場所に余裕があるなら、区分別にそれぞれの場所を準備するのが望ましいが、場所に余裕がない場合は、原材料における未試験品置場、適合品置場、不適合品置場の保管について判別できるようにする。これ以降の保管および管理については、製造部門が担当する。

●不適合品の取り扱い
不適合品となった原材料は、適合品に混入しないよう「廃棄物処理手順書」等により、速やかに廃棄もしくは、別処理するような取り扱いの規定をする。

１．３．４　原料受入
CPC において製品を製造するとき、出発原料である組織などの受入管理方法に関して定める。

●輸送
原料の輸送に関して、適正な輸送方法を記載する。例えば、病院で採取された組織は、生理食塩水に浮遊させ、滅菌カップに入れ、更にパッキング式の輸送容器に入れて、冷蔵で輸送される等の記載となる。
CPC へ搬入する際の規定もあれば、記載しておくとよい。

●受入検査
受け入れ検査は、品質部門が担当し、必要な検査内容を記載する。また、「原材料規格書」の試験検査項目に基づいて、各検査項目の判定を行うことを記載する。

●解析，評価
受入検査の結果を解析し、評価の合否判定することを記載する。結果における解析方法や判定方法を記載する。

１．３．５　細胞数と生存率の測定
CPC において製品を製造する際、製造工程内で実施される品質管理試験の一項目として「細胞数と生存率」がある。この手順について定めることが必要である。また、工程検査としての生存率なども規定するとよい。通常の細胞の生存率としては、80%以上が望ましい。

１．３．６　微生物試験，無菌試験
品質試験等として「微生物試験、無菌試験」がある。この方法について手順を示す必要がある。また、その判定方法についても規定する。外部へ委託する場合も、それについて記載する。

１．３．７　検体及び中間体の保管
CPC において製品を製造する工程で生じる保管検体の手順、保管条件、保管期間について定める必要がある。また、培養細胞の一部を凍結保存する場合は、その凍結保存方法および取り扱いについて規定することが必要である。

１．３．８　試験の委託
品質管理者及び品質部門責任者は GMP に適合していることを評価し、委託先を選定する。また、秘密保持契約及び委受託契約を締結させ、委託先の施設を監査する権利を有することを記載する。試験検査にかかる時間や搬送時間等における検体の影響を考慮し、選定を行うことが必要である。

１．３．９　製品出荷判定
CPC で製造した製品の出荷判定方法について規定する。例えば、以下の規格を満たす場合、出荷することを品質部門責任者が判断する。
・原材料が規格を満たしていること。
・製造工程が正常に実施されていること。
・製造工程内の品質試験が規格を満たしていること。
・CPC 内の環境清浄度が保たれていること。
・最終製品の品質試験が規格を満たしているこ

と。
記録などを確認し、製品の規格が満たしていることを保障するものとなる「製品出荷報告書」などについても、記載する。

１．３．10　製品不適合品の取り扱い
製品の製造の不適格品取り扱いの管理（廃棄や移動など）、品質管理の試験不適合時取り扱いに関する基本的要件を定め、記載する。また、不適合品の廃棄に関する手順についても規定して記載することが望ましい。

１．３．11　品質管理教育訓練
CPC 内で製造する製品について、直接品質管理試験に従事する品質部門の者は、その作業内容に関して、適切な教育訓練を受けた上で実務に当たる必要がある。そのため、必要な知識及び技術の習得手順を示す必要がある。また、品質管理試験に従事する者は定期的に力量を評価・判定することが望ましい。

１．３．12　各種機器取り扱い
品質試験に必要な機器の取り扱いについて、手順を定める必要がある。例えば、定量用 PCR、フローサイトメトリー、マイコプラズマ否定試験用 PCR、エンドトキシン濃度測定用機器 等 について使用方法の手順を定める必要がある。

１．４　製造管理

１．４．１　製造標準書
製造する製品についての基本的な基準や製法を記載する書類である。
以下に記載必要項目を挙げる。

●品名
製品の名称で、1 品につき、1 つの名称とすること。

●形状及び特徴
形状としては、細胞形状や採取条件などがある。特徴としては、生存率や特徴的なマーカーなどがある。

●原料、中間体、製品の規格及び試験方法
原料は細胞の最初の状態のもので、体性幹細胞などは採取した組織などになり、その規格や試験項目を記載する。中間体は各工程で得られる状態のもので、各々の工程における規格や試験項目を記載する。最終製品についての規格や試験項目も記載する。

●製造方法
概要を記載するが、工程を明確にする。詳細については、各 SOP 等に記載する。

●製品の保管及び使用期限
最終製品や場合によっては原料・中間体の保管条件及び使用期限の規格を記載する。

１．４．２　製造依頼
CPC において製品を製造するため、ユーザーからの製造依頼の受理から製造開始までの工程の管理に関する基本的要件を定める。製造を開始する元となるべき情報の管理である。例えば、「製造依頼書」などを持って依頼の管理を行い、「製造計画書」を持って開始の管理とする。

１．４．３　CPC の環境確認
CPC において製品の製造を開始する際、CPC 内の環境が基準値を満たしているか確認する方法を規定する必要がある。つまり、製造部門責任者が製造計画書を発行する際に確認する手順である。製造部門責任者は、CPC 環境が基準値を満たしていることを確認してから「製造計画書」を発行する。また、CPC 環境を清浄に保つため日頃より清掃・消毒を実施することが必要である。

１．４．４　製造計画書の作成

CPC において製品を製造する際、製造依頼の受理、製造計画の立案及び日程作成、製造終了までの工程管理に関する基本的要件を定め、作成するものである。

１．４．５　製造指図書及び記録書

製造指図書・記録書の発行、承認、保管について規定する必要がある。

●製造指図書

指図書は、製造番号ごとに発行され、製造部門責任者の指示を受け、製造部門担当者が発行する。指図書には少なくとも、製造番号、原材料のロット番号、サンプリングする指図量、その他製造において必要な事項などを記載することが必要である。製造部門責任者は、記入内容を確認し、指図に誤りがないことを確認および承認する。

●製造記録書

製造工程から出荷までの全ての作業内容は、製造記録書に記録される。当日の作業内容は、製造部門の記録担当者（確認者）が記録する。すべての作業終了後、記録された記録書は、製造部門責任者によって照査した上で承認される。

記録された記録書は承認後、製造部門にて 10 年間保管される。

●ラベル

CPC において製品の製造工程内で採取される検体に貼付するラベルの発行及び使用について定めるものである。ラベルは、製造工程内で採取する検体のクロスコンタミネーションを防止するためのものである。

ラベルに限定する必要性はないが、CPC 内での作業で塵埃が出ないことやエタノールなどの消毒液で文字が消えないこと等を考慮して、ラベルを使用する方が良いと考える。

１．４．６　製造時環境測定

CPC において製品を製造する際、製造作業時の CPC 内環境モニタリング及び無菌操作終了直後の作業者のモニタリング方法について規定する必要がある。基本的には、浮遊性微粒子(パーティクル)と落下菌・付着菌・浮遊菌の測定を行う手順について記載する。

例えば、浮遊性微粒子については、作業室内と安全キャビネット内の作業前と作業後に測定を 3 回ずつ行い、その平均値を規格値と比較して判断する。浮遊菌については、作業後に安全キャビネット内の空気を回収し、培養の後、判定する。落下菌については、作業前に安全キャビネット内に寒天培地をセットし、作業後に回収して、培養の後、判定する。付着菌については、作業後に細胞を扱う手などから採取し、培養の後、判定する等の手順を定める。

