Differentiated Embryonic Stem (ES) Cells Produce Functional Platelets In Vitro

Short title for running head: Functional platelets from ES cells

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Abstract

Megakaryocytes and functional platelets were generated in vitro from murine embryonic stem (ES) cells using a coculture system with stromal cells. Morphologically two distinctive megakaryocytes were observed sequentially. Small megakaryocytes rapidly produced proplatelets on day 8 of the differentiation and large hyperploid megakaryocytes developed after day 12, suggesting primitive and definitive megakaryopoiesis. Two waves of platelet production were consistently observed in the culture medium. A larger number of platelets was produced in the second wave; $10^4$ ES cells produced up to $10^8$ platelets. By transmission electron microscopy, platelets from the first wave were relatively rounder with a limited number of granules but platelets from the second were discoid shaped with well-developed granules that were indistinguishable from peripheral blood platelets. ES-derived platelets were functional since they bound fibrinogen, formed aggregates, expressed P-selectin upon stimulation, and fully spread on immobilized fibrinogen. These results show the potential utility of ES-derived platelets for clinical applications. Furthermore, production of gene-transferred platelets was achieved by differentiating ES cells that were transfected with genes of interest. Overexpression of the cytoplasmic domain of integrin $\beta_3$ in the ES-derived platelets prevented the activation of $\alpha_{IIb}\beta_3$, demonstrating that this system will facilitate platelet functional studies.

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Introduction

Various culture systems demonstrating megakaryocyte maturation and proplatelet formation from hematopoietic progenitor cells have been described. CD34-positive stem cells from various sources including bone marrow, peripheral blood and cord blood cells have been successfully differentiated into megakaryocyte lineages in vitro.\textsuperscript{1-4} Such culture systems include liquid culture or co-culture systems with stromal cells, and most require the addition of a cytokine, thrombopoietin (TPO).\textsuperscript{5,6} A few reports have also demonstrated platelet release into a culture medium.\textsuperscript{4} Such methods have been used for studies of developmental biology of megakaryocytes or lineage specific gene expression. Potential gains from these studies would be therapeutic applications, such as in transfusions or cell transplantation. However, the number of obtained CD34 stem cells and the difficulties in expansion of these cells in vitro are factors that limit such strategies from being able to generate sufficient amounts of megakaryocytes or platelets for clinical application or basic research.

Embryonic stem (ES) cells are another good source, as these cells can rapid proliferate and are able to differentiate to a variety of cell types.\textsuperscript{7} Several techniques have been established to promote in vitro differentiation of murine ES cells to hematopoietic cell lineages including megakaryocytes. In vitro differentiation has been performed either by formation of embryoid bodies,\textsuperscript{8} coculture with stromal cell lines\textsuperscript{9,10} or cultured on matrix-coated plates.\textsuperscript{11} Differentiation of human ES cells is also reported.\textsuperscript{12} However, there have been no reports focusing on the production of ES cell-derived functional platelets as a
terminal differentiation of megakaryocytes.
The role of several gene products in platelet function has also been investigated by transfection into heterologous or megakaryocytic cell lines that could not undergo terminal differentiation or produce platelets. There are recent reports that primary megakaryocytes or megakaryocytes derived from ES cells can be utilized for such an approach.\textsuperscript{13,14} However, platelets expressing extrinsic gene products have not been generated in vitro. The forced expression of genes of interest in functional platelets could be a useful method for research in the field of platelet biology.

In this study, we utilized a co-culture system with the stromal cell line OP9 to generate mature megakaryocytes from ES cells, and identified the functional platelets produced in the culture supernatants. Further, we demonstrated that expression of extrinsic gene products could be achieved in platelets derived from ES cells. Our data suggests the potential utility of ES cell-derived platelets as a substitute for platelet transfusion. Combined with the genetic manipulation ability of ES cells, this system should facilitate functional studies using gene-transferred platelets, and be a future approach for treatment of platelet dysfunction.

Methods

Culture and Differentiation of ES cells

A murine ES cell line TT2,\textsuperscript{15} established from an F1 embryo of a C57B2/6 female and a CBA male mouse, was maintained as undifferentiated cells by coculture with mitomycin C-treated mouse embryonic fibroblast cells or STO cells in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 15% Knockout SR serum
replacer (Invitrogen) and 1000 units/ml leukemia inhibitory factor (Invitrogen).

Differentiation induction to hematopoietic progenitors was performed according to the method described by Nakano T. et al. ES cells were dissociated with 0.25% trypsin/EDTA (Sigma, St. Louis, MO), seeded onto confluent OP9 stromal cells derived from M-CSF-deficient mice, and cultured in alpha-MEM medium (Invitrogen) supplemented with 20% fetal bovine serum (FBS). Differentiation was started with 1.0 x 10^4 ES cells in a well of a six-well plate or 1.0 x 10^5 ES cells in a 10 cm culture dish. In 5 days, the ES cells were differentiated into hematopoietic progenitors without formation of embryonic bodies. For differentiation into megakaryocytes, the cells were trypsinized on day 5 and passed on the fresh mitomycin C-treated OP9 cells in the same culture medium containing with 10 ng/ml thrombopoietin (TPO; Kirin Brewery, Tokyo). 1.0 x 10^4 cells were seeded in a well of a six-well plate for the experiments of megakaryocyte colony formation or for determination of platelet numbers. To obtain the large number of platelets needed for functional assay, 1.0 x 10^5 cells were seeded in a well of a six-well plate or 1.0 x 10^6 cells were seeded in a 10 cm culture dish. From two days later (from day 7 of differentiation), the culture medium was changed everyday.

