厚生労働科学研究費補助金（難治性疾患等克服研究事業）
分担研究報告書

再生医療製品の品質・安全性確保において「最低限必要とされる要求事項」MCP (Minimum Consensus Package)の提案

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研究要旨

再生医療製品の製造販売承認へ切れ目のない展開に向け、前臨床研究から開発、評価を効率的、効果的、合理的に行い、再生医療実用化を加速する基盤を示すことが課題である。その課題解決方策の一つとして、再生細胞治療製剤等において「最低限必要とされる要求事項」を明示することが挙げられる。これは、学問技術の進歩、開発動向、関係法規指針等をふまえて、必須かつ共通と考えられる製造施設、製造工程、製品評価、製品管理面での具体的留意事項について明示されるべきものであって、産官学が理解と解釈を共有できるミニマムコンセンサスであり、かつそれがパッケージング化されることが望ましい。

A．研究目的

近年の再生医学、発生医学の進歩は眼を見張るものであり、それが革新的治療法として難治性疾患への光明として話題に上らない日はない。世界を見渡すと、骨髄、末梢血、臍帯血中の造血幹細胞を用いた細胞治療が盛んに行われており、また心筋梗塞などで壊死に陥った組織の機能を補う再生医療臨床研究も行われている。我が国でも、数多くのヒト幹細胞を用いた臨床研究が実施されており、その数は米国のそれを凌駕している。

基礎的研究シーズの社会還元への迅速化にむけ、臨床研究が応用開発に連結しやすい仕掛けが、今、まさに求められている。解決すべき課題はあまたあるが、これまでの再生細胞医療臨床研究での経験を踏まえ、再生細胞医療研究開発から臨床利用における共通プラットフォームとしての「最低限に必要とされる要求事項」明示がその１つではないかと考える。医師法・医療法と薬事法という規制の目的方向性が異なる法の下での実施あっても、諸外国と法制度の差異はあっても、First-in-Manへの最低限確認しなければならないことは一貫しているはずである。本分担研究報告では、米国と我が国の再生医療臨床応用にむけた規制を比較し、再生細胞医療研究開発から臨床利用において「最低限必要とされる要求事項」について求めるべき考え方について述べたい。

B．研究方法

米国の関連規制については、FDC ACT 及び FDA HP を調査した。日本における規制動向については、いわゆる５指針を基盤に議論を進めた。

（倫理面への配慮）

該当なし

C．研究結果

米国での規制とその緩和

米国では、すべての細胞製剤についてはPublic Health Service Act Article 351&361およびFood, Drug and Cosmetic Actの諸条項により、BLAを受けるまでInvestigational New Drug（IND)として規制を受ける（機器の場合はInvestigational Device Exemption; IDE)（図１)。INDは申請者により commercial IND（企業が申請するIND）とnon-commercial IND（大学等研究機関研究者が申請するIND）に分けられるものの、phase Iに相当するclinical trialへの要求水準が同じであるため大学等研究者から不満があった。米国では、基礎的研究のすみやかな臨床応用を目指し、国家を挙げてCritical Path Initiative政策を掲げているところである。Critical Path Initiativeは速やかな基礎的シーズの産業化という目的をもった科学技術産業政策であり、その本質はとにかく成功体験を作り、それにより問題点・課題を抽出・明確化するということにある。加えて、成功体験を作るには基礎的シーズのBLAに行き着く確率が一定であれば、phase Iに入るシーズが多いほど産業化期待値が大きくなるはずであるから、基礎的シーズを多く保有する大学等によるnon-commercial IND申請・承認を増やさねばならない。再生医療周辺産業が産業として確立している米国においてさえ、研究者からはINDによる過剰な審査と脱落が由々しき問題であると認識されていた。確かに、米国においても研究費獲得のためのIND申請も行われていると仄聞しており、すべてのIND申請の質が良好であるわけではないが、上記背景から、phase Iにおいて求められる要求事項を“discount”したPhase I-GMP Guidanceがreleaseされた。米国をはじめ我が国を含めたICH参画国においてFirst-in-Manで求められる水準は収束してきていると思われ、興味深い。なお、産業化がなされ市場として成熟していくのであれば、OECDがガイドラインを作成すると思われるが、現在のところ市場が確立しているわけでもなくそのような動きはないのも現実である。

これまでの我が国における基準明確化の流れ

細胞・組織利用医薬品等による再生医療は、ヒトの臓器確保が難しいわが国の医療状況下において強く期待されており、研究の進歩に伴う技術的な実現可能性の高まりとともに、医療としての実用化を望む声がますます強くなっている。我が国では、医師法・医療法の下で行われる臨床研究として数多くの再生細胞治療臨床研究が試みられてきた[1)]。再生細胞医療はいまだ未解明な部分もあることから、臨床研究の質を向上させ、患者さんの不利益とならないように臨床研究を実施する必要があることから、平成18年9月1日に「ヒト幹細胞を用いる臨床研究に関する指針」が施行された。厳しいのでは、という指摘もあるところであるものの、厚生労働大臣が意見を述べるまでのtime clockも短縮してきており、申請プロトコールの質の向上は明白であり、ヒト幹細胞臨床研究プロトコールの質も世界標準に近づきつつあると認識される。

これまで、厚生労働省においても、わが国の再生医療を適正な規制のもと推進するため安全性評価基準の作成など規制のあり方について検討を加えており、平成12年医薬発第1314号別添2「ヒト由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針」の改定を行い、薬食発第0208003号通知並びに0912006号通知、ついでいわゆる5指針として薬事法上の規制に反映されてきた。臨床研究として医師法・医療法の規制下で行われてきた再生細胞治療に関しては、従前なんら規制などはなかったが、平成18年に厚生労働省告示第425号「ヒト幹細胞を用いる臨床研究に関する指針」が告示され、同年9月1日より施行された。同告示はあくまで医師が自らの医行為の一環として、自ら組織を採取、培養、投与することを前提としている。投与される細胞の品質を確保し、その有用性を担保するための基準として薬

事法関連規制としての平成12年医薬発第1314号を援用しているところであるが、同通知はFirst-in-Manにむけた確認申請で求められる水準のものから、製造販売後に求められる水準まで記載されており、いわゆる1314号通知に記載されているすべての項目を援用する必要はないはずであり、過剰な要求がなされているのではないかと研究者側が過剰に反応しているとの危惧を覚える。薬事法関連規制としての改定5指針において、もとめられる水準を明確にすべく改定されていることから、ヒト幹細胞を用いる臨床研究に関する指針」の改定委員会においても議論され、改定時に反映されたものと認識している。

なぜ「最低限必要とされる要求事項」の明示が必要なのか？

広く再生医療にかかる安全性および有効性評価項目・基準を研究早期から示すことは、大学等でFirst-in-Manとして開始される臨床研究を迅速に実用化するためにも必須である。大学発シーズが速やかにかつ広く社会還元されるには、医師法下にて行われるヒト幹細胞臨床研究から薬事法下での確認申請・治験・承認審査・製造販売承認までの制度を超越、一貫した共通して申請資料に使用できる「最低限必要とされる項目」が示されるべきである。すべての再生医療製品（細胞組織利用医薬品医療機器）に共通したFirst-in-Manに求められる水準が明示され、それがヒト幹細胞を用いる臨床研究に関する指針の運用で用いられれば、薬事法関連規制へと一貫連結した前臨床研究・非臨床研究データを得られることとなり、それらプロトコールを引き受ける企業も「安心して」引き受けることが可能となるのである[4]。

