule proteins (Borregaard et al., 2001; Friedman, 2007) were first detected faintly on day 10 + 10 and upregulated thereafter. MPO and lactoferrin, which were expressed at the highest levels in myeloblasts/promyelocytes and myelocytes/metamyelocytes, respectively (Cowland and Borregaard, 1999; Borregaard et al., 2001), were detected on day 10 + 10. Gelatinase, which was expressed at the highest level in band and segmented neutrophilic cells (Cowland and Borregaard, 1999; Borregaard et al., 2001), was first detected on day 10 + 20 and upregulated thereafter. Altogether, these results suggested that the neutrophil differentiation in this co-culture system might recapitulate the orderly differentiation process in bone marrow.

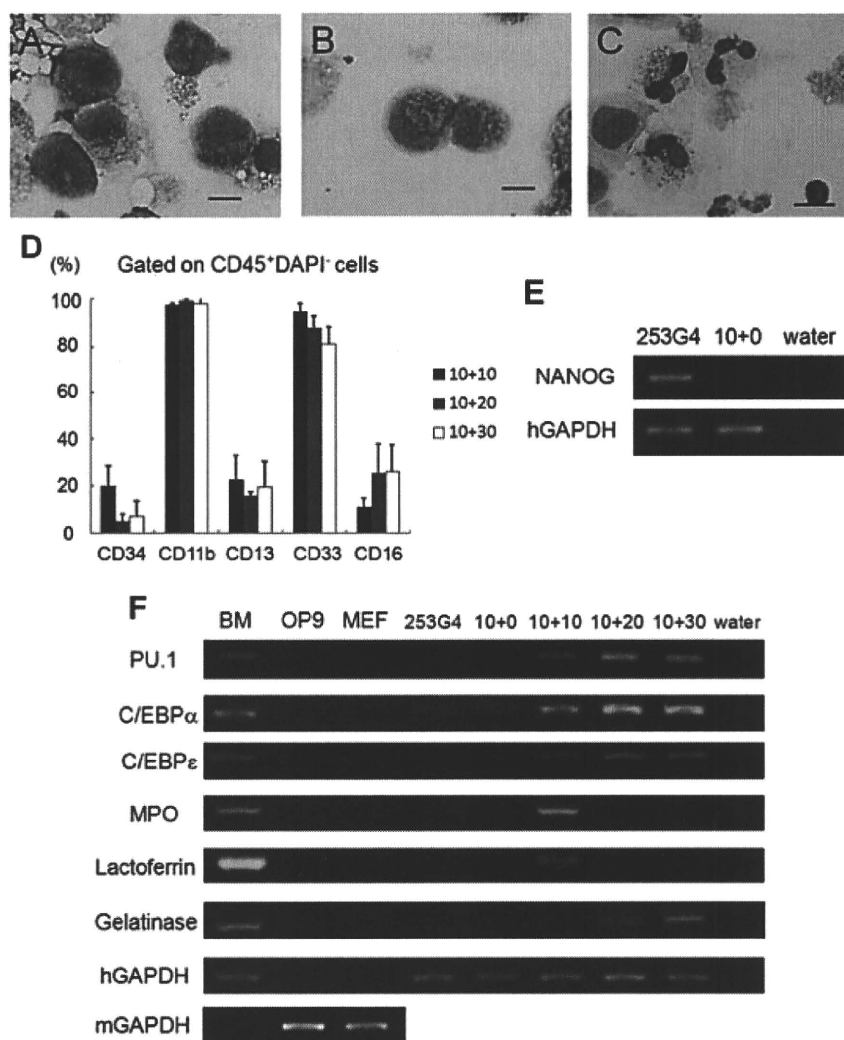

Fig. 4. Sequential analysis of neutrophil differentiation from hiPSCs. (A–C) Sequential morphological analysis of day10 + 10 (A), day10 + 20 (B) and day10 + 30 (C). Scale bars: 10 μm. (D) Surface antigen expression at each level of differentiation of hiPSC-derived cells was analyzed by flow cytometry. All adherent cells including OP9 cells were harvested and stained with antibodies. Human $CD45^+DAPI^-$ cells were gated as hiPSC-derived viable leukocytes (n = 3; bars represent SDs). (E–F) Sequential RT-PCR analysis of a pluripotency marker (E), genes associated with neutrophil development and neutrophils-specific granules (F) during differentiation. Human GAPDH was used as a loading control. Abbreviations: BM, human bone marrow cells; 253G4, undifferentiated 253G4 cells; 10 + 0, sorted $VEGFR2^{high}CD34^+$ cells after 10 days differentiation; 10 + 10, 20, 30, all cells after 10, 20, 30 days differentiation after cell sorting; hGAPDH, human GAPDH; mGAPDH, mouse GAPDH. The figures are representative of three independent experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Discussion