Characterization of megakaryocytes differentiated from ES cells

Megakaryocytes derived from ES cells were determined by morphology, immunocytochemistry and Wright-Giemsa and acetylcholinesterase (AChE) stainings.

For immunostaining, a cytospin preparation or cultured cells in a 6 well plate were
fixed with 4% formaldehyde-acetone solution and stained with anti-mouse CD41 (GPIIb/αIIIb) monoclonal antibody, MWReg30\textsuperscript{17} (Pharmingen, San Diego, CA) followed by a polymeric alkaline phosphatase-conjugated secondary antibody (ENVISION/AP polymer; Daco, Glostrup, Denmark). Visualization was performed using a substrate mixture of naphthol AS-BI phosphate sodium salt (Sigma) and new fuchsin solution (Merck, Darmstadt, Germany) according to the manufacturer’s instructions.

AChE staining was performed as described previously.\textsuperscript{18} Unfixed cells were incubated in 0.1 mol/L PBS (pH 6.0) containing 0.05% acetylthiocholine iodide, 0.1mol/L sodium citrate, 30 mmol/L copper sulfate, and 5 mmol/L potassium ferricyanide at room temperature for 3 hours.

Megakaryocyte diameters were measured by comparing CD41 positive megakaryocytes in the immuno-stained preparations and normal human erythrocytes from peripheral blood samples. Microscope photographs were taken at the same magnification, and the mean erythrocyte diameter was calculated as 7 µm.

DNA content of the differentiated cells was analyzed by flow cytometry as described previously.\textsuperscript{19} Cells were collected from the culture plate by mild pipetting and the detached cells were labeled with MWReg30 followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (Caltag, South San Francisco, CA). The cells were then washed, resuspended in hypotonic propidium iodide (50 µg/mL in 0.1% sodium citrate) containing 20 µg/mL RNAase (Sigma) and incubated for 30 minutes in the dark. The ploidy of the CD41-positive cells was analyzed by a flow cytometer, Epics XL (Coulter, Fullerton, CA).
Flow cytometry and determination of the number of platelets derived from ES cells

Platelets released into the culture supernatant were determined by flow cytometer. Culture medium was gently collected and centrifuged at 150 x g for 20 minutes to remove the nucleated large cells. The supernatant was fixed with 1% paraformaldehyde for one hour and centrifuged at 900 x g for ten minutes. The cells in the pellet were washed with Hank's balanced salt solution with Ca$^{2+}$ (HBSS) containing 1% FBS, and incubated with 10 µg/ml MWReg30 or anti-mouse glycoprotein (GP) V monoclonal antibody, 1C2 (Seikagaku Co., Tokyo, Japan), followed by FITC-goat anti-rat IgG; each incubation was performed on ice for 1 hour. Finally, the cells were washed again and then analyzed by a flow cytometer. A single platelet gate was created by analyzing adult mouse peripheral platelets in the same manner.

The number of platelets produced from ES-derived megakaryocytes was counted by flow cytometer. Culture supernatants were collected from the same well of a 6-well plate everyday from day 7 to day 16 of differentiation, and each day the cells in the supernatant were stained as described above. The cells were finally suspended in 500 µl HBSS. By flow cytometer, data from 50 µl aliquots were precisely collected several times during a continuous flow, and the numbers of platelet-sized and CD41-positive cells were determined.

Electron microscopy of platelets derived from ES cells

Culture medium containing ES-derived platelets was collected as described above. Mouse, control peripheral blood was obtained from the retro-orbital plexus. Blood
was collected in a tube containing ACD solution (2.5% trisodium citrate, 1.5% citric acid and 2% glucose), and platelet-rich plasma (PRP) was obtained by centrifugation at 150 x g for 20 minutes at room temperature. 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) was added to the medium or PRP and centrifuged at 1000 x g for ten minutes. Platelet pellets were then fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 60 min at 4°C. The samples were washed, postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer for 60 min at 4°C, dehydrated with a graded ethanol series, and embedded in Epon (TAAB Laboratories, Berkshire, UK) as described previously.21 Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and then examined with a JEM1200EX transmission electron microscope (JEOL, Ltd., Tokyo, Japan) at an accelerating voltage of 80 kV.

