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ヒトＥＳ細胞を用いた
安全な人工血液の開発に関する研究

平成２０年度　総括研究報告書

研究代表者　湯尾　明

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Ⅰ．総括研究報告

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厚生労働科学研究費補助金（創薬基盤推進研究事業（政策創薬総合研究事業））

総括研究報告書

ヒトＥＳ細胞を用いた安全な人工血液の開発に関する研究

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研究要旨　本研究では動物由来成分を少しでも排除するために、ヒトＥＳ細胞（KhES-3株）から無フィーダー環境において血液細胞生成を試みた。我々の分化培養系は、前半のsphere形成浮遊培養と、後半の平面接着培養の２段階から成り立っており、全体を通じて無フィーダーである。第１段階のsphere形成は、６種類の増殖因子・サイトカイン（IGF-II, VEGF, SCF, Flt3-L, TPO, G-CSF）存在下に３日間行われ、その後、ゼラチンコート培養皿の上にて第２段階の接着平面培養に移行した。接着したsphereは円盤状に増殖して１２日程度でその中心部に嚢状構造物（sac-like structure）が形成された。構造物の内部に球状細胞（血液細胞）が充満し、構造物の壁を繰り返し切開して、血球産生を持続させた。産生された血液細胞は、CD45陽性、CD11b、CD33陽性の比較的成熟した骨髄系（食細胞系）の血液細胞で、形態、細胞組織化学染色、表面抗原などから30-50%が好中球と考えられた。これらの好中球は、in vitroでは遊走能、貪食能、活性酸素産生能を示し、in vivoでも遊走能を発揮した。このような成熟好中球が効率よく産生できるＥＳ細胞の分化誘導システムは、ヒトでは世界初であり、顕著な成果が得られたと言える。さらに、本年度においては、ヒトiPS細胞からも同様の無フィーダー２段階分化誘導システムを駆使して、嚢状構造体を介した血液細胞の産生が誘導できた。今後は、サルＥＳ細胞の分化誘導系で実現しているhemogenic endotheliumを介する成体型造血幹細胞に近い造血をヒトの系において実現してゆく計画である。

A．研究目的

血液成分の輸注は現代医療において不可欠であるが善意のボランティアに供給を依存しているため供給量に限りがあり、また、感染症の媒体となる危険をかかえている。従って、病原体混入がないクリーンな環境で安定して血液細胞成分を生成する技術を開発していくことは極めて重要である。

骨髄の造血幹細胞から血液細胞成分を増幅する試みは長年研究されてきたが、造血幹細胞は体外では充分に増幅しないことも確認された。一方、ＥＳ細胞は無限増殖能と多能性分化能を持つために再生医療における優れた材料として近年注目されている。即ち、ヒトＥＳ細胞から安定して試験管内で血液細胞成分を作成することが可能になれば輸血医療・移植医療において革命的な進歩がもたらされる。とりわけ、寿命が短いために現行の輸血療法では効果が乏しい「顆粒球」に関してはその恩恵は大きい。尚、ヒトを含めた霊長類ＥＳ細胞はマウスＥＳ細胞とかなり異なる性質を持つので、医療応用を目指す研究は霊長類ＥＳ細胞においてなされる必要がある。さらには既存の霊長類ＥＳ細胞の培養技術ではマウスフィーダー細胞の混入が避けられず新しい培養技術の確立が要求されている。

これらのことを踏まえて、本研究ではヒトＥＳ細胞から無血清無フィーダー環境において未分化維持と血液細胞生成を試みる。特に、白血球産生のための培養条件の決定を目指すとともに、ヒトＥＳ細胞の未分化維持と血液細胞への分化を制御する分子を網羅的な手法も駆使して見出すことにより、一層効率的な分化システムの構築を目指す。

当該研究における成果は、免疫抑制剤投与方法の地道な工夫、組織適合抗原の発現制御技術、間葉系幹細胞を用いた免疫不適合緩和技術、ヒトiPS細胞やヒトクローン胚由来ＥＳ細胞を用いた培養技術、などと組合わすことで、常時万人に血液細胞成分を安全に供給するシステムが確立されることとなる。

B．研究方法

1．細胞など研究材料

マウス胎児線維芽細胞（murine embryonic fibroblasts, MEF）はマイトマイシンC（MMC）処理またはX線照射によって増殖を停止させて未分化維持用のフィーダー細胞として用いた。カニクイザルＥＳ細胞（CMK-6）、ヒトＥＳ細胞（KhES-3）、ヒトiPS細胞（201B6, 201B7, 253G1）は、MMC処理MEF上で20%KSR存在下に無血清培養により継代した。継代は週２回、コラゲナーゼ処理にて行い、細胞密度を２－４倍に希釈した。OP9細胞は、20%牛胎児血清存在下で継代培養した。

2．分化誘導プロトコール

未分化ヒトＥＳ細胞、ヒトiPS細胞をコラゲナーゼ処理によりMEFの混入を避けて回収した後に、2-methacryloyloxyethyl phosphorylcholineコート低接着培養皿にて３日間スフェア（sphere）形成させた。分化培養液には、15%牛胎児血清の他に、６種類のサイトカイン・増殖因子（insulin-like growth factor II（IGF-II), vascular endothelila growth factor（VEGF), stem cell factor（SCF), Flt3 ligand（Flt3-L), thrombopoietin（TPO), granulocyte colony-stimulating factor（G-CSF））を添加した。その後、スフェアはゼラチンコート培養皿での平面培養に移行した。サイトカイン・増殖因子は同様の６種類である。平面培養後、２週間以内に敷石状の細胞が増殖して、スフェアが着地した箇所に嚢状構造物が形成され、その中に球状の細胞が充満した。サルＥＳ細胞の分化誘導においては、高濃度のSCF、Flt3-Lを含むBhatiaらのプロトコール（BMP-4（bone morphogenic protein 4）、SCF、Flt3-L、IL-3（interleukin 3）、IL-6（interleukin 6）、G-CSF）を用いた。

3．コロニーアッセー

造血コロニーアッセーは、市販のキット（Methocult TM GF H4535）を用いて、メチルセルロース中にて造血因子のカクテル存在下（SCF、IL-3、IL-6、GM-CSF、G-CSF、erythropoietin）で行い、２週間後のコロニー形成を倒立顕微鏡にて観察した。

4．RT-PCR

市販のキット（RNeasy Mini Kit）によりRNA抽出後にc DNAを作成して行った。各種のグロビン遺伝子（α、ζ、ε、γグロビン）の同定を行った。

5．形態学的組織化学的観察方法

生細胞は、培養皿や培養フラスコのまま倒立顕微鏡により形態観察した。浮遊状態の血液細胞はスライドグラス表面にサイトスピン固定した後に、ライトギムザ染色、ミエロペルオキシダーゼ染色、エステラーゼ染色、好中球アルカリフォスファターゼ染色を行い、正立顕微鏡により観察した。

6．フローサイトメトリー

細胞膜表面抗原の同定は、細胞をPBS中で３０分間１次抗体と反応させた後にFACSCaliburを用いて解析した。解析した抗原は、CD45、CD11b、CD33、CD66、GPI-80である。

7．遊走能

遊走能は、２４穴プレートン設置したケモタキセルを用いて測定した。遊走因子は、菌体成分由来走化性ペプチドformyl-methionyl-leucyl-phenylalanine（FMLP）、ケモカインinterleukin 8（IL-8）を用いた。

8．貪食能

貪食能は、FMLP存在下でのザイモザンの貪食により定量した。細胞はライトギムザ染色し、正立顕微鏡により貪食を観察した。

9．NBT還元能

NBT還元能は、FMLP存在下での活性を測定した。細胞浮遊液をマツナミスライドグラスに滴下させ、formazan blue-black deposits陽性細胞を正立顕微鏡下で観察した。

１０．ケミルミネッセンス

ルミノール結合粒子の貪食による蛍光発色を蛍光分光光度計により定量した。

１１．空気嚢炎モデルによるin vivo遊走能の測定

ヒトＥＳ細胞由来好中球のNOGマウスへの移植実験と、空気嚢炎の組み合わせで実験を行った。マウス皮下に空気嚢を作成して３日後に、$2x10^6$の細胞を経静脈的に移植し、空気嚢内に起炎剤としてザイモザンを注入した。１６時間後に、空気嚢に集積してくる好中球を回収して、フローサイトメトリーによりヒトＥＳ好

中球を同定した。

（倫理面への配慮）

本研究ではヒト検体は使用しないし、臨床研究もない。また、動物実験を行う計画はない。さらに、ヒトのクローンなどの生命倫理に抵触するような実験、研究はいっさい含まれない。