また、CPC 内の温･湿度に関しても、作業前にが設定された範囲内であることを確認の上、作業にあたることが望ましい。

１．４．７　原材料の発注・受入・出庫管理

CPC において製品を製造するために使用する原材料等の発注・出庫及び保管、管理に関する事項を定める。例えば、以下のような内容となる。

・発注　→　・入荷　→　・規格試験（受入検査）　→　・入庫　→　・出庫

上記の内容を管理し、特に品質及び数量について、しっかり把握することが必要である。

１．４．８　検体採取

製造工程内で採取される製品の品質管理試験用検体・工程検査用サンプルに関して、分注、識別および検体の搬送方法について記載する。

１．４．９　細胞培養

培地調整・原料からの分離もしくは採取・細胞培養・培地交換・細胞継代・分化・凍結等の全般について、SOP を用意し、各工程の詳細を記載する。各工程に使用される原料，副原料，資材，機器については、品名，メーカー，ロットや製品番号（製品を特定する番号）等を記載する。

1．4．10　製品出荷包装・輸送

CPC において製品を製造した最終製品の出荷方法である梱包、輸送方法および製品引渡し方法について定める。包装及び梱包に関しては、グレードA の環境下である細胞処理室の安全キャビネット内で無菌的に実施するところから記載する。

1．4．11　廃棄物処理

CPC において製品を製造する際に発生する廃棄物処理の手順について記載する。例えば、廃棄物の定義や種類（感染性、非感染性廃棄物など）、廃棄物搬出手順等について、記載する。

1．4．12　製造管理教育訓練

新たに CPC 内で作業する者はもちろんのこと、通常CPC内で製造作業に従事する者も教育を受け、技能の判断・判定することが必要ある。これにより、作業者による製品の劣化や逸脱を防ぎ、高品質な製品を製造し続けることができるのである。

製品となる細胞の種類や使用 CPC によっては、必要ない項目もある可能性もあるが、基本的に上記の衛生・品質・製造管理において、記載のある項目は、標準作業手順書（SOP）を作成する必要がある。

D．考察

GMP ではハードウエアとソフトウエアの両立が必要で、ハードとしての施設・設備・機器と、ソフトとしての文書・製造・試験方法・清掃・組織・教育訓練を両輪として初めて成り立つ品質保証システムであると言える。試験物製造と非臨床試験をそれぞれの段階（相）に応じて品質管理・品質保証を行うことが肝要で、いわゆる品質の作りこみのため、本調査結果を参考にして研究開発が進められると信じる。

E．結論

承認申請書の記載事項はすなわち承認事項となるため、申請書の製造方法欄に操作条件などの具体的な管理値やパラメーターを記載してしまうと逸脱が薬事法違反になってしまうため、承認申請書と製品標準書（GMP）の違いには、配慮が必要である。申請書には製造工程の一連の操作手順のうち品質の恒常性確保の為に必要な事項を選択して記述すべきで、申請書には「目標値/設定値」を記載し、実際の管理範囲は製品標準書に記載しGMP で管理することを考慮すべきであろう。

F．健康危険情報

該当なし

G．研究発表

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２．学会発表

該当なし

H．知的財産権の出願・登録状況(予定を含む)

１．特許取得

該当なし

２．実用新案登録

該当なし

３．その他

該当なし

Ⅲ．研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表

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Ⅳ. 研究成果の刊行物・別刷

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Intracoronary artery transplantation of cardiomyoblast-like cells from human adipose tissue-derived multi-lineage progenitor cells improve left ventricular dysfunction and survival in a swine model of chronic myocardial infarction

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Transplantation

ABSTRACT

Transplantation of human cardiomyoblast-like cells (hCLCs) from human adipose tissue-derived multi-lineage progenitor cells improved left ventricular function and survival of rats with myocardial infarction. Here we examined the effect of intracoronary artery transplantation of human CLCs in a swine model of chronic heart failure. Twenty-four pigs underwent balloon-occlusion of the first diagonal branch followed by reperfusion, with a second balloon-occlusion of the left ascending coronary artery 1 week later followed by reperfusion. Four weeks after the second occlusion/reperfusion, 17 of the 18 surviving animals with severe chronic MI (ejection fraction <35% by echocardiography) were immunosuppressed then randomly assigned to receive either intracoronary artery transplantation of hCLCs hADMPCs or placebo lactic Ringer's solution with heparin. Intracoronary artery transplantation was followed by the distribution of DiI-stained hCLCs into the scarred myocardial milieu. Echocardiography at post-transplant days 4 and 8 weeks showed rescue and maintenance of cardiac function in the hCLCs transplanted group, but not in the control animals, indicating myocardial functional recovery by hCLCs intracoronary transplantation. At 8 week post-transplantation, 7 of 8 hCLCs transplanted animals were still alive compared with only 1 of the 5 control ($p = 0.0147$). Histological studies at week 12 post-transplantation demonstrated engraftment of the pre DiI-stained hCLCs into the scarred myocardium and their expression of human specific alpha-cardiac actin. Human alpha cardiac actin-positive cells also expressed cardiac nuclear factors; nkx2.5 and GATA-4. Our results suggest that intracoronary artery transplantation of hCLCs is a potentially effective therapeutic strategy for future cardiac tissue regeneration.

1. Introduction

End-stage heart failure remains a major cause of death worldwide, mainly due to myocardial ischemia. Cardiac transplantation and mechanical support using implantation of the left ventricular assist system (LVAS) were established as the ultimate means of support for these patients [1,2]. However, these treatment entities have certain limitations including donor shortage, rejection, and LVAS durability, and alternative strategies are needed in such circumstances.

Cellular cardiomyoplasty was developed as a new approach to restore normal heart function, [3,4] using a variety of cell types [3–5]. Mesenchymal stem cells (MSC) seem particularly advantageous for cellular therapy in general because they are multipotent, potentially immune privileged [6]. MSC also proliferate rapidly and differentiate into cardiomyogenic cells [7–10]. MSC can be isolated from human adipose tissue, which can be resected easily and safely in most patients [11,12]. In fact, we have reported that adipose tissue-derived multilineage progenitor cells (ADMPCs), which met the criteria as mesenchymal stem cells [13], can differentiate into hepatocytes both *in vitro* and *in vivo* [14,15]. Recently, we demonstrated that human cardiomyoblast-like cells (hCLCs) from human adipose tissue-derived multi-lineage progenitor cells transplanted into rats with chronic myocardial infarction reversed wall thinning

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in the scarred area with the engrafted cells forming a thick stratum, and that the hCLCs reversed left ventricular dysfunction in the long term and survival of rats with experimentally-induced myocardial infarction [16].

The present study is an extension to the above study and was designed to accelerate the clinical application of hCLCs. Specifically, we examined in pre-/non-clinical studies the effects of hCLCs transplantation on cardiac dysfunction and on long-term survival with swine chronic myocardial infarction model. We also documented the histological regeneration of damaged myocardium after transplantation of hCLCs *in vivo*.

2. Materials and methods

2.1. Adipose tissue

Adipose tissue samples were resected from five human subjects during plastic surgery (all females, age, 20–60 years) as excess discards. Ten to 50 g of subcutaneous adipose tissue were collected from each subject after obtaining of informed consent. The protocol was approved by the Review Board for Human Research of Kobe University Graduate School of Medicine, Osaka University Graduate School of Medicine and Foundation for Biomedical Research and Innovation.