Functional studies of the platelets derived from ES cells

For functional studies of the ES-derived platelets, the culture medium was changed to the same medium containing 1 µM prostaglandin E1 (PGE1; Ono pharmaceutical, Osaka) one day before assaying. Culture medium was collected and centrifuged at 150 x g for 20 minutes. To the supernatant, 1 µM PGE1, 1 U/mL apyrase (Sigma) and a 1/9 volume of ACD solution were added and centrifuged at 900 x g for ten minutes. The cells in the pellet were resuspended and washed twice in 85 mM sodium citrate, 111 mM dextrose, 71 mM citric acid, pH 7.0, containing PGE1 and apyrase, and then resuspended in a modified Tyrode-HEPES buffer (138 mM NaCl, 0.36 mM NaH₂PO₄, 2.9 mM KCl, 12 mM NaHCO₃, 10 mM HEPES, 5 mM glucose, and 1 mM MgCl₂ and 1 mM CaCl₂, pH 7.4).
Fibrinogen binding was determined by flow cytometric analysis using Alexa Fluor 488-labeled human fibrinogen (Molecular Probes, Eugene, OR). It is known that mouse platelets can bind human as well as mouse fibrinogen. ES-derived platelets in 50 µl of each aliquot were stimulated with 10 µM ADP (Biopool, Ventura, CA) or 500 µM PAR4 thrombin receptor-activating peptide, AYPGFK (BioSynthesis, Lewisville, TX) in the presence of 100 µg/ml Alexa Fluor 488-labeled human fibrinogen for 10 min at 37°C without stirring. The aliquot was then diluted with 500 µl of the HEPES buffer and the mixture was directly analyzed by a flow cytometer. Single platelets were gated and analyzed. For controls, platelets were incubated with fluorescence-labeled fibrinogen without stimulation or stimulated in the presence of 5 mM EDTA instead of CaCl₂ or 100 µg/ml MWReg30 in the same manner. Further, some samples were stimulated in the presence of unlabeled fibrinogen and the aggregate formation was observed by microscopy.

To determine the expression of P-selectin upon agonist stimulation, platelets were stimulated with 500 µM AYPGFK for 10 min. After fixation with 2% paraformaldehyde for 1 hour, the cells were washed, incubated with anti-P-selectin rabbit polyclonal serum followed by FITC-conjugated anti-rabbit secondary antibody, and then analyzed by flow cytometer. Control cells were fixed without stimulation, stained and analyzed simultaneously.

Platelet spreading on the immobilized fibrinogen was analyzed. An 8-well chamber slide was coated with 100 µg/mL fibrinogen over night. After blocking with 1mg/mL bovine serum albumin (BSA) for 1 hour, platelets were then added to each well with 20 µg/ml epinephrine (Biopool, Ventura, CA) and 1 U/mL apyrase and incubated at 10°C.
room temperature for 30 min. After fixation with 2% paraformaldehyde, platelets were treated with 0.1% Triton X-100 for 10 minutes, and the actin filament was stained with 50 units/ml FITC-conjugated phalloidin (Molecular Probes). Platelet spreading was observed by fluorescence microscopy.

**Production of gene-transferred platelets**

An expression vector pCX-EGFP\textsuperscript{24} (provided by Dr. M. Okabe, Osaka University) containing chicken beta-actin promoter and green fluorescence protein (GFP) cDNA was used for the expression of GFP. For lineage specific expression of megakaryocytes and platelets, an expression vector (pBK-PF4-GFP) was constructed. 1.6Kbp human platelet factor 4 (PF4) promoter\textsuperscript{25} was amplified by polymerase chain reaction (PCR) and cloned into pBluscript II (Stratagene, La Jolla, CA). GFP cDNA and a polyadenylation signal was excised from pEGFP-C1 (Clontech, Palo Alto, CA) and linked after the PF4 promoter. After linearization by enzyme digestion, the plasmids were co-transfected into TT2 cells with a vector, pKJ2\textsuperscript{26} (GIBCO), containing phosphoglycerate kinase-1 promoter and a neomycin-resistance gene. Transfection was performed by electroporation using Gene Pulser Apparatus (Bio-Rad, Richmond, CA). Cells were cultured with G418 (Invitrogen) for 10 days and the colonies picked up. Positive clones were screened by PCR using primer sets for amplification of GFP cDNA. Differentiation into megakaryocytes was started with the positive clones.

Mutant cDNA for a fusion protein of extracellular and transmembrane domains of IL2 receptor \(\alpha\) chain (Tac antigen/CD25) and cytoplasmic domain of integrin \(\beta_3\) was
constructed by PCR according to a previously described strategy. Primer sets were prepared in which half of the sequence was identical to the end of transmembrane domain of the IL2 receptor until Leu187, and the other half was to the beginning of the cytoplasmic domain of β3. Two separate PCR were performed to amplify the extracellular and transmembrane domains of the IL2 receptor and the cytoplasmic domain of β3. The PCR products were mixed and a final PCR was performed using outside primers and then cloned into an expression vector pBK-EF28 that contained human elongation factor-1α promoter. The construct was verified by nucleotide sequencing using an automated DNA sequencer (ABI 310, Applied Biosystems, Foster City, CA). Platelets expressing the fusion protein (Tac-β3) were produced from ES cells as described above. The expression of the protein on cell surfaces was determined by anti-IL2 receptor antibody, Tac (provide by Dr. T. Uchiyama, Kyoto University).

