chromosome, we detected no additional chromosomal abnormalities after performing the feeder- and cytokine-free cultures as demonstrated by G-banding karyotypipng studies (Fig. 1F). This finding excludes the possibility that a particular subclone of primate ES cells with growth advantages had been selected during the feeder- and cytokine-free cultures. Our novel culture protocol not only enables the maintenance of primate ES cells with lower costs but also excludes possible artificial effects by excess amounts of cytokines, providing more natural conditions for ES cell growth.

Formation of a sac-like structure is a critical step for the subsequent hematopoietic differentiation

Next we tried to induce the *in vitro* hematopoietic differentiation of ES cells maintained under the natural condition described above. Total process of differentiation culture sytem is summarized in Figure 1A. For this purpose, embryoid bodies (EBs) were generated (Fig. 2A and B) by culturing the dissociated ES cells in a floating state for 2 weeks in the presence of cytokine cocktail including BMP-4, SCF, Flt3-L, IL-3, IL-6, and G-CSF as reported by Chadwick et al. (2003). Because cell spheres including EBs can hardly proliferate as long as they are under floating conditions, we transferred the EBs onto gelatin-coated plates and subsequently cultured the cells under attached conditions (Fig. 2A). The EBs became flattened after overnight culture and continued to spread (Fig. 2A). Interestingly, after 2-week adherent culture, a sac-like structure emerged at the center of flattened EB, and in a few days, it became filled with abundant round cells (Fig. 2C). At this point, total cells including sac-like structures and peripheral spreading cells, were removed by trypsin/EDTA treatment and transferred onto new gelatin-coated plates. The cells actively proliferated and grew to confluence within a few days (Fig. 3A). After another few day culture, round cells appeared again, either floating in the supernatant or softly grounding on the adherent cells (Fig. 3B). The number of floating cells increased dramatically during the following culture (Fig. 3C). The floating round cells (Fig. 3D, upper) expressed high levels of CD45, a pan-hematopoietic cell marker (Fig. 3D, lower), whereas the adherent spindle-shaped cells (Fig. 3E, upper) expressed only low levels of CD45 (Fig. 3E, lower). This condition, where an expansion of $CD45^{low}$-adherent cells to confluence after subculture onto new gelatin-coated plates was followed by an emergence of abundant $CD45^{high}$-floating cells, was steadily repeated at every passage for about 3 months.

Evaluation of hematopoietic cells induced from primate ES cells in our culture method

The floating cells were collected from the culture supernatant and were analyzed morphologically and histologically. Wright-Giemsa (WG) staining indicated the presence the cells with various stages of granulocyte- and monocyte/macrophage-lineage (Fig. 4A, upper left). Because ES cells preferentially differentiate into monocyte-macrophage lineage (Borzillo et al., 1990), a detection of granulocytic differentiation would be an important point for the evaluation our differentiation system. Although percentages of polymorphonuclear neutrophils varied between 10 to 20 depending on the timing and/or the experiments, their functional maturation was reproducibly demonstrated by following studies.

First, we performed special staining studies specific for neutrophilic granulocytes. A myeloperoxidase (MPO) staining study showed the presence of granulocyte- and monocyte/macrophage-lineage cells (Fig. 4A, upper right), confirming the results of WG staining. An esterase double-staining study (α-naphthyl butyrate esterase and naphthol-ASD-chloroacetate esterase) (Fig. 4A, lower left) further showed the presence of granulocyte-lineage cells, whose cytoplasm were stained blue by naphthol-ASD-chloroacetate esterase. Neutrophil alkaline phosphatase (NAP) staining study showed the presence of NAP-positive neutrophils (Fig. 4A, lower right). In addition, flow cytometric analysis for leukocyte marker was performed. As shown in Figure 4B, majority of the floating cells (>90%) were positive for CD11b, a leukocyte-specific adhesion molecule.

Finally, we evaluated the capacities related to bactericidal functions. First, the floating cells showed high phagocytic activities against latex beads (Fig. 4C and D). A combination of a zymosan-uptaking test and WG staining further showed the presence of phagocytic activities in polymorphonuclear neutrophils (Fig. 4E and F). Moreover, the respiratory burst activities were demonstrated by NBT-reducing assay (Fig. 4G and H), where approximately 80% of the cells

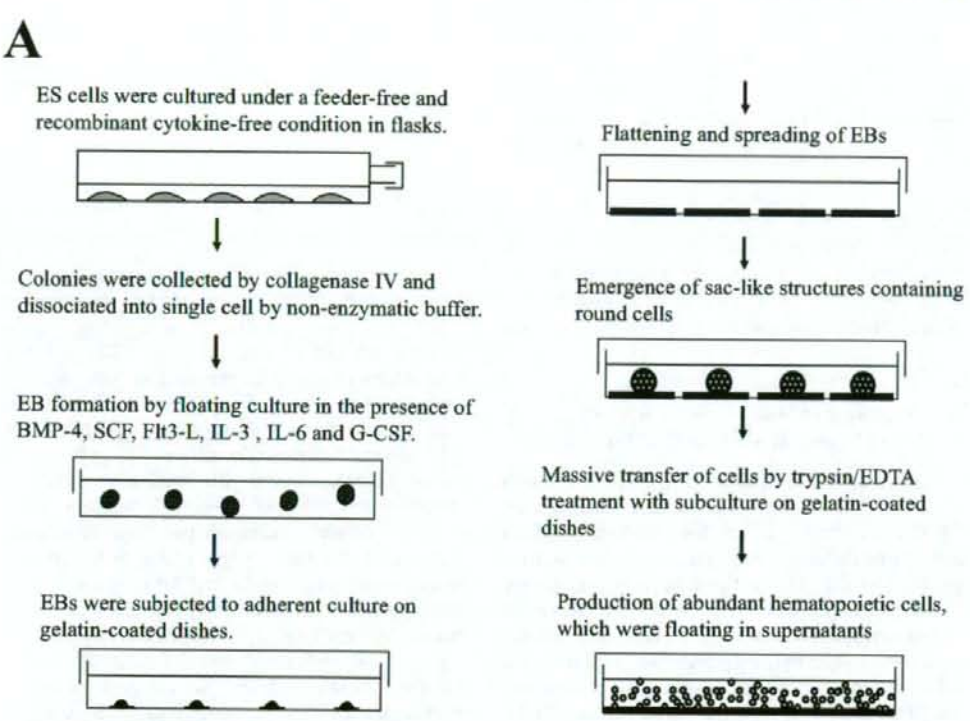

FIG. 2. Embryoid body (EB) formation and subsequent adherent culture process. (**A**) Schematic presentation of the differentiation process. (**B**) Phase contrast microscopy of EBs, which were generated by a 2-week floating culture as described in Materials and Methods. The scale bar indicates 100 μm. (**C**) EBs were transferred onto gelatin-coated plates *en bloc* without cell dissociation. After about a 2-week culture, sac-like structures filled by abundant round cells were generated. The scale bar indicates 100 μm.

were positively stained with reduced blue-black formazan particles. We also examined the chemotactic activity of ES-derived neutrophils by the transwell migration assay. The floating cells were incubated in the upper chamber and chemotaxis in response to bacterial chemoattractant fMLP was quantified by counting the number of polymorphonuclear neutrophils attached on the lower side of the membrane that separated the upper and lower chambers. Although there were a few polymorphonuclear neutrophils that transmigrated through the membrane in the absence of fMLP due to random movement, the presence of fMLP markedly increased the numbers of transmigrated polymorphonuclear neutrophils as detected by WG staining (Fig. 5A). The total number of polymorphonuclear neutrophils on the membranes in fMLP-untreated samples and those in fMLP-treated samples were 5.6 ± 3.2 ($n = 3$) and 174.7 ± 79.8 ($n = 3$), respectively ($p = 0.022$)

Thus, our feeder-free differentiation system enables preparation of high percentage CD45-positive hematopoietic cell populations from primate ES cells without a cell-sorting procedure, guaranteeing the generation of mature functional neutrophils.

Gene expressions during the differentiation process

In regard to the hematopoietic differentiation of ES cells, gene expression profiles have been investigated using RT-PCR analyses (Keller et al., 2003). To understand the gene expression during primate ES cell differentiation toward neutrophils, we prepared total RNAs from primitive ES cells (Fig. 6, lane 1), sac-like structures (Fig. 6,

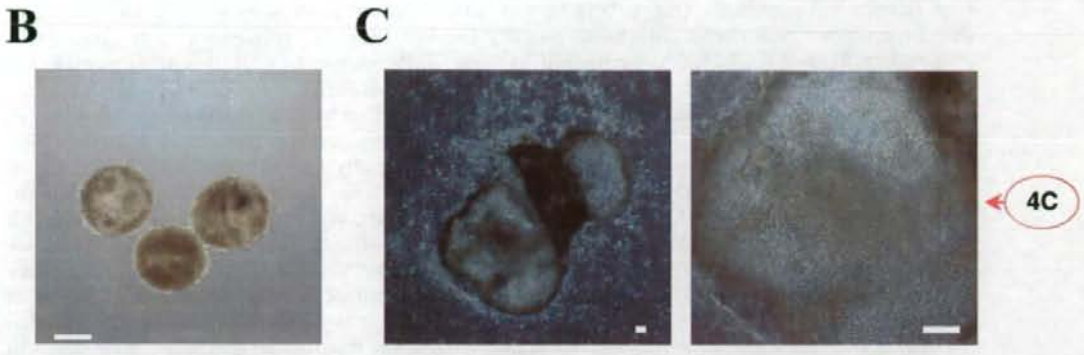

FIG. 2. (*Continued*).