The analysis of the differentiation process of neutrophils can provide helpful information for the elucidation of the pathogenesis of hematopoietic diseases that affect neutrophils and/or myeloid differentiation, including inherited bone marrow failure syndromes and neutrophil function disorders. Traditionally, HL-60, an acute promyelocytic cell line, has been used as a neutrophil differentiation model (Collins et al., 1978; Newburger et al., 1979). Although this cell line grows well and differentiates easily into neutrophils, the neutrophil differentiation model is not suitable for the analysis of neutrophil-affected disorders because of its leukemic cell-origin. Development of a neutrophil differentiation system based on iPS cells would provide a better model for the analysis of such diseases, because iPS cells can be generated from the somatic cells of patients suffering from these diseases.

The current study aimed to investigate two issues in hiPSC-derived neutrophil differentiation: tracking the step-wise maturation in vitro and evaluating the wide spectrum of neutrophil functions. Through the use of a modified OP9 co-culture system, the directed and step-wise differentiation from hiPSCs to mature neutrophils containing neutrophil specific granules was first accomplished. The expression of surface antigens, transcription factors and granule proteins during differentiation exhibited the characteristic pattern of normal granulopoiesis. The biological functions of hiPSC-derived neutrophils were demonstrated through the quantitative assessment of granule enzyme activities and biological bactericidal activities such as chemotaxis and phagocytosis.

Defects in the maturation and function of neutrophils are associated with certain blood diseases including inherited bone marrow failure syndromes and neutrophil function disorders.

Among bone marrow failure syndromes, certain conditions affect a specific maturation stage, such as the maturation arrest at the plomyelocyte/myelocyte stage seen in severe congenital neutropenia. Neutrophil function disorders can affect specific bactericidal activities, such as the absence of MPO activity characteristic of MPO deficiency disorders. The use of hiPSCs for the investigation of these diseases requires sequential analyses that can identify each neutrophil maturation stage and include a functional analysis to evaluate each bactericidal activity separately on disease-specific, iPSC-derived neutrophils. Although previous studies have reported neutrophil differentiation models from hESCs (Choi et al., 2009; Saeki et al., 2009; Yokoyama et al., 2009) and hiPSC-derived neutrophils have been shown before (Choi et al., 2009), evidence showing that hiPSCs, which are artificially reprogrammed somatic cells, can follow the normal developmental pathway into fully functional mature neutrophils is of great significance, and the description of methods for identifying each neutrophil maturation step and analyzing each bactericidal pathway separately is important for clinical applications.

Although flow-cytometric analysis combined with RT-PCR identified the neutrophil maturation step relatively successfully, discrepancies between the neutrophil differentiation system in this study and normal granulopoiesis were noted such as the lower expression of CD16 than that shown by previous reports on hESC-derived neutrophils (Choi et al., 2009; Saeki et al., 2009; Yokoyama et al., 2009). As CD16 is a mature neutrophil marker in peripheral blood, two reasons could explain this phenomenon. First, residual precursors could have been more significant contaminants in the present system than in previously reported methods due to the function of cytokines and stroma supporting immature hematopoietic cells. Another possible reason is the shift of protein types between membrane-bound and soluble forms. Calluri previously reported that G-CSF is not only a myeloid cell growth factor, but also a modulator of neutrophil behavior (Carulli, 1997), and its stimulation decreases the membrane bound CD16 and increases its soluble form. Low CD16 expression has been documented in neutrophils derived in vitro from bone marrow $CD34^{+}$ cells by stimulation with G-CSF (Kerst et al., 1993b), and it has been observed in vivo when G-CSF is administered to healthy volunteers (Kerst et al., 1993a). This phenomenon, which is also documented in a report of hESC-derived neutrophils (Yokoyama et al., 2009), is unavoidable in differentiation culture systems using recombinant cytokines. The combination of flow cytometric and PCR analyses enables a more accurate staging of progenitors that could be of importance in the investigation of maturation arrest in future studies.

