処置が必ずしも必要でないというメリットもある.

そもそもヒツジ胎仔への幹細胞移植実験は,アメリカのZanjaniらによって始められた.彼らは,1990年代,ヒトの造血幹細胞をヒツジの胎仔に移植して,ヒトの血液をもつキメラヒツジの作製に成功している.なぜヒツジなのか? ヒト造血幹細胞をさまざまな動物胎仔に移植して得られたキメラ動物を見ると,ヒトキメラ率には種差がある.ヒト-ヒツジ間で特に高いことが知られているからである(ヒト-サル,ヒト-イヌ,ヒト-ブタ,ヒト-ヤギ,ヒト-ラット,ヒト-マウス間に比較して).ヒトとヒツジはよほど相性がいいらしい.

さて,生まれたキメラヒツジでは,末梢血のサル/ヒツジキメラ率が低いという問題が残った.この解決が今後の最大の課題である.末梢キメラ率アップに並んで(あるいはそれ以上に)重要なことは,安全性,とりわけ水平感染に関する問題である.この問題に対しては,キメラヒツジからサルの血液細胞を取り出して,同種サルへの接種実験を行い,水平感染の有無などを地道に調べていく必要があると考えている.

今までわれわれが使用したES細胞はサルES細胞である.しかし,もちろんヒトES細胞を使用することも可能である.ヒトES細胞を使えば,家畜動物であるヒツジを,ヒトへ移植するための血液細胞の提供動物とすることができる(「動物工場」).これは決して夢物語ではない.事実,アメリカGTC Biotherapeutics社はヤギを「動物工場」に使って生産した医薬品を2005年半ば発売予定であるという.

E. 結論

SeVベクターによって導入したGFP遺伝子の発現は,抗ウイルス剤リバビリン添加によって調節できる可能性が示された.しかし,サルES細胞に対する毒性も観察され,その軽減に向けて更なる検討が必要である.

試験管内でサルES細胞を初期中胚葉細胞(ヘマンジオブラスト)に分化させてからヒツジ胎仔肝臓に移植すると,サルの造血を有するキメラヒツジが誕生した.本研究においてはサルES細胞を用いて実験を行ったが,ヒトES細胞を同様の方法で用いた場合,ヒツジの体内でヒトの血液を作ることが可能になるかもしれない.さらに末梢キメラ率をもっと上げることができれば,ヒツジをヒトの血液を作らせるための「動物工場」として利用することが可能になるかもしれない.

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

第二世代選択的増幅遺伝子の開発と造血幹細胞への導入

分担研究者
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研究要旨

研究要旨　血液疾患の理想的な根本治療法と考えられる造血幹細胞遺伝子治療の実現のために、遺伝子導入した造血幹細胞を、体内で外部からの薬剤刺激によって選択的に増幅させるシステム「選択的増幅遺伝子：selective amplifier gene; SAG」を創案し、さらにより安全で効果的な増幅を可能とするために erythropoietin（EPO）受容体を利用した第二世代 SAG を開発してきた。現在カニクイザルを用いた、造血幹細胞移植試験を行っている。本研究では臨床プロトコール上必要と考えられる非骨髄抑制下で、SAG による増幅効果を最大限に利用する方法を探索しており、造血幹細胞の骨髄内への生着率の向上が期待される骨髄還流置換法（bone marrow replacement; BMR）との併用により、遺伝子導入幹細胞の効果的な増幅がもたらされることが明らかになった。一方で、通常の経静脈による移植では、遺伝子導入した細胞の生着率が低く、増幅効果もみられなかった。また、BMR のみでは、末梢血中に分化して流出する遺伝子導入細胞の割合が低く、SAG による増幅効果が治療に必須であることも明らかになった。現在までのところ外部からの EPO 投与に依存した増幅効果を示し、SAG が安全に制御しうることを示している。以上により SAG と BMR は、世界でも成功例の少ない造血幹細胞遺伝子治療の実現に有用であることを示唆している。

A. 研究目的

造血幹細胞遺伝子治療は、標的細胞に対する遺伝子導入効率が十分高くないため、治療効果が現れていないのが現状である。ただし、治療遺伝子自体が、その遺伝子を発現することで増殖あるいは生存に優位性をもたらす場合は、遺伝子導入率が徐々に上昇し、治療効果がもたらされると考えている。我々はこの考え方を応用し、遺伝子導入細胞を選択的に増幅する選択的増幅遺伝子を開発し、動物を用いてその効果を確認してきた。しかし、造血幹細胞の増幅には遺伝子導入した細胞が、造血の場であるニッチに移動、生着することが必要であることが明らかになってきた。移植細胞の生着率を上昇させるためには、骨髄への放射線照射や，抗癌剤等による骨髄制御が必要である考えられてきた。しかし、放射線や抗癌剤の副作用を考えた場合、患者への負担増が予想される。そこで我々は全く骨髄に対し放射線や化学物質による抑制を行わずに生着効率を高める BMR を考案した。本研究では、BMR と SAG の組み合わせによる非骨髄抑制下での遺伝子導入効率の上昇効果を確認し、さらにそれぞれ単独の技術での遺伝子導入効率を比較する。

B. 研究方法

(1) カニクイザル末梢血 CD34 陽性細胞の単離とベクター感染

筑波霊長類センターにおいて飼育されていたカニクイザル（*Macaca Fascicularis*）末梢血有核細胞を血液体外循環法(apherasis)により採

取し、抗ヒト CD34 抗体結合ビーズを用い、CD34 陽性細胞を単離した。サル CD34 陽性細胞は直ちにレトロネクチンにてコートしたディッシュ上にて、Stem Cell Factor (SCF)、Thrombopoietin (TPO)、FLT-3 リガンドをそれぞれ 100ng/ml を添加した、レトロウイルスベクターを含む培地中で4日間培養することで、遺伝子導入を行った。4日間は ベクターを一日ごとにフレッシュなものに置き換え、その後2日間は SCF を含む培地のみで培養し、細胞の調子を整えた後に、自家移植した。SAG を搭載したウイルスベクターは GALV のパッケージング細胞である PG13 細胞に MSCV-EPORMpl を繰り返し導入し作成した高力価の生産細胞の培養上清を用いた。また SAG の対照としては neomycin 耐性遺伝子の翻訳開始コドンを削除した非発現ベクター PLI を用いた。

(2) 移植方法

BMR は、以下の手順に従った。麻酔をかけたサルの大腿骨、および上腕骨それぞれの両端に針を刺し、片側の針に 50ml の生理食塩水入りの注射筒を装着し、もう一方の針に装着した空の注射筒にて吸引し髄腔を洗浄した。この洗浄を繰り返した後、遺伝子導入を行った細胞を 1ml の 10%の自己血清含有 PBS に懸濁し、髄腔内に注入した。BMR の対照実験法としては同様に調整した細胞を中心静脈より末梢血中に移植した(intravenous; IV)。いずれの場合も放射線等の前処置は全く行っていない。また、SAG による移植直後からの増幅を期待して、SAG を導入したサルは、移植直後より EPO を投与した。

(3) 遺伝子導入効率の評価

移植後の遺伝子導入効率の評価は、移植後動物より経時的に末梢血あるいは腸骨の骨髄血を採血し、その血液より DNA を抽出し、導入した SAG あるいは PLI を検出する特異的なプライマーセットを用いたリアルタイム PCR を行い、遺伝子導入効率を求めた。また、前駆細胞に対する遺伝子導入効率の算出は、移植時の細胞あるいは骨髄血の細胞を半固形培地中で血球コロニーを形成させ、コロニーごとに PCR を行い、導入遺伝子を検出した。

(4) その他の評価

SAG と BMR との組み合わせにより高い遺伝子導入効率が得られた動物は末梢血の *in situ* PCR や、抹消血より T 細胞・B 細胞・好中球を分取しそれぞれの PCR により解析した。また、導入遺伝子のインテグレーション部位よりクローン解析をする目的で末梢血 DNA の LAM PCR を行った。

(倫理面への配慮)

サルを用いた実験は筑波霊長類センターの動物実験指針に従って行い、できる限り苦痛を与えないよう配慮した。また、血液検査の実施し、移植、薬剤投与などにより頻繁にサルの身体状態をモニターし、異常に対して迅速に対応するようにした.

