特集

再生医療の現状を識る

治す 11-b

骨髄細胞移植による血管再生療法
心臓

Therapeutic angiogenesis by autologous transplantation of bone-marrow cells

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キーワード

再生医療，血管再生，心筋梗塞，心筋再生，幹細胞

心筋梗塞への再生医療として末梢血単核球あるいは骨髄単核球を利用した血管新生治療が臨床応用されている。急性心筋梗塞(acute myocardial infarction；AMI)のPCI(percutaneous coronary intervention)治療後に骨髄単核球を冠動脈から注入する血管新生治療が欧米で2001年ごろからスタートした。初期のオープンラベル臨床試験では半年後の心機能が10％前後と改善し世界中の注目を浴びたが，最近の二重盲検試験では有意な改善がみられないとの報告もあり，適応症例の選択が必要になった。造血性サイトカイン(granulocyte-colony stimulating factor；G-CSF)をAMI後に投与して，心機能を改善させる臨床試験も実施されている。一方，陳旧性心筋梗塞(old myocardial infarction；OMI)への骨髄単核球の直接心筋移植は，有効例が多く報告され，開胸・カテーテルを利用した再生医療が期待されている。ヒト心筋からの多能性幹細胞も分離され低心機能の重症心筋梗塞への移植もまもなくである。心筋梗塞への再生医療の最新の臨床試験の成績を中心に述べ，将来展望についても触れてみたい。

骨髄細胞による血管新生と心筋再生

骨髄細胞中には造血系や間葉系幹細胞が含まれる。血管内皮細胞は造血系・間葉系幹細胞の両細胞群から分化可能とされる。造血系・間葉系幹細胞を含む骨髄単核球移植は虚血下肢や心筋において血管新生を誘導するが，新生血管のすべてが移植骨髄細胞から派生した(vasculogenesis)ものではなく，移植細胞から分泌されるVEGF(vascular endothelial growth factor)，bFGF(basic fibroblast growth factor)などの血管内皮増殖因子が血管新生(angiogenesis)に大きな役割を演じている。虚血下肢への骨髄単核球移植による血管新生細胞治療の有効性は国際

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的に承認され，わが国だけでなく世界中において実施されている[1]。

骨髄造血系幹細胞からの心筋細胞分化は現在では否定されている。まれに観察されたとしても既存心筋細胞との融合現象であろう。骨髄間葉系幹細胞にはMAPC(multipotent adult progenitor cell)とよばれる多能性幹細胞群が存在し心筋細胞に分化可能とされる。骨髄中に存在するとされる間葉系多能性幹細胞(MAPC)の存在数の低さを考えると，OMIやAMIへの骨髄単核球移植による心臓ポンプ機能の改善効果は心筋再生によるものとは考えにくく，移植細胞からの血管新生誘導因子や心筋保護因子の分泌などの関与と考えるのが正しいであろう。

急性心筋梗塞への細胞治療

急性心筋梗塞の際には急性期4～7日目をピークとして骨髄から末梢血に血管内皮前駆細胞が動員されることや，幹細胞のhoming factorであるstromal cell-derived factor 1(SDF-1)が心筋に発現し，SDF-1を導入した線維芽細胞を移植しておくと梗塞心に骨髄幹細胞のhomingが促進され，血管新生効果と心機能改善効果が増強されることが示されている[2,3]。

最近，ST上昇型AMIのPCI再灌流成功後に骨髄単核球細胞または末梢血内皮前駆細胞を採取し，さらに梗塞責任冠動脈より低圧バルーン拡張カテーテル先端より注入移植することで心筋血流分布，冠予備能や左室駆出率が10%前後改善されるという興味ある結果が報告された[4-6](図1)。最初の報告では6ヵ月後の心臓収縮機能の有意な改善(10%前後，$p<0.001$)が発表され[4-6]，世界中の注目が集まり，症例数を増やして，randomized研究が実施された(表1)。このうち，double-blind(骨髄採取を全例に実施し細胞・生食投与の二重盲検2アーム)はフランクフルト大学

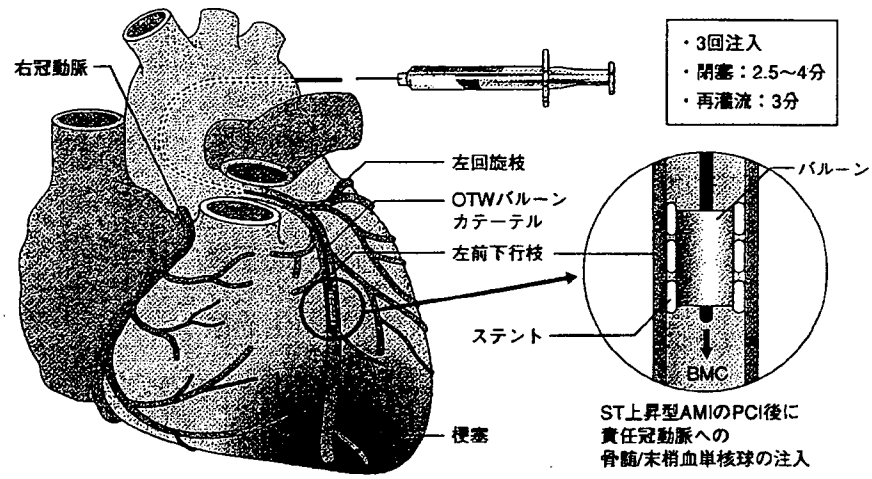

図1 AMIへの末梢血単核球移植

PCIにて閉塞部再疎通に成功したST上昇型AMI症例を対象とする。再疎通した冠動脈にバルーンを挿入し，約5億個の末梢血単核球分画を低圧膨張させたバルーン先端より3回に分けて2分かけて注入する。
OTW：over the wire，BMC：骨髄細胞。

表1 急性心筋梗塞への冠動脈を介した骨髄単核球移植の大規模臨床成績

臨床試験	改善	非改善	試験デザイン
デュッセルドルフ (Strauer, et al)	regional LV-function infarct size	LVEDV	
フランクフルト (TOPCARE)	regional LV-function global EF infarct size	LVEDV	
ハノーバー (BOOST-1)	regional LV-function global EF infarct size	LVEDV	randomized
スペイン	regional LV-function global EF	LVEDV	
ベルギー (Janssens, et al)	infarct size	regional LV-function global EF LVEDV	randomized double-blind
フランクフルト (REPAIR-AMI)	global EF+2.5% (better Tx>5 days, EF<49%)	LVEDV double-blind	randomized
ベルギー (ASTAMI)		global EF infarct size LVEDV	randomized

(ドイツ，REPAIR-AMI研究)[7]，Leuven大学(ベルギー，ASTAMI研究)[8]の2つの臨床研究だけであるが，前者は対照群と比較して2.5%のEF増加(左室造影で評価)，後者は有意差なし(MRI評価)と報告している。ただし，REPAIR-AMIではPCI 5日以降の移植やEF＜49%の症例では7.5%とその効果は倍増していることが報告され，心機能低下例や心筋リモデリング開始時への移植が有効であることは興味深い。ASTAMI研究はPCI翌日に細胞移植を行っているので，REPAIR-AMIの移植タイミングのデータと合致する。二重盲検ではないが，randomized trialデザインでなされた同様の臨床試験では，有意差なし(MRI評価)と報告されている[9]。骨髄単核球の調整方法がREPAIR-AMIとは異なり，回収細胞数や細胞の遊走能の違いが，臨床結果の違いを説明するともいわれている。

AMIに対するこれら3つの臨床試験の結果を受けて発表されたeditorialコメントは，再生医療におけるプラセボを用いた二重盲検試験の重要性がクローズアップされたとともに，AMIへの骨髄単核球を使った血管新生治療の有効性は事実だとしても，標準治療としては否定的な意見が述べられている[10]。しかしながら，筆者はPCI後の心機能低下症例への適用については，有用性が再検討されるべきではと考えている。広範囲梗塞などのショック症例はこれらの臨床試験では除かれている。まさに，これらの重症AMI症例が，再生医療の対象になるのではと，考えている。これら3つの試験の結果を参考にして，患者病態を考慮したAMIに対する骨髄単核球を使った新たな臨床試験が欧州を中心に進んでいる(BOOST-2 trial)。

急性心筋梗塞に対する末梢血単核球を利用した血管新生治療

急性心筋梗塞の際には急性期4～7日目をピークとして骨髄から末梢血に血管内皮前駆細胞が動員されることが室原博士のグループから報告された[2]。筆者らはブタの慢性狭心症モデルに末梢血由来単核球をカテーテルで心内膜側から心筋内移植すると局所血流が改善するとともに，低下した心筋壁運動が改善することを報告した[11]。

これらの報告をもとに，筆者らは左前下行枝(left anterior descending artery；LAD)に限局したST上昇型AMIのPCI再灌流成功後に，末梢血由来単核球を責任冠動脈から注入する血管新生の臨床研究を奈良県立医大との共同研究にて2004年2月より開始した(図2)。細胞移植時期はAMI後3日以内であるが，非細胞治療群に比較して(7.4%EF改善)，末梢血単核球の注入群では13.4%ものEFの有意な改善が見られている(図3)。REPAIR-AMI試験と同様に，移植前の心機能の悪い症例のほうが改善度が高く，適用症例の選択が必要と考えられる[12]。

PCIにて閉塞部再疎通後の急性心筋梗塞に対して骨髄や末梢血単核球の移植を行うことで心機能がよくなるメカニズムとしては，移植細胞から放出されるVEGF，FGF，IGF，PDGFなどの因子(それ以外の未知因子？)により，虚血心筋部位での血管新生促進や虚血心筋細胞への抗アポトーシス効果が生じる結果，心筋の保護が促されたり，血管新生や線維芽細胞からのコラーゲン産生が梗塞巣のexpansionを抑制することで，梗塞心のリモデリングが抑制され心機能が改善する可能性が考えられている[13]。移植骨髄細胞からの心筋再生の可能性についてはいまだ明らかではないが，骨髄間葉系幹細胞からのtrans-differentiationやfusionの問題とともに今後解明されなければならない課題が残っているといえる。

陳旧性心筋梗塞に対する骨髄細胞移植治療

ブタ動物を用いた基礎的研究に基づき[14]，筆者らはこれまでに，わが国において4例の重症狭心症の患者に外科バイパスと併用しない虚血冬眠心筋への骨髄細胞移植のみの治療を行った。提示する症例は64歳の男性で，心筋梗塞発症後8年を経過し，バイパス手術2回，冠動脈形成術を5回受けられている。CCS classⅣの重症狭心症であり，安静時狭心痛が頻発し，1日15回程度のニトログリセリンスプレーを使用している。肋間小切開にてNOGA mappingシステムで同定された虚血冬眠心筋に心外膜側より，自家骨髄単核球を30ヵ所に移植した。経カテーテル的に

