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【Upshaw-Schulman 症候群の ADAMTS13 遺伝子解析】

ADAMTS13 genetic analysis of Upshaw-Schulman syndrome

小亀 浩市・樋口（江浦）由佳

Koichi Kokame　　Yuka (Eura) Higuchi

Key words
ADAMTS13，遺伝子解析，
ダイレクト・シーケンシング，定量 PCR

要　約

von Willebrand 因子切断酵素 ADAMTS13 の活性著減は，血栓性血小板減少性紫斑病（TTP）の発症につながる。常染色体劣性遺伝形式を示す先天性 TTP は Upshaw-Schulman 症候群（USS）とも呼ばれ，ADAMTS13 遺伝子異常が原因となる。我々はこれまで，臨床的に USS が強く疑われた 49 患者に対してダイレクト・シーケンシング法を実施し，46 患者に複合ヘテロあるいはホモ接合性の原因変異を同定した。残り 3 患者のうち 2 患者には 1 アレルのみ変異が検出された。これら 3 患者に対してゲノム定量 PCR 法を行った結果，2 患者に 1,782 bp あるいは 729 bp の欠失を見出した。ダイレクト・シーケンシング法で異常が全く検出されなかった 1 患者では，ゲノム定量 PCR 法でも正常であった。USS の遺伝子解析においてはダイレクト・シーケンシング法が最も効率よく，それで変異が確定しなかった場合，ゲノム定量 PCR 法が有効になると思われる。

1．Upshaw-Shulman 症候群

血栓性血小板減少性紫斑病（Thrombotic Thrombocytopenic Purpura：TTP）は，血小板減少と溶血性貧血を呈し，時に腎障害や発熱，動揺性精神神経障害を併発する全身性の疾患である。TTP の病態は溶血性尿毒症症候群と類似しており，両者とも血栓性微小血管障害症に属するが，TTP は von Willebrand 因子（VWF）切断酵素 ADAMTS13 の活性が著減している点で特徴づけられる。TTP の大半を占める後天性 TTP の患者血漿には，ADAMTS13 活性を阻害する自己抗体（インヒビター）が検出される。一方，先天性 TTP では ADAMTS13 遺伝子の異常が原因で ADAMTS13 活性が著減する[1]。先天性 TTP は Upshaw-Shulman 症候群（USS）とも呼ばれ，先天性 ADAMTS13 欠損症とほぼ同義である。

ADAMTS13 は VWF マルチマーを切断することで血小板凝集を調節する血漿プロテアーゼであり，その活性が低下すると高分子量 VWF マルチマーの断片化が不十分となる。VWF マルチマーは分子量が大きいほど高い血小板凝集能をもつため，その断片化不全は血小板血栓の形成につながる[2]。

日本における TTP 診断ガイドラインは未完成であるが，これまでの研究などから，5 徴候（血小板減少・溶血性貧血・腎障害・発熱・動揺性精神神経障害）が揃っていれば TTP と診断され，5 徴候が揃っていなくても，血小板減少と溶血性貧血に加え，血漿 ADAMTS13 活性が 5-10% 未満で，インヒビターが陽性であれば後天性 TTP，陰性であれば USS と診断される。USS を確定するには，2 回以上の検査で常に ADAMTS13 活性が低くインヒビターが検出されないことや，家族歴から遺伝性が疑われることなどが重要である。ADAMTS13 遺伝子異常の確認は確定診断の支持に大きく寄与する。

2．ADAMTS13 の構造

ヒトゲノムには ADAMTS（A Disintegrin And Metalloproteinase with Thrombospondin type I motif）ファミリー遺伝子が 19 個存在する。いずれもプロテアー

国立循環器病研究センター 分子病態部
Department of Molecular Pathogenesis, National Cerebral and Cardiovascular Center
〒 565-8565　大阪府吹田市藤白台 5-7-1　TEL：06-6833-5012（内線 2460）

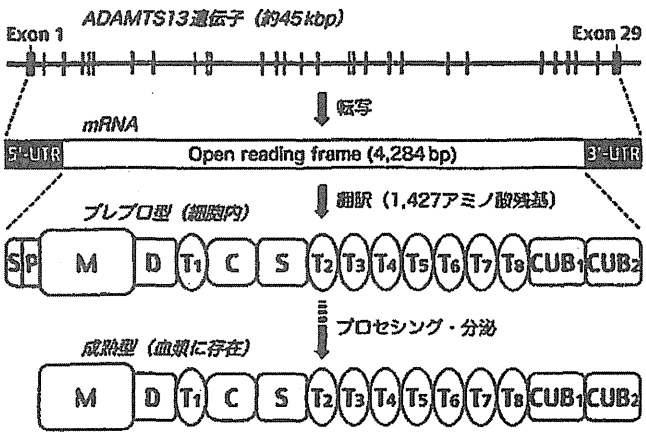

図1　ADAMTS13の遺伝子構造および蛋白質ドメイン構成

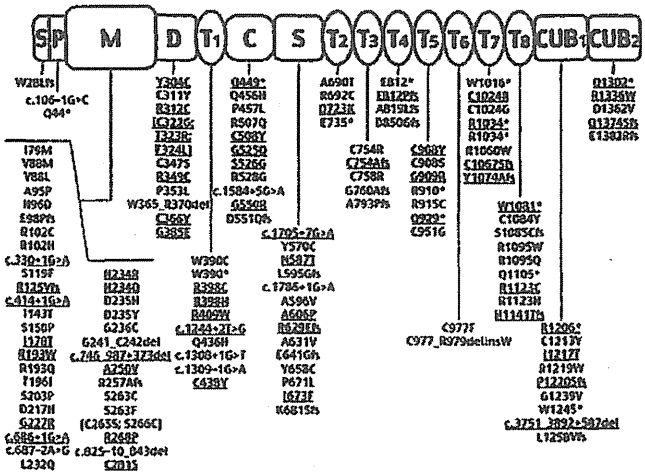

図2　ADAMTS13に同定されたUSS原因変異。
下線は日本人に同定された変異。

ゼであり，細胞外マトリクスの構築や分解，血管新生，生殖，臓器形成などに関与するが，それぞれの分子機能には不明な点が多い。

ADAMTS13遺伝子は染色体9q34に位置し，約45 kbpにおよぶ29個のエクソンからなる（図1）[3]。翻訳領域ORF（4,284 bp）の開始コドンはexon 1に，終止コドンはexon 29にある。

翻訳されたポリペプチド鎖は1,427アミノ酸残基からなり，N末端にシグナル配列とプロペプチドをもつプレプロ型として翻訳される[4]。血液中の成熟型ADAMTS13のN末端はメタロプロテアーゼ（M）ドメインであり，そのC末端側にDドメイン，Tドメイン，Cドメイン，Sドメインといったadamtsに特徴的な構造が続く。ADAMTSの多くはさらに複数のTドメインをもち，ADAMTS13では7個連続する。そのC末端側に続く2個のCUBドメインは他のADAMTSにない構造である。

3．ダイレクト・シーケンシング法による変異探索

遺伝子解析を行う場合，まず必要となるのはインフォームド・コンセントの取得や倫理審査委員会での承認などの倫理的手続きである。その後，患者や家族の血液からDNAを抽出し，解析を開始する。USS疑い患者のADAMTS13遺伝子解析では，すべてのエクソンに対してダイレクト・シーケンシング法による変異探索を行う[5]。ダイレクト・シーケンシング法とは，検体DNAを鋳型にして解析対象領域をPCRで増幅し，PCR産物の塩基配列をサンガー法で直接（クローニングせずにという意味）配列決定する方法である。

USSは常染色体劣性遺伝形式の疾患であるため，患者はADAMTS13遺伝子異常の複合ヘテロ接合体（各アレルに別の変異）あるいはホモ接合体（両アレルに同じ変異）である。我々はこれまでにUSS疑い49患者（43家系）を解析し，46患者（40家系）に複合ヘテロあるいはホモ接合性の原因変異を同定した。世界的には140を超える原因変異が報告されており，我々の報告はその4割近くを占める（図2）。ミスセンス変異が最も多いが，ナンセンス変異やフレームシフト変異，スプライシング異常なども多数報告されている。

我々は患者の遺伝子解析に加え，日本人一般住民の解析も行い，健常者にも見出されるミスセンス多型として，T339R，Q448E，P475S，P618A，S903L，G1181Rを同定した[6]。したがって，これらがUSS患者に見つかっても原因変異とは考えない。ただし，P475SはADAMTS13活性を若干低下させる[7]。

4．ゲノム定量PCR法による変異探索

USS疑い49患者のうち46患者に複合ヘテロあるいはホモ接合性の原因変異を同定したと述べた。残り3患者のうち2患者では1つの変異しか見つからず，1患者では全く見つからなかった。そこで，これら3患者はダイレクト・シーケンシング法で検出できない変異を

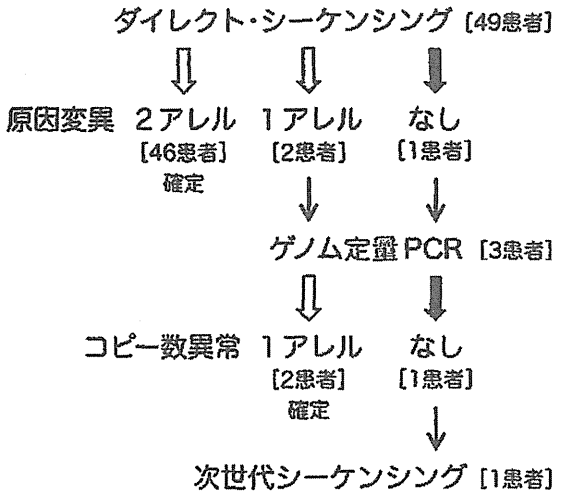

図3　USS患者を対象としたADAMTS13遺伝子解析の流れ

もつと想定した。例えば，あるエクソン領域において一方のアレルは正常で，もう一方のアレルにはPCRプライマーがハイブリダイズできない変異があると，正常アレルのみ増幅される。それをサンガー法で解析すると正常アレルの配列のみが読めるため，両アレルとも正常と誤解される。