C. 研究結果

(1) SAG×BMR

移植直後より EPO を三期に分割して投与した結果、EPO の投与後に末梢血中の遺伝子導入効率が上昇し、EPO 投与中断後、その率が低下する直前の 1/100 から 1/10 に低下した。上昇したときは、移植後 350 日に約 9%にまでに達した。

またこの時の in situ PCR でも数%の陽性率を示した。細胞種ごとに PCR 解析をした結果、T 細胞、B 細胞、好中球いずれも増幅されていた。さらに LAM PCR ではこの増幅がポリクローナルなオリジンに基づくものであることが明らかになった。

(2) SAG×IV

SAG 導入細胞を中心静脈から移植したサルでは移植2日後には末梢血中に 1.2%の導入効率が確認されたものの, 移植7日後には検出限界以下に低下し、その後も回復がなかった。

(3) PLI×BMR

移植直後より、非移植骨髄である腸骨骨髄中から遺伝子導入前駆細胞が検出され、その後も移植 300 日以上経っても、数%から 10%の前駆細胞に対する導入効率がみられた。しかしながら、末梢血中では移植2週後には導入効率が 0.1%以下に低下した。

(4) PLI×IV

移植後骨髄中には数%から 10%程度あった前駆細胞に対する遺伝子導入効率が 100 日以上経過するとほとんど0%に低下した。末梢血中の遺伝子導入効率はほとんど検出以下だった。

D. 考察

(1) 骨髄還流置換法の意義

SAG と BMR の組み合わせで末梢血中の遺伝子導入効率が約９%に上昇したが、SAG のみでは末梢血中の遺伝子導入効率はほとんど検出されなかった。従って、非骨髄抑制条件の下では骨髄還流置換法によって、始めて SAG が効果を発揮できる細胞環境に達することができたと考えられる。これまでの動物実験で骨髄廃絶を行った場合は、既存の造血幹・前駆細胞が分化増殖に適した領域を明け渡すため、そのニッチへの移動、その場での増殖分化が起こるが、無処理の場合は通常の移植法では、既に細胞が存在しているニッチに到達できないのであろう。

(2) SAG の意義

骨髄還流置換法を行った場合、確かに骨髄内にとどまるあるいは移動しやすい傾向が見られた。しかし、末梢血に分化してくる割合は非常に低い、ここに増殖優位性を持っていると、分化して末梢血中に娘細胞を送り込めるようになるのではないだろうか。

いずれにせよ骨髄中の遺伝子導入効率と末梢血中の遺伝子効率の違いは骨髄中の特定の細胞が選択されて幹細胞として機能していると考えられる。

E. 結論

骨髄抑制を行わずに造血幹細胞への高効率の遺伝子導入は、現時点ではまだ困難であるが、SAG と BMR の組み合わせによって末梢血の 10%近くの導入効率を実現することが可能になった。これは療法の技術の併用によるものであり、一方の技術のみでは実現できなかったものである。

BMR の効能が移植細胞の生着率の向上やニッチへの到達を促進する技術であれば、この方法を改善することにより、より高い遺伝子導入効率を得ることが可能になると思われる。

F. 健康危険情報

なし.

G. 研究発表

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H. 知的財産権の出願状況

特許取得

A method for transplanting lympho-hematopoietic cells into a mammal

PCT 国際出願済み

国際出願番号:PCT/JP2004/009370 (2004/6/25)

国際公開番号：WO2005/000890　(2005/1/6)

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

サルを用いた造血系再生

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研究要旨

体内微小環境を利用した ES 細胞からの造血系再生システムを構築するため、カニクイザルの ES 細胞から in vitro でヘマンジオブラストに分化させた細胞を用いてカニクイザル胎児に同種移植を試みた。GFP 標識したカニクイザル ES 細胞（CMK6/G）を MMC 処理した OP9 細胞をフィーダーとして種々のサイトカイン存在下で 6 日間培養したところ、60ー80%の細胞が未分化マーカー（SSEA4）陰性となった。これらの細胞をエコーガイド下で 60ー80 日齢のカニクイザル胎児肝臓内に移植した結果、骨髄 CFU あたり 4%前後のキメラ率が確認できたが、全例でテラトーマの形成が認められた。次に、in vitro で同様に分化誘導した細胞から未分化マーカーSSEA4 陽性細胞を FACS でのネガティブ・セレクションで除去し、純化した GFP+/SSEA4-細胞を 50ー70 日齢の胎児肝臓内に移植した結果、全例でテラトーマの形成は認められず、CFU あたりのキメラ率は平均 3.2%であった。このことから、以下の結論を得た。

1) 今回用いた in vitro 分化誘導系では胎児でテラトーマ形成能を有する SSEA4 陽性細胞が残存する。
2) SSEA4 陽性細胞を除去した場合にはテラトーマ形成が認められないことから SSEA4 は臨床的な未分化マーカーであると判断した。
3) キメラ率を向上させるためには、SSEA4 を指標としたネガティブセレクションよりも、ヘマンジオブラストに特異的な初期分化マーカーを確立し、ポジティブセレクションにより初期分化細胞を精製する必要がある。

キーワード：カニクイザル、ES 細胞、初期分化誘導、胎児、同種移植

A. 研究目的

ES 細胞を用いた細胞治療、再生医療技術の開発では、体外で分化誘導した組織前駆細胞を移植し、体内微小環境を利用して最終分化させる戦略が基本となる。そのためにはより高率に組織細胞へ分化させる体外分化誘導系の開発と同時に、分化誘導した細胞の分化能、体内分布、キメラ率などを同種移植により確認しておく必要がある。今年度は、カニクイザルの ES 細胞から in vitro でヘマンジオブラストに分化誘導した

細胞をカニクイザル胎児に同種移植し、造血系再生能の確認実験をおこなった。

B. 材料と方法

1) カニクイザル ES 細胞から造血前駆細胞への分化誘導：

GFP 遺伝子導入カニクイザル ES 細胞（CMK6/G）はマウス胎児繊維芽細胞（MEF）をフィーダーとして ES 培地で継代培養した。

継代培養 ES 細胞を 0.25%trypsin で処理し、剥離した細胞を OP9 細胞をフィーダーとしたフラスコに移し、以下の培地で 6 日間培養した。

分化誘導培地（IMDM 基礎培地）

8% house serum
8% FCS
5x10e-6M hydrocortisone
20ng/mL BMP-4
20ng/mL SCF
20ng/mL IL-3
10ng/mL IL-6
20ng/mL VEGF
20ng/mL G-CSF
10ng/mL Flt3 ligand
2U/mL EPO

2) 分化誘導した細胞の胎児への同種移植：

胎児への同種移植実験には 2 種類の細胞を用いた。ひとつは、培養 6 日目に trypsin 処理して回収した細胞（1ー4x10^7 個/頭）をそのままエコーガイド下で 60ー80 日齢のカニクイザル胎児 3 頭に移植した。移植後 76ー92 日目に帝王切開で胎児を摘出し、安楽殺後に主要臓器を病理解析した。

他は培養 6 日目に回収した細胞を抗ヒト SSEA4 抗体（PE-anti-SSEA4）で染色し、GFP+/SSEA4-細胞分画をフローサイトメトリーにより精製した。精製後の細胞純度は 95%以上であった。精製した GFP+/SSEA4-細胞（2ー7x10^5 個/頭）を 50ー70 日齢の胎児 6 頭の肝臓内に移植した。移植後 85ー100 日後に帝王切開により胎児を摘出し、主要臓器での ES 細胞由来組織を検索した。