The culture system presented in this study is considered ineligible for clinical applications due to the use of xenogeneic factors such as OP9 cells and FCS. To overcome this problem, a xeno-free hematopoietic differentiation system from pluripotent cells is currently being established.

In conclusion, the present study shows the establishment of a fully functional mature neutrophil differentiation system from hiPSCs and the detailed analysis of their function and differentiation process. This system could become a useful tool for the investigation of various hematological diseases with defects in maturation and function of neutrophils.

Acknowledgments

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Predicting response to immunosuppressive therapy in childhood aplastic anemia

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ABSTRACT

In aplastic anemia, predictive markers of response to immunosuppressive therapy have not been well defined. We retrospectively evaluated whether clinical and laboratory findings before treatment could predict response in a pediatric cohort from the multicenter AA-97 study in Japan. Between 1997 and 2006, 312 newly diagnosed children were enrolled and treated with a combination of antithymocyte globulin and cyclosporine. In multivariate analyses, lower white blood cell count was the most significant predictive marker of better response; patients with white blood cell count less than 2.0×10^9/L showed a higher response rate than those with white blood cell count of 2.0×10^9/L or more (P=0.0003), followed by shorter interval between diagnosis and therapy (P=0.01), and male sex (P=0.03). In conclusion, pre-treatment clinical and laboratory findings influence response to therapy. The finding that response rate worsens with increasing interval between diagnosis and treatment highlights the importance of prompt immunosuppressive therapy for patients with aplastic anemia.

Key words: aplastic anemia, children, immunosuppressive therapy, predictive marker.

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Introduction

Aplastic anemia (AA) is defined as peripheral blood pancytopenia caused by bone marrow failure, and the pathogenesis is thought to involve autoimmune processes.[1-3] Several studies have confirmed immunosuppressive therapy (IST) with antithymocyte globulin (ATG) and cyclosporine (CyA) as a promising therapeutic option for patients lacking HLA-identical related donors.[4-8] Although several potential markers of IST response that appear to reflect the immune pathophysiology of aplastic anemia have been suggested, mainly from adult studies,[9-11] none have been widely accepted. We have already investigated the clinical relevance of HLA, a minor population of paroxysmal nocturnal hemoglobinuria-type cells, and a specific autoantibody associated with aplastic anemia in pediatric patients, finding no correlation between these markers and response to therapy.[12]

Some groups have recently shown that pre-treatment laboratory variables are associated with good response to immunosuppressive therapy, but those results remain controversial, as the numbers of children included in the study was relatively small and the drugs used for immunosuppressive therapy have not been consistent.[13-15] The present study, therefore, evaluated whether clinical and laboratory findings before treatment could predict immunosuppressive therapy response in a large population of children with aplastic anemia enrolled in a multi-center study.

Design and Methods

Patients

Between October 1997 and September 2006, a total of 312 Japanese children with aplastic anemia (AA) from 118 hospitals were enrolled in the AA-97 multicenter study conducted by the Japan Childhood Aplastic Anemia Study Group. Patients with acquired AA were eligible if the following criteria were met: age under 18 years; newly diagnosed disease (≤180 days) without specific prior treatment; and moderate to very severe AA. The disease was considered severe if at least 2 of the following were noted: neutrophil count less than 0.5×10^9/L; platelet count less than 20×10^9/L; or reticulocyte count less than 20×10^9/L with hypocellular bone marrow.[16] AA was considered very severe if the criteria for severe disease were fulfilled and neutrophil count was less than 0.2×10^9/L. Moderate disease was defined by at least 2 of the following: neutrophil count less than 1.0×10^9/L; platelet count less than 50 $\times10^9$/L; or reticulocyte count less than 60×10^9/L.[6] Patients with congenital AA or paroxysmal nocturnal hemoglobinuria were excluded. Allogeneic stem cell transplantation was recommended for patients with severe or very severe disease who had an HLA-matched sibling, so these patients were not included in the AA-97 study. Written informed consent was obtained from all parents and all patients over the age of ten years. All study protocols were approved by the ethics committee of each participating hospital. The study also conforms to the recently revised Declaration of Helsinki.

