Novel Approach for Formation of Platelet-like Particles from Mouse Embryonic Stem Cells without Using Feeder Cells

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Summary: Megakaryocytes (MKs) and platelet-like particles (PLPs) have generally been obtained by culturing embryonic stem (ES) cells over feeder cells. However, using feeder cells need many labor-consuming processes and the MK and PLP fractions obtained are often contaminated by such cells and their fragments. Here we describe our new culture system for differentiating mouse ES cells to MKs and PLPs without using feeder cells. ES cells are differentiated to cells with MK-like morphology and properties, including proplatelet formation, high ploidy (>8N), and CD41 expression. The culture medium contained PLPs expressing platelet glycoproteins, CD41 and GPIb. Integrin $\alpha_{IIb}\beta_3$ of PLPs can be activated by thrombin. Addition of the metalloproteinase inhibitor TAPI-2 to the culture increased the surface expression of GPIb α and augmented the adhesion of PLPs to immobilized von Willebrand factor through decreasing the shedding of GPIb α . Thus our mouse ES cells culture system is a suitable and efficient method for obtaining MKs and functional PLPs that obviates the need for feeder cells.

Key words megakaryocyte, embryonic stem cell, proplatelet formation, GPIb, metalloproteinase inhibitor, platelet

INTRODUCTION

Platelets, small anucleate blood cells, normally circulate in the blood stream in a quiescent state, but upon blood vessel injury, platelets adhere to the exposed subendothelial tissues, become activated, and then form aggregates, leading to thrombus formation to arrest bleeding. Platelet transfusion is a widely applied therapy for thrombocytopenic patients, particularly those undergoing chemotherapy, so supplying safe platelets in sufficient amounts is a major concern in the clinic. Platelets are produced from megakaryocytes (MKs), multinucleate cells derived from the hemat-

opoietic stem cells (HSCs) [1]. Mature MKs produce elongated shaft-like cytoplasmic protrusions containing platelet-size swellings in a process called proplatelet formation [2]. And, recently, Junt et al. [3] succeeded to observe MKs extending dynamic proplatelet-like protrusions into microvessels in vivo. Human bone marrow was the initial source of MKs, but their low content in this tissue, <0.5% of the total cells [4], made it difficult to characterize these cells, particularly the process of how platelets are formed from them. Fortunately, discovery of a growth factor of MK, thrombopoietin (TPO), made it possible to obtain MKs by cell culture [5,6]. HSCs from bone mar-

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Abbreviations: EB, embryoid body; ES, embryonic stem; HSC, hematopoietic stem cell; MK, megakaryocyte; PBS, phosphate buffered saline; PLP, platelet-like particle; vWF, von Willebrand factor.

row [7], peripheral blood [8], cord blood [9], and fetal liver [10] were successfully differentiated to MKs and further to platelets after proplatelet formation by culture with TPO. In addition, mouse embryonic stem (ES) cells cultured in the presence of feeder cells, MS-5 or OP9 stromal cells, could be successfully differentiated into MKs [11,12]. Mouse or human ES-derived MKs were reported to differentiate into platelet-like particles (PLPs) using the culture system with OP9 cells [13,14]. Fujimoto et al. [13] first reported differentiation of ES cells to PLPs using OP9 feeder cells; in their system, TT2 ES cells were most successfully differentiated to PLP, but J1 and D3 ES cells showed less efficient differentiation. E14tg2A, another ES cell line, was derived from 129/Ola-derived HPRT-negative cells and reported to multiply in the absence of feeder cells [15]. Nishikii et al. [16] demonstrated that they can be grown and differentiated to PLPs in the presence of OP9 feeder cells.

Platelet formation from ES cells is an attractive method because it can be easily applied to mutational analysis in basic research and the obtained PLPs could be used instead of platelet transfusion, but the necessity for feeder cells make it questionable since it results in contamination by stromal cells and their fragments as well as being a complicated, labor-intensive procedure.

Here we report our new culture system in which mouse ES cells can be differentiated to MKs and to PLPs without using feeder cells. The analysis of ES-derived PLPs indicates that the obtained PLPs have properties similar to those of native platelets.