C. 結果及び考察

図 1 はカニクイザル ES 細胞（図左）から造血前駆細胞に分化誘導した細胞（図右）での未分化マーカー（SSEA4）の発現を示す。未分化な細胞は霊長類の ES 細胞に特徴的な円形コロニーの形態を示すが、分化誘導した細胞では円形コロニーの形態が失われている。GFP 陽性のカニクイザル ES 細胞に由来する細胞集団での SSEA4 発現細胞の割合は、未分化な ES 細胞ではほぼ 80%であった。一方、分化細胞では約 10ー20%の細胞が SSEA4 を発現していた。SSEA4 陽性細胞を含む分化誘導細胞をすべて胎児肝臓内に同種移植したところ、臍帯血、肝臓、脾臓、胸腺などの臓器に 0.2ー1.1%のキメラ率で ES 細胞由来と思われる GFP 陽性細胞が検出された。これらの細胞と周囲の細胞とで形態学的な差は認められなかった。しかしながら、肝臓内移植したにもかかわらず、3 頭全例の胸腹腔内に腫瘍の形成が確認された。これらの腫瘍は GFP 蛍光を発することから、GFP 標識 ES 細胞由来であると結論した。

ヘナンジオブラストを含む初期中胚葉系に分化誘導した細胞を胎児に同種移植するとテラトーマが形成されることから、分化誘導した細胞中にテラトーマ形成能を有する未分化 ES 細胞が混在している可能性が

強く示唆された。そこで、代表的な未分化マーカーである SSEA4 を指標として分化誘導した細胞集団からネガティブセレクションにより未分化 ES 細胞を除去した細胞（GFP+/SSEA4-）を妊娠初期の胎児肝臓内に移植し、テラトーマ形成の有無と移植細胞の分化能を検討した。ネガティブセレクションで回収した GFP+/SSEA4-細胞の純度はいずれも 95%以上であった。全分化誘導細胞移植の場合には 1ー4x10^7 個/頭の細胞が移植できたが、FACS によるセレクション操作のため移植細胞数は 1/10ー1/50 に減少した。少ない移植細胞にもかかわらず、現在までに解析を終えている 3 頭の胎児の造血キメラ率（CFU ベース）は、平均 3.2%と全分化誘導細胞移植の場合（4%）と有意な差は認められなかった。注目すべき事は、純化した GFP+/SSEA4-細胞を移植した 6 頭すべてでテラトーマ形成が認められなかったことである。このことは、SSEA4 が臨床的な意味で未分化マーカーとなり得ることを示している。

ES 細胞を用いた再生医療戦略では in vitro、ex vivo で目的とする細胞を効率的に分化誘導する技術と、分化誘導した細胞の移植によるテラトーマ形成などのリスクを取り除くことが重要である。今回得られた GFP+/SSEA4-細胞移植の結果は、霊長類で初めて ES 細胞の Clinical stemness marker を実証した結果であり、今後の ES 細胞を用いた再生医療技術の開発において重要な知見となる。一方、今回採用した in vitro 分化誘導系は、少なくともテラトーマ形成能を有する未分化細胞が混在すると言う意味で不完全なシステムであり、今後より効率的にヘマンジオブラストが誘導できる培養システムの開発が必要である。細胞表面抗原を利用して細胞を純化する方法には、今回用いた未分化マーカーを指標としたネガティブセレクションと初期分化マーカーを指標としたポジティブセレクションとの二つの方法がある。初期分化マーカー陽性細胞の純度という観点からは、ポジティブセレクションがはるかに優れている。GFP+/SSEA4-細胞集団には当然ヘマンジオブラスト以外の細胞も含まれており、これが移植後のキメラ率が低い要因とも考えられる。今後は効率的な分化誘導系を開発すると同時に、ヘマンジオブラストに特異的な初期分化マーカーを確立し、効率的で安全な造血系再生技術の開発を行うことが重要である。

D. 結　論

GFP で標識したカニクイザル ES 細胞（CMK6/G）を種々のサイトカイン存在下で 6 日間培養した全ての細胞を胎児肝臓内に同種移植すると、一部の細胞が定着分化したが全例でテラトーマの形成が認められた。分化誘導細胞にテラトーマ形成能を有する未分化 ES 細胞が混在する可能性を考え、FACS により GFP+/SSEA4-細胞を精製し、同様に同種移植を行った結果、すべての移植例でテラトーマの形成は認められなかった。この結果は霊長類 ES 細胞の臨床的未分化マーカーを特定した初めての結果である、ES 細胞を用いた再生医療技術開発に重要な情報である。

E. 健康危険情報

なし

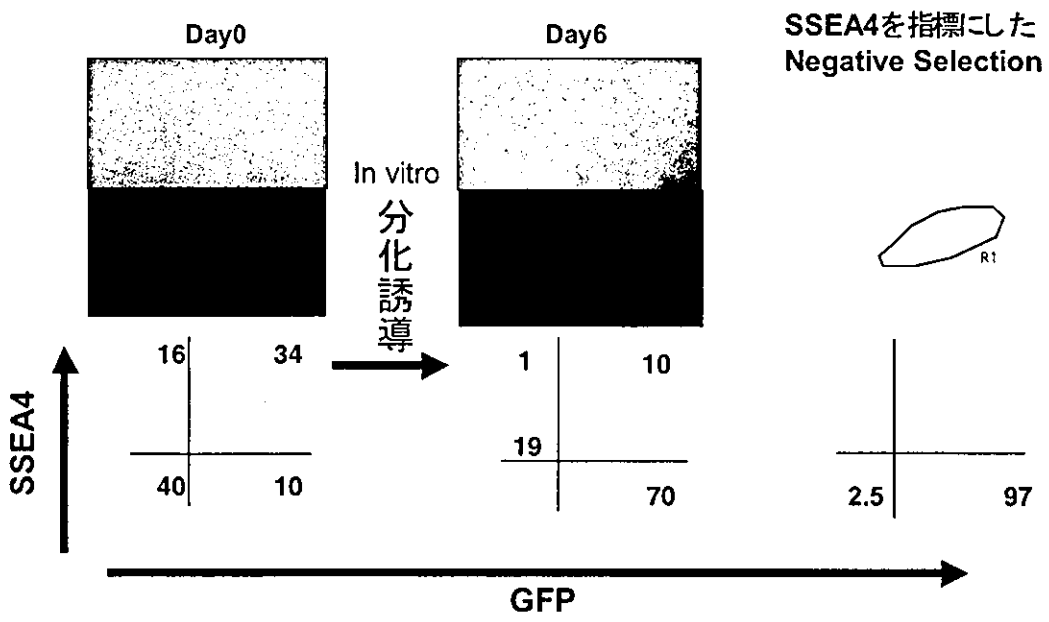
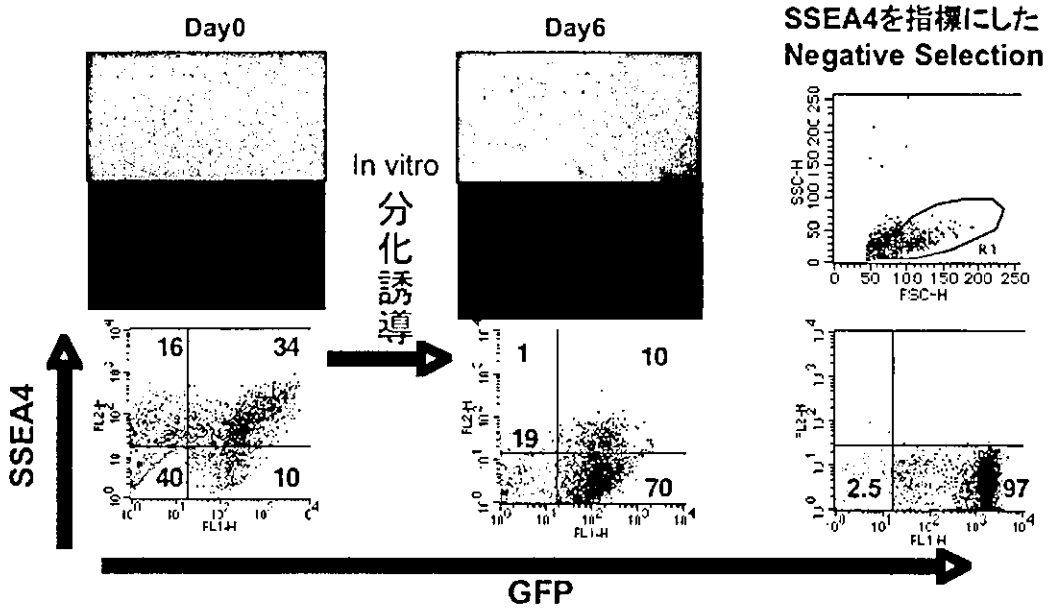