そのような弱点を補完するには，ゲノムにおける各エクソンの相対的コピー数を分析することが有効である。その目的でしばしばMultiplex Ligation-dependent Probe Amplification（MLPA）法やアレイComparative Genomic Hybridization（CGH）法が行われるが，ADAMTS13用の試薬やキットは市販されていない。そこで我々は，エクソン領域ごとに相対的コピー数を分析するゲノム定量PCR法を導入した。さらに，プライマーペアのうち少なくとも一方をダイレクト・シーケンシング法と同じ配列にするという独自の工夫を加えた[8]。ダイレクト・シーケンシング法と組み合わせれば，エクソン領域の変異を見逃す可能性がなくなり，その点でMLPA法やアレイCGH法にまさる。この解析を行った結果，上述3患者のうち2患者にそれぞれc.746_987+373del（1,782 bp欠失）とc.3751_3892+587del（729 bp欠失）を発見することができた。ADAMTS13遺伝子にこのような長い欠失が見つかったのは初めてである。この2患者にはすでにダイレクト・シーケンシング法でそれぞれp.G550Rとp.C281Sを同定しており，両親の遺伝子解析から，いずれの患者も複合ヘテロ接合体であることが確認された。

おわりに

ダイレクト・シーケンシング法とゲノム定量PCR法を行うことにより，49患者のうち48患者に原因変異を特定することができた（図3）。しかし，残る1患者ではいずれの方法でも異常が見られなかった。現在，次世代シーケンサーを利用して，全イントロンを含むADAMTS13遺伝子全体のシーケンシングを開始したところである。これで新たな変異が見つかるか否か。もし見つからなければ，ADAMTS13遺伝子全体を含む規模の転座や逆位といった異常や，ADAMTS13以外の遺伝子の異常も視野に入れた解析が必要となる。

これまでの経験上，USSのADAMTS13遺伝子解析では，まずダイレクト・シーケンシング法を行うことが効果的であり，9割以上は解決する。原因変異が一つしか見つからない場合，ゲノム定量PCR法で別の変異を発見できる可能性がある。ダイレクト・シーケンシング法で変異が一つも見つからない場合，原因の特定は難渋するかもしれない。

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ORIGINAL ARTICLE *Laboratory science*

Production of functional coagulation factor VIII from iPSCs using a lentiviral vector

Y. KASHIWAKURA,*† T. OHMORI,* J. MIMURO,* S. MADOIWA,* M. INOUE,‡ M. HASEGAWA,‡ K. OZAWA§ and Y. SAKATA*

**Research Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan; †Department of Immunology, Dokkyo Medical University School of Medicine, Tochigi, Japan; ‡DNAVEC Corporation, Ibaraki, Japan; and §Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan*

Summary. The use of induced pluripotent stem cells (iPSCs) as an autologous cell source has shed new light on cell replacement therapy with respect to the treatment of numerous hereditary disorders. We focused on the use of iPSCs for cell-based therapy of haemophilia. We generated iPSCs from mesenchymal stem cells that had been isolated from C57BL/6 mice. The mouse iPSCs were generated through the induction of four transcription factor genes Oct3/4, Klf-4, Sox-2 and c-Myc. The derived iPSCs released functional coagulation factor VIII (FVIII) following transduction with a simian immunodeficiency virus vector. The subcutaneous transplantation of iPSCs expressing FVIII into nude mice resulted in teratoma formation, and significantly increased plasma levels of FVIII. The plasma concentration of FVIII was at levels appropriate for human therapy at 2–4 weeks post transplantation. Our data suggest that iPSCs could be an attractive and prospective autologous cell source for the production of coagulation factor, and that engineered iPSCs expressing coagulation factor might provide a cell-based therapeutic strategy appropriate for haemophilia.

Keywords: haemophilia, induced pluripotent stem cells, lentiviral vector, cell therapy, gene therapy, blood coagulation

Introduction

Haemophilia is an X-linked inherited bleeding disorder, caused by mutations within the *coagulation factor VIII* (*F8*) or *coagulation factor IX* (*F9*) genes. This results in a longer than average time for blood to clot, which can lead to significant bleeding. Haemophilia is considered suitable for gene therapy, as it is caused by a single gene abnormality, and therapeutic coagulation factor levels can vary across a broad range [1]. Recently, therapeutic levels of coagulation factor have been achieved in haemophilia B patients through the direct administration of adeno-associated virus vectors in clinical trials [2]. Another gene therapy strategy for haemophilia is the application of cells transduced *ex vivo*, as a delivery vehicle for coagulation factor [3]. Cell-based therapy reduces the risk of unwanted virus dissemination, and ensures the selection of highly expressing clones prior to commencement of the procedure. However, cell-based therapies for haemophilia have been hampered in animals and during human clinical trials, because of the short life span of transplanted cells, difficulties in obtaining therapeutic plasma levels, and elimination of the transduced cells by immune reactions [4].

Induced pluripotent stem cells (iPSCs) are artificially generated stem cells, made by reprogramming somatic cells through the expression of defined transcription factors [5,6]. These iPSCs are pluripotent, with the ability to differentiate into cells of the three germ lineages *in vitro*. Mouse-derived iPSCs can be passaged through the germ line, as is the case with embryonic stem cells (ESCs). Using iPSCs has a distinct advantage over ESCs, as cells differentiated from iPSCs exhibit limited immunogenicity, and are therefore more easily tolerated by a recipient following transplantation. Accordingly, iPSC technology offers the possibility of patient-specific cell therapy for haemophilia, in which the use of genetically identical cells would prevent immune rejection. In this study, we examined whether iPSCs could release

Correspondence: Tsukasa Ohmori, MD, PhD, Research Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, 3111-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan.
Tel: +81-285-58-7397; fax: +81-285-44-7817;
e-mail: tohmori@jichi.ac.jp

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functional coagulation factor using lentiviral transduction. We then focused on the possible clinical application of the engineered iPSCs to cell-based gene therapy for haemophilia.

Materials and methods

Mice

C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan). BALB/cAJcl-*nu/nu* mice (nude mice) were obtained from CLEA Japan, Inc. (Shizuoka, Japan). All animal procedures were approved by the Institutional Animal Care and Concern Committee at Jichi Medical University, and animal care was in accordance with the committee's guidelines.

Generation of iPSCs and cell culture

Murine mesenchymal stem cells (MSCs) were isolated and maintained as described previously [7]. We selected MSCs for the establishment of iPSCs because MSCs have a higher reprogramming efficiency compared with fibroblasts [8,9]. Two plasmid vectors to generate iPSCs (pCX-OKS-2A and pCX-cMyc) were obtained from Addgene (Cambridge, MA). MSCs were transduced with the plasmid vectors by nucleofection as described previously [10]. Colonies that were ES-like were cloned at 30 days post transduction, and MSC-derived iPSCs were maintained on mouse embryonic fibroblasts with knockout Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 15% foetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 0.5 mM monothioglycerol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 1000 U mL^{-1} ESGRO (Merck Millipore, Billerica, MA).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was prepared using an RNeasy Mini Kit (QIAGEN, Montgomery, MD). The RT-PCR assays were conducted using a SuperScript One-Step RT-PCR System (Invitrogen). Primer pairs for the RT-PCR assays used in this study have been previously reported [5].

Lentiviral vector construct and production

The cDNA for human B-domain-deleted FVIII (hBDD-FVIII) was generated as previously described [11]. The *hBDD-FVIII* gene, under the control of a chicken β-actin promoter coupled with cytomegalovirus promoter early enhancer element (CAGp), was cloned into a self-inactivating simian immunodeficiency virus (SIV) lentiviral vector [12]. The SIV lentiviral vectors were generated as previously described [13].

Measurement of FVIII activity and antigen

The hFVIII antigens (FVIII:Ag) were measured using an anti-hFVIII-specific enzyme-linked immunosorbent assay (ELISA) kit (ASSERACHROM VIII:Ag; Diagnostica Stago, Seine, France). The functional activity of FVIII (FVIII:C) was measured using a one-stage clotting time assay on an automated coagulation analyser (Sysmex CA-500 analyser; Sysmex Corp., Kobe, Japan). We used pooled normal human plasma as a reference to measure both FVIII:C and FVIII:Ag.

Results and discussion

We first attempted to establish iPSCs from C57BL/6 mice. Bone marrow-derived MSCs were transduced with plasmid vector expressing the defined transcription factors. We cloned ESC-like cell colonies after transduction, and SSEA-1 positive cells were sorted by flow cytometry (Fig. 1a). The sorted cell colonies exhibited typical ESC morphology and alkaline phosphatase activity (Fig. 1b). The mRNA expression patterns of endogenous pluripotent-specific genes (*Sox2, Oct3/4, Nanog* and *c-Myc*) in the cells were similar to those in E14tg2a mouse ESCs (Fig. 1c). Furthermore, subcutaneous transplantation of these cells (1×10^6 cells) into nude mice resulted in the formation of teratomas containing tissues derived from the ectoderm, mesoderm, and endoderm (Fig. 1d and e). This would suggest that the cells possess the potential to differentiate into cells and tissues of the three germ layers. Therefore, we used these iPSCs for further experiments in this study.