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IST

All patients were treated with a combination of intravenous ATG (Lymphoglobulin; Genzyme, Cambridge, USA) at 15 mg/kg/day for five days and oral CyA at 6 mg/kg/day. The dose of CyA was adjusted to maintain trough levels between 100 and 200 ng/mL, and the appropriate dose was administered for at least six months. Granulocyte colony-stimulating factor (Filgrastim; Kirin, Tokyo, Japan) was administered intravenously or subcutaneously at 400 $\mu g/m^2$ for three months only to patients with very severe disease.[17] Response to IST was evaluated at six months after initiation of therapy. Complete response (CR) was defined as a neutrophil count more than 1.5×10^9/L, a platelet count more than 100×10^9/L, and a hemoglobin level more than 11.0 g/dL.[17] Partial response (PR) was defined as a neutrophil count more than 0.5×10^9/L, a platelet count more than 20×10^9/L, and a hemoglobin level more than 8.0 g/dL in patients with severe or very severe AA, and as a neutrophil count more than 1.0×10^9/L, a platelet count more than 30×10^9/L, and a hemoglobin level more than 8.0 g/dL in patients with moderate AA.[17] Overall response was defined as CR or PR at six months after IST.

Statistical analyses

Parameters for univariate analyses to determine predictors of response to IST included age at diagnosis, sex, interval between diagnosis and treatment, etiology, severity of disease, white blood cell (WBC) count, neutrophil count, lymphocyte count, hemoglobin level, reticulocyte count, and platelet count. Pre-treatment laboratory values were defined as the lowest value without transfusions during the four weeks preceding IST. Continuous variables were divided into quartile categories, and these cut offs were used for categorical analysis. To evaluate correlations between these parameters and response, differences in continuous variables were analyzed using the Mann-Whitney U-test and differences in frequencies were tested using the χ^2 or Fisher's exact test. For multivariate analyses, logistic regression modeling was performed. Important covariates in the multivariate models were chosen using stepwise variable selection procedures. Values of $P<0.05$ were considered statistically significant.

Results and Discussion

Patients' characteristics are shown in Table 1. A total of 312 patients fulfilled the eligibility criteria. Median age at diagnosis was eight years. Severity of AA was considered very severe in 156 patients, severe in 107 patients, and moderate in 49 patients. The median interval between diagnosis and treatment was 15 days. A total of 176 of the 312 (56.4%) patients improved with IST and achieved PR (n=131) or CR (n=45) at six months. All of them achieved transfusion independence.

To determine predictors of IST response, we compared differences in potential pre-treatment variables between IST responders and non-responders. The following were analyzed both for prevalence in categorical variables and differences in continuous variables: age at diagnosis, interval between diagnosis and treatment, WBC count, neutrophil count, lymphocyte count, hemoglobin level, reticulocyte count, and platelet count. In univariate analyses, WBC count, lymphocyte count, interval between diagnosis and therapy, and gender showed associations with IST response (Table 2). We also performed multivariate logistic regression analysis to assess the simultaneous contributions of each of the variables in predicting response. In these analyses, lower WBC count (P=0.0003), shorter interval between diagnosis and therapy (P=0.012), and male sex (P=0.036) represented significant predictors of better response (Table 2).

Boys displayed better response than girls (Figure 1A). This relationship was also observed in a retrospective European study in which a young female cohort experienced delayed recovery of bone marrow function following IST.[18] Median WBC count before treatment was significantly lower in patients who achieved response (1.9×10^9/L) than in those who did not (2.3×10^9/L; P= 0.007). In addition to the analysis with continuous variable, lower WBC count according to categorical analysis also associated with favorable response, with 93 of 144 patients (65%) with WBC less than 2.0×10^9/L and 83 of 168 patients (49%) with WBC of 2.0×10^9/L or more showing improvement with IST (P=0.009; Figure 1B). When lymphocyte count was applied to the analysis instead of WBC count, a correlation between lower lymphocyte count and response to IST was also observed (Table 2); 82 of 123 patients (67%) with lymphocyte count less than 1.5×10^9/L improved with IST, a significantly higher frequency than the 94 of 189 patients (50%) with lymphocyte count of 1.5×10^9/L or more who improved with IST (P=0.004). Neither neutrophil count nor severity of disease was predictive of response.

Regarding the association between pre-treatment neutrophil count and response, conflicting results have been reported. A European study reported superior response rates in children with very severe AA compared to severe AA[5] but, in contrast, some studies including a recent report of a Korean cohort of adult patients have produced the opposite results.[13,19] The present findings differ from those published studies, with favorable responses correlating well with lower WBC count rather than neutrophil count or disease severity. Indeed, WBC count was the strongest predictor of response to IST in multivariate analysis. In patients with AA, pre-treatment WBC count may mainly reflect the size of lymphocyte populations, due to the severe neutropenia in this condition. These results suggest that poor response to IST might possibly be ascribed to higher WBC

Table 1. Patients' characteristics.