2.2. Isolation of hADMPCs and preparation of hCLCs

Human adipose tissue-derived multi-lineage progenitor cells (hADMPCs) were prepared as described previously [13–17]. After passaging 5 to 6 times, the hADMPCs were replated and treated with 0.1% dimethyl sulfoxide (DMSO) (Cryoserve, GE Healthcare Biosciences, Uppsala, Sweden) for 48 h.

2.3. Reverse transcriptase–polymerase chain reaction

Total RNA was isolated from hADMPCs and cardiomyoblasts using an RNAeasy kit (Qiagen, Hilden, Germany). After treatment with DNase, cDNA was synthesized from 500 ng total RNA using Superscript III reverse transcriptase RNase H minus (Invitrogen, Carlsbad, CA). Real-time PCR was performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). 20X Assays-on-Demand™ Gene Expression Assay Mix for *nkx2.5* (Hs00231763_m1), *islet-1* (Hs00158126_m1), *GATA-4* (Hs00171403_m1), *alpha-cardiac actin* (Hs01109515_m), *cardiac troponin I* (Hs00165957_m1), *myosin light chain* (*MLC*) (Hs00166405_m1), *myosin heavy chain* (*MHC*) (Hs00411908_m1) and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) (Hs99999905_m1) were obtained from Applied Biosystems. TaqMan® Universal PCR Master Mix, No AmpErase® UNG (2X), was also purchased from Applied Biosystems. Reactions were performed in quadruplicate and the mRNA levels were normalized relative to human GAPDH expression. Then the fold-inductions of hCLCs were compared to hADMPCs.

2.4. Animal model of myocardial infarction and cell transplantation

Five weeks before transplantation, the first diagonal branch (D1; #9) of the coronary arteries of 24 pigs (8-week-old female, 30.5 ± 0.7 kg, mean ± standard error of the mean) was balloon-occluded for 60 min followed by reperfusion, (Fig. 1A). One week later, the left ascending coronary artery of the same animals was balloon-occluded just proximal of the first septal branch divergence (#6), followed by reperfusion (Fig. 1A). To rescue the better baseline survivals and to obtain severe old myocardial infarction swine model, two separate reperfused infarcts one week apart were performed. From 5 days before cell transplantation to the end of the experiment, the swine received tacrolimus as an immunosuppressant (0.1 mg/kg/day intramuscularly) (Fig. 1B) as previously reported [18] with modification. Four weeks after the second occlusion/reperfusion (day 0), we examined 17 animals with chronic severe MI (ejection fraction <35% by echocardiography) of only 18 survivors. The tacrolimus-immunosuppressed chronic MI swine were randomly assigned to receive intracoronary transplantation of hCLCs (3×10^5 cells/mL concentration of cell

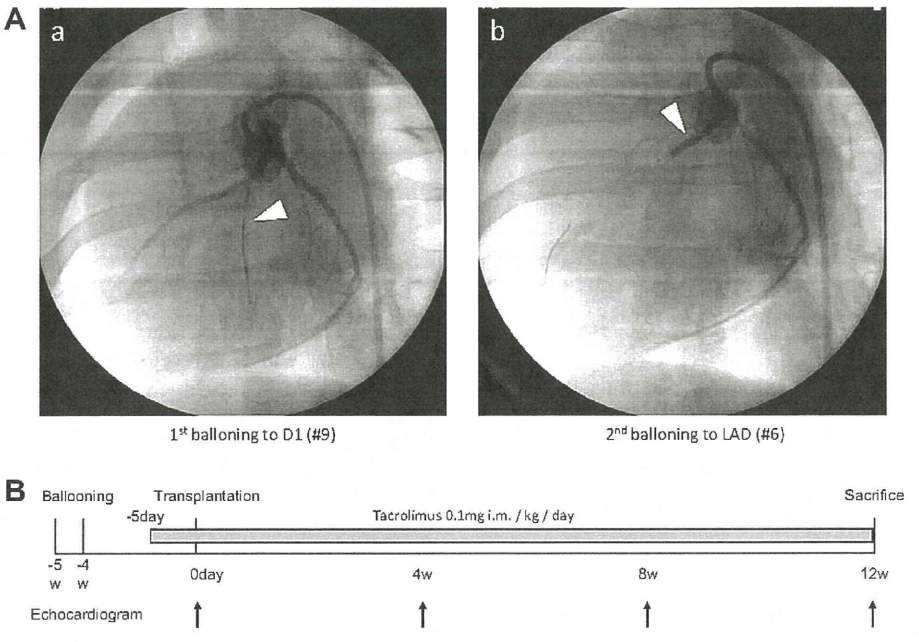

Fig. 1. Study protocol and angiographic demonstration of transient coronary artery occlusion. (A) Five weeks before transplantation, the first diagonal branch (D1; #9) of the coronary arteries was balloon-occluded followed by reperfusion (a, arrowhead). One week later, the left ascending coronary artery of the same animals was balloon-occluded just proximal of the first septal branch divergence (#6), followed by reperfusion (b, arrowhead). (B) From 5 days before cell transplantation to the end of the experiment, the swine received tacrolimus as an immunosuppressant. At day 0, 17 animals with chronic severe MI were applied for the experiment.

Table 1
Cardiocytic induction of hCLCs.

	Fold induction	
	Mean	SE
nkx2.5	2.49	1.02
islet-1	1.32	0.36
GATA-4	6.84	1.47
Alpha-Cardiac actin	1.46	0.22
Cardiac troponin I	2.36	0.47
Myosin light chain	1.89	0.49
Myosin heavy chain	109.89	6.13

suspension, 1 mL/kg cell suspension was transplanted.) ($n = 8$), hADMPCs (3×10^5 cells/mL concentration of cell suspension, 1 mL/kg cell suspension was transplanted.) ($n = 4$), or placebo lactic Ringer's solution with heparin ($n = 5$), at 4 weeks after the second occlusion/reperfusion. Transplantation procedure was performed as following, the transarterial catheter was placed in the left coronary artery, and then the cell-suspensions or placebo control solutions were transplanted into LAD (#6). The Osaka University Graduate School of Medicine Standing Committee on Animals approved all experimental protocols.

2.5. Assessment of swine cardiac function and histological analysis

Cardiac ultrasound studies were performed before cell-transplantation and at 4, 8 and 12 weeks after transplantation using a VIVID 7 system (GE Healthcare Biosciences, Uppsala, Sweden) and the data at the day transplantation, 4- and 8-week-after transplantation were applied for the statistical analysis. The studies were shown as M-mode with short axis view observed from left fifth intracostal space.

For histological analysis, the swine hearts were dissected out at the end of the experiment and immediately fixed overnight in 4% paraformaldehyde and processed for embedding in paraffin wax. Sections were cut at 3-μm thickness, deparaffinized and then rehydrated through a graded ethanol series into distilled water. The sections were then immersed in Target Retrieval Solution (Dako, Glostrup, Denmark) and boiled, followed by cooling at room temperature for 20 min. Sections were incubated overnight with 10% blocking solution (Nacalai tesque) in TBS-T, and then in a humidity chamber for 16 h at 4 °C with mouse monoclonal antibodies to human alpha-cardiac actin (American Research Products., Belmont, MA), human myosin heavy chain (MHC) (mouse monoclonal anti-human myosin heavy chain cardiac antibody, Cat: 05-833., Upstate, NY) and CD34 (ab81289 [EP373Y], Abcom) diluted in blocking solution, followed by Alexa Fluor 488-labeled anti- IgG (Molecular Probes, Eugene, OR) with counter DAPI-staining. Hematoxylin and eosin stain, Masson trichrome stain and Sirius red stain were also performed. The stained all slides were viewed on a Bio-Zero laser scanning microscope (Keyence, Osaka, Japan). The scarred area percentages of the middle portion and apex side of LV were calculated by area stained blue with Masson's trichrome staining/total of 10 each independent sections using software Dynamic Cell Count (Keyence, Osaka, Japan).

