て，心拍数パターンを中心とした所見と胎児健康度の解釈および臨床的対応に対してガイドラインが提案された。それぞれ 3 段階（3-tier system）に分類されているが，中間分類が広すぎる欠点がある。そこで，わが国においては独自に 5 段階（5-tier system）の評価が推奨[8)10)]された。

12 日産婦新基準の医学的根拠

8 文献を検討した報告は以下の結論を得ている。① 基線細変動が正常であれば，98％にアシドーシス（pH＜7.10）がない。② 基線細変動が減少または消失すれば，その 23％にアシドーシスがある。この結論を考慮すると臨床現場においては，基線細変動は胎児 well-being を予測するうえで最重要視すべき項目と考えられる。

また，遅発一過性徐脈（late deceleration）と変動一過性徐脈（variable deceleration）は，徐脈の程度や徐脈持続時間に規定される重症度が増すにつれて，有意の胎児血 pH 低下が観察されている[15)16)]。すなわち，遅発一過性徐脈においては，一過性徐脈の心拍数下降度が 45 bpm 以上，15-45 bpm，15 bpm 未満と軽度になるに従って，胎児血 pH が上昇する。また，変動一過性徐脈においては，高度（持続時間 60 秒以上，かつ最下点 70 bpm 未満），中等度（持続時間 60 秒以上，かつ最下点 70-80 bpm，持続時間 30-60 秒，かつ最下点 70 bpm 未満），そして軽度（それ以外の変動一過性徐脈）になるに従って胎児血 pH が上昇する。文献的報告はないが，遷延一過性徐脈（prolonged deceleration）においても同様であると推定される。

■ おわりに

周産期医療が再構築されようというこの時期に，さらなる産科医と麻酔科医の連携を深めるために，周産期医療から見た“外のインフラ”の話題として妊産婦死亡の問題，“内のインフラ”の話題として分娩時胎児管理の問題を述べ，その中でどのような連携法があるのか可能性を述べた。行った方策が，どのように反映されているのかという，ショートフィードバック体制を確立することもきわめて重要であることを付記する。

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総　説

全国1～4歳児死亡小票から見た我が国の小児重症患者医療体制の問題点

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要　旨

はじめに：

1～4歳児死亡率は，他の先進諸国と比較して我国で高い．本稿では我が国で初めて全国死亡小票を用いてこの年齢層の死亡に関する地域差を中心とした問題点を検討した．

対象と方法：

対象は，死亡小票を用いて得られた2005～2006年度2,245件の全国の1～4歳児死亡症例である．まず，2006年の年齢別各都道府県人口と上記データを用いて各地の全対象死亡率及び基礎疾患のない児（1,035件：全体の46%）の死亡率を算出した．次に，各地の搬送先病院を種別してその搬送先比率を検討した．

結果：

1）関東以外の地域では，大学病院，小児病院より一般病院で1～4歳児の死亡症例を主に診療していた．

2）全対象死亡率に比し基礎疾患のない児の死亡率において，より地域格差が認められる傾向にあった．

3）全国的に全対象死亡症例に比較して基礎疾患のない児の死亡症例には，大学病院や小児病院の関与が有意に減少していた（28±10% vs 18±9%，$p<0.05$）．

考察：

1）全対象死亡率と基礎疾患のない児の死亡率のどちらも上位10位以内に入っている7県には重症患者の受け皿となる小児集中治療室はなかった．

2）全国的に1～4歳児の死亡に大学病院，小児病院は主に関与しておらず，基礎疾患のない児の死亡への関与は更に減少し基礎疾患のない児の死亡率の地域格差との因果関係が推測された．

3）中核病院となるべき大学病院，小児病院に小児重症患者の受け皿である小児集中治療室が十分整備されていないことが，重症患者の集約化の妨げの原因の1つと推察される．従って人的物的資源の揃った大学病院や小児病院に重症患者を集約化できるように，常時院外に開かれた小児集中治療室の全国整備の検討を考慮すべきと考えられた．

4）基礎疾患のない児の死亡の4割が外因性疾患であり，小児集中治療室では小児の外傷に対応できる体制を検討する必要性が考えられた．

キーワード：小児集中治療，救命救急センター，小児救急，医療体制，死亡率

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はじめに

我が国の小児死亡の特徴は，他の年齢層と比較して1～4歳までの死亡率が先進諸国の中で高いことであ

都道府県名	全対象死亡率（対人口千人）
秋田	**0.38**
山口	**0.35**
鹿児島	**0.35**
青森	**0.33**
石川	**0.33**
山梨	0.32
岐阜	0.31
北海道	**0.31**
愛媛	**0.31**
福岡	0.31
全国平均（±SD）	0.25±0.06

図1　全対象死亡率上位10都道府県
（太字は，基礎疾患のない児の死亡率でも上位10位以内を占める都道府県を示す．）

る[1]．その原因を究明するには小児医療関係者が全国規模で小児死亡データを解析する必要があるが，今までは多くの法律上の制約から実行するのが困難であった．今回我々は日本小児科学会の強力な支援の下，1～4歳児の2005，2006年度の死亡小票の全国データを収集し，解析することができた．本稿では，特にこの年齢層の死亡の地域差に焦点を当てて問題点を解析したのでここに報告する．

対象と方法

2005年度及び2006年度の死亡小票の調査をして得られた総計2,245件の1～4歳児の死亡例を対象とした．

まず，2006年の年齢別各都道府県人口[2]と上記データを用いて各地の全対象死亡率を算出した．

次に，全対象から悪性腫瘍，先天性心疾患，染色体異常，奇形症候群，脳性麻痺，低出生体重児（極小，超低出生体重児），死亡まで半年以上の慢性疾患及び不詳の死亡を除いた基礎疾患の認められない児（1,035件：全体の46%）の死亡率を算出した．

最後に各地域における搬送先病院を種別してその搬送先比率を検討した．

統計学的検定はt検定を用いて行った．また，死亡率の地域格差に関してはGini係数を使用した．

結　果

1）1～4歳児死亡率上位都道府県について

北海道，青森県，秋田県，石川県，愛媛県，山口県，鹿児島県以上7県は，全対象死亡率，基礎疾患のない児の死亡率ともに上位10位以内を占めていた（図1，2）．

都道府県名	基礎疾患のない死亡率（対人口千人）
愛媛	0.23
秋田	0.22
山口	0.20
石川	0.18
鹿児島	0.18
青森	0.17
岡山	0.17
高知	0.17
北海道	0.16
宮崎	0.16
全国平均（±SD）	0.12±0.04

図2　基礎疾患のない児の死亡率上位10都道府県
（太字は，全対象死亡率でも上位10位以内を占める都道府県を示す．）

2）全対象と基礎疾患のない児の死亡率の地域差の比較

格差を示す統計量であるGini係数で比較すると全対象死亡が47都道府県で0.13に対して基礎疾患のない児の死亡が0.18と高いことより後者の死亡率により地域格差があることが認められた．

3）基礎疾患のない児の死亡原因

基礎疾患のない児の死亡原因は，主な内因性疾患である感染症，呼吸器疾患，循環器疾患，神経疾患で44%を占め，不慮の事故などの外因性疾患で41%を占めていた（図3）．

4）大学病院，小児病院における1～4歳児死亡の割合

1～4歳児の全死亡の中で大学病院，小児病院での死亡比率は全国平均28%に留まっていた．基礎疾患のない児の死亡に限定すると大学病院，小児病院での死亡の割合は全国平均18%と全死亡に比較して有意に（$p<0.05$）減少していた（図4）．尚，上記病院以外に個人病院，野外，自宅で死亡するケースがあるが明らかな地域差は認められなかった．

考　察

我が国の小児重症患者医療体制を構築する上で『小児死亡の詳細な情報』を知ることは重要である．なぜならこの情報から小児重症患者の集約化の程度や主な搬送先病院群の質あるいはどのような疾患で亡くなっているかなどが判明して将来の医療体制構築のための基礎データとなるからである．しかし，この情報は死亡診断書から作られた死亡小票から得られた情報でありこのデータを使うためには法律上の大きな制約を乗り越える必要があった．そして，その制約を乗り越えるためだけで数年を要するためいままでこのデータを現場の小児科医が解析することはなかった．今回初めて解析できたのは日本小児科学会，小児救急委員会の

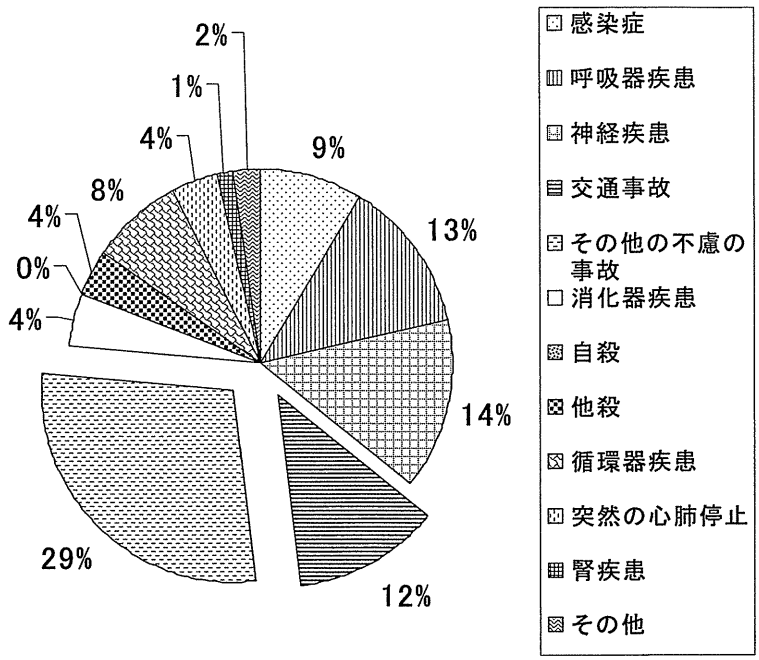

図3　基礎疾患のない児の死亡原因

	全患者群		基礎疾患のない患者群	
地域名	大学病院/小児病院	一般病院	大学病院/小児病院	一般病院
北海道	24%	53%	10%	60%
東北	27%	51%	18%	54%
関東	54%	32%	40%	40%
北陸	25%	60%	11%	64%
中部	21%	66%	17%	65%
関西	32%	44%	18%	47%
中国	19%	58%	14%	59%
四国	18%	61%	17%	60%
九州	32%	55%	23%	59%
沖縄	28%	58%	10%	70%
平均	28±10%	54±10%	18±9%	58±9%

図4　地域別搬送先病院の比率
（全国平均は平均±SDで示した.）

強力な支援を受けて，厚生労働省「幼児死亡の分析と提言に関する研究班」が中央行政に辛抱強い働きかけを行い，調査許可を得るだけで2年を要したが，死亡小票データの使用許可を得て解析まで漕ぎ着けたわけである. 将来的には, このような小児死亡に関するデータは小児医療専門家が必要な時に使用できるように死亡診断書の情報とは別に日本小児科学会が小児死亡症例の登録体制を構築し，データを独自に蓄積すべきではないかと考える．これにより我々小児医療専門家がこのような情報を管理，解析して今後の医療体制について独自の見解を作成してゆくことが可能になると思われる.

全対象及び基礎疾患のない児の死亡率がどちらも上位10位以内に入る7県には小児集中治療室は存在しなかった．欧米では小児集中治療室のある施設の方がそうでない施設より重症患者の死亡率が低下すること[3]. 小児の外傷に関しても小児集中治療室の方が一般の救命救急センターよりも死亡率が改善すること[4]などのデータからその有用性が示されている．また我が国では，小児集中治療室の有用性を示唆するデータとして小児集中治療室がある地域ではその他の地域に比較して外傷の小児死亡率が低下すること[5]. 同じ重症度の疾患における救命率が小児集中治療室のある病院の方がない施設より上昇する[6]などのデータがある. 日本小児科学会では，将来の小児医療体制グランドデザイン[7]の中で大学病院, 小児病院を中心とした中核病院に小児集中治療室を設置することを推奨しており，日本集中治療学会と合同で小児集中治療室の設置基準も定めている[8].