Results

Formation of megakaryocyte colonies from ES cells in vitro

In our initial experiments, where we tried to differentiate several murine ES cell lines (e.g., J1 and D3 cells), it was observed that TT2 were the cells most effectively differentiated to megakaryocytes. Therefore, all studies were performed with TT2 cells, which were cultured on OP9 layers that induced the differentiation to mesodermal-like hematopoietic progenitors. From day 5, a combination of several cytokines including IL3, IL6, IL11 and TPO was tried for differentiation. Although other cytokines enhanced the number of megakaryocytes, megakaryocyte purity was
highest with a single cytokine, TPO. Furthermore, low concentration of TPO induced sufficient differentiation into megakaryocytes and production of platelets even if TPO was present at all time. Thus, cells were thereafter differentiated in the presence of just 10 ng/ml TPO. Initially, cells formed isolated colonies with unclear borders (Fig. 1a), which showed a clear contrast with the distinct borders of colonies of undifferentiated ES cells. On day 8 of the differentiation, some colonies produced individually identifiable cells, with the majority of the colonies starting to produce such cells after day 12. Typically, large cells appeared at the periphery of the colonies and migrated outward thereafter (Fig. 1b). The large cells were positive for immunostaining with anti-CD41 (GPIIb/αIIb) antibody and staining AChE (Fig. 1, c-e). Subsequently, almost all cells within the colonies became large and CD41-positive (Fig. 1, f and g). Then, some of the cells spontaneously floated into the culture medium and were easily detached by gentle pipetting; suggesting only a loose adhesion between differentiated and OP9 cells. Wright-Giemsa staining showed that the cells recovered from the culture supernatant exhibited morphological features of mature megakaryocytes (Fig. 1h), indicating that mature megakaryocytes were produced from ES cells. These megakaryocytes were quite viable because, by flow cytometric analysis with propidium iodide (PI) staining, almost all CD41-positive cells were PI negative.

After day 13 or 14, the cells showed a dramatic morphological change (Fig. 1, i-k). Numerous cells exhibited long beaded projections, so-called proplatelets, from which platelets were produced. Proplatelets were observed in cells attached on the layer and those floating in the medium. By immunostaining, the proplatelets
were strongly CD41-positive up to the top of the projections.

**Two wave differentiation to megakaryocytes and platelets from ES cells**

We determined whether platelets were produced from the proplatelet-bearing megakaryocytes. Culture medium was collected and nucleated cells removed by low speed sedimentation. Cells in the supernatant were analyzed by flow cytometer. A gate was fixed in the forward and side scatter histogram using peripheral blood platelets from adult mouse. The size of the most cells was within the platelet gate (Fig. 2A), and almost all were positive for CD41 and another platelet-specific antigen, GPV (Fig. 2B). We concluded that platelets were released to the culture medium from mature megakaryocytes derived from ES cells. To determine the number produced, platelets were collected from medium of the same well of a 6-well culture plate every day from day 7 to day 16, and the number of platelet-sized and CD41-positive cells accurately counted by flow cytometer (Fig. 2C). Two waves of platelet production were observed; the first wave appeared on day 8 of the induction and reached a peak on day 9, and thereafter the number of platelets decreased gradually. Subsequently, a second wave of platelet production appeared on day 13. These two waves were reproducibly observed in more than 5 independent experiments. In the second wave, larger numbers of platelets were produced than in the first. Approximately 1.8 x 10⁶ platelets were produced from 1 x10⁴ cells on day 5 (3 x 10⁵ platelets from day 8 to 12 and 1.5 x 10⁶ platelets from day 13 to 16). Since an initial culture of 1 x 10⁴ ES cells typically yielded 1 x 10⁶ cells on day 5, 1 x 10⁴ ES cells could finally produce as many as 1.8 x 10⁸ platelets.
The phenomenon of the two waves of platelet production reminded us of primitive and definitive hematopoiesis. Therefore, the time course of megakaryocyte maturation was observed in greater detail. Consistent with the first wave of platelet production, small megakaryocytes were observed in some colonies around day 8. However, because these small cells rapidly produced proplatelets and disappeared by day 12, they were not likely to be precursors of the megakaryocytes produced after day 12. After this, large megakaryocytes were observed in other colonies, consistent with the second wave. The two megakaryocytes showed distinctive morphologies (Fig. 3A). Megakaryocytes on days 9 and 13 were both positive by immunostaining with anti-CD41 antibody and by AChE staining, but the cells on day 9 showed an obvious weaker intensity in immunostaining and AChE staining than those on day 13. Furthermore, the size of the day 9 megakaryocytes was smaller than those of days 13 or 16 (Fig. 3B: means of diameters; day 9=13.1 µm, day 11=13.8 µm, day 13=20.2 µm, day 16=24.2 µm). On day 9, most cells were uniformly about 12-15 µm, whereas cells of day 13 and 16 were more than 20 µm and some large megakaryocytes with diameters more than 30 µm were observed. In parallel with cell size, the DNA content was also different (Fig. 3C). The ploidy of day 9 cells was predominantly 4n, whereas day 13 megakaryocytes contained hyperploid cells with 8n-128n. These results indicated that the cells from the second wave were morphologically close to a mature adult type of megakaryocytes, and that the platelets in the first wave were released from qualitatively different small megakaryocytes. The results suggested primitive and definitive megakaryopoiesis from ES cells.
Transmission electron micrograph of ES-derived platelets

