厚生労働科学研究費補助金（ヒトゲノム・再生医療等研究事業）
分担研究報告書

ES細胞を利用する移植・再生治療の安全性に関する研究

分担研究者
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研究要旨

目的：ES細胞を利用する移植・再生治療の安全性の評価および向上.
方法：ヒトES細胞に近いサルES細胞を用いた同種移植実験を行う．移植細胞に対する免疫拒絶を避けるために，免疫能未成立のサル胎仔に細胞を移植する.
期待される成果：ES細胞を利用する移植・再生治療における腫瘍形成のリスクを明らかにする．また，腫瘍形成リスクを軽減して安全性を高める技術を開発し，この技術の有用性を明らかにする.
当該年度の成果：免疫能が正常な成体サルへ未分化ES細胞を移植すると，移植部位や移植細胞数にかかわらず，生着しないし腫瘍もできない．サル胎仔への移植が安全性の評価には必要である.核型異常のあるES細胞と核型異常のないES細胞が混じった細胞を移植した場合，出来た腫瘍細胞の核型は両者が混じっており，核型が正常だからといって腫瘍形成の危険性が下がる事実はなかった.

A. 研究目的

ES細胞を利用する移植・再生治療の腫瘍形成リスクをサル同種移植の系で評価する．さらに，リスクを軽減する技術を開発し，本治療法の安全性の向上をめざす.

ヒトES細胞を利用する治療の安全性や有効性は，もっぱら齧歯類で評価されているのが現状である．言うまでもなくヒトES細胞を用いる同種移植実験は行えないからである．齧歯類で得られた結果が必ずしもヒトに外挿できるわけではないので，ヒトES細胞を利用する治療の安全性や有効性の評価のためには，ヒトES細胞に近いサルES細胞を用いた同種移植の系が望まれる.

B. 研究方法

(1) **移植細胞**：カニクイザルES細胞を至適条件下（OP9 フィーダー細胞上，各種サイトカイン存在下）で6日間培養した．分化培養6日目の細胞はCD31, CD34, VE-cadherin, VEGFR-2の発現が高まるが，CD45の発現はまだ無い．従来の報告から（Wang L et al. Immunity 2004;21:31-41），この時期の細胞中に前造血細胞（将来，造血細胞になる細胞）が含まれると考えられた．この培養6日目の細胞を，造血再構築のための移植実験に用いた.

(2) **遺伝子標識**：移植後の細胞の運命を追跡できるように，GFP 遺伝子を恒常的に発現するサルES細胞を用いた（田辺製薬より供与）.

(3) **移植法**：移植細胞に対する免疫拒絶を避けるために，免疫能が未成立（妊娠1/3期前後）のカニクイザル胎仔をレシピエントとした．細胞は胎仔の肝臓内にエコーガイド下で移植した．この時期の胎仔は肝臓が造血器官だからである．

(4) **セレクション法**：安全性を高めるために，未分化細胞を除去してから移植した．具体的には，セルソーターを使って未分化マーカーである SSEA-4 が陽性の細胞を除去した．

(5) **評価**：移植後，満期帝王切開を行い，生まれたサル新生仔における腫瘍形成の有無，および移植細胞の生着・分化についてGFPを指標にして調べた．

(6) **倫理面への配慮**

組換えDNA実験：以下の通り承認が得られている．

・花園豊申請「幹細胞を利用する再生医療の基盤技術の開発」自治医科大学 平成16年 6月 1日承認（H16-51）

・花園豊申請「幹細胞治療法のサルを用いた有用性と安全性の評価」医薬基盤研究所 平成17年4月1日承認（DNA-070）

・長尾慶和申請「緑色蛍光タンパク質遺伝子（GFP）を組み込んだサルES細胞をin vitroで造血系へ初期分化させ，この細胞を妊娠ヒツジ子宮内の胎子の肝臓内へ外科的に移植する」宇都宮大学 平成17年7月27日承認

動物実験倫理：サルを用いる動物実験については，以下の通り承認を受けた．

・花園豊申請「サルを用いた幹細胞治療法の開発」自治医科大学 平成18年3月31日承認（No.150）

・花園豊申請「サルの幹細胞を用いた治療法の有効性と安全性の評価」医薬基盤研究所 平成18年7月21日承認（DS18-031号）

ヒツジを用いる実験は，実験実施機関から以下の通り承認を受けた．

・花園豊申請「ヒツジを利用するES細胞の分化技術の開発」自治医科大学 平成18年3月31日承認（No.172）

マウスを用いる実験は，実験実施機関から以下の通り承認を受けた．

・花園豊申請「ES細胞の増殖・分化の解析」自治医科大学 平成18年3月31日承認（No.175）

C. 研究結果

(1) **未分化ES細胞を移植した場合**：まず，サルES細胞を未分化のまま，サル胎仔の肝臓内に移植した（n＝3）．満期に相当する移植3ヶ月後に胎仔を取り出して調べてみると，案の定，奇形腫形成を認めた．これらの腫瘍はGFPの蛍光を発しており，移植したES細胞由来であることは明らかであった．腫瘍形成は，注射針軌跡上の胸腔または腹腔内のみに認められ，実質臓器内には認められなかった．

実質臓器を定量的PCRで調べると，全ての組織で約1%の移植したES細胞由来の細胞を認めた．in situ PCRでこれらの細胞は集落を作らず単独で存在し，周囲の細胞と同じ形態を示していた．

(2) **前造血細胞を移植した場合**：次に，前造血細胞（ES細胞分化培養6日目の細胞）をサル胎仔肝臓内に移植したところ，生まれたサル新生仔体内で，サルES細胞由来の造血細胞を確認した（n＝3）．コロニーアッセイで4-5%がES細胞由来であった．ところが，移植した全例で奇形腫の形成を認めた．腫瘍はGFPの蛍光を発しており，移植細胞由来であることは明らかだった．腫瘍形成は，やはり注射針軌跡上の胸腔または腹腔内のみに認められ，実質臓器内には認められなかった．

移植細胞（培養6日目の細胞）をFACS解析すると，未分化細胞（SSEA-4陽性細

胞）がまだかなり残存していること（約40%）が判明した．移植細胞中に残存した未分化細胞が奇形腫を形成したと考えられた．

(3) SSEA-4 陽性細胞を除去して移植した場合：そこで，分化培養した 6 日目の細胞から SSEA-4 陽性細胞を除去したものを，サル胎仔に移植する実験を行った(n = 7)．その結果，全例で，腫瘍形成は認められず，かつ移植細胞からの造血細胞への分化を確認した．この際，移植由来の造血キメラ率は低下していなかった（2-5%）．

(4) 免疫不全マウスやヒツジ胎仔に移植した場合：サル胎仔に移植したのと同じ分化培養 6 日目の細胞をヒツジ胎仔や免疫不全マウス（NOD/SCID）に移植した．いずれの場合も腫瘍形成の頻度はサル胎仔に移植した場合に比べてずっと少なかった（腫瘍形成率：免疫不全マウス 3/10，ヒツジ胎仔 1/10）．研究協力者：長尾慶和（宇都宮大学農学部），北野良博（国立成育医療センター外科），林聡（同産科）

(5)成体サルへ移植した場合：免疫能が正常な成体サルの脳内および皮下に未分化 ES 細胞を移植すると（n=2），移植部位や移植細胞数にかかわらず，拒絶されて生着しないし腫瘍もできない．サル胎仔への移植が腫瘍形成など安全性の評価に必要である．

(6) ES 細胞の品質と腫瘍形成：核型異常のある ES 細胞と核型異常のない ES 細胞が混じった細胞を移植した場合，出来た腫瘍細胞の核型は両者が混じっており，核型が正常だからといって腫瘍形成の危険性が下がる事実はなかった．（注：本実験は，同種移植の系ではレシピエントの正常細胞核型が混入しても判別不可能なため，ヒツジ胎仔への異種移植の系で実施した．）