ヒトES細胞研究を開始するための国立国際医療センターにおける生命倫理に対する取り組み

1．主任研究者による使用計画書とその概要の作成
2．機関内倫理委員会（「ヒトES細胞研究倫理　審査委員会」）の人選と確定
3．当機関としての倫理規定、倫理委員会運営規定などの作成
4．生命倫理に関する勉強会、講演会の開催と参加
5．主任研究者が提出した使用計画に対する機関内倫理審査員会の審査
　第1回：平成17年4月18日
　第2回：平成17年5月30日
　第3回：平成17年8月　2日
6．使用計画書一式、機関内倫理委員会審査経過を文部科学省に提出
7．文部科学省特定胚及びヒトES細胞研究専門委員会にて当機関の使用計画が審査され承認。（平成17年9月30日）

最終的には、平成17年11月9日に当機関長宛に文部科学大臣の確認の文書（17諸文科振第734号）が送付され、ヒトES細胞使用が認められた。

その後、研究者の追加・削除と研究業績の変更、使用期間と使用の方法の変更、使用機関の基準に関する説明の変更についても平成18年11月24日に文部科学大臣の確認（18諸文科振第743号）を得た。

さらにその後、文部科学省指針の改定に伴う変更と使用の方法の変更についても平成19年12月18日に文部科学大臣の確認（19国文科振第26号）を得た。

さらにその後、研究者の追加・削除について機関内倫理委員会と機関長の了承を得て、平成20年3月11日、10月27日に文部科学省に届け出た。

C．研究結果

1．ヒトES細胞からの好中球分化誘導

マウス胎児線維芽細胞（MEF）との共培養で維持中のヒトES細胞（KhES-3）をコラゲナーゼ処理で回収し、サイトカインカクテル（6種類のサイトカイン・増殖因子（IGF-II, VEGF, SCF, Flt3-L, TPO, G-CSF））を含む分化培地を用いて低吸着培養皿上で3日間浮遊培養を行い、形成された大小不同のshereをまとめてゼラチンコート皿で培養した。

接着後にsphereは平板化し、活発な細胞増殖に伴って円盤状の細胞層が形成された。しばらくすると円盤状細胞層中心付近（sphereの接着部位付近）が重層化し、12日前後で球状細胞を包含する嚢状の構造物が形成された。この嚢状構造物はカニクイザルES細胞からの造血細胞分化過程でも観察されており、我々はこれを嚢状構造物（a sac-like structure; SLS）と命名した。球状細胞はSLS内に充満し、次第にSLS外側の円盤状細胞層の上にも載積するようになった。培地交換の際にはまずSLS壁面をマイクロピペットで切開して球状細胞を上清中に放出させ、上清を回収して遠心後に沈殿した球状細胞をフレッシュな分化培地に懸濁させるようにして行った。切開したSLS壁は一晩で塞がり2～3日後には再び球状細胞が充満していった。このような球状細胞は、以下の記述するように高率に好中球に分化したが、マクロファージや赤芽球（胎児型、成人型）にも分化しうる多能性の造血前駆細胞であることが、コロニーアッセー、グロビン遺伝子発現解析（RT-PCR）から明らかとなった。

SLS壁の切開、培養上清の遠心、球状細胞の回収、培地交換、という手順を繰り返しながら合計30～40日の培養を行なった。最終的に回収された球状細胞は95%以上で汎血球細胞マーカーであるCD45の発現を認めた。また骨髄系細胞マーカーであるCD33や単球/顆粒球系マーカー（CD11b, GPI-80）の発現も高く、また過半数の細胞で顆粒球マーカーであるCD66bが陽性であった。これらの結果は、細胞形態（ライトギムザ染色標本で判定）や特殊染色（ミエロペルオキシダーゼ染色、エステラーゼ染色、好中球アルカリフォスファターゼ染色）およびコロニーアッセイによる評価からも確認された。さらに産生された血球をinterleukin 8（IL-8）で遊走させることで成熟機能（貪食能、活性酸素産生能）を持つ好中球を濃縮することもできた。また、産生された血球

の in vivo での機能は、免疫不全マウス（NOG マウス）を用いた空気嚢モデル実験により確認された。

我々の分化誘導法においては、SLS 内に球状細胞が徐々に充満していくが、この状態を放置したまま培地交換を行なうと SLS 内の球状細胞はマクロファージ主体になる。この理由は不明であるが、元来 ES 細胞はマクロファージを産生しやすい性質を持つものであることに加えて、産生されたマクロファージが造血前駆細胞を貪食する可能性も推測される。我々は SLS が充満したら壁面を切開して球状細胞を上清中に放出して充満状態の持続を避けるとマクロファージの優勢化が遅延されることに気づき、これを実行した。これにより分化誘導開始後約 40 日までは顆粒球優位状態を保持しながら血球産生を行なうことが可能になった。

2．ヒト iPS 細胞を用いた血液細胞分化誘導

京都大学・山中伸弥教授の樹立したヒト iPS 細胞を用いて造血細胞分化を行なったが、試した 2 株（201B7、253G1）のどちらでも SLS の形成が確認された。有効株数を比較するならば、ヒトＥＳ細胞（３株中１株）よりヒト iPS 細胞（２株中２株）の方が成功率は高かった。ただし、ヒトＥＳ細胞（KhES-3）では全ての sphere から円盤状細胞層が形成され、その過半数で SLS が形成されたのに対して、ヒト iPS 細胞で円盤状細胞層の形成を認めたのは一部（＜3 割）の sphere であった。我々の印象では、ES 細胞では株の選択が、iPS 細胞では sphere の選択が、効率良い分化誘導のための鍵になると思われる。なお iPS 細胞では SLS 壁切開後の球状細胞の再生産は認められなかった。

3．サルＥＳ細胞を用いた造血前駆細胞の分化誘導

本研究の過程で、ヒトＥＳ細胞からの好中球産生に成功したが、成体型の造血幹細胞の産生は困難であった。しかにしながら、本年度において、カニクイザルＥＳ細胞からの血液細胞分化誘導の系において、将来につながる成果を得たので報告する。近年の著しい研究の進歩によって、発生過程においては hemogenic endothelium（CD34 陽性、VE-cadherin 陽性）から最初の造血幹細胞（CD34 陽性、CD45 陽性）が派生することが明らかとなっている。我々は高濃度の SCF や Flt3-L を含むサイトカインカクテルを用いてカニクイザル ES 細胞を無フィーダー培養したところ $CD34^{high}$ $CD45^{middle}$ $VE\text{-}cadherin^{high}$ 細胞（接着細胞）が作製された。これらの細胞は活発に増幅しながら大量に浮遊細胞（$CD34^{high}$ $CD45^{middle}$ $VE\text{-}cadherin^{middle}$）を産生し続けた。ＥＳ細胞からの造血幹細胞がこのような時期を経るとすれば、成体型の造血幹細胞に近い表現型を得ることに成功したことになり、今後の進展が期待される。

D．考察

好中球は細菌や真菌からの感染防御に極めて重要である。好中球減少症の患者が難治性感染症に罹患し、G-CSF、抗生物質、抗真菌剤、外科的処置等の治療に反応しない場合に顆粒球輸血が考慮される。ヒト白血球型抗原適合性顆粒球輸血の有効性は実証されているが、治療効果を得る充分量の顆粒球を常に確保することは難しく、顆粒球採取時に使用される G-CSF、副腎皮質ステロイド、赤血球沈降促進剤等のリスクも問題となる。我々は現行の顆粒球輸血療法に対する補助手段の提供を目的に、ヒトＥＳ細胞からの高純度好中球分化誘導法を開発すべく検討を行なった。

ヒトＥＳ細胞からはフィーダー細胞を用いた共培養法、および無フィーダー培養法により多系統の成熟血球の産生が報告されているが、好中球産生に関する報告はまだなかった。またマウスＥＳ細胞で報告されていた好中球産生法はマウスフィーダー細胞を用いた共培養系であった。我々は臨床応用の観点から異種動物細胞を用いない無フィーダー培養法での好中球分化誘導について検討を重ねた。そして浮遊培養とそれに続く接着培養の２段階からなる培養法により、高効率な血液細胞の産生、並びに成熟機能をもつ好中球の産生に成功した。この２段階培養法はサイトカインの種類を変えることで純粋に血管内皮細胞を作製することも可能であり、血球・血管内皮細胞の分化誘導に極めて有効な手法と考えられる。

sphere や胚様体などの細胞凝集塊の形成プロセスは無フィーダー分化誘導法の要となっているが、細胞凝集塊を single cell レベルに分散してセルソーターや磁気ビーズを用いて特定細胞集団を選別する操作がとられることが多い。我々の方法は sphere をそのまま接着培養をすることに特徴があり、セルソーティングが不要であるという技術的メリット（簡便化と低コスト化）に加えて、接着培養により細胞凝集塊が