The morphology of platelets derived from ES cells was examined by transmission electron microscopy. Control normal platelets from mouse peripheral blood exhibited discoid forms with two major granules (alpha-granules that predominated and dense granules), an open canalicular system and other organelles (Fig. 4a). ES cell-derived platelets from the first wave (day 10) were mostly rounder than normal platelets. Granules and other organelles were present but the numbers and the sizes of the granules were smaller compared with normal peripheral platelets (Fig. 4b). In contrast, platelets from the second wave (day 15) were relatively larger than the peripheral platelets but exhibited well-developed granules and normal organelles (Fig. 4c). Some platelets attached to each other, probably by spontaneous activation. The morphology of the platelets from the second wave was indistinguishable from mouse peripheral platelets.

This result suggested that cells released into the culture medium were not a simple fragmentation of megakaryocytes, but were platelets produced from ES cell-derived megakaryocytes through a physiological process.

Platelets derived from ES cells were functional

Platelets were obtained from the second wave of production (days 14 and 15), and a functional assay performed. By flow cytometry, platelet particles were shifted to a higher level in the forward scatter after stimulation of PAR4 thrombin receptor-activating peptide, AYPGFK without stirring, indicating the formation of
aggregates (Fig. 5A). When platelets were stimulated with vigorous stirring, most of the platelets were shifted to a higher level. Numerous platelet aggregates were observed under microscopy, whereas unstimulated platelets showed no aggregation. Aggregation was likely mediated by fibrinogen binding since the binding of Alexa Fluor 488-labeled fibrinogen to the platelets was detected in response to AYPGFK or ADP (Fig. 5B). Binding of fibrinogen depended on $\alpha_{\text{IIb}}\beta_3$ because it was blocked by EDTA or the anti-CD41 (GPIIb/\alpha_{\text{IIb}}) inhibitory antibody, MWReg30.17 Surface expression of P-selectin was also observed after the stimulation, but not in unstimulated platelets (Fig. 5C), suggesting that release reaction occurred. These platelets fully spread and exhibited formation of actin stress fibers on the immobilized fibrinogen, whereas they kept a round morphology on the control BSA surface (Fig. 5D). These results indicated that platelets derived from ES cells in vitro were as functional as the platelets from peripheral blood.

**Gene-transferred platelets derived from ES cells for functional assay**

To generate platelets expressing extrinsic gene products, platelets expressing green fluorescence protein (GFP) were produced as a test case. We prepared constructs in which actin (pCX-EGFP) or megakaryocyte-specific PF4 (pBK-PF4-GFP) promoters were linked to GFP cDNA. ES cells were transfected with the constructs and positive clones selected. When differentiation was started with these cells, GFP-positive megakaryocytes were detected. Differentiation was started with the stably transfected ES clones and the percent of the transfected cells was 100%, which was confirmed by the positivity of GFP. In case of pCX-EGFP, strong green fluorescence was observed in
the megakaryocytes, and also in proplatelets under fluorescence microscopy (Fig. 6A). GFP-positive platelets were observed in the culture medium. However, numerous platelets were attached on the OP9 layer or were possibly present under the layer; although such platelets were hardly recognized under phase-contrast microscopy (Fig. 6A, lower panel). By flow cytometry, platelets in the supernatant were found both GFP and anti-CD41-positive (Fig. 6B). With pBK-PF4-GFP, lineage specific expression of GFP was observed because only mature megakaryocytes and platelets were GFP positive by flow cytometry, although the expression was not strong enough for microscopic observation (Fig. 6C).

To verify the utility of the gene-transferred platelets in functional studies, a construct, which produced a fusion protein of IL2 receptor and the cytoplasmic domain of integrin β3 (Tac-β3), was tested. It has been reported, in CHO cells or megakaryocytes, that the overexpression of the cytoplasmic domain of β3 by the same construct prevented the conversion of αIIbβ3 to the active form; probably because of the interference of the interaction of intracellular associated factors to the cytoplasmic domains of αIIbβ3.13,32 However, such effects were not confirmed in platelets. Platelets from ES cells transfected with Tac-β3 cDNA were strongly positive for anti-IL2 receptor antibody, Tac (data not shown). When these platelets were stimulated with AYPGFK, the binding of Alexa Fluor 488-labeled fibrinogen was dramatically reduced compared with control platelets (Fig. 7). Therefore, it was demonstrated that in platelets too, the overexpression of the cytoplasmic domain of β3 prevented activation of integrin αIIbβ3.