N. of patients		312
Age at diagnosis, years, median (range)		8 (1-17)
Gender	male / female	186 /126
Etiology	n. of patients (%)	
Idiopathic		261 (83.7)
Hepatitis		44 (14.1)
Others		7 (2.2)
Severity of AA	n. of patients (%)	
VSAA		156 (50.0)
SAA		107 (34.3)
MAA		49 (15.7)
Peripheral blood data at diagnosis		
Median WBC count, $\times10^9$/L (range)		2.02 (0.20-8.70)
Median neutrophil count, $\times10^9$/L (range)		0.22 (0.00-3.13)
Median lymphocyte count, $\times10^9$/L (range)		1.82 (0.10-8.50)
Median Hb level, g/dl (range)		6.9 (2.1-13.2)
Median reticulocyte count, $\times10^9$/L (range)		16.0 (0.0-98.0)
Median platelet count, $\times10^9$/L (range)		11.0 (1.0-109.0)
Interval from diagnosis to treatment, days, median (range)		15 (1-180)

VSAA: very severe aplastic anemia; SAA: severe aplastic anemia; MAA: moderate aplastic anemia; WBC: white blood cell; Hb: hemoglobin.

Table 2. Univariate and multivariate analysis for IST response in 312 patients with AA.

Univariate variables	Responder	Non-responder	P
N. of patients (%)	176 (56.4)	136 (43.6)	
Median age at diagnosis, years	8	8	NS
Gender, male / female	115/61	71/65	0.025
Etiology, n. of patients (%)			
Idiopathic	141(80)	120(88)	NS
Hepatitis	29(17)	15(11)	
Others	6(3)	1(1)	
Severity of AA, n. of patients (%)			
VSAA	90(51)	66(49)	NS
SAA	62(35)	45(33)	
MAA	24(14)	25(18)	
Median WBC count, $\times10^9$/L	1.900	2.255	0.007
$\geq 2.0\times10^9$/L, n. of patients (%)	87(47)	85(63)	0.009
$< 2.0\times10^9$/L, n. of patients (%)	93(53)	51(37)	
Median lymphocyte count, $\times10^9$/L	1.600	2.016	0.006
Median neutrophil count, $\times10^9$/L	0.218	0.200	NS
Median Hb level, g/dl	6.8	6.8	NS
Median reticulocyte count, $\times10^9$/L	15.730	17.600	NS
Median platelet count, $\times10^9$/L	10.000	11.000	NS
Interval from diagnosis to treatment, days	13	19	0.002

Multivariate variables	Odds ratio	95% CI	P
WBC count, $< 2.0\times10^9$/L	3.219	1.707-6.070	0.0003
Interval from diagnosis to treatment, < 30 days	2.571	1.225-5.396	0.012
Gender, male	1.873	1.042-3.366	0.036
Reticulocyte count, $>25\times10^9$/L	1.589	0.843-2.997	NS
Platelet count, $>20\times10^9$/L	1.362	0.657-2.826	NS
Etiology, hepatitis/others	1.223	0.504-2.966	NS

VSAA: very severe aplastic anemia; SAA, severe aplastic anemia; MAA, moderate aplastic anemia; WBC, white blood cell; Hb, hemoglobin.

count, that is, a relative increase in lymphocytes. Given the dramatic effects of T-cell suppressants including ATG and CyA on *in vivo* hematopoiesis, autoreactive T-cell responses against hematopoietic stem cells have been suggested to play a major role in the pathogenesis of AA, and *in vitro* studies have also supplied supportive evidence for this idea. Early experiments demonstrated inhibitory effects of autologous lymphocytes on hematopoietic progenitor cell growth through overproduction of cytokines such as interferon-γ and tumor necrosis factor-α by activated cytotoxic T cells in AA patients.[20-22] More recently, oligoclonal T-cell expansions have been described in AA patients, disappearing with clinical improvement following IST.[23] Taking our results and previous findings together, a higher WBC count before treatment may indicate the presence of numerous autoreactive T cells that need to be eliminated and thus a high potential to destroy marrow function through lymphocytes, rather than better residual marrow function. In this scenario, patients with a lower WBC count could be seen to have a better probability of hematopoietic recovery following IST.