MATERIALS AND METHODS

Animals

C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan). All experiments using animals were approved by the Ethical Committee for the Research of Life Science of Kurume University.

Materials

The following were obtained from the indicated commercial sources: FITC-conjugated and PE-conjugated anti-mouse CD41 (integrin α_{IIb}) antibodies (BD Biosciences, San Jose, CA, USA); PE-conjugated JON/A, PE-conjugated and non-conjugated anti-mouse GPIb α (CD42b), FITC-conjugated anti-mouse GPIb β (CD42c) antibodies (Emfret, Würzburg, Germany); purified human fibrinogen (Enzyme Research Laboratories, Swansea, UK); mouse leukemia inhibi-

tory factor (LIF) (Millipore, Billerica, MA, USA); recombinant murine thrombopoietin (TPO) (Pepro Tech, Rocky Hill, NJ, USA); fetal bovine serum (FBS), Glasgow minimum essential medium, and apyrase (Sigma-Aldrich, St. Louis, MO, USA); MEM non-essential amino acids solution (NEAA), Iscove's modified Dulbecco's medium, MEM- α medium, and L-glutamine (Invitrogen, Carlsbad, CA, USA); prostaglandin I_2 (Funakoshi, Tokyo, Japan); TAPI-1, GM6001, TAPI-2, and von Willebrand Factor (vWF) (Merck, Darmstadt, Germany).

Cell culture conditions

Mouse E14tg2A cells (HPRT-negative ES cell line derived from 129/OlaHsd mouse) were kindly provided by Dr. H. Niwa, Riken, Japan. ES cells were maintained with Glasgow minimum essential medium containing 10% FBS, 1% NEAA, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, 100 units/ml penicillin, 100 μ g/ml streptomycin, 292 μ g/ml L-glutamine, and 1000 units/ml LIF in gelatin-coated cell culture dishes (IWAKI, Tokyo, Japan). For embryoid body (EB) formation, 2×10^5 ES cells were cultured in 90-mm bacterial culture dishes (Sterilin, London, UK) using Iscove's modified Dulbecco's medium containing 15% FBS, 0.45 mM monothioglycerol, 50 μ g/ml ascorbic acid, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 292 μ g/ml L-glutamine. After 6 days of culture, many EBs floating in the medium were observed. EBs were separated by sedimentation and the cells in the EBs were suspended by pipeting with 0.25% trypsin/EDTA/PBS (Nacalai Tesque, Kyoto, Japan). The cells were incubated with PE-conjugated anti-CD41 antibody, and the cells expressing CD41 antigen were separated by anti-PE antibody conjugated magnetic immunobeads (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). Sorted cells ($5-10 \times 10^6$ cells per dish) were cultured further in 90-mm bacterial culture dishes with MEM- α medium/15% FBS/50 ng/ml TPO. At the 10th and 13th days of culture, the culture medium was centrifuged ($200 \times g$, 5 min) to precipitate MKs; the PLP-containing supernatant was further centrifuged ($1000 \times g$, 15 min) to obtain PLPs. In some experiments, TAPI-2 (100 μ M final), to prevent GPIb α shedding, or H_2O (final concentration of 0.83%), a control, was added on day 10 to the culture medium, and PLPs were obtained as described above.

Preparation of PLPs

PLPs were prepared as described by Nishikii et al. [16], with some modifications. PLPs in culture supernatant were collected and acid citrate dextrose solu-

tion was added to yield final concentrations of 8.5 mM sodium citrate, 6.5 mM citric acid, and 10.4 mM glucose. The combined suspension was centrifuged ($200\times g$, 5 min) to eliminate large cells. The supernatant was added with prostaglandin I_2 ($0.1\text{ }\mu\text{g/ml}$, final) and apyrase (0.01 unit/ml , final) and then centrifuged ($1000\times g$, 15 min). The obtained pellets were washed once with citrate-buffered saline (6.85 mM citrate, 130 mM NaCl, 4 mM KCl, and 5.5 mM glucose, pH 6.5/ $0.1\text{ }\mu\text{g/ml}$ PGI₂/0.01 unit/ml apyrase) and resuspended in modified HEPES-Tyrode's buffer, pH 7.4 (10 mM Hepes, 12 mM NaHCO₃, 138 mM NaCl, 5.5 mM glucose, and 2.9 mM KCl)/ 2% bovine serum albumin (BSA). The obtained PLPs were counted by a cell counter (Sysmex, Hyogo, Japan), applying mouse platelet size for counting, and then applied for the following analyses.