These results demonstrated that the gene-transferred platelets derived from ES cells
would be a powerful tool in the field of biological research with platelets.

Discussion

In this study, megakaryocyte differentiation and functional platelet production from murine ES cells were demonstrated using a co-culture system with a stromal cell line, OP9, derived from M-CSF-deficient mice. We demonstrated for the first time that ES-derived platelets, especially those produced at a late phase of differentiation induction, were morphologically and functionally comparable with normal murine peripheral blood platelets.

In vitro platelet production was demonstrated from CD34-positive progenitor cells from human peripheral blood. Choi E.S. et al. demonstrated that such platelets were ultrastructurally identical to plasma-derived platelets, and functional in several assays. However, CD34-positive progenitor cells are not suitable as starting materials for clinical applications or functional studies of platelets because of the number of obtained cells and the difficulties of expansion in vitro. ES cells are a good source since they can rapidly proliferate and might enable an unlimited supply of platelets in vitro to be produced.

Nakano T. et al. first reported that ES cells could give rise to hematopoietic cells when cultured with OP9 cells. They demonstrated erythroid, myeloid, B lymphoid and megakaryocyte lineages developed by this system (the OP9 system). Recently, Eto K. et al. reported that megakaryocytes were generated in quantity from murine ES cells by the OP9 system, and that forced expression of genes and functional assay of \( \alpha_{\text{Iib}} \beta_3 \) can be achieved with these megakaryocytes. However, the authors in these
two studies observed megakaryocytes maturation only until day 12, but did not give attention to platelets released into the culture medium. Our data clearly demonstrated that quantitative platelets were generated after day 12; therefore being missed by the previous reports. We also consider that the utility of the ES-derived platelets has advantages for the study of platelet-specific gene products, rather than using megakaryocytes, because upon stimulation they can undergo more dynamic changes such as aggregate formation.

TT2 cells were the best starting cell lines for in vitro platelet production in ES cell lines used for the differentiation. Other cell lines (J1 and D3 cells) similarly differentiated to hematopoietic cells but, for reasons unknown, ultimately produced lower numbers of platelets (approximately $1 \times 10^7$ total platelets) than did TT2 cells (not shown). However, TT2 cells have different characteristics from other commonly used murine ES cells. For instance, when TT2 cells were injected into blastocysts, the colonization into tissues was very low, but when injected into eight-cell embryos, the cells efficiently colonized each tissue of pups. Furthermore, pups were disproportionately male, and TT2-derived cells were dominant, accounting for over half of the total cells. It remains to be determined whether such characteristics of ES cells could affect the potency of the platelet production in vitro.

Two morphologically distinctive megakaryocytes appeared sequentially over the time course of differentiation. Small megakaryocytes rapidly produced proplatelets on day 8 and large hyperploid megakaryocytes developed thereafter. Consistently,
two waves of platelet production were observed; with the two waves producing two types of untrastructurally distinctive platelets. The data indicates there are two distinct megakaryopoiesis during differentiation from ES cells in vitro, possibly demonstrating primitive and definitive megakaryopoiesis. It is well known that erythropoiesis originates in the yolk sac, and then migrates to fetal liver, where primitive and definitive erythrocytes are respectively produced. However, such transitions in other cell lineages, including myelocytic and megakaryocytic cells, are not well understood. Nakano T. et al. demonstrated that ES cells gave rise to primitive and definitive erythropoiesis in an OP9 system, and two types of erythrocytes that differ in their morphology and hemoglobin types were sequentially produced. Compared with the time course of erythropoiesis in their report, the two megakaryopoiesis observed in our study appeared rather slowly; maybe indicating that megakaryocyte maturation needed more time until platelet production. In our preliminary study, RT-PCR analysis of hemoglobin types in erythrocytes obtained from ES-derived mixed colonies containing the megakaryocytes showed that embryonic hemoglobin was detected in the first wave and adult type was detected in the second wave (not shown). However, molecular markers that define primitive and definitive megakaryocytes need to be identified. Xu M.J. et al. reported the presence of murine primitive megakaryopoiesis in the early yolk sac. When hematopoietic cells from the yolk sac were cultured, megakaryocyte colonies were observed that corresponded with the first wave megakaryocytes in our study; because megakaryocytes from yolk sacs rapidly produced proplatelets as early as day 3 of culture, much earlier than those from
adult bone marrow, and their ploidy class was lower than that of bone marrow
megakaryocytes. Furthermore, these megakaryocytes disappeared by 13.5 days
postcoitum. The similarities between megakaryocytes from yolk sacs and early
megakaryocytes from ES cells further suggest the presence of ES-derived primitive
megakaryopoiesis in vitro. We observed that early megakaryocytes disappeared by
day 12 and that large megakaryocytes developed in the other colonies thereafter.
Therefore, it is also suggested that the two types of megakaryocytes developed from
different precursors by distinct differentiation pathways.