We next examined whether iPSCs could produce functional coagulation factor after transduction by a lentiviral vector. The iPSCs were transduced with the SIV vector expressing hBDD-FVIII under the control of CAGp (SIV-CAG-hFVIII) (Fig. 2a). We cloned iPSC colonies from the cells transduced with SIV-CAG-hFVIII at a multiplicity of infection (MOI) of 30, and selected three iPSC clones that stably produced hFVIII in the supernatant (Fig. 2b). The iPSC clones expressing hFVIII were subcutaneously transplanted into nude mice. Following transplantation, plasma levels of hFVIII:Ag in nude mice gradually increased according to teratoma formation derived from the transplanted iPSCs (Fig. 2c and d). Although the plasma level of hFVIII:Ag in the nude mice was at 20%, we could not measure hFVIII:C in nude mice because of the existence of mouse FVIII. We simultaneously measured hFVIII:C and hFVIII:Ag produced from transduced

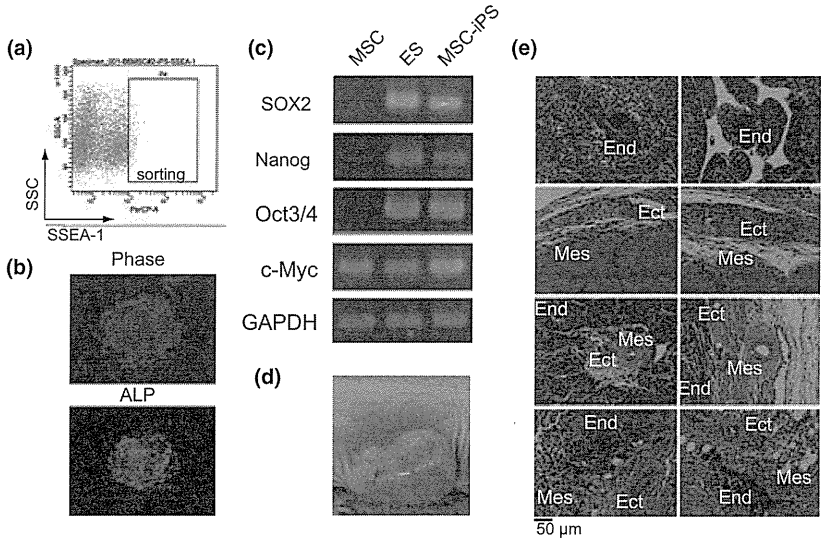

Fig. 1. Characterization of induced pluripotent stem cells (iPSCs) derived from mesenchymal stem cells (MSCs). MSCs isolated from C57BL/6 mice were transduced with plasmid vectors expressing Oc3/4, Klf4, Sox2 and c-Myc. (a) Stage-specific embryonic antigen 1 (SSEA-1) expression was examined by flow cytometry following transduction. SSEA-1-positive cells (shown in the square) were sorted as MSC-derived iPSCs. (b) Morphology of iPSC colonies derived from SSEA-1-positive cells. Alkaline phosphatase activity in iPSC colonies was detected using HNP/Fast Red TR. (c) The mRNA expression levels of the pluripotency markers SOX2, Nanog, Oct3/4 and c-Myc were determined by reverse transcription polymerase chain reaction. (d) Teratoma formation in nude mice after subcutaneous transplantation of iPSCs. (e) Differentiation into cells and tissues of the three germ lineages were confirmed by histological analysis. End, endoderm; Ect, ectoderm; Mes, mesoderm.

iPSCs, and calculated the ratio of hFVIII:C to hFVIII:Ag (0.30 ± 0.041). Accordingly, hFVIII:C levels seemed to reach the therapeutic level of FVIII required for haemophilia A (6%) in transplanted mice.

Recent studies have reported the potential of iPSCs for the treatment of many human diseases; iPSCs possess the ability to differentiate into cardiovascular [14–16], haematopoietic [16,17], neural [18], and hepatic [19,20] progenitor cells. Treatment of inherited disorders using iPSCs has been proposed for animal models of sickle cell anaemia [17]. The haemophilic mouse model could also be used to examine the potential of iPSC therapy. Xu *et al.* reported that transplantation of endothelial progenitor cells derived from iPSCs into the liver increased FVIII levels, resulting in a corrected bleeding phenotype for haemophilia A mice [21,22]. The results reported by Xu *et al.* were surprising because the differentiated endothelial progenitor cells that were transplanted only contained one copy of *F8*, yet they were able to increase plasma levels of FVIII. In contrast, lentiviral transduction using our procedure resulted in 5–10 proviral integration sites in the diploid genome at an MOI of 30, suggesting that the same procedure using engineered iPSCs expressing coagulation factor would result in a more potent therapeutic effect.

In our preliminary experiments, we used nude mice to verify the following: the net production of FVIII from iPSCs by excluding the role of the immune system; and pluripotency of iPSCs by teratoma formation. However, tumourigenesis by iPSCs, should be

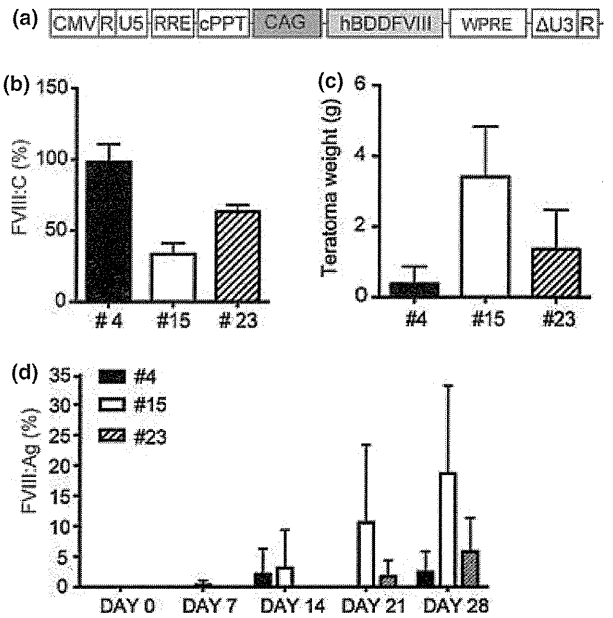

Fig. 2. Increase in FVIII levels after transduction with the simian immunodeficiency virus (SIV) lentiviral vector. The induced pluripotent stem cells (iPSCs) were transduced with SIV vector expressing human B-domain deleted coagulation factor VIII (hBDD-FVIII) under the control of a CAG promoter (SIV-CAG-hFVIII) at an MOI of 30. (a) Schematic of the SIV vectors used in our experiments. (B) FVIII activities in the supernatants derived from cloned iPSCs (#4, #15, and #23) transduced with SIV-CAG-hFVIII were measured using a one-stage clotting time assay. Values are presented as means ±SD (n = 4 for each experiment). (c, d) iPSC clones expressing hFVIII were subcutaneously transplanted into nude mice. (c) Teratoma weight at 4 weeks after subcutaneous transplantation of cloned MSC-iPSCs transduced with SIV-CAG-hFVIII. Values are presented as means ±SD (n = 3–5). (d) Plasma FVIII antigen levels in nude mice at the indicated times after subcutaneous transplantation of the iPSC clones. Values are means ±SD (n = 3–5).

completely avoided in their application. To reduce tumourigenicity and to improve the safety of iPSCs, the use of non-integrative vectors and changes of defined factors has been widely examined [6,23–25]. We also should differentiate iPSCs into the appropriate cells before transplantation, and plan to establish a more realistic cell therapy approach using immunocompetent FVIII-deficient mice.

One strategy to increase the safety of iPSCs for cell therapy would be to administer anucleated cells, such as red blood cells and platelets, differentiated from iPSCs. Integration of transgenes into genomic DNA during iPSC induction and lentiviral transduction might be negligible in the transplantation of anucleate cells. We have previously reported, along with other researchers, that expression of coagulation factor in red blood cells [26] and platelets [13,27] using lentiviral vectors has corrected the phenotype of mouse models of haemophilia. The use of blood cells to deliver coagulation factor is attractive as it avoids interference from circulating inhibitors. Recent reports have suggested the production of functional platelets from human iPSCs [28]; the transfusion of these blood cells expressing coagulation factor produced from iPSCs *in vitro* is possibly the most efficient and effective method for treating haemophiliacs.

In conclusion, we have proposed a new cell-based treatment for haemophilia involving iPSCs. Our proposed approach appears to be feasible, as transplantation of iPSCs resulted in increased and therapeutically appropriate FVIII plasma levels. Further investigations are needed to explore the risks of tumourigenicity from iPSC-derived cells, and to efficiently increase plasma levels of coagulation factor following cell therapy.

Acknowledgements

Y. Kashiwakura and T. Ohmori designed and performed the experiments, analysed the data and wrote the manuscript; M. Inoue and M. Hasegawa provided vital reagents and critically reviewed the manuscript; and J. Mimuro, S. Madoiwa, K. Ozawa and Y. Sakata analysed the data and revised the manuscript. We thank Naoko Ito and Masanori Ito (Jichi Medical University) for their technical assistance. This study was supported by a Grant from the Japan Baxter Hemophilia Scientific Research & Education Fund; Grants-in-Aid for Scientific Research (23591427, 21591249 and 23591426); Special Project Award from Bayer Hemophilia Award Program 2011; the Support Program for Strategic Research Infrastructure from the Japanese Ministry of Education and Science, and Health, Labour and Science Research Grants for Research on HIV/AIDS and Research on Intractable Diseases from the Japanese Ministry of Health, Labour and Welfare.