「最低限必要とされる要求事項」策定の考え方と具体的な検討の方向性

「最低限必要とされる要求項目」は、科学的合理的で拠って立ちうる最低限かつ不可欠な評価項目・基準とそのパッケージとして提示される必要がある。これらパッケージの提示は、「サイエンス」という共通かつ普遍言語をもってして初めて可能となる。「サイエンス」からみれば、医師法・医療法下での臨床研究も薬事法下での治験もなく、本質的不可欠な項目は普遍でなければならない。そうでなければ、我々研究者が拠って立つサイエンスは無力と言われてしまう。すべての再生医療製品（細胞組織利用医薬品医療機器）に共通したFirst-in-Manに求められる水準は、疾患ごとのケースバイケースの上乗せ評価項目の基盤として、階層構造を念頭においたパッケージとして構築されるべきであろう。

そのため、ヒト幹細胞臨床研究において投与・移植される細胞・組織利用製剤等に関して、学問・技術の進歩、倫理上の重要ポイント、国際的動向等を調査・検討、さまざまな研究者などから提言されている安全性評価基準を広く収集し、当該評価項目・基準の科学的合理性および妥当性に関して検討、「最低限必要とされる要求事項」の適切な安全性評価項目立てを行い基準の作成を行われるべきである。特に、ヒト幹細胞臨床研究に際して、臨床応用にむけた一貫した評価指標との観点から、研究、開発、評価等を効率的、効果的、合理的に行う上で、学問技術の進歩、開発動向、倫理上の重要ポイント、関係法規指針等、関連する諸要素をふまえて、必須かつ共通と思われる技術、細胞・組織利用製剤等の製造施設、製造方法、特性解析方法、品質管理方法及び安定性評価に関する具体的留意事項について明示されるべきものであって、産官学が理解と解釈を共有できるミニマムコンセンサスであり、かつそれがパッケージング化されることが望ましい。

ヒト幹細胞を利用した細胞・組織利用製剤等に係る国内外のガイダンスや最新の情報収集を行い、その科学的合理性に関しては追試を行うことも必要となるかもしれない。研究者などから提言されている有用性評価基準・項目を収集することも、研究から開発、社会還元にむけた一貫性確保の観

点から重要である。これらをとりまとめのうえ科学的に合理性があると想定される事項に関して再現検討を含めて評価、「最低限要求項目」として設定し、わが国における規制の枠組み、特に再生医療実現化の入口であるヒト幹細胞臨床研究審査基準に反映させるため、「最低限要求項目」に階層構造を付与し、「最低限要求事項ポートフォリオ」として策定されるべきである。

D．考察

これらにより、再生医療実現化の入口であるヒト幹細胞臨床研究から薬事法上の製造販売承認までシームレスかつ一貫した研究開発の方向性が示される。ヒト幹細胞利用製剤の評価等を効率効果的、合理的に行う上での基本的考え方、必要と思われる技術、データ、安全性評価指標の主要項目等、すなわち「最低限必要とされる要求事項」が示されれば、再生医療を活用する新規治療技術の実用化に関連した、細胞・組織等を用いる治療技術の安全性・品質の確保に関する技術開発に資することとなる。ヒト幹細胞を用いる臨床研究に関する指針でも取り込む必要があろうし、この基盤をいわゆる 5 指針の実務運用に活用すべきである。これらを国際標準とするための働きかけも重要である。また、これら「最低限必要とされる要求事項」作成の課程を公表することで、すべては患者さんのためというその哲学を示すことが可能となる。要求項目の一つ一つに込められた規制側の「思い」を理解することで、Phase が進行したときに上乗せされる規制についても合理的に想像ができるようになると思われ、ケースバイケースとされている判断項目・基準がいかに合理的に運用されているかを理解できよう。研究と開発の非連続性を解決し、数多くの臨床研究のなかから本当に患者さんに有用な細胞組織利用医薬品等が、患者さんの手に届く日が一日でも早く来るよう、その結果国民の保健医療の向上に大いに貢献するよう願いたい。

E．結論

再生医療製品の製造販売承認へ切れ目のない展開に向け、前臨床研究から開発、評価を効率的、効果的、合理的に行い、再生医療実用化を加速する基盤を示すことが課題である。その課題解決方策の一つとして、再生細胞治療製剤等において「最低限必要とされる要求事項」を明示することが挙げられる。これは、学問技術の進歩、開発動向、関係法規指針等をふまえて、必須かつ共通と考えられる製造施設、製造工程、製品評価、製品管理面での具体的留意事項について明示されるべきものであって、産官学が理解と解釈を共有できるミニマムコンセンサスであり、かつそれがパッケージング化されることが望ましい。

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

別紙４

研究成果の刊行に関する一覧表レイアウト（参考）

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Myoblast Sheet Can Prevent the Impairment of Cardiac Diastolic Function and Late Remodeling After Left Ventricular Restoration in Ischemic Cardiomyopathy

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Background. Impairment of diastolic function and late remodeling are concerns after left ventricular restoration (LVR) for ischemic cardiomyopathy. This study aims to evaluate the effects of combined surgery of myoblast sheets (MS) implantation and LVR.

Methods. Rat myocardial infarction model was established 2 weeks after left anterior descending artery ligation. They were divided into three groups: sham operation (n=15; group sham), LVR by plicating the infracted area (n=15; group LVR), and MS implantation with LVR (n=15; group LVR+MS).

Results. Serial echocardiographic study revealed significant LV redilatation and decrease of ejection fraction 4 weeks after LVR in group LVR. MS implantation combined with LVR prevented those later deteriorations of LV function in group LVR+MS. Four weeks after the operation, a hemodynamic assessment using a pressure-volume loop showed significantly preserved diastolic function in group LVR+MS; end-diastolic pressure (LVR vs. LVR+MS: 9.0±6.6 mm Hg vs. 2.0±1.0 mm Hg, $P<0.05$), end-diastolic pressure-volume relationship (LVR vs. LVR+MS 42±23 vs. 13±6, $P<0.05$). Histological examination revealed cellular hypertrophy and LV fibrosis were significantly less and vascular density was significantly higher in group LVR+MS than in the other two groups. Reverse transcription polymerase chain reaction demonstrated significantly suppressed expression of transforming growth factor-beta, Smad2, and reversion-inducing cysteine-rich protein with Kazal motifs in group LVR+MS.

Conclusions. MS implantation decreased cardiac fibrosis by suppressing the profibrotic gene expression and attenuated the impairment of diastolic function and the late remodeling after LVR. It is suggesting that MS implantation may improve long-term outcome of LVR for ischemic heart disease.

Keywords: Ischemic cardiomyopathy, Left ventricular restoration, Regenerative therapy, Myoblast sheet, Diastolic function.

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Ischemic heart disease is one of the leading causes of death and disability in most of the industrialized countries and recognized as a major public health issue. Progression to end-stage heart failure involves massive loss of cardiomyocyte, massive fibrosis, and progressive remodeling of the ventricles. Left ventricular (LV) volume reduction surgery or LV restoration (LVR) surgery has been introduced as a surgical treatment of patients with dilated LV and chronic heart failure (*1*, *2*), and has been shown to reduce the LV volume, increase the ejection fraction, and improve ventricular function (*3*, *4*).