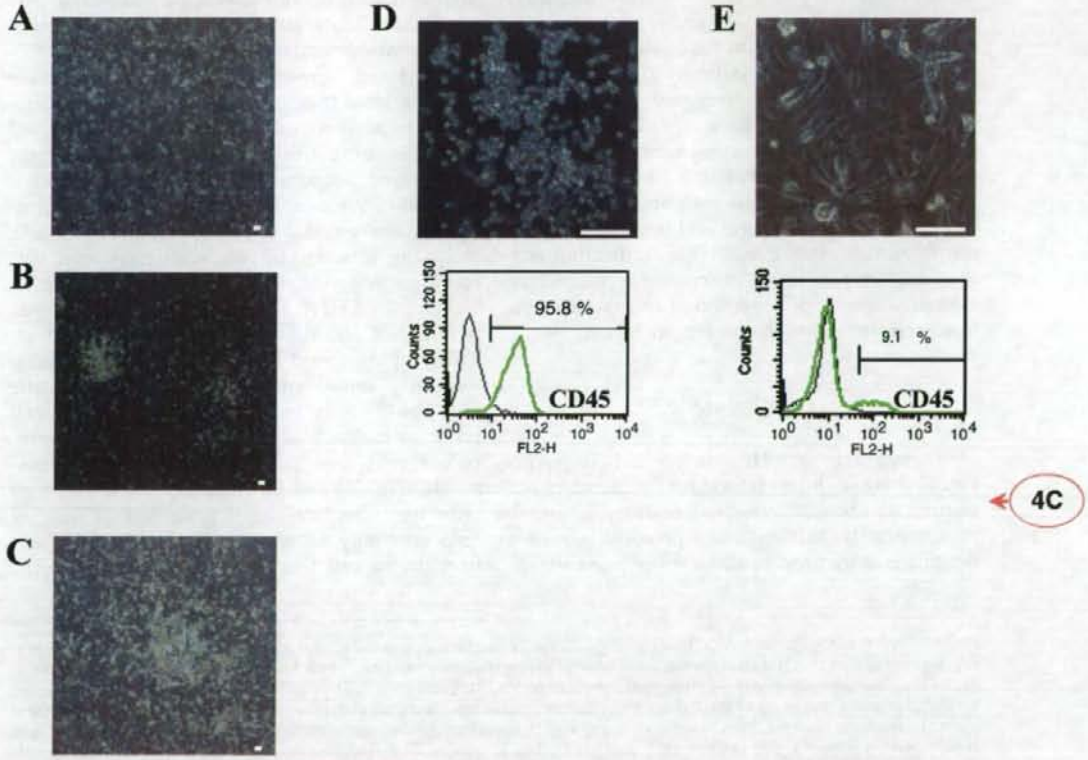

FIG. 3. Adherent subculture of EB-derived cells and subsequent hematopoiesis process. (A) A phase contrast microscopy of the adherent cells that expanded to confluence after massive transfer of EB-derived cells onto new plates. (B, C) Phase contrast microscopies of the cells at the next day (B) and the 6th day (C) of the culture shown in (A). (D) A phase-contrast microscopy of floating round cells with higher magnification (upper) and the flow cytometric analysis of these cells on the CD45 expression (lower). (E) A phase-contrast microscopy of adherent spindle-shaped cells with higher magnification (upper) and the flow cytometric analysis of these cells on the CD45 expression (lower). The scale bar indicates 100 μm.

lane 2), adherent population (Fig. 6, lane 3), and floating endpoint cells used in functional assays (Fig. 6, lane 4), and RT-PCR was performed using primate-specific primers for Oct-4 and Nanog, primitive ES cell markers, Flk-1, an immature mesoderm marker, GATA-2 and PU.1, hematopoietic cell-specific transcription factors, CD10, an immature B cell marker, neutrophil cytosolic factor 1 (NCF-1), a 47-kDa component of phagocytic NADPH oxidase complex, embryonic hemoglobin subunits (ε- and ζ-globins) and fetal hemoglobin subunit (γ-globin) as well as α-globin subunit.

As shown in Figure 6, a gradual reduction of Oct-4 throughout the differentiation process and an abrupt disappearance of Nanog in the phases later than the sac stage were observed. The expressions of Flk-1 and GATA-2 were definitely upregulated at the sac stage and remained at constant levels thereafter. On the other hand, the expression of CD10 was induced at the sac stage and declined thereafter. In regard to the myeloid and/or phagocytic markers, a gradual upregulation in PU.1 and NCF-1 expressions was observed throughout the differentiation process. The expressions of ε-, ζ-, γ-, and α-globins were readily detectable at the sac stages and remained high in the floating hematopoietic cells, indicating erythroid differentiation in our culture system. Thus, hematopoietic differentiation of our culture system is confirmed at the molecular level.

DISCUSSION

We reported a novel feeder-free and exogenous cytokine-free culture method for the maintenance of primate ES cells. We also presented a unique hematopoietic differentiation protocol, where a floating culture process and a subsequent attachment culture process were combined. This protocol enables high efficiency (>90%) production of CD45-positive or CD11b-positive hematopoietic cells; majority of the cells belonged to granulocyte or monocyte/macrophage lineages. In addition, this system guarantees the production of functionally mature neutrophils. By virtue of the application of monolayer adherent culture in later phases, we, for the first time, identified a unique cell construction of sac-like structure filled with abundant round cells as a precursory organization of hematopoiesis from ES cells *in vitro*. This construction also produces endothelial cells, under a particular condition (manuscript in preparation by K. Saeki). Thus, the sac-like structure contains precursors of both hematopoietic and endothelial cells, although the existence of bipotential hemangioblasts remains elusive. Our preliminary data showed that the inner round cells inevitably underwent cell death as they proliferated and were packed solid in a sac. So we had to release them into culture supernatant before they proliferated to fullness via dissection of sac walls using a microknife under microscopic observation. Because this manual operation is not so feasible, we adopted a total cell passage protocol as we reported in this paper, where all cells including round cells, sac wall cells, and surrounding adherent cells were massively collected by trypsin–EDTA treatment, transferred onto new dishes, and subcultured. By this method, viability of the round hematopoietic cells was significantly improved. Moreover, hematopoietic cells could easily be segregated from other cell types as majority of the CD45-positive hematopoietic cells were floating in the culture supernatant (Fig. 3D and E). Although the transferred cells are subculturable, the number of floating cells gradually decreases; they finally disappear from the supernatant after several passages while

FIG. 4. Morphological and functional examinations of the hematopoietic cells. (**A**) Wright-Giemsa (WG) staining, myeloperoxidase (MPO) staining, esterase double staining (Esterase) and neutrophil alkaline phosphatase (NAP) staining of the floating cells at day 32 culture are shown. In WG staining picture (upper left), a black arrowhead indicates myeloblast, a blue arrowhead indicates promyelocyte, red arrows indicate polymorphonuclear neutrophils and a blue arrow indicates monocyte. MPO-positive (upper right), naphthol-ASD-chloroacetate esterase-positive (lower left) and NAP-positive (lower right) cells were indicated by black arrows. The fractions of polymorphonuclear neutrophils vary between 5 to 20% depending on experiments and time points. Scale bars indicate 20 μm. (**B**) CD11b expression was determined by flow cytometric analysis using the floating cells at day 25. (**C, D**) The round cells at day 35 were subjected to the phagocytic assay using latex beads. Similar results were obtained in other time points (data not shown). The scale bars indicates 40 μm in (**C**) and 25 μm in (**D**). (**E, F**) The round cells at day 108 (**E**) and 95 (**F**) were subjected to the phagocytic assay using zymosan with WG back-stain. Similar results were obtained in other time points (data not shown). Arrows indicate the phaocytised zymosan granules by polymorphonuclear neutrophils. The scale bars indicates 25 μm. (**G, H**) The round cells at day 33 were subjected to the NBT-reducing assay. Similar results were obtained in other time points (data not shown). The scale bars indicates 40 μm in (**G**) and 25 μm in (**H**).

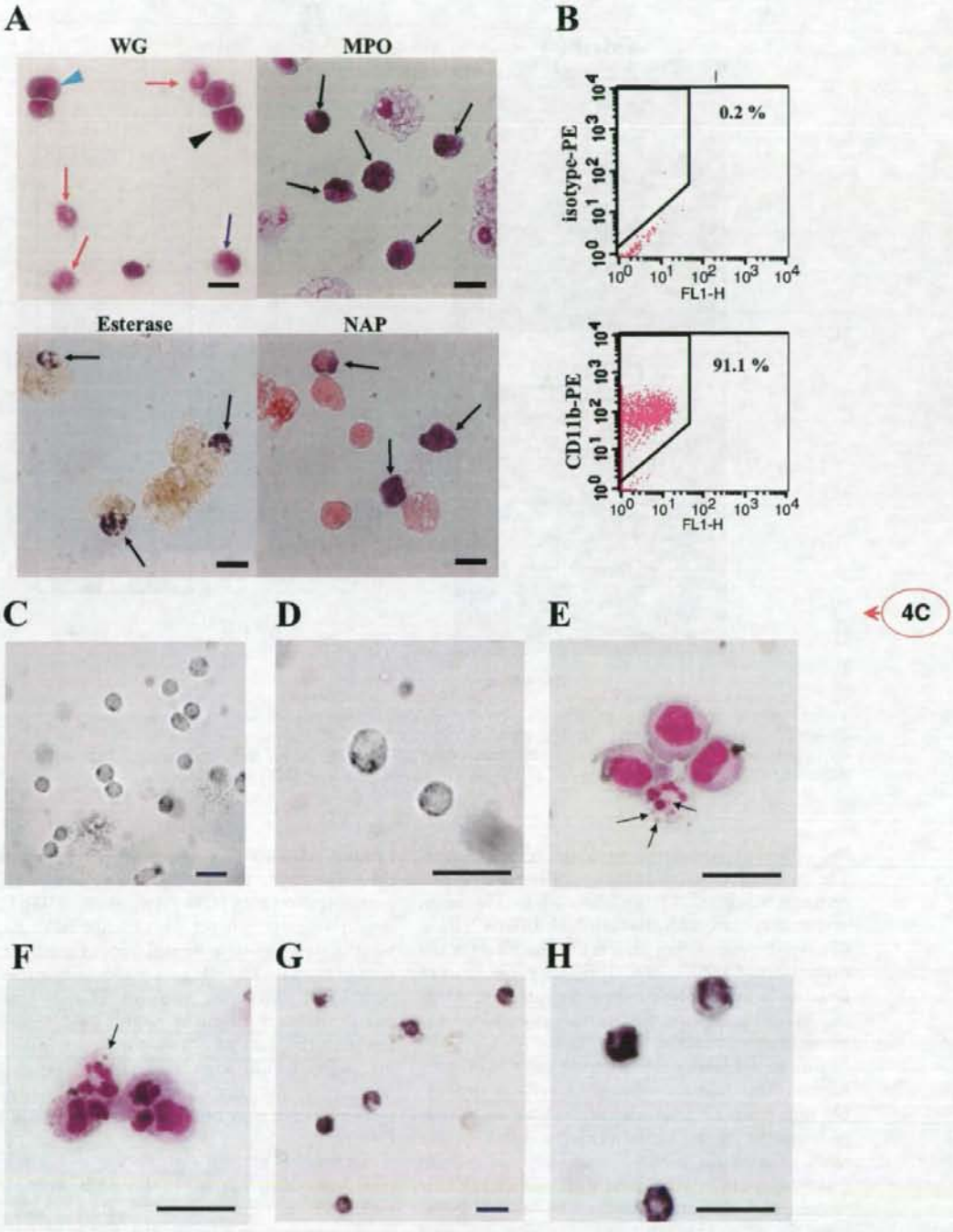
A
WG
MPO
Esterase
NAP
B
0.2 %
isotype-PE
FL1-H
91.1 %
CD11b-PE
FL1-H
C
D
E
4C
F
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FIG. 4.