We identified a significantly inverse correlation between response and interval between diagnosis and treatment; median intervals among responders and non-responders were 13 and 19 days, respectively (*P*=0.002). In categorical analysis, response rates of patients with intervals less than 30 and of 30 days or more were 60% and 43%, respectively (*P*=0.013). Figure 1C clearly indicates the inverse relationship. Notably, response rates to IST were considerably low among AA patients with long-standing disease; only 35% of patients treated 90 days or more after diagnosis responded, suggesting that patients with this condition may receive irreversible damage to hematopoietic progenitor cells or stromal elements that progresses over time, possibly due to

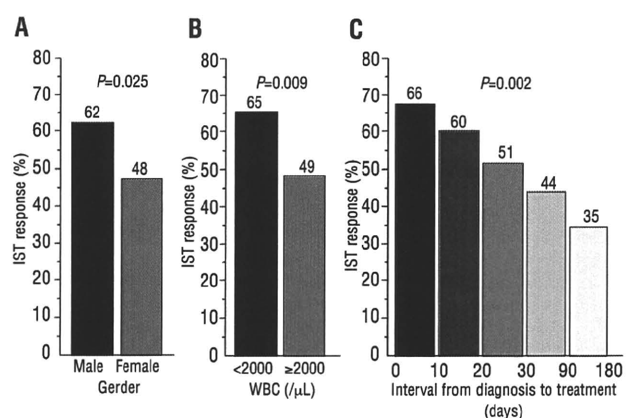

Figure 1. Response to IST in 312 patients according to WBC count, gender, and interval from diagnosis to treatment. (A) Response rate according to gender. Boys showed better response than girls (62% *vs.* 48%, respectively; *P*=0.025). (B) Response rate according to WBC counts. Patients with WBC count <2.0×10⁹/L displayed a significantly higher response rate than patients with WBC ≥2.0×10⁹/L (65% *vs.* 49%, respectively; *P*=0.009). (C) Response rate according to the interval between diagnosis and treatment. Response rate was inversely associated with the interval between diagnosis and treatment (*P*=0.002).

immune attack through autoreactivated lymphocytes. The present study indicates the importance of prompt IST therapy for patients with AA. We, therefore, recommend offering IST as soon as possible in all children with AA who lack a matched sibling donor.

Other variables did not differ significantly between responders and non-responders (Table 2). Particularly with regard to reticulocyte count, 122 patients showed reticulocyte count more than 25×10^9/L, of whom 67 (55%) responded to IST, and 186 patients had reticulocyte count of 25×10^9/L or less, of whom 107 (58%) responded to IST. Correlations of higher reticulocyte count and higher lymphocyte count at initial diagnosis with better response to IST in patients of all ages have recently been described by the National Institutes of Health (NIH) group.[15] However, when the same analysis was applied to their 77 pediatric patients, lymphocyte count was not predictive.[14] More recently, another relatively small study in adults with AA found no such association.[13] These studies were limited by inconsistency of regimens used for IST. The current study investigated a large cohort of children with AA treated using a unified regimen, but failed to confirm any correlation between reticulocyte count and response to IST, suggesting a limited contribution of this clinical parameter to the prediction of hematopoietic recovery, at least in children.

In conclusion, pre-treatment clinical and laboratory findings influence response to IST. Favorable response correlates better with lower WBC count than with neutrophil count or disease severity, and this blood count parameter might help in clinically assessing bone marrow function. Unlike the situation in adult AA, reticulocyte count is not predictive of response to IST in pediatric patients. IST should be started as soon as possible after diagnosis of AA, given that the response rate worsens as the interval between diagnosis and treatment increases.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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Brief report

Autoimmune lymphoproliferative syndrome–like disease with somatic *KRAS* mutation

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Autoimmune lymphoproliferative syndrome (ALPS) is classically defined as a disease with defective FAS-mediated apoptosis (type I-III). Germline *NRAS* mutation was recently identified in type IV ALPS. We report 2 cases with ALPS-like disease with somatic *KRAS* mutation. Both cases were characterized by prominent autoimmune cytopenia and lymphoadenopathy/splenomegaly. These patients did not satisfy the diagnostic criteria for ALPS or juvenile myelomonocytic leukemia and are probably defined as a new disease entity of RAS-associated ALPS-like disease (RALD). (*Blood*. 2011;117(10):2887-2890)