D. 考察

ヒトとサルの遺伝的近縁性から言って，サル ES 細胞をサルへ移植する実験は，ヒト ES 細胞をヒトに移植する治療の，もっとも忠実な疑似モデルといえる．しかし，このような同種移植では，移植後の免疫反応のことを考えないといけない．サル ES 細胞をサルに接種しても生着することなく迅速に拒絶された．腫瘍さえも作らなかった．したがって，今のところサル同種移植の実験系は限られてくる．一つは，免疫学的に比較的隔離されている脳内移植実験である．この場合，適当な免疫抑制剤を使用すれば移植後の生着が可能であることが別の研究で示されている．もう一つは我々が進めている子宮内移植，つまり動物胎仔への移植である．胎仔への移植には３つの大きなメリットがある．まず，胎仔は，とくに第１トリメスターの胎仔は，免疫学的に未成熟であり，たとえ異種の細胞を移植しても拒絶されることなく生着し，免疫抑制がいらないこと．第２は，胎仔は日に日に大きくなるため，生着のためのスペースが自然に生まれ，移植前処置がいらないこと．そして第３は，子宮内はそもそも無菌環境なので，移植前後の無菌管理がいらないこと．

未分化のままのサル ES 細胞を成体サルに移植してもそもそも生着しないし腫瘍も作らなかったが，サル胎仔に移植すれば予想通り奇形腫を作った．興味深いのは，腫瘍形成は注射針軌跡上の腹腔および胸腔に限られ，実質臓器に腫瘍はできなかったことである．すなわち，漏れた細胞が腫瘍を作る可能性が高い．漏れずに移植する技術が腫瘍形成予防のために実は重要である．さらに興味深いのは，各組織に ES 由来細胞の“生着”が見られたことである．実質臓器には腫瘍は出来

ないが，どの組織にも約1%のES細胞由来の細胞を認めた．しかも，周囲の細胞と同じ形態を示していたことから，移植したES細胞は，生着の場に応じて分化したか，または既存の周囲細胞と融合した結果，周囲と同じ表現型を得たものと考えられる．胎仔がレシピエントの場合，未分化ES細胞は本来，各組織への生着能をもつが，それが漏れた場合は奇形腫を作るらしい．

未分化のままでなくES細胞を分化培養後に移植したらどうなるか？　そこでサルES細胞を前造血細胞に分化させてからサル胎仔に移植した．生後，期待通り造血系を一部再構築できたものの(2-5%)，全例で奇形腫が見られた．分化培養後の細胞を移植したのに腫瘍を形成したわけで，腫瘍形成リスクは高いと言わざるを得ない．ところが，同じ分化培養後の細胞を免疫不全マウスやヒツジ胎仔に移植した場合，腫瘍形成は稀であった．とすれば，マウスやヒツジを使った異種移植実験では腫瘍形成の危険性を過少評価してしまう．ES細胞移植治療の安全性は，サルの同種移植実験を行なわなければ正しく評価できないことになる．

さて，移植後の腫瘍形成を予防できないか？　ES細胞は，長期培養によって核型異常など「品質」の劣化が報告されている．これが腫瘍形成を促すのではないかという考えがある．しかし，我々の実験結果からは，核型が正常だからといって腫瘍形成の危険性が下がる事実はなかった．一方，霊長類ES細胞の未分化表面マーカーであるSSEA-4の陽性細胞を除去してから移植すると，移植後の造血再構築を損なうことなく，腫瘍形成は全く認められなかった．SSEA-4は臨床的なステムネス・マーカーといってよい．(マウスES細胞ではSSEA-1が陽性だが，霊長類ES細胞ではSSEA-4が陽性である．)SSEA-4陽性細胞を除去するネガティブ・セレクション法は，ES細胞を用いる移植・再生医療の安全性の向上のために普遍的な応用が期待できる．

以上のように，サルES細胞の同種移植実験は，マウスをレシピエントとして用いた従来の移植実験では得られない知見を生む．そしてそれは，ES細胞治療の有効性（ベネフィット）と安全性（リスク）を評価するにあたって有用性がきわめて高く，我が国でもこの種の実験を今後も継続していく必要があると考える．

最近，体細胞や生殖母細胞から多分化能をもったES様細胞の樹立が報告されている．これらの中には免疫不全マウスで腫瘍を形成しないものもあって，ES細胞の代替として期待されている．ほんとうに腫瘍形成リスクがないのかどうかは，我々のサル同種移植の系で確認する必要がある．

E. 結論

(1) 未分化のサルES細胞をサル胎仔肝臓に移植すると，各組織にES由来細胞の“生着”が見られ，腫瘍形成は実質臓器外の注射針軌跡上に限られた．漏れた細胞が腫瘍を作る可能性が高い．

(2) サルES細胞を前造血細胞に分化させてから移植すると，造血系を一部再構築できたが，全例腫瘍を作った．分化培養後であっても腫瘍形成リスクは高い．

(3) しかし，免疫不全マウスやヒツジ胎仔へ同じ細胞を移植しても腫瘍形成は稀である．サル同種移植の系が安全性の厳密な評価に適している．

(4) SSEA-4陽性細胞を除去すると，生着能を妨げずに，腫瘍形成は完全に予防できた．SSEA-4は臨床的なステムネス・マーカーと言える．

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

なし.

厚生労働科学研究費補助金（ヒトゲノム・再生医療等研究事業）
分担研究報告書

霊長類ES細胞の品質管理と同種移植の安全性確保に関する研究

－霊長類ES細胞の移植で生じる病理変化と指標蛋白の発現－

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研究要旨：霊長類 ES 細胞を SCID マウスに移植すると三胚葉の構成成分を持つ奇形腫が形成された。各構成成分は免疫組織化学的に、形態的特徴に合致する適切な蛋白を標榜していた。移植後２ヶ月と３ヶ月の奇形腫は概ね類似した構造を示し、３ヶ月の奇形腫には外胚葉組織の毛包組織、中胚葉の横紋筋細胞、内胚葉の杯細胞様細胞およびクロム親和性細胞様細胞など、より分化した組織が多く観察された。免疫二重染色によって、内胚葉および外胚葉組織の一部で AFP とケラチンまたは Nestin と GFAP といった、それぞれの胚葉の低分化マーカーと高分化マーカーの共存が確認された。霊長類 ES 細胞が様々な組織に分化する可能性が示唆された一方、ES 細胞に由来する組織は形態的に分化していても、低分化な状態が潜伏している可能性があり、安全性確保の上では重要な事象と考えられた。

Ａ．研究目的

ES 細胞による再生医療を成功させるためには、増殖能を保持したまま、細胞が三胚葉すべての組織へ分化する能力を持ち、かつ適切に分化した細胞が適切な場所に定着する必要がある。本研究で用いられているカニクイザル由来 ES 細胞が移植された宿主で、いかなる増殖または分化態度を示すか、形成された奇形腫の性質を病理組織学的に検索した。

Ｂ．研究方法

分化誘導されていないカニクイザル由来 ES 細胞 CMK6 を SCID マウスの皮下に接種し、接種２ヶ月後の腫瘤(n=12)、３ヶ月後の腫瘤（n=10）を得た。摘出組織をホルマリン固定、パラフィン包埋し、組織学的ならびに免疫組織化学的に検索した。組織学的検索では HE 染色を施した。免疫組織化学的検索では外胚葉に対して Glial Fibrillary Acidic Protein (GFAP)、Nestin、Neurofilament (NF)、Neuron Specific Enolase (NSE)、S-100、Anti-Synaptophysin (Syn)、中胚葉を Desmin、Sarcomeric Actin (SA)、Smooth Muscle Actin (SMA)、Vimentin (Vim)、Von Willebrand Factor (Fac.8)、内胚葉を・1-Fetoprotein (AFP)、内胚葉と外胚葉の共通のマーカーとして Chromoglanin A (CA) 、Cytokeratin (CK)を用いた。さらに免疫二重染色として、外胚葉の低分化マーカーの Nestin と高分化マーカーの GFAP または NSE、中胚葉の低分化マーカーの Vim と高分化マーカーの SMA または Fac.8、

内胚葉の低分化マーカーの AFP と高分化マーカーのCKを一次抗体とする蛍光染色を行った。

（倫理面への配慮）

当研究では、この研究班に属する各研究員が、それぞれの研究施設の動物実験委員で承認された動物実験に則って、ES 細胞の性質を確認するために作製した奇形腫を用いたものである。従って、倫理面に関して何ら問題はない。