図１：カニクイザル ES 細胞のヘマンジオブラストへの分化誘導と SSEA4 発現



研究成果の刊行に関する一覧表

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研究成果の刊行物・別刷

STEM CELLS®
Original Article

Repair of Infarcted Myocardium Mediated by Transplanted Bone Marrow–Derived CD34+ Stem Cells in a Nonhuman Primate Model

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Key Words. Nonhuman primate • Acute myocardial infarction • Stem cell transplantation Genetic marking • Lentivirus vector • Plasticity • Neoangiogenesis

ABSTRACT

Rodent and human clinical studies have shown that transplantation of bone marrow stem cells to the ischemic myocardium results in improved cardiac function. In this study, cynomolgus monkey acute myocardial infarction was generated by ligating the left anterior descending artery, and autologous CD34+ cells were transplanted to the peri-ischemic zone. To track the in vivo fate of transplanted cells, CD34+ cells were genetically marked with green fluorescent protein (GFP) using a lentivirus vector before transplantation (marking efficiency, 41% on average). The group receiving cells ($n = 4$) demonstrated improved regional blood flow and cardiac function compared with the saline-treated group ($n = 4$) at 2 weeks after transplant. However, very few transplanted cell–derived, GFP-positive cells were found incorporated into the vascular structure, and GFP-positive cardiomyocytes were not detected in the repaired tissue. On the other hand, cultured CD34+ cells were found to secrete vascular endothelial growth factor (VEGF), and the in vivo regional VEGF levels showed a significant increase after the transplantation. These results suggest that the improvement is not the result of generation of transplanted cell–derived endothelial cells or cardiomyocytes; and raise the possibility that angiogenic cytokines secreted from transplanted cells potentiate angiogenic activity of endogenous cells.
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INTRODUCTION

Recent clinical studies have shown that the introduction of bone marrow cells can restore blood flow in ischemic myocardium and ameliorate cardiac function [1–6]. Despite enthusiasm for these studies, it is unclear how transplanted bone marrow cells contribute to the clinical improvement. Because endothelial progenitor cells are identified in bone marrow cells [7], these cells might participate in the repair

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of vascular tissue. On the other hand, it has been reported that hematopoietic stem cells differentiate into endothelial cells and cardiomyocytes when transplanted into the ischemic myocardium in mice [8]. More recently, however, it has been reported that hematopoietic stem cells do not give rise to nonhematopoietic cells in the ischemic myocardium in murine models [9–11].

In vivo tracking and plastic properties of hematopoietic stem or progenitor cells have not been examined in primate cardiac ischemia. We have transplanted genetically marked autologous CD34+ cells to the ischemic myocardium in a nonhuman primate (cynomolgus macaque) model and tracked the in vivo fate of the cells. We have used CD34+ cells because the cells are widely used as a fraction of hematopoietic stem cells in clinical and nonhuman primate studies [12]. In addition, CD34+ cells contain vascular endothelial progenitor cells [7]. Thus, the present study can address the question of whether transplanted CD34+ cells really give rise to endothelial cells and cardiomyocytes in ischemic myocardium in primates.

Materials and Methods

Animals

Eight cynomolgus macaques bred in the Tsukuba Primate Center (Ibaraki, Japan) were enrolled in the present study. This study strictly adhered to the rules for animal care and management of the Tsukuba Primate Center, as well as the guiding principles for animal experiments using nonhuman primates formulated by the Primate Society of Japan. The protocols of animal experiments were approved by the animal welfare and animal care committee of the National Institute of Infectious Diseases (Tokyo).

Preparation of CD34+ Cells

Cynomolgus bone marrow (50 ml) was aspirated from the iliac crest under an isoflurane-induced general anesthesia. From the bone marrow, a nucleated cell fraction was obtained after red blood cell lysis with addition of ACK buffer (Biosource, Camarillo, CA). CD34+ cells were isolated using magnetic beads conjugated with anti-human CD34 (clone 561; Dynal, Lake Success, NY), which cross-reacts with cynomolgus CD34 [13]. The purity of CD34+ cells at harvest ranged from 90% to 95%, as assessed with another anti-human CD34 (clone 563; PharMingen, San Diego) that cross-reacts with cynomolgus CD34 [13]. The purity remained at the same levels after the 1-day transduction culture, which is discussed next.

Lentiviral Transduction

A simian immunodeficiency virus (SIV)-based lentivirus vector carrying enhanced jellyfish green fluorescent protein (GFP) (Clontech, Palo Alto, CA) was used for transduction. The vector was prepared as previously reported [14, 15]. All recombinant DNA experiments were approved by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

CD34+ cells (1×10^6) were seeded in six-well plates in 2 ml of StemSpan serum-free expansion medium (Stem Cell Technologies, Vancouver) supplemented with recombinant human thrombopoietin (100 ng/ml; Kirin, Tokyo), recombinant human stem cell factor (100 ng/ml; Biosource, Camarillo, CA), recombinant human Flt-3 ligand (100 ng/ml; Research Diagnostics, Flanders, NJ), and antibiotics (100 U/ml of penicillin and 0.1 μg/ml of streptomycin; Meiji, Tokyo). The cells were transduced twice each for 12 hours (total, 24 hours) with the SIV vector at 50 transducing units per target cell. After transduction, cells were cryopreserved with 10% dimethylsulfoxide (Wako, Osaka, Japan) and 1% Dextran 40 (Yoshitomi, Osaka, Japan) in a controlled-rate programmable freezer (Kryo 10; Planer Biomed, Middlesex, UK) until transplantation. The viability of cells after thawing was 53.0 ± 6.5%, as assessed by trypan blue staining. An aliquot of transduced cells was assessed for GFP expression at 48 hours after transduction by flow cytometry using a FACScan (Becton Dickinson, Franklin Lakes, NJ) with excitation at 488 nm and fluorescence detection at 530 ± 30 nm.

In Vitro Endothelial Differentiation

CD34+ cells were seeded on fibronectin-coated plates (Becton Dickinson) in M199 medium (Invitrogen, Carlsbad, CA) with 20% fetal calf serum and bovine pituitary extracts (Invitrogen) as previously described [7]. After 7 days in culture, cells were examined for the uptake of DiI-acetylated low-density lipoprotein (LDL) and for the expression of CD31, von Willebrand factor (vWF), vascular endothelial (VE)-cadherin, and vascular endothelial growth factor receptor (VEGFR)-2. Briefly, adherent cells were incubated with 1 μg/ml of DiI-acetylated LDL (Molecular Probes, Eugene, OR) for 4 hours at 37°C. For immunofluorescence staining, after fixation in ice-cold 4% paraformaldehyde for 10 minutes and blocking in 1% bovine serum albumin (BSA) for 15 minutes, cells were incubated with a primary antibody: mouse anti-human CD31 (VM-59; Becton Dickinson), rabbit anti-human vWF (DakoCytomation, Glostrup, Denmark), mouse anti-human VE-cadherin (55-7H1; Becton Dickinson), or rabbit anti-mouse VEGFR2 (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room

temperature. Cells were then incubated with a secondary antibody, Texas red–conjugated horse anti-mouse immunoglobulin G (IgG) (Vector, Burlingame, CA) or goat anti-rabbit IgG (Vector) for 30 minutes at room temperature.