Analysis of megakaryocytes

The cells were stained with May-Gruenwald Giemsa reagent and their morphology was determined. DNA ploidy of cells was analyzed as follows: cells were isolated by centrifugation ($200\times g$, 5 min), washed with PBS, and suspended in the modified HEPES-Tyrode's buffer/2% BSA. The cells were incubated with FITC-conjugated anti-CD41 antibody or FITC-conjugated control IgG for 30 min at room temperature, fixed/permeabilized by gradually adding ice-cold methanol with constant stirring, followed by a 30-min incubation at 4°C. Then the cells were isolated by centrifugation and then further incubated in propidium iodide (PI) buffer (PI at $50\text{ }\mu\text{g/ml}$ in PBS containing 100 units/ml RNase) for 30 min at 4°C, followed by 15-min incubation at 37°C. DNA ploidy of cells was determined by flow cytometry (EPICS XL; Beckman Coulter, Fullerton, CA, USA) [17].

Flow cytometric analysis of PLPs

The cultured whole cell medium was centrifuged ($200\times g$, 5 min) to eliminate large cells and the supernatant was further centrifuged ($1000\times g$, 15 min) to obtain PLPs. The PLP precipitate was suspended in modified HEPES-Tyrode's buffer/ 2% BSA and incubated with FITC-conjugated anti-mouse GPIIb β antibody and PE-conjugated anti-mouse CD41 antibody for 30 min at room temperature followed by the analysis with flow cytometer. Platelet-specific marker expression on PLPs was analyzed after PLPs were selected by gating from whole cells with the forward- and side-scatter gates used for murine platelets. Ability to undergo integrin $\alpha_{\text{IIb}}\beta_3$ activation was determined by incubating the cells with PE-conjugated anti-activated

$\alpha_{\text{IIb}}\beta_3$ antibody (JON/A) instead of anti-CD41, followed by addition of 2 units/ml bovine thrombin/1 mM CaCl₂. Non-specific binding was determined in the presence of 10 mM EDTA.

Measurement of PLP adhesion to immobilized vWF

Glass slides were reacted with $30\text{ }\mu\text{g/ml}$ human vWF in modified HEPES-Tyrode's buffer overnight at 4°C. VWF-coated slides were blocked with 2% BSA solution for >1 h at room temperature and then washed with modified HEPES-Tyrode's buffer. PLPs (1×10^7 /ml) were suspended in modified HEPES-Tyrode's buffer/ 0.2% BSA/biotinocetin (kindly provided from Dr. M. C. Berndt, Monash University, Melbourne, Australia; $1\text{ }\mu\text{g/ml}$) and placed on vWF-coated glass slides for 30 min at 37°C. Nonspecific binding was determined in the presence of $20\text{ }\mu\text{g/ml}$ of anti-mouse GPIIb β antibody (Emfret, clone Xia.B2), which blocks the vWF binding site of GPIIb β . Slides were washed 3 times with modified HEPES-Tyrode's buffer and adhered cells were stained with FITC-conjugated anti-mouse CD41 antibody and then observed under a fluorescence microscope (Eclipse TE2000-U; Nikon, Tokyo, Japan). The images were acquired by the Nikon Digital Sight System, and the extent of adhesion was calculated as a percent of the surface area covered by the adhered cells using the software Lumina Vision (Mitani, Fukui, Japan).

Statistical Analysis

Statistical significance of the difference between each data set was evaluated by Student's *t*-test with Prism software (Graph Pad Software, San Diego, CA, USA).