C．研究結果及び考察

1．組織学的検索

外胚葉由来の神経系組織として、神経管様構造は2ヶ月の腫瘍で12例中9例に、3ヶ月の腫瘍で10例すべてに、神経網様構造は2ヶ月の動物の12例中11例に、3ヶ月では10例すべてに、グリア様細胞は2ヶ月で12例中6例、3ヶ月で10例すべてに確認された。表皮系組織として、メラニン顆粒を有する細胞が2ヶ月で12例中2例、3ヶ月で10例中4例、扁平上皮様組織が2ヶ月で12例中10例、3ヶ月で10例中8例に、毛包は2ヶ月の動物には認められず3ヶ月の10例中3例に確認された。中胚葉組織の線維組織は2ヶ月、3ヶ月ともにすべての動物で、脂肪組織は2ヶ月で12例中4例、3ヶ月で10例中5例、脈管組織は2ヶ月、3ヶ月ともにすべての動物で、平滑筋組織も2ヶ月、3ヶ月ともにすべての動物で、軟骨組織は2ヶ月で12例中2例、3ヶ月で5例、骨組織は2ヶ月で12例中9例、3ヶ月で10例中4例、横紋筋組織は2ヶ月で12例中1例、3ヶ月で10例中4例に認められた。内胚葉組織の未熟な腺腔が2ヶ月、3ヶ月ともにすべての動物で、杯細胞様細胞を持つ腺腔が2ヶ月で12例中2例、3ヶ月で10例中5例に、大型細胞の集塊が2ヶ月で12例中2例、3ヶ月で10例中3例に認められた。

2．免疫組織化学的検索

外胚葉の神経管様構造は Nestin、一部 S100 に陽性だった。この周囲の神経網様構造は NSE、Syn、一部で CA に陽性だった。神経網構造の間には NF 陽性の軸索様突起構造が見られた。これらの構造の間に見られた突起を持つ星型細胞は主に GFAP、一部で Nestin に陽性だった。

中胚葉の線維構造は Vim に、一部が SMA および Desmin に陽性だった。脂肪構造および軟骨構造は Vim と S100、一部が NSE に陽性だった。骨構造は Vim に陽性、脈管構造は Fac.8 陽性、一部で Vim 陽性だった。平滑筋構造は SMA 陽性で、一部 Vim、Desmin、わずかに SA が陽性だった。横紋筋構造は SA と Desmin が陽性、一部 SMA が陽性だった。

内胚葉の未熟な腺構造は AFP 陽性、一部で CK 陽性だった。杯細胞様細胞を持つ腺構造は CK 陽性、一部で AFP 陽性だった。これらの細胞の間に CA、一部で syn 陽性のクロム親和性細胞様細胞が認められた。大型円形細胞の集塊は AFP、CK ともに陽性だった。

3．免疫二重蛍光染色

外胚葉組織については Nestin と GFAP の二重染色で、一部の星形細胞が共陽性だった。NSE との共陽性像は見られなかった。

中胚葉組織については Vim と SMA の共陽性像は頻繁に観察された。Fac.8 との共陽性像はやや丈の高い扁平細胞に裏打ちされる一部の脈管様構造に見られた。

内胚葉組織については約半数の未熟な腺構造と少数の分泌物を持つ腺構造

にAFPとCKの共陽性像が認められた。

D．考察

以上の結果から、本研究で用いているカニクイザル由来ES細胞移植には三胚葉の、さらにそれぞれ胚葉のあらゆる組織に分化することが明らかとなった。これらの構造はそれぞれに相応した分化マーカー蛋白も発現しており、形状とともに細胞の分化の方向が適切であることを意味していた。一方でそれぞれの胚葉において、高分化と低分化を示すマーカー蛋白の共存が確認された。Nestinは神経幹細胞、AFPは胎性肝細胞のマーカーで、一般的に健常組織では見られない蛋白である。外および内胚葉組織については、ES細胞に由来する組織や細胞は形態的に高分化となっていても、低分化な状態の細胞が潜伏している可能性があり、安全性確保の上では重要な事象と考えられた。中胚葉のVimとSMAおよびFac.8の共存は一般的に肉芽組織形成などで見られる現象である。

E．結論

本研究で用いているカニクイザル由来ES細胞移植の多分化能は確実に保証された。ES細胞から分化した組織中には、高分化と低分化のマーカーが共存する可能性があるので、安全性確保の上で注意する必要がある。

F．健康危機情報

G．研究発表

1．論文発表

なし。

2．学会発表

なし。

H．知的財産権の出願・所得状況

1．特許取得

なし。

2．実用新案登録

なし。

3．その他

なし。

研究成果の刊行に関する一覧表

雑誌
（花園　豊）

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Shibata H, Ageyama N, Tanaka Y, Kishi Y, Sasaki K, Nakamura S, Muramatsu S, Hayashi S, Kitano Y, Terao K, Hanazono Y	Improved safety of hematopoietic transplantation with monkey ES cells in the allogeneic setting	Stem Cells	24巻6号	pp.1450-1457	2006年
Ageyama N, Hanazono Y, Shibata H, Ono F, Nagashima T, Ueda Y, Yoshikawa Y, Hasegawa M, Ozawa K, Terao K.	Prevention of immune responses to human erythropoietin in cynomolgus monkeys (*Macaca fascicularis*)	J Vet Med Sci	68巻５号	pp.507-510	2006年
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Improved Safety of Hematopoietic Transplantation with Monkey Embryonic Stem Cells in the Allogeneic Setting

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Key Words. Cynomolgus monkey • Hematopoiesis • Embryonic stem cell • In utero transplantation • Teratoma • Purging Tumor prevention

ABSTRACT

Cynomolgus monkey embryonic stem cell (cyESC)-derived in vivo hematopoiesis was examined in an allogeneic transplantation model. cyESCs were induced to differentiate into the putative hematopoietic precursors in vitro, and the cells were transplanted into the fetal cynomolgus liver at approximately the end of the first trimester (n = 3). Although cyESC-derived hematopoietic colony-forming cells were detected in the newborns (4.1%–4.7%), a teratoma developed in all newborns. The risk of tumor formation was high in this allogeneic transplantation model, given that tumors were hardly observed in immunodeficient mice or fetal sheep that had been xeno-transplanted with the same cyESC derivatives. It turned out that the cyESC-derived donor cells included a residual undifferentiated fraction positive for stage-specific embryonic antigen (SSEA)-4 (38.2% ± 10.3%) despite the rigorous differentiation culture. When an SSEA-4-negative fraction was transplanted (n = 6), the teratoma was no longer observed, whereas the cyESC-derived hematopoietic engraftment was unperturbed (2.3%–5.0%). SSEA-4 is therefore a clinically relevant pluripotency marker of primate embryonic stem cells (ESCs). Purging pluripotent cells with this surface marker would be a promising method of producing clinical progenitor cell preparations using human ESCs. STEM CELLS *2006;24:1450–1457*

INTRODUCTION

Human embryonic stem cells (hESCs) hold great potential in the treatment of a variety of diseases and injuries because embryonic stem cells (ESCs) have the ability to proliferate indefinitely in culture and to differentiate into any cell type [1, 2]. Because ESCs are able to form teratomas when transplanted into immunodeficient mice, safety concerns would be raised against the clinical application of hESCs [3, 4]. It will be necessary to test the safety of these cells in animal transplantation models before clinical application. Nonhuman primate transplantation models would be desirable for this purpose; however, there have been only a few reports on these models [5–7]: The successful engraftment of transplanted cells in primates will not be achieved unless the immune rejection of transplanted cells is circumvented (e.g., through immunosuppressive treatment) [6]. The early gestational fetus may be a good recipient with which to circumvent immune rejection because the immune system is premature [8]. In addition, in the animal fetus, transplanted cells would engraft without conditioning of recipients such as irradiation or immunosuppressive treatment [9–12]. We have previously established a system for allogeneic transplantation of cynomolgus ESCs (cyESCs) using preimmune fetal monkeys as recipients [5].

We have also reported a novel method for hematopoietic engraftment from cyESCs in sheep [13]. The method is a combination of three steps: (a) differentiation in vitro to generate the putative hematopoietic precursors [14]; (b) transplantation of the cells in utero [15]; and (c) development into hematopoietic cells in vivo using the hematopoietic microenvironment of the fetal liver [16]. In the present study,

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we have examined the safety as well as the efficacy of hematopoietic engraftment of cells derived from cyESCs in the allogeneic transplantation model.