We could obtain as many as 10^8 platelets in the culture supernatant from 10^4 ES cells.
The number was below the theoretical range proposed as the number of platelets
released from mature megakaryocytes, but was possibly an underestimate of the
capacity of ES-derived megakaryocytes since not a few platelets were observed on
or under the stromal layers in the case of GFP-positive platelets.

A report has shown that the thrombin/antithrombin III (AT III) complex can
stimulate proplatelet formation of megakaryocytes. However, addition of AT III in
the culture medium in the late phase of differentiation did not dramatically
increase the number of platelets produced (not shown). The discovery of factors that
stimulate proplatelet formation and platelet release from megakaryocytes would
thus improve the efficiency of platelet production in this system.

Another approach might be the recovery of the unactivated platelets in the medium
of this culture system. Some platelets were still observed on the stromal layers even
after the addition of PGE1 to the culture medium to suppress platelet activation.
The precise mechanism by which bone marrow megakaryocytes shed platelets in physiological conditions is not fully understood. It was postulated that the circulatory shear force within the marrow supports the fragmentation of proplatelets into platelets. Some reports show that proplatelets projected through the endothelial cell layer of marrow venous sinusoids and into circulation. The bone marrow environment is composed of a complex adherent cell population, which regulates platelet release and might help to keep platelets unactivated just after release from proplatelets. We also tested coculture with endothelial cell lines instead of OP9 at a late phase of differentiation, but did not obtain a higher amount of platelets than with OP9 cells. The combination of our culture system with an artificial capillary culture system with rheostatic shear forces or a three dimensional culture system with other types of cell lines might be useful.

The hematopoietic differentiation of ES cells has important therapeutic implications, including the derivation of platelets for transfusion. For clinical purposes, the utility of human ES cells has advantages. Kaufman D.S. et al. demonstrated that the co-culture of human ES cells with murine stromal cell lines lead to differentiation into hematopoietic cells. Human platelets will possibly be generated from human ES cells in vitro by a similar approach. If in the future cloned ES cells were available from patients’ somatic cells, platelets from such ES cells could be applied to autologous platelet transfusion. Further, combined with the methods to generate gene-transferred platelets described here, platelets of self-origin expressing a necessary gene would be an ideal substitutive therapy for
platelet disorders.

Further, the forced expression of the genes of interest in functional platelets could be a useful method for basic research into platelet biology. So far, such gene-transferred functional platelets have only been obtained from gene-modified animals such as transgenic or targeted mice,\textsuperscript{40} which need complicated techniques and timing. The method we describe here could make it possible to generate gene-transferred platelets in easier ways and shorter times.

In conclusion, the method established here to produce functional platelets from ES cells in vitro will facilitate functional studies using gene-transferred platelets, and might provide a future approach for clinical treatments of thrombocytopenia and platelet dysfunctions.

Acknowledgements

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References


Figure Legends

Figure 1

**Megakaryocyte colony and proplatelet formation by ES-derived hematopoietic cells.**

A hematopoietic colony with unclear borders was formed on day 6 of differentiation (a). On day 12, a megakaryocyte colony with individually identifiable large cells appeared (b). These cells were visualized by immunostaining with anti-CD41 (GPIIb) antibody (c, d) and AChE staining (e). CD41-positive mature megakaryocytes appeared at the periphery of the colonies. On day 13, almost all cells within the colonies became large (f) and were CD41-positive (g). Cytospin preparation of the culture medium on day 13 was stained by Wright-Giemsa staining (h). On day 14 of culture, cells attached on the layer (i), floated in the medium (j), and in cytospin preparation (k) displayed proplatelet formation from mature megakaryocytes. The cytospin preparation (k) was immunostained with anti-CD41 antibody. The proplatelets were CD41-positive up to the top of the projections. Original magnifications were x 100 for (a)-(e), x 200 for (f), (g), x 600 for (h) and x 400 for (i)-(k).

Figure 2

**Two wave platelet production from ES cells.**

(A) Cells released to the culture medium on day 15 were analyzed by flow cytometry. A platelet gate was fixed in the forward and side scatter profiles of peripheral blood platelets from adult mouse (left panel). Most cells in the culture medium of ES cells were within the gate (right panel). (B) Cells in culture supernatant were labeled with megakaryocyte and platelet-specific monoclonal antibodies. Almost all cells
were positive for CD41 (left) and GPV (right) as shown by the gray histogram. The open histogram represents cells stained with control antibody. (C) Cells were collected from medium of the same well of a 6-well culture plate every day (days 7-16) and the numbers of platelets counted as platelet-sized and CD41-positive cells by flow cytometry. Two waves of platelet production were observed. The values shown are the mean±SD from five independent experiments.

Figure 3

Primitive and definitive megakaryopoiesis from ES cells.