Myocardial Infarction and Transplantation

All operations on cynomolgus monkeys were performed under an isoflurane-induced general anesthesia. Thoracotomy was conducted, the pericardium was opened, and the left anterior descending coronary artery was ligated with 5-0 prolene sutures. One to 2 hours after the ligation, GFP-transduced, autologous $CD34^+$ cells in normal saline were injected with a microsyringe through a 27-gauge needle into 10 sites (5 μl/site) in the peri-ischemic zone. In the control group, saline alone was injected in the same way. The pericardium and chest were closed. The animals then received butorphanol tartrate (0.5 mg/kg, intramuscularly) daily for 5 days to alleviate the pain associated with the operation and myocardial infarction.

Echocardiography

Echocardiographic imaging was obtained using a Sonos 5500 system (Philips Medical Systems, Andover, MA) before transplantation and at 2 weeks after transplant. The echocardiography was conducted by independent technicians irrelevant to our study group. In one animal (BM97080), it was additionally performed at 12 weeks. Short-axis two-dimensional images at the midpapillary level of the left ventricle were stored, and percent fractional shortening (%FS) was calculated to assess cardiac function.

Myocardial contrast echocardiography (MCE) was performed at day 0 (just before transplantation) and at 2 weeks after transplant to assess regional blood flow and blood flow defect size. In one animal (BM97080), chronic assessment was performed at 12 weeks after transplant. The electrocardiograph-triggered end-systolic intermittent imaging was conducted in short-axis views at incremental pulsing intervals (triggering intervals of 1, 2, 3, 4, and 8 beats) using an S12 probe. Once optimized, the settings of depth (4 cm), mechanical index (0.9), and focus (3 cm) were fixed. The contrast agent (perflutren; Yamanouchi, Tokyo) consisted of lipid-coated microbubbles of perfluorocarbon [16]. Perflutren diluted with saline (1:10) was administered intravenously at a constant rate (0.01 ml/kg per min). For the assessment of regional blood flow, MCE images were analyzed using ORIGIN 6.0J (Lightstone, Tokyo), and the blood flow was calculated as previously described [17]. Data are presented as a blood flow ratio (the peri-infarct versus nonischemic control region or the infarct versus nonischemic control region). For the assessment of blood flow defect, MCE images obtained at triggering interval of four beats were analyzed using National Institutes of Health Image software (version 1.61). Data are presented as percent defect compared with the total blood flow.

Microspheres

Colored microspheres (15 μm ± 2% diameter; E-Z Trac, Los Angeles) were used to evaluate regional blood flow 2 weeks after transplant [18], with the exception of one animal (BM97080), in which evaluation was performed 12 weeks after transplant. A set of microspheres (2×10^6) was diluted in 2 ml of saline and injected into the left ventricle over 30 seconds. A reference blood sample was withdrawn at a constant rate of 5 ml/min through the femoral artery. After the collection of blood samples, monkeys were irrigated with saline for mercy killing and blood was completely washed out. The heart was excised from each monkey. Tissue samples from the infarct, peri-infarct, and nonischemic regions (one sample per region) were digested, microspheres were collected, and the blood flow was calculated according to the manufacturer's instructions. Data are presented as blood flow ratio (the peri-infarct versus nonischemic control region or the infarct versus nonischemic control region).

Immunohistochemistry

Tissue samples from the infarct, peri-infarct, and nonischemic regions at 2 weeks after transplant were embedded in optimal cutting temperature compound (Sakura, Zoeterwoude, Netherlands) and frozen in liquid nitrogen. Sections were prepared (6 μm), fixed for 10 minutes at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS), and blocked with 1% BSA in PBS. The sections were incubated at room temperature with a primary antibody, monoclonal mouse anti-human CD31 (1:200; Becton Dickinson), followed by a secondary antibody, biotin-conjugated horse anti-mouse IgG (1:500; Vector). The sections were then treated with avidin-alkaline phosphatase (ABC AP kit; Vector) for 30 minutes. The reaction was developed with a Vector Red substrate kit (SK-5100; Vector). In the case of double staining of CD31 and GFP, the above sections were further incubated with polyclonal rabbit anti-GFP (1:200; Clontech) followed by biotin-conjugated anti-rabbit IgG (1:500; Vector) and treated with avidin-peroxidase (ABC Elite kit; Vector). The reaction was developed with a Vector SG substrate kit (SK-4700; Vector). The sections were counterstained with hematoxylin, mounted in glycerol, and examined under a light microscope.

In Situ Polymerase Chain Reaction

In situ detection of transduced cell progeny was performed by amplifying proviral sequences as previously reported [19]. The following primer set for the GFP gene was used:

5'-CGT CCA GGA GCG CAC CAT CTT C-3' and 5'-GGT CTT TGC TCA GGG CGG ACT-3'. The polymerase chain reaction (PCR) mixture consisted of 420 μM dATP, 420 μM dCTP, 420 μM dGTP, 378 μM dTTP, 42 μM digoxigenin-labeled dUTP (Roche, Mannheim, Germany), 0.8 μM of each GFP primer, 4.5 mM $MgCl_2$, 1 × PCR buffer (Mg^{2+} free), and 4 U of Takara Taq DNA polymerase (Takara, Kyote). Sections were prepared with a Takara slide frame (Takara) from the infarct, peri-infarct, and nonischemic regions at 2 weeks after transplant. PCR was performed using a PTC100 thermal cycler (MJ Research, Watertown, MA) with the following conditions: 94°C for 1 minute and 57°C for 1 minute with 10 cycles. The digoxigenin-incorporated DNA fragments were detected using horseradish peroxidase (HRP)-conjugated rabbit F(ab') anti-digoxigenin antibody (DakoCytomation). The sections were then stained for HRP using a Vector SG substrate kit (Vector). Finally, the sections were counterstained with a Kernechtrot solution (Muto, Tokyo) that stains nucleotides, mounted in glycerol, and examined under a light microscope.

ELISA

Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) levels in tissue lysate or medium were assessed by ELISA (R&D Systems, Minneapolis) according to the manufacturer's instructions. Tissue lysate was obtained from the peri-infarct region (three samples from each monkey) at 2 weeks after transplant. Briefly, tissue was homogenized and suspended in lysis buffer containing 10 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 150 mM NaCl, and protease inhibitor cocktail tablets (Complete Mini, Roche). The suspension was rocked at 4°C for 20 minutes and centrifuged at 16,000g and 4°C for 30 minutes. The supernatant was used for ELISA. The protein concentration of lysate was determined with DC Protein Assay (Bio-Rad, Hercules, CA).