Introduction

Autoimmune lymphoproliferative syndrome (ALPS) is a disease characterized by dysfunction of the FAS-mediated apoptotic pathway,[1,2] currently categorized as: type Ia, germline *TNFRSF6/FAS* mutation; type Ib, germline *FAS ligand* mutation; type Is, somatic *TNFRSF6/FAS* mutation; and type II, germline *Caspase 10* mutation. Patients exhibit lymphadenopathy, hepatosplenomegaly, and autoimmune diseases, such as immune cytopenia and hyper-γ-globulinemia. An additional subclassification has been proposed that includes types III and IV, whereby type III has been defined as that with no known mutation but with a defect in FAS-mediated apoptosis and type IV as one showing germline *NRAS* mutation.[3] Type IV is considered exceptional because the FAS-dependent apoptosis pathway is not involved in the pathogenesis, and this subclass is characterized by a resistance to interleukin-2 (IL-2) depletion-dependent apoptosis. Recent updated criteria and classification of ALPS suggested type IV ALPS as a RAS-associated leukoproliferative disease.[4]

Juvenile myelomonocytic leukemia (JMML) is a chronic leukemia in children. Patients show lymphadenopathy, hepatosplenomegaly, leukocytosis associated with monocytosis, anemia, thrombocytopenia, and occasional autoimmune phenotypes. Approximately 80% of patients with JMML have been shown to have a genetic abnormality in their leukemia cells, including mutations of *NF1*, *RAS* family,[5] *CBL*, or *PTPN11*. The hallmarks of the laboratory findings of JMML include spontaneous colony formation in bone marrow (BM) or peripheral blood mononuclear cells (MNCs) and hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) of $CD34^+$ BM-MNCs.[6]

Germline RAS pathway mutations cause Costello (*HRAS*), Noonan (*PTPN11*, *KRAS*, and *SOS1*), and cardio-facio-cutaneous syndromes (*KRAS*, *BRAF*, *MEK1*, and *MEK2*). Patients with Costello and Noonan syndromes have an increased propensity to develop solid and hematopoietic tumors, respectively[7]; among these tumors, the incidence of JMML in patients with germline mutation of *NF1* or *PTPN11* is well known.

We present 2 cases with autoimmune cytopenia and remarkable lymphadenopathy and hepatosplenomegaly, both of which were identified as having a somatic KRAS G13D mutation without any clinical features of germline *RAS* mutation, such as cardio-facio-cutaneous or Noonan syndrome.

Methods

All studies were approved by the ethical board of Tokyo Medical and Dental University.

Case 1

A 9-month-old boy had enormous bilateral cervical lymphadenopathy and hepatosplenomegaly (supplemental Figure 1A-B, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Blood test revealed the presence of hemolytic anemia and autoimmune thrombocytopenia. Hyper-γ-globulinemia with various autoantibodies was also noted. ALPS and JMML were nominated as the diseases to be differentially diagnosed. Detailed clinical history and laboratory data are provided as Supplemental data. The patient did not satisfy the criteria for the diagnosis of ALPS or JMML as discussed in "Results and discussion."