平面化するために形態観察が容易になること、細胞分化に重要である細胞間相互作用を破壊せずに培養ができること、などの利点がある。

従来からsphere形成は高指向性分化誘導のために有利とされてきたが、それは目的系列の細胞だけでなくフィーダーとなる細胞種も同時に産生されるためと推測される。発生過程では分化細胞とフィーダー細胞（またはストローマ細胞）とが密接な関係をもって発生するが、sphereという3次元構造に起因する位置依存的シグナルが発生類似の環境を提供するのかもしれない。

現在の我々の課題は、1）長期の好中球産生法の開発、2）ヒトES細胞の分化挙動に関する株間差への対応、3）産生された好中球の臨床使用における有用性の検証、である。1）については、胎児造血で見られるような「自己増幅能のある造血幹細胞」の作製が必要になるかもしれない。HOXB4遺伝子導入が造血幹/前駆細胞の作製に有効であることがマウスＥＳ細胞で示されているが、ヒトＥＳ細胞に関しては臨床応用を可能とするような成果はまだ報告されていない。HOXB4に加えてRunx1など造血幹細胞の発生に重要な転写因子群をセットで導入することが有効なのかもしれない。2）については、当面はヒトＥＳ細胞株の数を増やすことで対応するのが近道であると思われる。しかし網羅的解析などを通じて分化指向性を決定づけているファクターが同定されれば分化誘導操作の幅が広がるかもしれない。3）については、我々の解析はヒトＥＳ細胞由来好中球のin vivoでの遊走能を免疫不全マウスへの移植実験で確認したにとどまっているが、将来的には多剤耐性菌への感染防御能についても評価できるようにしたい。

E．結論

本研究では、異種動物成分の混入を回避する培養法の開発のために、ヒトＥＳ細胞を用いて、無フィーダー分化誘導系による血液細胞の産生を試みた。培養法の基本は、前半のsphere形成浮遊培養と後半の平面接着培養であった。敷石状の細胞の増殖、中心部での嚢状構造物の形成を経て、血液細胞が産生された。産生された血液細胞は比較的分化した骨髄系の細胞で、好中球を多く含んでいた。これらの好中球は、in vitroでもin vivoでも十分な機能を発揮出来る成熟好中球であった。このような成熟好中球が効率よく産生できるＥＳ細胞の分化誘導システムは、霊長類では世界初であり、顕著な成果が得られたと言える。また、今年度は成果をヒトiPS細胞にも展開して、臨床応用へ向けて着実に進展した。

F．健康危険情報

なし

G．研究発表

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H．知的財産権の出願・登録状況

１．特許取得
発明の名称：血管平滑筋細胞増殖を抑制する血管狭窄部挿入用基材
発明者：佐伯久美子、湯尾　明、安田和基、佐藤千香子、五香麻衣子
出願人：財団法人ヒューマンサイエンス振興財団
出願番号：特願２００８－２８０１４８
出願日：平成２０年１０月３１日

２．実用新案登録
なし

３．その他
なし

Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

なし

雑誌

発表者氏名	論文タイトル	発表誌名	巻号	頁	出版年
Saeki K, Yogiashi Y, Nakahara M, Nakamura N, Matsuyama S, Koyanagi A, Yagita H, Koyanagi M, Kondo Y, Yuo A	Highly efficient and feeder-free production of subculturable vascular endothelial cells from primate embryonic stem cells.	J Cell Physiol	217	261-280	2008
Nakahara M, Matsuyama S, Saeki K, Nakamura N, Saeki K, Yogiashi Y, Yoneda A, Koyanagi M, Kondo Y, Yuo A	A feeder-free hematopoietic differentiation system with generation of functional neutrophils from feeder and cytokine-free primate embryonic stem cells.	Cloning Stem Cells	10	341-354	2008
Saeki K, Saeki K, Nakahara M, Matsuyama S, Nakamura N, Yogiashi Y, Yoneda A, Koyanagi M, Kondo Y, Yuo A	A feeder-free and efficient production of functional neutrophils from human embryonic stem cells.	Stem Cells	27	59-67	2009
Nakahara M, Saeki K, Nakamura N, Matsuyama S, Yogiashi Y, Yasuda K, Kondo Y, Yuo A	Human embryonic stem cells with maintenance under a feeder-free and recombinant cytokine-free condition.	Cloning Stem Cells	11	5-18	2009

Ⅲ. 研究成果の刊行物・別刷

Journal of Cellular Physiology

Highly Efficient and Feeder-Free Production of Subculturable Vascular Endothelial Cells From Primate Embryonic Stem Cells

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The vascular endothelial cell (VEC) differentiation from primate embryonic stem (ES) cells has critical problems: low differentiation efficiencies (<2%) and/or subculture incapability. We report a novel feeder-free culture method for high efficiency production of subculturable VECs from cynomolgus monkey ES cells. Spheres, which were generated from ES cells in the presence of cytokine cocktail, were cultured on gelatin-coated plates. Cobblestone-shaped cells spread out after a few days, which were followed by an emergence of a sac-like structure containing hematopoietic cells. All adherent cells including sac walls cells and surrounding cobblestone cells expressed vascular endothelial cadherin (VE-cadherin) at intercellular junctions. Subculture of these cells resulted in a generation of homogeneous spindle-shaped population bearing cord-forming activities and a uniform acetylated low density lipoprotein-uptaking capacity with von Willbrand factor and endothelial nitric oxide synthetase expressions. They were freeze–thaw-tolerable and subculturable up to eight passages. Co-existence of pericytes or immature ES cells was ruled out. When introduced in a collagen sponge plug implanted intraperitoneally in mice, ES-derived cells recruited into neovascularity. Although percentages of surface VE-cadherin-positive population varied from 20% to 80% as assessed by flow cytometry, the surface VE-cadherin-negative population showed intracellular VE-cadherin expression and mature functions, as we call it as atypical VECs. When sorted, the surface VE-cadherin-positive population expanded as almost pure (>90%) VE-cadherin/PECAM-1-positive VECs by 160-fold after five passages. Thus, our system provides pure production of functional, subculturable and freeze–thaw-tolerable VECs, including atypical VECs, from primate ES cells.

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Embryonic stem (ES) cells are a valuable resource in regenerative medicine because of their high capacity to differentiate into a broad range of cell types. It has been revealed that primate ES cells have characteristics distinct from those of murine ES cells. For example, leukemia inhibitory factor is ineffective for maintaining the immaturity of human ES cells (Sato et al., 2004). The gene expression patterns also differ between primate and murine ES cells. For example, undifferentiated primate ES cells express kinase insert domain receptor (KDR, Flk-1, a type 2 receptor for vascular endothelial growth factor (VEGF-R2)) although undifferentiated murine ES cells do not (Sone et al., 2003; Vodyanik et al., 2005). KDR is known as an excellent marker for sorting the vascular endothelial precursor population in murine ES differentiation system (Yamashita et al., 2000). However, the presence of KDR in undifferentiated primate ES cells limits a direct application of the murine system to the primate one (Sone et al., 2003). Eventually, by contrast to the highly efficient production (>90%) of vascular endothelial cells from murine ES cells (Hirashima et al., 1999), that from primate ES cells is significantly low; the efficiency of the production of vascular endothelial cadherin (VE-cadherin)-positive (Sone et al., 2003, 2007) or platelet endothelial cell adhesion molecule-1 (PECAM-1)-positive (Levenberg et al., 2002) cell are not higher than two percents. Although the human ES cell study is essential for clinical application, basic researches using monkey ES cells still has great importance because they provide good transplantation models that must be performed in the pre-clinical study (Takagi et al., 2005). Moreover, the use of monkey ES cells avoids ethical issues and thus the biotechnological manipulation of them, including gene transfer, can immediately be applied, which will contribute to understand human ES cells.