全対象死亡率に比較して基礎疾患のない児の死亡率により地域格差が認められる傾向にあった．この傾向は，全国的に1～4歳児死亡に対して大学病院，小児病院での死亡は限定されており（全国平均28%），基礎疾患のない児の死亡への関与は更に（$p<0.05$）減少している（全国平均18%）事実との因果関係が疑われる. つまり，基礎疾患のない児は小児病院，大学病院と比較してより人的・物的資源のあまり整備されていない一般病院へ搬送されている可能性がある.

今まで小児病院，大学病院は，専門病院ということで特殊な疾患を中心に扱ってきた[9][10]．一般病院にある程度の人的資源がそろっている時期にはこれらの病院で重症患者を診療することも可能であると思われるが新研修医制度により大都市圏以外の地域の一般病院が

人的に極めて厳しい状況に追い込まれている．この現状を直視すれば，将来的には多くの大学病院，小児病院で疾患の種類にかかわらず24時間いつでも院外から重症患者を受けいれる体制を整備することを検討すべきと考えられた．

本研究の限界としては，まず1～4歳の死亡原因の詳細に言及していない点である．欧米と比較して我が国特有の疾患が1～4歳の死亡率の上昇に関与している可能性は否定できない．次に，小児集中治療室の有効性を直接示すまでには至っていない点がある．これは，我が国の小児集中治療室の多くが小規模でその有効性が限られていること[11]や術後管理を中心として常時院外からの救急患者に対応する体制をとっていないなど我が国の小児集中治療室は，いまだ発展途上で数だけの問題だけでなく質の問題も抱えていることも考慮に入れる必要があると考える．最後に，大学病院，小児病院での収容比率の地域差にはその地域の大学，小児病院数が影響していると考えられる．しかし，大学，小児病院数が同じ同一地域で全対象死亡患者収容率に比較して基礎疾患のない児の死亡患者収容率が大学病院，小児病院で減少している事実は，大学，小児病院が基礎疾患のない児の死亡に積極的に関わっていない可能性を示している．

以上より人的物的資源の揃った大学病院や小児病院を中心に急変時の小児を集約化することを検討する必要がある．現在年間約1,100人の1～4歳児死亡がおり，15歳未満の全死亡数でも5,000人前後[2]である．全国に大学病院・小児病院が100以上あるため十分集約化は可能である．ただ，そのための受け皿である常時院外に開かれた小児集中治療室の整備が不十分であり，その整備は急務である．我々の試算では，現在の大学病院，小児病院の半数の約50施設に各10床の小児集中治療室を整備すれば我が国の小児重症患者に十分対応可能な状況となる[5][12]．

また，基礎疾患のない児の死亡の4割が外因性疾患である事を考慮して外因性疾患にも対応できるように小児集中治療室の体制を検討する必要がある．これは，小児集中治療室が救命救急センターとしての役割も持つ必要があるということである．つまり，小児集中治療室は今後外傷に対応できるように人的・物的資源を揃える必要がある．例えば，小児集中治療室を設置する施設の選定に当たっては当面救命救急センターを併設している施設を優先し，そこでは小児科医が中心となって小児集中治療室を運営し，救命救急医，小児外科医，脳外科医などと小児外傷チームを編成して協力しながら同じ小児集中治療室で内因性疾患以外に外傷などの外因性疾患も管理してゆくことが合理的である[13][14]．また，集約化ということを考慮すればドクターヘリの7割が救命救急センターに配置されていることも考慮すべき点と思われる[12]．しかしながら，現状では成人救命救急センターは小児患者に十分対応しているとは言えず，上記したように独立した小児集中治療室を併設して将来的にそちらに機能を移行することが重要と考える[13][14]

結　論

2005～2006年度1～4歳児の死亡小票を解析した．その結果，2年間に2,245人の死亡がありそのうちの約3割の患者しか大学病院，小児病院に搬送されていなかった．このことより全国的に人的・物的資源の整備されている大学病院，小児病院に小児重症患者が集約化されていないことが明らかとなった．1～4歳児の死亡率を減少させるためには，重症患者の受け皿となるべき常時院外に開かれた小児集中治療室を大学病院，小児病院に整備することが必要と考えられた．

謝辞　死亡小票の閲覧調査に参加された，東京大学大学院医学系研究科小児医学講座の井田孔明，土田晋也，五石圭司，康　勝好，小野　博，小寺美咲，自見英子，関　正史，林　郁子，谷口留美，山口真由美，資料解析に参加された青谷裕文（京都きづ川病院），松浪　桂（大阪府立母子保健総合医療センター），統計解析に参加された林　啓一（帝京大学医学部小児科）の各位に深謝いたします．

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The Problems of the Pediatric Emergency System Based on the National Infant Death Report (Aged from 1y.o through 4y.o) in Japan

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Preface：

The mortality of the infants aged from 1y.o through 4y.o in Japan is higher than that in the other OECD countries. We have analyzed the national death report of the infants for the first time in Japan. Because of the complicated legal hurdle, it has been difficult for the pediatricians to analyze the data. Herein, we show the regional difference of the infant mortality based on the national death report.

Subject & Method：

We analyzed 2,245 deaths aged from 1y.o through 4y.o in Japan based on the national death report from 2005 through 2006.

Then, we calculated the mortality of all infants & the infants without underlying diseases (1,035 cases, 46% of 2,245) based on the above data & the population aged from 1y.o through 4y.o of each prefecture in 2006.

Finally, we analyzed what kind of hospitals those infants died in.

Results：

1) More infants aged from 1y.o through 4y.o died in the general hospitals rather than the university & children's hospitals in all regions except for the Kanto region.

2) The regional mortality difference of the infants without underlying diseases is more various than that of all infants.

3) Compared with all infants, the infants without underlying diseases died less in the university & children's hospitals than in the general hospitals ($28 \pm 10\%$ vs $18 \pm 9\%$, $p < 0.05$).

Discussion：

1) There were no PICUs in the 7 prefectures which ranked in the top 10 of both all infants mortality & the infant mortality without underlying diseases.

2) The fact that the regional mortality difference of the infants without underlying diseases is more various than that of all infants could be due to the fact that the infants without underlying diseases died less in the university & children's hospitals than in the general hospitals.

3) The shortage of PICU is considered to be one of the causes for the inadequate rationalization of the severe sick children in Japan. Therefore, we should plan to distribute Picas in the university & children's hospitals throughout the country.

4) About 40% of the infants without underlying diseases died of the injuries. Therefore, PICU should be able to take care of injured infants so that we should adjust the PICU system for the purpose.

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Original article

Allogeneic administration of fetal membrane-derived mesenchymal stem cells attenuates acute myocarditis in rats

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ABSTRACT

We reported previously that the autologous administration of bone marrow-derived mesenchymal stem cells (BM-MSC) significantly attenuated myocardial dysfunction and injury in a rat model of acute myocarditis by stimulating angiogenesis and reducing inflammation. Because BM aspiration procedures are invasive and can yield low numbers of MSC after processing, we focused on fetal membranes (FMs) as an alternative source of MSC to provide a large number of cells. We investigated whether the allogeneic administration of FM-derived MSC (FM-MSC) attenuates myocardial injury and dysfunction in a rat myocarditis model. Experimental autoimmune myocarditis (EAM) was induced in male Lewis rats by injecting porcine cardiac myosin. Allogeneic FM-MSC obtained from major histocompatibility complex-mismatched ACI rats (5×10^5 cells/animal) were injected intravenously into Lewis rats one week after myosin administration. At day 21, severe cardiac inflammation and deterioration of cardiac function were observed. The allogeneic administration of FM-MSC significantly attenuated inflammatory cell infiltration and monocyte chemoattractant protein 1 expression in the myocardium and improved cardiac function. In a T-lymphocyte proliferation assay, the proliferative response of splenic T lymphocytes was significantly lower in cells obtained from FM-MSC-treated EAM rats that reacted to myosin than in cells obtained from vehicle-treated rats with EAM. T-lymphocyte activation was significantly reduced by coculture with FM-MSC. The allogeneic administration of FM-MSC attenuated myocardial dysfunction and inflammation, and the host cell-mediated immune response was attenuated in a rat model of acute myocarditis. These results suggest that allogeneic administration of FM-MSC might provide a new therapeutic strategy for the treatment of acute myocarditis.

1. Introduction

Acute myocarditis is a nonischemic heart disease characterized by myocardial inflammation and edema. This disease is associated with rapidly progressing heart failure, arrhythmia, and sudden death [1,2]. Although the early evidence showing the efficacy of immunoglobulin and interferon therapies appears promising, these results have yet to be demonstrated in randomized controlled clinical trials. The current options are restricted to supportive care for patients with heart failure and arrhythmia. The lack of a specific treatment and the potential severity of the illness emphasize the importance of new effective therapeutic strategies for myocarditis.

Mesenchymal stem cells (MSC) are multipotent stem cells present in the bone marrow (BM), adipose tissue, and many other tissues, and these cells can differentiate into a variety of cells, including

Abbreviations: MSC, mesenchymal stem cells; BM, bone marrow; BM-MSC, bone marrow-derived mesenchymal stem cells; FMs, fetal membranes; FM-MSC, fetal membrane-derived mesenchymal stem cells; MHC, major histocompatibility complex; ACI, August–Copenhagen–Irish; GFP, green fluorescent protein; EAM, experimental autoimmune myocarditis; PBS, phosphate-buffered saline; α-MEM, α-minimal essential medium; FBS, fetal bovine serum; TGF-β3, transforming growth factor-β3; FITC, fluorescein isothiocyanate; LVSP, left ventricular systolic pressure; LVDs, left ventricular systolic dimension; LVDd, left ventricular diastolic dimension; H&E, hematoxylin and eosin; MCP1, monocyte chemoattractant protein 1; HRP, horseradish peroxidase.

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adipocytes, osteocytes, chondrocytes, endothelial cells, and myocytes [3–5]. MSC are a promising cell source for regenerative therapies. We have reported that the autologous administration of BM- or adipose tissue-derived MSC improves cardiac function in rat models of dilated cardiomyopathy and myocardial infarction [6–8]. We also recently demonstrated that the administration of autologous BM-derived MSC (BM-MSC) attenuates myocardial injury and dysfunction in rats with acute myocarditis [9].

However, there are limitations to the application of autologous BM in clinical situations. BM procurement procedures in humans may be painful and may yield low numbers of MSC after processing. An alternative source of MSC that could provide large quantities of cells would be advantageous. To address this issue, we focused on fetal membranes (FMs), which are generally discarded as medical waste after delivery, as an alternative source of autologous MSC. Several studies have reported that human FMs contain multipotent cells similar to BM-MSC, which are easy to expand [10,11]. We demonstrated recently that the allogeneic transplantation of FM-derived MSC (FM-MSC) and BM-MSC induces therapeutic angiogenesis in a rat hind-limb ischemia model [12]. MSC have been reported to induce immune tolerance [13,14], and we confirmed that the transplantation of FM-MSC did not elicit any lymphocyte proliferative response despite their allogeneic origin.

In this study, we investigated whether the intravenous allogeneic administration of FM-MSC improves cardiac function and decreases myocardial inflammation in rats with myosin-induced myocarditis, and the mechanisms underlying the changes induced by allogeneic FM-MSC administration.

2. Materials and methods

2.1. Animals

Different strains of rats were used, based on their major histocompatibility complex (MHC) antigen disparities: Lewis rats (MHC haplotype: RT-1A^{l}; Japan SLC, Hamamatsu, Japan), and August–Copenhagen–Irish (ACI) rats (MHC haplotype: RT-1A^{a}; Japan SLC). Green fluorescent protein (GFP)-transgenic Lewis rats (Institute of Laboratory Animals, Kyoto University, Japan) were also used to investigate the distribution of the transplanted FM-MSC. Adult rats, aged 8–12 weeks, were used for the induction of experimental autoimmune myocarditis (EAM) and were maintained in our animal facility. The experimental protocols were approved by the Animal Care Committee of the National Cardiovascular Center Research Institute.