RESULTS

Characterization of megakaryocytes derived from mouse ES cells

Mouse ES cells, E14tg2A cells, were cultured for EB formation as described in the Methods. After 6 days of culture, there was usually formation of cell aggregates (EBs) in the culture medium; these EBs were dispersed to cell suspension and CD41-positive cells were separated from this suspension by magnetic beads. The sorted cells were incubated in the medium containing 50 ng/ml TPO, and the growth of MK-like cells was observed. No feeder cells were used for the differentiation to MKs because we observed that the number of mononuclear cells were increased in later culture stages with OP9 (data not shown). However,

even without feeder cells, we still observed formations of MK-like cells and platelet-like small particular cells. We characterized the cells taken at 10 and 13 days of culture (time table is shown in Fig. 1A).

Phase-contrast microscopy (Fig. 1B) shows the formation of large MK-like cells (arrow) in day-10 culture, and proplatelet formation is observed at day 13 (Fig. 1B, C, arrowhead). Proplatelet formation occurs from day 9-10 of culture, and increased up to day 12-13. Cultured cells stained with May-Gruenwald Giemsa reagent show the development of morphology characteristic to MKs, a large cytoplasm and large nucleus (arrow), which is different depending on culture length (Fig. 1C).

DNA ploidy and CD41 expression was determined for cells collected on day 6, 10, and 13. Cells with high ploidy (Gate-2, >8N) are increased from 33% (day 10) to 42% (day 13) (Fig. 1D). Whole cells (Gate-1) were analyzed for their expression of CD41, specific marker of platelets, MKs and primitive erythroid (Fig. 1E). On day 6, only a small number of cells expressed CD41, but there is higher expression in day-10 and -13 cells. These results demonstrate that most of cells obtained from our feeder cell-free culture system have

the characteristics of MKs.

Characterization of platelet-like particles obtained from ES-derived megakaryocytes

In our culture system, proplatelet formation was observed for cells in the later stage of culture, accompanied by the appearance of PLPs in the medium. These PLPs were collected from culture medium and subjected to flow cytometry to analyze their expressions of platelet-specific markers, in comparison to mouse platelets. In flow cytometry, PLPs were gated as the same size as mouse platelets (Fig. 2A) for their expressions of CD41 and GPIIb/IIIa (Fig. 2B). Some PLPs expressed both CD41 and GPIIb/IIIa, similar to platelets, and these cells increased in the later days of culture : day 10 (65.8%) and day 13 (71.8%). Although no PLPs expressing only GPIIb/IIIa and no CD41, 17% of PLPs expressed only CD41 and no GPIIb/IIIa. Since GPIIb/IIIa was indicated to be expressed at a later stage of MK maturation than CD41 [18], these PLPs expressing only CD41 are suggested to be immature cells. The number of PLPs obtained from culture medium of day 10 and day 13 calculated as a number relative to the number of original ES cells (Table 1). Results from 3

Fig. 1. (A) Time schedule of the culture system for ES-cell differentiation to megakaryocytes. (B) Phase-contrast microscopic images of ES cell-derived megakaryocytes. Bar=50 μ m. (C) Cultured cells stained with May-Gruenwald Giemsa. Bar=50 μ m. (D) Flow cytometric analysis of DNA ploidy of ES-cell-derived MKs. FITC-conjugated anti-CD41 antibody and propidium iodide (PI) staining. DNA content was shown by PI fluorescence and cells having >2N were gated as Gate-1 and analyzed for CD41 expression (E).

Fig. 2. Flow cytometric analysis of CD41 and GPIIb/IIIa expressions of PLPs. (A) Patterns of forward- (x-axis) and side-scattering (y-axis) of mouse platelets and PLPs. PLPs having the same scattering properties as mouse platelets were gated for further analysis. (B) The PLPs gated as in (A) were further analyzed for the surface expression of GPIIb/IIIa (x-axis, FITC-conjugated anti-mouse GPIIb/IIIa antibody) and CD41 (y-axis, PE-conjugated anti-mouse CD41 antibody).