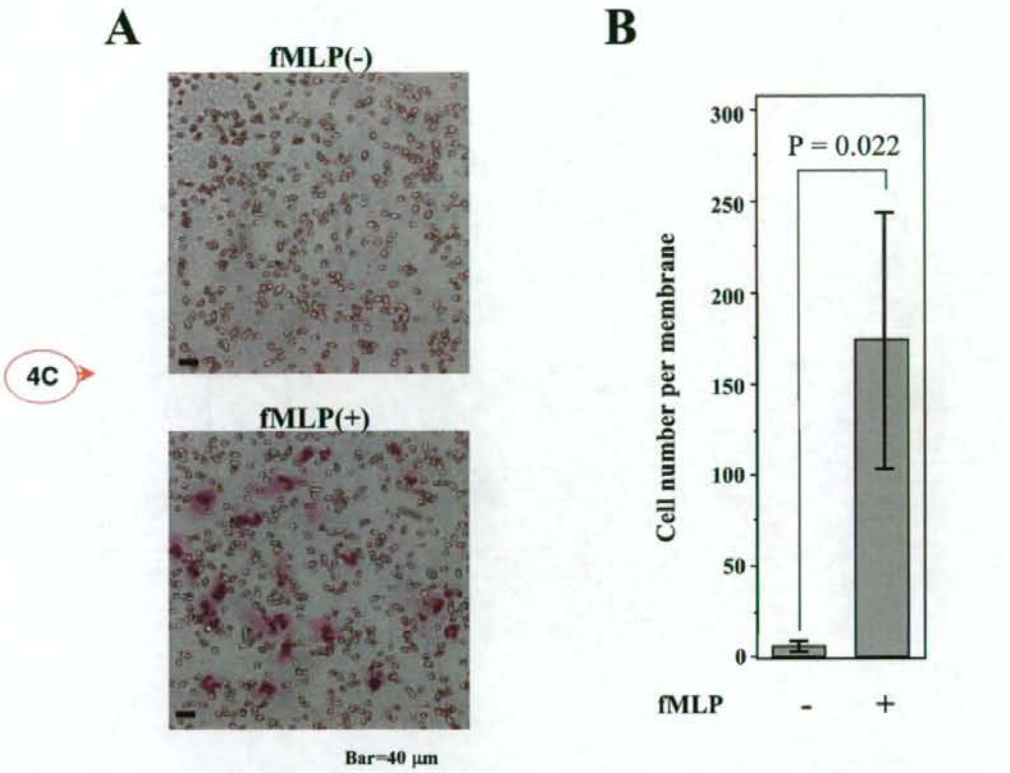

FIG. 5. Chemotaxis assays of the floating cells. Chemotactic activity toward fMLP was determined using 3-μm pore Chemotaxel as described in Materials and Methods. After a 2-h culture, internal membranes were removed and stained by Wight-Giemsa solutions (**A**), and the numbers of the polymorphonuclear cells were counted (**B**). Three independents experiments were performed and statistically analyzed (means ± SD).

the adherent population remains to proliferate. The reason for this diminution of hematopoiesis remains unclear. As we showed in Figure 6, serum is required for the terminal differentiation of granulocytes, although it is not essential for the formation of sac-like structures or generation of floating hematopoietic cells after total cell transfer. Which component of serum is required for granulopoiesis awaits further investigation.

Our novel feeder-free method for the maintenance of nonhuman primate ES cells is applicable to human ES cells, although there are differences in the optimal conditions for densities and sizes of colonies between monkey and human (manuscript in preparation by M. Nakahara). Our system has also an advantage in searching the possible autocrine factor or factors that assumed to play roles for the maintenance of primate ES cells. The existence of such factors has been suggested (Hishikawa 2006; Niwa et al., 2004). However, they have not yet been identified due to, at least in part, possible disturbance of intrinsic signals of primate ES cells by exogenously added recombinant cytokines such as FGF-2. Protein-based analyses might be useful in determining the autocrine factors. For example, preparation of monoclonal antibodies that hinder the maintenance of immaturity of primate ES cells cultured by our system may be helpful in identifying such factors.

As for the clinical application of human ES cells, xenogenic materials should be completely excluded. In our culture system, only one xenogenic material is used; we use Matrigel™,

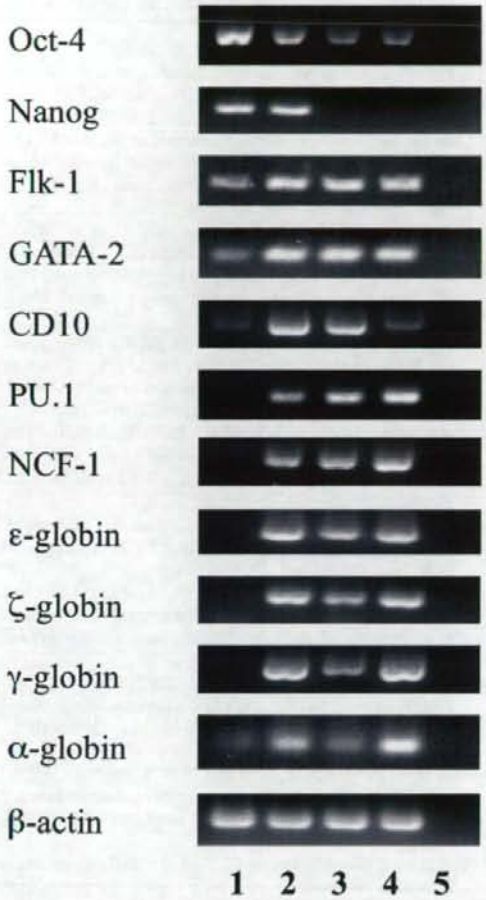

FIG. 6. Gene expressions. Total RNAs were extracted from undifferentiated primate ES cells (lane 1), sac-like structures (lane 2), adherent population (lane 3) and floating endpoint cells used in functional assays (lane 4) and RT-PCR was performed concerning indicated genes using primers shown in Table 1. In lane 5, water was used in place of cDNA template solutions. For internal control, β-actin gene was used. Abbreviations: neutrophil cytosolic factor 1; NCF-1,

QU1

which is the solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma, as a coating material for the culture flasks. We are now trying to replace Matrigel™ by human-derived materials, such as human placenta-derived collagen, or synthetic products such as peptide fragments whose amino acid sequences are derived from laminin or fibronectin. Our preliminary observation indicates that human ES cells can be maintained in an undifferentiated state even using these materials over several passages (unpublished observation by M. Nakahara). Refinement of experimental conditions including densities and sizes of ES colonies would make it possible to maintain human ES cells under exogenous cytokine-free and xenogenic material-free conditions.

In regard to the culture system for neutrophil differentiation from ES cells, there is a well-known report by Lieber et al. (2004), in which *in vitro* production of murine neutrophils from murine ES cells was reported. On the other hand, this report represents the first description of effective production of functional neutrophils from the *in vitro* differentiation of primate ES cells. In addition, our culture system consists of two novel technologies, that is, feeder-free, serum-free, and cytokine-free maintenance of immature primate ES cells and effective feeder-free hematopoietic differentiation. We are now trying to make efforts to improve effectiveness of neutrophils production to the level obtained in murine ES cell culture system.

AUTHOR DISCLOSURE STATEMENT

AU1

The authors declare that no competing financial interests exist.

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STEM CELLS

EMBRYONIC STEM CELLS/INDUCED PLURIPOTENT STEM CELLS

A Feeder-Free and Efficient Production of Functional Neutrophils from Human Embryonic Stem Cells

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Key Words. Embryonic stem cells • Neutrophils • Feeder-free culture • Differentiation

ABSTRACT

A novel, feeder-free hematopoietic differentiation protocol was established for highly efficient production of neutrophils from human embryonic stem cells (hESCs). For the induction of differentiation, spheres were generated in the presence of serum and cytokine cocktail and subjected to attachment culture on gelatin-coated plates. After approximately 2 weeks, a sac-like structure filled with abundant round cells emerged at the center of flattened spheres. After cutting off this sac-like structure, round cells actively proliferated, either floating in the supernatant or associated weakly with the adherent cells. Almost all of these round cells were CD45-positive hematopoietic cells with myeloid phagocytic markers (CD33 and CD11b), and approximately 30%–50% of the round cells were mature neutrophils, as judged from morphology, cytochemical characteristics (myeloperoxidase and neutrophil alkaline phosphatase), and neutrophil-specific cell surface markers (CD66b, CD16b, and GPI-80). In addition, hESC-derived neutrophils had chemotactic capacity in response to the bacterial chemotactic peptide formyl-methionyl-leucyl-phenylalanine and neutrophil-specific chemokine interleukin (IL)-8. Using "semipurified" neutrophils migrated to IL-8, both phagocytic and respiratory burst activities were demonstrated. Finally, it was shown that hESC-derived neutrophils had chemotactic activity in vivo in a murine air-pouch inflammatory model. The present results indicate successful induction of functional mature neutrophils from hESCs via highly efficient feeder-free differentiation culture system of human hematopoietic cells. STEM CELLS *2009;27:59–67*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Human neutrophils, a primary constituent of peripheral blood leukocytes, play an important role during host defense against invading microorganisms [1]. The decreased number or attenuated function of neutrophils results in serious infections in several pathological situations, such as congenital leukocyte function deficiencies or myelosuppression caused by chemotherapy [2, 3]. Granulocyte transfusion therapy can be effective for life-threatening infections unresponsive to conventional antimicrobial therapies in severely neutropenic cancer patients [1, 3, 4]. Granulocyte collection is performed by apheresis devices using hydroxyethyl starch (HES), and granulocyte induction is performed by the combined use of granulocyte colony-stimulating factor (G-CSF) plus dexamethasone [5, 6]. After HES, deposits may last for months, causing pruritus and acquired lysosomal storage disease [7]. Repeated dosage of steroid may increase the risk of cataracts [8]. In addition, G-CSF itself has been reported to exert several side effects in clinical settings [9].