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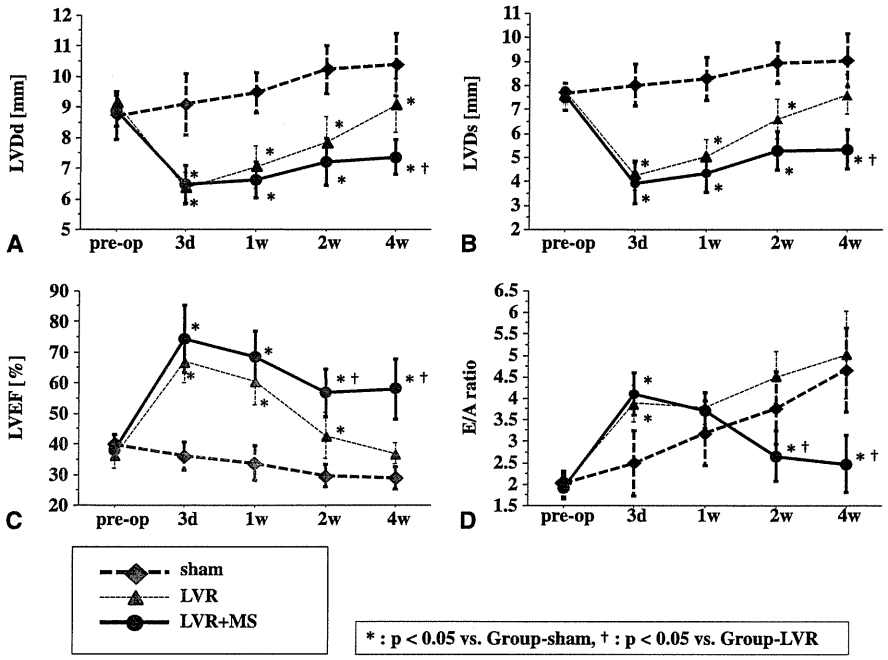

FIGURE 1. Serial echocardiographic study revealed significant decrease in left ventricular chamber size and significant increase in left ventricular ejection fraction by left ventricular restoration (LVR) in group LVR and in group LVR+ myoblast sheets (MS). However, gradual redilatation of left ventricular chamber and decrease of ejection fraction was observed in group LVR. Those later deteriorations were prevented in group LVR+MS, and the differences in chamber size and ejection fraction were significant between group LVR and group LVR+ MS 4 weeks after the operation. Changes in echocardiographic parameters before and after the operation. (A) Left ventricular dimension at end-diastole (LVDd), (B) left ventricular dimension at end-systole (LVDs), (C) Left ventricular ejection fraction (LVEF), (D) mitral valve E/A ratio. *P0.05 vs. group sham; †P0.05 vs. group LVR.

However, impairment of diastolic function and late remodeling are great concerns after LVR for ischemic cardiomyopathy (*5–7*), and the long-term effect of LVR is still controversial. Although LVR that is performed together with coronary artery bypass grafting (CABG) has been suggested to reduce the rate of hospitalization and improve ventricular function to a greater degree than CABG alone on the basis of a small, nonrandomized, case-control study (*8*), recently conducted multicenter, nonblinded, randomized trial (the Surgical Treatment for Ischemic Heart Failure [STICH] trial) have revealed that LVR does not improve the symptoms, exercise tolerance, rate of death, or hospitalization in patients with ischemic heart disease and severe LV dysfunction compared with CABG alone (*5*).

On the other hand, cell transplantation into impaired myocardium, also known as cellular cardiomyoplasty, has been investigated (*9, 10*). Recently, we have developed a new cell delivery method by the means of cell sheet, in which autologous skeletal myoblasts were transplanted in sheet form, and reported that this method was effective especially in the attenuation of LV dilatation and the improvement of LV diastolic function (*11–14*). On the basis of these findings, we hypothesized that skeletal myoblast sheet (MS) implantation may attenuate the disadvantageous effects and enhance the advantageous effects of LVR. Using a rat model of chronic myocardial infarction model, we investigated whether MS implantation combined with LVR can attenuate the redilatation and diastolic dysfunction of LV after LVR.

RESULTS

Changes in Cardiac Function by LVR and LVR Combined With MS

Two weeks after left anterior descending coronary artery (LAD) ligation, severe dilatation of the LV chamber and severe asynergy of the anterior wall were observed in all the rats. By excluding the large akinetic or dyskinetic area of the LV anterior wall, LV dimension at end-diastole (LVDd) and end-systole (LVDs) significantly decreased and left ventricular ejection fraction (LVEF) significantly increased in group LVR and in group LVR+MS 3 days after treatment (Fig. 1). However, gradual LV redilatation and decrease of LVEF were observed in group LVR. MS implantation combined with LVR attenuated those later deteriorations of LV function significantly in group LVR+MS (Fig. 1). Mitral valve *E/A* ratio showed significant restrictive pattern after LVR. In group LVR, the restrictive pattern progressed even further with time. However, addition of the MS implantation attenuated the progression of the restrictive pattern (Fig. 1).

Hemodynamic Improvement by LVR Combined With MS

Table 1 shows the results of the hemodynamic study by cardiac catheterization 4 weeks after the second operation. The basic hemodynamic indices revealed that LV end-diastolic pressure (EDP) and the time constant of isovolumic relaxation (τ) were significantly lower in group LVR+MS than in group LVR or group sham. Load-independent parameters measured by pressure-volume loop analysis revealed that end-systolic pressure (ESP) volume relationship was significantly higher in group LVR+MS than in the other two groups. EDP volume relationship (EDPVR) was significantly lower in group LVR+MS than in the other two groups.

Histological Impact of the MS on the Failing Heart

Figure 2 shows the typical cross section of the whole hearts 4 weeks after the operation from each group. Severe dilatation of the LV chamber and thinning of the LV wall were observed in group sham (Fig. 2A). In group LVR, although infracted area was excluded and smaller than that in group sham, LV chamber was markedly dilated (Fig. 2B). Also severe dilatation of the right ventricular chamber was observed. In group LVR+MS, the size of the LV chamber and the thickness of the LV wall were well preserved compared

TABLE 1. Hemodynamic indices 4 weeks after the operation

Group	Sham	LVR	LVR+MS
Basic hemodynamic indices			
HR (bpm)	219 ± 37	206 ± 20	231 ± 32
ESP (mm Hg)	60.9 ± 7.7	63.0 ± 13.9	73.0 ± 11.3[a]
EDP (mm Hg)	5.1 ± 2.2	9.0 ± 6.6	2.0 ± 1.0[a,b]
τ (msec)	21.3 ± 2.4	19.8 ± 2.2	14.4 ± 1.2[a,b]
Load independent parameters analyzed by pressure-volume loop			
ESPVR (mm Hg/ml)	1896 ± 906	1364 ± 661	4722 ± 2416[a,b]
EDPVR (/ml)	50 ± 36	42 ± 23	13 ± 6[a,b]
PRSW (mm Hg)	37.1 ± 24.3	33.0 ± 24.2	45.2 ± 32.7

[a] $P < 0.05$ vs. group sham.
[b] $P < 0.05$ vs. Group-LVR.
HR, heart rate; ESP, end-systolic pressure; EDP, end-diastolic pressure; τ, time constant of isovolumic relaxation; ESPVR, end-systolic pressure-volume relationship; EDPVR, end-diastolic pressure-volume relationship; PRSW, preload-recruitable stroke work.

with the other groups (Fig. 2C). The LV wall thickness was significantly larger in group LVR+MS than in the other two groups (vs. group sham and group LVR, $P<0.05$) (Fig. 3A). The degree of cardiac fibrosis was significantly smaller in group LVR+MS than in the other two groups (vs. group sham and group LVR, $P<0.05$) (Figs. 3B and 4A–C). Myocyte size was also significantly smaller in group LVR+MS than in the other two groups (vs. group sham and group LVR, $P<0.05$) (Figs. 3C and 4D–F). Vascular density of the LV lateral wall, the area where MS were applied in group LVR+MS, was significantly higher in group LVR+MS than in the other two groups (vs. group sham and group LVR, $P<0.05$) (Figs. 3D and 4G–I).