図2 AMIへの末梢血単核球移植の実際

末梢血(PB-MNCs)を患者大腿静脈より採取し(a)，単核球分画(5×10^8個)を血球分離器にて分離する(b)，再疎通した冠動脈にバルーンを挿入し，約5億個の細胞を低圧膨張させたバルーン先端より細胞を3回(1回2分間注入，2分間バルーン圧減圧)に分けて注入する(c，d)。評価項目として移植後3ヵ月，6ヵ月後の心機能をLVG，MRI，心エコーで評価する。
PB-MNC：peripheral blood mononuclear cells

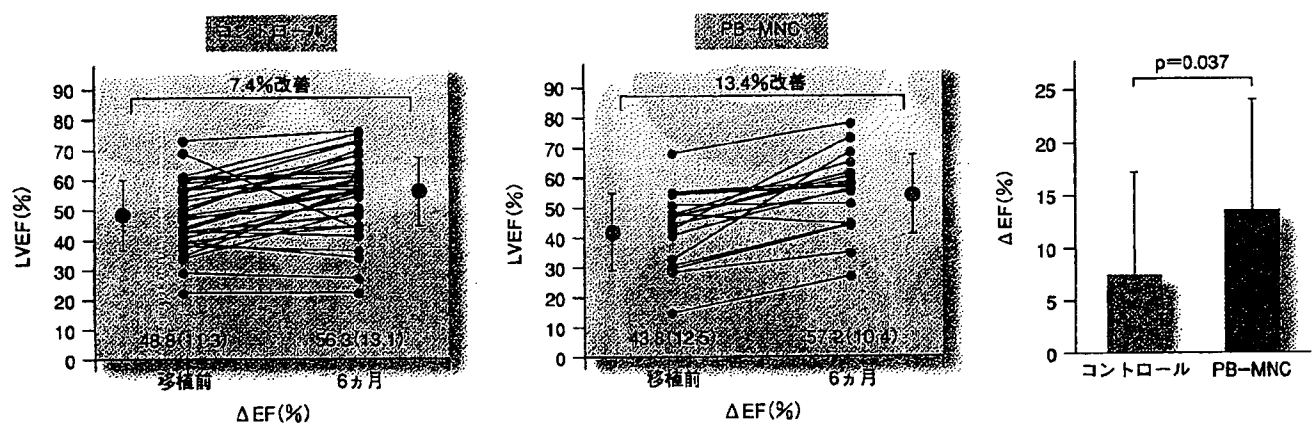

図3 AMIへの末梢血単核球移植による心機能改善

非細胞治療群(コントロール)に比較して(7.4%改善)，末梢血単核球の注入群(PB-MNC)では13.4%もの左室収縮能(left ventricular ejection fraction；LVEF)の有意な改善が移植前と比較して6ヵ月後に観察されている。

冬眠心筋に骨髄単核球を移植した部位は著しく運動低下部位が改善した。14日以内に狭心痛はまったく消失した。4ヵ月間，週1回24時間Holter心電図フォローした不整脈の出現は認めなかった。CPK，トロポニンで評価される心筋傷害は最小限であり，4日以内に正常域に復帰した。左心室収縮率は43%から52%へと増加した。心筋シンチでは負荷後再分布現象は消失し，運動対応能は3倍も亢進した(図4)。その他の症例も胸痛の消失，心機能の改善がみられている。特異的な副作用は出現していない。

米国では同じく経カテーテル的に重症の虚血性心不全患者21人に自家骨髄単核球を移植する治療が行われ，安全性とともに虚血部血流増大や心機能の改善が認められている[15]。この成績をベースに米国FDAは慢性虚血性心筋症の患者への自家骨髄単核球の心筋内移植治療の臨床応用を2004年許可した。現在，NOGAナビゲーションのもとでの MYOSTARカテーテルを用いた二重盲検臨床試験が米国で実施されている。

心筋梗塞に対する骨髄細胞移植治療の将来展望

TOPCAREなどの初期の臨床成績からは通常のPCI後に10%前後の心機能や心筋リモデリング改善効果が得られ，インターベンション治療と再生医療の組み合わせが新しい標準治療となる可能性が示唆され，世界中の循環器内科医の注目を集めた。その後，2つのrandomized，double-blind試験が実施され，最近発表された[7-8]。その結果は期待とは反するものであり，有効であったとしても2～5%のEF改善であり，梗塞巣の縮小，リモデリング抑制効果も小さく，骨髄採取の侵襲度を考えると標準治療として拡大する見込みは少ないと思われる。一方，慢性虚血性心臓病の冬眠領域への骨髄細胞の心筋内移植は狭心症の軽減も含めて，心機能改善の点では非常に有効との報告が多い。しかしながら，重症疾患患者が多いため，randomized，double-blind試験は困難と考えられたが，2005年3月より米国で実施されており，その結果が待たれる。

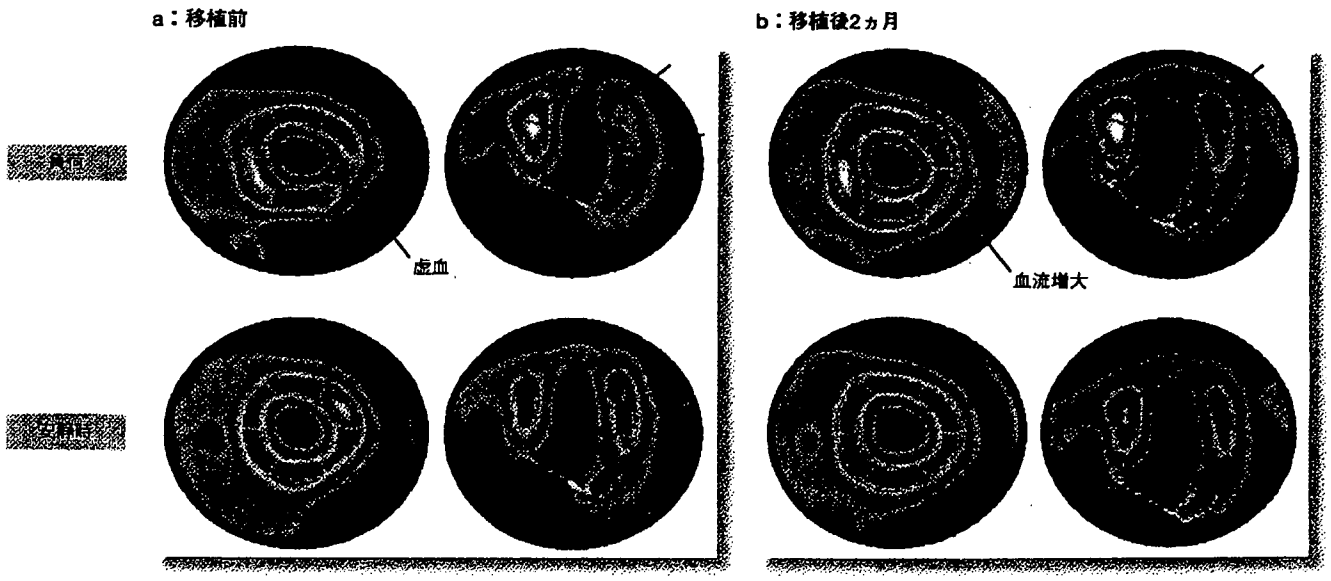

図4　SPECT-sestamibi心筋シンチによる狭心症症例への骨髄単核球移植効果の評価
左室下壁側壁の負荷後再分布領域(→)は骨髄単核球移植の2ヵ月後には消失している。

心筋梗塞への心筋再生医療（ヒト心筋由来心筋幹細胞の発見と心筋再生治療）

広範囲の梗塞巣を有する心筋梗塞や心筋破壊の進んだ心筋症では心筋細胞の移植・補充が心筋収縮能の改善には必要である。心筋再生医療を実施するためにはヒト心臓から心筋前駆細胞を採取・増殖させ病態心筋へ移植する必要がある。心筋幹細胞マーカーとしてc-kit，sca-1，isl-1が報告されている。筆者らは臨床応用を目的に，手術時に得られたヒト心房，肺動脈組織や心筋生検組織から単クローン幹細胞の単離に成功した。この幹細胞は無血清培地下でsphereとよばれる間葉系幹細胞の表現系を強く呈する浮遊系の細胞塊を形成し，高い増殖能を示した。特異的成長因子の存在で神経細胞，上皮細胞，脂肪細胞に分化可能な間葉系由来の多能性幹細胞であった。電気生理学的にも成熟心筋と同じイオン電流・活動電位をもち，心筋移植後にはconnectin-43などのgap juntion蛋白も正常に発現する心筋細胞へ分化しており，心筋創生に向けた探索医療に十分適合した幹細胞ソースである。虚血心筋に移植されたときには，移植後の生存度が大きな問題となるが，筆者らはゲラチンシートを用いて特異的な幹細胞維持因子を除放させ，移植後の生存率が大きく改善することを発見した。このシートとヒト心筋由来幹細胞を用いたハイブリット療法が現在では最もすぐれた心筋再生治療と考えられ，現在はOMIのブタモデルにて前臨床試験を京都大学探索医療センターで実施している。

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Human cardiac stem cells exhibit mesenchymal features and are maintained through Akt/GSK-3β signaling

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Abstract

Recent evidence suggested that human cardiac stem cells (hCSCs) may have the clinical application for cardiac repair; however, their characteristics and the regulatory mechanisms of their growth have not been fully investigated. Here, we show the novel property of hCSCs with respect to their origin and tissue distribution in human heart, and demonstrate the signaling pathway that regulates their growth and survival. Telomerase-active hCSCs were predominantly present in the right atrium and outflow tract of the heart (infant > adult) and had a mesenchymal cell-like phenotype. These hCSCs expressed the embryonic stem cell markers and differentiated into cardiomyocytes to support cardiac function when transplanted them into ischemic myocardium. Inhibition of Akt pathway impaired the hCSC proliferation and induced apoptosis, whereas inhibition of glycogen synthase kinase-3 (GSK-3) enhanced their growth and survival. We conclude that hCSCs exhibit mesenchymal features and that Akt/GSK-3β may be crucial modulators for hCSC maintenance in human heart.

Keywords: Cardiac stem cells; Mesenchymal cells; Proliferation; Survival; Akt/GSK-3β

The postmitotic heart was shown to exhibit a previously unappreciated self-renewing phenotype, in which primitive cells proliferated and differentiated into specific progeny under acute or chronic workloads [1,2]. Recent studies have challenged this paradigm and shown the existence of intrinsic cardiac stem or progenitor cells in the mammalian heart [3–5]. CSCs expressing c-kit were clonogenic and multipotent [4,6], and were also able to be isolated from human heart in the floating culture system [7]. Furthermore, hCSCs were reported to be activated in response to myocardial ischemia and increased workload [8,9]. These cells have a significant impact on future clinical application to treat patients with heart failure. However, it is necessary to further examine the property and regulatory mechanism of hCSC growth to obtain a sufficient number of stem cells from a small amount of tissue samples to achieve an efficient regenerative-therapy.