Results

Lentiviral Marking

The CD34+ fraction of autologous bone marrow cells was used for transplantation in our study (Table 1). Before transplantation, CD34+ cells were genetically marked with GFP using an SIV-based lentivirus vector. The ex vivo transduction results are summarized in Table 1. The transduced cells were frozen until transplantation. An aliquot of the transduced cells was examined in vitro for the endothelial differentiation ability. After the differentiation culture, a vessel-like structure was observed (Fig. 1A). The ability of cells to take up DiI-acetylated LDL and the expression of CD31, vWF, VE-cadherin, and VEGFR-2 suggested the endothelial lineage (Fig. 1B). We and others have already confirmed the ability of hematopoietic differentiation of the cells [20, 21]. Taken together, the SIV-mediated GFP gene transfer does not spoil the differentiation abilities of CD34+ cells. In addition, on average, 41% of cells fluoresced 48 hours after transduction, and 56% of

Table 1. Summary of ex vivo transduction and transplantation

	Sex	Age (y)	Body weight (kg)	Harvested bone marrow cell number	Isolated CD34+ cell number	Transplanted cell number	% GFP expression Before[a]	% GFP expression After[b]
Saline group								
CTR01061[c]	M	3	4.1			NA		
CTR99056	M	3	3.4					
CTR96116	F	5	3.2					
CTR99051	M	5	5.9					
CD34+ cell group								
BM01052	M	3	3.9	213×10^6	1.00×10^6	0.47×10^6	49	87
BM01051[d]	M	3	4.1	396×10^6	5.14×10^6	2.20×10^6	51	54
BM97080[e]	M	5	3.9	330×10^6	2.35×10^6	1.04×10^6	49	67
BM90047	M	13	5.8	343×10^6	3.10×10^6	1.07×10^6	16	14
Average		5	4.3	321×10^6	2.90×10^6	1.20×10^6	41	56

[a]Before endothelial differentiation of GFP-transduced CD34+ cells.
[b]After the in vitro endothelial differentiation.
[c]CTR01061 died of heart failure 5 days after myocardial infarction.
[d]BM01051 developed a ventricular aneurysm after myocardial infarction.
[e]BM97080 was killed 12 weeks after the treatment. All other animals were killed 2 weeks after the treatment.
Abbreviations: GFP, green fluorescent protein; NA, not applicable.

endothelial cells still fluoresced after in vitro differentiation (Table 1), showing that the GFP expression is stable during the in vitro differentiation to endothelial cells. Thus, GFP was expected to serve as a good genetic tag after transplantation.

Acute Myocardial Infarction and Autologous Transplantation

Cynomolgus acute myocardial infarction was generated by ligating the left anterior descending artery. One to two hours after the ligation, GFP-transduced, autologous CD34$^+$ cells were injected in the peri-ischemic zone at 10 sites (total, $1.20 \pm 0.73 \times 10^6$ cells; $n = 4$). In the control group, saline was injected in the same way ($n = 4$). We conducted contrast echocardiography immediately after the coronary ligation and found no significant differences in the blood flow defect size (percent blood flow defect compared with the total) between the cell-treated and saline-treated groups ($13.0 \pm 2.1\%$ versus $12.3 \pm 3.5\%$, $p = .75$), suggesting that the initial risk of infarction did not differ between the two groups. In addition, we tried to assess the cardiac isozyme of serum creatine kinase (CK) to evaluate the infarct size; however, either the immuno-inhibition assay or chemical luminescence immunoassay did not work well for cynomolgus monkey samples. We were at least able to show that total CK values at 24 hours after the ligation did not significantly differ between the two groups ($p = .83$).

One of the control monkeys (CTR01061) died of heart failure 5 days after myocardial infarction, and the other control monkeys showed a decrease in %FS at 2 weeks after infarction (Fig. 2). Thus, all four control animals showed the deteriorated cardiac function. In the cell-treated group, one monkey (*, BM01051) underwent ventricular fibrillation immediately after the ligation and survived after cardiopulmonary resuscitation but eventually developed a ventricular aneurysm. Only this animal showed a decrease in %FS despite CD34$^+$ cell treatment; the other animals receiving CD34$^+$ cells showed an increase in %FS (Fig. 2). CD34$^+$ cell treatment may not be able to rescue such a heavily impaired heart but otherwise had a significant effect on cardiac function. Even an old monkey (BM90047, Table 1) showed improved %FS.

The relative blood flow in the peri-infarct to nonischemic control region was also significantly ameliorated in the CD34$^+$ cell–treated monkeys compared with the saline-treated ones, as assessed using contrast echocardiography (Fig. 3A) and colored microspheres (Fig. 3B). An excellent correlation was found between the two methods (Fig. 3C; correlation coefficient = 0.93). Two groups (CD34$^+$ cell–treated and saline-treated) were well separated on the panel, showing an obvious positive effect of CD34$^+$ cell injection on the blood flow in the peri-infarct zone after acute myocardial infarction. In fact, the average myocardial blood flow in the peri-infarct region in the absolute value was 0.988 ml/g per minute and 0.383 ml/g per minute for the cell-treated and saline-treated groups, respectively. Of note, the blood flow in the peri-infarct zone was ameliorated even in the animal with a ventricular aneurysm. On the other hand, the relative blood flow in the infarct to nonischemic region did not show

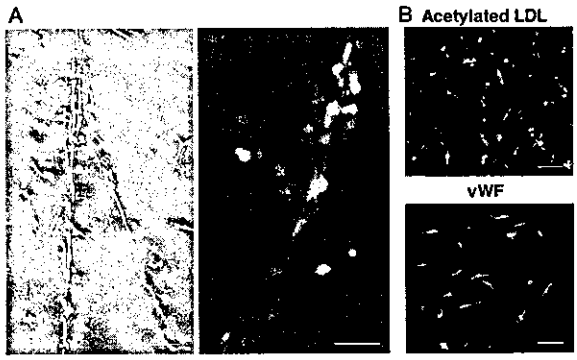

Figure 1. In vitro endothelial differentiation of cynomolgus CD34$^+$ cells lentivirally transduced with GFP. The transduced CD34$^+$ cells were differentiated to endothelial cells after 7 days in culture. **(A)**: Representative vessel-like structure derived from CD34$^+$ cells observed under a phase-contrast microscope (left) and a fluorescent microscope (right). **(B)**: The transduced CD34$^+$ cells differentiated into fluorescent cells (green) positive for the cellular intake of acetylated LDL and immunostaining for von Willebrand factor (vWF) (stained in red). Bar = 100 μm. Abbreviations: GFP, green fluorescent protein; LDL, low-density lipoprotein.

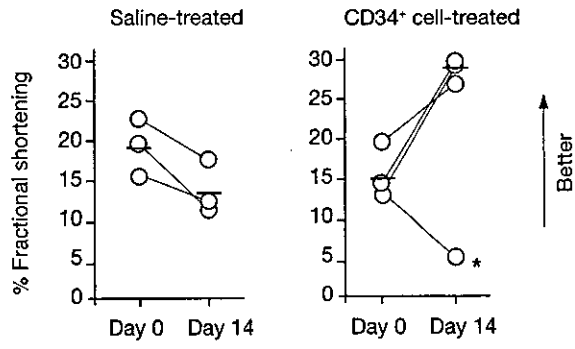

Figure 2. Improved cardiac function after CD34$^+$ cell transplantation. Cardiac function was assessed by echocardiography in terms of percent fractional shortening (%FS) before and 2 weeks after treatment. One monkey in the saline-treated group (CTR01061) died of heart failure 5 days after myocardial infarction and is not included in the figure. One monkey in the CD34$^+$ cell–treated group (*, BM01051) developed a left ventricular aneurysm after myocardial infarction. If this animal was excluded from the statistical analysis, the cardiac function was significantly improved in the CD34$^+$ cell–treated compared with the saline-treated group in terms of the ratio of %FS at day 14 versus day 0 after transplant ($p = .017$).

a significant difference between the CD34+ cell–treated and saline-treated groups. The peri-infarct region was the injection site, and thus the highest degree of change would be expected there.

All monkeys except one CD34+ cell–treated monkey (BM97080) were examined for cardiac function and blood flow at 2 weeks after transplantation, and their tissue sections were finally prepared at this time point (see below). BM97080 was examined at 12 weeks, at which time the cardiac function was still improved compared with immediately after infarction (data not shown) and the blood flow data were in a position similar to the cell-treated group at 2 weeks (Fig. 3C).