Human embryonic stem cells (hESCs) are pluripotent cells derived from the inner cell mass of embryos cultured to the blastocyst stage [10, 11]. hESCs have the capacity to differentiate into a wide variety of somatic cells, including hematopoietic cells, and it has been reported that the coculture system with murine stromal cell lines, such as OP9 cells, efficiently induce multilineage hematopoietic differentiation of murine and human embryonic stem (ES) cells [12–15], indicating potential neutrophil production from ES cells. Although neutrophils were successfully and efficiently derived from murine embryonic stem cells [14], the regulated and directed differentiation toward neutrophils from hESCs and complete characterization of hESC-derived human neutrophils have not been reported.

This study produced neutrophils from hESCs via a feeder-free culture system and carried out granulocyte transfusion into

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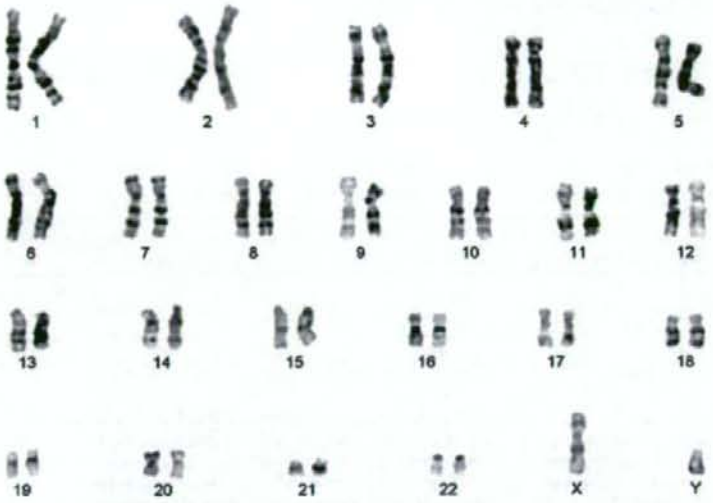

Figure 1. Karyotype analysis of human embryonic stem cells. A chromosomal analysis with G band staining was performed using KhES-3 cells.

mice. The results showed clearly that mature neutrophils with sufficient function were induced efficiently from hESCs and that these "human neutrophils" were capable of migrating to inflammatory sites in vivo in this mouse model.

Materials and Methods

Cell Culture

The use of hESCs was performed in accordance with the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science and Technology of Japan, after approval by the Institutional Review Board of International Medical Center of Japan (IMCJ). hESCs (KhES-1, KhES-2, and KhES-3) [11], provided by Kyoto University (Kyoto, Japan), were maintained on dishes coated with γ-irradiated murine embryonic fibroblasts (MEFs) in Dulbecco's modified Eagle's medium/Ham's F-12 medium (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) supplemented with 20% Knockout Serum Replacement (Invitrogen), 5 ng/ml fibroblast growth factor 2 (Peprotech, Rocky Hill, NJ, http://www.peprotech.com), 1% nonessential amino acids solution (Invitrogen), 1 mM sodium pyruvate solution (Invitrogen), 100 μM 2-mercaptethanol (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), 2 mM L-glutamine (Invitrogen), 20 U/ml penicillin (Invitrogen), and 20 μg/ml streptomycin (Invitrogen). hESCs were passaged twice a week by collagenase treatment and seeded at split ratios of 1:2 to 1:4 into new MEF-coated dishes. As described below, KhES-3 cells were used mainly in this study, and KhES-3 cells maintained as described above showed a normal karyotype (Fig. 1). Murine stromal OP9 cells [15] were maintained with α-minimal essential medium (Invitrogen) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (PAA Laboratories, Linz, Austria, http://www.paa.at), 100 μM 2-mercaptoethanol (Sigma-Aldrich), 1 mM L-glutamine (Invitrogen), 20 U/ml penicillin (Invitrogen), and 20 μg/ml streptomycin (Invitrogen). Human myeloid HL-60 and erythroid UT-7 cells were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS.

Neutrophil Differentiation of hESCs in Nonfeeder Culture

For sphere formation, hESCs were detached with 1 mg/ml collagenase IV (Invitrogen) and transferred to a 6-cm-diameter low-attachment dish (Nalge Nunc International, Tokyo, http://www.nalgenunc.com) coated with 2-methacryloyloxyethyl phosphorylcholine in 5 ml of Iscove's modified Dulbecco's medium (Sigma-Aldrich) supplemented with 15% FBS (PAA Laboratories), 2 mM L-glutamine, 100 μM 2-mercaptethanol, 20 U/ml penicillin, and 20 μg/ml streptomycin in the presence of 20 ng/ml insulin-like growth factor II (IGF-II; Peprotech), 20 ng/ml vascular endothelial growth factor (VEGF; Peprotech), 100 ng/ml stem cell factor (SCF; Peprotech), 100 ng/ml Flt3 ligand (Flt3-L; Peprotech), 50 ng/ml thrombopoietin (TPO; Kirin Brewery Co., Tokyo, http://www.kirin.co.jp/english), and 100 ng/ml G-CSF (Kirin Brewery Co.) (differentiation medium) at a density of 4×10^5 cells per milliliter. After primary differentiation for 3 days, the spheres were transferred to 10-cm-diameter dish coated with gelatin and cultured in differentiation medium for up to 40 days. Although these feeder-free hematopoietic differentiation cultures were performed using three lines of hESCs (KhES-1, KhES-2, and KhES-3), only KhES-3 showed sufficient hematopoietic differentiation. Therefore, only data on KhES-3 are presented.

Colony Assays

Colony assays were performed using Methocult TM GF^+H4535 (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) in accordance with the manufacturer's recommendations. In brief, 0.3 ml of cell suspension, which contained 10 cells, was mixed in 3 ml of methylcellulose solution consisting of 1% methylcellulose, 30% FBS, 1% bovine serum albumin, 100 μM 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml SCF (Peprotech), 20 ng/ml interleukin (IL)-3 (Peprotech), 20 ng/ml IL-6 (Peprotech), 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; Peprotech), 20 ng/ml G-CSF, and 3 U/ml erythropoietin (Kirin Brewery Co.) in 3.5-cm culture dishes. After 2 weeks, the number of colonies was counted. The morphology of the colonies was observed using an inverted light microscope (Olympus, Tokyo, http://www.olympus-global.com).

Reverse Transcription-Polymerase Chain Reaction

RNA was extracted from 5×10^6 cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, http://www1.qiagen.com), and cDNA was synthesized using a Superscript II Kit (Invitrogen) in accordance with the manufacturer's protocol. Polymerase chain reaction (PCR) was carried out using globin-α primers (forward, 5′-TGCACGCGCACAAGCTTCGG-3′; reverse, 5′-GCACGGTGCTCACAGAAGCCAG-3′), globin-ζ primers (forward, 5′-TTCCTCAGCCACCCGCAGAC-3′; reverse, 5′-AGCAGGCAGTGGGACAGGAG-3′), globin-ε primers (forward, 5′-TGCATTTTACTGCTGAGGAGA-3′; reverse, 5′-AAGAGAACTCAGTGGTACTT-3′), and globin-γ primers (forward, 5′-AGACGCCATGGGTCATTTCACA-3′; re-

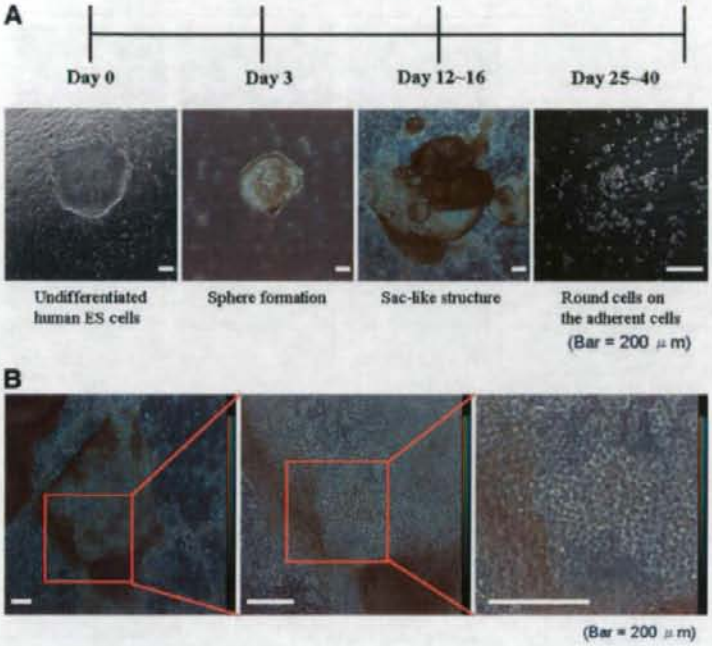

Figure 2. Schematic presentation of feeder-free production of neutrophils from human ES cells. **(A):** Undifferentiated human ES cells were induced to differentiate into hematopoietic cells using a two-step culture system with initial sphere formation followed by the secondary adherent culture. During an early phase of the secondary adherent cultures, sac-like structures with abundant round cells appeared. Thereafter, the round cells proliferated, either floating in the supernatant or associated weakly with the adherent cells. **(B):** Phase-contrast microscopy of a sac-like structure. Higher-magnification photographs clearly show abundant round cells in the structure. Scale bars = 200 μm. Abbreviation: ES, embryonic stem.

verse, 5′-GCCTATGGTTGAAAGCTCTGTAT-3′). As the internal control, β-actin primers were used (forward, 5′-GCAGGAGATGGCCACGGCGCC-3′; reverse, 5′-TCTCCTTCTGCATCCTGTCGGC-3′).

Neutrophil Differentiation of Human Cord Blood CD34-Positive Cells

For neutrophil differentiation, human cord blood CD34-positive cells were cultured under the same conditions as the hESCs described above except that the cells were cocultured with γ-irradiated murine stromal OP9 cells to induce the effective and optimal differentiation of these cells into neutrophils.

Determination of Cell Surface Molecules by Flow Cytometric Analysis

Cell surface markers of hESC-derived differentiated cells were analyzed by flow cytometric analysis. Cells were collected by 0.2% EDTA treatment, and after being washed in phosphate-buffered saline (PBS), 1×10^6 cells were reacted with the first antibody on ice for 30 minutes. The expression level of each protein was analyzed using a FACSCalibur (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com). The antibodies used were mouse anti-human CD34-phycoerythrin (PE) (BD Biosciences), mouse anti-human CD45-PE (BD Biosciences), mouse anti-human CD11b-PE (BD Biosciences), mouse anti-human CD33-PE (BD Biosciences), mouse anti-human CD66b-fluorescein isothiocyanate (FITC) (Beckman Coulter, Fullerton, CA, http://www.beckmancoulter.com), mouse anti-human CD16b-PE (BD Biosciences), and mouse anti-human GPI-80-PE (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan, http://www.mbl.co.jp/e/index.html).