Recent reports have suggested that bone marrow-derived mesenchymal stem cells (MSCs) enhanced with Akt, a serine/threonine protein kinase, can repair infarcted myocardium, prevent remodeling, and normalize cardiac performance through the prevention of apoptosis as well as a paracrine effect on resident cells [10,11]. Recently, insulin-like growth factor-1 (IGF1) has been shown to maintain murine CSC (mCSC) viability and growth through activation of Akt [12,13]; however, the downstream signals of

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Akt pathway in hCSC growth remain to be investigated. In the present study, we characterized the property of hCSCs and clarified the role of Akt/GSK-3β signaling pathway in hCSC growth and survival. These results suggest that pharmacological inhibition of GSK-3β may have practical application in hCSC transplantation therapy in human heart failure.

Materials and methods

Tissue samples. The heart samples were obtained from 18 patients undergone cardiac surgery (9 males and 9 females aged from 9 days to 77 years old) in confirmation with the guidelines of the Kyoto University Hospital and Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Isolation of hCSCs. The heart samples were excised, minced, and digested with 0.4% type II collagenase and 0.01% DNAse. Obtained cells were then plated at 20 cells/μl in ultra-low culture dishes to generate cardiospheres with growth medium containing DMEM/F12, 5% FBS, 20 ng/ml EGF (Sigma), and 40 ng/ml bFGF (Promega). For the analyses described below, generated cardiospheres were dissected into single cells to obtain hCSCs by exposure to a 0.05% Trypsin/EDTA solution.

hCSC differentiation. For cardiac differentiation, hCSCs were cultured in differentiation medium containing 10% FBS, insulin-transferrin-selenium, and 10 nM dexamethasone. Differentiation medium containing DMEM/F12 supplemented with 10 ng/ml VEGF or 50 ng/ml PDGF-BB (R&D Systems) and 10% FBS was used to induce endothelial or smooth muscle cell differentiation, respectively. For the assay of cell proliferation and survival, specific inhibitors for Akt and GSK-3 (BIO) were purchased from Calbiochem.

FACS analysis. hCSCs were labeled with the following antibodies; phycoerythrin-conjugated antibodies against c-kit, CD45, CD34, CD31, CD90, CD29, CD73, CD71 (BD Biosciences), CD105 (Ancell Corp), and Stro-1 (R&D Systems). Cell events were collected by FACS Calibur flow cytometer and data were analyzed by Cell Quest (BD Biosciences).

RT-PCR and telomerase activity. Total RNA was extracted from cells using TRIzol and RT-PCR was performed with a SuperScript III First-Strand Synthesis System. The primer sequences are available upon request. Telomerase activity was measured with a TRAP assay kit, TRAPEZE (Chemicon).

Immunochemistry. Fixed cells and sections were stained with primary antibodies against cardiac troponin-I (Scripps), CD31, Ki67 (DAKO), α-SMA, connexin 43 (Sigma), collagen typeI (LSL), vimentin, and human nuclei (Chemicon). Secondary antibodies were conjugated to Alexa 488 and Alexa 555, and nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI). Apoptotic hCSCs were evaluated by TUNEL assay with ApopTag kit (Chemicon). Images were captured with a BZ-8000 (Keyence) and IX71 (Olympus Corporation).

Myocardial infarction (MI) and cell grafting. MI was created in 12- to 24-week-old NOD/*scid* mice (Jackson Laboratories) in accordance with the animal care and use guidelines at Kyoto University Hospital. MI was induced by ligation of the left anterior descending coronary artery. One hour after MI, 3×10^5 hCSCs were injected into two sites of the infarcted border zone. In the control group, mice were sham-operated on receiving a thoracotomy but no ligation of coronary artery.

Echocardiography. Two-dimensional and M-mode recordings (Sonos 5500, PHILIPS) were obtained from the short-axis view at the midpapillary muscle level.

Western blotting. Cell lysates were extracted with lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.25% sodium deoxycholate, 1 mM EDTA, 1% Nonidet P-40, 1 mM PMSF, 1× protease inhibitor, 1 mM Na_3VO_4, and 1 mM NaF. Transferred membranes were incubated with primary antibodies against GSK-3β (BD Biosciences), phospho-GSK-3β (Ser9), phospho-Akt (S473), and Akt (Cell Signaling). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG and HRP-conjugated anti-rabbit IgG were used as secondary antibodies.

Statistics. Data are means ± SE. and were analyzed by ANOVA and Scheffe's test, using a significance level of $p < 0.05$ (StatView).

Results

Identification and distribution of hCSCs in human heart

To characterize the hCSCs in human heart, primary heart-derived cells from patients were cultured at low density with low serum condition in a floating culture system using a modification of the method previously reported [7]. At day-14, spherical colonies were generated at a frequency of 63.1 ± 16.5 spheres per 200,000 viable cells (Fig. 1A). The initial yield of digested cells was proportional to the number of spheres, and the number of isolated cells was significantly increased in heart tissues from the right atrium (RA) and outflow tract (OFT) than in tissue from the left ventricle (LV) (Fig. 1B). Moreover, the isolated cells were 5-fold greater and had higher telomerase activity in the infant heart than the adult heart (Fig. 1C and D).

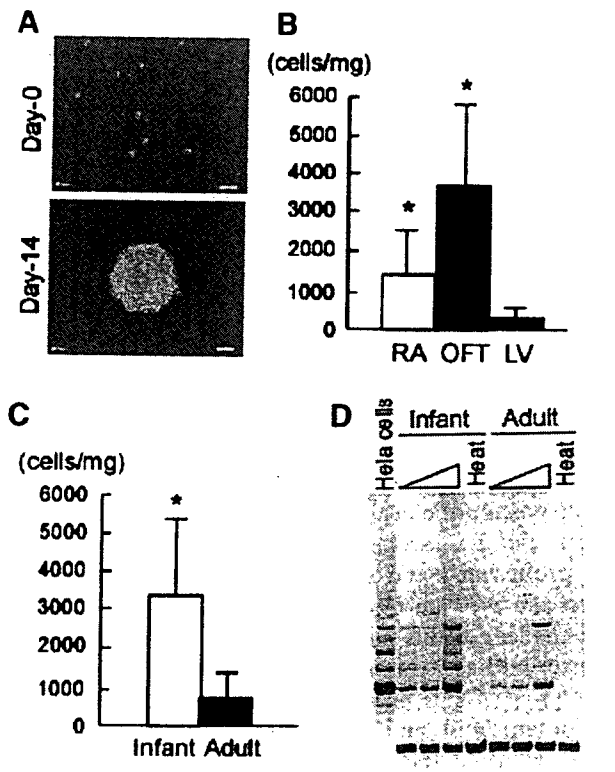

Fig. 1. Isolation and distribution of hCSCs. (A) Generation of cardiosphere from human heart. Bars, 20 μm. (B, C) The initial progenitor cell number harvested by primary isolation as indicated. Total yield was corrected by tissue weight (mg). Distribution of hCSCs corresponding to the parts of the heart (B) or the patients' age (C). *$p < 0.05$ versus LV in (B); *$p < 0.01$ versus adult in (C). (D) Telomerase activity in hCSCs. Threefold serial dilutions of hCSCs isolated from infant and adult hearts were treated with or without heat and used as templates. HeLa cells were used as a positive control ($n = 3$).

hCSCs exhibit mesenchymal features

Immunophenotyping revealed that hCSCs rarely expressed c-kit and did not express the hematopoietic and endothelial progenitor cell-specific surface antigens: CD45, CD34, and CD31, while they were positive for typical MSC surface antigens: CD105, CD90, CD29, CD73, CD71, and Stro-1 (Fig. 2A) [14,15]. Human cardiospheres also expressed both vimentin and collagen type I (Fig. 2B), and had a spindle shaped morphology in attached cell-culture experiments (Fig. 2C). RT-PCR showed that hCSCs expressed ATP-binding cassette transporter subfamily G member 2 (ABCG2), which was associated with Hoechst's efflux properties prerequisite for the side population cells [16]. Human cardiospheres also expressed Rex1, Nanog, and Sox2, although Oct4 was not detectable (Fig. 2D), suggesting that hCSCs express the embryonic stem cell markers and contain the mesenchymal cell-like population.

hCSCs give rise to cardiovascular lineages in vitro and in vivo

To determine the differentiation potential of hCSCs *in vitro*, hCSCs were cultured in differentiation medium. Immunostaining showed that hCSCs gave rise to smooth muscle cells, endothelial cells, and cardiomyocytes co-expressing connexin-43 (Fig. 3A). Furthermore, cardiac-specific transcriptional factors such as Nkx2.5 and GATA4, ANP, and structural genes, including α-cardiac-actin, cardiac troponin-T, MLC2a, MLC2v, α-MHC, and β-MHC, were detected in the differentiated cardiomyocytes by RT-PCR (Fig. 3B).

To investigate the regenerative potential of hCSCs *in vivo*, we performed cell transplantation into MI using NOD/*scid* mice. The injected cells formed a successful engraftment within the border and infarcted regions. The differentiation of hCSCs into the cardiovascular-lineage cells was verified by the presence of smooth muscle cells, endothelial cells, and cardiomyocytes, colocalized with human nuclei (Fig. 3C). Capillary density was also increased in the implanted hearts compared with the PBS-treated hearts (Fig. 3D).

After the transplantation of hCSCs, cardiac function was analyzed by echocardiography (Fig. 3E). In PBS-treated mice, the ejection fraction (EF) and fractional shortening (FS) were significantly decreased (EF: $81.5 \pm 2.0\%$ to $46 \pm 2.0\%$, $p < 0.01$; FS: $43.7 \pm 2.0\%$ to $20.2 \pm 1.0\%$, $p < 0.01$), and LV diastolic dimension (Dd) was expanded (35.2 ± 2.0 to 47.0 ± 3.0 mm, $p < 0.01$) at day-14 after MI compared with baseline. In contrast, the implantation of hCSCs effectively ameliorated the cardiac dysfunction (EF: $46 \pm 2.0\%$ vs $58 \pm 2.0\%$, $p < 0.01$; FS: $20.2 \pm 1.0\%$ vs $26.2 \pm 2.0\%$, $p < 0.01$) and reduced LV dilatation (Dd: $47.0 \pm 3.0\%$ vs $40.7 \pm 2.0\%$, $p < 0.01$) compared with PBS-injected mice. These parameters showed that the