2.2. Preparation of FM-MSC

The isolation and expansion of FM-MSC were performed as described previously [12]. In brief, pregnant ACI rats (15 days postconception) were sacrificed, and their uteri were harvested and placed in phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA, USA). We chose 15 days postconception as the day of FM retrieval because that point was the best in terms of cell isolation and reproducibility in rats. After separation from the placenta, the FMs were minced with scissors and digested with type II collagenase solution (300 U/mL; Worthington Biochemicals, Lakewood, NJ, USA) for 1 h at 37 °C in a shaking water bath. Enzyme activity was neutralized with α-minimal essential medium (α-MEM; Invitrogen) containing 10% fetal bovine serum (FBS; Invitrogen). After filtration through a mesh filter (100 μm; BD Biosciences, Bedford, MA, USA) and centrifugation at 300×*g* for 5 min, the dissociated FM cells were suspended in α-MEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen), plated onto 100-mm uncoated culture dishes, and incubated at 37 °C in 5% CO_2. The population of adherent, spindle-shaped MSC was expanded. Almost all of non-adherent cells were hematocytes in the morphological observation. The isolation of FM-MSC was repeated three times to evaluate its reproducibility. In all experiments, the FM-MSC were used at passages 5–7.

2.3. Differentiation of FM- and BM-MSC into adipocytes, osteocytes, and chondrocytes

The multipotency of FM-MSC was assessed as described previously [12]. FM-MSC were seeded into six-well plates, and the differentiation into adipocytes and osteocytes was induced at 40–50% confluence. To induce differentiation into adipocytes, MSC were cultured with adipocyte differentiation medium: 0.5 mM 3-isobutyl-1-methylxanthine (Wako Pure Chemical Industries, Osaka, Japan), 1 μM dexamethasone (Wako Pure Chemical Industries), 50 μM indomethacin (Wako Pure Chemical Industries), and 10 μg/mL insulin (Sigma-Aldrich, St. Louis, MO) in α-MEM. After two weeks of differentiation, adipocytes were identified by the existence of lipid vesicles stained with oil red O (Sigma-Aldrich).

To induce differentiation into osteocytes, MSC were cultured in α-MEM with MSC osteogenesis supplements (Dainippon Sumitomo Pharma, Osaka, Japan), according to the manufacturer's instructions. After two weeks of differentiation, osteocytes were identified by the existence of mineral nodule deposition stained with alizarin red S (Sigma-Aldrich).

To induce differentiation into chondrocytes in three-dimensional culture, the pellet culture method was used. MSC were centrifuged at 150×*g* for 5 min and resuspended at a density of 1×10^6 cells/mL in an hMSC Differentiation BulletKit-Chondrogenic (Cambrex Bio Science, Walkersville, MD) supplemented with transforming growth factor-β3 (TGF-β3; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, 5×10^5 cells were placed in a 15-mL polypropylene tube and centrifuged at 150×*g* for 5 min. Fresh medium was added every third day. After three weeks of differentiation, cell pellets were fixed with 4% paraformaldehyde and embedded in paraffin. Differentiation into chondrocytes was identified by the existence of proteoglycan deposition stained with Safranin O (Sigma-Aldrich). Differentiation of FM-MSC was repeated three times to evaluate reproducibility.

2.4. Flow cytometry

Cultured FM-MSC were analyzed by flow cytometry (FACSCalibur, BD Biosciences) as described previously [12]. Fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies against rat CD34 (clone ICO-115, Santa Cruz Biotechnology, Santa Cruz, CA), CD45 (clone OX-1, BD Biosciences), CD73 (clone 5F/B9, BD Biosciences), CD90 (clone OX-7, BD Biosciences), RT1A^{a,b,l} (clone B5, BD Biosciences), and RT1B (clone OX-6, BD Biosciences) were used. Isotype-identical antibodies served as controls. Flow cytometric analysis of FM-MSC was repeated three times to evaluate reproducibility.

2.5. Acute myocarditis model

Purified cardiac myosin was prepared from the ventricular muscles of pig hearts according to a previously described procedure [15]. The antigen was dissolved at a concentration of 20 mg/mL in PBS containing 0.3 M KCl and mixed with an equal volume of complete Freund's adjuvant containing 11 mg/mL *Mycobacterium tuberculosis* (Difco Laboratories, Sparks, MD, USA). The rats were anesthetized with an intraperitoneal injection of 20 mg/kg sodium pentobarbital, and 0.1 mL of the antigen–adjuvant emulsion was injected into each footpad.

Forty-five Lewis rats were assigned randomly into the following three groups and treated with: 1) 0.2 mL of PBS only (Sham group,

$n=15$); 2) 0.2 mL of cardiac myosin only (MyoC group, $n=15$); and 3) 0.2 mL of cardiac myosin and FM-MSC (MyoC + FM-MSC group, $n=15$). One week after the myosin injection, allogeneic FM-MSC (5×10^5 cells/animal) or vehicle (PBS) was administered intravenously via the tail vein. We chose an intravenous route for the administration of FM-MSC because of its clinical applicability. We chose the time for the cell injection of one week after myosin injection on the basis of our previous report showing that this time was the most effective compared with other schedules [9]. In this study, we used the 5×10^5 cell administration because this cell number did not elicit the significant and persistent rise in pulmonary arterial pressure.

To assess the distribution of the injected cells, FM-MSC (5×10^5 cells/animal) derived from GFP-transgenic Lewis rats were administered intravenously via the tail vein seven days after the myosin injection. One day, one week or four weeks after the injection of the cells, the rats were sacrificed, and sections of tissues were obtained from the heart, lung, spleen, and liver, and embedded in paraffin ($n=4$ for each tissue).

2.6. Hemodynamic studies

Hemodynamic studies were performed on day 21 after the myosin injection. Anesthesia was maintained with isoflurane (1.5–2.0 vol.% in air), and a polyethylene catheter (model PE-50, BD Biosciences) was placed into the left ventricle through the right carotid artery. The heart rate was monitored by electrocardiography. Heart rate, mean arterial pressure, left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure, maximum dP/dt, and minimum dP/dt were used as the hemodynamic indices and were recorded simultaneously during ventilation after a minimum equilibration period of 20 min. The hemodynamic studies were performed with the investigators blinded to the treatment group and the analyses were performed offline.

2.7. Echocardiographic studies

Echocardiography was performed on day 21 after the myosin injection. The rats were anesthetized with isoflurane (1.5–2.0 vol.% in air). A 12-MHz probe was placed at the left fourth intercostal space for M-mode imaging using two-dimensional echocardiography (Sonos 5500, Philips, Bothell, WA, USA). The left ventricular systolic dimension (LVDs), left ventricular diastolic dimension (LVDd), anterior wall thickness, posterior wall thickness, and ejection fraction were measured and recorded as the average for three beats. Fractional shortening (%) was calculated as $([LVDd-LVDs]/LVDd)\times100$. The echocardiography studies were performed with the investigators blinded to the treatment group and the analyses were performed offline.

2.8. Histopathological studies

The hearts were excised above the origin of the great vessels on day 21 after the myosin injection, and the heart and body weight were recorded. The heart, spleen, pancreas, kidney, and liver were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned to 4-μm thickness, and stained with hematoxylin and eosin (H&E) or Masson's trichrome. H&E-stained sections were evaluated by a cardiovascular pathologist (H.I.-U.) with no knowledge of the experimental groups, who characterized the myocardial injury and inflammation using the following scale: 0, absent or questionable presence; 1, limited focal distribution; 2–3, intermediate severity; and 4, coalescent and extensive foci throughout the entire transversely sectioned ventricular tissue. To evaluate fibrosis, the collagen volume fraction was analyzed with image-processing software (Win ROOF, Mitani Co. Ltd., Tokyo, Japan).

2.9. Immunohistochemical studies

Deparaffinized sections were incubated with Protein Block (DakoCytomation, Glostrup, Denmark) and then with mouse anti-rat CD68 (clone ED-1; Millipore, Bedford, MA, USA), CD3 (BD Biosciences), monocyte chemoattractant protein 1 (MCP1; BD Biosciences), or rabbit anti-GFP antibody (Invitrogen) in diluent for 40 min, followed by incubation with horseradish peroxidase (HRP)-linked rabbit anti-mouse IgG or DakoCytomation Envision+ System-HRP Labeled Polymer (DakoCytomation) for 30 min. The sections were visualized with 0.5% diaminobenzidine (DakoCytomation) and 0.03% hydrogen peroxide, and counterstained with hematoxylin. Five random fields from each rat were photographed (Biorevo BZ-9000; Keyence, Osaka, Japan). The numbers of CD68- and CD3-positive cells and the MCP1-positive areas were analyzed with the image-processing software. The number of GFP-positive cells was counted in 20 randomly selected fields per section.

2.10. T-lymphocyte proliferation assay

T lymphocytes were isolated from the spleens of rats with myocarditis on day 21 using a previously described procedure [12]. The responder T lymphocytes were isolated from untreated control Lewis rats (sham-TL), myosin-treated Lewis rats with myocarditis (MyoC-TL), or allogeneic FM-MSC-administered myosin-treated Lewis rats with myocarditis (MyoC + FM-MSC-TL). The responder T lymphocytes (1×10^5 cells/well) were cultured in a 96-well culture plate with 50 μg/mL of purified porcine heart myosin (Sigma-Aldrich) as the stimulator, with or without the modulator cells. The modulator cells were allogeneic FM-MSC obtained from MHC-mismatched ACI rats (1×10^5 cells/well). When no allogeneic FM-MSC were added, T lymphocytes isolated from normal Lewis rats were added to adjust the cell number (1×10^5 cells/well). The modulator cells were irradiated at 30 Gy before they were cultured. A total of 2×10^5 cells were cocultured in 0.2 mL of tissue culture medium (RPMI 1640; Invitrogen) supplemented with 20% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in 96-well flat-bottomed plates for three days. The proliferation of the responding cells was assessed using the Cell Proliferation Biotrak ELISA System (GE Healthcare, Piscataway, NJ, USA), according to the manufacturer's instructions. T-lymphocyte proliferation is presented as the percentage of the relative proliferation response, as follows: % change in proliferative response = $(\text{absorbance}_{\text{each group}}/\text{absorbance}_{\text{sham-TL without modulator}})\times100$.

2.11. Statistical analysis

Data are expressed as mean ± standard error of the mean. Analysis of variance was used to compare each variable between groups, and the post hoc Tukey test was used to locate the significant differences. Differences were considered significant at $P<0.05$.

3. Results

3.1. Distribution of intravenously administered FM-MSC in rats with myocarditis

We obtained FM-MSC from ACI rats (MHC haplotype: RT-$1A^a$) to examine whether the allogeneic administration of FM-MSC could attenuate myocarditis in Lewis rats (MHC haplotype: RT-$1A^l$). FM-MSC differentiated into adipocytes, osteocytes, and chondrocytes ($n=3$, each) (Figs. 1(B)–(D)). Flow cytometric analysis of cultured FM-MSC at passage 5 ($n=3$) demonstrated that FM-MSC were positive for CD73, CD90, and MHC class I (i.e., RT1A) but were negative for CD34, CD45, and MHC class II (i.e., RT1B) (Fig. 1(E)).