Wright-Giemsa Staining and Special Staining Procedures

Viable cells in the dishes were observed directly using an inverted phase-contrast light microscope (Olympus). Alternatively, the cells were fixed on glass slides using a cytospin centrifuge (Cytospin 2; Thermo Shandon Inc., Pittsburgh, http://www.thermo.com), stained with Wright-Giemsa solution (Muto Pure Chemical Co., Tokyo, http://www.mutokagaku.com), and then observed using a light microscope (Olympus). Myeloperoxidase (MPO) staining and neutrophil alkaline phosphatase (NAP) staining were performed using the corresponding staining kits (Muto Pure Chemical Co.) in accordance with the manufacturer's protocols.

Chemotaxis Assay

Chemotaxis was assessed using Chemotaxel (3-μm pore; Kurabo Industries Ltd., Osaka, Japan, http://www.kurabo.co.jp/english/index.html), which was set in a 24-well dish. The lower chamber, a 24-well dish, was filled with 500 μl of Hanks' balanced saline solution (HBSS) supplemented with 2.5% FBS per well, and the upper chamber, a Chemotaxel cup, was filled with hESC-derived hematopoietic cells suspended in 500 μl of HBSS supplemented with 2.5% FBS per well (4×10^5 cells per milliliter). As chemoattractants, 100 nM formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma-Aldrich) and 10 ng/ml IL-8 (Peprotech) were added to the lower chambers. After the cells were incubated at 37°C for 4 hours, the cells in three randomly selected areas of the lower chamber were counted.

Phagocytosis

hESC-derived neutrophils attracted to the lower chamber of Chemotaxel were suspended in HBSS containing 2.5% FBS and incubated at 37°C for 1 hour with 5 μl of zymosan (1 mg/ml) in the presence of 100 nM fMLP. Subsequently, the cells were collected using a cytospin apparatus and stained with Wright-Giemsa solution. Phagocytosis was determined using a microscope by counting at least 100 cells and was defined as the percentage of cells containing more than two phagocytosed particles of zymosan.

Nitroblue Tetrazolium Reduction Assay for Respiratory Burst Activity

The floating cells were collected by mild centrifugation of the culture supernatant. After being washed with PBS, the cells were resuspended in 1 ml of RPMI 1640 supplemented with 10% FBS

containing 1 mg/ml nitroblue tetrazolium (NBT) (Nacalai Tesque Inc., Kyoto, Japan, http://www.nacalai.co.jp/en) and 100 nM fMLP for 30 minutes at 37°C. After being washed with PBS, the cells were resuspended in 10 μl of PBS and dropped onto Matsunami Adhesive Silane-coated glass slides (Matsunami Glass Ind., Ltd. Osaka, Japan, http://www.matsunami-glass.co.jp/english/index_e.html), and the formazan blue-black deposit-containing cells were observed using a light microscope (Olympus).

Chemiluminescence Measurements

Chemiluminescent microspheres (luminol-binding carboxyl hydrophilic microspheres), prepared as described previously [16], were purchased from TORAY Industries Inc. (Tokyo, http://www.toray.com). Chemiluminescence was measured in a single-channel Biolumat LB 9507 (Berthold Co., Wildbad, Germany, http://www.bertholdtech.com) using disposable 4-ml polypropylene tubes with a 200-μl reaction mixture (1×10^5 cells per milliliter suspended in HBSS). The tubes were placed in the Biolumat and allowed to equilibrate at 37°C for 5 minutes. To activate the system, 20 μl of chemiluminescent microspheres was added, and light emission was recorded continuously.

Transplantation of hESC-Derived Neutrophils into Mice and Air Pouch Chemotaxis Assay

Nine-week-old female nonobese diabetic/severe combined immunodeficiency (NOD/SCID)/γc^{null} (NOG) mice (Central Institute of Experimental Animals, Kanagawa, Japan) were made neutropenic by a single intraperitoneal injection of 5-fluorouracil (5-FU; 150 mg/kg; Wako Pure Chemical Co., Tokyo, http://www.wako-chem.co.jp/english) [17]. A subcutaneous air pouch was formed on the back of NOG mice, as described previously [18]. After 3 days, mice received i.v. transplants of 2×10^6 cells or vehicle (saline). Five hundred microliters of PBS containing zymosan (1 mg/ml) and/or human IL-1β (10 ng/ml) was injected into the air pouch. Sixteen hours after the injection of PBS containing zymosan and/or human IL-1β, mice were sacrificed under sevoflurane anesthesia, and the air pouch was washed with 1 ml of ice-cold PBS to obtain the accumulated leukocytes. All animal care procedures, including this experimental protocol, were approved by the Animal Care and Use Committee of the Research Institute, IMCJ, and complied with the procedures of the Guide for the Care and Use of Laboratory Animals of IMCJ. All mice were kept under specific pathogen-free conditions at the animal laboratory of the Research Institute of IMCJ in accordance with the guidelines of the Central Institute of Experimental Animals.

In some experiments, SCID mice (CLEA Japan, Tokyo, http://www.clea-japan.com) were used instead of NOG mice. In these experiments with SCID mice, similar procedures, including 5-FU injection and air pouch formation, were performed except that anti-asialo-GM1 polyclonal antibody (40 μl/mouse) (Wako Pure Chemical Co.) was injected into SCID mice before transplantation, and human IL-8 (20 ng/ml) and human IL-1β (20 ng/ml) were used as chemoattractants for hESC-derived human neutrophils.

RESULTS

Two-Step Culture Methods for Hematopoietic Differentiation of hESCs

In the first step of differentiation, spheres (Fig. 2A) were formed in the presence of cytokine cocktail (20 ng/ml IGF-II, 20 ng/ml VEGF, 100 ng/ml SCF, 100 ng/ml Flt3-L, 50 ng/ml TPO, and 100 ng/ml G-CSF). After primary differentiation of the spheres for 3 days, they were transferred onto gelatin-coated dishes to begin adherent culture. In the secondary adherent culture, the spheres became flattened after overnight culture and continued to spread. After 2 weeks of adherent culture, a sac-like structure emerged at the center of each flattened sphere, and in a few days, it became filled with

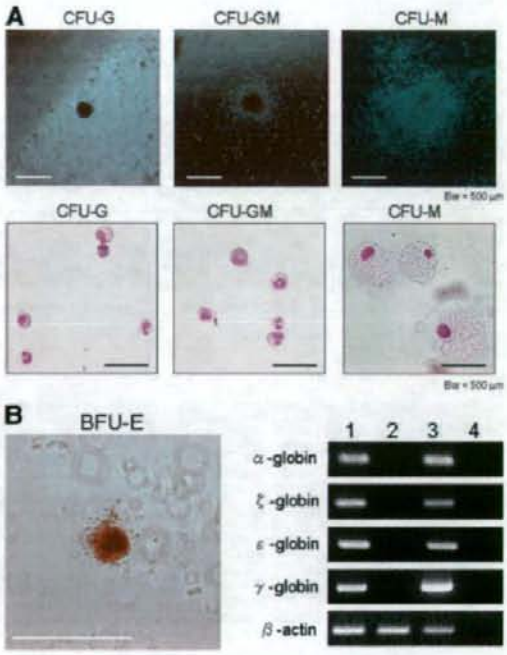

Figure 3. Colony assay and transcriptional analysis of human embryonic stem cell-derived hematopoietic round cells. Shown is the potential of the hematopoietic round cells to differentiate into granulocyte/macrophage (**A**) and erythroid (**B**) cells. The round cells from the sac-like structure were subjected to hematopoietic colony assays. Phase-contrast microscopic observation ([**A**], upper panels) showed the presence of granulocyte (CFU-G), granulocyte/macrophage (CFU-GM), and macrophage (CFU-M) colonies. Wright-Giemsa staining of the cells from the colonies ([**A**], lower panels) showed the presence of granulocytes and/or macrophages in each of the types of colony. Under the same culture conditions as colony assay, red erythroid colonies (BFU-E) were also observed ([**B**], left panel). The presence of primitive erythroid cells was shown by the existence of mRNA for embryonic hemoglobins (globin-ζ and globin-ε), as determined by reverse transcription-polymerase chain reaction (RT-PCR) ([**B**], right panel). Each lane of the RT-PCR analysis indicated round cells in the sac-like structure (lane 1), human myeloid HL-60 cells (lane 2), human erythroid UT-7 cells (lane 3), and negative control (water) (lane 4).

abundant round cells (Fig. 2B). At this point, some of but not all of the sac-like structures were cut off with a stem cell knife, and the round cells in the sac were induced into the medium. The round cells proliferated actively, either floating in the supernatant or associated weakly with the adherent cells (Fig. 2A). Although sac cutting at this stage was not essential for the proliferation of round cells and subsequent induction of hematopoietic cells and neutrophils, it apparently amplified the differentiation processes; therefore, sac cutting was performed in almost experiments. The results of colony assays of the round cells at this stage showed their sufficient capacity to develop into granulocyte and/or macrophage colonies (Fig. 3A), indicating granulocyte-macrophage dominant development in the present differentiation culture system. In addition, these round cells also infrequently formed red erythroid colonies (Fig. 3B, left panel), suggesting multilineage hematopoietic differentiation in the present culture system. The presence of erythroid cells, particularly