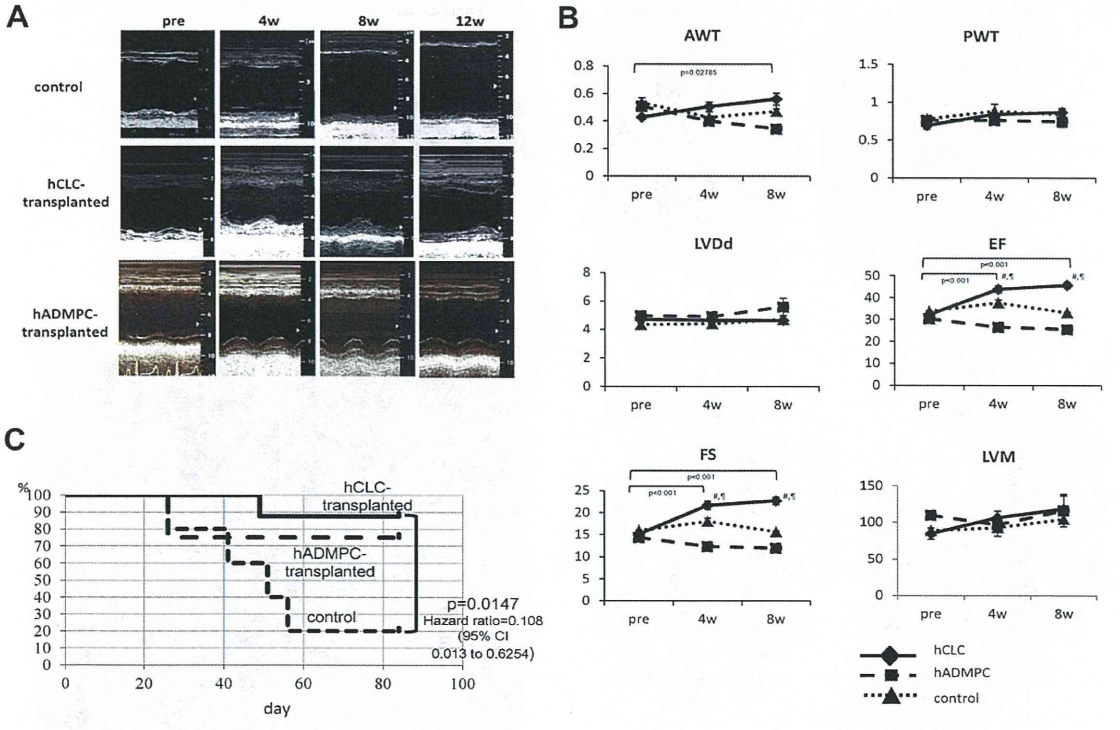

Fig. 2. Effects of hCLCs transplantation on cardiac function and survival rate. (A) In the hCLCs transplanted group, M-mode echocardiography showed improved wall motion within 4 weeks of transplantation. In contrast, worsening of the wall motion was noted in the mock-transplanted control swine. (B) Anterior wall thickness (AWT), ventricular ejection fraction (EF) and fractional shortening (FS) improved significantly in the hCLCs transplanted group, as confirmed by echocardiography. In the hCLCs transplanted swine, cardiac functions were recovered from transplantation to the end of the study. In the hADMPCs transplanted swine, cardiac functions were maintained from transplantation to the end of the study. In contrast, worsening of these cardiac function parameters was noted after mock-transplantation. The left ventricular diastolic dimension (LVDd) was maintained during the course of the experiment in hCLCs transplanted swine, but increased in the control groups. Posterior wall thickness (PWT) and left ventricular mass (LVM) showed no significant difference in the groups. Solid lines and squares indicated the transplanted group and the dashed lines and open squares indicated the control group. The symbol # indicated $p < 0.01$ hCLCs -transplanted vs control and indicated $p < 0.01$ hADMPC-transplanted vs control, respectively. Bars indicated mean ± standard error of the mean (SEM). (C) Effect of hCLCs transplantation ($n = 8$), hADMPCs transplantation ($n = 4$) and lactic Ringer's solution injection ($n = 5$) on long-term survival rates of swine. Kaplan–Meier survival curve analysis demonstrated significant difference in the survival rates between the hCLCs group and the lactic Ringer's solution group.

2.6. Statistical analysis

Longitudinal changes between groups were tested with the use of mixed-model repeated-measures analysis of variance, with adjustment for baseline values. When the overall *P* value for the main effect of group or time, or interaction between group and time was less than 0.05, the post hoc multiple comparisons with the use of the single-step adjustment method as implemented by Hothorn et al. were performed [19]. Survival curves were constructed by the Kaplan–Meier method and survival among groups was compared using the Log-Rank test (StatMate III for Windows, Atoms, Tokyo).

3. Results

3.1. Cardiocytic commitment of hADMPCs into hCLCs

The potential for hADMPCs to commit into CLCs was evaluated from the mRNA expression of several cardiocytic markers by quantitative reverse transcriptase-PCR before and after DMSO induction, as follows: *islet-1* is a cardiac stem cell marker; *nkx2.5* and *GATA-4* are transcription factors required for subsequent cardiac differentiation; and *alpha-cardiac actin*, *myosin light chain* (*MLC*), and *myosin heavy chain* (*MHC*) are markers of cardiocytic commitment (Table 1). After induction, hADMPCs expressed all markers with increment, indicating that hADMPCs could be successfully committed into cells of the cardiac lineage, hCLCs.

3.2. Effects of hCLCs transplantation on cardiac function and survival rate

Cardiac function was assessed by echocardiography. Four weeks after intracoronay transplantation of hCLCs, wall motion was improved but not in the placebo group (Fig. 2A). The wall motion of control swine worsened at 12 weeks after transplantation, while the improved motion was maintained after the hCLCs transplant (Fig. 2A). In the early post-transplantation period, there was no significant difference in left ventricular diastolic dimension (LVDd) between hCLCs -transplanted swine and the control. During the course of the study, LVDd exacerbated gradually in the control swine while it did not change significantly in the transplant swine (Fig. 2B). Likewise, the left ventricular ejection fraction (EF) and fractional shortening (FS) improved in the implanted group, but not in control swine (Fig. 2B). After hCLCs transplantation via left anterior descending (#6), the anterior wall thickness improved in the implanted group, but not in control swine. These results indicate that intracoronary transplantation of hCLCs resulted in recovery of cardiac function.