To investigate the distribution of the intravenously injected FM-MSC in the rats with myocarditis, we intravenously administered

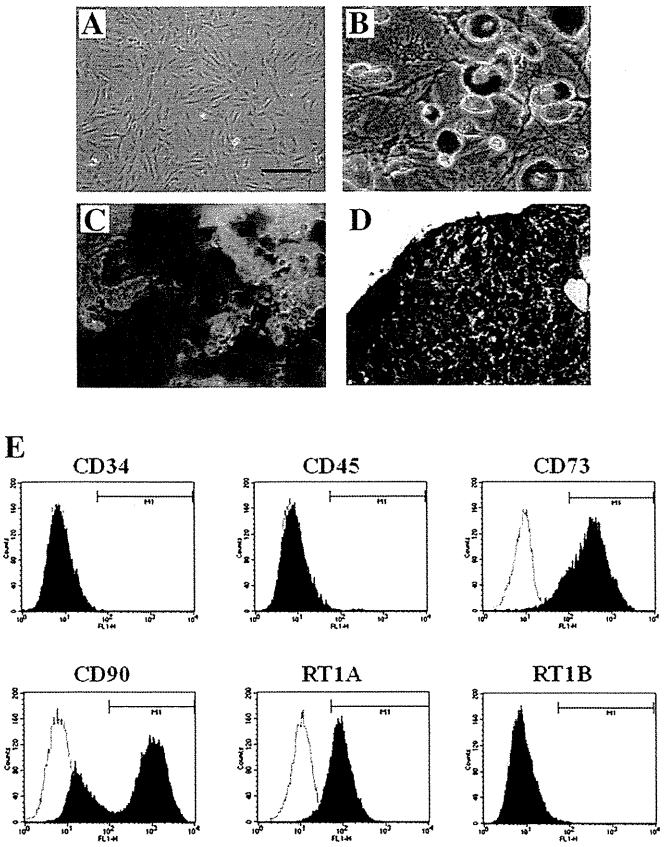

Fig. 1. Characterization of FM-MSC. (A) Morphology of FM-MSC. Scale bar = 100 μm. (B–D) Multipotency of FM-MSC. FM-MSC differentiated into adipocytes (B), osteocytes (C), and chondrocytes (D) ($n=3$ each). Scale bars = 50 μm. (E) Flow cytometric analysis of FM-MSC ($n=3$ each).

GFP-expressing FM-MSC obtained from GFP-transgenic Lewis rats one week after the myosin injection. GFP immunostaining demonstrated that GFP-positive transplanted FM-MSC were present in the heart, lungs, spleen, and liver at both one day and one week after FM-MSC injection (Fig. 2(A)). Semiquantitative analysis demonstrated that a significant number of GFP-positive cells were observed in the lung (day 1, 4.0 ± 0.4 cells/mm^2; and day 7, 2.4 ± 0.4 cells/mm^2), whereas only a few GFP-expressing engrafted cells were observed in the other organs at day 1 (heart, 1.1 ± 0.5 cells/mm^2; spleen, 0.3 ± 0.2 cells/mm^2; and liver, 0.2 ± 0.1 cells/mm^2) and one week (heart, 0.7 ± 0.3 cells/mm^2; spleen, 0.8 ± 0.1 cells/mm^2; and liver, 1.0 ± 0.5 cells/mm^2) after the FM-MSC injection ($n=4$ for each) (Fig. 2(B)). We found no GFP-positive cells four weeks after the FM-MSC injection.

3.2. Improvement in cardiac function by allogeneic administration of FM-MSC

All rats with myocarditis survived the 21-day observation period. On day 21, the heart weight/body weight ratio was significantly lower in the MyoC + FM-MSC group than in the MyoC group (3.7 ± 0.1 vs 4.3 ± 0.1, $P<0.05$). Hemodynamic analysis revealed significant improvements in the MyoC + FM-MSC group compared with the MyoC group in LVSP (119.1 ± 3.4 vs 102.0 ± 4.3 mmHg), mean arterial pressure (84.3 ± 3.2 vs 69.0 ± 3.1 mmHg), and maximum dP/dt (8202 ± 516 vs 6445 ± 373 mmHg/s) ($P<0.05$ for all; $n=15$ in each group) (Figs. 3(A) and (B), and Table 1).

Echocardiographic analysis revealed significant improvements in the MyoC + FM-MSC group compared with the MyoC group in LVDs (3.8 ± 0.2 vs 4.7 ± 0.1 mm), fractional shortening (45.4 ± 1.8 vs 36.8 ± 1.2%), and ejection fraction (83.2 ± 1.6 vs 74.2 ± 1.4%) ($P<0.05$ for all). Wall thickness was also significantly thinner in the MyoC + FM-MSC group than in the MyoC group (anterior wall thickness diastole, 2.1 ± 0.2 vs 2.5 ± 0.2 mm; and posterior wall thickness diastole, 2.2 ± 0.1 vs 2.5 ± 0.1 mm) ($P<0.05$ for all; $n=15$ in each group) (Figs. 3(C)–(E) and Table 2).

3.3. Attenuation of myocardial inflammation after allogeneic administration of FM-MSC

Histological analysis on day 21 after the induction of experimental myocarditis showed severe myocardial inflammatory changes. The semiquantitative grading of H&E-stained heart sections by a pathologist (H.I.-U.) using a blinded method showed significantly lower in the MyoC + FM-MSC group than in the MyoC group for tissue granulation (2.7 ± 0.2 vs 3.1 ± 0.1), eosinophil infiltration (1.8 ± 0.1

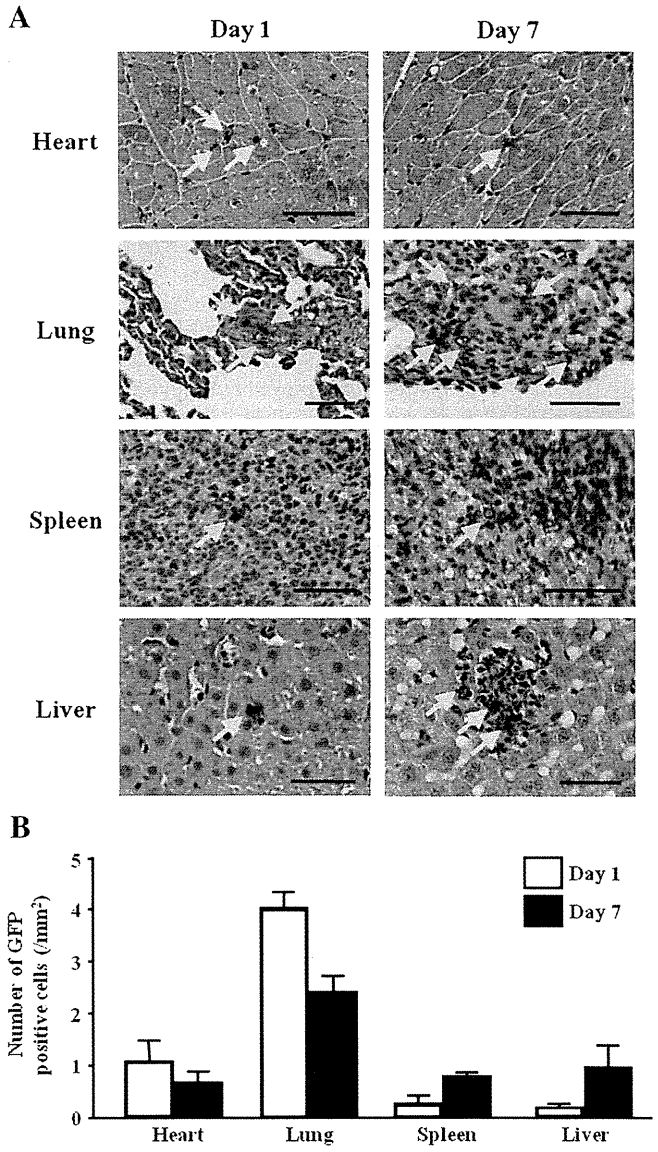

Fig. 2. Distribution of intravenous-administered FM-MSC in acute myocarditis. (A) GFP-positive-administered FM-MSC were present in the heart, lung, spleen, and liver one day and one week after cell administration (brown stain; yellow arrows). Scale bars = 50 μm. (B) Semiquantitative analysis demonstrated that a significant number of GFP-positive cells were observed in the lung, whereas only a few GFP-expressing engrafted cells were observed in the other organs at one day and one week after the FM-MSC injection ($n=4$ for each organ).

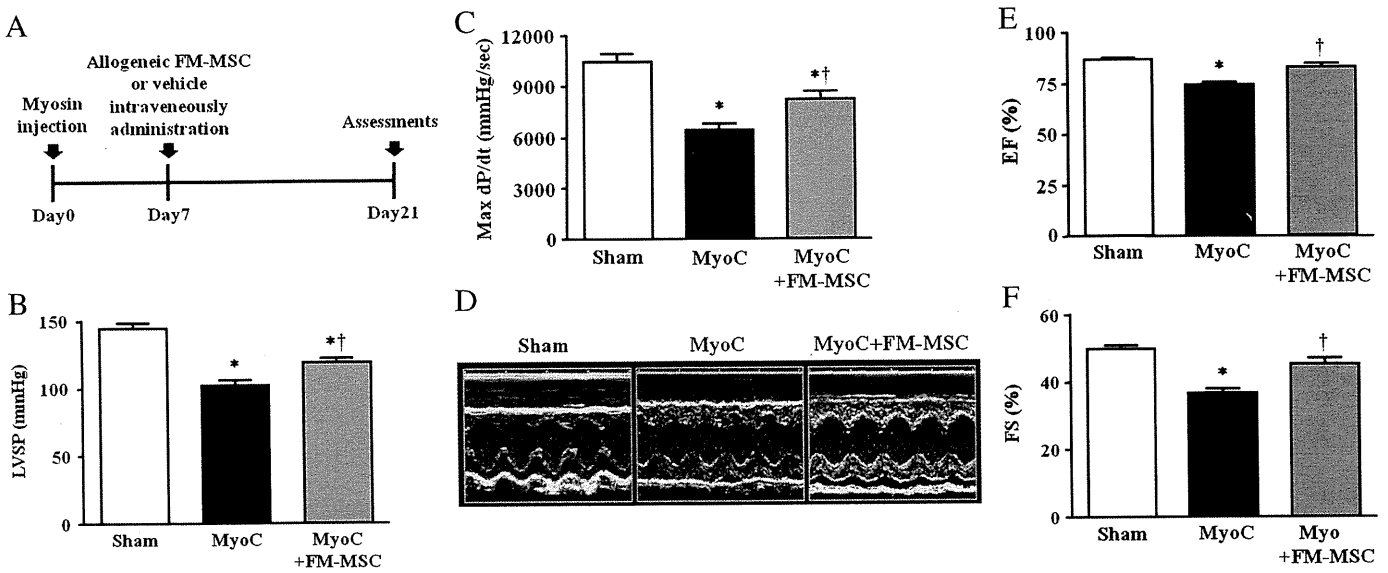

Fig. 3. Effects of administration of allogeneic FM-MSC on hemodynamic and echocardiographic parameters in acute myocarditis. (A) The study flowchart. (B) Left ventricular systolic pressure (LVSP) and (C) maximum dP/dt (max dP/dt) were measured in sham-treated rats given vehicle (Sham group), myosin-treated rats given vehicle (MyoC group), and myosin-treated rats given FM-SC (MyoC + FM-MSC group; $n = 15$ in each group). (D) Representative echocardiographic images showing wall thickening and poor movement in the MyoC group, and improved cardiac contractility in the MyoC + FM-MSC group. (E, F) Allogeneic FM-MSC administration significantly improved the ejection fraction (EF) and fractional shortening (FS; $n = 15$ for each group). Data are expressed as mean ± SEM. *$P<0.05$ vs the Sham group; †$P<0.05$ vs the MyoC group.

vs 2.7 ± 0.1), and edema (2.4 ± 0.1 vs 3.1 ± 0.1) ($P<0.05$ for all; $n = 15$ in each group) (Figs. 4(A) and (B)).

Masson's trichrome staining of the myocardium derived from the MyoC group on day 21 demonstrated prominent and diffuse interstitial fibrosis, which was attenuated dramatically in the MyoC + FM-MSC group (Fig. 4(C)). Quantitative assessment of myocardial fibrosis showed that the Masson's trichrome-stained collagen volume fraction was significantly smaller in the MyoC + FM-MSC group than in the MyoC group (3.1 ± 1.4 vs 7.7 ± 1.9%) ($P<0.05$; $n = 15$ in each group) (Fig. 4(D)).