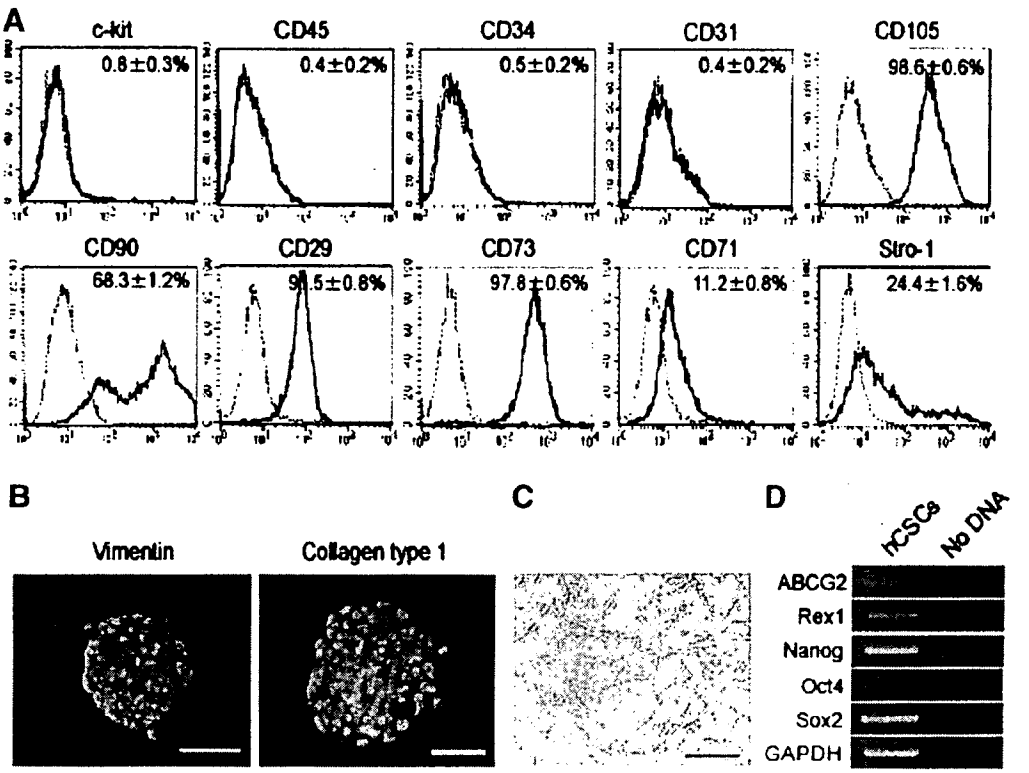

Fig. 2. Characterization of hCSCs. (A) FACS analysis of hCSCs. Black line, control IgG; red line, corresponding antibody ($n = 3$). (B) Immunostaining of human cardiospheres. Red signals show the expression of vimentin (left) and collagen type I (right). Scale bars, 50 μm. (C) Phase contrast image of hCSCs in attached cell-culture. Scale bars, 100 μm. (D) Gene expression profile by RT-PCR examined in hCSCs. No DNA template was used as a negative control ($n = 6$).

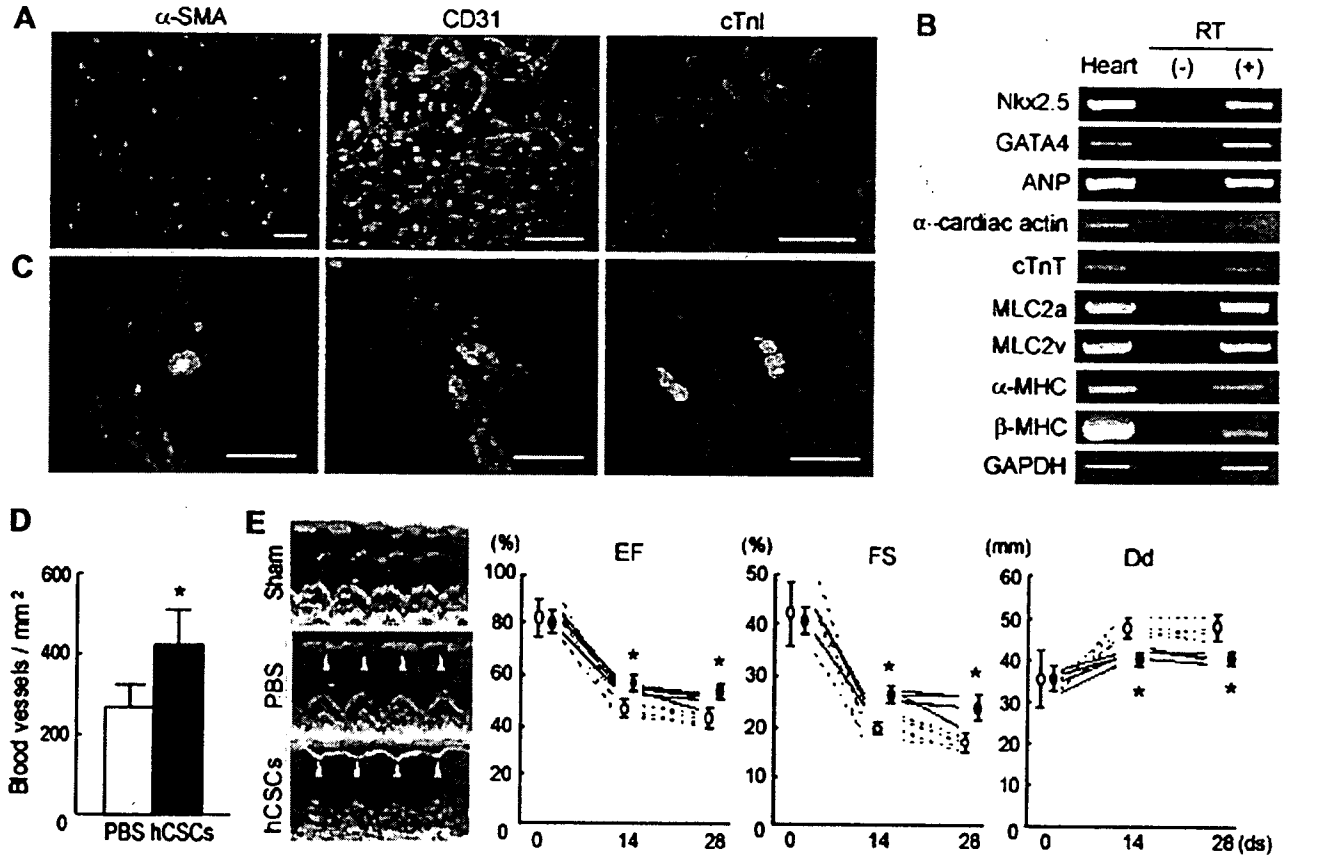

Fig. 3. Functional differentiation of hCSCs *in vitro* and *in vivo*. (A) *In vitro* differentiation of hCSCs into smooth muscle cells (left: α-SMA, red), endothelial cells (middle: CD31, green), and cardiomyocytes (right: cardiac troponin-I, red; connexin-43, yelow). DAPI, blue. (B) RT-PCR shows cardiac differentiation of hCSCs. Heart tissue was used as positive control ($n = 3$). (C) *In vivo* differentiation of hCSCs. Smooth muscle cells (left: α-SMA, red), endothelial cells (middle: CD31, red), and cardiomyocytes (right: cardiac troponin-I, red), counterstained with human nuclei (green) are shown. DAPI, blue ($n = 4$). (D) Capillary density was assessed by CD31 immunohistochemistry in the border zone. $^*p < 0.01$ versus PBS treated mice. (E) Serial assessment of cardiac function by echocardiography. Representative M-mode images of sham-operated, PBS-injected, and hCSC-transplantated hearts at 28 days after MI. Closed circles, hCSC transplanted hearts; open circles, PBS-injected hearts ($n = 8$). Arrowheads indicate significantly improved anterior wall movement on stem cell implantation. $^*p < 0.01$ versus PBS-treated mice. Scale bars, 50 μm in (A); 20 μm in (C).

significant recovery was observed 2 and 4 weeks after hCSC implantation.

The proliferation and survival of hCSCs depend on Akt/GSK-3β pathway

Akt pathway plays a crucial role to mediate the proliferation activity in mCSCs [13]. To verify whether Akt pathway was involved in hCSC proliferation, we examined the activation of Akt in hCSCs and found that EGF/bFGF treatment of hCSCs caused a rapid activation of Akt (Fig. 4A) and also augmented sustained phosphorylation of GSK-3β, which is one of the downstream targets of Akt, to inactivate GSK-3β function (Fig. 4B). The EGF/bFGF-induced activation of Akt in hCSCs was inhibited by Akt inhibitor, Akt-I, in a dose-dependent manner (Fig. 4C). In contrast, the levels of phosphorylated GSK-3β (inactive form of GSK-3β) could be enhanced by the treatment of 10 nM GSK-3-inhibitor, BIO (Fig. 4D), as previously reported in renal epithelial cells [17].

If Akt mediates hCSC proliferation through the inhibition of GSK-3β, the pharmacological inhibition of Akt/GSK-3β signaling pathways may affect the growth of hCSCs. To test this hypothesis, the diameter of cardiospheres was measured in the presence or absence of 10 μM Akt-I or 10 nM BIO, the minimal doses needed to achieve an effect shown above (Fig. 4C and D). Our results demonstrated that Akt-I significantly decreased the diameter of EGF/bFGF-expanded cardiospheres (Fig. 4E), whereas addition of BIO significantly increased their growth at the range of sphere size more than 100 μm (Fig. 4F).

We next determined the underlying mechanisms by which Akt/GSK-3β pathway modulated sphere formation and growth of hCSCs. $TUNEL^+$ cells were significantly increased in cardiospheres treated with Akt-I compared with control, whereas BIO apparently reduced the number of $TUNEL^+$ cells (Fig. 4G). In contrast, Ki67-positive cells were apparently decreased in cardiospheres treated with Akt-I compared with control, whereas a significant