The Kaplan–Meier survival curves showed higher long-term survival rates for the hCLCs transplanted group than the control (Fig. 2C). Notably, only 1 of 8 swine died after transplantation of hCLCs. Survival at 12 weeks after transplantation was significantly higher in the hCLCs group (87.5%) than the control group (20%, 1 of 5) (Log-rank test: $p = 0.0147$. Hazard ratio = 0.108: 95% CI 0.013 to 0.625). These results suggest that transplantation of hCLCs

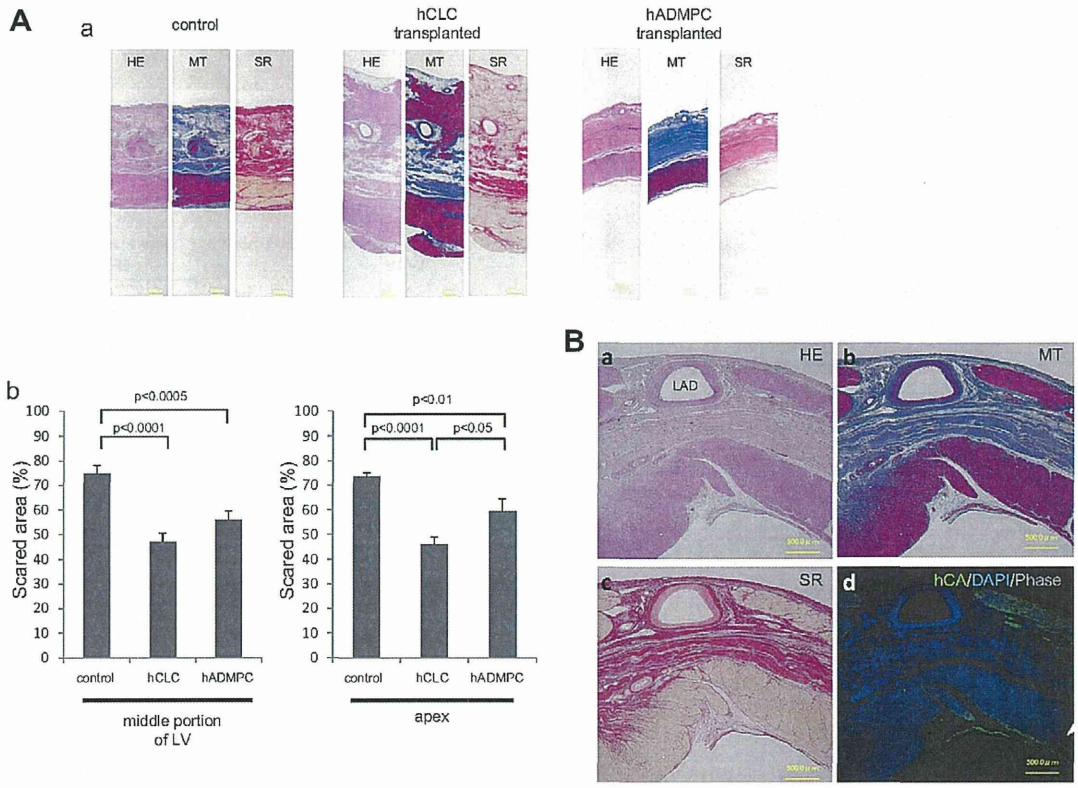

Fig. 3. Effects of hCLCs transplanted via coronary artery on cardiac structure. (A) (a) Photomicrographs of representative myocardial sections of the scarred area stained with hematoxylin/eosin (HE), Masson trichrome (MT) and Sirius red (SR) in the hCLCs-, hADMPC-transplantation and mock-transplanted control groups. Transplantation of hCLCs improved myocardial wall thickness in the infarcted myocardium and resulted in the development of new cardiac muscles on the surface. Bars = 500 μm. HE; hematoxylin and eosin staining, MT; Masson trichrome staining, and SR; Sirius red staining. (b) The scarred area percentages of the middle portion and apex side of LV. The scarred area percentages of hCLCs-, hADMPC-transplantation and control groups were calculated by area stained blue with Masson's trichrome staining /total of 10 independent sections. The error bar indicated SEM. (B) Photomicrographs of representative myocardial sections of apical side of the anterior wall stained with HE (a), MT (b), SR (c) or phase contrast merge image of neighboring sections stained with anti-human alpha-cardiac actin (hCA; green), and DAPI as counter staining (d). In the HE-, MT-, and SR-stained sections, cardiac muscles were distributed on the scarred areas, and some parts of these muscles expressed human alpha-cardiac actin (green). Bars = 500 μm. LAD; left anterior descending.