Materials and Methods

Animals

Pregnant cynomolgus monkeys (16–22 years old) were obtained by mating and were reared at the Tsukuba Primate Research Center in accordance with Rules for Animals Care and Management set forth by the Research Center and Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan. Experimental procedures were approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases. The animals were free of intestinal parasites and were seronegative for herpes virus B, varicella-zoster-like virus, measles virus, and simian immunodeficiency virus.

Cell Preparation

A cyESC line (CMK6G) stably expressing green fluorescent protein (GFP) was established after transfection of the parental cyESC line (CMK6) with the enhanced GFP gene (Clontech, Palo Alto, CA, http://www.clontech.com) [17]. cyESCs were maintained on a feeder layer of mitomycin C (Kyowa, Tokyo, http://www.kyowa.co.jp)-treated mouse (ICR or BALB/c; Clea Japan, Tokyo, http://www.clea-japan.com) embryonic fibroblasts as previously described [18]. The mouse bone marrow stromal cell line OP9 was maintained in α-minimum essential medium (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) supplemented with 20% fetal calf serum (FCS; Invitrogen) [19].

cyESCs were induced to differentiate into the putative hematopoietic precursors as previously described [13]. Briefly, undifferentiated cyESCs were transferred onto mitomycin C-treated confluent OP9 cells and cultured for 6 days in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 8% FCS, 8% horse serum (Invitrogen), 5×10^{-6} M hydrocortisone (Sigma, St. Louis, http://www.sigmaaldrich.com), and multiple cytokines, including 20 ng/ml recombinant human (rh) bone morphogenetic protein-4 (R&D Systems, Minneapolis, http://www.rndsystems.com), 20 ng/ml rh stem cell factor (Biosource, Camarillo, CA, http://www.biosource.com), 20 ng/ml rh vascular endothelial growth factor (VEGF; R&D Systems), 20 ng/ml rh Flt-3 ligand (PeproTech, Rocky Hill, NJ, http://www.peprotech.com), 20 ng/ml rh interleukin-3 (PeproTech), 10 ng/ml rh interleukin-6 (PeproTech), 20 ng/ml rh granulocyte colony-stimulating factor (PeproTech), and 2 IU/ml rh erythropoietin (Roche, Basel, Switzerland, http://www.roche.com). The cells were resuspended in 0.1% human serum albumin (Sigma)/Hanks' balanced saline solution (Sigma) for transplantation.

Flow Cytometry

Primary antibodies (Abs) used in the present study were anti-human CD34 monoclonal Ab (mAb; BD Pharmingen, San Diego, http://www.bdbiosciences.com/pharmingen), anti-human CD31 mAb (Pharmingen), anti-human CD45 mAb (Pharmingen), anti-human vascular endothelial (VE) cadherin mAb (Pharmingen), rabbit anti-human VEGF receptor (VEGFR)-2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com), and anti-stage-specific embryonic antigen (SSEA)-4 mAb (Chemicon, Temecula, CA, http://www.chemicon.com). All of them cross-reacted to cynomolgus counterparts as previously demonstrated [18, 20–22]. Secondary Abs were phycoerythrin (PE)-conjugated rabbit anti-mouse immunoglobulins (Ig) Ab (DakoCytomation, Glostrup, Denmark, http://www.dako.com) and Alexa Fluor 647-conjugated goat anti-mouse IgG Ab (Molecular Probes, Eugene, OR, http://probes.invitrogen.com). Cells stained with unlabeled primary Abs were incubated with fluorescence-labeled secondary Abs. Cells were incubated with either primary or secondary Ab for 20–60 minutes at 4°C. Regarding staining with the anti-VEGFR-2 Ab, the cells were incubated with biotin-conjugated goat anti-rabbit IgG Ab (Beckman Coulter, Miami, http://www.beckmancoulter.com), followed by PE-conjugated streptavidin (Beckman Coulter). Fluorescence-labeled cells were analyzed with a FACS Calibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). Data analysis was performed using the CellQuest software (Becton, Dickinson and Company). Isotype-matched, irrelevant mAbs (DakoCytomation or Beckman Coulter) served as negative controls. Nonviable cells were excluded from analysis by propidium iodide (Sigma) costaining.

Cell Sorting

Cell sorting was performed to purge SSEA-4^+ cells from among the cultured cyESCs in vitro. Cells were incubated with the anti-SSEA-4 mAb for 1 hour at 4°C and washed twice with Dulbecco's modified Eagle's medium supplemented with 10% FCS. The cells were then incubated with the PE-conjugated anti-mouse Ig Ab for 1 hour at 4°C and washed twice again. GFP-positive and SSEA-4-negative cells were sorted using an Epics Elite cell sorter (Beckman Coulter). Data acquisition was performed using the Expo2 software (Beckman Coulter).

Transplantation and Delivery

Transplant procedures were previously described [5]. Briefly, animals were anesthetized via an intramuscular administration of ketamine hydrochloride (Ketalar, 10 mg/kg; Sankyo, Tokyo, http://www.sankyo.co.jp) and received 0.5%–1.0% isoflurane by inhalation by means of an endotracheal tube. Cells ($0.16–46 \times 10^6$ cells per fetus; Table 1) were injected into the fetal liver through a 23-gauge needle using an ultrasound-guided technique at approximately the end of the first trimester. The fetuses were delivered by cesarean section at 2–3 months after transplant (gestation 120–157 days, full term 165 days).

Colony Polymerase Chain Reaction

Cynomolgus clonogenic hematopoietic colonies were produced as previously described [20]. After cells were cultured in methylcellulose medium for 10–14 days, well-separated individual colonies were plucked into 50 μl of distilled water and digested with 20 μg/ml proteinase K (Takara, Shiga, Japan, http://www.takara-bio.com) at 55°C for 1 hour, followed by 99°C for 10 minutes. Each sample (5 μl) was used for a nested polymerase chain reaction (PCR) to detect the *GFP* gene sequence. The outer primer set was 5′-AAGGACGACGGCAACTACAA-3′ and 5′-ACTGGGTGCTCAGGTAGTGG-3′, and the inner primer set was 5′-GCATCGACTTCAAGGAGGAC-3′ and 5′-GTTGTGGCGGATCTTGAAGT-3′. Amplification conditions for both the outer and inner PCR were 30 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. The

Table 1. ESC-derived hematopoiesis and tumor formation

Animals	Animal no.	Transplanted cells	Purging SSEA-4$^+$ cells	Cell number per fetus ($\times 10^6$)	Donor-derived CFU in recipients[a] at birth (donor/total colony number)	Tumor formation	Observation period (months)
Monkeys	0031	Undifferentiated	−	3.90	n.d.	+	3
	2311	ESCs	−	0.16	n.d., Dead	+	2
	0321		−	0.21	n.d., Dead	+	2
	0841	Day-6 ESC-	−	10	4.1% (2/49)	+	3
	1551	derived cells	−	46	n.d., Dead	+	2.5
	0021		−	46	4.7% (4/85)	+	3
	0691	Day-6 ESC-	+	0.16	3.2% (2/62)	−	3
	0381	derived cells	+	1.40	5.0% (4/80)	−	3
	0022		+	0.17	2.3% (2/86)	−	3
	0981		+	0.31	4.1% (3/73)	−	3
	0051		+	0.31	n.d., Dead[b]	−	3
	1552		+	0.75	4.4% (2/45)	−	4
Sheep[c]	57	Day-6 ESC-	−	50	1.1% (1/91)	−	18
	55	derived cells	−	50	1.1% (1/91)	−	26
	141		−	78	1.1% (1/91)	−	26
	182		−	14	1.6% (1/63)	−	21

[a]Percentage of donor-derived CFU was calculated by dividing the number of CFU positive for the green fluorescent protein gene sequence by the number of CFU positive for the β-actin gene sequence. Donor-derived CFU were analyzed at delivery.
[b]Death due to ablation of placentae. Other deaths were presumably tumor-related.
[c]As published by Sasaki et al. [13].
Abbreviations: CFU, colony-forming units; ESC, embryonic stem cell; n.d., not done; SSEA, stage-specific embryonic antigen.

outer PCR products were purified using a QIA quick PCR purification kit (Qiagen, Valencia, CA, http://www.qiagen.com). Simultaneous PCR for the β-actin sequence was also performed to ensure DNA amplification of the sample in each colony. The primer set for β-actin was 5′-CATTGTCATGGACTCTGGCGACGG-3′ and 5′-CATCTCCTGCTCGAAGTCTAGGGC-3′. Amplification conditions for β-actin PCR were 40 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. Amplified GFP (131 bp) and β-actin (234 bp) products were resolved on 2% agarose gel (Sigma) and visualized by ethidium bromide (Invitrogen) staining.