In Vivo Tracking of Transplanted Cells

Two weeks after the transplantation, tissue sections were prepared from the infarct, peri-infarct, and nonischemic regions. Immunostaining of an endothelial marker CD31 demonstrated more vessels in the peri-infarct region of the CD34+ cell-treated than saline-treated myocardium (Fig. 4A). In fact, the capillary density of the peri-infarct region was significantly better preserved in the cell-treated than saline-treated group, although there was no significant difference in the capillary density of the nonischemic control regions between the two groups (Fig. 4B).

Double immunostaining with anti-CD31 and anti-GFP showed that some cells in vessels were positive for both CD31 and GFP in the peri-infarct region (Fig. 5A). The result clearly indicates that at least some transplanted CD34+ cells gave rise to endothelial cells. However, we found that the transplanted cell progeny accounted for only a small fraction of endothelial cells after examining more than 100 sections of the peri-infarct region. In situ PCR for proviral GFP sequences also showed that few CD31-positive endothelial cells contained the GFP-provirus (Fig. 5B). There were no GFP-positive cardiomyocytes in more than 100 sections. Most of the transplanted cell progeny were found not incorporated in vessels (Fig. 5C). Hematoxylin-eosin staining did not show any noncardiac tissue regeneration in the myocardium.

On the other hand, we found that in vitro conditioned medium of CD34+ cell culture for endothelial differentiation contained high levels of VEGF, whereas unconditioned medium did not contain detectable VEGF, as assessed

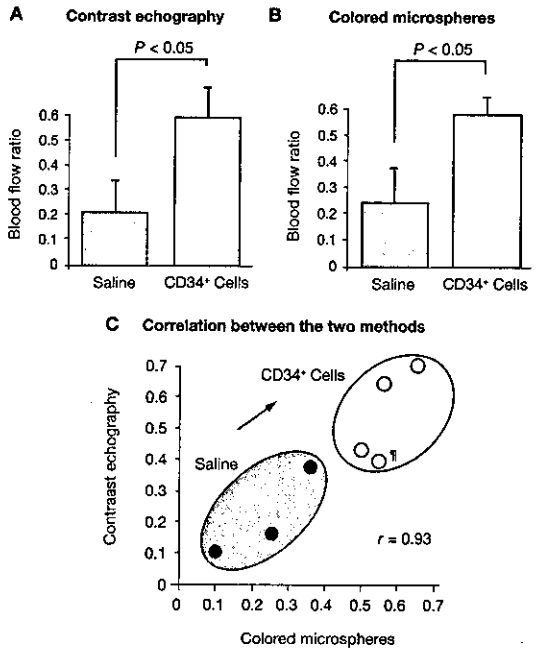

Figure 3. Improved regional blood flow after CD34+ cell transplantation. Myocardial contrast echocardiography (**A**) and colored microspheres (**B**) showed a significantly ameliorated blood flow ratio (the peri-infarct to nonischemic control region) in the CD34+ cell–treated monkeys ($n = 3$) compared with the saline-treated monkeys ($n = 3$) at 2 weeks after treatment. (**C**): An excellent correlation was found between the two methods. A CD34+ cell–treated monkey (¶, BM97080) that was examined at 12 weeks after transplant is included in the panel (**C**) but excluded from the statistical analysis in (**A**) and (**B**).

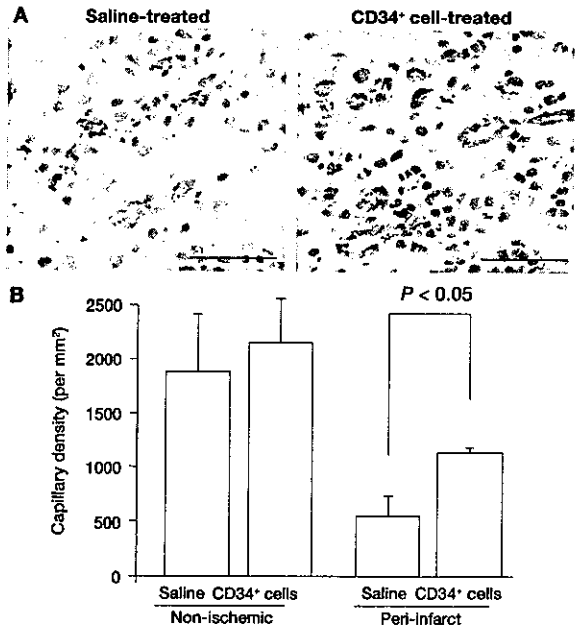

Figure 4. Neoangiogenesis in the ischemic myocardium. Tissue sections were prepared at 2 weeks after the treatment. (**A**): Representative results of immunostaining with anti-CD31 (stained in brown) in the peri-infarct region of the saline-treated and CD34+ cell–treated myocardium. Bar = 50 μm. (**B**): The density of CD31-positive capillaries in the peri-infarct and control nonischemic regions in the saline-treated and CD34+ cell–treated groups. Five fields for each section were randomly selected ($n = 3$ for the saline injection, $n = 3$ for the CD34+ cell injection), and the number of CD31-positive capillaries was counted (average ± standard deviation).

by ELISA (Fig. 6A). In addition, in vivo VEGF levels in the peri-infarct tissue were significantly higher in the CD34⁺ cell–treated than saline-treated group (Fig. 6B, left), although in vivo levels of bFGF differed little between the two groups (Fig. 6B, right).

Discussion

Although gained with small numbers of cynomolgus monkeys, our data suggest that the direct transplantation of bone marrow CD34⁺ cells, even without coronary bypass grafts or percutaneous coronary intervention, results in improved regional blood flow and cardiac function after myocardial infarction in nonhuman primates. Furthermore, we have tried to see the contribution of transplanted CD34⁺ cells to the repair of ischemic myocardium. To this end, we genetically marked CD34⁺ cells with GFP using an SIV-based lentiviral vector before transplantation. Lentiviral vectors can transduce nondividing cells unlike oncoretroviral vectors, and thus the ex vivo culture period with multiple cytokines to allow cell cycling can be reduced to 1 day or less [20, 22, 23]. This is the great advantage of using lentiviral vectors over oncoretroviral vectors for transduction of multipotent stem cells, given that extended ex vivo culture of stem cells may result in loss of multilineage differentiation and engraftment abilities [24]. Human immunodeficiency virus (HIV)-1–based lentiviral vectors can efficiently transduce human cells, but not Old World monkey cells [25]. According to a recent report, a species-specific cytoplasmic component confers the innate

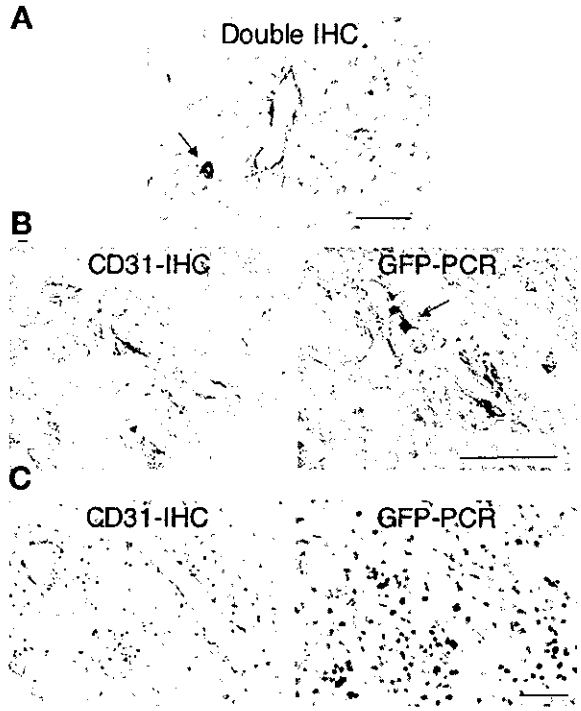

Figure 5. In vivo fate of transplanted cells. Cardiac sections were prepared at 2 weeks after transplantation. **(A)**: Double immunohistochemistry (IHC) with anti-CD31 and anti–green fluorescent protein (GFP) in the peri-infarct region of the CD34⁺ cell–treated myocardium. Some cells (arrow) were positive for both CD31 (stained in brown) and GFP (stained in black), but such cells were rare. **(B, C)**: Serial sections from the peri-infarct region of the CD34⁺ cell–treated myocardium. One section (left) was stained with anti-CD31 (stained in brown), and the other (right) was assessed by in situ polymerase chain reaction (PCR) for proviral GFP sequences (stained in black). **(B)**: Some CD31-positive endothelial cells contained the GFP-provirus (arrow, right panel), but such cells were rare. **(C)**: Transplanted cell progeny (cells positive for GFP-provirus in the right panel) were not incorporated in vessels (cells positive for CD31 in the left panel). Bar = 50 μm.