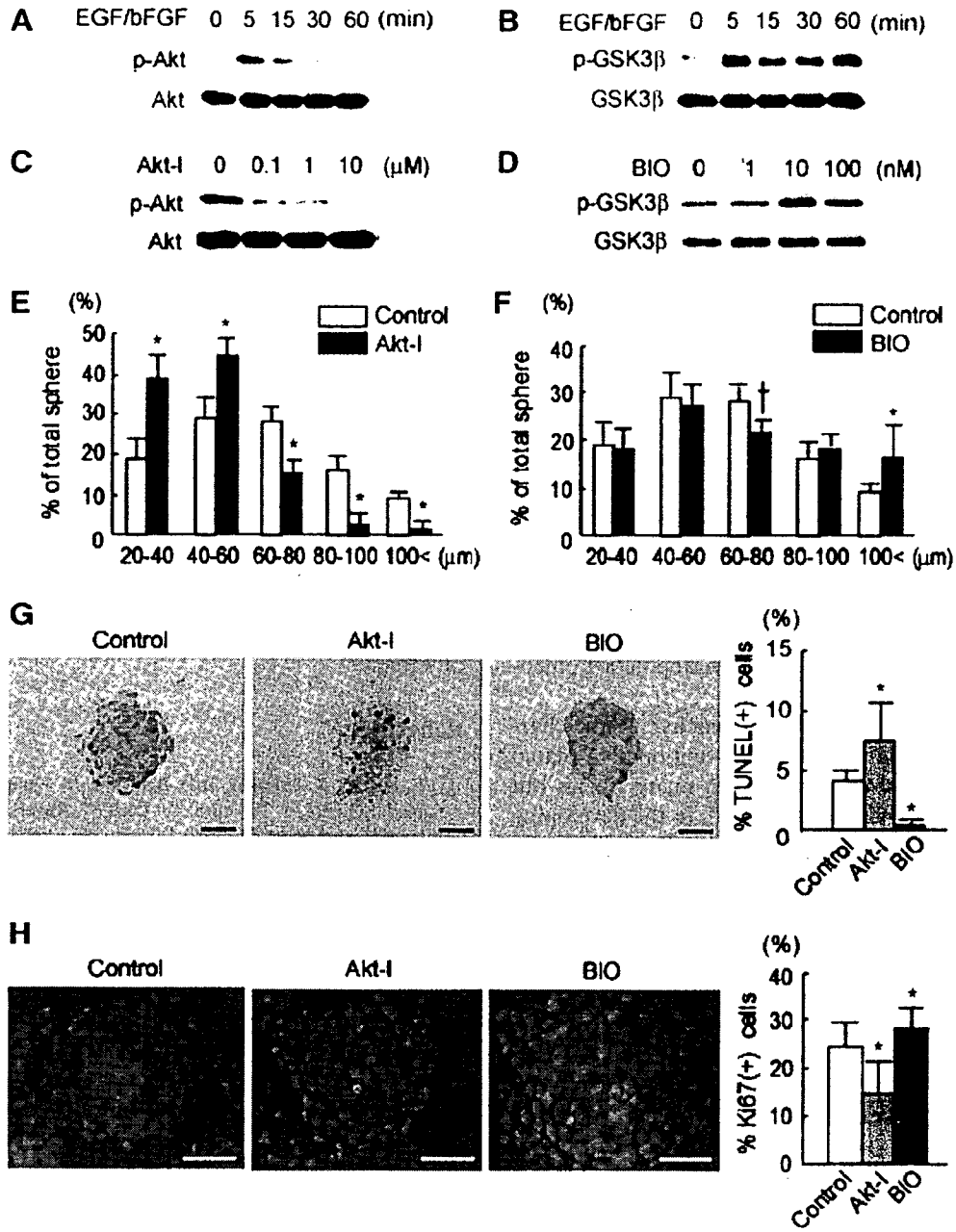

Fig. 4. Akt/GSK-3β signaling regulates the proliferation and survival of hCSCs. (A,B) Phosphorylation of Akt (A) and GSK-3β (B) induced by EGF/bFGF in hCSCs. After serum starvation for 2 h, hCSCs were treated with EGF/bFGF for the period of time indicated. (C) Activation of Akt induced by EGF/bFGF treatment for 5 min was abolished by the pretreatment of Akt-I for 4 h in a dose-dependent manner. (D) Phosphorylation of GSK-3β (inactive) induced by EGF/bFGF treatment for 5 min was enhanced by the pretreatment of 10 nM BIO for 4 h. (E,F) Size distribution of cardiospheres cultured in EGF/bFGF-containing medium in the presence of either 10 μM Akt-I (E), or 10 nM BIO (F) for 6 days ($n = 7$). $^{*}p < 0.01$ and $^{\dagger}p < 0.05$ versus DMSO control. (G,H) TUNEL assay (G) and Ki67 staining (H) of cardiospheres exposed to 10 μM Akt-I and 10 nM BIO. Ki67, red. DAPI, blue. $^{*}p < 0.01$ versus DMSO control ($n = 3$). Scale bars, 50 μm.

increase in the number of Ki67-positive cells was observed in cardiospheres exposed to BIO (Fig. 4H).

Discussion

Our present study provides the novel evidence that hCSCs exhibit a mesenchymal cell-like property and Akt/GSK-3β signaling is involved in their proliferation and survival. Furthermore, our study shows that hCSCs are predominantly present in the right atrium and outflow tract of the heart (more expressed in infant heart rather than adult heart).

A recent report has suggested that cellular aging induces a functional impairment of mCSC growth that may result from the reduction in Akt phosphorylation and telomerase inactivation [18]. Consistent with these data, we showed

here less telomerase activity in hCSCs from the adult heart than that from the infant heart. The abundance of hCSCs isolated from RA and OFT may reflect their specific distribution in the human heart. Although there is a possibility of bias caused by the patients' background, including disease, age, and sex, our results are consistent with a recent report showing that the stem cell niches are predominantly present in the atrium in the murine heart [19].

Messina et al. [7] have demonstrated that hCSCs can be isolated from human heart using floating culture system. We employed the essentially similar method to isolate hCSCs. However, in contrast to the previous report, we found that c-kit expression was extremely low in the isolated cells from both infant and adult hearts. Several reports demonstrated that c-kit expression was diminished on the lineage-committed cardiac progeny as observed in murine cardiac progenitor cells and cardioblasts [3,5,19]. It is possible that cardiospheres contain a mixed population of cells that, as in the niche, can promote the viability of c-kit progenitors and contribute to their proliferation [7]. Our observation suggest that mixed progenitor populations may exist during the process of lineage-commitment of hCSCs in the human heart as during hematopoietic homeostasis [20].

It is notable that hCSCs have a mesenchymal-like character. Mesenchymal stem cells were conventionally isolated from bone marrow and the presence in many tissues but not heart has recently been reported [21]. In the developing heart, the neural crest cells are known to migrate into the cardiac outflow tract to supply the cells from the primitive epicardial epithelium through a process of epithelial-to-mesenchymal transition [22]. These epicardially derived cells have a mesenchymal phenotype and stem cell property in human adult hearts [23]. Thus, it may be conceivable that hCSCs isolated from the human heart might be originated from the primitive epicardial epithelium.

The mechanism to regulate the proliferation and survival of stem cells has been examined. Akt is a nodal signaling kinase linked to both the proliferation and survival of somatic stem or progenitor cells in neural tissue and blood [24,25]. Our studies demonstrate that the proliferation of hCSCs appears to be dependent on the activation of Akt in response to EGF/bFGF stimulation. Furthermore, we have documented that inhibition of Akt pathway impairs cell growth and survival. Our observations are consistent with two independent studies demonstrating that *ex vivo* transduction of Akt prevents bone marrow-derived MSCs from the oxidative stress-induced apoptosis [10] and that the nuclear-targeting of Akt leads to an acceleration of mCSC expansion [13].

The novel finding we showed here that GSK-3β is also associated with the proliferation and survival of hCSCs may provide the new prospect for stem cell therapy. GSK-3β is one of the substrates of Akt and participates in regulating the cell cycle in various cell types [26]. We found that BIO stimulated the growth kinetics of hCSCs consistent with the observation seen in BIO-mediated proliferation of differentiated cardiomyocytes [27]. Thus, our findings suggest that Akt/GSK-3β pathway is crucial in hCSC growth and survival as well as mCSCs.

In conclusion, the present study demonstrates that the resident CSCs in human hearts have mesenchymal characteristics and proliferate through Akt/GSK-3β pathway. Understanding whether pharmacological inhibition of GSK3β by BIO may act through direct activation of the Wnt signaling pathway for stem cell maintenance [28] will provide a new insight into the signaling pathways required for hCSC expansion and engraftment *in vivo*. These novel findings may enable practical applications for establishing hCSC lines and provide an advanced cell therapy for patients with heart failure.

Acknowledgments

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Intracoronary Transplantation of Non-Expanded Peripheral Blood-Derived Mononuclear Cells Promotes Improvement of Cardiac Function in Patients With Acute Myocardial Infarction

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Background Transplantation of non-expanded peripheral blood mononuclear cells (PBMNCs) enhances neovessel formation in ischemic myocardium and limbs by releasing angiogenic factors. This study was designed to examine whether intracoronary transplantation of PBMNCs improves cardiac function after acute myocardial infarction (AMI).

Methods and Results After successful percutaneous coronary intervention (PCI) for a ST-elevation AMI with occlusion of proximal left anterior descending coronary artery within 24 h, patients were assigned to either a control group or the PBMNC group that received intracoronary infusion of PBMNCs within 5 days after PCI. PBMNCs were obtained from patients by COBE spectra-apheresis and concentrated to 10 ml, 3.3 ml of which was infused via over-the-wire catheter. The primary endpoint was the global left ventricular ejection fraction (LVEF) change from baseline to 6 months' follow-up. The data showed that the absolute increase in LVEF was 7.4% in the control group and 13.4% (p=0.037 vs control) in the PBMNC group. Cell therapy resulted in a greater tendency of ΔRegional ejection fraction (EF) or significant improvement in the wall motion score index and Tc-99m-tetrofosmin perfusion defect score associated with the infarct area, compared with controls. Moreover, intracoronary administration of PBMNCs did not exacerbate either left ventricular (LV) end-diastolic and end-systolic volume expansion or high-risk arrhythmia, without any adverse clinical events.

Conclusion Intracoronary infusion of non-expanded PBMNCs promotes improvement of LV systolic function. This less invasive and more feasible approach to collecting endothelial progenitor cells may provide a novel therapeutic option for improving cardiac function after AMI. (*Circ J* 2007; **71**: 1199–1207)

Key Words: Acute myocardial infarction; Angiogenesis; Cardiac function; Peripheral blood-derived mononuclear cells

Differentiation of mesodermal cells to angioblasts and subsequent endothelial differentiation was believed to exclusively occur in embryonic development[1] but this dogma was overturned when human adult peripheral blood mononuclear cells (PBMNCs) were demonstrated to differentiate into the endothelial lineage[2] These cells named "endothelial progenitor cells" (EPCs) expressed endothelial markers, and were incorporated into the sites of ischemia[3,4] We have recently demonstrated that bone marrow mononuclear cells (BMMNCs) contain EPCs in the CD34+ cell fraction and various proangiogenic factors, such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and angiopoietin 1 in the CD34− cell fraction, and that implantation of BMMNCs into the site of ischemia enhances angiogenesis via harmonic supply of EPCs and angiogenic factors[5,6] This technique has been used clinically and developed as a useful therapeutic option for human critical limb ischemia[7]

The concept of the heart as an organ composed of terminally differentiated myocytes incapable of regeneration is also being challenged[8–10] Although attempts to replace necrotic tissue by transplanting other cells (eg, fetal cardiac myocytes or skeletal myoblasts) succeeded in reconstituting heart muscle, these cells failed to completely integrate structurally and to display characteristic physiological function[11–13] In contrast, bone marrow cells (BMCs) have the ability to differentiate into various tissue and are likely to

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regenerate myocardium by inducing myogenesis and angiogenesis, as shown by improved cardiac function and myocardial perfusion in recent accumulating evidence from animals and humans![14–17] In particular, cardiac transfer of BMC-derived stem/progenitor cells can have a favorable impact in patients with acute myocardial infarction (AMI)![18,19] Clinical efficacy of intracoronary transplantation of BMCs after AMI has been the focus of recent large scale, randomized, and controlled trials. The potential benefit of intracoronary injection of BMCs for left ventricular (LV) function was reported in the randomized Bone marrow transfer to enhance ST-elevation infarct regeneration (BOOST) trial[20] and in the Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI) trial?[21] In contrast, intracoronary injection of BMCs after AMI did not significantly improve LV function in the Autologous Stem-Cell Transplantation in Acute Myocardial Infarction (ASTAMI) trial[22] or in the trial reported by Janssens et al?[23] Thus, the latest randomized clinical studies for transplantation of BMCs against AMI retain discrepancies that must be resolved in future trials.