RNA PCR

Total RNA was extracted from cells of interest using the EZ1 RNA universal tissue kit (Qiagen). RNA was reverse-transcribed at 50°C for 30 minutes using the RNA LA PCR kit (Takara) with oligo dT primer. The resulting cDNA was then subjected to PCR. Regarding PCR for Oct-4, the primer set was 5′-GGACACCTGGCTTCGGATT-3′ and 5′-TTCGCTTTCTCTTTCGGGC-3′. The PCR conditions were 35 cycles of 95°C for 30 seconds, 67°C for 45 seconds, and 68°C for 1.5 minutes. Regarding PCR for Scl, the primer set was 5′-GGGCGGAAAGCTGTTTGCGATT-3′ and 5′-TCGCTGAGAGGCCTGCAGTT-3′. The PCR conditions were 35 cycles of 95°C for 30 seconds, 63°C for 1 minute, and 72°C for 1 minute. A simultaneous PCR for β-actin was also conducted on each cDNA sample as an internal control as described above. Amplified Oct-4 (697 bp), Scl (201 bp), and β-actin (234 bp) products were resolved on 2% agarose gel and visualized by ethidium bromide staining.

RESULTS

In Utero Transplantation and Delivery

cyESCs stably expressing GFP were used in this study [17]. In the setting of allogeneic transplantation, GFP was used as a genetic tag to track transplanted cell progeny. We employed the OP9 stromal cell coculture method instead of the embryoid body formation method to facilitate the hematopoietic differentiation [19, 23, 24] (Fig. 1A, 1B). According to the flow cytometric analysis, CD34, CD31 (platelet/endothelial cell adhesion molecule-1 [PECAM-1]), CD144 (VE-cadherin), and VEGFR-2 (Flk-1) were all upregulated on day 6 but decreased thereafter (Fig. 1C–1E, 1G). Among the markers examined, CD34 is a widely used surface marker of hematopoietic stem cells in both human and monkey subjects [25–27]. The others are key markers of hemangioblasts (which generate endothelial and hematopoietic lineages) in both mice and humans [14, 28]. Cells positive for both VEGFR-2 and VE-cadherin emerged on day 6 (Fig. 1H). CD45, however, was not detected until day 12 (Fig. 1F). Despite the hemangioblast marker expression on day 6, the hematopoietic *Scl* gene was upregulated at this time point as assessed by RNA PCR (Fig. 1I), implying that the hematopoietic commitment might have already occurred on day 6 [29, 30]. We therefore designated the day 6 cyESC-derived progenitor cells as putative hematopoietic precursors. The time course profiles presented here were similar to those of hESCs [14, 24]. The GFP expression was stable during the 6-day culture (Fig. 1A, 1B) and afterward (data not shown).

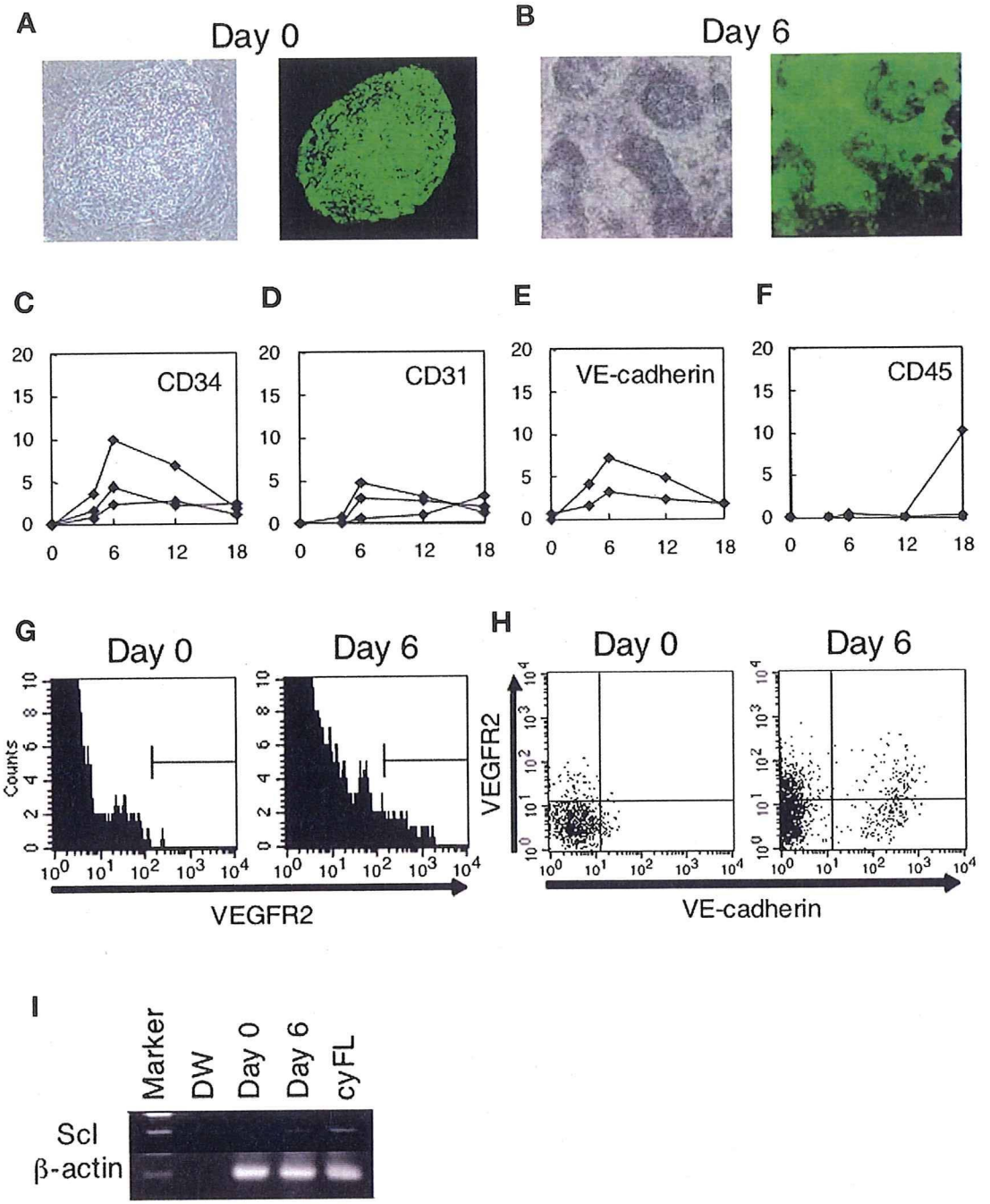

Figure 1. Flow cytometric analysis during the in vitro differentiation of cyESCs. Undifferentiated cyESCs expressing green fluorescent protein were cultured on OP9 cells with multiple cytokines (see Materials and Methods). **(A):** Cells on day 0 are shown in bright (left) and dark (right) fields. **(B):** Cells on day 6 are shown in bright (left) and dark (right) fields. **(C):** Cells on days 0, 4, 6, 12, and 18 were stained for CD34. **(D):** Cells on days 0, 4, 6, 12, and 18 were stained for CD31. **(E):** Cells on days 0, 4, 6, 12, and 18 were stained for VE-cadherin. **(F):** Cells on days 0, 4, 6, 12, and 18 were stained for CD45. The vertical axis shows the fraction (percentage) of cells that were stained positive. **(C–F):** Results of two or three independent experiments are shown. **(G):** Although cells on day 0 already express low levels of VEGFR-2, a VEGFR-2^{high} population did not emerge until day 6. **(H):** Dot-plot profiles for VEGFR-2 and VE-cadherin expression indicate that cells positive for both VEGFR-2 and VE-cadherin emerged until day 6. **(G, H):** Representative results from three independent experiments are shown. **(I):** The *Scl* gene expression was upregulated on day 6 to a level similar to that in the cynomolgus fetal liver as assessed by RNA polymerase chain reaction. Day-6 cells (putative hematopoietic precursors) were used for transplantation. Abbreviations: cyESC, cynomolgus embryonic stem cell; cyFL, cynomolgus fetal liver; DW, distilled water; VE, vascular endothelial; VEGFR, vascular endothelial growth factor receptor.