Currently, there are three major methods for the in vitro differentiation of ES cells into specific lineages. One is co-culturing ES cells with stromal cells as their feeders (Hirashima et al., 1999; Sone et al., 2003, 2007). Feeder cells have large capacities to promote directed differentiation and to support viability of differentiated cells. Nevertheless, contamination of the final product by feeder cells is inevitable, and thus, an especially strict cell-sorting technique is required for purification of the final products. Concerning vascular endothelial differentiation of primate ES cells, however, the efficiency of cell surface VE-cadherin-positive cell production is considerably low (<2%) even when murine OP9 feeder cells were used (Sone et al., 2003, 2007). Besides, massive

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contamination of pericytes reportedly occurs in this system. Pericytes are important players for angiogenesis, and thus, the co-generation of pericytes might be beneficial in some aspects. However, domination of pericytes after passages considerably hinder the pure expansion of vascular endothelial cells even after sorting of VE-cadherin-positive cells (Sone et al., 2007). The second method is a feeder-free culture via a generation of embryoid bodies (EBs) or spheres (Levenberg et al., 2002). It is beneficial that it can exclude the risk of contamination of xenogenic materials. Yet, directions of differentiation in EBs or spheres are principally random and cannot easily be focused on a specific lineage. As a result, fairly large volumes of starting ES cells are required to obtain sufficient amounts of differentiated cells. Additionally, these two methods described above share a common disadvantage; they do not provide a clear microscopic field for observation and manipulation due to co-existing feeder cells or the compact three-dimensional structures of EBs or spheres. The third method is a simple adherent culture of ES cells. There have been two reports concerning this method. One is the continual culture of rhesus monkey ES (rmES) cells on mouse embryonic fibroblasts (MEFs) using a vascular endothelial cell-specific culture medium of EGM®-2 MV BulletKit (Kaufman et al., 2004). This system provided a good microscopic field and effectively produced the subculturable vascular endothelial cells. However, these cells are not the conventional or canonical vascular endothelial cells, but rather, seem to be "atypical" vascular endothelial cells in that neither the expression of VE-cadherin, a vascular endothelial cell-specific and pan-vascular endothelial marker, nor PECAM-1, a mature vascular endothelial cell marker, was detected by flow cytometry. Nonetheless, they have mature endothelial functions including in vitro cord-forming and acetylated low density lipoprotein (Ac-LDL)-uptaking activities and in vivo neovascularization activity along with expressions of von Willbrand factor (vWF) and endothelial nitric oxide synthetase (eNOS). The other one is an adherent culture of human ES-derived CD34-positive cells, which were spontaneously generated by overgrown ES cells on MEFs in the presence of serum, in EGM®-2 MV BulletKit (Wang et al., 2007). This system requires the enrichment of CD34-positive cells (<10%) by cell sorter before subsequent culture in EGM®-2 MV BulletKit. Although this system provides VE-cadherin-positive functional vascular endothelial cells, they are not subculturable. Thus, the most urgent task for the vascular endothelial differentiation from primate ES cells is to establish a method for "feeder-free" and "high efficiency" production for cell surface "VE-cadherin-positive" and "subculturable" vascular endothelial cells.

Here we report a novel method for highly efficient production of functional, subculturable, freeze–thaw-tolerable and cell-surface-VE-cadherin-positive vascular endothelial cells from cynomolgus monkey ES (cmES) cells in feeder-free culture system. Furthermore, it does not require a step to enrich the progenitor fractions, such as CD34-positive and/or KDR-positive fractions, by cell sorter. To our knowledge, this is the highest efficiency system for the production of vascular endothelial cells. Indeed, our system provides almost two pure populations: the cell surface VE-cadherin-positive canonical vascular endothelial cells and cell surface VE-cadherin-negative "atypical" vascular endothelial cells. The characterization and significance of these atypical vascular endothelial cells is referred and discussed in this report.

Materials and Methods

Cells and reagents

Murine embryonic fibroblasts (MEFs), which had been treated with Dulbecco's modified Eagle's medium (DMEM) containing mitomycin C (MMC) (Sigma Chemical Co., St. Louis, MO) for 3 h, were seeded on the dishes coated with 0.1% gelatin. The cmES cells (CMK-6) (Suemori et al., 2001) were maintained on MMC-treated MEF-coated dishes in DMEM/F12 medium (Invitrogen Corp., Carslbad, CA) supplemented with 20% Knockout™ Serum Replacement (KSR™, Invitrogen Corp.), 1% Non-essential amino acids solution (Invitrogen Corp.), 1 mM Sodium Pyruvate Solution (Invitrogen Corp.), 2 mM L-glutamine (Invitrogen Corp.), 10 U/ml penicillin (Invitrogen Corp.) and 10 μg/ml streptomycin (Invitrogen Corp.). ES cells were passed twice a week by collagenase treatment and seeded at split ratios of 1:2 to 1:4 on new MEF-coated dishes. Human aortic endothelial cells (HAEC) and human umbilical vein endothelial cells (HUVEC) were purchased from Lonza Group Ltd. (Basel, Switzerland), and maintained on gelatin-coated dishes using EGM®-2 BulletKit (Lonza Group Ltd.). Normal Human Aortic Smooth Muscle cells (AOSMC)) were purchased from Lonza Group Ltd. and maintained on gelatin-coated dishes using SmGM®-2 BulletKit® (Lonza Group Ltd.). The human leukemic UT-7 and HL-60 cells were cultured RPMI 1640 medium (Sigma Chemical Co.) supplemented with 10% heat-inactivated fetal bovine serum (FBS).

Differentiation procedure

ES colonies were collected by collagenase treatment without a contamination of MEFs and further disaggregated by trypsinization. Cell aggregates were generated by a hanging drop culture, where 3,000 cmES cells were incubated in a 30 μl drop of differentiation medium (DM) consisting of Iscove's modified Dulbecco's medium (IMDM) (Sigma Chemical Co.) supplemented with 15% heat-inactivated FBS (PAA Laboratories GmbH, Linz, Austria), 0.1 mM β-mercaptoethanol (Sigma Chemical Co.), 3 mM L-glutamine (Invitrogen Corp.), 10 U/ml penicillin (Invitrogen Corp.), 20 ng/ml vascular endothelial growth factor (VEGF; Pepro Tech Inc., Rocky Hill, NJ), 20 ng/ml bone morphogenic protein 4 (BMP-4; R&D Systems Inc., Minneapolis, MN), 20 ng/ml stem cell factor (SCF; Pepro Tech Inc.), 10 ng/ml Flt3 ligand (Flt3-L; Pepro Tech Inc.), 20 ng/ml interleukin 3 (IL-3; Pepro Tech Inc.) and 10 ng/ml interleukin 6 (IL-6; Pepro Tech Inc.). After incubating the drops for 3 days at 37°C under a 100% humidified condition in a 5% CO_2 gas incubator, cell aggregates generated from 72 hanging drops were subjected to adherent culture on a 100 mm × 20 mm gelatin-coated dishes using differentiation medium described. Media were changed twice a week. After about 2 weeks, a VE-cadherin-positive sac-like structure filled with round cells along with surrounding VE-cadherin-positive cobblestone cells emerged. Before sacs were fully packed by inner round cells, sac walls were manually cut by using stem cell knife (Vitrolife AB, Kungsbacka, Sweden) under microscopic observation. After releasing the inner round cells into culture supernatant, total adherent cells including fragmented sac walls and surrounding cobblestone cells were massively transferred onto new gelatin-coated dishes via trypsin/EDTA treatment. These cells were subcultured by 1:3 dilution twice a week. In some experiments, EGM®-2 BulletKit (Lonza Group Ltd.) was used in place of the differentiation medium described above throughout the differential processes including hanging drop culture and subsequent adherent culture.

Morphological and cytochemical examinations

Viable cells were directly observed under an inverted phase contrast light microscope (Olympus Optical Co. Ltd., Tokyo, Japan), or alternatively, cells were fixed on slide glasses using a cytospin apparatus (Cytospin 2, SHANDON, Pittsburgh, PA), stained with Wright-Giemsa solution, myeloperoxidase staining kit or esterase staining kit (Muto Pure Chemical Co., Tokyo, Japan), and then observed under the light microscope (Olympus Optical Co. Ltd.).

Flow cytometric analyses and cell sorting

Cells were collected by 0.2% EDTA treatment and, after a wash in phosphate-buffered saline (PBS), 1×10^6 cells were reacted with first antibodies on ice for 30 min. The expression level of each protein was analyzed using a FACSCalibur™ (BD Biosciences, San Jose, CA). The antibodies used were a mouse monoclonal anti-human VE-cadherin (clone TEA1/31)-phycoerythrin (PE) antibody (Beckman Coulter Inc., Fullerton, CA), a mouse monoclonal anti-human CD31 (PECAM-1)-fluorescein isothiocyanate (FITC) antibody (clone WM59) (BD Biosciences), a mouse monoclonal anti-human CD34 (clone 563)-PE antibody (BD Biosciences), a mouse monoclonal anti-human Tie-2 (clone 83715)-PE (R&D Systems Inc.), a mouse monoclonal anti-human VEGF-R1 (Flt-1) (clone 49560)-PE antibody (R&D Systems Inc.), a mouse monoclonal anti-human VEGF-R2 (KDR, Flk-1) (clone 89106)-PE antibody (R&D Systems Inc.), a mouse monoclonal anti-human VEGF-R3 (Flt-4) (clone 54733)-PE antibody (R&D Systems Inc.), a mouse monoclonal anti-human CD14 (clone M5E2)-PE antibody (BioLegend, San Diego, CA), a mouse monoclonal anti-human CD18 (clone 6.7)-FITC antibody (BD Biosciences), a mouse monoclonal anti-human CD11b (clone ICRF44)-PE antibody (BD Biosciences) and a mouse monoclonal anti-human CD45 (clone TÜ116)-PE antibody (BD Biosciences). As for the anti-VE-cadherin antibody reaction, secondary antibody reaction was performed using a goat anti-mouse IgG-PE (Calbiochem Co., La Jolla, CA). After antibody-staining procedures, cells were stained with TO-PRO3 fluorescent dye (Invitrogen Corp.) for 10 min. During analysis, dead cells were gated out as the FL4-higher fraction. The cell surface VE-cadherin-positive and VE-cadherin-negative fractions were sorted using as FACSAria™ (BD Biosciences) after cells were stained VE-cadherin (clone TEA1/31)-phycoerythrin (PE) antibody (Beckman Coulter Inc.).