TABLE 1.
Numbers of PLPs expressing CD41 and GPIIb/IIIa produced from one ES cell

	CD41+	CD41+GPIIb/IIIa+	CD41+GPIIb/IIIa-
Day 10	5.66±1.47	4.48±1.06	1.19±0.41
Day 13	10.35±3.47	8.64±2.94	1.70±0.53
total	16.02±3.40	13.13±2.86	2.89±0.56

PLPs in the medium of day 10 and day 13 were analyzed for the expression of CD41 and GPIIb/IIIa by flow cytometer and the number of PLPs expressing these antigen were calculated as a number corresponding to one original ES cell. Data show the mean $\pm$ SEM of three independent experiments.

experiments indicate that about 16 PLPs expressing CD41 and 13 PLPs expressing both CD41 and GPIIb/IIIa were obtained from each ES cell. More PLPs expressing these platelet-specific marker proteins were obtained on day 13 than day 10 (Table 1).

Activation of integrin $\alpha_{IIb}\beta_3$ on PLPs

We determined the ability of the $\alpha_{IIb}\beta_3$ expressed in the PLPs to undergo activation by determining binding of JON/A antibody, specific for the active form of

the integrin $\alpha_{IIb}\beta_3$, after addition of thrombin and Ca^{2+} to the PLPs. Thrombin induced JON/A binding to PLPs (Fig. 3), which was prevented by the presence of EDTA. These results suggested that $\alpha_{IIb}\beta_3$ on PLPs has a functional conformation similar to the one on platelets.

Increased expression of GPIIb/IIIa on PLPs by inhibiting its shedding

We observed very low expression of GPIIb/IIIa com-

Fig. 3. Flow cytometric analysis of $\alpha_{IIb}\beta_3$ of PLPs. (A) Mouse platelets and PLPs from day 13 culture were activated with 2 unit/ml thrombin in the presence of FITC-conjugated anti-mouse GPIIb β antibody and PE-conjugated JON/A antibody. Mouse platelets and PLPs were gated at first with size and anti-GPIIb β antibody binding analyzed. (B) GPIIb β -positive cells were gated as Gate-3 and JON/A binding analyzed. JON/A-positive cells were gated as Gate-4. (C) Number of cells in Gate-4 was calculated as a percent of cells in Gate-3. A significant number of PLPs were activated by thrombin. The data shown the mean $\pm$ SEM, $n=3$.

pared to the level of GPIIb β on PLPs by flow cytometry. Since GPIIb is composed of one GPIIb α and one GPIIb β chain covalently connected by disulfide bonding, this discrepancy could be explained by the shedding of GPIIb α by ADAM family proteases, as reported for platelets [19].

To determine if this is the case, we tested several metalloproteinase inhibitors, GM6001 (100 μ M), TAPI-1 (20 μ M), and TAPI-2 (100 μ M), the former two being reported to increase GPIIb α expression in MKs cultured in the presence of OP9 [19]. Because matrix metalloproteinase-9 was reported to facilitate megakaryopoiesis [16], we added metalloproteinase inhibitor at the later culture stage, day 10. In prelimi-

nary experiments, GM6001 showed only a weak effect, but TAPI-1 and TAPI-2 increased GPIIb α expression on PLPs (data not shown). So, we compared the effects of TAPI-1 and TAPI-2 on GPIIb α expression and found that 30.7 and 36.6% of CD41-positive PLPs become GPIIb α -positive after TAPI-1 and TAPI-2 treatment, respectively (Fig. 4A), suggesting that TAPI-2 is the most effective inhibitor to maintain the level of GPIIb α expression. Because TAPI-2 is water-soluble, it can be added to the cell culture without affecting cell growth. In contrast, TAPI-1, as well as GM6001, must be dissolved in DMSO, which showed an inhibitory effect on the cell growth even at 0.2% in our culture system, making it unsuitable for use in this system. We performed three more experiments using TAPI-2 and verified that this inhibitor significantly increased GPIIb α expression on PLPs from day 13 culture (Fig. 4B). These data confirm that TAPI-2 increases expression of GPIIb α on PLPs by inhibiting the shedding by metalloproteinase.