Immunohistochemical analysis of the myocardial tissue on day 21 showed significantly attenuated infiltration of CD68-positive monocytes/macrophages in the MyoC + FM-MSC group compared with the MyoC group (3713 ± 426 vs 6528 ± 590 cells/mm^2) ($P<0.05$; $n = 15$ in each group) (Figs. 5(A) and (B)).

Immunohistochemical staining of the myocardial tissue for MCP1 on day 21 showed a few positive cells in the myocardial interstitium of the normal heart (Fig. 5(C)). In the rats with myocarditis, increased MCP1 expression was observed in the myocardial interstitium and in the vascular wall of the heart tissue. The hearts of the MyoC + FM-MSC group showed a partial reduction in MCP1 expression. Quantitative analysis demonstrated less MCP1 expression in the MyoC + FM-MSC group than in the MyoC group (0.85 ± 0.1 vs 1.46 ± 0.2%) ($P<0.05$; $n = 15$ in each group) (Fig. 5(D)).

Table 1
Physiological parameters in the three experimental groups.

	Sham	MyoC	MyoC + FM-MSC
HW/BW (g/kg)	2.5 ± 0	4.3 ± 0.1*	3.7 ± 0.1*,**
HR (bpm)	371.9 ± 7.0	337.9 ± 7.6*	364.3 ± 8.5**
MAP (mmHg)	100.1 ± 2.7	69.0 ± 3.1*	84.3 ± 3.2*,**
LVEDP (mmHg)	4.5 ± 1.0	5.7 ± 0.8	6.5 ± 1.0
Min dP/dt (mmHg/s)	−8258.9 ± 422.2	−4980.5 ± 278.6*	−6135.6 ± 375.9*

Sham, sham-operated rats given vehicle; MyoC, myosin-treated rats given vehicle; MyoC + FM-MSC, myosin-treated rats given FM-MSC (5×10^5 cells/animal); FM-MSC, fetal membrane-derived mesenchymal stem cells; HW/BW, heart weight-to-body weight ratio; HR, heart rate; MAP, mean arterial pressure; LVEDP, left ventricular end-diastolic pressure; Min dP/dt, minimum dP/dt.
* $P<0.05$ vs the Sham group.
** $P<0.05$ vs the MyoC group.

3.4. Attenuation of T-cell infiltration by allogeneic administration of FM-MSC

Marked T-cell infiltration was demonstrated by CD3 immunostaining of the cardiac tissues of the MyoC group on day 21. By contrast, T-cell infiltration was attenuated significantly in the MyoC + MSC group (2014 ± 196 vs 3068 ± 455 cells/mm^2) ($P<0.05$ vs the MyoC group; $n = 15$ in each group) (Figs. 5(E) and (F)).

3.5. Suppression of T-lymphocyte activation by allogeneic treatment with FM-MSC demonstrated in a T-lymphocyte proliferation assay

To examine whether allogeneic FM-MSC suppress T-lymphocyte activation, we performed a T-lymphocyte proliferation assay [12]. T lymphocytes collected on day 21 from the MyoC group with myocarditis were cocultured with porcine heart myosin and with irradiated T lymphocytes derived from normal Lewis rats. The proliferative response was significantly higher in the MyoC group with myocarditis than in the sham group T lymphocytes (183.2 ± 2.5 vs 100.0 ± 2.6%, $P<0.05$; $n = 8$ in each group). However, on day 21, the proliferative response was significantly lower in the T lymphocytes derived from the MyoC + FM-MSC group with myocarditis (164.2 ± 0%) than in the MyoC group ($P<0.05$, $n = 8$). T-cell activation was significantly reduced when T lymphocytes from the MyoC group or MyoC + FM-MSC group were cocultured with allogeneic ACI-derived

Table 2
Echocardiographic findings in the three experimental groups.

	Sham	MyoC	MyoC + FM-MSC
LVDs (mm)	3.6 ± 0.1	4.5 ± 0.1*	3.8 ± 0.2**
LVDd (mm)	7.1 ± 0.1	7.2 ± 0.1	7.0 ± 0.2
AWT diastole (mm)	1.4 ± 0	2.5 ± 0.2*	2.1 ± 0.2*,**
PWT diastole (mm)	1.7 ± 0	2.5 ± 0.1*	2.2 ± 0.1*,**

Sham, sham-operated rats given vehicle; MyoC, myosin-treated rats given vehicle; MyoC + FM-MSC, myosin-treated rats given FM-MSC (5×10^5 cells/animal); FM-MSC, fetal membrane-derived mesenchymal stem cells; LVDs, left ventricular systolic dimension; LVDd, left ventricular diastolic dimension; AWT, anterior wall thickness; PWT, posterior wall thickness.
* $P<0.05$ vs the Sham group.
** $P<0.05$ vs the MyoC group.

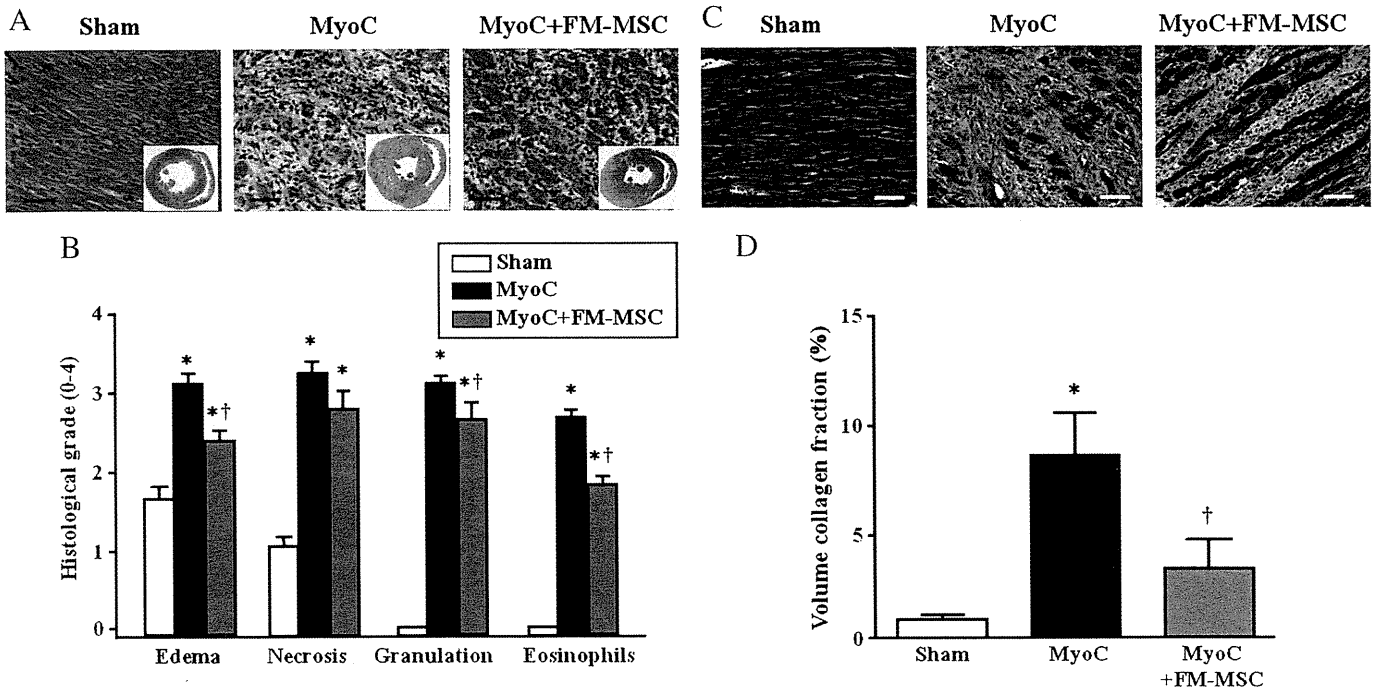

Fig. 4. Histopathological changes of acute myocarditis induced by administration of allogeneic FM-MSC. (A) Myocardial sections show markedly less inflammation in the rats given allogeneic FM-MSC than in the MyoC group. Insets are transverse sections of the myocardium. (B) The semiquantitative histological grading of edema and eosinophil infiltration were markedly lower in the MyoC + FM-MSC group than in the MyoC group ($n = 15$ in each group). (C) Myocardial fibrosis was markedly lower in the allogeneic FM-MSC group than in the MyoC group. (D) The semiquantitative area of fibrosis was smaller in the MyoC + FM-MSC group than in the MyoC group ($n =$ for 15 each group). Scale bars = 50 μm. Data are expressed as mean ± SEM. *$P<0.05$ vs the Sham group; †$P<0.05$ vs the MyoC group.

FM-MSC instead of autologous normal Lewis T cells; the values were 145.1 ± 4.6% for MyoC-TL with allogeneic FM-MSC vs 183.2 ± 2.5% for MyoC-TL without allogeneic FM-MSC and 136.2 ± 3.6% for MyoC + MSC-TL with allogeneic FM-MSC vs 164.2 ± 3.7% for MyoC + MSC-TL without allogeneic FM-MSC ($P<0.05$, $n = 8$ in each group) (Fig. 6). However, in the sham-TL group, proliferation did not differ in the presence or absence of allogeneic FM-MSC. These results show that allogeneic FM-MSC had both an acute suppressive effect on T-lymphocyte proliferation in vitro and a chronic suppressive effect on T-lymphocyte proliferation in the pathological studies.

4. Discussion

In this study, we investigated the therapeutic potential of allogeneic FM-MSC in the acute phase of myocarditis. In our rat model of acute myocarditis, intravenous allogeneic administration of FM-MSC one week after the myosin injection significantly improved cardiac function and the pathological findings in the heart three weeks after the myosin injection. The pathological findings included the attenuated expression of the proinflammatory factor MCP1 and a reduction in the infiltration of T cells and macrophages into the hearts treated with FM-MSC. The T-lymphocyte proliferation assay demonstrated that splenic lymphocytes from rats with myocarditis treated with FM-MSC reacted to myosin less strongly than did the splenic lymphocytes from untreated rats with myocarditis.

Experimental autoimmune myocarditis (EAM) is induced by immunization with cardiac myosin in Lewis rats and is characterized by severe myocardial dysfunction and the appearance of multinucleated giant cells. EAM has been used as an animal model of human giant cell myocarditis [15,16]. This myocarditis model is triphasic and comprises an antigen-priming phase on days 0–13, an autoimmune response phase on days 14–21, and a reparative phase thereafter, which is associated with a chronically dilated cardiomyopathy phenotype. Although the pathogenesis of EAM has not been clarified fully, severe inflammatory cell infiltration is a characteristic of the disease [15,16].

In a recent study, we tried to determine whether MSC improve the function and pathological findings in the affected heart in EAM. The intravenous administration of autologous BM-MSC one week after myosin injection significantly attenuated the myocardial dysfunction in rats with acute myocarditis [9]. Moreover, the intramyocardial transplantation of autologous BM-MSC five weeks after the myosin injection, corresponding to the reparative phase, improved cardiac function in a model of chronic dilated cardiomyopathy [7]. These findings suggest that autologous BM-MSC are an attractive source of cells for transplantation. However, there are several limitations in using an autologous cell source, including their invasiveness, inadequate cell numbers, and donor-site morbidity [17]. In addition, a waiting period is required for transplantation of autologous BM-MSC to prepare an adequate number of cells. Because acute myocarditis is often associated with rapidly progressive heart failure, time-consuming transplantation of autologous BM-MSC is not suitable. Human FMs, which are generally discarded as medical waste after delivery, have been shown recently to be rich sources of MSC [18,19]. Previous reports have suggested that FM-MSC might have regenerative potential for cell therapy. Using a rat hind-limb ischemia model, we demonstrated that allogeneic FM-MSC and autologous BM-MSC are suitable cell sources for tissue regeneration [12]. We infer that, as an alternative source to autologous BM-MSC, allogeneic FM-MSC might be a promising candidate for the cell therapy-based treatment of acute myocarditis.