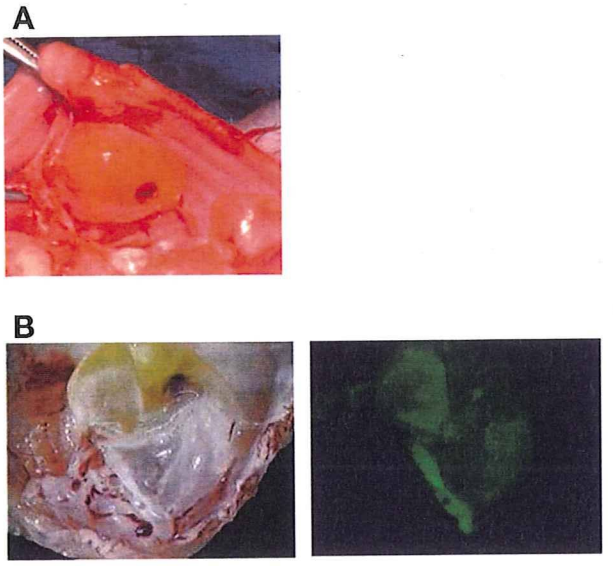

Figure 2. Tumor formation after the transplantation of cynomolgus embryonic stem cell (cyESC)-derived progenitor cells. Tumors formed in all three monkey fetuses transplanted with the day-6 cyESC-derived progenitor cells (putative hematopoietic precursors). **(A):** A representative tumor in the thoracic cavity at 3 months after transplantation (monkey no. 0841). **(B):** The tumor was observed in bright (left) and dark (right) fields under a fluorescence microscope.

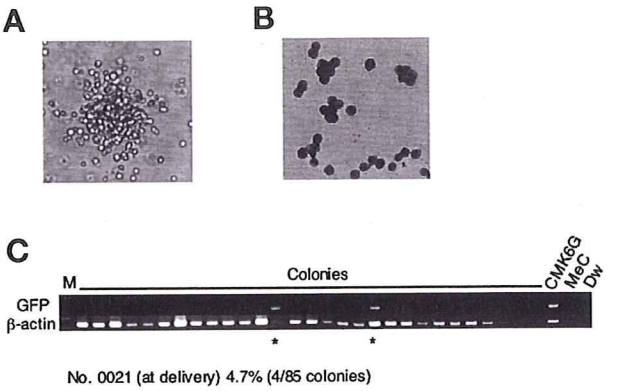

Figure 3. cyESC-derived hematopoiesis in vivo. **(A):** Bone marrow, cord blood, and liver cells were harvested from newborn monkeys and placed in methylcellulose medium to produce clonogenic hematopoietic colonies. **(B):** A cytospin specimen (stained with the May-Giemsa method) of plucked colonies reveals mature neutrophils. To identify cyESC-derived colonies, well-separated individual colonies were plucked and examined for the GFP sequence by PCR. Plucked MeC alone (not containing colonies) served as a negative control. PCR of the β-actin sequence in the same colonies was simultaneously performed as an internal control. Colony PCR was repeated at least twice. **(C):** Representative colony PCR results for monkey no. 0021. Asterisk indicates bands positive for the GFP sequence. Abbreviations: CMK6G, positive control green fluorescent protein-expressing cynomolgus cells; cyESC, cynomolgus embryonic stem cell; DW, distilled water; GFP, green fluorescent protein; M, molecular weight marker; MeC, methylcellulose; PCR, polymerase chain reaction.

Teratoma Formation

The undifferentiated cyESCs ($n = 3$) or cyESC-derived putative hematopoietic precursors ($n = 3$) were transplanted in utero into allogeneic fetuses in the liver under ultrasound guidance at approximately the end of the first trimester (49–66 days, full term 165 days) (Table 1). Regardless of whether the undifferentiated cyESCs or putative hematopoietic precursors were transplanted, tumors were found in the thoracic or abdominal cavities in all the six animals at 2–3 months after transplant (Table 1; Fig. 2A). The tumors fluoresced (Fig. 2B) and consisted of three germ layer cells. Thus, they were teratomas derived from transplanted cells. However, tumors were hardly observed in fetal sheep (1/10; [13] and our unpublished data) (Table 1) and immunodeficient (nonobese diabetic/severe combined immunodeficient) mice (3/10; our unpublished data) after the same putative hematopoietic precursors were transplanted.

In Vivo cyESC-Derived Hematopoiesis

Regarding the newborn monkeys that had been transplanted with the putative hematopoietic precursors, we harvested cells from the femur, cord blood, and liver and plated the cells in methylcellulose medium to produce clonogenic hematopoietic colonies (colony-forming units [CFU]) (Fig. 3A). The monkey cells generated colonies of clear hematopoietic morphology in this assay (Fig. 3B). To detect transplanted cell-derived, GFP-positive colonies, we tried to observe GFP fluorescence of colonies under a fluorescent microscope but were hampered by the high autofluorescence. We then conducted PCR for the *GFP* gene sequence in DNA isolated from each colony (colony PCR) (Fig. 3C). The transplanted cell-derived CFU were clearly detected in the animals (4.1% and 4.7%; Table 1). We repeated the colony PCR and confirmed that the results were reproducible. We detected both granulocytic and erythroid cynomolgus CFU. In the peripheral blood, however, we were not able to detect cells expressing GFP by flow cytometry. It turned out that, as assessed by quantitative PCR, the fractions of GFP-positive cells in the peripheral blood were very small (<0.1%). Low peripheral "chimerism" has been reported more than once in other in utero transplantations of ESCs or hematopoietic stem cells such as in mice, sheep, and pigs [13, 31–33].

Purging SSEA-4$^+$ Cells of the Putative Hematopoietic Precursors

We examined the expression of an undifferentiated primate ESC marker, SSEA-4, in the undifferentiated cyESCs (day 0) and putative hematopoietic precursors (day 6). The proportion of SSEA-4$^+$ cells was 93.4% ± 8.1% and 38.2% ± 10.3% among the day-0 and -6 cells, respectively (Fig. 4A). A substantial number of cells were still positive for SSEA-4 after the rigorous differentiation culture. In addition, a considerable number of cells expressing another undifferentiated marker, Oct-4, remained among the day-6 population as assessed by RNA-PCR (Fig. 4B). Those residual undifferentiated cells might be responsible for the formation of teratomas in the recipients.

To prevent teratomas from forming in recipients, we purged SSEA-4$^+$ cells of the putative hematopoietic precursors and transplanted the SSEA-4$^-$ population into the fetal monkey liver ($n = 6$) (Fig. 4C). At delivery, tumors were no longer observed in the six animals that had been transplanted with the sorted SSEA-4$^-$ cells (Fig. 4D). The transplanted cell-derived CFU were clearly detected in the newborns, and