We have previously reported that NOGA-catheter based implantation of non-expanded PBMNCs alone can significantly improve systolic function in ischemic hibernating myocardium of pigs?[24] and that intramuscular injection of human PBMNCs markedly increases regional blood flow in hindlimb ischemia by releasing potent angiogenic factors such as VEGF and bFGF?[25] Moreover, it has been demonstrated that EPCs are indeed mobilized in patients with AMI, peak at 7 days after the onset?[26] and that stromal-cell-derived factor-1 (SDF-1), an important stem cell homing factor, is expressed in the myocardium immediately after AMI?[27] Because the invasiveness of BMC collection in the acute phase of AMI limits its clinical application, we hypothesized that transplantation of non-expanded PBMNCs would even improve the cardiac function in patients with AMI. In this context, we started a clinical trial named the "Japan Trial for Therapeutic Angiogenesis by Cell Transplantation of Peripheral Blood-derived Mononuclear Cells for Acute Myocadial Infarction (TACT-PB-AMI)" in 2004. The primary aim of our study was to examine whether intracoronary injection of non-expanded PBMNCs results in an improvement in LV function, as measured by LV ejection fraction (LVEF), after AMI. Additional objectives were to test the feasibility and safety of this treatment, as well as to assess the effectiveness on regional wall motion, cardiac volumes, and arrhythmias.

Methods

Patients and Study Protocol

Patients between 18 and 80 years of age were eligible for inclusion in the study if they had a first acute ST-elevation myocardial infarction with occlusion of the proximal left anterior descending (LAD) coronary artery and a creatine kinase (CK) level >1,000 IU, which was successfully treated by percutaneous coronary intervention (PCI) within 24 h. According to previous observations?[28,29] CK values were serially measured every 4 h for 24 h after the onset of AMI. Exclusion criteria were the presence of cardiogenic shock requiring intravenous pressors or intra-aortic balloon counterpulsation, pulmonary edema, advanced hepatic or renal dysfunction, evidence of malignant diseases, or unwillingness to participate. Because the patients were best suited for an evaluation of LV function by angiographic imaging, we decided to include only patients with anterior wall infarction. This study protocol was approved by the Ethics Review Board of Kyoto Prefectural University School of Medicine, and written informed consent was given by each patient.

The study was designed as an open-label and non-randomized clinical trial. Briefly, after successful PCI (TIMI III), patients were assigned to either the control (PCI alone) group or non-expanded PBMNC group that received intracoronary infusion of PBMNCs within 5 days after PCI. Intracoronary cell transplantation was performed by over-the-wire balloon catheter. Neither collection of PBMNCs nor sham injection was performed in the control group. The primary endpoint was the global LVEF change from baseline to 6 months' follow-up.

Catheterization Procedure for Progenitor Cell Transplantation

A mean of 2.5±0.5 days after the AMI, an over-the wire balloon catheter was advanced into the infarct-related artery (eg, LAD). To allow for adhesion and transmigration of the infused cells through the endothelium, the balloon was inflated inside the stent previously implanted during the acute reperfusion procedure with low pressure to block blood flow for 3 min while 3.3 ml of the PBMNCs suspension was infused distally to the occluding balloon through the central port of the balloon catheter, as previously described![19] This maneuver was repeated 3 times to accommodate infusion of the total 10-ml cell suspension, interrupted by 3 min of reflow by deflating the balloon to minimize extensive ischemia. After completion of intracoronary cell transplantation, coronary angiography was repeated to ascertain vessel patency and unimpeded flow of contrast material.

Preparation of Progenitor Cells

A cell separator apheresis system with computer software (COBE Spectra, software version 6.1, Gambro BCT, Lakewood, Co, USA) was used to collect all PBMNC products via the standard MNC program. Acid citrate dextrose-A (ACD-A, Baxter Healthcare Corporation, Deerfield, IL, USA) was used as the anticoagulant at a whole body-to-ACD ratio of 20–25:1 in combination with 2,000 IU heparin sulfate. Apheresis was performed through central venous access from the femoral vein in all patients in the PBMNC group. With the aim of processing the largest amount of blood in the shortest possible time, apheresis procedures were performed with the highest possible but still tolerable blood flow rate, such as 55 ml/min. No more than 2.5-fold of the donor's blood volume was processed on a single day. We usually obtain PBMNCs ($\approx 5\times10^9$ cells) from patients by COBE spectra-apheresis and concentrate them to 10 ml by density gradient centrifugation (Kubota 9810, Japan).

After PBMNCs were harvested by COBE Spectra, 100 μl of the cell suspension was diluted by PBS(–) containing 0.5% bovine serum albumin (Fraction V, Sigma, St Louis, MO, USA) used for flow cytometric analysis. The cells were stained with allophycocyanin-conjugated (APC)-anti-human CD34 (Becton Dickinson, San Jose, CA, USA) and phycoerythrin-conjugated (PE)-anti-human VEGFR2 (KDR) (R&D Systems, Minneapolis, MN, USA).

Appropriate isotype controls were used for each staining procedure; 1×10^5 cells were gated within the lymphocyte region on forward-scatter vs side-scatter plots using a FACS Calibur (BD Bioscience). Next, the percentages of cells in

Table 1 Clinical Characteristics of the Study Population

	Control group (n=36)	PBMNC group (n=18)	p value
Age, years	60.4±11.3	61.8±8.7	0.65
Male sex, no. (%)	32 (88.9)	15 (83.3)	0.57
Hypertension, no. (%)	16 (44.4)	11 (61.1)	0.25
Hyperlipidemia, no. (%)	20 (55.6)	12 (66.7)	0.43
Diabetes, no. (%)	12 (33.3)	5 (27.8)	0.68
Smoking, no. (%)	20 (55.6)	12 (66.7)	0.43
PAD, no. (%)	3 (8.3)	1 (5.6)	0.71
Killip class on admission	1.20±0.48	1.22±0.43	0.66
Infarct segment	6.3±0.5	6.6±0.5	0.06
Vessel diameter, mm	3.24±0.38	3.22±0.31	0.89
Peak creatine kinase, IU/dl	3,764±2,506	4,255±1,615	0.47
Time to revascularization, h	6.1±5.7	5.2±2.4	0.49
Mean transplanted cells, no.	-	$4.92\pm2.82\times10^{9}$	
Medication			
ACEI, no. (%)	26 (72.2)	15 (83.3)	0.37
ARB, no. (%)	11 (30.6)	5 (27.8)	0.83
β-blocker, no. (%)	19 (52.8)	10 (55.6)	0.85
Diuretics, no. (%)	6 (16.7)	3 (16.7)	1.00
Statins, no. (%)	22 (61.1)	11 (61.1)	1.00

Values are expressed as mean±SD.
PBMNC, peripheral blood mononuclear cell; PAD, peripheral arterial disease; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin-receptor blocker.

each population described below were calculated using CELLQuest software (BD Bioscience).

LV Angiography

LV angiograms were obtained according to standard acquisition guidelines immediately after PCI and at 6 months' follow-up. LVEF and LV volumes were calculated by the area–length method, and regional wall motion was determined with the use of the centerline chord method.

Measurement of Other Parameters

For the assessment of regional LV wall motion, echocardiography was carried out before cell transplantation and at 6 months' follow-up. Two-dimensional resting echocardiography was performed in the 4 standard views (parasternal long-axis and short-axis views and apical 4- and 2-chamber views) and regional LV wall motion analysis was performed as described by the Committee on the Standards of the American Society of Echocardiography, dividing the left ventricle into 16 segments and scoring wall motion as 1=normal, 2=hypokinesis, 3=akinesis, 4=dyskinesis for each segment. The wall motion score index (WMSI) was calculated as the sum of the scores of the segments divided by the number of the segments evaluated at the day of cell transplantation and 6 months' follow-up.

We performed resting Tc-99m (^{99m}Tc)-tetrofosmin gated single photon emission computed tomography (SPECT) before hospital discharge and at 6 months' follow-up. In all patients, 592 MBq of ^{99m}Tc-tetrofosmin was intravenously injected at rest. Immediately after the injection, each patient drank a glass of milk to accelerate tracer clearance from the hepatobiliary system. Data acquisition for SPECT imaging was performed at 30 min after ^{99m}Tc-tetrofosmin injection, using a rotating digital gamma camera (Picker PRISM IRIX) equipped with a low energy, high resolution, and parallel-hole collimator. Reconstructed transaxial images were reoriented in the vertical long-axis and short-axis of the LV. The basal and midventricular segments on short-axis views of the LV myocardium were divided into 8 segments each, and 16 segments were taken. An apical region on the vertical long axis was also taken, and a total of 17 segments were analyzed. The ^{99m}Tc-tetrofosmin perfusion defects were visually evaluated by 2 experienced observers, who had no knowledge of the patient's clinical information, with a 5-point grading system (0=normal, 1=mildly decreased uptake, 2=moderately decreased uptake, 3=severely decreased uptake, 4=defect). The grading was decided on by consensus between the 2 observers, and the sum of the scores for all segments was used as the defect score.

To assess whether intracoronary cell transplantation was associated with proarrhythmic effects, we performed 24-h Holter recording for all patients before hospital discharge and at 6 months' follow-up, and estimated the Holter Lawn class by calculating premature ventricular complexes and ventricular tachycardias.

Follow-up Examinations

Six months after progenitor cell therapy, cardiac catheterization was repeated; left ventriculography was performed with identical projections and adequate contrast opacification for quantitative analysis according to standard guidelines and coronary angiograms were analyzed for the presence of restenosis in the infarct-related artery. Echocardiography, Holter ECG, and perfusion scintigraphy were also repeated after 6 months.

Statistical Analysis

Continuous variables are presented as means±SD. Control and cell therapy groups were compared using the chi-square test for discrete variables and unpaired Student's t-test for continuous variables according to standard statistical methods. Statistical significance was assumed at a value of $p<0.05$. All statistical analysis was performed with SPSS (Version 9.0, SPSS Inc, Chicago, IL, USA).

Results

Baseline Characteristics and Procedural Results of Cell Infusion

The clinical characteristics of the study population are

Table 2 Clinical Characteristics of the Patients' Measurements of Hemodynamics at the Time of AMI (Baseline) and 6-Months' Follow-up

	Control group (n=36)	PBMNC group (n=18)	p value
LVSP (mmHg)			
Baseline	121.7±13.0	120.3±13.9	0.73
6 months	125.0±19.2	126.9±8.7	0.67
p value (baseline vs 6 months)	0.47	0.10	
LVDP (mmHg)			
Baseline	3.9±4.8	4.6±5.1	0.67
6 months	3.1±2.4	4.1±3.5	0.27
p value (baseline vs 6 months)	0.4	0.73	
LVEDP (mmHg)			
Baseline	20.1±6.1	19.8±4.8	0.89
6 months	13.6±3.7	14.3±5.3	0.57
p value (baseline vs 6 months)	<0.0001	<0.0001	
AOMP (mmHg)			
Baseline	90.3±13.4	88.4±9.7	0.61
6 months	89.5±14.2	90.2±9.7	0.85
p value (baseline vs 6 months)	0.84	0.58	
HR (beats/min)			
Baseline	88.3±7.7	88.0±10.9	0.91
6 months	65.8±6.8	65.0±7.2	0.69
p value (baseline vs 6 months)	<0.0001	<0.0001	

Values are mean±SD.
AMI, acute myocardial infarction; LVSP, left ventricular systolic pressure; LVDP, left ventricular diastolic pressure; LVEDP, left ventricular end-diastolic pressure; AOMP, aortic mean pressure; HR, heart rate. Other abbreviation see in Table 1.