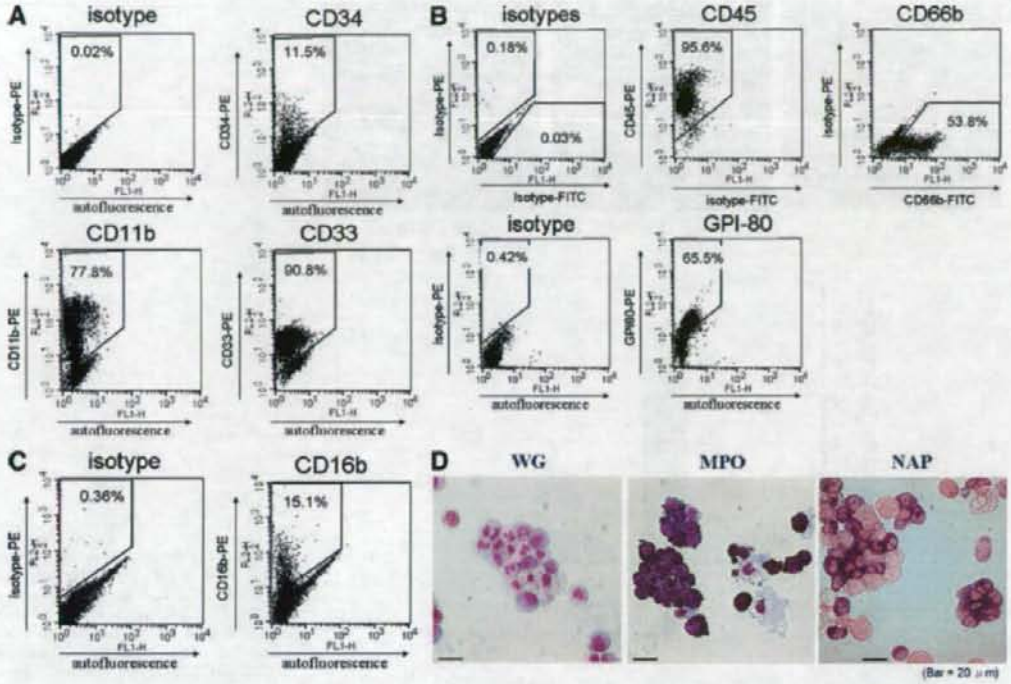

Figure 4. Flow cytometric analysis and morphological and cytochemical characterization of human embryonic stem cell (hESC)-derived myeloid cells. hESCs were cultured as shown in Figure 2. Flow cytometric analysis of hESC-derived hematopoietic cells at day 30 was performed for the cell surface expression of CD34 **(A)**, CD45 **(B)**, CD11b **(A)**, CD33 **(A)**, and neutrophil-specific markers, including CD66b **(B)**, CD16b **(C)**, and GPI-80 **(B)**. As morphological and cytochemical characterization of hESC-derived hematopoietic cells, WG staining, MPO staining, and NAP staining of hESC-derived hematopoietic cells at day 30 were performed and are shown in **(D)**. Scale bars = 20 μm. Abbreviations: FITC, fluorescein isothiocyanate; MPO, myeloperoxidase; NAP, neutrophil alkaline phosphatase; PE, phycoerythrin; WG, Wright-Giemsa.

from primary yolk-sac erythropoiesis, was further confirmed by the PCR analysis (Fig. 3B, right panel).

Thus, a two-step differentiation culture was performed in which hematopoietic progenitors produced in spheres during primary differentiation were expanded in the secondary adherent culture. This enabled effective induction of mature neutrophils without the use of cell sorting, as described below. It was estimated roughly that 4×10^6 neutrophils were obtained from 4×10^6 immature hESCs. After producing neutrophils, the culture system stopped producing hematopoietic cells within 2–3 months.

Evaluation of Hematopoietic Cells Induced from hESCs by the Present Culture Method

After 4–6 weeks of adherent culture, cell surface markers were evaluated for hematopoietic and myeloid differentiation of round cells in the culture. As shown in Figure 4, almost all round cells expressed CD45 (96.2% ± 2.0%; $n = 6$), a hematopoietic specific cell surface antigen, whereas these cells only minimally expressed CD34 (13.2% ± 4.1%; $n = 3$), a hematopoietic stem cell markers, indicating that the round cells were relatively mature hematopoietic cells. In addition, approximately 70%–90% of round cells were positive for CD11b (75.9% ± 9.3%; $n = 4$), an adhesion molecule of mature phagocytes [19], and CD33 (88.5% ± 6.5%; $n = 3$), a myeloid cell marker [20] (Fig. 4), suggesting that almost all of these hematopoietic cells were phagocytic myeloid cells.

Myeloid cells in the present culture system included immature myeloid cells, neutrophils, and monocyte/macrophages, as judged from morphological evaluation after Wright-Giemsa staining (Fig. 4D). Approximately 30% of cells were polymorphonuclear neutrophils, 30% were macrophages, and the other 40% were immature granulocytic and/or monocytic cells. Consistent with these findings, 80%–90% of the cells were positive for MPO staining. In addition, approximately 30%–50% of cells were positive for NAP.

To further evaluate the production of neutrophils in the present culture system, flow cytometric analysis was performed for the expression of neutrophil-specific markers of hematopoietic cells. As neutrophil-specific markers, CD66b [21], CD16b [22], and GPI-80 [23] were selected, and 20%–70% of the cells were positive for these neutrophil markers (Fig. 4). More precisely, the percentages of CD66b-, CD16b-, and GPI-80-positive cells were 50.5% ± 13.2% ($n = 4$), 18.9% ± 8.1% ($n = 4$), and 60.3% ± 11.0% ($n = 3$), respectively. Thus, taken together with the results of morphological and cytochemical studies (Fig. 4D), 30%–50% of the cells were considered to be mature neutrophils.

Then, the chemotactic activity, one of the most critical functions of mature leukocytes, including neutrophils, was evaluated using bacterial chemotactic peptide fMLP and neutrophil-specific chemokine IL-8 as chemoattractants. As shown in Figure 5, human leukocytes differentiated from hESCs showed chemotactic activity in response to these chemoattractants. Although less potent, IL-8 more specifically attracted neutrophils

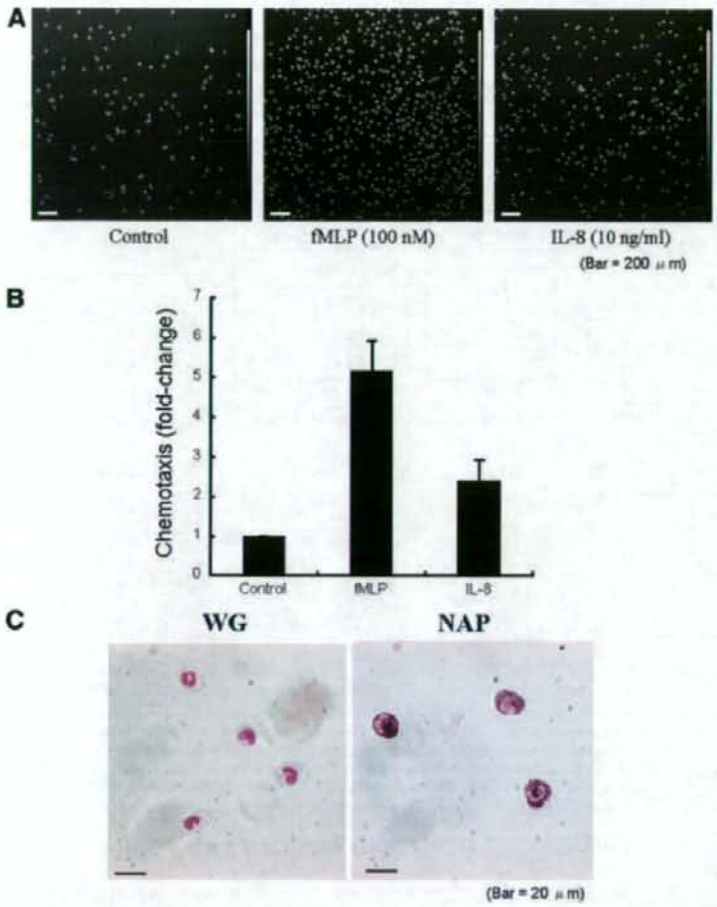

Figure 5. Chemotactic response of human embryonic stem cell-derived hematopoietic cells to fMLP or IL-8 in bare transwell assays. **(A, B):** Chemotactic activity of round cells in response to fMLP and IL-8 was determined using Chemotaxel as described in Materials and Methods. After a 2-hour culture, the transwell inserts were removed, and the cells in the lower chamber were observed and counted by phase-contrast microscopy. Scale bars = 200 μm **(A)**. **(C):** After chemotaxis assay, IL-8-induced cells in the lower chamber were collected and subjected to WG and NAP staining. Scale bars = 20 μm **(C)**. Abbreviations: fMLP, formyl-methionyl-leucyl-phenylalanine; IL, interleukin; NAP, neutrophil alkaline phosphatase; WG, Wright-Giemsa.

as compared with fMLP. Leukocytes attracted to IL-8 consisted mainly of segmented neutrophils and were positive for NAP (Fig. 5C).

Using these "semipurified" human neutrophils, further evaluation of neutrophil functionality was performed. As shown in Figure 6A, hESC-derived neutrophils have the capacity to phagocytose zymosan in the presence of fMLP. In addition, approximately 90% of cells were positive in the fMLP-stimulated NBT reduction assay (Fig. 6B), indicating the respiratory burst activity of hESC-derived neutrophils. Finally, phagocytosis-induced respiratory burst activity in hESC-derived neutrophils was determined using luminol-bound microspheres. As shown in Figure 6C, hESC-derived neutrophils produced reactive oxygen species in response to the phagocytosis of microspheres, which was completely abolished in the presence of cytochalasin B, a phagosome-destruction agent, and was potently inhibited by its later addition.

In Vivo Evaluation of Chemotactic Activity of Neutrophils Induced from hESCs

Leukocytes that accumulated in the zymosan-induced air pouch inflammation model in normal mice were shown to be predominantly neutrophils, along with small numbers of monocytes and lymphocytes [18]. Previous studies demonstrated that CD66b-positive human neutrophils appeared in the air pouch of NOG mice that had received transplants of cord blood CD34-positive cells at 6 weeks after transplantation [18]. In the present study, it was investigated whether the zymosan-induced accumulation of hESC-derived neutrophils in the air pouch occurred in NOG mice that received a transplantation of hESC-derived hematopoietic cells.