(A) Megakaryocytes on day 9 (upper panels) and 13 (lower panels) are shown pairwisely. Cells are observed under the phase-contrast microscopy (a and b). Consistent with the first wave platelet production, small megakaryocytes on day 9 already display numerous proplatelets. These megakaryocytes are stained with anti-CD41 (c and d) and with AChE staining (e and f). Original magnification in all photographs is x 200. (B) The diameter of the megakaryocytes on day 9, 11, 13 and 16 was measured as described under the method. The values shown are the mean±SD in 50 cells. (C) The DNA content of the megakaryocytes on day 9 (upper) and day 13 (lower) was analyzed by flow cytometry. Wright-Giemsa staining of the typical analyzed cells was shown on the right.

Figure 4

Transmission electron microscopy (magnification × 10000) of platelets derived from ES cells.
The morphology of platelets derived from ES cells was examined by transmission electron microscopy. Control normal platelets from mouse peripheral blood (A), ES cell-derived platelets from the first wave (day 10) (B), and platelets from the second wave (day 15) (C) are shown.

**Figure 5**

**Functional assay of the platelets derived from ES cells.**

Platelets were obtained from the culture medium on days 14-15, and functional assays were performed. (A) Unstimulated platelets (left) and stimulated platelets by PAR4 thrombin receptor-activating peptide, AYPGFK without stirring (right) were analyzed by flow cytometry. In the stimulated platelets, particles with higher forward scatter are observed (indicated by an arrow), indicating aggregate formation. A microscopic photograph of the analyzed platelets is shown below each histogram. (B) Platelets were stimulated by AYPGFK (left) or ADP (right) in the presence of Alexa Fluor 488-labeled fibrinogen, and the fibrinogen binding determined by flow cytometry (gray histograms). Simultaneous binding to unstimulated platelets and control binding in the presence of EDTA or the anti-CD41 inhibitory antibody, MWReg30 are shown as open histograms. (C) Platelets were stimulated by AYPGFK and stained with anti-P-selectin antibody followed by FITC-secondary antibody. Surface expression of P-selectin was analyzed by flow cytometry (gray histogram). The open histogram represents the control experiment with unstimulated platelets. (D) Platelets were allowed to adhere and spread on the immobilized fibrinogen (left) and BSA surface (right),
stained with FITC-conjugated phalloidin and observed under the fluorescence microscopy.

**Figure 6**

**GFP-expressing megakaryocytes and platelets derived from ES cells.**

In A and B, megakaryocytes and platelets expressing GFP were produced from ES cells transfected with pCX-EGFP. (A) Paired photographs of phase-contrast microscopy (left) and fluorescence microscopy (right) are shown. Proplatelet-bearing megakaryocytes are strongly GFP-positive up to the top of the proplatelets (upper and middle panels, day 14). GFP-positive platelets are observed on or under the stromal layer, although platelets are hardly recognized under the phase-contrast microscopy (lower panel, day 15). (B) Platelets in the culture supernatants (day 15) were stained with anti-CD41 antibody followed by PE-conjugated secondary antibody, and two-color flow cytometric analysis (PE and GFP) was performed. Platelets derived from GFP-transfected ES cells are both GFP and anti-CD41-positive (right panel). Results of the platelets stained with a control antibody (middle), and of the platelets derived from untransfected ES cells (left) are shown. (C) Platelets on day 15 derived from ES cells transfected with GFP linked to megakaryocyte-specific PF4 promoter (pBK-PF4-GFP) are also GFP positive by flow cytometry.

**Figure 7**

**Gene-transferred platelets derived from ES cells for functional assay.**
A cDNA construct which produced a fusion protein of IL2 receptor and cytoplasmic domain of integrin $\beta_3$ (Tac-$\beta_3$) was transfected into ES cells and platelets produced. (A) Platelets from the transfected ES cells (day 15) were stimulated with AYPGFK and the binding of Alexa Fluor 488-labeled fibrinogen was analyzed by flow cytometry (gray histogram of the right panel). Results obtained with control platelets produced from ES cells transfected with pKJ2 are shown in the left panel. Open histograms represent the fibrinogen binding in the presence of EDTA. (B) Fibrinogen binding is expressed as mean fluorescence intensity. Data represent the mean $\pm$ SEM from platelets produced by three independently transfected ES clones.
Figure 1
Figure 2

A  Blood platelets  Culture supernatant

B  CD41-FITC  GPV-FITC

C

<table>
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<th>Number of Platelets (x10^4)</th>
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<tr>
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(Day)
Figure 3

A

Day 9

Day 13

B

C

Day 9

Day 13

DNA content

Counts

Counts
Figure 4
Figure 6

A

B  Untransfected  GFP-Transfected

Control Ab  Control Ab  Anti-CD41

GFP  GFP  GFP

C  PF4-GFP

GFP
Figure 7

A

Control

Tac-β

+Fibrinogen-Binding

AYPGFK

+EDTA

B

Fibrinogen Binding
(mean fluorescence intensity)

AYPGFK

Control

Tac-β

- + - +
Differentiated embryonic stem (ES) cells produce functional platelets \textit{in vitro}

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