Immunostaining

The cells were fixed on slide glasses with a cytospin apparatus (Cytospin 2) with further fixation with acetone/methanol solution (1:3). The immunostaining procedure was performed as described elsewhere (Saeki et al., 2003) with first antibody reactions using a mouse anti-human VE-cadherin antibody (clone55-7H1) (BD Biosciences), a rabbit anti-human N-cadherin antibody (H-63) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), a rabbit polyclonal anti-human Nanog antibody (ReproCELL Inc., Tokyo, Japan), a mouse monoclonal anti-human Actin, α smooth muscle (SMA) (clone 1A4) (Sigma Chemical Co.), a mouse monoclonal anti-platelet-derived growth factor receptor β (PDGF-Rβ (clone 28) (BD Biosciences), a rabbit polyclonal anti-human eNOS antibody (H-159) (Santa Cruz Biotechnology Inc.), a goat polyclonal anti-human vWF antibody (C-20) (Santa Cruz Biotechnology Inc.) or a mouse monoclonal anti-CD68 antibody (Transgenic Inc., Kobe, Japan) followed by second antibody reactions using Alexa Fluor® 488 chicken anti-mouse IgG (H + L), Alexa Fluor® 568 goat anti-rabbit IgG (H + L) or Alexa Fluor® 594 chicken anti-goat IgG (H + L) (Molecular Probes, Inc., Eugene, OR).

Cord formation assays

Matrigel (BD Biosciences) was loaded into the 24 multi-well dishes (95 μl/well). After the dishes were incubated for 30 min at 37°C, 1×10^4 cells per well were seeded in 1 ml of EGM®-2 BulletKit supplemented with cytokines and growth factors according to the manufacturer's instructions (Cambrex Bio Science Walkersville, Inc., Walkersville, MD). Cell morphologies were observed after overnight culture under an inverted light microscope (Olympus Optical Co. Ltd.).

Colony assays

Colony assays were performed using Methocult TM GF$^+$H4535 (Stemcell Technologies Inc., Vancouver, Canada) according to the manufacturer's recommendations. In brief, 0.3 ml of cell suspension, which contained 10 cells, was mixed in 3 ml of methylcellulose solution consisting of 1% methylcellulose, 30% FBS, 1% bovine serum albumin, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml SCF (Pepro Tech Inc.), 20 ng/ml IL-3 (Pepro Tech Inc.), 20 ng/ml IL-6 (Pepro Tech Inc.), 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; Pepro Tech Inc.), 20 ng/ml granulocyte colony-stimulating factor (G-CSF; Kirin Brewery Company, Ltd., Tokyo, Japan) and 3 U/ml erythropoietin (Kirin Brewery Company, Ltd.) in 3.5-cm culture dishes. After 2 weeks, the number of colonies was counted. The morphology of the colonies was observed under an inverted light microscope (Olympus Optical Co. Ltd.).

Western blotting

Western blotting was performed as described previously (Saeki et al., 2003) using a rabbit anti-human VE-cadherin antibody (C-19) (Santa Cruz Biotechnology Inc.) or a mouse monoclonal anti-tubulin β (D-10) antibody (Santa Cruz Biotechnology Inc.). The second antibody reaction was performed using a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Cell Signaling Technology, Inc., Beverly, MA). The final detection procedure was performed using ECL Western blotting detection reagents (GE Healthcare UK Ltd., Buckinghamshire, England).

Uptake of acetylated low-density lipoprotein (Ac-LDL)

Cells were transferred in 4-well chamber slide system (Nalge Nunc International Corp., Naperville, IL). After overnight culture, cells were washed by Hank's balanced salt solution (HBSS) twice and incubated in serum-free medium containing 10 μg/ml of low-density lipoprotein from human plasma, acetylated, DiI complex (DiI Ac-LDL) (Molecular Probes) for 4 h. After washing the cells by HBSS for three times, the cells were observed under the fluorescence microscope (Olympus Optical Co. Ltd.).

Polymerase chain reaction (PCR)

Genomic PCR. Genomic DNA was extracted from ES-derived vascular endothelial cells (ESdEC), HUVECs (Cambrex Bio Science Walkersville, Inc.) or MEFs of the ICR stain (CLEA Japan, Inc., Tokyo, Japan). 1×10^6 cells were lysed with a buffer (50 mM Tris–HCl (pH 8.0), 0.1 M NaCl, 20 mM EDTA and 0.1% SDS) supplemented with 75 μg/ml Proteinase K (WAKO Pure Chemical Industries, Osaka, Japan) for 8 h at 55°C. After phenol/chloroform extraction and isopropanol precipitation, dried pellets were solubilized by 100 μl of water containing 50 ng/ml DNase-free RNase (Invitrogen Corp.) and incubated for 1 h at 37°C. The genomic DNA solution was kept at −80°C. PCR was performed using 2 μl of the 50× diluted DNA template, SP-Taq (Hokkaido System Science Co., Ltd., Hokkaido Japan) and following primers. For detecting the murine ly 9.2 genomic fragment, a forward primer 5′-gtaaattcccccagcctttgt-3′ and a reverse primer 5′-atgccatgtcctcttcatccs-3′ were used (the product length is 433 bp). For detecting the primate CD34 genomic fragment, a forward primer 5′-CGACAGTCAAATTCACATCTACC-3′ and a reverse primer 5′-GAGATGTTGCAAGGCTAGTGC -3′ were used (the product length is 254 bp). The PCR procedure was carried out using a DNA Thermal Cycler PJ2000 (PerkinElmer Corp., Foster City, CA) with the following program: 95°C; 5 min, 94°C; 30 sec, 57°C; 30 sec, 72°C; 1 min at 27 cycles for murine ly9.2 and at 34 cycles for primate CD34. The product was separated by agarose gel electrophoresis and the DNA was visualized by ethidium bromide staining. A 100 bp ladder marker 4 (Nippon Gene Co. Ltd., Tokyo, Japan) was used to evaluate the molecular weights of the PCR products.

Reverse transcription-polymerase chain reaction (RT-PCR). RNA was extracted from 5×10^6 cells with RNeasy Mini Kit (Qiagen Inc., Valencia, CA) and cDNA was synthesized by Superscript II Kit (Invitrogen Corp.) according to a manufacture's protocol. The PCR procedure was carried out as described above using primate globin-ε primers; a forward primer 5′-TGCATTTTACTGCTGAGGAGA-3′ and a reverse primer 5′- AAGAGAACTCAGTGGTACTT-3′, primate globin-ζ primers; a forward primer 5′-TTCCTCAGCCACCCGCAGAC-3′ and a reverse

primer 5′-AGCAGGCAGTGGGACAGGAG-3′, primate VE-cadherin primers; a forward primer 5′-TGGGCTCAGACATCCACATA-3′ and a reverse primer 5′-TCACAGTCTCCCTTGGGAAT-3′. For internal control, primate β-actin primers were used: a forward primer 5′-gCAggAgATggCCACggCgCC-3′ and a reverse primer 5′-TCTCCTTCTgCATCCTgTCggC-3′.

Collagen plug assay

About 10 blocks of dried honeycomb collagen sponge (Koken Co. Ltd., Tokyo, Japan) were mixed with 500 μl of cmES-derived cells at passage number 4, which were suspended in differentiation medium at the density of 4×10^5/ml, and were then cultured for 2 days in vitro. Then they were transplanted intraperitoneally into SCID mice. After 35 days, 0.2 ml of FITC-dextran (500,000 average molecular size, Sigma Chemical Co.) solution (100 mg FITC-dextran suspended in 5 ml PBS) was injected into mice from a tail vein. After several minutes, mice were sacrificed and the blocks were fixed by 10% formaldehyde and paraffin embedded. The 4 μm sliced specimen were further subjected to immunostaining using a mouse monoclonal anti-human HLA-A, B, C antibody (BD Biosciences) or a rabbit polyclonal anti-PECAM-1 antibody (H-300) (Santa Cruz Biotechnology Inc.), and also to histological examination using hematoxylin and eosin solutions.