Disclosures

The authors stated that they had no interests that might be perceived as posing a conflict or bias.

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OP9 Bone Marrow Stroma Cells Differentiate into Megakaryocytes and Platelets

Yumiko Matsubara[1]*, Yukako Ono[2], Hidenori Suzuki[3], Fumio Arai[4], Toshio Suda[4], Mitsuru Murata[1], Yasuo Ikeda[5]

1 Department of Laboratory Medicine, Keio University School of Medicine, Tokyo, Japan, **2** Division of Hematology, Department of Medicine, Keio University School of Medicine, Tokyo, Japan, **3** Department of Morphological and Biomolecular Research, Nippon Medical School, Tokyo, Japan, **4** Department of Cell Differentiation, Keio University School of Medicine, Tokyo, Japan, **5** Faculty of Science and Engineering, Life Science and Medical Bioscience, Waseda University, Tokyo, Japan

Abstract

Platelets are essential for hemostatic plug formation and thrombosis. The mechanisms of megakaryocyte (MK) differentiation and subsequent platelet production from stem cells remain only partially understood. The manufacture of megakaryocytes (MKs) and platelets from cell sources including hematopoietic stem cells and pluripotent stem cells have been highlighted for studying the platelet production mechanisms as well as for the development of new strategies for platelet transfusion. The mouse bone marrow stroma cell line OP9 has been widely used as feeder cells for the differentiation of stem cells into MK lineages. OP9 cells are reported to be pre-adipocytes. We previously reported that 3T3-L1 pre-adipocytes differentiated into MKs and platelets. In the present study, we examined whether OP9 cells differentiate into MKs and platelets using MK lineage induction (MKLI) medium previously established to generate MKs and platelets from hematopoietic stem cells, embryonic stem cells, and pre-adipocytes. OP9 cells cultured in MKLI medium had megakaryocytic features, i.e., positivity for surface markers CD41 and CD42b, polyploidy, and distinct morphology. The OP9-derived platelets had functional characteristics, providing the first evidence for the differentiation of OP9 cells into MKs and platelets. We then analyzed gene expressions of critical factors that regulate megakaryopoiesis and thrombopoiesis. The gene expressions of p45NF-E2, FOG, Fli1, GATA2, RUNX1, thrombopoietin, and c-mpl were observed during the MK differentiation. Among the observed transcription factors of MK lineages, p45NF-E2 expression was increased during differentiation. We further studied MK and platelet generation using p45NF-E2-overexpressing OP9 cells. OP9 cells transfected with p45NF-E2 had enhanced production of MKs and platelets. Our findings revealed that OP9 cells differentiated into MKs and platelets *in vitro*. OP9 cells have critical factors for megakaryopoiesis and thrombopoiesis, which might be involved in a mechanism of this differentiation. p45NF-E2 might also play important roles in the differentiation of OP9 cells into MK lineages cells.

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* E-mail: yumikoma@a7.keio.jp

Introduction

Platelets play critical roles in hemostatic plug formation and thrombosis [1–3]. Platelets are released from terminally differentiated megakaryocytes (MKs). The underlying molecular mechanisms of megakaryopoiesis and subsequent thrombopoiesis are only partially understood [4–10]. Current advances in a culture system to generate MKs and platelets *in vitro* help us to clarify the mechanism underlying MK differentiation and platelet production [11]. Also, studies on new strategies to manufacture MKs and platelets pursue to develop a donor-independent source for platelet transfusion [11].

MKs and platelets have been differentiated from hematopoietic stem cells (HSCs), embryonic stem (ES) cells, fetal liver cells, induced pluripotent stem (iPS) cells, and fibroblasts transfected with a combination of p45NF-E2, Maf G, and Maf K, *in vitro* [12–19]. Moreover, we reported the generation of MKs and functional platelets from both normal human subcutaneous adipose tissues and mouse pre-adipocyte cell line 3T3-L1 [20–22]. Differentiation of pre-adipocytes into MKs and platelets has been observed when the culture medium is switched from maintenance medium to MK lineage induction (MKLI) medium previously established to generate platelets from HSCs and ES cells [21–24].

The mouse bone marrow stroma cell line OP9 was established from macrophage colony stimulating factor deficient osteopetrotic mice [25]. OP9 cells have been widely used as feeder cells for the differentiation of ES cells and iPS cells into hematopoietic cells as well as for the differentiation of these stem cells into MKs and platelets [12–15,25–27]. Also, OP9 cells were reported to be pre-adipocytes [28,29]. In the present study, we examined whether OP9 cells differentiate MKs and platelets, based on our previous observations that pre-adipocytes differentiated into MKs and platelets [20–22], and then investigated the involved mechanisms of MK differentiation and platelet production from OP9 cells.

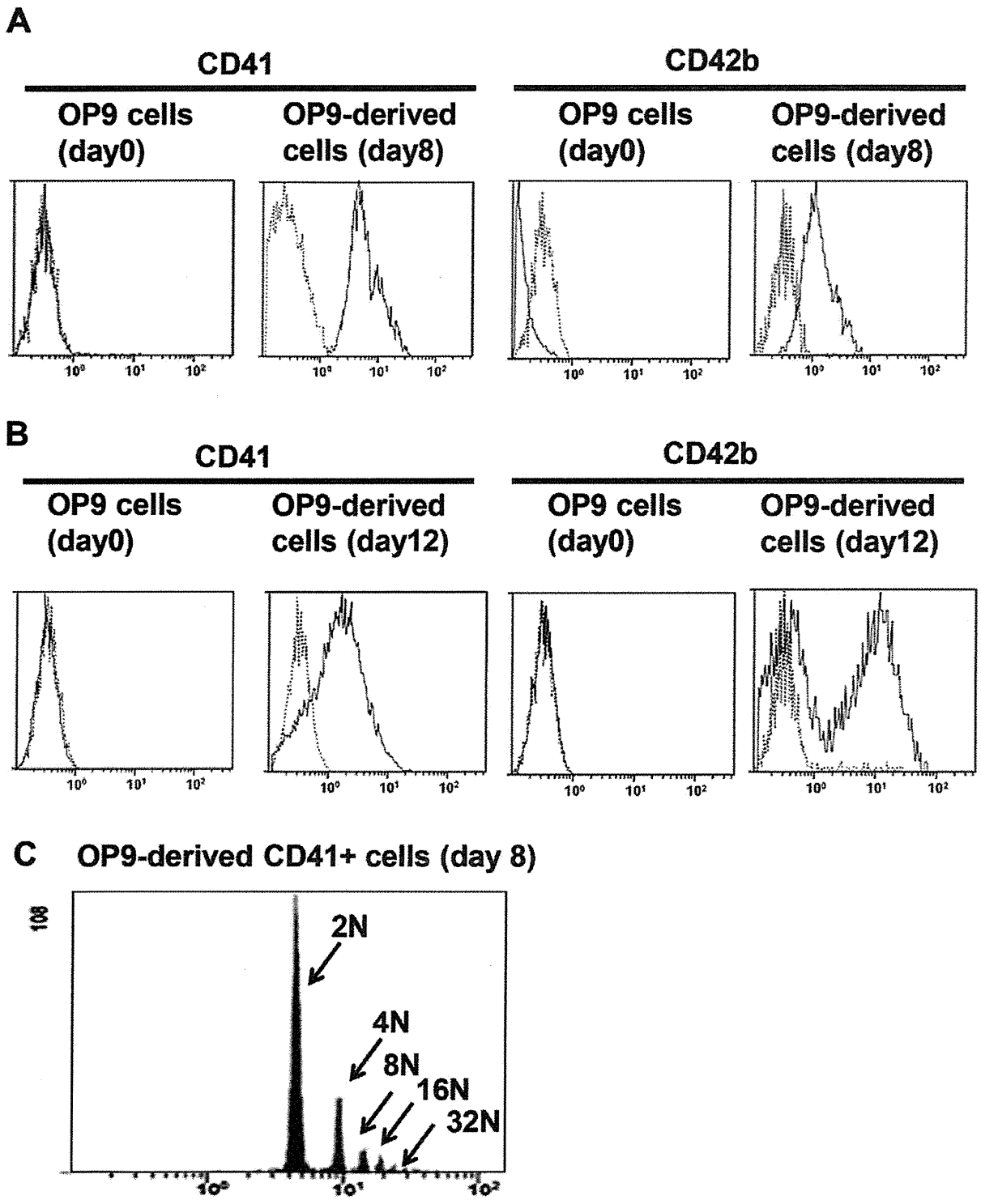

Figure 1. Flow cytometric analyses for megakaryocytes and platelets differentiated from OP9 cells. A, Representative flowcytometry histogram of CD41 and CD42b expression on megakaryocyte-sized cells. Dot-line shows data using isotype control. **B,** Representative flowcytometry histogram of CD41 and CD42b expression on platelet-sized cells. Dot-line shows data using isotype control. **C,** DNA ploidy analysis in OP9-derived CD41+ cells on day 8.
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Methods

Cell Culture for Differentiation into MKs and Platelets

OP9 cells were maintained as described previously [12]. To induce the differentiation of OP9 cells into MK lineages, OP9 cells were cultured for 12 to 14 days using MKLI medium comprised of Iscove's Modified Dulbecco's Medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin G sodium, 0.1 mg/ml streptomycin sulfate, 0.5% bovine serum albumin, 4 μg/ml LDL cholesterol, 200 μg/ml iron-saturated transferrin, 10 μg/ml insulin, 50 μM 2-β-mercaptoethanol, 20 μM each nucleotide (ATP, UTP, GTP, and CTP), and 50 ng/ml thrombopoietin (TPO; a gift from Kyowa Hakko Kirin CO., Ltd.) [19–24]. Mouse ES cells (gifted by Dr. Niwa H, RIKEN Center, Kobe, Japan) were established as described previously [30]. We maintained the ES cells as described previously [21,31]. Primary mouse low-density bone marrow mononuclear cells (mBMMNCs) were obtained as described previously [23] and were then cultured in MKLI medium.