Table 3 Clinical Characteristics of Patients' Baseline Cardiac Function

	Control group (n=36)	PBMNC group (n=18)	p value
LVEF (%)	48.8±11.3	43.8±12.5	0.20
EDVI (ml/m²)	60.9±14.8	70.0±15.0	0.07
ESVI (ml/m²)	32.0±13.1	39.9±14.9	0.054
Regional EF (%)			
Segment #2	11.7±6.1	12.3±7.0	0.77
Segment #3	8.4±6.5	5.5±5.8	0.11

LVEF, left ventricular ejection fraction; EDVI, end-diastolic volume index; ESVI, end-systolic volume index; EF, ejection fraction. Other abbreviation see in Table 1.

shown in Table 1. The PBMNC group and control group were well matched with respect to baseline characteristics and procedural characteristics, such as age, sex, and coronary risk factors, Killip class, infarct segment, and vessel diameter. Although particularly important factors influencing cardiac function are considered to be peak CK and time to revascularization, there were no significant differences in these factors between the 2 groups. All patients were treated with aspirin (100 mg/day), ticlopidine (200 mg/day for at least 4 weeks after PCI) or cilostazol (200 mg/day at least 4 weeks after PCI), statin, β-blocker, and angiotensin-converting enzyme inhibitor (ACEI) or angiotensin-receptor blocker (ARB) during the hospitalization for AMI and continued until the 6-months' follow-up examination, unless these agents were contraindicated. There were no significant differences between the control and PBMNC groups in the administration of ACEIs, ARBs, β-blockers or statins (Table 1).

No patient had either bleeding complications through the central venous access from the femoral vein or systemic blood pressure fall during the apheresis procedures. Although a transient further ST elevation associated with balloon occlusion was seen in the infarct-related ECG leads in most of the patients receiving PBMNCs, there were no serious symptomatic complaints or circulatory disturbances during or after cell transplantation. Neither intracoronary infusion nor the stop-flow procedure was performed in the control group. There were no fatal cardiac events during the follow-up period, and no patient in either group had any clinical manifestation of heart failure.

Endothelial Progenitors in AMI Patients

FACS analysis showed that the percentage of $CD34^+$ (0.12±0.2) or $CD34/KDR^+$ (0.05±0.1) cells in the PBMNCs from AMI patients tended to be higher (2–10-fold, but not statistically significant), compared with healthy volunteers ($CD34^+$; 0.06±0.1, $CD34/KDR^+$; 0.003±0.001), and that these cells also possess the characteristics of EPCs, as demonstrated by DiI-acetylated LDL uptake and lectin binding.

Hemodynamics and LV Function by Angiography

Table 2 shows the hemodynamic measurements in the control and PBMNC groups at the time of AMI (baseline) and at 6-months' follow-up. Although LV end-diastolic pressure (LVEDP) and heart rate (HR) were significantly reduced from baseline to 6-months' follow-up in both groups, there were no significant differences in LV systolic pressure (LVSP), LV diastolic pressure (LVDP), or aortic mean pressure (AOMP). Moreover, there were no statistically significant differences in LVSP, LVDP, LVEDP, AOMP, or HR between groups at either baseline or 6-months' follow-up.

Table 3 shows the clinical characteristics of baseline LV cardiac function. There were no significant differences in

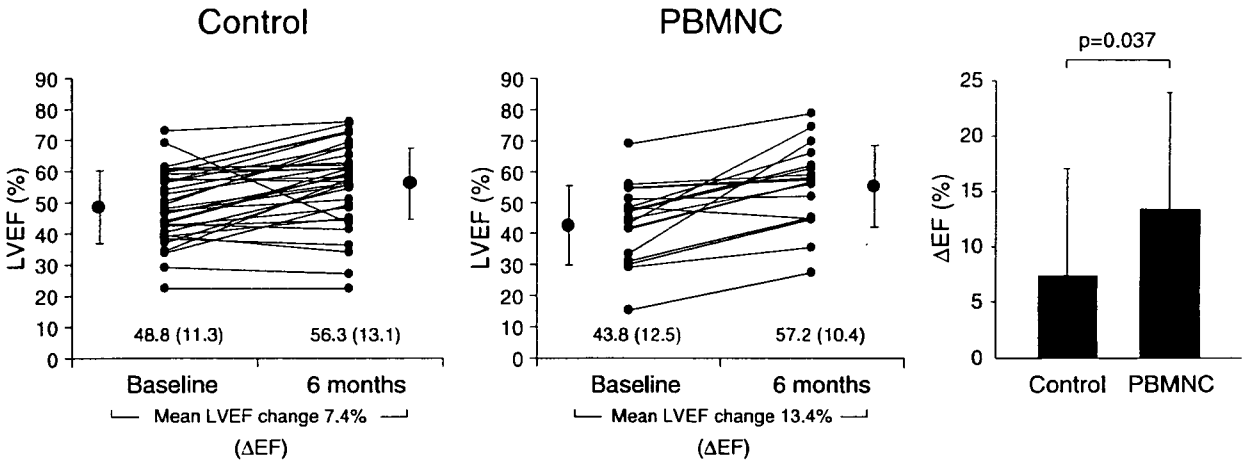

Fig 1. Global left ventricular ejection fraction (LVEF) at baseline and 6 months' follow-up, and the absolute increase in LVEF (Δejection fraction (EF)) in the control and peripheral blood mononuclear cell (PBMNC) groups. Small dots show data for individual patients; large dots show mean values. Vertical bars show SD.

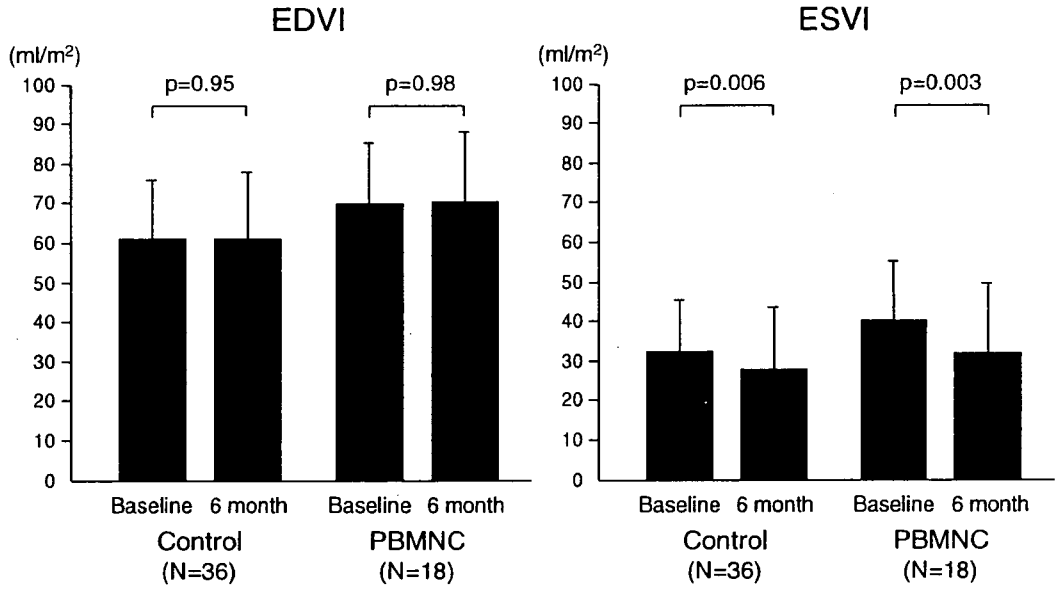

Fig 2. End-diastolic volume index (EDVI) and end-systolic volume index (ESVI) at baseline and 6 months' follow-up. Data are mean ± SD. PBMNC, peripheral blood mononuclear cell.

LVEF, end-diastolic volume index (EDVI), end-systolic volume index (ESVI), or regional ejection fraction (EF) between the control and PBMNC groups. Fig 1 illustrates LV function as assessed by cineventriculography at baseline and 6-months' follow-up. In the control group, LVEF was 48.8% at baseline and gradually increased to 56.3% after 6 months. In contrast, LVEF was 43.8% at baseline and increased to 57.2% after 6 months in the PBMNC group. Although the baseline measurement of LVEF did not differ significantly between the 2 groups, the absolute increase in LVEF (ΔEF) was 7.4% in the control group and 13.4% in the PBMNC group. Our data therefore show that cell transplantation significantly improved LVEF, and there was a modest but significant increase in global LVEF, even in the patients with PCI alone (ie, controls). It is notable that the ΔEF value in thePBMNC group was significantly greater than that in the control group (PCI alone) (Fig 1).

There were no significant difference in EDVI between baseline and 6 months' follow-up in either control or PBMNC group (Fig 2) and also no significant difference in the absolute difference in EDVI (ΔEDVI) between the control and PBMNC groups (p=0.90). ESVI was significantly decreased from baseline to 6 months' follow-up in both groups, such that the absolute difference in ESVI (ΔESVI) was −4.3% in the control group and −8.2% in the PBMNC group. Thus, although there was no statistically significant difference in ΔESVI value between the 2 groups (p=0.09), the ΔESVI value tended to be lower in the PBMNC group, compared with controls. Selective analysis of the infarcted zone showed that baseline measurements of regional wall motion (regional EF), that is, segments #2 and #3 of the AHA classification, did not differ significantly between the control and PBMNC groups (Table 3). Although regional wall motion in the infarct area was significantly improved from baseline to 6 months' follow-up in both groups, the absolute value of regional EF (ΔRegional EF)

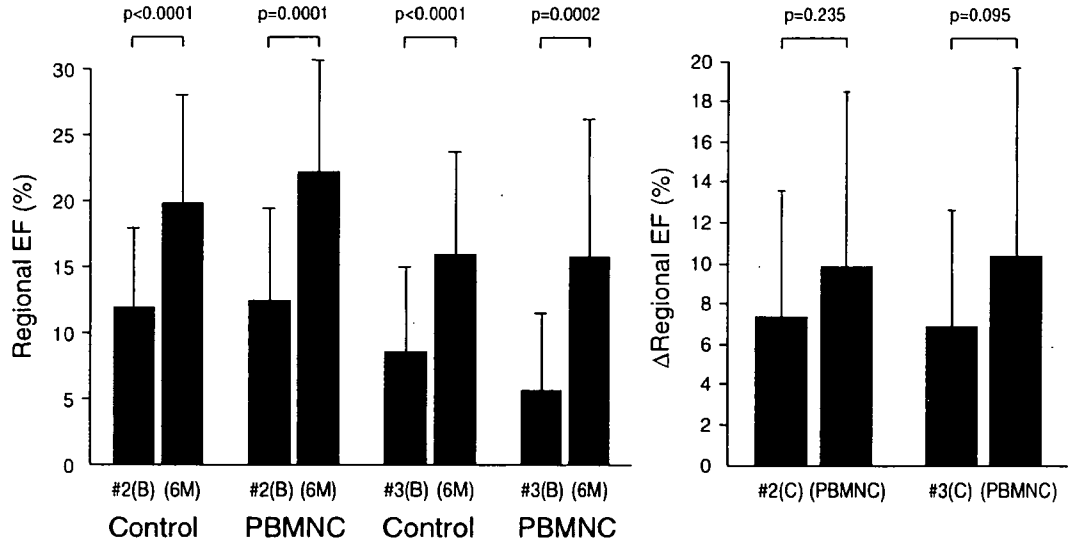

Fig 3. Regional ejection fraction (EF) at baseline and 6 months' follow-up, and the absolute increase in regional EF (ΔRegional EF) in the control and peripheral blood mononuclear cell (PBMNC) groups. Regional EF in the infarct area (segments #2 and #3) was estimated as described in Methods. Data are mean±SD.