To analyze the function of GPIIb expressed on PLPs, we measured PLP adhesion to immobilized von vWF. PLPs from day 13 culture, which were treated with TAPI-2 or vehicle, were incubated on a vWF-coated glass slide in the presence or absence of anti-GPIIb α antibody for 30 min at 37°C. The adhered PLPs were stained with FITC-conjugated anti-mouse CD41 antibody and the adhered PLPs were observed by fluorescence microscopy. Visual inspection of the images suggests that TAPI-2-treated PLPs show more adhesion to vWF than the vehicle-treated PLPs (Fig. 4C: i). Quantitation of the adhesion as a percent of surface area covered by adhered PLPs demonstrated that the surface area coverage was significantly increased in TAPI-2-treated PLPs ($P<0.05$) compared to the vehicle-treated PLPs (Fig. 4C: ii). Taken together, these results indicate that the addition of TAPI-2 at day 10 in our culture system prevents the shedding of GPIIb α on PLPs and increases GPIIb-specific adhesion of PLPs to vWF.

DISCUSSION

Until now, MKs were produced from mouse or human ES cells cultured in the presence of TPO over a layer of feeder cells [see ref. 5 for a review], which could contaminate the MK preparation as well as being a laborious procedure. Here we report the first culturing system for producing MKs and PLPs from E14tg2A cells without using feeder cells. We tried ES cells from another cell line, BRUCE-4 (Millipore), under similar conditions, but the efficiency to obtain

Fig. 4. (A) Flow cytometric analysis of CD41 (x-axis) and GPIb α (y-axis) of PLPs from day-13 culture treated with TAPI-1 (20 μ M), TAPI-2 (100 μ M), or vehicle. TAPI-1 or TAPI-2 was added at day 10. (B) Flow cytometric analysis of GPIb α and GPIb β expression of PLPs from day-13 culture. (i) Mouse platelets and PLPs gated as in Fig. 2 were analyzed for the expressions of GPIb β (x-axis) and GPIb α (y-axis). (ii) Ratio of GPIb α -positive PLPs against total PLPs in TAPI-2- or vehicle-treated cultures. TAPI-2 increases the percent of GPIb α -expressing PLPs. (C) Adhesion of PLPs to vWF-coated surface. (i) PLPs treated with TAPI-2 or vehicle from day-13 culture medium were incubated on vWF-coated glass slides with botrocetin, with or without anti-GPIb α antibody (20 μ g/ml) for 30 min at 37°C. Adhered PLPs stained with FITC-conjugated anti-mouse CD41 antibody are shown. Bars=50 μ m. (ii) Surface coverage areas of the adhered PLPs were measured as described in the text. Anti-GPIb α antibody almost completely inhibits PLP adhesion to vWF and TAPI-2 treatment increases the adhesion of PLPs. Data show the mean $\pm$ SEM, n=3.

MKs was very low comparing to that of E14tg2A cells. Thus unique feature of our culture system, no requirement for feeder cells, would be mainly attributed to the original characteristics of the E14tg2A cells. Our feeder-cell-free culture provides many advantages, including reducing time to culture feeder cells and preventing the feeder-cell contamination to the MK and PLP fractions.

Because the EB formation method was indicated to be effective for obtaining MKs [20], we isolated EBs from the culture supernatant, dissociated them, and selected the CD41-positive cells among the dissociated

cells for the second culture in the presence of TPO, instead co-culturing ES cells with feeder cells as indicated by Nishikii et al. [16]. This specific selection of CD41-positive cells increased the efficiency to obtain MKs and PLPs.

Many types of growth factors, including TPO, IL-3, IL-6, IL-11, and stem cell factor (SCF), have been added to culture medium to stimulate differentiation of hematopoietic stem cells to MKs. IL-3, IL-6, IL-11, and SCF are not essential for megakaryopoiesis, but contribute to megakaryopoiesis synergically with TPO [21], a primary regulator of megakaryopoiesis [6].

However, neither IL-6 nor SCF significantly increased MK formation in our system (data not shown). However, future studies may find an appropriate combination of growth factors for increasing PLPs in our culture system.