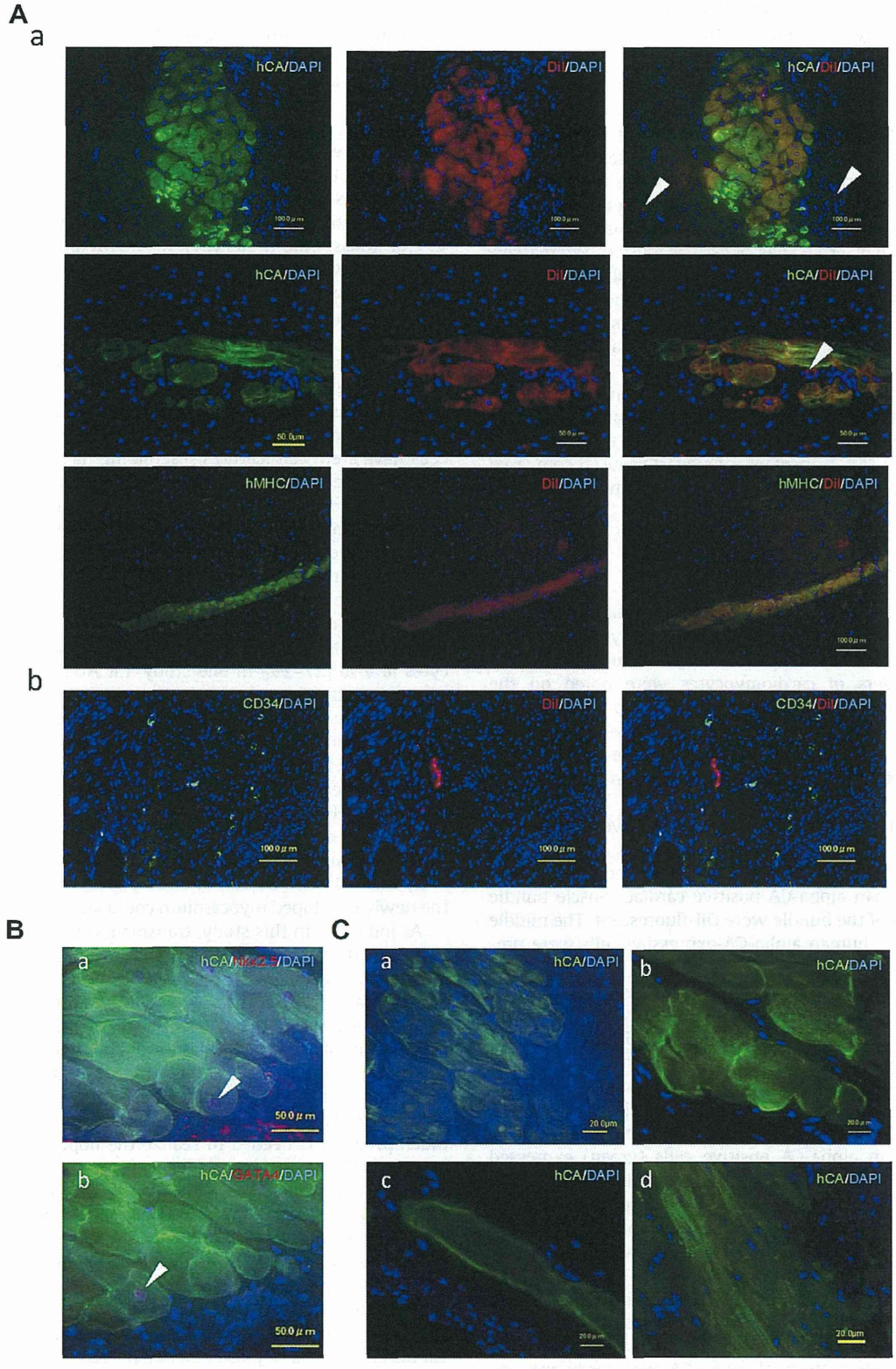

Fig. 4. hCLCs survive *in situ*. (A) (a) *In situ* survivals of the fluorescent DiI-prestained hCLCs into cardiomyocytes at 12-week- after transplantation. Note the presence of human alpha-CA positive cardiac muscle bundles or cells and that almost all cells exhibit DiI-fluorescence. Only minor part of DiI-positive cells did not express human alpha-CA (arrowheads). DiI-prestained cells were also positive for human myosin heavy chain (lower panel). (b) Survivals of hCLCs outside of vessel capillaries. The vessel capillaries were stained with anti-CD34 antibody and localization of DiI-positive cells were examined using fluoromicroscpy. DiI-positive cells exist outside of vessel capillaries which were stained with anti-CD34 antibody (B) Co-expression of human alpha-CA (green) and Nkx2.5 (purple) (a) or GATA-4 (purple) (b) in the nuclei of human alpha-CA positive cells. (C) Typical expression patterns of human alpha-CA on the cells. Human alpha-CA exhibited a brush pattern in oval cells (a), a spot pattern in cell-to-cell contact areas (b), as a sarcomeric structure beneath around the cell surface (c), and in a pattern resembling cardiomyocytes (d). Bars = 20 μm.

improves long-term survival rate of swine with heart failure induced by chronic myocardial infarction.

3.3. Effects of hCLCs transplantation on cardiac structure

Twelve weeks after transplantation, the treated swine were sacrificed and cardiac tissues prepared for histological examination for further analysis of cardiac structure and delineate the difference between hCLCs transplanted animals and controls (Fig. 3). Hematoxylin/eosin, Masson's trichrome and Sirius red staining showed the presence of a thin layer of cardiac muscles and massive fibrosis in the scarred anterior left ventricular wall of the control and hADMPCs transplanted swine (Fig. 3Aa). In contrast, the same staining techniques in hCLCs -transplanted swine showed significant thickening of the infarcted myocardium and layers of cardiomyocytes on the anterior ventricular wall (Fig. 3Aa). Next, to confirm the hCLCs could rescue from the fibrosis on cardiac structure, the scarred area percentages of the middle portion and apex side of LV were calculated. As shown in Fig. 3Ab, the percentage of scarred area of hCLCs -transplantation heart reduced compared to the control swine heart and hADMPC-transplanted one in both middle portion of LV and apex side.

3.4. hCLCs integrated in situ with the cardiac milieu

The *in situ* differentiation capacity of the implanted hCLCs into cardiomyocytes after grafting onto the scarred myocardium was assessed by immunohistochemical staining for human alpha-CA (Fig. 3B). Thin layers of cardiomyocytes were noted on the scarred myocardium by hematoxylin and eosin staining and Masson trichrome staining. Furthermore, clusters of human alpha-CA-positive cells were identified on the scarred myocardium (Fig. 3B; Green, arrowhead), indicating that hCLCs might integrate *in situ* with the cardiac milieu.

To confirm that the transplanted hCLCs survived *in situ*, we chased the fluorescent DiI-prestained hCLCs *in situ* 12 weeks after transplantation using histochemical technique. The top panel of Fig. 4Aa shows human alpha-CA positive cardiac muscle bundle and almost all cells of the bundle were DiI-fluorescent. The middle panel shows that all human alpha-CA-expressing cells were prestained DiI-fluorescent. DiI-prestained cells were also positive for human myosin heavy chain (Fig. 4Aa lower panel). On the other hand, DiI-positive cells exist outside of vessel capillaries which were stained with anti-CD34 antibody (Fig. 4Ab). Since cardiomyocytes are known to express the nuclear transcriptional factors; Nkx2.5 and GATA-4, we examined the expression of these molecules on human alpha-CA positive cells. The nuclei of human alpha-CA positive cells (green) expressed Nkx2.5 (purple) (Fig. 4Ba) and those of human alpha-CA positive cells (green) expressed GATA-4 (purple) (Fig. 4Bb), adding further confirmation that hCLCs might differentiate into cardiac marker positive cells.

The expression patterns of human alpha-CA on the cells were presented in Fig. 4C. The first pattern of human alpha-CA expression was the brushed pattern in oval-shaped cells (Fig. 4Ca). Alpha-CA also showed a spot pattern in the cell-to-cell contact areas (Fig. 4Cb). Resident alpha-CA-like immunoreactivity also appeared as sarcomeric structure beneath and around the cell surface (Fig. 4Cc). The fourth pattern of alpha-CA was cardiomyocyte structure-like pattern (Fig. 4Cd). These results indicate that hCLCs survive *in situ* and integrate into the cardiac milieu.

4. Discussion

There are several advantages to intracoronay transplantation of hCLCs for regeneration therapy. First, the source of adipose-derived cells is easily and safely accessible and large quantities of the cells can be obtained without serious ethical issues. Second, hCLCs can survive *in vivo* within the myocardial milieu. Finally, the reconstruction of a thick myocardial wall rescued cardiac dysfunction after chronic myocardial infarction and improved long-term survival in our swine model.

The choice of cell source is critical for realizing success in cellular therapy [19,20]. The adipose tissue is easily and safely accessible without serious ethical issues, and the cells can be obtained in large quantities since liposuction surgeries yield from 100 ml to >3 L of lipoaspirate tissue [21]. In the literature, isolation of cells from adipose tissue was first described by Bjorntorp et al. [22]. This procedure was then modified for the isolation of cells from human adipose tissue specimens [23–25]. In this context, Zuk et al. [11] reported the presence of cells with properties resembling those of mesenchymal stem cells resident in adipose tissue and they renamed the cell populations as adipose tissue-derived stromal/stem cells (ADSC). Recently, we have reported hADMPC as a novel cell population in human adipose tissue and indicated that these cells have stem cell features resembling mesenchymal stem cells including their ability to differentiate into cardiomyocytes in rat infracted cardiac milieu, into hepatocytes in rabbit hepatic milieu *in situ*, and into clusters of islet-like cells and hepatocytes *in vitro* [13–16]. Based on the above advantages, hADMPCs represent a potentially promising source of cells for cellular therapy, including patients with severe heart failure.

While the differentiation of ADSCs *in vitro* has been reported [26], only a few studies reported their differentiation into cardiomyocytes *in vivo* [27–29]. In one study, rat ADSCs were isolated and grown in intact monolayer sheets using temperature-responsive culture dishes. Placement of the rat ADSC sheets onto scarred myocardium in rats reduced the scarring and enhanced cardiac structure and function. Histological analysis demonstrated that the engrafted rat ADSC sheets grew to form a thickened layer that included newly formed vessels and few cardiomyocytes. In this context, Gimble et al. [20] suggested that hADSCs might secrete angiogenic factors. In our previous study, hCLCs survived within the rat myocardial milieu *in vivo*, as indicated by immunohistological results, suggesting that the newly developed myocardium could augment cardiac function.