Suppression of Probrotic Agent Gene Expression by MS

Reverse transcription polymerase chain reaction analysis 4 weeks after the second operation revealed significantly suppressed expression of the profibrotic gene transforming growth factor-beta (TGF-β), Smad2, and reversion-inducing cysteine-rich protein with Kazal motifs (RECK) in group LVR+MS than in the other two groups (vs. group sham and group LVR, $P<0.05$) (Fig. 5A–C).

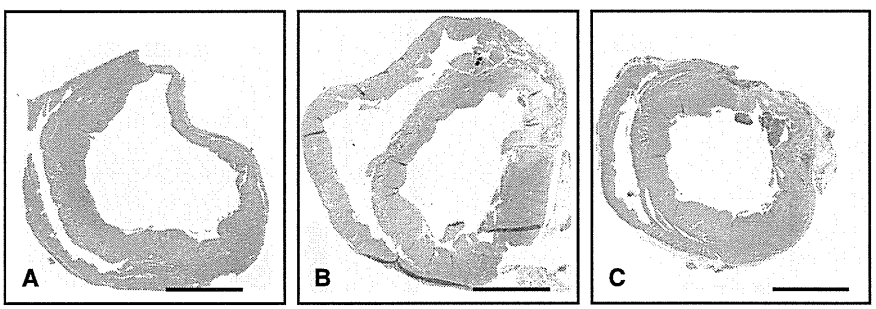

FIGURE 2. Cross section of the whole hearts 4 weeks after the operation from each group (hematoxylin-eosin staining). (A) group sham, (B) group LVR, (C) group LVR + MS.

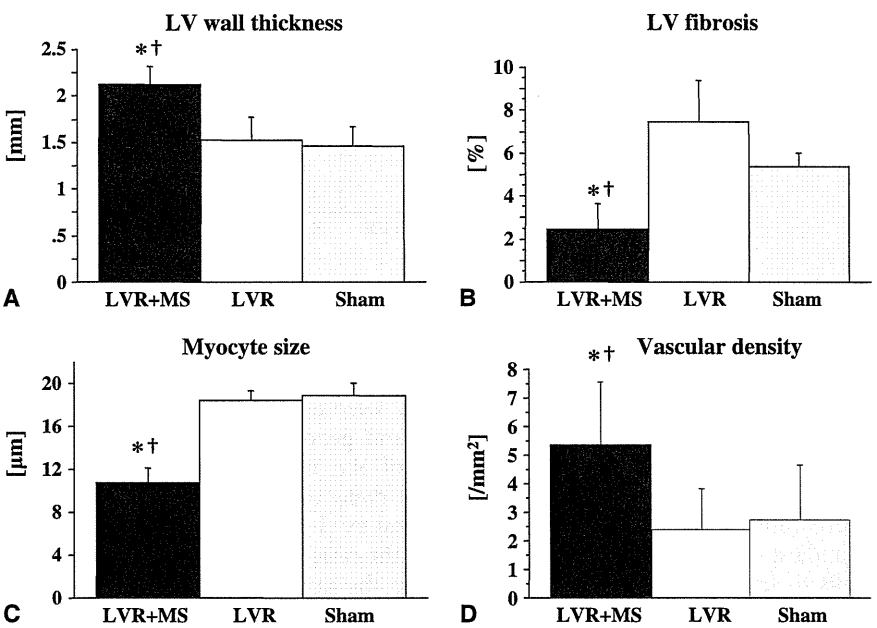

FIGURE 3. The left ventricular (LV) wall thickness was significantly larger in group left ventricular restoration (LVR)+myoblast sheets (MS) than in the other two groups 4 weeks after the operation (A). The degree of cardiac fibrosis (B) and myocyte size (C) were also significantly smaller in group LVR+MS than in the other two groups. The vascular density in the LV lateral wall, where MS were applied in group LVR+MS, were significantly higher in group LVR+MS than in the other two groups (D).

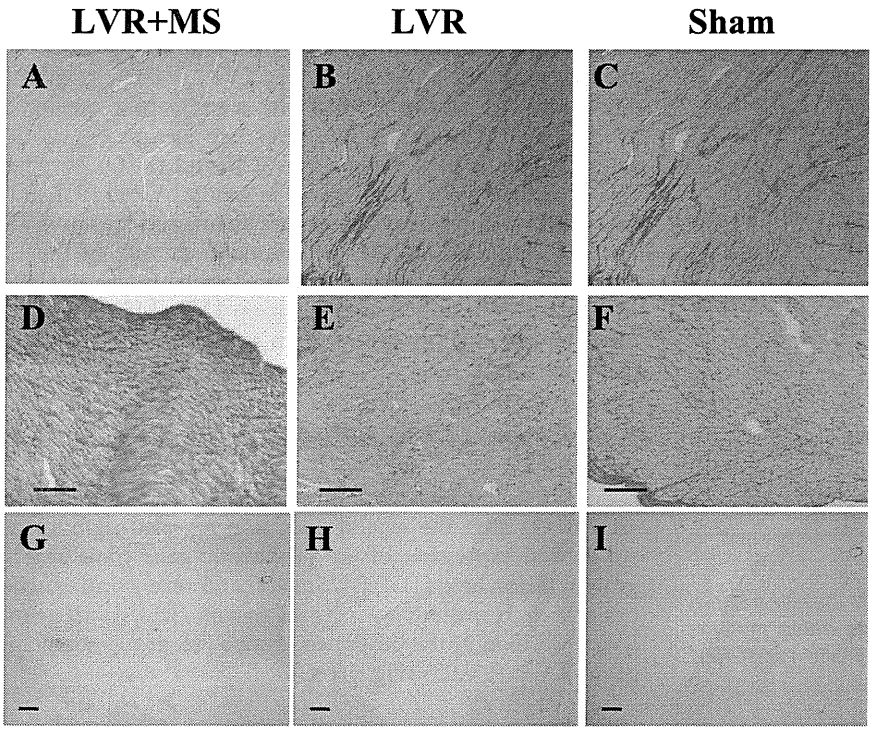

FIGURE 4. Picrosirius-red staining of myocardium from noninfarcted regions (A, B, C) and periodic acid-Schiff-stained myocardium from noninfarcted regions (D, E, F, bar=200 μm). Picrosirius-red staining of myocardium from noninfarcted regions. Sections of myocardium stained with antibody to von Willebrand factor (G, H, I, bar=300 μm).