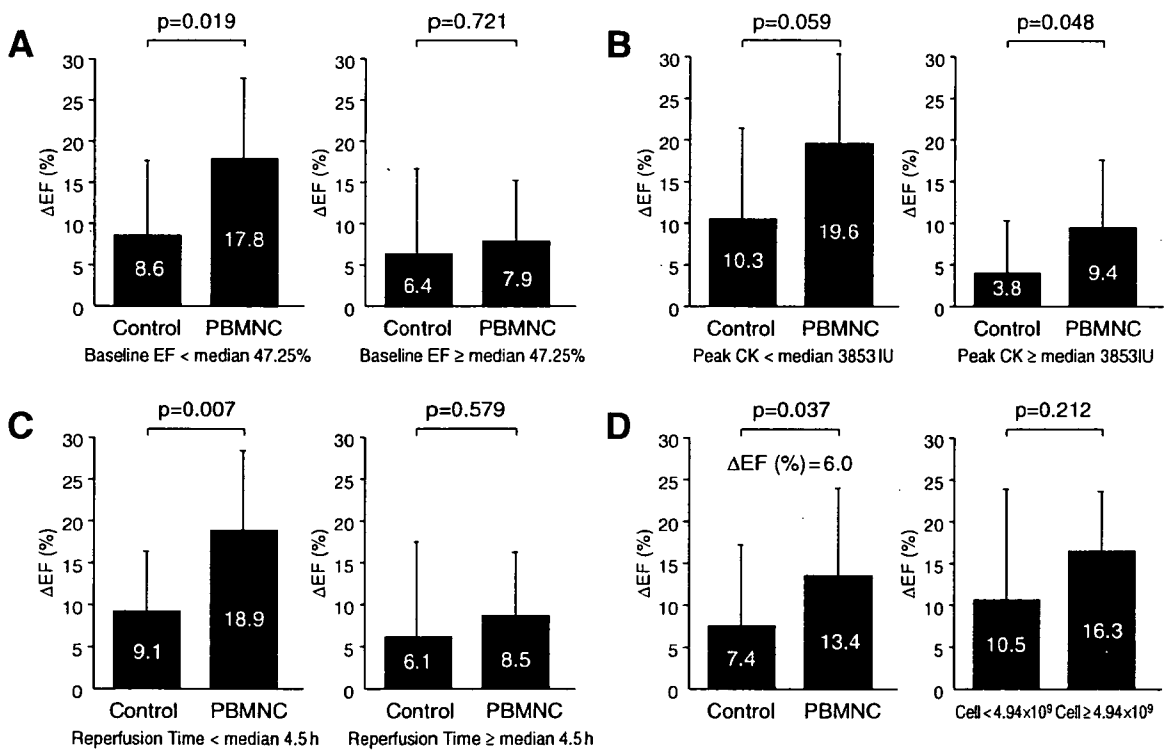

Fig 4. Impact of baseline ejection fraction (EF), peak creatine kinase (CK), reperfusion time, and transplanted cell number on cardiac function. All patients were divided into 2 groups by median baseline EF (A), median CK (B), median reperfusion time (C), and median transplanted cell number (D), and the treatment effect of peripheral blood mononuclear cell (PBMNC) infusion on the absolute increase in left ventricular EF (ΔEF) was analyzed. Data are mean±SD.3

tended to be greater in PBMNC group, compared with control (Fig 3).

Effects of Cell Transplantation on Other Parameters

Resting echocardiography indicated that cell transplantation significantly decreased the WMSI from baseline (1.67±0.18) to 6 months' follow-up (1.50±0.34) and this improvement in regional wall motion was especially seen in the infarct-related area of 15 of 18 patients. Resting ^{99m}Tc-tetrofosmin gated SPECT also showed that cell transplantation significantly decreased the perfusion defect score from baseline (20.4±9.0) to 6 months' follow-up (14.1±9.4), and this improvement in regional myocardial perfusion was seen in the infarct-related area of 14 of 18 patients. In con-

trast, cell transplantation did not significantly exacerbate but rather tend to decrease the Holter Lawn Class (data not shown).

Impact of Baseline Parameters and on Cardiac Function

We observed that cell transplantation significantly improved the ΔEF value, compared with controls (Fig 1). We further analyzed the effect of baseline parameters on the absolute increase in LVEF. In order to avoid our "arbitrary decision" on subgroup analysis, the total patient population was dichotomized according to the "median values" of baseline EF, peak CK, reperfusion time, and transplanted cell number at baseline, as previously reported[21] We then reanalyzed the data for addressing the clinical relevance of PBMNC administration.

We first examined the impact of baseline EF on cardiac function. When we divided all the patients into 2 groups by median baseline EF 47.25%, there was a significant interaction between the treatment effect of PBMNC infusion and the baseline EF. Among patients with a baseline EF below the median value, patients in the PBMNC group had an absolute increase in LVEF (ΔEF value) that was 2-fold that of the control group (Fig 4A) (absolute difference, 9.2%; 95% confidence interval (CI), 5.3 to 13.1). In contrast, among patients with a baseline EF at or above the median, the absolute difference between the 2 groups was only 1.5% (absolute difference, 1.5%; 95% CI, −2.1 to 5.1), suggesting that cell transplantation preferentially improved LV function in patients with relatively depressed contractility.

We next examined the impact of peak CK on cardiac function. When we divided all the patients into 2 groups by median peak CK 3,853 IU/dl, there was again a significant interaction between the treatment effect of PBMNC infusion and the peak CK. Among patients with a baseline peak CK at or above the median value, those in the PBMNC group had an absolute ΔEF value that was more than 2-fold the value for the control group (Fig 4B) (absolute difference, 5.6%; 95% CI, 2.8 to 8.4). Among patients with a baseline peak CK below the median, those in the PBMNC group also had an absolute ΔEF value that was ≈2-fold that in the control group, although there was not a significant difference in the ΔEF value between the 2 groups. The data suggest that cell transplantation preferentially improved LV function irrespective of infarct size.

We also examined the impact of reperfusion time on cardiac function. When we divided all the patients into 2 groups by a median reperfusion time of 4.5 h, there was again a significant interaction between the treatment effect of PBMNC infusion and the reperfusion time. Among patients with a baseline reperfusion time below the median value, those in the PBMNC group had an absolute ΔEF value that was almost 2-fold that in the control group (Fig 4C) (absolute difference, 9.8%; 95% CI, 6.1 to 13.5). In contrast, among patients with a baseline reperfusion time at or above the median, the absolute difference between the 2 groups was only 2.4% (absolute difference, 2.4%; 95% CI, −1.6 to 13.8), suggesting that cell transplantation preferentially improved LV function in patients with relatively early reperfusion.

We further examined the impact of transplanted cell number on cardiac function. When we divided the patients receiving cell therapy into 2 groups by a median cell number of 4.94×10^9, there was no significant interaction between the treatment effect of PBMNC infusion and the number of transplanted cells, suggesting that cell number did not significantly affect LV function, at least in our study (Fig 4D).

Clinical Manifestations and Adverse Effects

The occurrence of individual major adverse cardiac events of death, recurrence of myocardial infarction, or rehospitalization for heart failure did not differ significantly between the control and PBMNC groups. The rate of in-stent restenosis at the culprit lesion in patients who received PBMNC transplantation was 22.2%, which was not significantly different from that in the control patients (p=0.21).

Discussion

The major finding of the present study is that the intracoronary administration of non-expanded PBMNCs significantly enhanced the recovery of LV contractile function in patients optimally treated for AMI. After 6 months, the absolute increase in LVEF (ΔEF) was significantly higher in the PBMNC group than in controls. The enhanced recovery of LV contractile function after the administration of PBMNCs appeared to be related to a reduction in regional LV dysfunction within the territory of the infarct, because cell therapy resulted in a greater tendency of ΔRegional EF or significant improvement of WMSI and ^{99m}Tc-tetrofosmin perfusion defect score associated with the infarct area, compared with controls. Moreover, intracoronary administration of PBMNCs did not exacerbate LV expansion or high-risk arrhythmia after the infarction. Taken together, our findings indicate that when combined with optimal reperfusion therapy and standard medical treatment, intracoronary administration of PBMNCs is able to enhance the recovery of global and regional LV function after AMI.

Our results of subgroup analysis also provide some meaningful suggestions in the choice of patients for cell therapy against AMI; cell transplantation preferentially improved LV function in patients with relatively depressed contractility, irrespective of infarct size, and with relatively early reperfusion. Thus, patients with relatively early reperfusion and depressed LV contractile function had better improvement in contractile function after the intracoronary administration of PBMNCs. Our data therefore suggest that PBMNC transplantation may rescue dying myocytes that were severely stunned in the infarct border zone, irrespective of the infarct size.

Several lines of evidence suggest that the level of circulating CD34+ EPCs is predictive of future cardiovascular events;[30] and that bone marrow-derived CD34+ cells could be important for cardiovascular repair.[31] In the present study, we used a mean of 4.92×10^9 PBMNCs containing $\approx6\times10^6$ CD34+ cells for intracoronary injection and obtained an increase of 6% in ΔEF value. In the BOOST and REPAIR-AMI trials, $\approx2.5\times10^9$ unfractionated BMCs and $\approx2.4\times10^8$ Ficoll-separated BMCs (≈2–3×10^6 CD34+ cells) were transplanted, with increases of 6% and 2.5% in ΔEF values, respectively. In contrast, in Janssens's report and the ASTAMI trial, $\approx3\times10^8$ Ficoll-separated BMCs ($\approx2.8\times10^6$ CD34+ cells) and $\approx7\times10^7$ Ficoll-separated BMCs ($\approx0.7\times10^6$ CD34+ cells), respectively, were used, and there was no significant increase in ΔEF value. These data therefore indicate that the total number of injected cells or CD34+ cells does not always correlate with the improvement in cardiac performance after cell transplantation, although trans-