Flow Cytometric Analyses

Surface marker analyses were performed on OP9 cells before the MK induction (day 0) and OP9 cells cultured in MKLI medium (OP9-derived cells). We used the directly labeled fluorescein isothiocyanate (FITC)-conjugated antibodies for CD41 (also known as platelet glycoprotein IIb) (BD bioscience), and CD42b (also known as platelet glycoprotein Ib alpha) (EMFRET Analytics Gmbh and Co.). The positive values (%) were calculated using cell number of binding to isotype control and cell number of binding to anti-CD41-antibody or anti-CD42b-antibody. Fluorescence-activated cell sorting was used to obtain CD41+ population from OP9-derived cells. OP9-derived MKs and platelets were counted as large-sized CD41+ cells and small-sized CD41+ cells, respectively [23]. The "platelet-sized" particles were defined using mouse platelets, and the plot is shown in Figure S1. DNA ploidy was assessed by interaction with propidium iodide (Sigma) as described previously [22].

Morphologic Analyses

The ultrastructure for OP9-derived CD41+ cells on day 8 and mouse bone marrow cells was analyzed. These studies were done by transmission electron microscopy as described previously [32]. To examine proplatelet formation in OP9-derived cells, the OP9-derived CD41+ cells were plated on the fibrinogen-coated glass (100 μg/ml) were incubated for 6 hours at 37 degree C. These cells were observed using scanning electron microscopy as described previously [33]. Also, the cells were fixed with 4% paraformaldehide in Ca^{++}- and Mg^{++}-free Phosphate Buffered

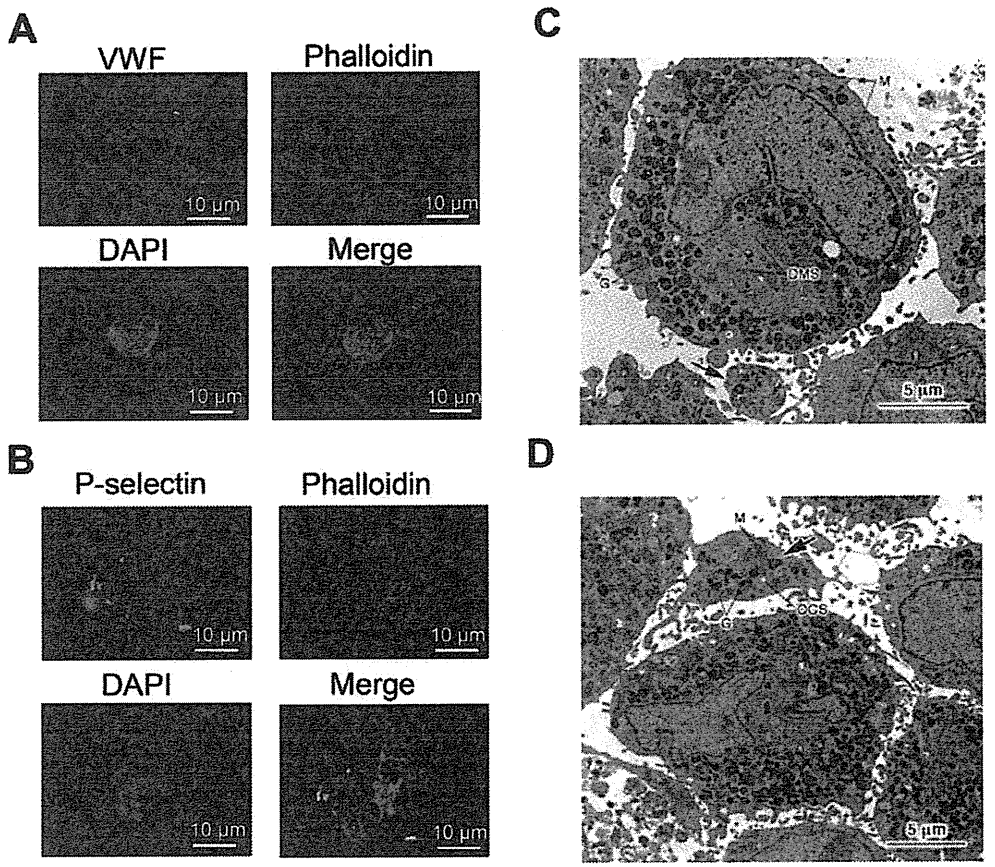

Figure 2. Cell staining and morphologic analyses. A, Cells stained with antibody for von Willebrand factor (green), Phalloidin (red) and DAPI (blue). **B,** Cells stained with antibody for P-selectin (green), Phalloidin (red) and DAPI (blue). **C, D,** Transmission electron micrograph of OP9-derived CD41+ cells. Megakaryocyte and platelet (arrow indicated in black): G, granule, DMS, demarcation membrane system, M, mitochondria, and OCS, open canalicular system.
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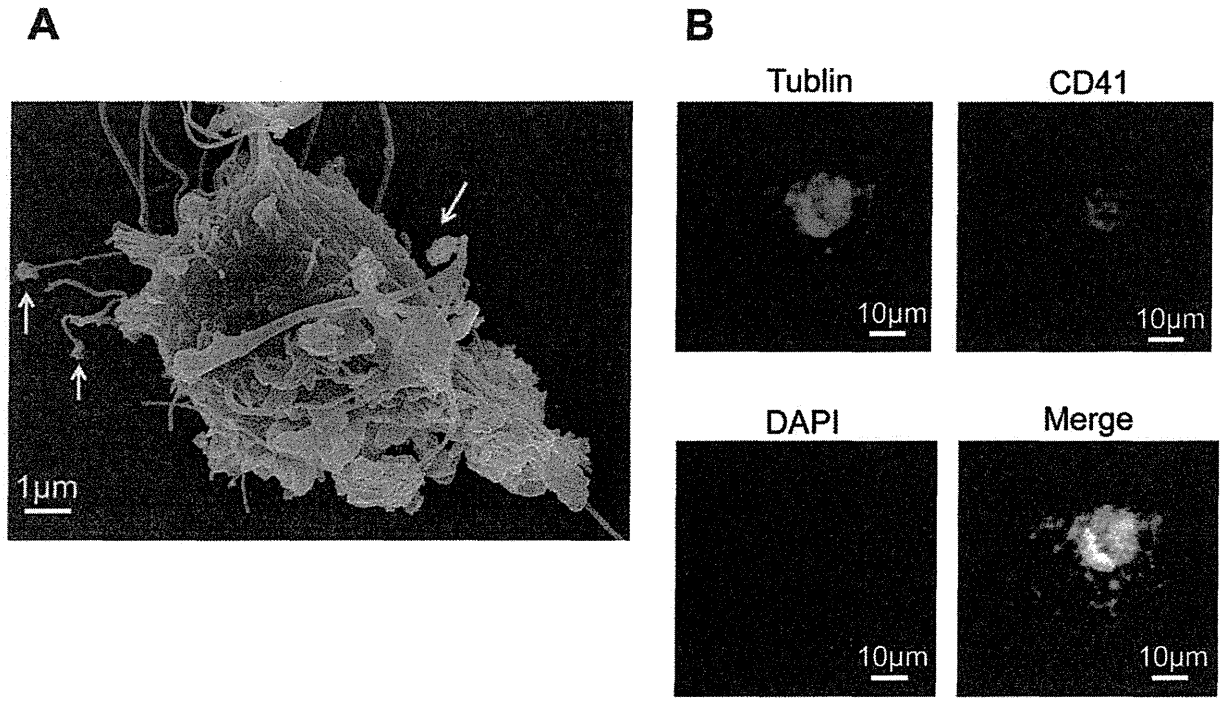

Figure 3. Proplatelet formation in OP9-derived megakaryocyte. A. Scanning electron micrograph of proplatelets (arrow indicated in yellow) forming OP9-derived CD41+ cells. **B.** Proplatelet-forming megakaryocyte was stained with antibody for CD41 (red) and alpha-tubulin (green), and DAPI (blue).
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Saline (PBS) for 15 minutes at room temperature and then permeabilized by 0.2% Triton X 100 in PBS for 5 minutes at room temperature. The samples for proplatelet formation were stained with phycoerythrin-conjugated antibody for CD41 (BD bioscience), unlabeled anti-alpha-tubulin antibody (Lab Vision Co.) and FITC-conjugated anti-rabbit antibody, and DAPI blue.

Immunohistochemical Studies

To analyze the expression of von Willebrand factor (VWF) and P-selectin in OP9-derived CD41+ cells on day 8, the cells stimulated with 10 μM ADP and 10 μM epinephrine were plated on the fibrinogen-coated glass (100 μg/ml, coating concentration). The cells were fixed with 4% paraformaldehide in Ca^{++}- and Mg^{++}-free PBS for 10 minutes at room temperature and then permeabilized by 0.2% Triton X 100 in PBS for 5 minutes at room temperature. The samples from OP9-derived CD41+ cells were stained with FITC-conjugated anti-VWF antibody (EMFRET Analytics Gmbh and Co.) and FITC-conjugated anti-P-selectin antibody (EMFRET Analytics Gmbh and Co.) for 60 min at room temperature. Cells were also stained DAPI blue and Texas Red Phalloidin (Invitrogen).