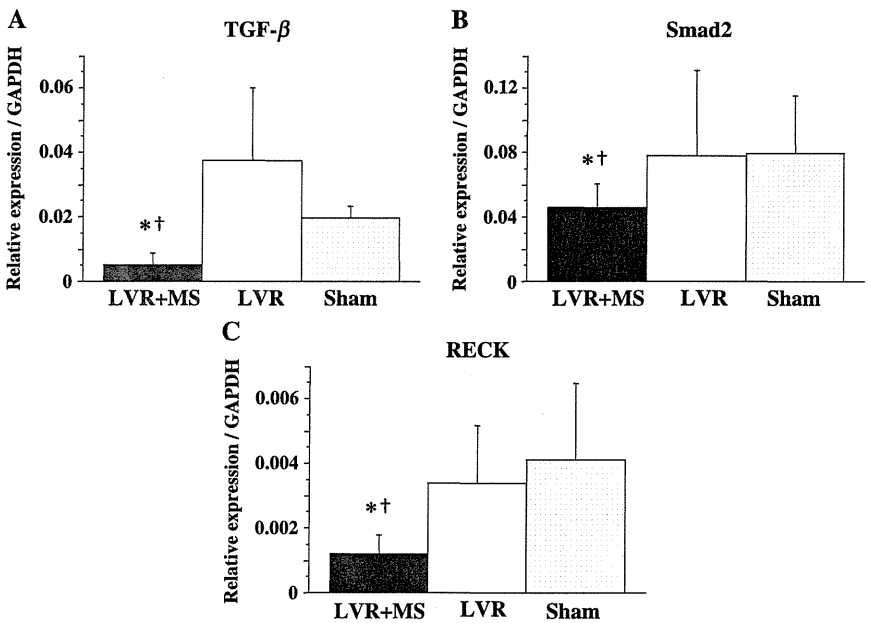

FIGURE 5. Reverse transcription polymerase chain reaction (RT-PCR) analysis 4 weeks after the second operation revealed significantly suppressed expression of the profibrotic gene transforming growth factor-beta (TGF-β) (A), Smad2 (B), and RECK (C) in group left ventricular restoration (LVR)+myoblast sheets (MS) than in the other two groups.

Engrafted Cell Survival

To evaluate the survival of engrafted cell on the recipient LV, MS made from male rats were implanted on the female LV, and surviving cell numbers were examined by detecting the Y chromosome-specific and gender consensus genes. To confirm the accuracy of the measurements, MS made from known numbers of male myoblasts were implanted on the LV wall of a female rat ex vivo, and a standard curve was prepared to determine the ratio of male cells to female cells and the relationship to the number of male cells. The correlation coefficient for the standard curve was 0.9716, indicating a significant correlation. The number of surviving engrafted cells was calculated using this standard curve (*15*). The number of cells detected on the day of implantation was approximately 64% of the engrafted cells (five layers of MS, with 3.0×10^6 myoblasts in each sheet). Surviving cells decreased to 69% of those in day 0. Although the number continued to decrease with time, 13% of those cells were still surviving on the LV wall 4 weeks after MS implantation (Fig. 6A).

Immunostaining of the green fluorescent protein (GFP) revealed that myoblasts sheets made from GFP transgenic rats

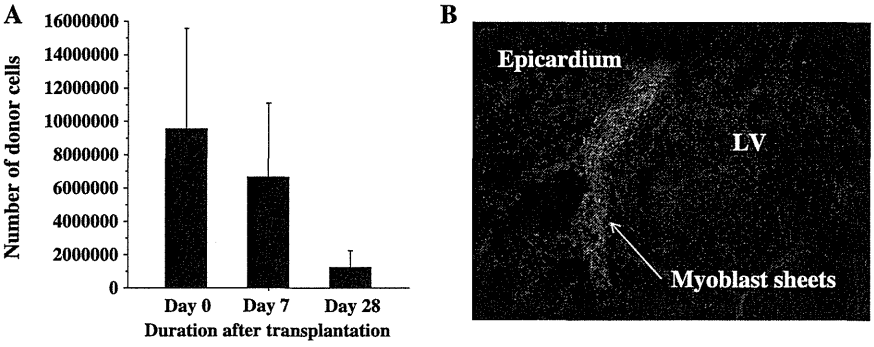

FIGURE 6. Survival of donor cells in recipient hearts. (A) Number of surviving engrafted cells in recipient hearts. Although the number of donor cells decreased with time, the surviving engrafted cells were still detectable 28 days after transplantation. (B) Immunostaining of the green fluorescent protein. Transplanted myoblast sheets were still detectable 28 days after the surgery.

were still detectable on the epicardium of LV wall 28 days after implantation (Fig. 6B).

DISCUSSION

Impairment of diastolic function and late remodeling are concerns after LVR for ischemic cardiomyopathy (*5–7*). Dor et al. (*6*) have reported the late redilatation of LV after LVR in their clinical experiences, and Nishina et al. (*16*) have developed a rat model that reproduces this clinical situation, in which model an infracted area of the LV anterior wall was simply plicated. Although LV configuration and function improved after the operation, LV chamber gradually redilated and LV function decreased, and the initial improvement almost disappeared in 4 weeks.

Using this same model, we implanted the skeletal MS concomitantly with LVR to investigate the ability of MS to overcome the drawbacks of the LVR. In this study, MS implantation attenuated the LV redilatation and decrease in EF after LVR. It was also shown by echocardiographic study and pressure-volume loop analysis that MS attenuated the impairment of diastolic function after LVR. Histological examination revealed that MS induced the angiogenesis in the myocardium where they were applied, and decreased the degree of myocardial fibrosis. MS controlled the gene expression that may regulate the myocardial fibrosis (TGF-β, Smad2, and RECK), and suppressed myocardial fibrosis. The number of viable myoblasts implanted on the LV wall concomitantly with LVR decreased with time, but they were still detectable on the LV wall 28 days after implantation. The surviving cells detected on the LV wall 28 days after transplantation were only 13% of those detected on the day of transplantation. However, to enhance the survivability and effectiveness of implanted cells, we have developed new additional therapy such as transfection of the gene for hepatocyte growth factor (HGF) (*17*) or omentum flap (*18*) combined with cell transplantation, and reported the efficacies of these additional therapies in the previous studies.

The mechanism of recovery of cardiac function by autologous MS are considered as combination of restoration of the LV wall by the MS, that is "girdling effect," and biological effects of the cytokines such as stromal-derived factor 1 (SDF-1), HGF, and vascular endothelial growth factor (VEGF) paracrined from sheet-shaped autologous myoblasts, that is "paracrine effect." SDF-1 is known to mobilize and recruit stem cells and leads to neovascularization (*19, 20*) and is secreted in skeletal muscle tissue (*21*). HGF is an angiogenic and antifibrotic factor (*22*), and VEGF is also a potent angiogenic factor (*23*). In the previous reports with animal models, we have demonstrated that the gene expressions of SDF-1, HGF, and VEGF were significantly higher in the hearts treated with MS than in hearts treated with myoblasts injection or with medium injection (*11, 14, 24*). As results of those enhanced gene expression, the hearts treated with MS showed higher number of hematopoietic stem cells in the treated area (*11*), greater vascularity (*11, 12, 14*), decreased cardiac fibrosis (*11–14, 24*), decreased apoptotic cells (*13*), and increased proliferative cells (*13*). Moreover, those effects were enhanced as the number of transplanted MS increased (*14*). Sekiya et al. (*14*) reported that the effect of the MS was maximally enhanced when it was implanted on the impaired myocardium in five layers, compared with three or one layer. Based on these data and experiences in our own laboratory, we chose the skeletal myoblasts as donor of cell sheets in this study, and decided the cell number and the layer number of the MS. In this study, we reconfirmed that angiogenesis was induced and fibrosis was suppressed by MS. It is considered that the angiogenesis enhanced the myocardial microcirculation and improved the myocardial ischemia, and resulted in attenuation of myocardial fibrosis and late remodeling. Instead of the well-known key factors secreted by MS such as SDF-1, HGF, and VEGF, we investigated the other signals that are known to control the degree of tissue fibrosis such as TGF-β, Smad, and RECK. TGF-β is a known profibrotic cytokine that has been demonstrated to induce cardiac fibrosis (*25*). The effect of TGF-β in the heart is primarily mediated though Smad2 phosphorylation (*26*). The TGF-β-Smad pathway seems to be involved in the activation of collagen-gene promoter sites, increasing DNA translation of collagen I. In this study, it was clearly proved that MS suppress the TGF-β-Smad pathway leading to the attenuation of cardiac fibrosis. RECK is known to be one of the inhibitors of metalloproteinases (*27*) and believed to be an important regulator of cardiac extracellular matrix. Although in this study we could not evaluate the matrix metalloproteinase (MMP) and tissue inhibitors of metalloproteinase activity, MS may activate the MMP acting through the suppression of RECK, leading to the reduction of fibrosis. It was shown for the first time that MS suppressed the degree of myocardial fibrosis by regulating those signals. The mechanisms by which MS regulate those signals remain to be investigated.