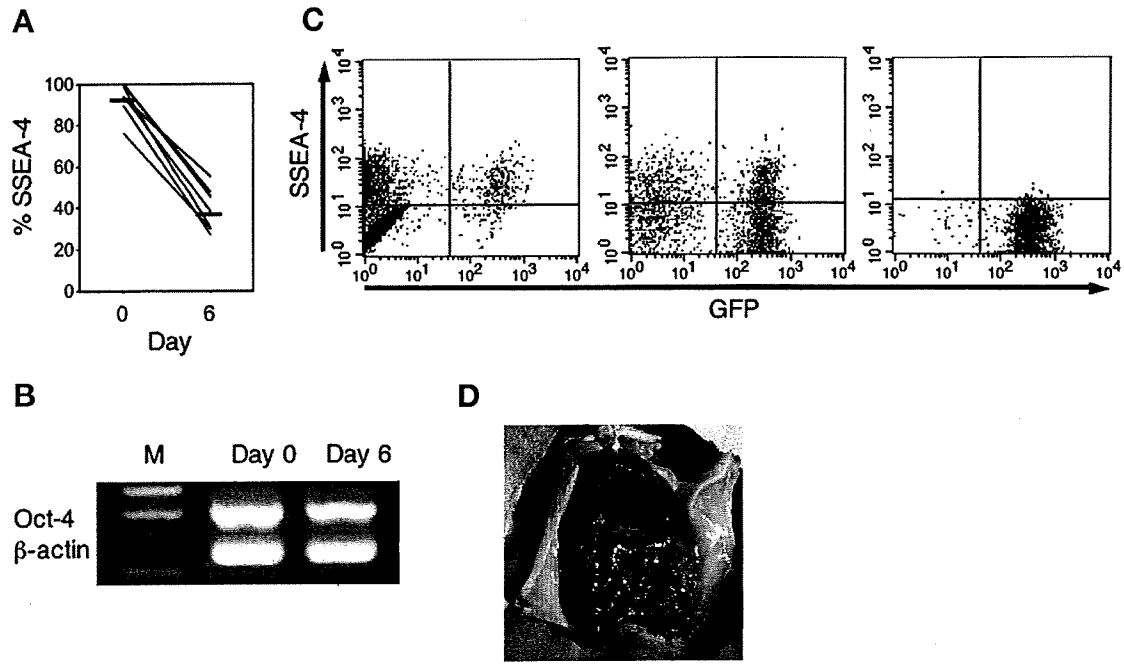

Figure 4. Purging SSEA-4$^+$ cells from among cyESC-derived progenitor cells. **(A):** Undifferentiated cyESCs (day 0) and cyESC-derivatives (day 6) were stained with anti-SSEA-4. The SSEA-4 expression (percentage of total) at day 0 and day 6 is shown (n = 8). **(B):** The Oct-4 expression at days 0 and 6 was also examined by RNA polymerase chain reaction. **(C):** Flow cytometric dot-plot profiles are shown for the SSEA-4 versus GFP expression at day 0 (left), at day 6 before the purge (middle), and at day 6 after the purge (right). Six independent experiments were conducted, and similar results were obtained. **(D):** No tumors were detected in any monkey after the transplantation of SSEA-4-negative day-6 cyESC derivatives (a representative monkey, no. 0981). Abbreviations: cyESC, cynomolgus embryonic stem cell; GFP, green fluorescent protein; M, molecular weight marker; SSEA, stage-specific embryonic antigen.

the fraction was not spoiled (2.3%–5.0%; Table 1), although the removed SSEA-4$^+$ fraction included some CD34$^+$ cells (data not shown).

Discussion

We have previously described a method for hematopoietic engraftment from cyESCs [13]. cyESCs were first cultured for 6 days in vitro, and the day-6 cyESC-derived putative hematopoietic precursors were transplanted in vivo into fetal sheep liver after the first trimester, generating sheep with cynomolgus hematopoiesis. We transplanted the day-6 cells because the CD34 expression level was highest at this time point (Fig. 1C). We transplanted the cells into the liver because the liver is the major hematopoietic organ at this stage of gestation in sheep [34]. In the present study, we tested this method in a cynomolgus monkey allogeneic transplantation model and successfully detected cyESC-derived hematopoietic cells in cynomolgus recipients, albeit at low levels. cyESC-derived chimerism was, however, higher in the primate allogeneic transplantation model (2.3%–5.0%) than in our recently reported sheep xeno-transplantation model (1.1%–1.6%; [13]) (Table 1). To enhance ESC-derived hematopoiesis, further consideration is required of the in vitro culture conditions (i.e., the cytokine milieu, coculture- or embryoid body-associated cellular microenvironment, culture period, and genetic manipulation) and the in utero transplantation conditions (i.e., the preconditioning, route, and timing).

Teratomas developed in all animals, even after the transplantation of ESC-derived progenitor cells that had been cultured for 6 days in the differentiation medium. The risk of tumor formation was high, given that we could hardly detect tumors in immunodeficient mice or fetal sheep that had been transplanted with the same day-6 cyESC derivatives ([13] and our unpublished data). Innate immune responses against cynomolgus-derived tumors might be more rigorous in xeno-transplanted mice and sheep than in allo-transplanted monkeys, resulting in a failure to detect tumorigenesis in the xeno-transplantation models. Similarly, Erdo et al. reported that tumors developed after ESC-derived progenitor cell transplantation in the mouse-to-mouse setting, but not in the mouse-to-rat setting [35]. Our monkey allogeneic transplantation setting would therefore allow the strict evaluation of the in vivo safety of transplantation therapies using ESCs. However, given that teratomas indeed form when undifferentiated cyESCs alone are xeno-transplanted into immunodeficient mice, it is unclear why residual undifferentiated cells included among the day-6 cyESC derivatives did not form teratomas in immunodeficient mice or fetal sheep.

SSEAs that are developmentally regulated during early embryogenesis are widely used as markers to monitor the differentiation of both mouse and human embryos and ESCs [36–38]. Undifferentiated ESCs of both human and cynomolgus origin are characterized by the expression of SSEA-4 and by a lack of SSEA-1 [1, 2, 18]. We have therefore used SSEA-4 as a marker for the negative selection of an undifferentiated fraction. As a result of this negative selection, tumors were no longer detected in the monkeys after transplantation. On the other hand, Bieberich et al. recently developed a method for selective apoptosis of residual pluripotent stem cells using the transcription

factor Oct-4 as a pluripotency marker to prevent teratoma formation [39]. They found that the expression of Oct-4 is colocalized with that of prostate apoptosis response-4, a protein mediating ceramide-induced apoptosis. Treatment of ESC-derived neural precursors with ceramide resulted in selective elimination of residual Oct-4-positive pluripotent cells. Our method, however, uses a cell surface marker to purge pluripotent cells. With this method, one can see the purging efficiency in real-time. This would be meritorious for clinical applications. Although we used a cell sorter to obtain the SSEA-4$^-$ fraction in the present study, selection with beads would be easier and more appropriate for clinical applications.

To generalize the use of SSEA-4 for eliminating undifferentiated cells from among donor cells, we differentiated cyESCs into neural stem cells. After the culture, approximately 10% of cells were still positive for SSEA-4. When all the cells were transplanted into the striatum of Parkinson's cynomolgus monkeys, teratomas developed. We then transplanted cyESC-derived neural stem cells without an SSEA-4$^+$ fraction into the cynomolgus striatum and successfully detected the engraftment without tumor formation (our unpublished data). The removal of SSEA-4$^+$ cells is useful at least for hematopoietic and neural lineages.

CONCLUSION

We are now able to prevent the formation of tumors in nonhuman primate recipients by purging SSEA-4$^+$ cells from among ESC-derived progenitor cells without spoiling the engraftment. SSEA-4 is therefore a clinically relevant pluripotency marker of primate ESCs. Purging pluripotent cells with this marker would be a promising method for producing clinical progenitor cell preparations using hESCs to improve safety in vivo.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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NOTE *Laboratory Animal Science*

Prevention of Immune Responses to Human Erythropoietin in Cynomolgus Monkeys (*Macaca fascicularis*)

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ABSTRACT. Genes and proteins of human origin are often administered to monkeys for research purposes, however, it can be difficult to obtain sufficient levels of the products *in vivo* due to immunological clearance. In this study, we showed that human erythropoietin (hEPO) induces generation of anti-hEPO antibody in cynomolgus macaques (n=2), although 92% of amino acid residues are common between the human and macaque EPO. The administered hEPO was thus eliminated from the animals. On the other hand, when an immunosuppressant, cyclosporin A (CyA), was administered (6 mg/kg) intramuscularly every other day in combination with hEPO (n=2), no anti-hEPO antibody was generated and high serum levels of hEPO were obtained during administration of hEPO, resulting in an increase in serum hemoglobin levels. No adverse effects associated with CyA were observed. Thus, CyA treatment is useful for prevention of immune responses associated with the administration of human proteins in monkeys.

KEY WORDS: cyclosporin A, cynomolgus monkey, erythropoietin.

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Macaque monkeys are widely used for preclinical testing of genes and proteins of human origin, taking advantage of their close phylogenetic relationship to humans [5, 11, 21]. Despite the genetic similarity between the two species, human gene products or proteins are often immunogenic to monkeys. An example is erythropoietin (EPO). EPO is a hematopoietic growth factor that stimulates the proliferation and differentiation of erythroid progenitor cells [10]. Recombinant human EPO (hEPO) has a variety of clinical uses [4, 6, 17, 22]. Although 92% of amino acid residues (142/166) are common between human and macaque EPO [12, 20], we showed here that hEPO induces potent immune responses in macaque monkeys, precluding its administration to monkeys.