The MK-like cells obtained in our system had a large cytoplasm and nucleus, DNA ploidy, and expression of CD41 marker protein, all characteristics of MKs (Fig. 1). However, CD41 is also primitive erythroid marker. In our study, CD41⁺GPIb β ⁻ cells are possible to be primitive erythroid cells. Otani et al. demonstrated that the CD41^{dim} but not CD41^{bright} population could serve as immediate precursor of primitive erythroid cells. More than 30% of ES cells-derived Flk-1⁺ cells differentiated to CD41^{dim} cells after coculture for 5 days (9 days from differentiated culture) with OP9 [22]. We did not distinguish CD41^{dim} and CD41^{bright} cells. Thus, in this study the cells with low ploidy (2N-4N) might include primitive erythroid progenitors. The maximum number of DNA ploidy of our MKs was 32N, which is lower than those of MKs from ES cells cultured with OP9 cells (128N) [12] and MKs from mouse bone marrow (64N) [23]. Fujimoto et al. reported two waves of PLP production from ES cells cocultured with OP9; first wave on day 8 and second wave on day 12 of the differentiation. The MKs derived from ES cells from day 8 culture have smaller size and lower ploidy (4-8N) and produced smaller number of PLPs comparing with those from MKs at second wave culture [13]. Presence of MKs with different maturation was also reported by Xu et al. [23]. They reported the presence of primitive megakaryopoiesis in the early yolk sac of murine. They showed that MKs cultured in vitro from E8.5 yolk sac of mouse embryos had a lower ploidy (maximum=32N, mean=6.8N) when compared with those derived from the mouse adult bone marrow (maximum=64N, mean=22.9N) [23]. However it is currently not possible to distinguish the products of primitive and definitive megakaryopoiesis due the lack of differentiating marker[24]. Megakaryopoiesis in feeder free system might be a primitive megakaryopoiesis resembling to that in yolk sac of mouse embryo. Thus MKs obtained from ES cells in our feeder cell free culture system might not be in a fully matured state, compared with those cultured with feeder cell. That might explain a lower yield of PLPs in our system. We obtained about 16 PLPs from one ES cell but Fujimoto et al. [13] and Takayama et al. [14] reported that they obtained 10⁴ and 48 PLPs from one ES cell, respectively.

Although we could detect GPIb α expression on MKs (data not shown), its expression on PLPs was

very low. Nishikii et al. [16] reported that the extracellular domain of GPIb α in PLPs was shed while in the culture, but this was prevented by adding the metalloproteinase inhibitor GM6001 to the culture medium. We tested the effect of three metalloproteinase inhibitors (GM6001, TAPI-1, and TAPI-2) on our system and found that TAPI-2 most efficiently prevented GPIb α shedding as well as having the advantage of being water-soluble, while GM6001 had little effect. By including TAPI-2 in the culture medium, we successfully obtained PLPs expressing two major platelet receptors, $\alpha_{IIb}\beta_3$ and GPIb.

Integrin $\alpha_{IIb}\beta_3$ expressed on PLPs obtained from our culture system could undergo activation in response to thrombin, as demonstrated by thrombin-treated PLPs to bind JON/A, an antibody specific for the activated form of the integrin, although the binding was weaker than that to activated mouse platelets (Fig. 3). Thus our PLPs could undergo integrin activation like those derived from ES cells with the OP9 co-culture system, which upon activation exhibited binding to antibody against the active form [14] and adhesion to fibrinogen [13,16]. Although the expression of GPIb on PLPs was previously indicated by several papers [14,16], they did not analyze its function. Our GPIb-expressing PLPs showed GPIb-specific adhesion to a vWF-coated surface. The adhesion of TAPI-2 treated cells was significantly increased compared to those without addition of the inhibitor (Fig. 4C), supporting the increased expression of GPIb by TAPI-2 treatment.

In conclusion, we successfully developed a new culture system in which mouse ES cells are differentiated to MKs and to platelet-like particles without using feeder cells. This culture system would be useful for analyzing the function of platelet proteins by inducing mutations to such proteins and analyzing their functions.

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