We also revealed that LV wall thickness was maintained and LV dilatation was attenuated by MS after LVR. From Laplace's law, this might have led to decrease in

LV wall stress and attenuation of the myocardial cellular hypertrophy.

In our previous study, we reported that MS increased elastin in the myocardium where the MS were implanted, and this might have contributed to the improvement in diastolic function (*14*). In this study, all the data acquired from echocardiography (mitral *E/A* ratio), catheter study (LVEDP, τ, and EDPVR), and histological study (fibrosis) revealed improvement of diastolic function by the MS.

One of the unique points of this study, compared with the previous studies with skeletal MS, was that the MS were applied to the viable area of the myocardium in this study. One of the most important mechanisms of the myocardial improvements by MS is considered paracrine effect of cytokines secreted from the skeletal myoblast. From this point of view, it is anticipated that the greater the number of the viable cells in the area of myocardium where the MS is attached, the greater the effect of the MS. This study is different from the previous studies in the point that the impaired myocardium was excluded by surgical LVR and the skeletal MS were attached to the remaining viable area of the myocardium. In the preliminary experiment of this study, we have also included the "MS only group" in the study groups. As reported in the previous studies, MS showed a certain effects and prevented the deterioration of the heart function compared with sham group. However, the comparisons between the group LVR+MS and "MS only group" were complicated because the conditions of the myocardium in which the MS were applied were different, so we excluded this group from the final design of this study.

Using the rat LVR model, other additional treatments such as administration of angiotensin-converting enzyme inhibitor (*28*), chymase inhibitor (*29*), or transplantation of fetal cardiomyocyte by needle injection (*30*) were reported to prevent the late remodeling after LVR in some extent. Not like the single medical treatments mentioned above, MS implantation affects on cardiac function by integrated pathway of angiogenesis, antifibrosis, mechanical unloading of the LV wall stress, and possibly other unknown mechanisms. MS implantation is supposed to be more effective than single medical treatment. As a cell delivery method, it is known that direct intramyocardial injection has several disadvantages, including cell loss caused by leakage of injected cells from the myocardium, poor survival of the grafted cells, myocardial damage after mechanical injury by the needle, and subsequent acute inflammation. MS implantation is a useful method to overcome these disadvantages, and we have reported the superiority of the myocardial sheets implantation to needle injection (*11–13*).

This study has some limitations. In this rat model, the area of myocardial infarction is not identical in all the rats 2 weeks after ligating the coronary artery, and thus the size of the LV and the degree of impairment of diastolic function are not identical in all the rats after LVR. Second, the surgery for excluding the infarction was carried out by imbrication stitches, and this is different from the actual procedure in the clinical setting, excision and re-sculpting of the left ventricle as described by Dor et al. (*6*). Additionally, we chose rats with large akinetic area as a myocardial infarction model and aggressively plicated this area to reproduce "the failing situation" after LVR. This situation may not be directly applied to clinical settings. However, we consider that the effectiveness of MS to attenuate impairment of diastolic function and late remodeling after LVR was shown by this model. We also recognize some limitations in our study with regard to the analysis of the mechanisms in which the MS reduce the cardiac fibrosis. Although we have demonstrated the enhanced gene expression of smad and RECK, further study is needed to analyze the level of gene expression of collagens, MMPs, and tissue inhibitors of metalloproteinases to show the activation of Smad2 and RECK protein.

In conclusion, skeletal MS implantation attenuated the impairment of diastolic function and the late remodeling after LVR in rat myocardial infarction model. It is suggested that MS implantation may improve the long-term outcome of LVR for ischemic heart disease.

MATERIALS AND METHODS

Animal Care

All experimental procedures and protocols used in this investigation were reviewed and approved by the institutional animal care and use committee and are in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996).

Isolation of Myoblasts and Construction of MS

Myoblasts were isolated from the skeletal muscle of the anterior tibialis from 3-week-old male Lewis rats and cultured as previously described (*11–14*). They were dissociated from the culture dishes with trypsin-ethylenediaminetetraacetic acid and reincubated on 35-mm temperature-responsive culture dishes (UpCell, Cellseed, Tokyo, Japan) at 37°C, with cell number adjusted to 3.0×10^6 per dish. More than 70% of these cells were actin-positive and 40% to 50% were desmin-positive (*14*). After 24 hr, the dishes were incubated at 20°C for 30 min. During that time, the MS detached spontaneously to generate free-floating, monolayer cell sheets. After detachment, the area of the sheets decreased to 1.00±0.05 cm^2, while the thickness increased to 100±10.0 μm (*14*). For the immunostaining of the engrafted MS, myoblasts were isolated from GFP transgenic Lewis rats and made into cell sheets in the same way as described earlier.

Myocardial Infarction Model

Eight-week-old male Lewis rats were used (220–250 g; Seac Yoshitomi Ltd. Fukuoka, Japan). The rats were anesthetized with ketamine (90 mg/kg) and Xylazine (10 mg/kg), and myocardial infarction was induced by ligation of LAD under mechanical ventilation. Two weeks after the ligation, baseline cardiac functions were measured by echocardiography, and rats that fulfill the following criteria were selected for further experiment: large akinetic or dyskinetic area in the anterior wall of the LV, LVDd 9.0±1.0 mm, and LVEF 35%±5%. For the quantitative study of the engrafted cell fate, 8-week-old female Lewis rats were used and myocardial infarction model was made in the same way as described earlier.

Experimental Groups

Male rats were randomized into three groups: 15 rats underwent only rethoracotomy (group sham), 15 underwent LVR (group LVR), and 15 underwent LVR, which was immediately followed by MS implantation (group LVR+MS). In group LVR and group LVR+MS, LVR was performed as follows: three to four mattress stitches with 7-0 polypropylene sutures were placed just onto the border line between infarcted and intact myocardium, and the infarcted myocardium was excluded (*16*). In group LVR+MS, five layers of MS were attached directly to the intact myocardium without sutures subsequently to LVR. After detachment from the temperature-responsive dish, each sheet was picked up individually and applied to the surface of the heart. After 3 to 5 min, subsequent sheets were applied and a total of five layers of MS were implanted. All the female rats underwent implantation of MS made from male rats concomitantly with LVR for the engrafted cell fate analysis. Additionally, three rats underwent implantation of the MS made from GFP positive

myoblasts after LVR in the same way as group LVR+MS for immunostaining of implanted MS.