Sixteen hours after i.v. transplantation of hESC-derived hematopoietic cells and injection of proinflammatory agent(s) into the air pouch, accumulated neutrophils were collected and evaluated for human CD66-positive cells by flow cytometric analysis. Irrespective of the transplantation of hESC-derived cells, massive accumulation of murine neutrophils in the air pouch was observed (data not shown), which was consistent with the results of the previous study [18]. When NOG mice were transplanted with hESC-derived hematopoietic cells and both zymosan and IL-1β were injected into the air pouch, hESC-derived CD66b-positive neutrophils in the air pouch of NOG mice were 0.54% of the total accumulated cells (Fig. 7A, middle panel), whereas human CD66b-positive cells were not detected significantly in the air pouch of control NOG mice that did not receive transplantation of hESC-derived cells even when both inflammatory agents were injected into the air pouch (Fig. 7A, left

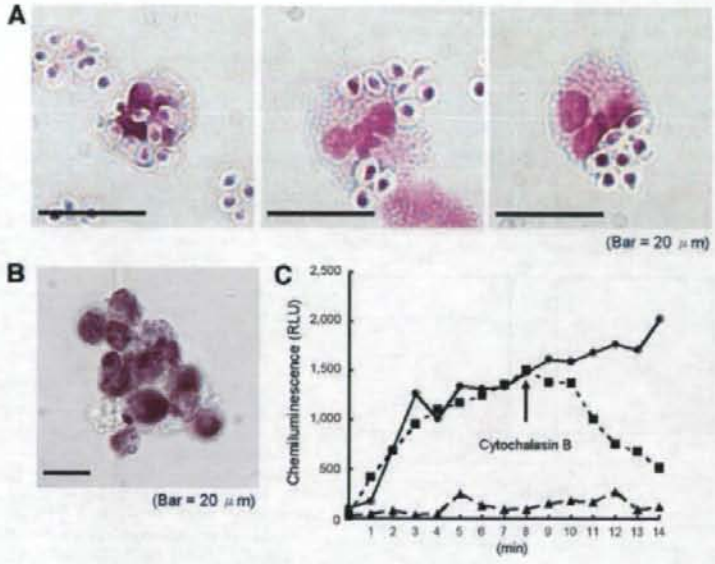

Figure 6. Phagocytosis and respiratory burst activity of human embryonic stem cell-derived neutrophils attracted to interleukin (IL)-8. **(A):** Neutrophils attracted to IL-8 were subjected to a phagocytic assay using zymosan in the presence of 100 nM formyl-methionyl-leucyl-phenylalanine (fMLP). Scale bars = 20 μm. **(B):** Neutrophils attracted to IL-8 were subjected to NBT reduction assays in the presence of 100 nM fMLP. Scale bars = 20 μm. **(C):** Neutrophils attracted to IL-8 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 (circles). The increase in chemiluminescence was almost completely abolished by the addition of cytochalasin B (triangles) and inhibited by its later addition (squares). Abbreviation: RLU, relative light units.

panel). Interestingly, without human IL-1β, the accumulation of hESC-derived CD66b-positive neutrophils in the air pouch was only minimal (Fig. 7A, right panel), suggesting that human IL-1β enhanced zymosan-induced accumulation of human ES cell-derived CD66b-positive neutrophils.

As a control, human neutrophils induced from human cord blood CD34-positive cells were used instead of hESC-derived cells, and the results were identical to those for hESC-derived cells (i.e., human cord blood cell-derived CD66b-positive neutrophils in the air pouch of NOG mice were 0.54% of the total accumulated cells) (Fig. 7B).

To further confirm the in vivo chemotactic function of hESC-derived neutrophils, the present study investigated whether IL-8-induced accumulation of hESC-derived neutrophils into the air pouch occurred in SCID mice receiving a transplantation of hESC-derived hematopoietic cells. When SCID mice were transplanted with hESC-derived hematopoietic cells and both IL-8 and IL-1β were injected into the air pouch, hESC-derived human CD45/CD66b double-positive neutrophils in the air pouch of SCD mice were 0.45% and 0.69% of the total accumulated cells (Fig. 7C), respectively, which was almost consistent with the results in NOG mice (Fig. 7A). Thus, the chemotactic function of hESC-derived neutrophils was demonstrated in vivo using air-pouch inflammation model systems of both NOG and SCID mice.

Discussion

To support hematopoietic differentiation, particularly to induce the production of neutrophils, ES cells have usually been cultured in the presence of several stromal cell lines, such as OP9 [13–15], S17 [12, 13], C166 [12], MS-5 [13], and CH3 10T1/2 [24] cells. To exclude any contamination of animal-derived factors, the induction of neutrophil differentiation was attempted with hESCs using a feeder-free culture system, which succeeded in establishing the present culture system.

A unique hematopoietic differentiation protocol was devised, in which a floating culture process and subsequent attachment culture process were combined. This protocol enabled the highly efficient (almost 100%) production of CD45-positive hematopoietic cells; the majority of the cells belonged to granulocyte or monocyte/macrophage lineages. In addition, this system guaranteed the production of functionally mature neutrophils. By virtue of the application of monolayer adherent culture in the later phase, this study identified a unique cell construction of a sac-like structure filled with abundant round cells as a precursory organization of hematopoiesis from human ES cells in vitro. This construction also produced endothelial cells under particular conditions (K. Saeki, manuscript in preparation). Thus, the sac-like structure contained precursors of both hematopoietic and endothelial cells, although the existence of bipotential hemangioblasts remained elusive.

In regard to the culture system for neutrophil differentiation from ES cells, there is a well-known report by Lieber et al. [14], in which in vitro production of murine neutrophils from murine ES cells was reported. On the other hand, the present report represents the first description of a highly effective protocol for the production of functional neutrophils from in vitro differentiated hESCs. In addition, this culture system provides a novel technology with highly efficient feeder-free hematopoietic differentiation.

The present study demonstrated the efficient production of human neutrophils from one cell line of hESCs, KhES-3. Although minor hematopoietic differentiation was observed in the other two lines tested (KhES-1 and KhES-2), sufficient and reproducible neutrophil production was observed only in KhES-3 cells. Certain modifications of this differentiation protocol might induce sufficient and reproducible neutrophil production from the other two lines (KhES-1 and KhES-2). Previous differentiation studies using hESCs often mainly or exclusively used one cell line [13, 25–31]. In addition, a recent report clearly showed that each line of hESCs had a specific tendency to differentiate into a specific lineage [32]. These problems might be overcome by the use of much additional lines of hESCs, or by a large number of patient specific pluripotent stem cells.

This study reported the production of functional neutrophils from the in vitro differentiation of hESCs. hESC-

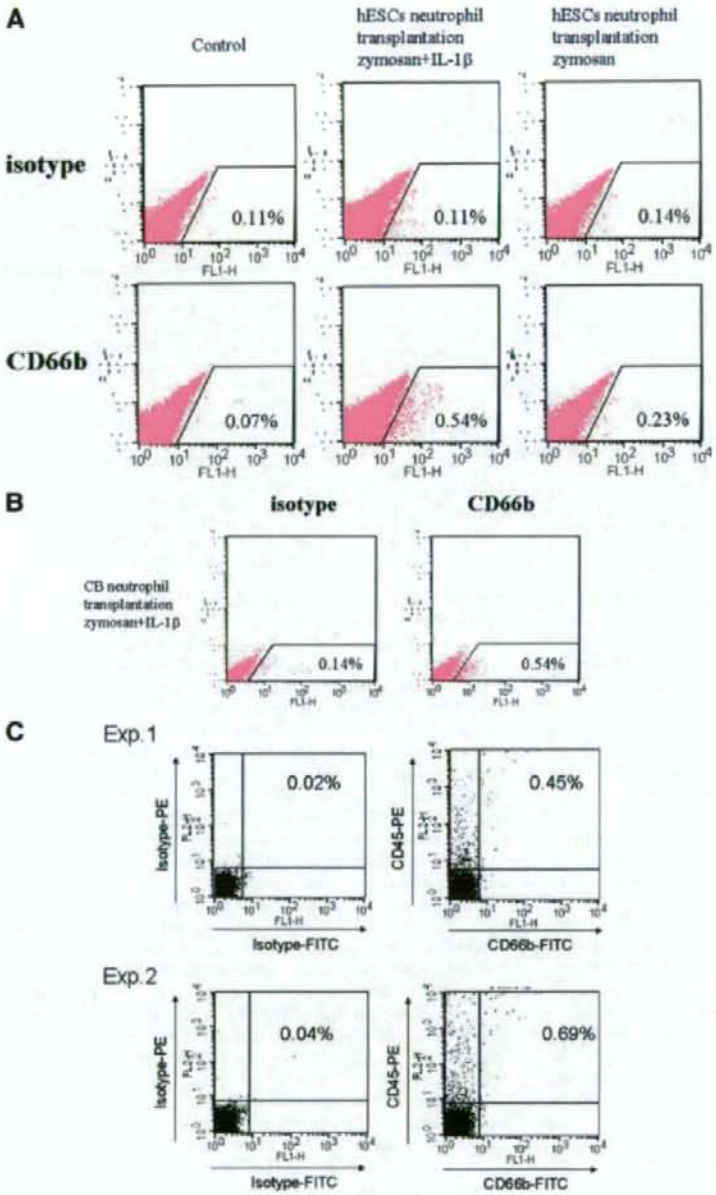

Figure 7. In vivo chemotactic activity of hESC-derived neutrophils in the air pouch inflammatory model of nonobese diabetic/severe combined immunodeficiency (NOD/SCID)/γc^{null} (NOG) and SCID mice. **(A):** hESC-derived neutrophils (middle and right panels) or vehicle (saline) (left panel) were transplanted into NOG mice intravenously. Phosphate-buffered saline (PBS) (500 μl) containing zymosan (1 mg/ml) in the presence (left and middle panels) or absence (right panel) of IL-1β (10 ng/ml) was injected into the air pouch to induce inflammation. After 16 hours, cells that had accumulated in the pouch were collected and subjected to flow cytometric analysis for the determination of cell surface expression of human neutrophil-specific human CD66b antigen. **(B):** As a control, human neutrophils induced in vitro from human CB CD34-positive cells were transplanted into NOG mice, and the air pouch inflammatory model was examined as in **(A)** using both zymosan and IL-1β as inflammatory agents. **(C):** hESC-derived neutrophils were transplanted into SCID mice intravenously. PBS (500 μl) containing IL-8 (20 ng/ml) and IL-1β (10 ng/ml) was injected into the air pouch to induce inflammation. After 16 hours, cells that had accumulated in the pouch were collected and subjected to flow cytometric analysis for the determination of cell surface expression of human hematopoietic-specific CD45 and human neutrophil-specific CD66b antigens. Abbreviations: CB, cord blood; Exp., experiment; hESC, human embryonic stem cell; IL, interleukin.

derived neutrophils had in vitro chemotactic, phagocytic and reactive oxygen-producing capacities. In addition, the in vivo chemotactic activity of hESC-derived neutrophils was demonstrated using an air pouch inflammatory model in NOG mice transplanted with hESC-derived neutrophils. In vivo chemotaxis of hESC-derived neutrophils was induced by the coinjection of zymosan and human IL-1β into the air pouch created in NOG mice. Although murine granulocytes migrated to the air pouch after the injection of zymosan alone, the migration of hESC-derived neutrophils seemed to depend on human IL-1β. NOG mice have multiple immunological defects in innate immunity, including a lack of IL-1α production after macrophage activation, complement-dependent hemolytic activity, and natural killer cell activity [33]. Previous studies have shown the reduction of neutrophils in *Listeria*-induced peritoneal exudates in SCID mice pretreated with anti-IL-1 antibodies [34]. These results suggest that endogenously produced IL-1 plays an important role in neutrophil migration. Furthermore, it has been reported that murine IL-1β is more active in murine cell bioassays whereas human IL-1β is more active in human cell bioassays [35]. Thus, human IL-1β might have a critical role in the migration

of hESC-derived neutrophils but not murine neutrophils in the present inflammatory model of NOG mice.