In this study, intravenous allogeneic administration of FM-MSC significantly improved cardiac function and the pathological findings in the hearts exhibiting EAM. The extent of the improvement was in the range of 30–60% in the indices of the dysfunction level, which are equivalent to those observed in our previous study of administration of autologous BM-MSC [9]. Our previous report focused on the angiogenesis effects and the paracrine action of MSC in EAM, and we focused on the immunomodulatory properties of MSC in this study.

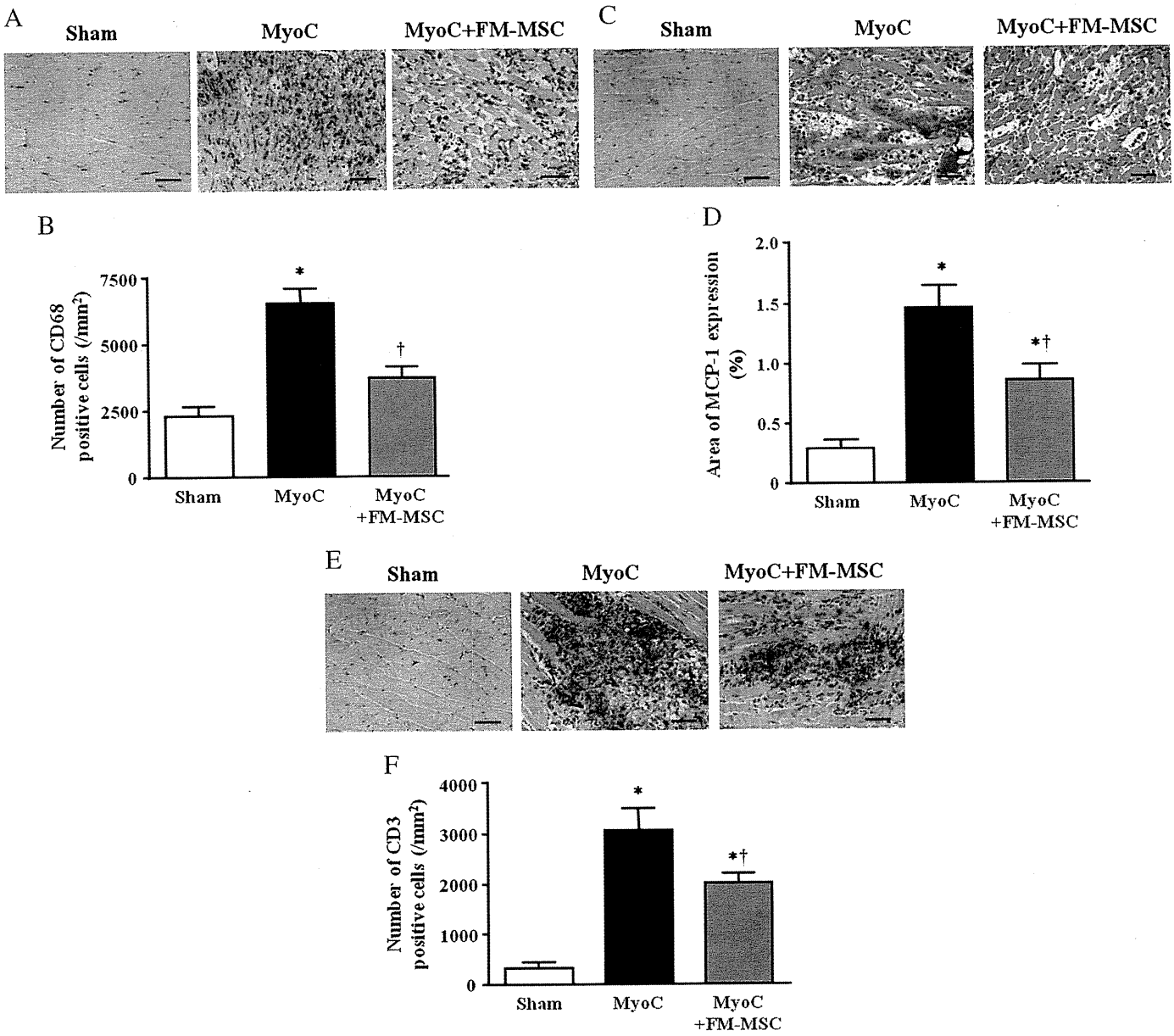

Fig. 5. Effects of administration of allogeneic FM-MSC on myocardial infiltration of inflammatory cells and MCP1 expression in acute myocarditis. (A, B) CD68-positive macrophage/monocyte infiltration was markedly lower in the MyoC + FM-MSC group than in the MyoC group. (C, D) The area of MCP1 expression was significantly smaller in the MyoC + FM-MSC group than in the MyoC group. (E, F) Significantly fewer infiltrating CD3-positive T cells were observed in the allogeneic FM-MSC group than in the MyoC group. Scale bars = 50 μm, $n = 15$ for each group. Data are expressed as mean ± SEM. *$P<0.05$ vs the Sham group; †$P<0.05$ vs the MyoC group.

Because, the migration of activated T cells into the myocardium is considered the initial process of EAM [20]. Subsequently, large numbers of inflammatory cells, including macrophages and T cells, infiltrate the myocardium where they induce severe inflammation.

In this study, GFP-positive cells were detected in the heart tissue one day and one week after the intravenous administration of GFP-expressing FM-MSC, although only a few engrafted cells were found, even one day after injection, which is consistent with a previous work [6]. The low percentage of cells migrating to the heart is in agreement with other reports [21–23]. In several clinical applications, MSC are administered preferentially by an intravenous route [24,25]. However, limited data are available regarding the fate of systemically infused MSC. Studies in rodents suggest that a broad distribution of transplanted MSC is observed initially, followed by a limited capacity for sustained engraftment [22,26]. In addition, the number of von Willebrand factor-positive capillaries in the heart did not increase in the EAM rats given FM-MSC compared with the untreated EAM group two weeks after cell administration (data not shown), and we found no GFP-positive cells and cardiomyocyte-differentiated cells four weeks after cell administration. A recent study reported that engraftment of MSC is very low and that transplanted MSC appear to differentiate into cardiomyocytes at a very low frequency [27]. These results suggest that the angiogenic effect and the cardiomyocyte differentiation of FM-MSC in the heart are not the main effects in EAM therapy.

We have demonstrated that some of the administered GFP-positive cells were found in the lung, spleen, and liver. It is interesting that the number of GFP-positive cells in the spleen, the hub of immunoreactions of T cells, was greater at one week after administration than at one day after administration. One may speculate that the systemically engrafted allogeneic FM-MSC can exert a therapeutic effect in the treatment of myocarditis.

Recent studies have highlighted the potential immunomodulatory or anti-inflammatory effects of MSC [28,29]. MSC can suppress T-cell

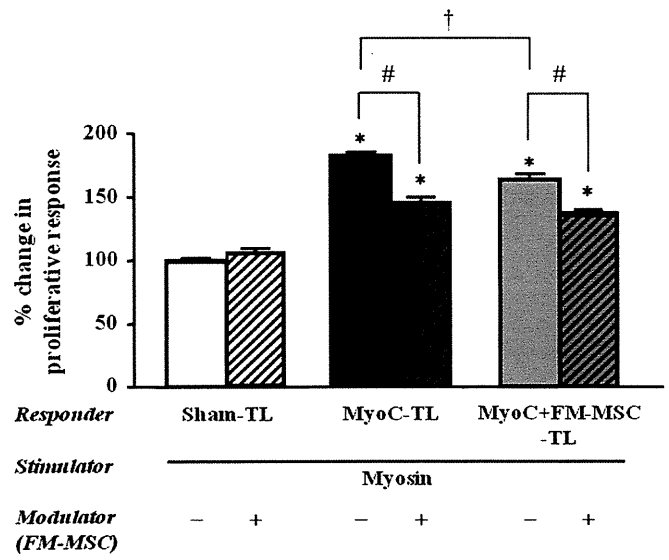

Fig. 6. Suppressive effects of T-lymphocyte activation by allogeneic FM-MSC. The T-lymphocyte proliferative response was significantly lower in the MyoC + FM-MSC group than in the MyoC group. The T-lymphocyte-activated proliferation was markedly attenuated by allogeneic FM-MSC ($n = 8$ each). Data are expressed as mean ± SEM. *$P<0.05$ vs Sham-TL; †$P<0.05$; #$P<0.05$.

activation and proliferation both in vitro and in vivo [30,31]. Recent clinical studies found that the intravenous injection of BM-MSC ameliorates acute graft-versus-host disease [13,14,32]. Interestingly, the suppression of T-cell proliferation by MSC causes no immunological restriction, insofar as similar suppressive effects were observed with cells that were either autologous or allogeneic to the responder cells [33,34]. Several independent reports have suggested multiple mechanisms by which MSC inhibit T-cell responses. Prostaglandin E2, nitric oxide, indoleamine 2,3-dioxygenase, and galectin are among the molecules postulated to be involved in the inhibition of T-cell proliferation by MSC [35–39]. Our present study demonstrated that allogeneic administration of FM-MSC significantly attenuates the infiltration of macrophages and T cells into EAM hearts. In the T-lymphocyte proliferation assay, splenic T lymphocytes derived from rats with myocarditis given allogeneic FM-MSC had a reduced activated proliferative response compared with the response of splenic T lymphocytes from untreated myocarditis rats, even two weeks after the injection of FM-MSC. In addition, activated T-lymphocyte proliferation was also suppressed by coculture with allogeneic FM-MSC in vitro. These results suggest that allogeneic FM-MSC reduce the severity of EAM by inhibiting T-cell activation and proliferation through both a direct effect and a systematic effect, and that these combined effects lead to the amelioration of impaired cardiac function.

However, GFP-positive cells could not be detected in the spleen, lung, and liver as with the heart at four weeks after administration. In this study, we administered a low number of FM-MSC to avoid pulmonary embolism. More effective results might be obtained with administration of more cells or multiple administrations of FM-MSC in EAM. One study reported that transplantation of allogeneic BM-MSC into a rat model of myocardial infarction improved cardiac functions at four weeks after transplantation, although this benefit was transient [40]. Currently, we are extending our observation up to three months in EAM. Although the results are an inadequate number to be presented, improvement of cardiac function appears to be maintained in the intravenous allogeneic FM-MSC administration group compared with the untreated EAM group.

In conclusion, this study showed that the intravenous allogeneic administration of FM-MSC ameliorated cardiac dysfunction in a rat model of acute myocarditis. These beneficial effects may be mainly attributable to the suppression of T-lymphocyte activation rather than to angiogenesis and cardiomyocyte differentiation of the administered allogeneic FM-MSC. Although further experiments are needed to apply the current results to human cardiomyoplasty, the allogeneic administration of FM-MSC may provide a new therapeutic strategy for the treatment of severe acute myocarditis.