Echocardiography

LV functions of all the treated rats were monitored by echocardiography at baseline (2 weeks after LAD ligation), 3 days, 1 week, 2 weeks, and 4 weeks after the second operation. Echocardiography was performed with a SONOS 5500 (Agilent Technologies, Palo Alto, CA) using a 12-MHz annular array transducer under anesthesia with inhalation of isoflurane. The hearts were imaged in short-axis 2D views at the level of the papillary muscles, and the LVDs and LVDd were determined. LVEF was calculated by Pombo's method, as EF (%)=$\{(LVDd^3-LVDs^3)/LVDd^3\}\times100$. All the echocardiographic studies were performed by a single investigator who was blinded to the treatment groups and the results were agreed by all the other investigators.

Hemodynamic Study and Data Analysis

Four weeks after the second operation, after the last echocardiographic study, all the rats were ventilated again. Re-re-thoracotomy was performed and the LV apex was dissected carefully to minimize hemorrhaging. A silk thread was placed under the inferior vena cava just above the diaphragm to change the LV preload. After a purse string suture was attached to the LV apex with 7-0 polypropylene, the conductance catheter (Unique Medical Co., Tokyo, Japan) was inserted through the LV apex toward the aortic valve along the longitudinal axis of the LV cavity and then fixed. A Miller 1.4 Fr pressure-tip catheter (SPR-719, Millar Instruments, Houston, TX) was also inserted from the LV apex and fixed. The conductance system and the pressure transducer controller (Integral 3 [VPR-1002], Unique Medical Co.) were set as previously reported (*31*). The pressure-volume loops and intracardiac electrocardiogram were monitored online, and the conductance, pressure, and intracardiac electrocardiographic signals were analyzed with Integral version 3 software (Unique Medical Co.) (*31*). Under stable hemodynamic conditions, the baseline indices were initially measured and then the pressure-volume loop was drawn during the inferior vena cava occlusion and analyzed.

The following indices were calculated as the baseline LV function: heart rate, ESP, EDP, and τ. ESP volume relationship and EDPVR were determined by pressure-volume loop analysis as load-independent measures of the LV function. All the catheter studies were performed by a single investigator who was blinded to the treatment groups and the results were agreed by all the other investigators.

Histological Study

After all measurements were finished, the rats were killed for histological study. In eight rats from each group, LV myocardial specimens were obtained and fixed with 10% buffered formalin and embedded in paraffin. Hematoxylin-eosin staining was performed for the measurement of the ventricular wall thickness. The thickness of the ventricular wall was measured at two points from the LV posterior area and two points from the interventricular septum, and results were expressed as the average of the four points. Picrosirius red staining was performed to detect myocardial fibrosis. Myocardial fibrosis was expressed as percent fibrosis, the fraction of red-stained area in total myocardium, with results obtained from 10 fields per section per animal from LV lateral and posterior wall. Also periodic acid-Schiff staining was performed to examine the degree of cardiomyocyte hypertrophy. Myocyte size was determined by point-to-point perpendicular lines drawn across the cross-sectional area of the cell at the level of the nucleus. The results were expressed as the average diameter of 40 myocytes randomly selected from the LV lateral and posterior wall. To label vascular endothelial cells, so that blood vessels could be counted, immunohistochemical staining for factor VIII-related antigen was performed according to a modified protocol. We used EPOS-conjugated antibody to factor VIII-related antigen coupled with HRP (Dako EPOS Anti-Human von Willebrand Factor/HRP, Dako) as primary antibody. The stained vascular endothelial cells were counted under a light microscope. Results were expressed as the number of blood vessels/mm^2.

Measurement Probrotic Agent Gene Expression 4 Weeks After LVR and MS Implantation

In the remaining seven rats from each group, the myocardium from the LV lateral wall, the area where MS were applied in group LVR+MS, were also stored in RNAlater solution (QIAGEN, Hilden, Germany). Total RNA was extracted with the RNeasy mini kit (QIAGEN), and relative levels of RNA transcripts were measured by the real-time quantitative reverse transcription polymerase chain reaction technique using the ABI PRISM 7700 Sequence Detection System. The measurement of the mRNA expression of TGF-β, Smad2, and RECK was performed in triplicate. The results are expressed after normalization for glyceraldehydes-phosphate dehydrogenase.

Quantitative and Histological Evaluation of Engrafted Cell Survival

Intact hearts from female Lewis rats were collected, freed of the right ventricular free wall, and transplanted with MS made from known numbers (3.0×10^2, 3.0×10^3, 3.0×10^4, 3.0×10^5, 3.0×10^6, or 3.0×10^7, n=3 each) of male Lewis rats myoblast. Samples were homogenized and analyzed for the levels of *sry* and *il2*, which are Y chromosome-specific and gender consensus genes, respectively. An estimate of the fraction of donor cells was calculated as $2\times$ *sry*/*il2*$\times100$, and standard curves were constructed to determine the myoblast number from the percentage of male cells (*15*). The amount of donor myoblasts was measured on the day of MS implantation (n=5), 7 days (n=6), and 28 days (n=5) after implantation. Genomic DNA was prepared using an Allprep kit (Qiagen). Quantitative polymerase chain reaction of *sry* and *il2* was performed with 1.2 μg of DNA using Taqman universal polymerase chain reaction master mix (Applied Biosystems) according to the manufacturer's instructions and an ABI PRISM7700 sequence detection system (Applied Biosystems).

To evaluate the surviving engrafted cell histologically, five layers of MS made from myoblasts of GFP transgenic Lewis rats were implanted on the LV of Lewis rats. They were killed 28 days after the surgery.

Data Analysis

All data were expressed as the mean±standard error of mean and subjected to analysis of variance (ANOVA). Time-course data were first analyzed by using repeated-measurements two-way ANOVA, and the other numeric data were analyzed by using one-way ANOVA. If significance was found, posthoc comparisons were performed. Findings were considered significant at P less than 0.05.

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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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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

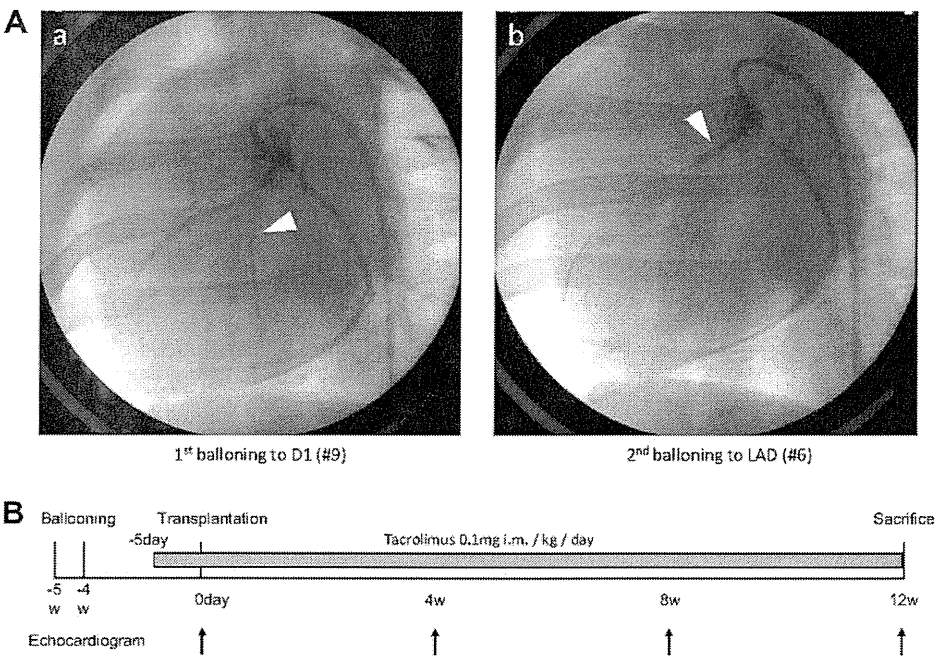

Fig. 1. Study protocol and angiographic demonstration of transient coronary artery occlusion. (A) Five weeks before transplantation, the first diagonal branch (D1; #9) of the coronary arteries was balloon-occluded followed by reperfusion (a, arrowhead). One week later, the left ascending coronary artery 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).