There is a barrier to be overcome before the clinical application of hESCs: the immunological hurdles of rejection. Several methods have been proposed to overcome this barrier (reviewed in [36]): the transplantation of mesenchymal stem cells as inducers of general immunotolerance [37], the establishment of an ES line library to meet the requirements of all individuals [38, 39], and the preparation of the individual patient's genome type ES cells by somatic cell nuclear transfer. All of these methodologies are promising, and thus hESCs will contribute greatly to the development of regenerative and transfusion medicine in the near future.

Conclusion

The present results indicate successful induction of functional mature neutrophils from hESCs via highly efficient feeder-free differentiation culture system of human hematopoietic cells.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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A Feeder-Free and Efficient Production of Functional Neutrophils from Human Embryonic Stem Cells

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Human Embryonic Stem Cells with Maintenance under a Feeder-Free and Recombinant Cytokine-Free Condition

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Abstract

We previously reported that cynomolgus monkey embryonic stem (ES) cells could be maintained under a feeder-free condition without using recombinant cytokines if sizes and numbers of ES colonies were kept within an appropriate range. Here we show that this finding is also true with human ES cells (hESCs). The two lines of hESCs, khES-1 and khES-3, were appropriately maintained in the absence of feeder layers or exogenous cytokines such as fibroblast growth factors, Noggin, transforming growth factor β, and Activin by closely controlling the size and number of hESC colonies. High-level expressions of immature markers including SSEA-4, Oct-4, and Nanog were detected in feeder-free and cytokine-free hESCs, and they formed teratomas when implanted into severe combined immunedeficiency (SCID) mice. No chromosomal abnormalities were observed over 20 passages, ruling out the possibility that special clones with growth advantages had been selected. Global protein expression profiles were quite similar among the hESCs maintained by our feeder- and cytokine-free method, by coculture with mouse embryonic fibroblasts (MEFs) and by a feeder-free method using conditioned media of MEFs. However, the activation level of Akt, an important player for the maintenance of ES cells, was highest and the activation level of extracellular signal-regulated kinase, a critical player for differentiation of ES cells, was lowest in the hESCs maintained by our cytokine-free method. Our results not only show a technical improvement for the maintenance of hESCs but also open a new avenue for the understanding of autocrine signaling networks of hESCs.

Introduction

HUMAN EMBRYONIC STEM (ES) cells (hESCs), which were established from inner cell mass of blastocysts via coculture with mouse embryonic fibroblasts (MEF), are generally maintained by coculture with MEFs. In aim of clinical application, however, usage of xenogenic cells should completely be avoided. The first success in performing the feeder-free culture of hESCs was reported by Xu et al. (2001), where hESCs were cultured on Matrigel™ Matrix-coated vessels using conditioned media (CM) of MEFs, namely, MEF-CM. Afterward, several MEF-CM-free methods were reported (Amit et al., 2004; Beattie et al., 2005; Chen et al., 2006; Ludwig et al., 2006; Sato et al., 2004; Wang et al., 2005; C Xu et al., 2005; RH Xu et al., 2005; Yao et al., 2006). In those methods, either small-molecular-weight chemical substances of yet undetermined safety or nonphysiological doses of cytokines including fibroblast growth factor (FGF)-2, Noggin, transforming growth factor (TGF)-β and Activin were used. Although hESCs were effectively maintained by using these substances in each experimental condition, the molecular bases for the self-renewal of hESCs remain controversial. For example, one report interpreted the roles of FGF-2 for the maintenance of hESCs as follows: it stimulates autologously derived human ES fibroblast-like cells (hdFs) to secret insulin-like growth factor (IGF)-II, which in turn, stimulate self-renewal of hESCs (Bendall et al., 2007). However, it is well known that Knockout™ Serum Replacement (KSR), which was added to the culture medium, has a sufficient IGF-like activity (Wang et al, 2007). Thus, it may be possible that KSR per se contributed to the maintenance of hESCs rather than the IGF-II produced by the FGF2-stimulated

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hdFs. Moreover, the existence of hdFs per se seems quite controversial because a few reports showed the absence of hdFs around the well-maintained human ES colonies (Ludwig et al., 2006; Wang et al., 2005; RH Xu et al., 2005). Recently, a very interesting finding on self-renewal pathways of murine ES cells was reported. It was shown that extrinsic stimuli were dispensable for the maintenance of murine ES cells as long as their spontaneous differentiation was blocked by using three categories of intracellular signaling inhibitors: FGF receptor inhibitor, glycogen synthase kinase (GSK)-3 inhibitor and extracellular signal-regulated kinase (ERK) inhibitor (Ying et al., 2008). Authors concluded that murine ES cells have an innate program for self-replication that does not require extrinsic instruction, which may account for their latent tumorigenicity. Considering culture technique, it is noteworthy that the cell density was reportedly a critical factor in the feeder-free maintenance of hESCs (Xu et al., 2001). Thus, we hypothesized that, if human ES colonies were carefully cultured at appropriate densities to inhibit spontaneous differentiation, they could be maintained even in the absence of exogenous stimuli.

We recently succeeded in maintaining the cynomolgus monkey ES cells without feeder layers or exogenous cytokines by closely controlling the size and number of ES colonies (Nakahara et al., 2008). To assess the effectiveness of this culture technique in the maintenance hESCs, we cultured hESCs without feeder layers or exogenous cytokines by a controlling of the size and number of colonies.

Here we show that two lines of hESCs, both of which have normal karyotype, can be maintained without feeder layers or recombinant cytokines by controlling the size and number of ES colonies. Over 20 passages, no chromosomal abnormality was detected. Our method proposes the most natural condition for the maintenance of hESCs, which is free from possible disturbing side effects of exogenously added cytokines and also provides ideal conditions for autocrine signaling networks in hESCs.

Materials and Methods

Cells and reagents

khES-1 and khES-3 (Suemori et al., 2001) were cultured on Matrigel™ Matrix (BD Biosciences, San Jose, CA)-coated culture dishes using DMEM/F12 medium (Invitrogen Corp., Carslbad, CA) supplemented with 20% Knockout Serum Replacement (KSR™) (Invitrogen Corp.), 1% nonessential amino acids solution (Invitrogen Corp.), 1 mM Sodium Pyruvate Solution (Invitrogen Corp.), 2 mM L-glutamin (Invitrogen Corp.), and 0.1 mM β-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) without using recombinant cytokines. The hESCs were maintained as a crop of colonies of similar sizes with diameters between 200 μm and 1000 μm at densities less than eight colonies per one cm^2. The hESCs were passed every 4 days. For detachment from the culture dish, cells were treated with a minimum volume of dissociation liquid including 0.25% trypsin (Invitrogen Corp.), 1 mg/mL collagenase IV (WAKO Pure Chemical Industries, Osaka, Japan), 20%KSR™, 1 mM $CaCl_2$ at 37°C for 5 to 15 min. Cells were gently collected without pipetting to avoid cell damage. They were seeded at split ratios of 1:2–1:4 on newly Matrigel™ Matrix-coated dishes, avoiding fusion between colonies. Culture medium was changed every day. In some experiments, hESCs were cultured on the layer of 40 Gy-irradiated MEFs (i.e., coculture with MEFs) using the previously described media or on Matrigel™ Matrix-coated dishes using conditioned media (CM) of MEF supplemented with 4 ng/mL of FGF-2 (PeproTech Inc., Rocky Hill, NJ). MEF-CM was prepared as reported by elsewhere (Xu et al., 2001).

Flow cytometry

Cell surface markers of hESCs were analyzed by flow cytometric analysis. Cells were collected by treatment with trypsin/EDTA (Invitrogen Corp.) and, after a wash in phosphate-buffered saline (PBS), 1×10^6 cells were treated with primary antibodies on ice for 30 min. The expression level of each protein was analyzed using a FACSCalibur (BD Biosciences, San Jose, CA). The antibodies used were a mouse antihuman SSEA-4-phycoerythrin (PE) (clone MA813-7) (R&D Systems Inc., Minneapolis, MN), a mouse antihuman E-cadherin-PE (clone #180224) (R&D Systems Inc.) and a rat antihuman Oct-4-PE (clone 240408) (R&D Systems Inc.). For studies on SSEA-4 and E-cadherin expressions, cells were further stained with TO-PRO3 fluorescent dye (Invitrogen Corp.) for 10 min to gate out the dead cell populations as FL4-higher fractions during analyses. For studies on Oct-4 expression, cells had been fixed and permeabilized by FIX & PERM® reagents (Invitrogen Corp.) according to the manufacturer's guidelines before the antibody reaction.

Immunostaining

Cells were fixed on slide glasses by using a cytospin apparatus (Cytospin 2) along with further fixation with acetone/methanol solution (1:3). The immunostaining procedure was performed as previously described (Saeki et al., 2003). The first antibody reactions were performed using a mouse monoclonal antihuman Oct-3/4 antibody (C-10, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and a rabbit polyclonal antihuman Nanog antibody (ReproCELL Inc., Tokyo, Japan). The second antibody reactions were performed using Alexa Fluor® 488 chicken antimouse IgG (H+L), Alexa Fluor® 568 goat antirabbit IgG (H+L) (Invitrogen Corp.).

Western blotting

Western blotting was performed as previously described (Saeki et al., 2003) using an antihuman extracellular signal-regulated kinase (ERK) (p44/p42 MAPK) antibody (Cell Signaling Technology Inc., Beverly, MA), antihuman phosphorylated ERK (phospho-p44/p42 MAPK (T202/Y204)) antibody (Cell Signaling Technology Inc.), a mouse monoclonal antihuman signal transducer and activator of transcription 3 (Stat3) (BD Biosciences), antihuman phosphor-STAT3 (Tyr705) antibody (Cell Signaling Technology, Inc.), antihuman phospho-Akt (Ser473) antibody (Cell Signaling Technology Inc.), an antihuman Akt antibody (Cell Signaling Technology Inc.), a rabbit polyclonal antihuman b-tubulin (H-235) antibody (Santa Cruz Biotechnology Inc.). The second antibody reaction was performed using a horseradish peroxidase-conjugated antirabbit or antimouse IgG (Cell Signaling Technology, Inc.). The final detection procedure was performed using ECL Western blotting detection reagents (GE Healthcare UK Ltd., Buckinghamshire, UK).