Results

A sac-like structure with surrounding cobblestone cells as a parental organization for vascular endothelial cell generation

During our attempt to generate hematopoietic progenitor cells from cmES cells under feeder-free conditions, we found that the condition we had tried to optimize for hematopoietic differentiation was unexpectedly fitted for the production of vascular endothelial cells. Our differentiation medium was prepared by modifying the one reported in a case of hematopoietic differentiation of rmES cells by co-culture method (Li et al., 2001). In the presence of VEGF, BMP-4, SCF, Flt3-L, IL-3, IL-6 and serum, we generated spheres from cmES cells (Fig. 1A,B, upper left). Then spheres were subjected to adherent culture on gelatin-coated dishes (Fig. 1A,B, upper middle and right). Time course observations revealed the differentiation processes as follows: spreading of cobblestone-shaped cells with leaving the center region rather amorphous in a few days (Fig. 1B, lower left); swelling of the center region at about 6 days (Fig. 1B, lower middle); ballooning out of the center region resulting in the formation of "a sac-like structure" filled with abundant round cells, some of which brimmed over the sac wall, around 12 days (Fig. 1B, lower right). At this time, the cobblestone cells were divided into two populations by their morphology. The cells located at proximal regions to the sac-like structure became densely packed, while the cells located at distal regions remained rather stretched (Fig. 1C). Although the early phased cobblestone cells (Fig. 1B, lower left) did not express VE-cadherin but expressed only N-cadherin at intercellular junctions (data not shown), VE-cadherin expression had become detectable in all adherent cells at the time when a sac-like structure was formed. As shown in Figure 1D, the late phased cobblestone cells, including proximal compact cells and distal extended cells, as well as sac wall cells expressed VE-cadherin at intercellular junctions. Thus, total adherent cells generated after the culture of ES-derived spheres on gelatin-coated dishes using our differentiation medium are regarded as vascular endothelial cell-committed populations although no special selection procedures were performed.

We also studied the characteristics of the non-adherent round cells, majority of which located within the sacs and minor portion of which resided around the sacs. They were collected from the culture supernatant after cutting the sac walls by micropipettes and releasing into the medium. The cytochemical analysis including Wright-Giemsa, myeloperoxidase and esterase staining showed that the majority of the round cells were myeloblasts and macrophages (Fig. 1E). This finding was further supported by the results of colony assays of round cells, where generation of granulocyte/macrophage colonies consisting of promyelocytes, myelocytes, and segmented neutrophils were observed (Fig. 1F). Because sac-like structures resembled the morphology of blood islands in the yolk sac, we investigated the possible existence of primitive hematopoietic cells. The RT-PCR studies showed that the messages for embryonic hemoglobins, globin-ε and globin-ζ, were expressed at the sac stage (Fig. 1G, lane 2 (cmES-derived cells, p1)) and subsequent first-passage-stage (Fig. 1G, lane 3 (cmES-derived cells, p2)). Thus, non-adherent round cells consist of myeloblasts and macrophages along with a small portion of primitive erythroid cells.

Hence, simple adherent culture of ES-derived spheres in the presence of hematopoietic cytokines resulted in generation of a unique construction: a sac-like structure and surrounding cobblestone cells that expressed VE-cadherin along with primitive and definitive hematopoietic cells localized within and around the sac.

Characterization of subcultured adherent cells

We next tried to expand the cell surface VE-cadherin-positive adherent cells by an ordinary subculture method. After cutting sac walls and releasing the inner hematopoietic cells into culture supernatant, residual adherent cell populations including sac wall-constituting cells and surrounding cobblestone cells were detached by trypsin/EDTA treatment and transferred massively onto new gelatin-coated dishes. During the cutting process of the sac walls, they were divided into small fragments but remained attached to dishes. After trypsin/EDTA treatment, the cells were dissociated almost into single cell and transferred to new gelatin-coated plates by 1:3 dilution. Cells actively proliferated and reached confluence in 3–4 days. They showed highly homogenous spindle-shaped morphology similar to HUVEC (Fig. 2A). Indeed, these cmES-derived cells showed cord-forming activities equivalent to HUVEC (Fig. 2B). Furthermore, all of the cells were positive for Ac-LDL-uptaking activities (Fig. 2C) with uniform expressions of eNOS and vWF (described in the following part of the manuscript and Fig. 4B, left parts).

To quantitatively evaluate the vascular endothelial differentiation, we determined the cell surface expression of VE-cadherin and PECAM-1 by flow cytometry. As shown in Figure 3A, limited populations were positive for VE-cadherin and PECAM-1 despite rather uniform expression of VE-cadherin before subculture (Fig. 1D). Although the reason for the reduction of VE-cadherin expression after subculture was not known, the expressions of VE-cadherin/PECAM-1 were well preserved during subsequent culture (Fig. 3B). The cells were subculturable up to eight passages, during which 2×10^5 cmES cells generated 7×10^9 of VE-cadherin/PECAM-1-positive vascular endothelial cells. Moreover, the cells were freeze–thaw-tolerable and re-cultured cells properly retained the VE-cadherin/PECAM-1 expressions (Fig. 3C). The average percents of VE-cadherin/PECAM-1-positive cells were $29.8 \pm 15.1\%$ ($n = 17$). The expressions of other surface markers for vascular endothelial cells were also examined. As shown in Figure 3D, the expression of VEGF-R1, a vascular endothelial cell-specific marker, was detected at a comparable level to HUVEC and HAEC. Tie-2 expression was also detected in cmES-derived cells although the expression levels were slightly lower than HUVEC and HAEC. On the other hand, CD34-positive populations were detected mainly in cmES-derived cells and HUVEC. Interestingly, the expression of