Therefore, it is necessary to develop a method to prevent such immune responses following administration of hEPO. Among many immunosupressants available, cyclosporin A (CyA) is widely used to suppress detrimental immune reactions associated with allogenic bone marrow and organ transplantation [1–3, 19]. CyA is a calcineurin inhibitor that inhibits nuclear factor of activated T cells (NFAT) activity and induces immunosuppression [9, 13]. In this study, we showed that hEPO can be successfully administered to cynomolgus monkeys (*Macaca fascicularis*) without immunological clearance by using CyA.

Four cynomolgus monkeys (4–6 years old, 2.5–5.5 kg) bred in the Tsukuba Primate Research Center (Ibaraki, Japan) were used in this study (Table 1). The animals were free of intestinal parasites, herpes-B, simian type-D retrovirus, and simian varicella virus. This study was conducted according to the Rules for Animal Care and Management of the Tsukuba Primate Research Center [8] and the Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan [14]. The protocols of the experimental procedures were approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases (Tokyo, Japan).

First, we administered hEPO (Chugai, Tokyo, Japan) subcutaneously to a cynomolgus monkey (099054) at a dose of 3,000 IU/kg three times a week and assessed the hEPO concentrations in the serum by enzyme-linked immunosorbent assay (ELISA; Roche Applied Science, Mannheim, Germany). Low levels (< 1.0 *ng*/m*l*) of hEPO were detected for the first 3 weeks, but thereafter the levels decreased to the lowest limit of detection (0.01 *ng*/m*l*) despite continued administration of hEPO (Fig. 1A). Assessment by ELIZA revealed that anti-hEPO antibody was being generated [7] (Fig. 1A), and the hEPO was cleared from the serum. A second cynomolgus monkey (001051) was intravenously (instead of subcutaneously) given a much lower dose of hEPO (200 IU/kg, three times a week). During administration, very low levels (< 0.1 *ng*/m*l*) of hEPO were detected with the exception of one time point (1.0 *ng*/m*l* at day 28), and the levels eventually decreased to zero (Fig. 1B). Despite the lower dose, anti-hEPO antibody was generated again (Fig. 1B), leading to clearance of hEPO from the serum. Although we did not try subcutaneous administration of 200 IU/kg hEPO in the present study (Table 1), we assumed that subcutaneous administration of 200 IU/kg hEPO would also result in anti-hEPO antibody generation

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Table 1. Characteristics of cynomolgus monkeys subjected to hEPO administration

	Animal (Sex)	Age (years)	Body Weight (kg)	hEPO		CyA		Hemoglobin Levels (g/dl)		Complication
				Dose (IU/kg)	Administration Route and Frequency	Dose (*ng*/kg)	Administration Route and Frequency	Day 0	Day 35	
hEPO Only	099054 (Male)	5	5.5	3000	Subcutaneous (3 times a week)	–	–	12.7	12.6	Antibody production
	001051 (Female)	4	2.5	200	Intravenous (3 times a week)	–	–	12.4	12.4	Antibody production
	Average	4.5	4.0	–	–	–	–	12.6	12.5	–
hEPO and CyA	396053 (Female)	6	3.2	200	Subcutaneous (3 times a week)	6	Intramuscular (every other day)	10.9	11.6	None
	396058 (Female)	6	4.0	200	Subcutaneous (3 times a week)	6	Intramuscular (every other day)	11.1	12.0	None
	Average	5.5	3.6	–	–	–	–	11.0	11.8	–

given that intravenous administration of the same dose of hEPO produced this result. The reason for this was subcutaneous administration is known to induce a stronger immune response than intravenous administration [16]. The hemoglobin levels did not increase in either animal (Table 1). Despite the genetic similarity of EPO between humans and macaques [12, 20], hEPO is a potent immunogen in macaque monkeys. This is the first report on the immune responses in monkeys following administration of hEPO.

On the other hand, two cynomolgus monkeys (396053, 396058) were given 6 mg/kg of CyA (Sandimmun; Novartis Pharma, Basel, Switzerland) intramuscularly every other day in combination with subcutaneous hEPO administration (200 IU/kg, three times a week) (Table 1). CyA concentrations in the plasma were assessed by radioimmunoassay according to a previously reported method [15], and it was found that the concentrations were maintained within an effective range of 200 to 400 *ng*/m*l*. As a result, no anti-hEPO antibody was generated in either monkey and high serum levels (around 10 *ng*/m*l*) of hEPO were obtained during administration of hEPO (Figs. 2A and 2B). A second trial of hEPO resulted in a similar elevation of the serum levels of hEPO (Figs. 2A and 2B). The hemoglobin levels apparently increased in response to administration of hEPO (Table 1), suggesting that the hEPO trial was effective when CyA was administered together. Blood biochemistry tests revealed no adverse effects associated with the CyA and hEPO treatment.

We have thus established a method to prevent immune responses to hEPO in cynomolgus monkeys using CyA. In fact, this method has successfully been applied to our preclinical monkey testing, and the long-term (around 1 year) efficacy and safety of CyA administration has been well demonstrated [18]. CyA administration will be useful in preventing immune responses when human proteins are administered to monkeys for research purposes.

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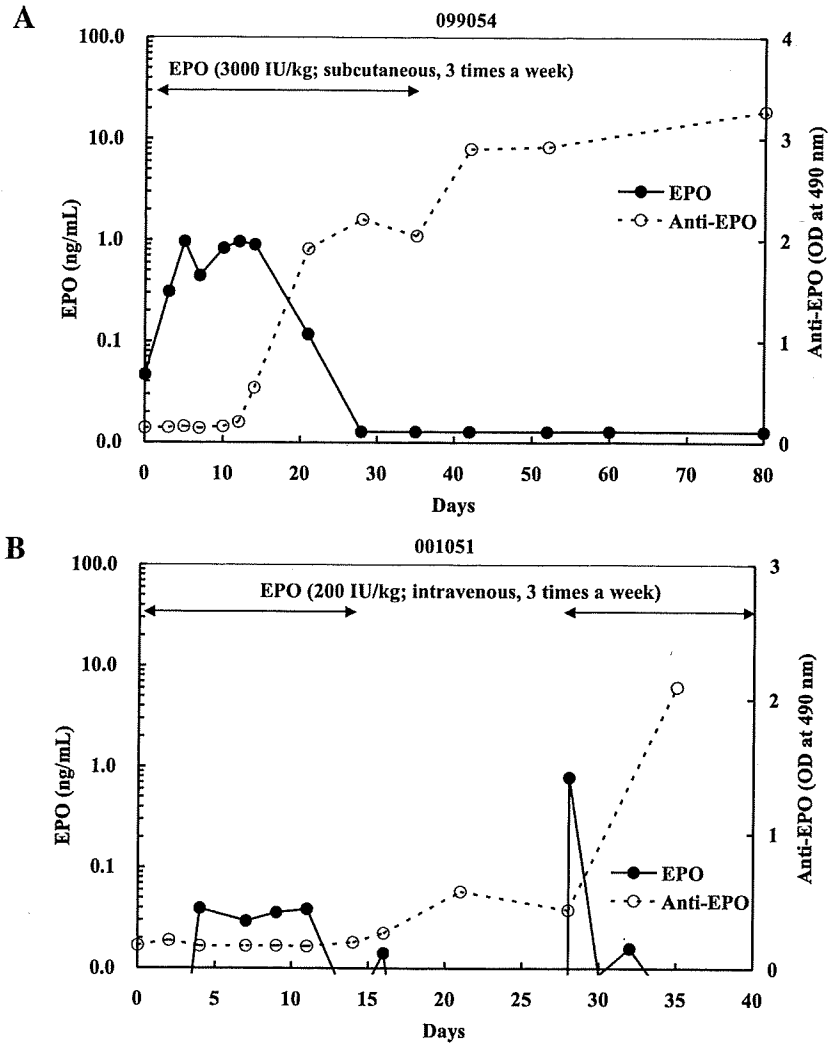

Fig. 1. Administration of only hEPO in cynomolgus monkeys. After subcutaneous administration of hEPO (3,000 IU/kg) to a monkey (099054), anti-hEPO antibody was generated and serum hEPO levels decreased to almost zero (A). Anti-hEPO antibody was also generated in another monkey (001051) receiving hEPO intravenously at a lower dose (200 IU/kg), leading to clearance of hEPO from the serum (B).

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