Acknowledgments

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Allogenic fetal membrane-derived mesenchymal stem cells contribute to renal repair in experimental glomerulonephritis

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Departments of [1]Regenerative Medicine and Tissue Engineering and [6]Biochemistry, National Cardiovascular Center Research Institute; Departments of [2]Advanced Technology for Transplantation, [3]Functional Diagnostic Science, Course of Health Science, and [7]Urology, Osaka University Graduate School of Medicine, Suita; [4]Department of Perinatology, National Cardiovascular Center, Osaka; [5]Second Department of Internal Medicine, Nara Medical University, Nara; and [8]Department of Cell Biology, Institute of Nephrology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

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Tsuda H, Yamahara K, Ishikane S, Otani K, Nakamura A, Sawai K, Ichimaru N, Sada M, Taguchi A, Hosoda H, Tsuji M, Kawachi H, Horio M, Isaka Y, Kangawa K, Takahara S, Ikeda T. Allogenic fetal membrane-derived mesenchymal stem cells contribute to renal repair in experimental glomerulonephritis. *Am J Physiol Renal Physiol* 299: F1004–F1013, 2010. First published August 25, 2010; doi:10.1152/ajprenal.00587.2009.—Mesenchymal stem cells (MSC) have been reported to be an attractive therapeutic cell source for the treatment of renal diseases. Recently, we reported that transplantation of allogenic fetal membrane-derived MSC (FM-MSC), which are available noninvasively in large amounts, had a therapeutic effect on a hindlimb ischemia model (Ishikane S, Ohnishi S, Yamahara K, Sada M, Harada K, Mishima K, Iwasaki K, Fujiwara M, Kitamura S, Nagaya N, Ikeda T. *Stem Cells* 26: 2625–2633, 2008). Here, we investigated whether allogenic FM-MSC administration could ameliorate renal injury in experimental glomerulonephritis. Lewis rats with anti-Thy1 nephritis intravenously received FM-MSC obtained from major histocompatibility complex-mismatched ACI rats (FM-MSC group) or a PBS (PBS group). Nephritic rats exhibited an increased urinary protein excretion in the PBS group, whereas the FM-MSC group rats had a significantly lower level of increase ($P < 0.05$ vs. PBS group). FM-MSC transplantation significantly reduced activated mesangial cell (MC) proliferation, glomerular monocyte/macrophage infiltration, mesangial matrix accumulation, as well as the glomerular expression of inflammatory or extracellular matrix-related genes including TNF-α, monocyte chemoattractant protein 1 (MCP-1), type I collagen, TGF-β, type 1 plasminogen activator inhibitor (PAI-1) ($P < 0.05$ vs. PBS group). In vitro, FM-MSC-derived conditioned medium significantly attenuated the expression of TNF-α and MCP-1 in rat MC through a prostaglandin E_2-dependent mechanism. These data suggest that transplanted FM-MSC contributed to the healing process in injured kidney tissue by producing paracrine factors. Our results indicate that allogenic FM-MSC transplantation is a potent therapeutic strategy for the treatment of acute glomerulonephritis.

prostaglandin E_2; cell therapy; anti-Thy-1 nephritis

MESENCHYMAL STEM CELLS (MSC) are multipotent stem cells present in bone marrow (BM), adipose tissue, and many other organs and have the ability to differentiate into a variety of lineages including adipocytes, osteocytes, and chondrocytes (9, 11, 42). Previous reports described that BM-derived stem cells including MSC contributed to the repair of several compartments of the kidney, including the endothelium (47), interstitium (16), epithelium (23), and the mesangium (21). Moreover, several studies have demonstrated that transplanted BM-derived MSC (BM-MSC) contribute to improve renal function in experimental glomerulonephritis induced by anti-Thy1 (31, 58). These features make BM-MSC an attractive therapeutic tool for the treatment of renal diseases.

However, there are limitations in using autologous BM-MSC as a source of regenerative medicine. BM aspiration may be painful and sometimes yields low numbers of MSC on processing (61). Therefore, an alternative source of MSC that can be obtained noninvasively and in large quantities would be advantageous. Fetal membrane (FM), which is generally discarded as medical waste, has been found to be a rich, easily expandable source of MSC (18, 43). Recently, we demonstrated that transplantation of allogenic FM-derived MSC (FM-MSC) as well as autologous BM-MSC induced therapeutic angiogenesis using a rat hindlimb ischemia model (20). These results suggest that allogenic FM-MSC are a potential alternative to autologous BM-MSC as a source of regenerative therapy.

In the present study, we investigated whether systemic administration of allogenic FM-MSC could improve the course of anti-Thy1 nephritis. Anti-Thy1 nephritis is a model of mesangioproliferative glomerulonephritis characterized by mesangiolysis followed by repair via mesangial cell (MC) proliferation, mesangial matrix accumulation, and monocyte/macrophage influx (25). Because we previously reported that MSC possessed paracrine angiogenic and anti-fibrotic effects (20, 39), we also examined the contribution of the paracrine effects to ameliorate anti-Thy1 nephritis after allogenic FM-MSC transplantation.

MATERIALS AND METHODS

Animals. All experimental protocols were approved by the Animal Care Committee of the National Cardiovascular Center Research Institute. Different strains of rats were used according to their major histocompatibility complex (MHC) antigen disparity: Lewis (MHC haplotype: RT-1l) and ACI (MHC haplotype: RT-1a) rats (Japan SLC, Hamamatsu, Japan). Green fluorescent protein

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(GFP)-transgenic Lewis rats (Institute of Laboratory Animals, Kyoto University, Kyoto, Japan) were used to investigate the distribution of injected FM-MSC.

Isolation and expansion of FM-MSC and glomerular MC. Isolation and expansion of FM-MSC were performed as previously described (20). In brief, FM was obtained from pregnant rats on *day 15* postconception. Minced FM was digested with type II collagenase solution (300 U/ml; Worthington Biochemical, Lakewood, NJ) for 1 h at 37°C. After filtration and centrifugation, FM-derived cells were suspended in α-MEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin (Invitrogen) and cultured in standard plastic dishes. The adherent MSC populations appeared by *days 5–7*, and these FM-MSC were used for the experiments at *passage 3–6*.

Glomerular MC were established as described elsewhere (29). MC obtained from Sprague-Dawley rats were cultured in standard medium (DMEM, high glucose, Invitrogen, 10% FBS, and 1% penicillin/streptomycin) and used for experiments at *passages 13–15*.

Experimental model and design. Mesangial proliferative glomerulonephritis was induced in 6-wk-old male Lewis rats (170–180 g) by intravenous injection of anti-Thy1 monoclonal antibody (mAb 1–22-3; 0.5 mg/rat) (24). Because FM-MSC reportedly express high levels of Thy1 (20), we administered FM-MSC on *day 2* after anti-Thy1 antibody injection when its antibody in plasma is undetectable (Supplemental Figure and Method; supplemental material for this article is available online at the journal website). On *day 2* after mAb injection, rats were randomized to two groups: *1*) FM-MSC injection (FM-MSC group; $n = 8$) and *2*) control PBS injection alone (PBS group; $n = 8$). A total of 5×10^5 FM-MSC obtained from MHC mismatched ACI rats or PBS (200 μl each) was injected into the tail vein of Lewis nephritic rats. Sham rats (Sham group; $n = 8$) received a PBS injection instead of mAb. On *days 7* and *14*, rats were placed in metabolic cages for collection of urine to determine the excretion of urine protein.

Histological examination. Kidney tissues were fixed with 4% phosphate-buffered formalin solution (Wako Pure Chemical Industries, Osaka, Japan), embedded in paraffin block, and cut into 2-μm sections. To quantify mesangial matrix accumulation, sections were stained with periodic acid-Schiff (PAS), photographed using a digital microscope (BIOREVO BZ-9000; KEYENCE, Osaka, Japan), and evaluated by assessing 30 randomly selected glomeruli/kidney and scoring each glomerulus on a semiquantitative scale (0–4) as described previously (19).

Immunohistochemical staining was performed with mouseanti-α-smooth muscle actin (α-SMA) antibody (clone 1A4; Dako, Glostrup, Denmark), mouse anti-CD68 antibody (clone ED-1; Serotec, Oxford, UK), and rabbit anti-GFP antibody (Invitrogen). Negative control staining was performed by replacing the primary antibody with normal IgG. Following antigen retrieval, endogenous peroxidase activity was quenched with 1.5% H_2O_2 for 10 min. The first antibodies were incubated for 1 h at room temperature, followed by incubation with Envision system-horseradish peroxidase-labeled polymer (Dako) for 30 min. The sections were visualized with 3,3′diaminobenzidine tetrahydrochloride (Dako) and counterstained with hematoxylin.

The α-SMA-positive area relative to the glomerular area was calculated as a percentage using a computer-aided manipulator (WinRoof; Mitani, Fukui, Japan). The α-SMA staining percentage of total glomerular area was determined, and the mean value of 30 randomly selected glomeruli was calculated. The number of ED-1-positive monocytes/macrophages was evaluated by counting stained cells per glomerulus in at least 30 randomly selected glomeruli. To evaluate the distribution of GFP-positive administered cells, we counted all the GFP-positive cells in one randomly selected section ($n = 4$) from each organ and an overall average for all rats was calculated.

Quantitative RT-PCR analysis. Glomeruli were isolated from rat kidneys using a graded sieving technique (19). Total RNA was extracted from isolated glomeruli using an RNeasy mini kit (Qiagen, Hilden, Germany). Obtained RNA was reverse-transcribed into cDNA using a Quantitect Reverse Transcription kit (Qiagen). PCR amplification was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). β-Actin transcript was used as an internal control. Primers used are listed in Table 1.

Western blot analysis. Western blotting was performed as previously described (36). Briefly, kidney tissues were homogenized in 0.1% Tween 20 with a protease inhibitor, loaded (30 μg) on a 10–20% gradient gel (Bio-Rad, Hercules, CA), and blotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking for 1 h, membranes were incubated with mouse anti-monocyte chemoattractant protein (MCP)-1 antibody (1:1,000; BD Biosciences Pharmingen, San Jose, CA), then incubated with peroxidase-labeled secondary antibody (1:1,000; Cell Signaling Technology, Danvers, MA). Positive protein bands were visualized with an ECL kit (GE Healthcare, Piscataway, NJ) and measured by densitometry. A mouse monoclonal antibody against β-actin (Sigma-Aldrich, St. Louis, MO) was used as a control ($n = 8$ in each group).

Assessment of paracrine effects of FM-MSC on glomerular MC. Conditioned medium was collected from 1×10^6 cells of FM-MSC cultured in 8 ml of standard medium (DMEM supplemented with 10% FBS and 1% penicillin/streptomycin) with or without the cyclooxygenase (COX) 2 inhibitor NS-398 (0.1 μM; Wako) for 48 h, and filtered through a 0.22-μm filtration unit (Millipore). MC were plated on six-well plates (2×10^5 cells/well) with standard medium for 24 h. The medium was then changed to serum-free DMEM for 24 h, followed by conditioned medium obtained from FM-MSC. After 8 h, total RNA was extracted from MC.

ELISA. The concentration of PGE_2 in the conditioned medium of FM-MSC was determined using an ELISA kit, according to the

Table 1. *Primers for qRT-PCR*

Gene	Forward	Reverse
Type I collagen	5′-AATGGTGCTCCTGGTATTGC-3′	5′-GGTTCACCACTGTTGCCTTT-3′
TGF-β	5′-CTACTGCTTCAGCTCCACAGAGA-3′	5′-ACCTTGGGCTTGCGACC-3′
PAI-1	5′-ACCTCGATCTTGACCTTTTG-3′	5′-GACAATGGAAGAGCAACATG-3′
MMP-2	5′-GATCTGCAAGCAAGACATTGTCTT-3′	5′-GCCAAATAAACCGATCCTTGAA-3′
MMP-9	5′-TGGAACTCACACAACGTCTTTCA-3′	5′-TCACCCGGTTGTGGAAACTC-3′
TIMP-1	5′-ATCAAGATGACTAAGATGCTCAAAGG-3′	5′-GGCCCGCGATGAGAAACT-3′
TNF-α	5′-TGCCTCAGCCTCTTCTCATT-3′	5′-CCCATTTGGGAACTTCTCCT-3′
MCP-1	5′-ATGCAGGTCTCTGTCACGCT-3	5′-GGTGCTGAAGTCCTTAGGGT-3′
HGF	5′-TGCAACGGTGAAAGCTACAG-3′	5′-AGCCCTTGGTCGGGATATCTT-3′
VEGF	5′-AGAAAGCCCAATGAAGTGGTG-3′	5′-ACTCCAGGGCTTCATCATTG-3′
β-Actin	5′-GCCCTAGACTTCGAGC-3′	5′-CTTTACGGATGTCAACGT-3′

TGF, transforming growth factor; PAI-1, type 1 plasminogen activator inhibitor; MMP, membrane-type matrix metalloproteinase; TIMP-1, tissue inhibitor of MMP-1; MCP-1, monocyte chemoattractant protein 1.

manufacturer's protocol (Cayman Chemical, Ann Arbor, MI). The absorbance was measured by a microplate reader (Bio-Rad) at 405 nm.