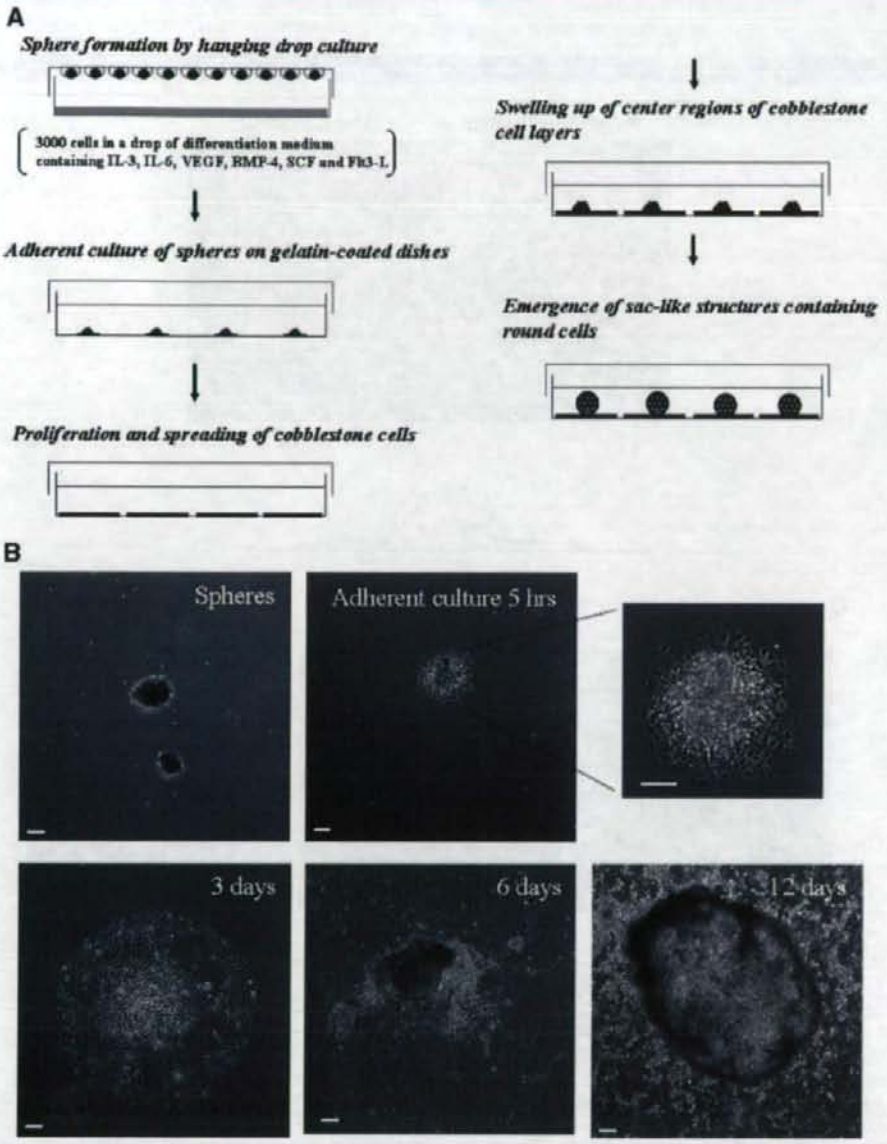

Fig. 1. Generation of a sac-like structure and cobblestone cells from feeder-free differentiation culture of cmES cells. The procedure for the production of a sac-like structure (A) and phase contrast microscopy (B). Spheres were generated by a hanging drop culture and subjected to subsequent adherent culture using gelatin-coated plates. Within a few days, spheres adhered on the plates became flattened and VE-cadherin-negative cobblestone cells spread out. At about 6 days, the center region began to swell up again. Around 12 days, the center regions ballooned out and sac-like structures were formed. Scale bars indicate 100 μm. C: The phase contrast microscopy of late phase cobblestone cells. Morphologies of proximal and distal cobblestone cells that surrounded a sac-like structure were shown. The scale bar indicates 100 μm. D: VE-cadherin expression in late phase cobblestone cells. Sac-like structure and surrounding cobblestone cells were stained with anti-VE-cadherin antibody. The scale bar indicates 50 μm. E: Morphological and cytochemical examinations of the round cells released from the sac-like structure. The Wright-Giemsa staining (WG) showed the presence of two types of the cells: the small cells with basophilic cytoplasm and high nucleus/cytoplasm ratio resembling myeloblasts and the large cells with abundant vacuolated cytoplasms resembling macrophages. The myeloperoxidase staining (MPO) showed that majority of the small cells were positive for myeloperoxidase. The esterase double staining (Esterase) showed the cells with blue granules (granulocyte lineage cells) and cells with brown granules (macrophage lineage cells). The scale bar indicates 100 μm. F: Hematopoietic colony assays. The round cells released from the sac-like structure were subjected to hematopoietic colony assays. The phase contrast microscopic observation (Phase) showed the presence of granulocyte/macrophage colonies. Wright-Giemsa staining (WG) of the cells from colonies showed the presence of myeloblasts (a black arrow), promyelocytes (a blue arrow) and polymorphonuclear granulocytes (a red arrow). The myeloperoxidase staining (MPO) showed the presence of a myeloperoxidase-rich smaller granulocyte population (a black arrow) and a myeloperoxidase-poor large macrophage population (a red arrow). Esterase double staining (Esterase) showed the presence of cells with blue granules (a black arrow) and cells with brown granules (a red arrow). The scale bar indicates 100 μm. G: RT-PCR. The presence of primitive hematopoietic cells was shown by the existence of messages for embryonic hemoglobins (globin-ε and globin-ζ) using primate-specific primers (pr-globin-ε, pr-globin-ζ, and pr-β-actin). "M" indicates 100 bp ladder marker. The subscript character of "p" of cmES-derived cells indicates the passage number. "H"; HUEVC, "E"; undifferentiated cmES cells, "U"; hematopoietic UT-7 cells as positive control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

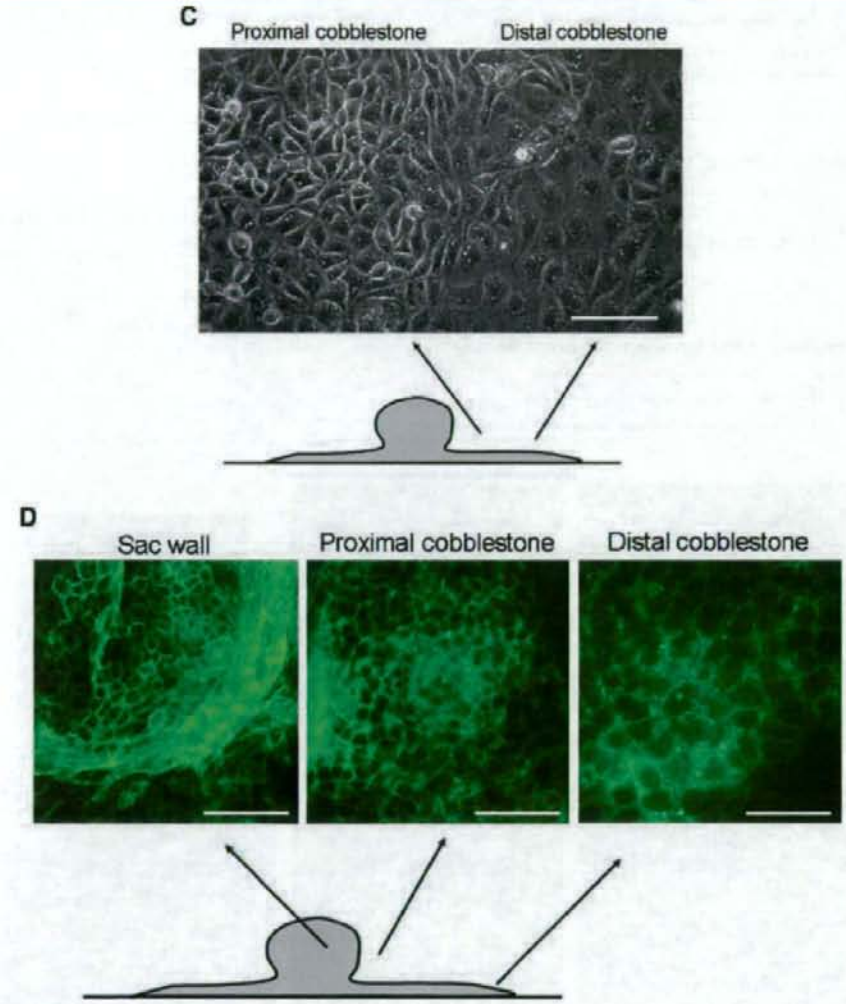

Fig. 1. (*Continued*)

VEGF-R3, a marker for lymphatic endothelial and embryonic vascular endothelial cells, was detected at fairly high levels in cmES-derived cells and HUVEC. The expression of VEGF-R2, a marker for vascular and lymphatic endothelial cells, was constantly detected at the same level as VE-cadherin, as we confirm it by detailed analyses in the following part of the manuscript (Fig. 5). From these flow cytometric analyses, it was concluded that cmES-derived cells resembled HUVEC rather than HAEC.

Although limited populations of cmES-derived cells were positive for cell surface VE-cadherin/PECAM-1, their uniform Ac-LDL-uptaking activities and uniform expressions of eNOS and vWF, taken together with their cord-forming activities and VEGF-R1 expression equivalent to HUVEC, suggest that the almost all of the cmES-derived cells are vascular endothelial cells or vascular endothelial endothelial-like cells and, therefore, "VE-cadherin/PECAM-1-negative" populations are also types of cells that are closely related to the vascular endothelial cells. This idea was analogous to the proposal by Thomson and colleagues (Kaufman et al., 2004). They showed that rmES-derived vascular endothelial cells were negative for VE-cadherin/PECAM-1 despite the presence of mature endothelial functions with eNOS and vWF expressions. They used commercially available EGM™-2 BulletKit for the differentiation of rmES cells. Eventually, usage of EGM™-2 BulletKit resulted in the production of almost pure VE-cadherin/PECAM-1-negative vascular endothelial cells by our method as the report by Thomson and colleagues. As shown in Figure 4, differentiation induction by our method using EGM™-2 BulletKit resulted in generation of cells with mature