As indicated in this study, transplantation of the hCLCs via the coronary artery resulted in the development of a new thick myocardial tissue, rescued cardiac dysfunction after MI in the swine model, and improved long-term survival rate compared to the control. Our findings suggest that hCLCs can be engrafted and survive within the myocardial infarct milieu, acquire phenotypic markers consistent with cardiomyocytic lineages, and have a positive impact on structural and functional endpoints. These are desirable outcomes for cardiac function and survival. Despite these encouraging results, much progress is needed to realize the hope of cell therapies for myocardial damage. First, delivery of the cell number to patients should be optimized for each given disease. Second, the risk–benefit based approach should be considered in the infracted or affected tissues after transplantation. Finally, the value and impact of hCLCs -transplantation should be confirmed in Investigational New Drug approval before embarking on clinical trials and applications.

In conclusion, we showed that the hCLCs were successfully engrafted into the scarred myocardium. The hCLCs -transplantation via the coronary artery also resulted in recovery of cardiac function and improved survival rate. Thus, transplantation of hCLCs in heart patients is a potentially effective therapeutic strategy for cardiac tissue regeneration within a few years.

Acknowledgments

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Human adipose tissue-derived multilineage progenitor cells exposed to oxidative stress induce neurite outgrowth in PC12 cells through p38 MAPK signaling

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Abstract

Background: Adipose tissues contain populations of pluripotent mesenchymal stem cells that also secrete various cytokines and growth factors to support repair of damaged tissues. In this study, we examined the role of oxidative stress on human adipose-derived multilineage progenitor cells (hADMPCs) in neurite outgrowth in cells of the rat pheochromocytoma cell line (PC12).

Results: We found that glutathione depletion in hADMPCs, caused by treatment with buthionine sulfoximine (BSO), resulted in the promotion of neurite outgrowth in PC12 cells through upregulation of bone morphogenetic protein 2 (BMP2) and fibroblast growth factor 2 (FGF2) transcription in, and secretion from, hADMPCs. Addition of *N*-acetylcysteine, a precursor of the intracellular antioxidant glutathione, suppressed the BSO-mediated upregulation of BMP2 and FGF2. Moreover, BSO treatment caused phosphorylation of p38 MAPK in hADMPCs. Inhibition of p38 MAPK was sufficient to suppress BMP2 and FGF2 expression, while this expression was significantly upregulated by overexpression of a constitutively active form of MKK6, which is an upstream molecule from p38 MAPK.

Conclusions: Our results clearly suggest that glutathione depletion, followed by accumulation of reactive oxygen species, stimulates the activation of p38 MAPK and subsequent expression of BMP2 and FGF2 in hADMPCs. Thus, transplantation of hADMPCs into neurodegenerative lesions such as stroke and Parkinson's disease, in which the transplanted hADMPCs are exposed to oxidative stress, can be the basis for simple and safe therapies.

Keywords: Human adipose-derived multilineage progenitor cells, Adult stem cells, Reactive oxygen species, p38 MAPK, Neurite outgrowth, BMP2, FGF2, Neurodegenerative disorders

Background

Mesenchymal stem cells (MSCs) are pluripotent stem cells that can differentiate into various types of cells [1-6]. These cells have been isolated from bone marrow [1], umbilical cord blood [2], and adipose tissue [3-6] and can be easily obtained and expanded *ex vivo* under appropriate culture conditions. Thus, MSCs are an attractive material for cell therapy and tissue engineering.

Human adipose tissue-derived mesenchymal stem cells, also referred to as human adipose tissue-derived multilineage progenitor cells (hADMPCs), are especially advantageous because they can be easily and safely obtained from lipoaspirates, and the ethical issues surrounding other sources of stem cells can be avoided [4-6]. Moreover, hADMPCs have more pluripotent properties for regenerative medical applications than other stem cells, since these cells have been reported to have the ability to migrate to the injured area and differentiate into hepatocytes [4], cardiomyoblasts [5], pancreatic cells [7], and neuronal cells [8-10]. In addition, it is known that hADMPCs secrete a wide variety of cytokines and

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growth factors necessary for tissue regeneration including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) [11-14].

Recently, several groups have reported that hADMPCs facilitate neurological recovery in experimental models of stroke [9,10,15] and Parkinson's disease [16]. Despite the superiority of hADMPCs over other stem cells, the potential use of hADMPCs for the treatment of these neurodegenerative disorders has not been fully investigated. It has been reported that administration of hADMPCs in animal models of acute ischemic stroke markedly decreased brain infarct size, improved neurological function by enhancing angiogenesis and neurogenesis, and showed anti-inflammatory and anti-apoptotic effects [9,10]. These effects were due in part to increased secretion levels of VEGF, HGF and bFGF under hypoxic conditions [13], indicating the role of hADMPCs in reducing the severity of hypoxia-ischemic lesions.

In addition to hypoxic stress, ischemic lesions are generally subject to inflammation, which leads to the generation of reactive oxygen species (ROS) [17,18]. ROS are