Functional Analyses for OP9-derived Platelets

Function for OP9-derived platelets was examined. The analyses of fibrinogen binding and P-selectin surface exposure after stimulation were performed on OP9-derived cells on day 12, as described previously [22]. FITC-conjugated anti-P-selectin antibody (EMFRET Analytics Gmbh and Co.) was used for the analysis of P-selectin surface exposure in the presence or absence of stimulation.

Gene Expression Analyses

To carry out gene expression analyses by reverse transcription-polymerase chain reaction (RT-PCR), total RNA samples were prepared from OP9-derived cells on days 0, 4, and 8, mES cells, and mBMMNC-derived cells on days 0 and 4 after treatment with Trizol reagent (Invitrogen). cDNA samples with genomic DNA removal were obtained by QuaniTect Reverse Transcription (QIAGEN) or by QuaniTect Whole Transcriptome (QIAGEN) with DNase (Promega), according to manufacturer's protocols. Primers of RT-PCR for GATA1, GATA2, Fli1, FOG, p45NF-E2, and GAPDH were used as described previously [16]. The pre-made primers (Applied Biosystems) were used for RT-PCR for OCT3/4, SOX2, RUNX1, TPO, c-mpl, KLF1, and PU1. Quantitative real time-PCR was also performed on samples from OP9 cells and OP9-derived cells using pre-made primers (Applied Biosystems). The amount of target normalized to GAPDH was determined by evaluating expression: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Cts - \Delta Ct$ cb, ΔCt was threshold cycle, ΔCts was the ΔCt value of a sample, ΔCt cb was that of the calibrator and meant the difference in threshold cycles between the target and reference.

Retroviral Vectors and Cell Culture

Retroviral vector for overexpression of p45NF-E2 in OP9 cells was used as described previously [19]. A CalPhos Mammalian Transfection Kit (Clontech) and AmphoPack-293 cells (Clontech), as packaging cells, were used according to the manufacture's protocol. AmphoPack-293 cells were transfected with p45NF-E2 expression vector or empty vector. After 48 hours of transfection, retroviral supernatants were collected. OP9 cells were infected with the p45NF-E2 expression vector or empty vector (p45NF-E2-

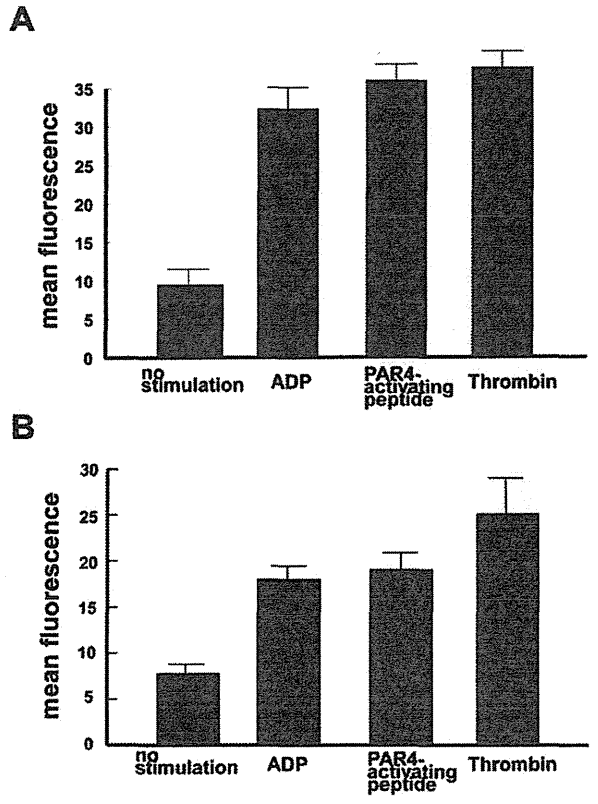

Figure 4. Functional analyses for OP9-derived platelets. A, By flowcytometry analysis, Alexa Fluor 488-labeled fibrinogen (100 μg/ml) binding to platelets derived from OP9 cells was examined in the presence or absence of platelet stimulation reagents. **B,** The surface exposure of P-selectin was analyzed in the presence or absence of platelet stimulation reagents.
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OP9 and empty-OP9). Each of cells was cultured in MKLI medium for 12 days.

Statistics

Mean values of two groups were compared using Student's t-test. Statistical analysis was performed using StatView (ver 5.0, for Macintosh, SAS Institute Inc., Cary, NC). A p value of less than 0.05 was considered statistically significant.

Results

Differentiation of OP9 Cells into MKs and Platelets

We examined whether OP9 cells differentiate into MKs and platelets by using MKLI medium previously established to generate MKs and platelets from HSCs, ES cells, and pre-adipocytes. OP9 cells cultured for 6 days in MKLI medium resulted in adherent cells and a few floating cells. The OP9-derived cells on day 8 had adherent, loosely adherent, and floating cells, and most of cells on day 12 had floating cells (Figure S2A). Among OP9-derived cells on day 8, floating and loosely adherent cells morphologically resembling MKs derived from mBMMNCs cultured in MKLI medium (Figure S2B). These OP9-derived cells were characterized using surface markers, DNA polyploidy with nuclear staining, morphology using electron microscopy, and immunohistochemistry.

By flow cytometric analysis with MK lineage specific markers, approximately 95% and 60% of these OP9-derived MK-sized cells on day 8 expressed CD41, a surface marker throughout MK differentiation, and CD42b, a surface marker for the late stage of MK differentiation, respectively (Figure 1A). Approximately 70% and 60% of OP9-derived platelet-sized cells on day 12 expressed CD41 and CD42b, respectively (Figure 1B). Regarding the number of the OP9-derived MKs and platelets, approximately 4×10^4 MKs and 1×10^5 platelets were generated from 1×10^6 OP9 cells before the MK induction. OP9 cells before the MK induction (day 0) did not express CD41 and CD42b (Figure 1A, 1B). DNA ploidy of OP9-derived CD41+ cells ranged from 2N to 32N (Figure 1C). Immunohistochemical analyses showed that VWF and P-selectin, cytoplasmic proteins of MK lineage cells, were positive in OP9-derived CD41+ cells (Figure 2A, 2B). Under electron microscopic observation, OP9-derived MK-sized CD41+ cells had typical organelles for MKs, such as granules, demarcation membrane system, and lobulated nuclei, and OP9-derived platelet-sized CD41+ cells showed typical features for platelets, such as granules, mitochondria, and open canalicular system (Figure 2C, 2D). The present observations were similar to what described in MKs derived from mBMMNCs (Figure S3). Also, the present observations were similar to what described in MKs and platelets derived from mouse ES cells, mouse pre-adipocyte cell line 3T3-L1, human bone marrow CD34-positive cells, and human adipose tissues [19,20]. OP9-derived MK-sized CD41+ cells were examined for proplatelet formation under scanning electron microscopic observation. We observed proplatelets forming OP9-derived MK-sized CD41+ cells (Figure 3A). Moreover, proplatelet-forming OP9-derived MK showing CD41 and alpha-tubulin was observed (Figure 3B). To examine whether OP9-derived platelets are functional, fibrinogen-binding assay was performed on OP9-derived platelet-sized cells on day 12. Binding of Alexa Fluor 488-labeled fibrinogen to OP9-derived platelet-sized cells was increased upon stimulation when assessed by mean fluorescence (mean±S.D.): 9.7±0.9 (no stimulation), 25.7±0.3 (10 μM ADP), $p<0.0001$ (vs no stimulation), 28.6±0.4 (1.5 mM PAR4-activating peptide), $p<0.0001$ (vs no stimulation), and 29.2±1.6 (0.5 U/ml thrombin), $p<0.0001$ (vs no stimulation) (Figure 4A). The representative data of dot-plots in this assay are shown in Figure S4. Moreover, P-selectin surface exposure, a marker for platelet activation, on OP9-derived platelet-sized cells on day 12 was examined in the presence or absence of stimulation, and mean fluorescence (mean±S.D.) was 7.8±1.1 (no stimulation), 17.9±1.5 (10 μM ADP), $p=0.0159$ (vs no stimulation), 19.1±1.8 (1.5 mM PAR4-activating peptide), $p=0.0162$ (vs no stimulation), and 25.1±3.8 (0.5 U/ml thrombin), $p=0.0162$ (vs no stimulation) (Figure 4B). These observations indicated that OP9 cells differentiated into MKs and platelets *in vitro*.