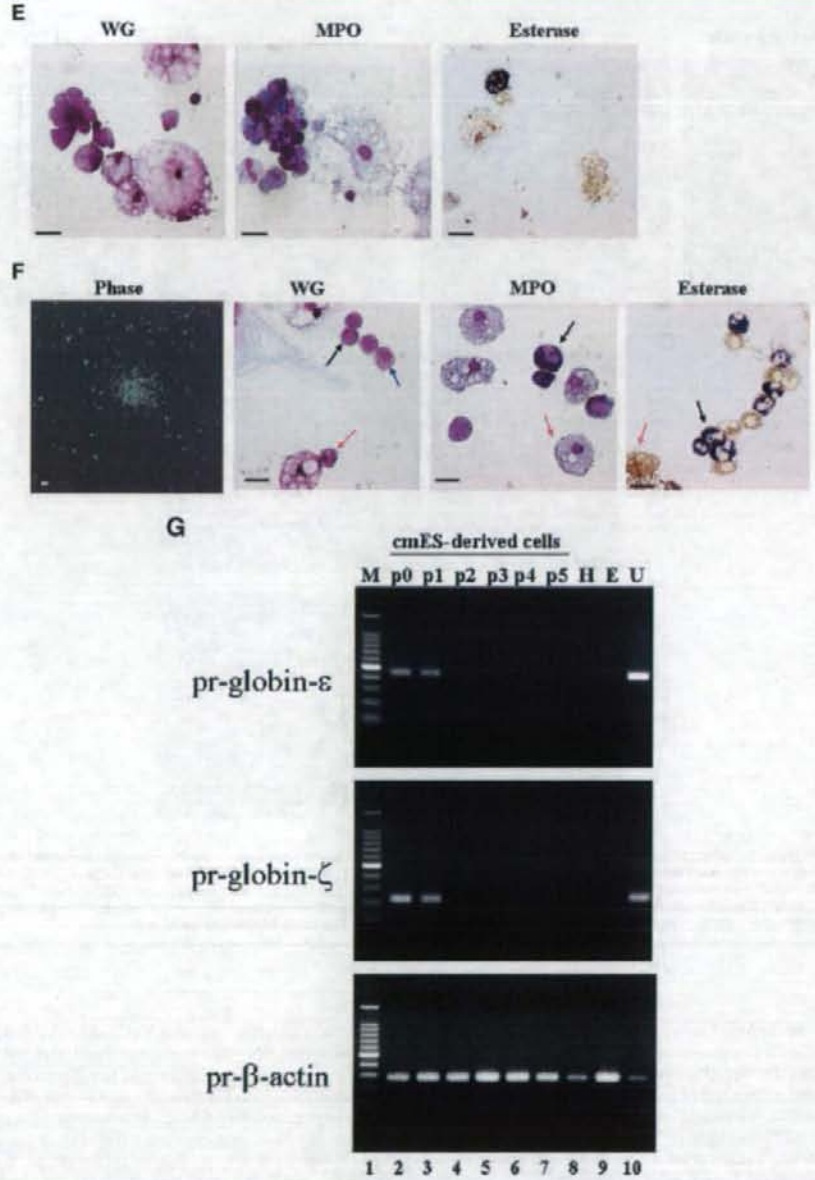

Fig. 1. (*Continued*)

functions including cord-forming activities (Fig. 4A, upper right in EGM2 part) and uniform Ac-LDL-uptaking activities (Fig. 4A, middle and lower right in EGM2 part) and with eNOS and vWF expressions (Fig. 4B, EGM2 part) despite the lack of VE-cadherin/PECAM-1 as assessed by flow cytometry (Fig. 4A, lower left in EGM2 part). In contrast to the findings by Thomson and colleagues (Kaufman et al., 2004), VE-cadherin expression was detected by Western blotting (Fig. 4C) in our culture system even by using EGM™-2 BulletKit, indicating that VE-cadherin protein was really produced though its membrane translocation seems to be blocked by unknown reason(s). Anyway, it can be concluded that there existed an "atypical"

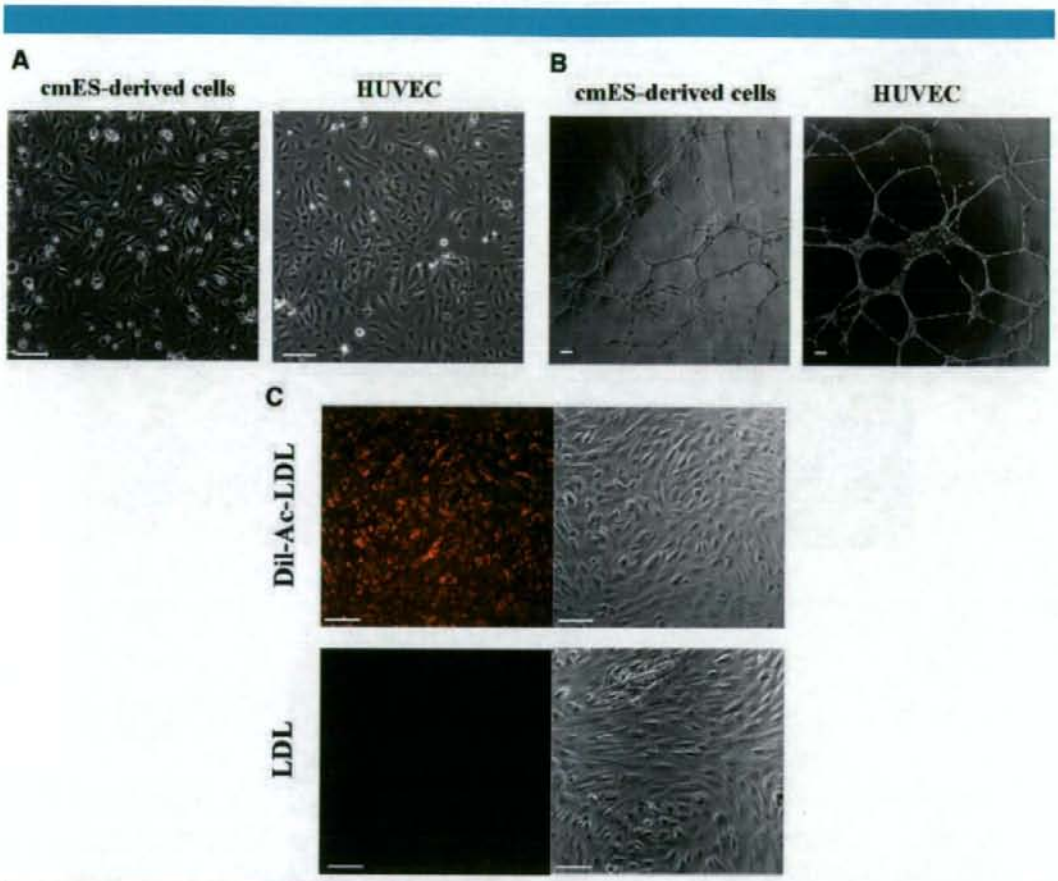

Fig. 2. Morphology and functions of cmES-derived subcultured cells. A: The phase contrast microscopy. Morphologies of cmES-derived cells (left) and HUVEC (right) were shown. The scale bar indicates 100 μm. B: Cord formation assays. A cord-forming activity of cmES-derived cells (left) and HUVEC (right) was shown. The scale bar indicates 100 μm. C: Ac-LDL-uptaking assays. Fluorescence (DiI)-labeled Ac-LDL (upper) or non-labeled LDL (lower) was added to cmES-derived cells. After overnight culture, cells were observed under fluorescence microscopy. Right parts indicate the photographs with Normarsky differentiated interference contrast. The scale bar indicates 20 μm.

type of vascular endothelial cells (i.e., cell surface VE-cadherin/PECAM-1-negative vascular endothelial cells) among primate ES-derived differentiated cells.

For further understanding of cell surface VE-cadherin-positive "canonical" vascular endothelial cells and cell surface VE-cadherin-negative "atypical" vascular endothelial cells, we fractionated those two populations by FACSAria. As shown in Figure 5C, cell surface VE-cadherin-positive populations proliferated as VE-cadherin-positive cells and expanded by 160-fold after five passages. Interestingly, cell surface VE-cadherin-positive populations were positive for cell surface CD34 expression as assessed by flow cytometry (Fig. 5D). The immunostaining study of cell surface VE-cadherin-positive cells clearly showed the localization of VE-cadherin at intercellular junctions in these cells (Fig. 5E). The functional maturation including cord-forming activities (Fig. 5F) and uniform Ac-LDL-uptaking activities (Fig. 5G) was also detected in cell surface VE-cadherin-positive populations. Expressions of other vascular endothelial markers were also detected including VEGF-R1, VEGF-R2, VEGF-R3, and Tie-2 (Fig. 5H). On the other hand, the cell surface VE-cadherin-negative populations proliferated as VE-cadherin-negative cells and were negative for cell surface CD34 expression (Fig. 6A–D). Interestingly, these cells showed obvious cord-forming capacities (Fig. 6E) and a uniform Ac-LDL-uptaking activity (Fig. 6F). Moreover, they expressed VEGF-R1, VEGF-R3, and Tie-2 despite the absence of VEGF-R2 as demonstrated by flow cytometric analyses (Fig. 6G). Immunostaining studies using an anti-VE-cadherin antibody demonstrated intracellular region-staining patterns (Fig. 6H), suggesting that the cell surface VE-cadherin-negative cells expressed VE-cadherin intracellularily. This finding was confirmed by Western blotting studies, which showed the presence of the 130-kDa VE-cadherin band (Fig. 6I). RT-PCR studies further demonstrated the presence of the VE-cadherin message (Fig. 6J). As monocytes/macrophages or other hematopoietic cells reportedly show various endothelial cell-like features and are positive for VEGF-R1 and VEGF-R3