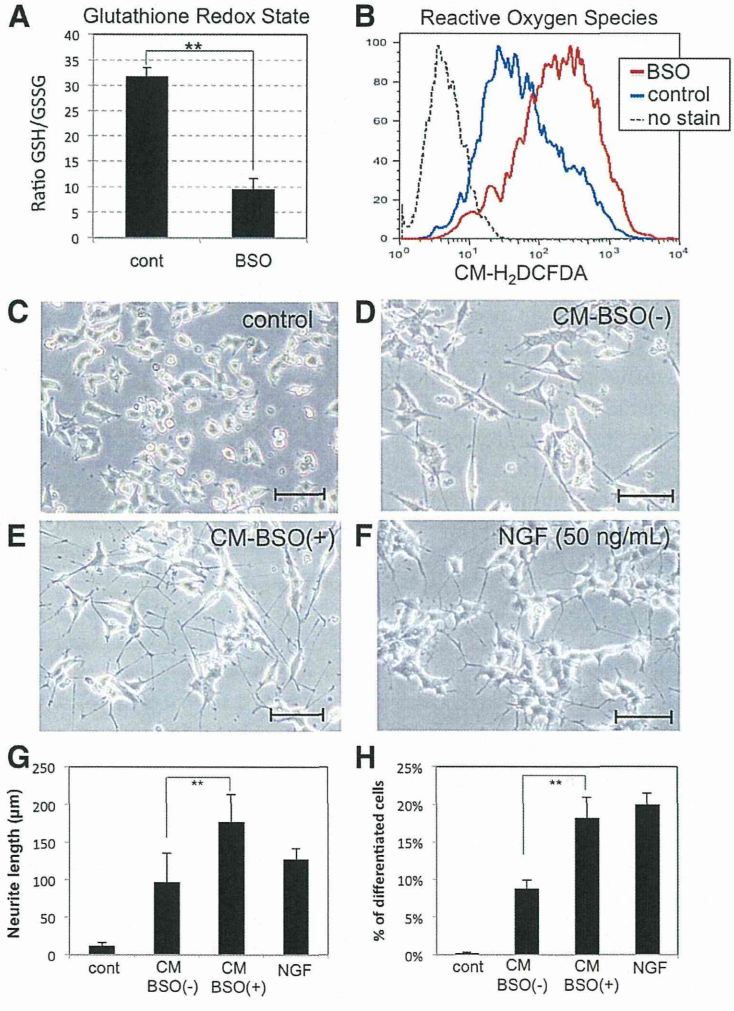

Figure 1 Conditioned medium from hADMPCs exposed to oxidative stress induces neurite outgrowth in PC12 cells. (A, B) Decrease of the reduced/oxidized glutathione ratios and increase in the intracellular ROS levels in hADMPCs treated with BSO. hADMPCs were treated with 1 mM BSO for 16 h, and cellular GSH/GSSG levels (**A**) or ROS (H_2O_2) levels (**B**) were analyzed. **(C-G)** Induction of neurite outgrowth in PC12 cells by conditioned medium from BSO-treated hADMPCs. PC12 cells were induced to differentiation by changing medium to differentiation medium alone (**C**), CM-BSO (–) (**D**), CM-BSO (+) (**E**), or differentiation medium with NGF (50 ng/mL) (**F**) for 2 days. Scale bars, 200 μm. (**G**) One hundred individual neurites were measured in each sample using Dynamic Cell Count Analyzer BZ-H1C (Keyence, Osaka, Japan) and average neurite length was calculated. **, $P < 0.01$ (Student's t test). **(H)** Percentage of neurite-bearing PC12 cells. A cell was scored positive for bearing neurites if it has a thin neurite extension that is double the length of the cell body diameter. A total of 500–600 cells in each sample were counted. **, $P < 0.01$ (Student's t test).

generated as a natural byproduct of normal aerobic metabolism, and mitochondrial respiration, together with oxidative enzymes such as plasma membrane oxidase, is considered to be the major intracellular source of ROS production [19]. Although appropriate levels of ROS play an important role in several physiological processes, oxidative damage initiated by excessive ROS causes many pathological conditions including inflammation, atherosclerosis, aging, and cancer. Neuronal cells are especially vulnerable to oxidative stress, and numerous studies have examined the crucial roles of oxidative stress in neurodegenerative disorders such as stroke [17,18], Alzheimer's disease [20,21], and Parkinson's disease [22,23]. In these diseases, microglia, the macrophages of the central nervous system (CNS), are activated in response to a local inflammation [24] and generate large amounts of reactive oxygen and nitrogen species, thereby exposing nearby neurons to stress [18,25]. Thus, the influence of oxidative stress generated by neurodegenerative lesion on hADMPCs needs to be further studied.

In this study, we examined the role of oxidative stress on hADMPCs in neurite outgrowth in cells of the rat pheochromocytoma cell line (PC12). Upon treatment with buthionine sulfoximine (BSO), an inhibitor of the rate-limiting enzyme in the synthesis of glutathione, hADMPCs accumulated ROS, which resulted in the upregulation of expression levels of the neurotrophic factors BMP2 and FGF2. Our present data thus provide new insights into understanding the mechanism of how hADMPCs exposed to oxidative stress contribute to neurogenesis, and this may explain the effects of stem cell transplantation therapy with hADMPCs in treating ischemic stroke.

Results

hADMPCs exposed to oxidative stress stimulate neurite outgrowth in PC12 cells

hADMPCs were treated with 1 mM BSO for 24 h; a group of hADMPCs that were not given any treatment was used as the control group. As shown in Figure 1A and B, BSO treatment resulted in significant reduction of intracellular reduced glutathione levels, followed by accumulation of intracellular reactive oxygen species (ROS) in hADMPCs. To investigate whether accumulation of ROS affects secretion of cytokines from hADMPCs, conditioned medium from BSO-treated (CM-BSO (+)) or BSO-untreated (CM-BSO (−)) hADMPCs was added to PC12 cells. As expected, addition of NGF significantly induced neurite outgrowth in the PC12 cells (Figure 1F, G, H). hADMPCs, like other mesenchymal stem cells derived from bone marrow or adipose tissue, may secrete many cytokines including NGF, BDNF and FGF2, and this may account for the slight induction of neurite outgrowth seen in the CM-BSO (−) treated cells (Figure 1D, G, H). In contrast, the number and length of neurite outgrowth of PC12 cells in CM-BSO (+) (Figure 1E) was markedly enhanced compared with those in CM-BSO (−) (Figure 1D, E, G, H).

Conditioned medium from BSO-treated hADMPCs activates Erk1/2 MAPK and Smad signaling in PC12 cells

To investigate which intracellular signaling pathways were involved in the neurite outgrowth of PC12 cells in CM-BSO (+), we used western blotting to determine the phosphorylation levels of Erk1/2 MAPK, p38 MAPK, Smad1/5/8 and Akt in PC12 cells in various culture conditions. NGF significantly activated Erk1/2 MAPK and Akt signaling pathway (Figure 2). In contrast, Erk1/2 MAPK was not activated in PC12 cells exposed to CM-BSO (−), while an increase in phosphorylated Smad1/5/8 was observed. Interestingly, CM-BSO (+) treatment led to both a significant increase in Smad1/5/8 phosphorylation levels as well as activation of the Erk1/2 MAPK

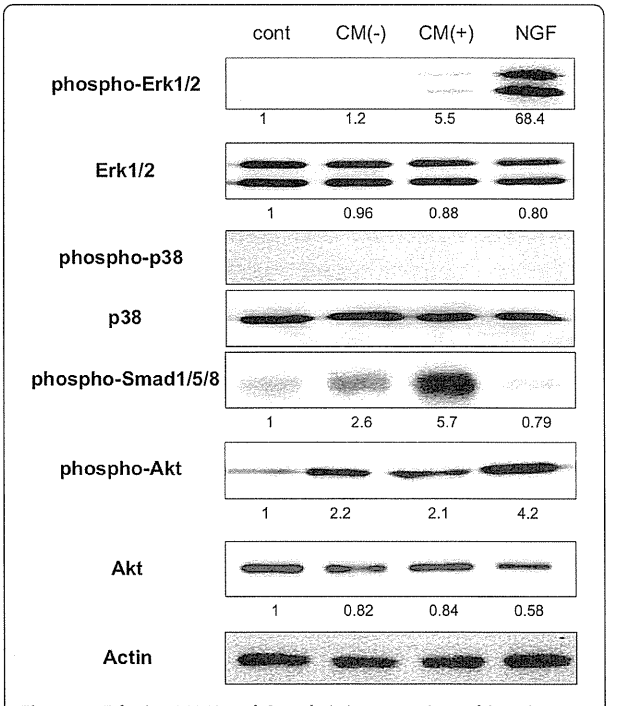

Figure 2 Erk1/2 MAPK and Smad1/5/8 are activated in PC12 cells cultured in conditioned medium from BSO-treated hADMPCs. Western blot analysis of PC12 cells cultured in differentiation medium alone (cont), CM-BSO (−), CM-BSO (+), or differentiation medium with NGF (50 ng/mL) for 1 h. Proteins extracted from each cell culture were resolved by SDS-PAGE, transferred to a membrane, and probed with anti-phosphorylated Erk1/2 (phospho Erk1/2), anti-Erk1/2, anti-phosphorylated p38 (phospho p38), anti-p38, anti-phosphorylated Smad1/5/8 (phospho Smad1/5/8), anti-phosphorylated Akt (phospho Ark) and anti-Akt. Actin was analyzed as an internal control. Numbers below blots indicate relative band intensities as determined by the ImageJ software.