Analyses of Gene Expressions of OP9 Cells during MK Differentiation

We then analyzed gene expression of candidate factors to elucidate mechanisms for the differentiation of OP9 cells into MKs and platelets. To examine whether this conversion goes through the status of cell pluripotency, the expressions of OCT3/4, specifically expressed in pluripotent cells, and SOX2, a key factor for the maintenance of cell pluripotency [34,35], were analyzed. Expression of both OCT3/4 and SOX2 was not detected in OP9 cells (day 0) and OP9-derived cells by RT-PCR (Figure 5). We also analyzed the gene expressions for transcription factors, p45NF-E2, FOG, Fli1, GATA1, GATA2, and RUNX1, that regulate megakaryopoiesis and thrombopoiesis [5–11]. These transcription factors, except for GATA1, were clearly detected in OP9 (day 0)

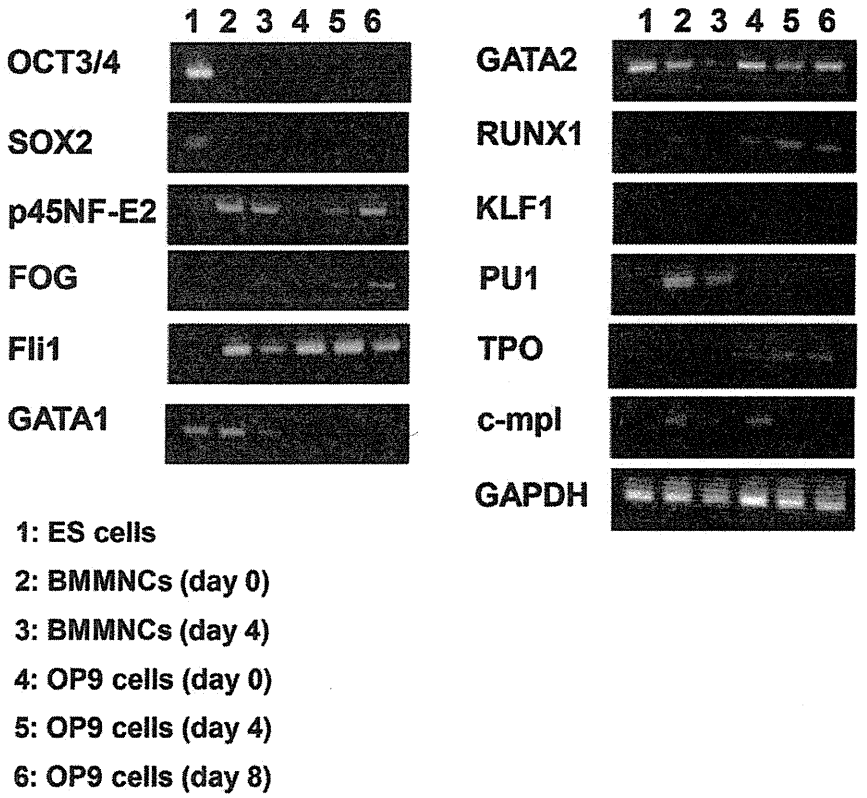

Figure 5. Analyses of gene expression of OP9 cells during megakaryocyte differentiation. Gene expression assessed by reverse transcription-PCR.
doi:10.1371/journal.pone.0058123.g005

and OP9-derived cells (days 4 and 8) (Figure 5). Because major cell population of BMMNCs on day 4 morphologically resembled that of OP9-derived cells on day 8, we used BMMNCs on days 0 and 4 as a control. OP9 cells (day 0) also showed gene expression of TPO and c-mpl, receptor for TPO (Figure 5). Regarding the key transcription factors for other hematopoietic cell lineages, PU.1 for leukocytes [36] and KLF1 for erythrocytes [37], were not detected in OP9 cells (day 0) and OP9-derived cells (days 4 and 8) (Figure 5). Among the observed transcription factors, p45NF-E2 expression was increased during the differentiation of OP9 cells into MKs, and expression levels of p45NF-E2 in OP9 cells (day 0) and OP9-derived cells (day 4) were measured by quantitative real-time PCR analysis. It was found that expression levels of OP9-derived cells (day 4) had 3.91±0.08-fold higher than that of OP9 cells (day 0). Other transcription factors were also measured. It was for 1.54±0.02-fold higher for FOG, 0.03±0.00 (0.0007)-fold higher for Fli1, 0.20±0.03-fold higher for GATA2, and 1.00±0.16-fold higher for RUNX1, as compared with those of OP9 cells (day 0). TPO expression levels of OP9-derived cells (day 4) had 3.59±0.70-fold higher than that of OP9 cells (day 0). c-mpl expression levels of OP9-derived cells (day 4) had 0.182±0.11-fold higher than that of OP9 cells (day 0). These results indicate that OP9 cells possess critical factors for MK differentiation and platelet production. Among them, the p45NF-E2 expression was increased during the differentiation of OP9 cells into MK lineages.

Enhanced Production of MKs and Platelets from OP9 cells Transfected with p45NF-E2

Based on increased expression of p45NF-E2 during differentiation of OP9 cells into MK lineages, we further studied the effect of OP9 cells transfected with p45NF-E2 on MK and platelet production. Transfection efficiency was estimated by DsRed expression, and approximately 50% of transfected OP9 cells was positive for DsRed expression, as assessed by flowcytometry (data not shown). Gene expression levels, assessed by qRT-PCR, of p45NF-E2 in p45NF-E2-OP9 cells on day 4 had 37.68±11.36-fold higher than that in empty-vector-OP9 cells. The p45NF-E2-OP9 cells and empty-vector-OP9 cells were cultured in MKLI medium for 12 days. By flow cytometric analysis, nearly all of the p45NF-E2-OP9-derived MK-sized cells on day 7 expressed CD41, whereas approximately 60% of empty-vector-OP9-derived MK-sized cells on day 7 expressed CD41 (Figure 6A). Also, CD42b expression was approximately 50% and 20% of p45NF-E2-OP9- and empty-vector-OP9-derived MK-sized cells on day 7, respectively (Figure 6A). The 1×10^6 OP9 cells (day 0) generated $3.3\pm1.8\times10^4$ MKs for p45NF-E2-OP9 cells and $2.2\pm1.6\times10^4$ ($p=0.4008$) MKs for empty-vector-OP9 cells. The effect of p45NF-E2-overexpressing OP9 cells on platelet production was also examined. CD41 expression was approximately 50% and 20% of p45NF-E2-OP9- and empty-vector-OP9-derived platelet-sized cells on day 12, respectively (Figure 6B), and CD42b expression was approximately 20% and 10% of p45NF-E2-OP9- and empty-vector-OP9-derived platelet-sized cells on day 12, respectively (Figure 6B). It was found that the number of p45NF-

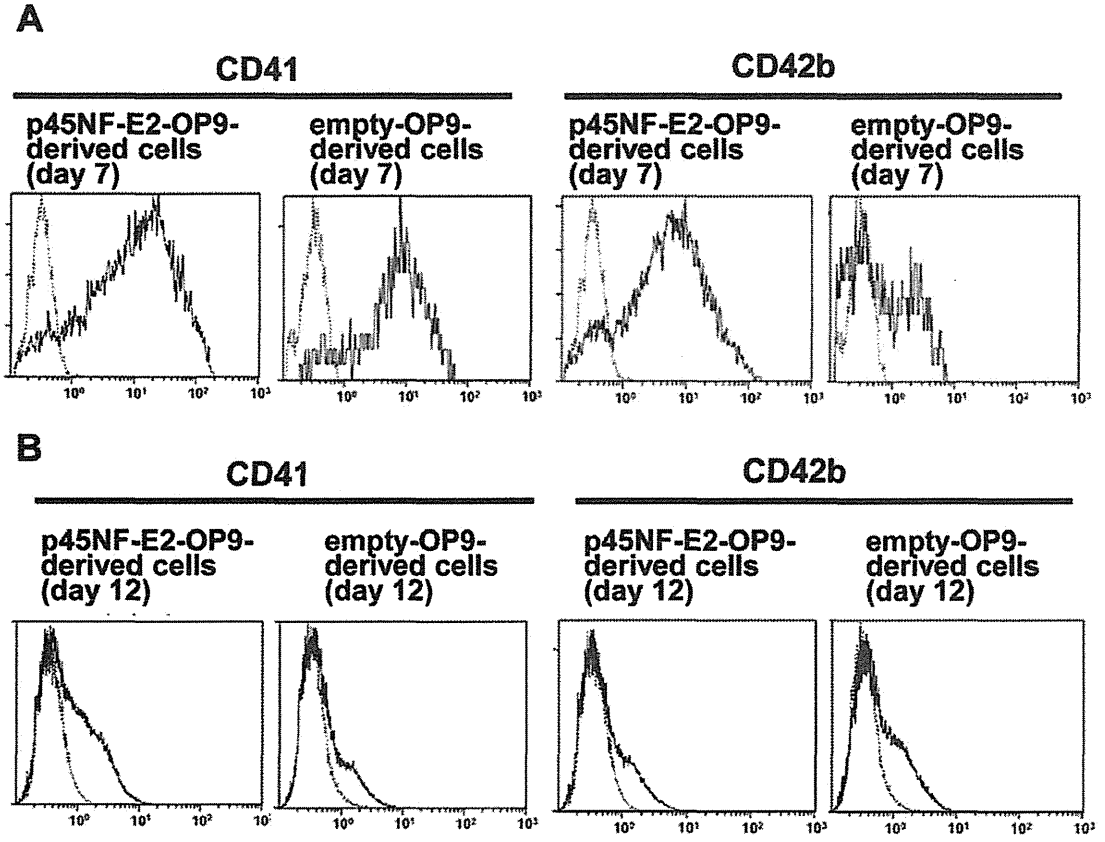

Figure 6. Production of megakaryocytes and platelets from OP9 cells transfected with p45NF-E2. A, Representative flowcytometry histogram of CD41 and CD42b expression on megakaryocyte-sized cells in each of samples introduced with the indicated vectors. Dot-line shows data using isotype control. **B,** Representative flowcytometry histogram of CD41 and CD42b expression on platelet-sized cells in each of samples introduced with the indicated vectors. Dot-line shows data using isotype control.
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E2-OP9-derived platelet-sized CD41+ cells on day 12 showed approximately 3-fold higher than those in the empty-vector-OP9-derived platelet-sized CD41+ cells on day 12. The number of platelets generated from 1×10^6 OP9 cells (day 0) was $2.9\pm2.6\times10^5$ for the p45NF-E2-OP9-derived platelets and $1.0\pm7.3\times10^5$ ($p=0.0518$) for the empty-vector-OP9-derived platelets. These findings indicate that p45NF-E2 has a critical role in the generation of MKs and platelets from OP9 cells, although the production of MKs and platelets was not significantly different between the p45NF-E2-OP9 cells and empty-vector-OP9 cells.