Statistical analysis. All data are expressed as means ± SE. Comparisons between two parameters were analyzed by using the unpaired Student's *t*-test. Comparisons of parameters among the three groups were made by one-way ANOVA, followed by Tukey's test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Reduction of proteinuria by FM-MSC transplantation. In the anti-Thy1 nephritic model, transient renal damage with massive proteinuria developed (25). On *day 7*, the 24-h urine protein excretion rate was significantly increased in rats with anti-Thy1 nephritis (84.9 ± 7.6 mg/24 h in the PBS group) compared with control (10.7 ± 0.5 mg/24 h in the Sham group), and FM-MSC treatment significantly reduced this increase (60.8 ± 8.0 mg/24 h, $P < 0.05$ vs. PBS group) (Fig. 1). On *day 14*, the 24-h urine protein excretion rate in these three groups fell to within the normal range. Creatinine clearance was significantly decreased in nephritic rats (2.14 ± 0.06 ml/min in the PBS group) compared with the Sham group (2.97 ± 0.15 ml/min, $P < 0.01$). However, no significant difference in creatinine clearance was observed between the PBS and FM-MSC groups (2.14 ± 0.09 ml/min in the FM-MSC group).

Inhibition of accumulation of activated MC and mesangial matrix by FM-MSC transplantation. Glomerular expression of α-SMA, a marker of activated MC, was increased after disease induction (Fig. 2, *D–F*). Expression of α-SMA in nephritic rats increased to a peak level on *day 7* and then gradually decreased thereafter (Fig. 2*M*). On *days 7* and *14*, α-SMA staining of the glomerular area in FM-MSC-treated rats (30.7 ± 0.8 and 22.4 ± 0.8%, respectively) was significantly decreased compared with the PBS group (37.5 ± 0.6 and 26.6 ± 0.7%, respectively, $P < 0.01$ vs. FM-MSC group).

PAS staining in rats with anti-Thy1 nephritis revealed the accumulation of mesangial matrix (Fig. 2, *G–I*). Similar to α-SMA expression, the glomerular PAS-positive area in nephritic rats reached its peak on *day 7* (PAS score; 2.72 ± 0.12 in the PBS group vs. 0.20 ± 0.05 in the Sham group, $P < 0.01$)

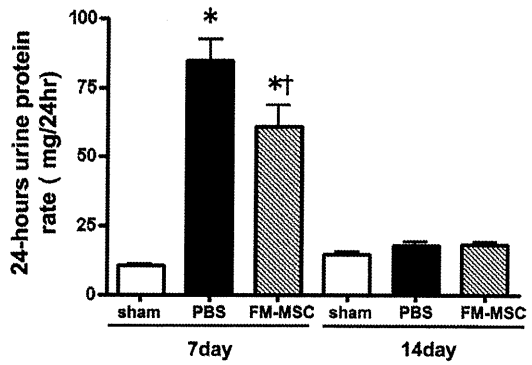

Fig. 1. Effect of fetal membrane-derived mesenchymal stem cells (FM-MSC) administration on proteinuria in anti-Thy1 nephritis. The 24-h urine protein excretion rate was measured in the Sham, PBS, and FM-MSC groups. On *day 7*, the rate was significantly increased in the PBS group compared with the FM-MSC group. On *day 14*, both groups showed remission of proteinuria, and no significant differences were seen among these 3 groups. *$P < 0.05$ vs. Sham group. †$P < 0.05$ vs. PBS group.

and remained elevated on *day 14* (2.97 ± 0.08 in the PBS group and 0.25 ± 0.05 in the Sham group, $P < 0.01$), which was significantly decreased by FM-MSC administration (FM-MSC group: 2.21 ± 0.08 on *day 7* and 1.54 ± 0.06 on *day 14*, $P < 0.05$ vs. PBS group) (Fig. 2*N*). qRT-PCR analysis revealed that the reduction of mesangial matrix accumulation in FM-MSC-treated rats was associated with decreased expression of glomerular type I collagen, transforming growth factor (TGF) -β, type 1 plasminogen activator inhibitor (PAI-1) ($P < 0.05$ vs. PBS group) (Fig. 3, *A–C*). However, FM-MSC treatment did not significantly affect the glomerular expression of membrane-type matrix metalloproteinases (MMPs) and tissue inhibitor of MMP-1 (TIMP-1) in anti-Thy1 nephritic rats (Fig. 3, *D–F*).

Attenuation of glomerular monocyte/macrophage influx by FM-MSC transplantation. Immunostaining of ED-1 in rats with anti-Thy1 nephritis revealed a significant monocyte/macrophage infiltration into the glomeruli (Fig. 2, *J–L*). On *day 7*, the number of infiltrating monocytes/macrophages in the PBS group (7.5 ± 0.2/glomerulus) was significantly higher than in the Sham group (0.4 ± 0.1/glomerulus), which was significantly lower than in the FM-MSC group (6.0 ± 0.2/glomerulus, $P < 0.01$ vs. PBS group) (Fig. 2*O*). A similar result was also observed on *day 14* (PBS group 5.1 ± 0.2/glomerulus vs. FM-MSC group 3.9 ± 0.1/glomerulus, $P < 0.01$).

Reduction of renal inflammatory cytokine/chemokine expression by FM-MSC transplantation. We examined the glomerular expression of inflammatory cytokines/chemokines in nephritic rats on *day 7*. QRT-PCR analysis showed that tumor necrosis factor (TNF)-α expression in glomeruli was significantly increased by 7.70 ± 0.54-fold in the PBS group ($P < 0.01$ vs. the Sham group), and this increase was significantly decreased in the FM-MSC group (5.92 ± 0.20-fold, $P < 0.05$ vs. PBS group) (Fig. 3*G*). Glomerular MCP-1 mRNA expression in the PBS group showed a 5.41 ± 0.38-fold increase compared with the Sham group ($P < 0.01$) (Fig. 3*H*), but FM-MSC transplantation reduced this increase by >30% (3.51 ± 0.51-fold, $P < 0.05$ vs. PBS group). Similarly, Western blot analysis showed that renal MCP-1 protein expression in the PBS group was significantly increased compared with the Sham group (7.65 ± 2.49-fold, $P < 0.05$) (Fig. 4), and FM-MSC administration showed a tendency of decreasing the expression of MCP-1 protein (6.44 ± 0.96-fold vs. the Sham group) (Fig. 4).

Renal expression of VEGF and HGF after FM-MSC transplantation. Previously, we reported that cultured FM-MSC secreted large amounts of angiogenic/antiapoptotic factors including VEGF and HGF (20). Because VEGF and HGF have been reported as renoprotective factors (34, 41, 53, 55), we analyzed glomerular expression of these factors in FM-MSC-transplanted nephritic rats. qRT-PCR analysis revealed that expression of VEGF mRNA in the PBS group was significantly decreased (0.36 ± 0.07-fold vs. Sham group, $P < 0.05$), and no significant upregulation was seen after FM-MSC administration (0.30 ± 0.08-fold vs. Sham group, $P < 0.05$) (Fig. 3*I*). Glomerular expression of HGF mRNA was significantly increased in the PBS group (2.64 ± 0.38-fold vs. Sham group, $P < 0.05$), but no significant difference was observed between PBS and FM-MSC groups (2.51 ± 0.34-fold vs. Sham group, $P < 0.05$) (Fig. 3*J*).

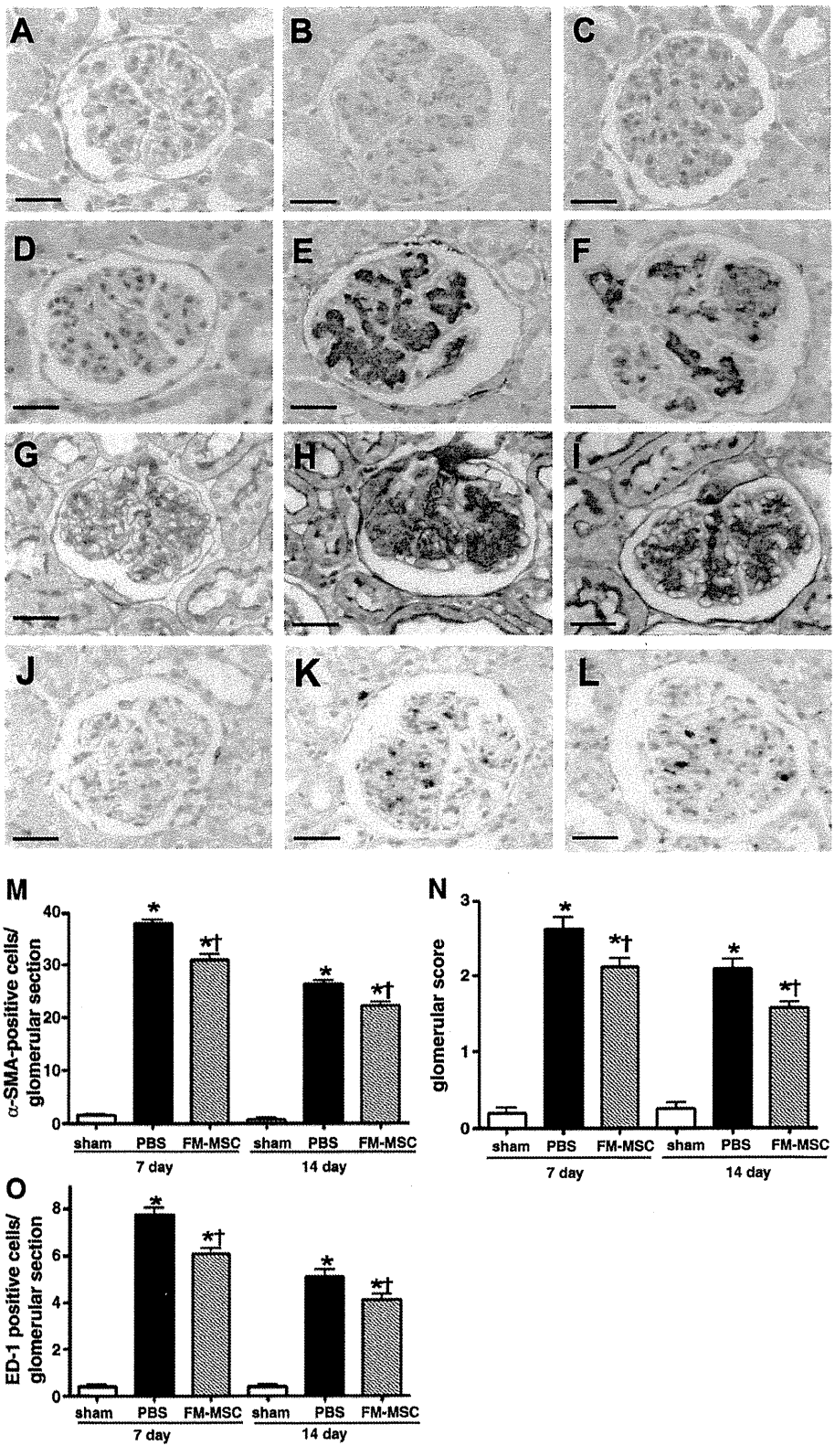

Fig. 2. Inhibition of the accumulation of activated MC, mesangial matrix, and glomerular monocyte/macrophages by FM-MSC transplantation in rats with anti-Thy1 nephritis. *A–L*: representative micrographs of negative control (*A–C*), α-smooth muscle actin (SMA; *D–F*), periodic acid-Schiff (PAS; *G–I*) and ED-1 (*J–L*) staining in the Sham (*A*, *D*, *G*, *J*), PBS (*B*, *E*, *H*, *K*) and FM-MSC (*C*, *F*, *I*, *L*) groups on *day 7*. *M*: quantitative analysis revealed that the number of α-SMA-positive activated MC was lower in the FM-MSC group compared with the PBS group on *days 7* and *14*. *N*: mesangial matrix accumulation was significantly reduced in the FM-MSC group compared with the PBS group on *days 7* and *14*. *O*: the number of infiltrated ED-1-positive monocytes/macrophages was significantly reduced in the FM-MSC group compared with the PBS group on *days 7* and *14*. Scale bars = 20 μm. *$P < 0.05$ vs. Sham. †$P < 0.05$ vs. PBS group.