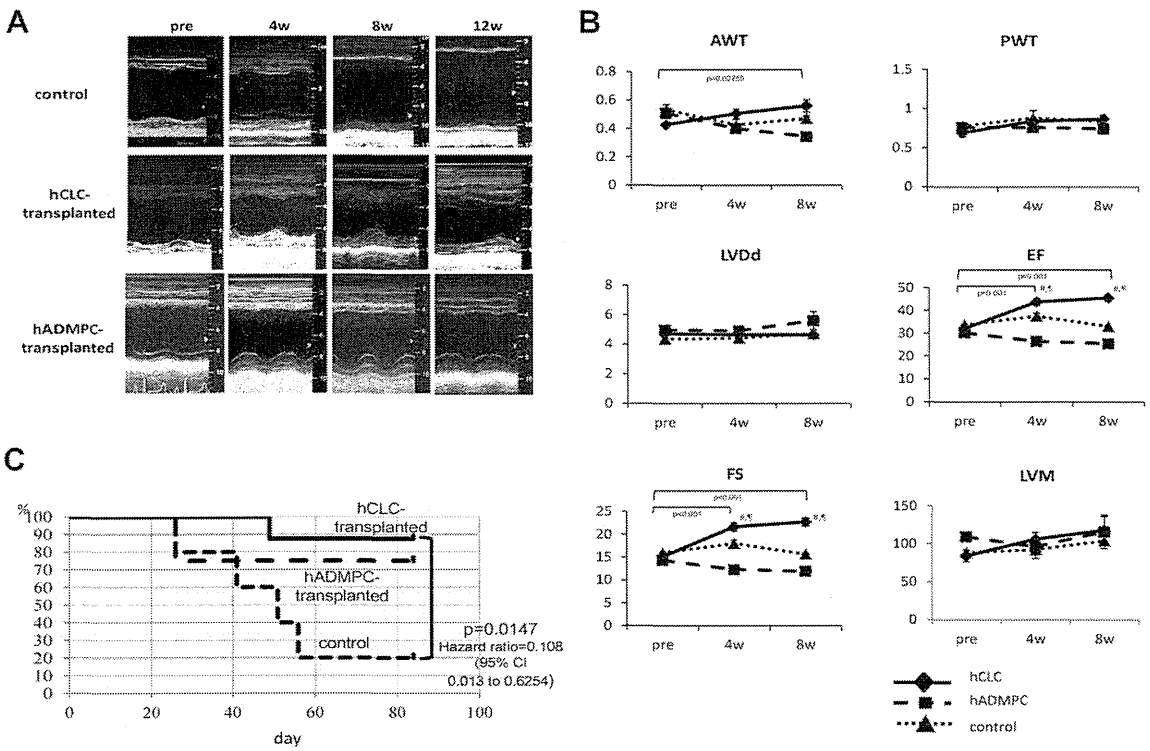

Fig. 2. Effects of hCLCs transplantation on cardiac function and survival rate. (A) In the hCLCs transplanted group, M-mode echocardiography showed improved wall motion within 4 weeks of transplantation. In contrast, worsening of the wall motion was noted in the mock-transplanted control swine. (B) Anterior 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

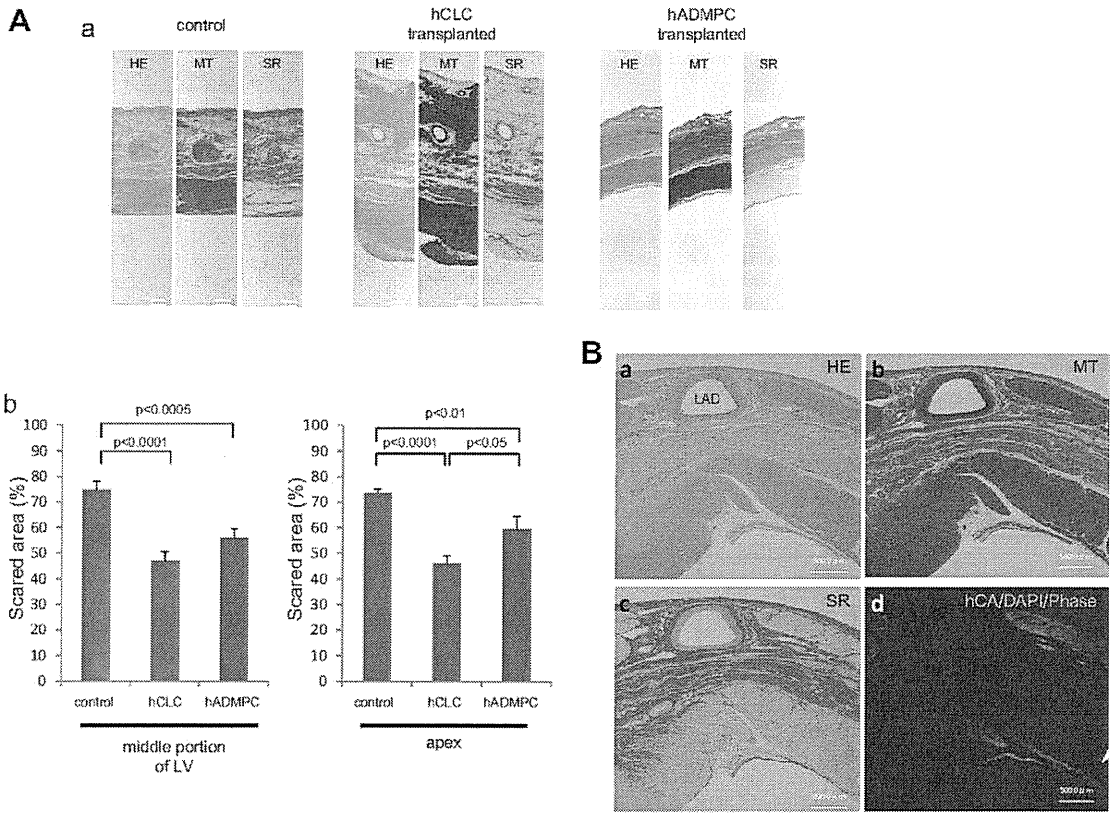

Fig. 3. Effects of hCLCs transplanted via coronary artery on cardiac structure. (A) (a) Photomicrographs of representative myocardial sections of the scarred area stained with hematoxylin/eosin (HE), Masson trichrome (MT) and Sirius red (SR) in the hCLCs-, hADMPC-transplantation and mock-transplanted 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.