Discussion

This study demonstrated that OP9 cells differentiated into MKs and platelets *in vitro* using MKLI medium previously established to differentiate HSC, ES cells, pre-adipocytes into MK lineages. The present findings provide the first evidence for the differentiation of OP9 cells into MK lineages. Regarding the efficiency of the MK and platelet production from OP9 cells, approximately 4×10^4 MKs and 1×10^5 platelets were generated from 1×10^6 OP9 cells. On the other hand, 1×10^6 human bone marrow mononuclear cells produced approximately 6×10^3 MKs and 3×10^3 platelets in a similar culture scale using MKLI medium [24]. Although it is difficult to compare precisely the efficiency of the MK and platelet production among various stem cell sources, our observations suggested that OP9 cells possess high capacity of the differentiation into MK lineages *in vitro*.

OP9 cells cultured in maintenance medium express specific surface marker for MSC and were reported to be pre-adipocytes [28,29]. These cell lineage fate of mesenchymal cells is distinct from that of HSCs, and OP9 cells are widely used as feeder cells for differentiation of iPS cells and ES cells into hematopoietic cells and MK lineages [12–15,25–27]. However, the present study shows that OP9 cells themselves are the source of MKs and platelets. OP9-derived MKs and platelets were characterized by specific surface markers, DNA polyploidy, morphology using electron microscopy, and immunohistochemistry. These analyses have been performed on *in vitro*-generated MKs and platelets beginning with cell sources including HSCs, ES cells, and iPS cells. When MKs and platelets derived from iPS cells or ES cells are harvested in the differentiation study using OP9 co-culture system, there is a possibility that some of MK lineage cells are derived from OP9 cells.

The gene expression analyses indicated that differentiation of OP9 cells into MK lineages did not share the common mechanism with pluripotent cells. OP9 cells possess the important factors related to megakaryopoiesis and thrombopoiesis, and these observations are compatible with our previous findings that 3T3-L1 pre-adipocytes possess GATA2, RUNX1, Fli1, FOG1, and

p45NF-E2. The expression of GATA1 was not detected in OP9 cells and 3T3-L1 cells [19]. Furthermore, we did not observe the GATA1 expression during differentiation of OP9 cells into MKs. Although GATA1 was reported to be a critical factor for the erythroid and MK development, previous studies demonstrated that GATA2 coordinates MK differentiation in GATA1 deficient and mutant cells [38]. Also, the present study revealed that the differentiation of OP9 cells into MK lineages involves in a p45NF-E2-mediated mechanism. The NF-E2 transcriptional factor is a basic-leucine zipper hetero-dimer complex consisting of p45 subunit, known as tissue-restricted subunit, and the small Maf proteins, Maf K and Maf G, known as widely expressed in many cells [39–45]. Observations in p45NF-E2 deficient MKs suggested that p45NF-E2 is important in the MK terminal differentiation and platelet release [46,47]. On the other hand, the *in vitro* and *in vivo* study using p45NF-E2-overexpressing bone marrow cells showed additional roles of p45NF-E2 in early megakaryopoiesis [48]. We previously reported that fibroblasts transfected with p45NF-E2, Maf G and Maf K differentiated into MKs and platelets, whereas fibroblast did not differentiate into MK lineage cells. These observations support p45NE-E2, Maf G, and Maf K as critical factors for megakaryopoiesis and thrombopoiesis. In the present study, OP9 cells have Maf G and Maf K, and thus cells were transfected with P45NF-E2. The present findings provide additional information for the importance of p45NF-E2 in megakaryopoiesis and thrombopoiesis. Further studies are definitely needed to elucidate the detailed pathways that cause OP9 cells to differentiate into the MK lineage ultimately leading to platelet production.

In summary, OP9 cells differentiated into MKs and platelets, although OP9 cells have been wildly used as feeder cells in differentiation of ES cells and iPS cells into MKs and platelets. OP9 cells possess critical factors related to megakaryopoiesis and thrombopoiesis. The generation of MKs and platelets from OP9 cells could have important implications for study on the underlying mechanisms of megakaryopoiesis and thrombopoiesis.

Supporting Information

Figure S1 The plot of mouse platelets in flow cytometric analysis.
(TIFF)

Figure S2 Megakaryocyte lineage cells were generated from OP9 cells in vitro. A, Schematic outline and pictures for OP9 cells and differentiated stages into megakaryocyte lineages. **B,** Mouse bone marrow mononuclear cells were cultured in megakaryocyte lineage induction media for 7 days.
(TIFF)

Figure S3 Transmission electron micrograph of mouse bone marrow mononuclear cells.
(TIFF)

Figure S4 Alexa Fluor 488-labeled fibrinogen binding to platelets derived from OP9 cells was examined in the presence or absence of platelet stimulation reagents.
(TIFF)

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Author Contributions

Conceived and designed the experiments: YM YI. Performed the experiments: YM YO HS. Analyzed the data: FA TS MM. Contributed reagents/materials/analysis tools: YM HS MM YI. Wrote the paper: YM YO YI.

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Regular Article

Studies of a microchip flow-chamber system to characterize whole blood thrombogenicity in healthy individuals

Yusuke Yamaguchi [a], Takanori Moriki [b,*], Atsuko Igari [a], Yumiko Matsubara [a], Tomoko Ohnishi [c], Kazuya Hosokawa [c], Mitsuru Murata [a]

[a] Department of Laboratory Medicine, Keio University School of Medicine, Tokyo, Japan
[b] Health Center, Keio University, Tokyo, Japan
[c] Research Institute, Fujimori Kogyo Co., Yokohama, Kanagawa, Japan

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ABSTRACT

Introduction: A whole blood flow-chamber system, the Total Thrombus-formation Analysis System (T-TAS), was developed for quantitative analysis of platelet thrombus formation (PTF) using microchips with thrombogenic surfaces (collagen, PL chip; collagen plus tissue thromboplastin, AR chip) under shear stress conditions. We evaluated the usefulness of the T-TAS for assessing individual thrombogenicity compared with other platelet function tests.

Materials and Methods: Blood samples from 31 healthy volunteers were applied to the T-TAS to measure PTF starting time (T_{10}: time to reach 10 kPa), occlusion time (T_{60} for PL chip; T_{80} for AR chip), and area under the curve (AUC_{10}, area under curve until10 min for PL chip; AUC_{30}, 30 min for AR chip) under various shear rates (1000, 1500, 2000 s^{-1} for PL chip; 300 s^{-1} for AR chip). Platelet functions were also tested using platelet aggregometry, the PFA-100 (collagen and epinephrine [C/EPI], collagen and adenosine diphosphate [C/ADP]), and the VerifyNow P2Y12 assay.

Results: Individual pressure waveforms, including PTF starting and ending points, varied among healthy subjects. In the PL chip, T_{10} and AUC_{10} showed a shear-dependent correlation with C/EPI or C/ADP. VerifyNow P2Y12 values were not significantly associated with the parameters of the T-TAS. Platelet counts were correlated with all AR measurements, and mostly with PL measurements.

Conclusion: The results of the T-TAS were associated with those of the PFA-100 in many respects, indicating that its characteristics are related to shear-induced PTF. The T-TAS showed few correlations with platelet aggregometry and the VerifyNow P2Y12 assay. The T-TAS may allow for the measurement of comprehensive parameters of individual thrombogenicity under whole blood flow conditions.

Introduction

The initial step of thrombus formation at the site of vascular injury in an artery is the adhesion of platelets to the exposed collagen-bound von Willebrand Factor (VWF), followed by platelet activation to bind stably to the collagen on the subendothelium under high-shear blood flow. Activated platelets undergo morphologic changes and aggregate together by bridging with VWF and fibrinogen, and release soluble agonists such as adenosine diphosphate, thromboxane A_2, and thrombin, which further enhance platelet activation. The coagulation pathway on the platelet plasma membrane is accelerated to generate localized thrombin, leading to enhanced platelet activation and fibrin formation, and resulting in the growth of platelet thrombus formation (PTF) [1,2]. This process, hemostasis, is essential for maintaining blood circulation *in vivo* to prevent blood loss due to bleeding.

Arterial thrombosis, however, is attributed to pathologic PTF at sites of atherosclerotic plaque rupture, which leads to ischemia or necrosis of the organs downstream, such as acute coronary syndrome, myocardial infarction, and ischemic stroke [3,4]. Arterial thrombotic diseases are a major cause of morbidity and mortality, particularly in industrialized countries, and the numbers continue to grow worldwide, increasing healthcare costs. Prothrombotic conditions, e.g., high platelet reactivity, enhanced coagulation, and reduced fibrinolysis, have been identified in disorders that lead to atherosclerotic thrombosis [5], such as diabetes [6,7], hypertension [8], dyslipidemia [9], obesity [10], and metabolic syndrome [11,12], as well as in smokers [13] and the elderly [14].

Evaluation of the prothrombotic status is expected to contribute to the primary prevention of arterial thrombosis by improving lifestyle habits, such as diet and exercise, or the consideration of antithrombotic medication, but the most appropriate test for evaluating thrombogenicity in an

* Corresponding author at: Health Center, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160–8582, Japan. Tel.: +81 3 5363 3973; fax: +81 3 3359 6963.
E-mail address: TakaMoriki@aol.com (T. Moriki).

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