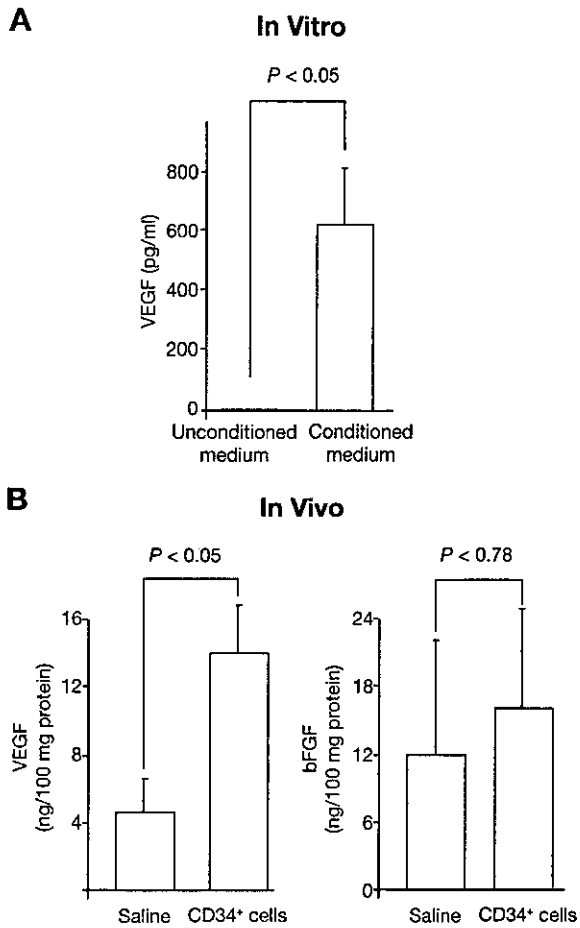

Figure 6. VEGF is implicated in the neoangiogenesis. **(A)**: Unconditioned and conditioned media of in vitro CD34⁺ cell cultures for endothelial differentiation were examined for VEGF by ELISA. The average ± standard error of six culture dishes is shown. **(B)**: Lysates (three samples per monkey) from the peri-infarct region of the CD34⁺ cell–treated (monkey, $n = 3$) and saline-treated (monkey, $n = 3$) myocardium were prepared and examined for VEGF and basic fibroblast growth factor (bFGF) by ELISA. Data are shown as the average ± standard error. Abbreviation: VEGF, vascular endothelial growth factor.

postentry restriction to HIV-1 infection in simian cells [26]. Unlike HIV-1–based lentiviral vectors, SIV-based ones can efficiently transduce simian hematopoietic stem/progenitor cells [21]. In this study, we also used an SIV-based lentiviral vector and achieved the efficient gene transfer into simian CD34+ cells.

As a result of this marking study, we found only a few GFP-positive cells incorporated into the vascular structure in the ischemic myocardium at 2 weeks after transplantation. GFP-positive cardiomyocytes were not detectable. The existence of GFP-positive endothelial cells can be explained by fusion events [27, 28]. However, if that is the case, GFP-positive cardiomyocytes should have also been detected, given that cardiomyocytes are even easier targets of fusion than endothelial cells [11, 29]. Whether fusion occurred or not, only a few transplanted cells gave rise to nonhematopoietic cells in our primate model.

There are several possible explanations for the very low prevalence of transplanted cell–derived endothelial cells or cardiomyocytes in the ischemic myocardium. First, 2 weeks was too short or the number of transplanted cells was too small to see the nonhematopoietic differentiation. However, the cardiac function and regional blood flow were ameliorated by this time point and with this number of transplanted cells. Thus, if transplanted cell–derived, nonhematopoietic differentiation was a reason for the improvement, transplanted cells at this number should have given rise to such cells by this time point. In fact, Orlic et al. [8] observed transplanted cell-derived endothelial cells and cardiomyocytes within 11 days after transplant in mice. In addition, we observed the endothelial differentiation from CD34+ cells within 7 days in vitro (Fig. 1). However, we cannot formally rule out a possibility that inflammatory responses after generation of infarction might have negative effects on engraftment of transplanted cells. Second, the SIV vector failed to transduce stem or progenitor cells that might be responsible for nonhematopoietic differentiation. Even if the transduction was successful, the cytokine treatment during the transduction or GFP expression in the cells spoiled the differentiation abilities. However, we have shown that the SIV vector successfully transduced cells that were capable of differentiating into GFP-expressing endothelial cells (Fig. 1). We have not examined the differentiation ability to cardiomyocytes, because the method to differentiate CD34+ cells to cardiomyocytes in vitro has not been well established. Thus, we cannot formally rule out the possibility that the ex vivo culture spoiled the ability to differentiate to cardiomyocytes or reduced the ability to differentiate to endothelial cells. Third, cells expressing xenogeneic GFP were rejected via immune responses. However, 2 weeks is too short to allow immune elimination of GFP-expressing cells in monkeys [30, 31]. Fourth, the GFP expression was shut down because of transcriptional silencing in vivo, resulting in negative immunostaining with anti-GFP. To examine this possibility, we tried to detect the provirus (vector integrated into genome) in the cardiac tissue by in situ PCR and found again that only a few CD31-positive endothelial cells contained the GFP-provirus (Fig. 5B), thus arguing against transcriptional silencing-based negative immunostaining with anti-GFP. Taken together, we concluded that most transplanted cell progeny were not incorporated into the repaired, nonhematopoietic tissues.

Our results are in agreement with recent reports that transplanted hematopoietic cells are unable to transdifferentiate into nonhematopoietic cells in ischemic myocardium in mice [9–11]. Our studies confirm and extend these findings in a couple of ways. First, we show that the cardiac function can be indeed significantly improved after injection of hematopoietic cells in a nonhuman primate model, although the above studies used murine myocardial infarction models and did not address the potential beneficial effects of hematopoietic cell injection. Second, the improvement is unlikely to be the result of generation of transplanted cell–derived endothelial cells or cardiomyocytes. Finally, we have found that cultured CD34+ cells secrete VEGF and that the CD34+ cell–treated myocardium contains a significantly higher level of VEGF than the saline-treated myocardium. This observation raises a possibility that some angiogenic cytokines secreted from transplanted cells (paracrine effects) potentiate angiogenic activity of endogenous cells. VEGF would be a candidate. Despite this, the delivery of a single agent (VEGF) failed in clinical trials for cardiac ischemia [32]. In situ multiple cytokine production and coordinated action may be essential for clinical benefits [33, 34]. It will be important to explore and identify cytokines responsible for the paracrine effect. If transplanted cells serve as cytokine factories rather than stem cells in ischemic tissues, it is not surprising that not only stem cells but other types of cells may also work [35]. The concept of stem cell therapeutics for ischemic diseases needs additional consideration.

Acknowledgments

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