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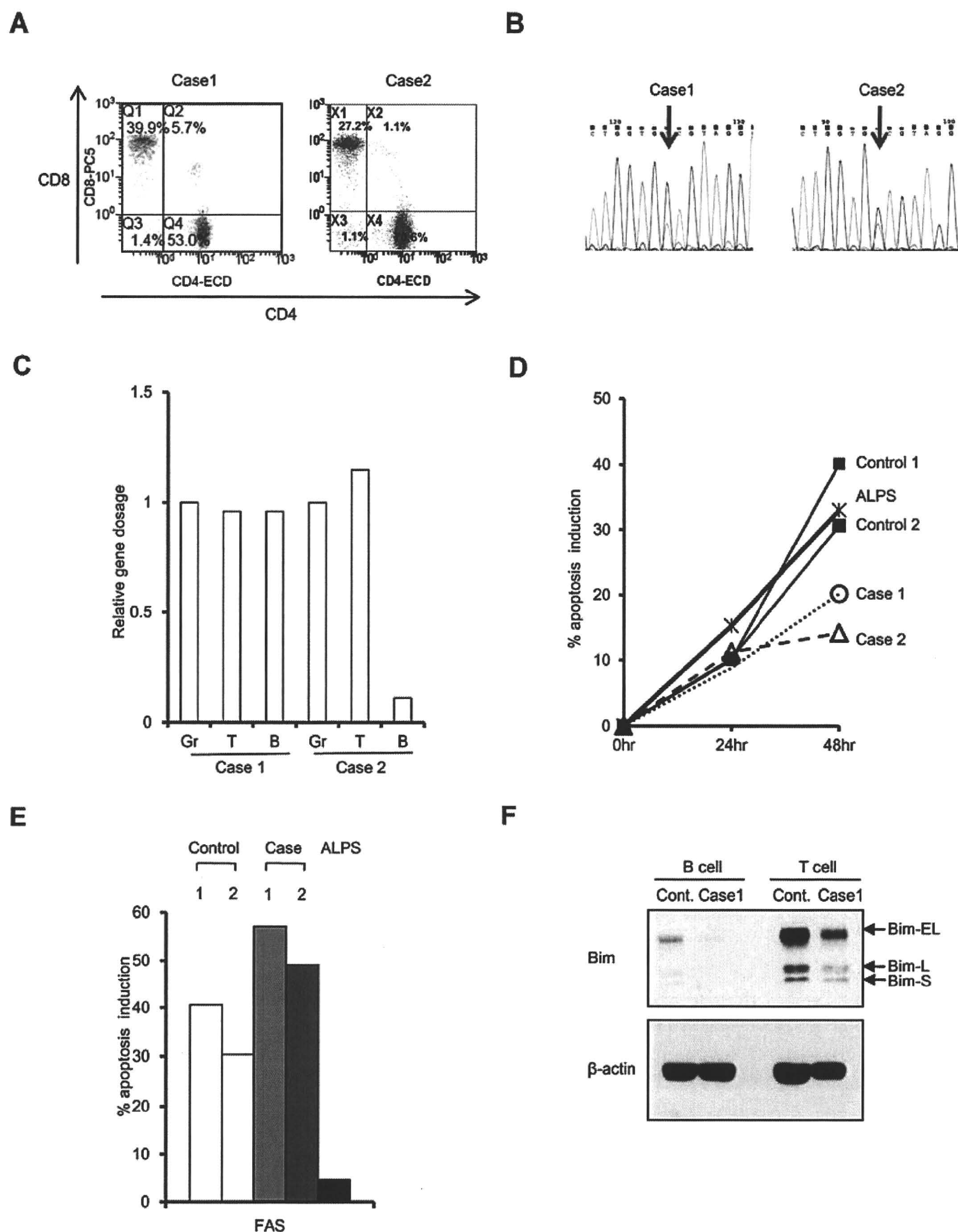

Figure 1. Molecular cell biologic assay of RALD. (A) Flow cytometric analysis of double-negative T cells. CD8 and CD4 double staining was performed in T-cell receptor-αβ-expressing cells. (B) Electropherogram showing KRAS G13D mutation in BM-MNCs in case 1 (left panel) and case 2 (right panel). (C) Gene dosage of mutated allele in granulocytes (Gr), T cells (T), and B cells (B). Relative gene dosage was estimated by a mutant allele-specific polymerase chain reaction method in cases 1 and 2 using albumin gene as internal control. (D) Apoptosis assay using activated T cells. Apoptosis percentage was measured by flow cytometry with annexin V staining 24 and 48 hours after IL-2 depletion. (E) Apoptosis percentage was measured 24 hours after addition of anti-FAS CH11 antibody (final 100 ng/mL). (F) Western blotting analysis of Bim expression.

Case 2

A 5-month-old girl had a fever and massive hepatosplenomegaly (supplemental Figure 1D). She was initially diagnosed with Evans syndrome based on the presence of hemolytic anemia and autoimmune thrombocytopenia with hyper-γ-globulinemia and autoantibodies. Spontaneous colony formation assay and GM-CSF hypersensitivity of BM-MNCs showed positivity. Then, tentative diagnosis of JMML was given, even though she showed no massive monocytosis or increased fetal hemoglobin. Detailed clinical history and laboratory data are provided in supplemental data.

Detailed methods for experiments are described in supplemental data.

Results and discussion

Case 1 showed a high likelihood of being a case of ALPS according to the symptoms and clinical data presented (supplemental Table 1), except for number of double-negative T cells, which was only 1.4% of T-cell receptor-αβ cells (Figure 1A). JMML was also nominated as a disease to be differentiated because remarkable hepatosplenomegaly with thrombocythemia and moderate monocytosis was