厚生労働科学研究費補助金医療機器開発推進研究事業
（医療機器開発（ナノテクノロジー等）総合推進研究事業）
分担研究報告書

幹細胞分離デバイスの医療機器としての早期認可推進に関する研究

研究分担者　山本 晴子　国立循環器病研究センター 先進医療・治験推進部 部長

研究要旨

現在、幹細胞を用いた細胞治療は、特定機能病院を中心としてごく少数の患者を対象に実施されているのみである。この原因の主なものとして、現状では細胞の分離、培養過程を細胞調整施設（CPC）で行わなければならず、CPCとその稼働体制が構築されたごく少数の施設でのみ対応が可能であり、一般診療への普及は困難である。我々は、CPCなしで細胞分離が可能な、完全閉鎖系で操作が容易、かつ生体に安全な分離液を用いた分離デバイスの開発を開始するとともに、医療機器として早期認可を可能とするための研究を開始した。本年度は専門家のアドバイス等を受けた上でPMDAの薬事戦略相談を実施し、クラス分類や臨床開発の道筋について一定の見解を得た。しかし、製品化するためには民間企業が採算性を確保できる形で開発する必要があるため、来年度以降臨床開発の方向性について一層の検討を行う予定である。

共同研究者

嘉田 晃子（国立循環器病研究センター 先進医療・治験推進部）

A. 研究目的

現在、幹細胞を用いた細胞治療は、特定機能病院を中心としてごく少数の患者を対象に実施されているのみである。この原因の主なものとして、現状では細胞の分離、培養過程を細胞調整施設（CPC）で行わなければならず、CPCとその稼働体制が構築されたごく少数の施設でのみ対応が可能であり、一般診療への普及は困難である。我々が臨床試験として実施している急性期脳梗塞患者に帯する骨髄間葉系幹細胞による治療は、分離液を用いて幹細胞を分離、洗浄するだけのプロセスであるため、完全閉鎖系で操作が容易、かつ生体に安全な分離液を用いた分離デバイスがあれば、CPCは必ずしも必要でなくなる。そこで、この条件を満たす分離デバイスの開発を開始するとともに、医療機器として早期認可を可能とするための研究を開始した。

B. 研究方法

共同研究を行っている医療機器企業とともに試作した幹細胞分離デバイスについて、1）医療機器のクラス分類の検討、2）臨床評価の必要性の有無、3）前記の2項目を踏まえた上で、臨床試験の実施の必要性および必要な場合はその計画について検討することとした。国立医薬品食品衛生機構や（財）医療機器センター等の専門家のアドバイスを頂きながら検討を重ね、医薬品医療機器総合機構（PMDA）の薬事戦略相談においてクラス分類等についての相談を行った上で、研究計画の検討に入ることとした。

（倫理面への配慮）

今年度については、臨床試験計画策定に至らず、倫理面への配慮を必要とする研究に該当しなかった。

C. 研究結果

国立医薬品食品衛生機構のアドバイザーの指導を受けつつ、共同研究を行っている医療機器企業の意向も考慮に入れながらクラス分類について検討を行った。類似の効果を持つ医療機器の検討等から、クラスⅡに該当すると考えられたため、その旨を確認することをPMDAの薬事戦略相談の主要目的として相談に赴いた。PMDAとは、クラスⅡに該当するということで意見の合致をみた。一方、当該デバイスを用いて分離された幹細胞を用いた脳梗塞の治療が有用な治療として

確立しているとまでは言いがたいとの判断が示され、分離された幹細胞の臨床的有用性を何らかの形で検証する必要があるという見解が PMDA より示された。また、現在CPCで分離している幹細胞と、当該デバイスを用いて分離された幹細胞との性質の類似性も示される必要があるとのことであった。また、当該デバイスに用いられる材質の生物学的安全性の説明が必要であることと、分離液の扱い（医療機器の付属品として扱うかどうか、等）の検討が必要であることも明らかとなった。

薬事戦略相談の結果を受けて、臨床試験の計画の検討に入る予定であったが、その後当該デバイスの製作を担当している共同研究企業より開発中断の連絡があり、今年度中の臨床試験計画の検討には至らなかった。

D. 考察

医療機器の認可は薬事法及び関連法令に基づいて行われているが、現状では関連法令が医薬品の許認可と類似の内容であり、医療機器の特徴が必ずしも正しく反映されていないという問題点が指摘されている。我々の開発している医療機器は、従来CPCで行われている細胞分離手技を簡便なデバイスで行うことで、CPCの使用可能な施設でしか実施できない治療行為をほぼ国内全地域に普及させることのできる画期的なコンセプトの医療機器である。操作が簡便で使用毎に遣い捨てるという性質から、機器自体は単純な構造であり、販売予想価格も非常に低いと考えている。そのこと自体は認可後の当該機器の普及に大きく寄与すると思われるが、逆に開発コストを低く抑えなければ採算性がなく、企業に導出することが困難という問題を抱えている。今年度、臨床試験計画の検討にまで至らなかった理由の一つに、採算性の見込みが立ちにくく共同研究を行っている企業が開発を中断したことが挙げられるが、その遠因として、当該機器で分離した細胞の臨床的有用性まで臨床試験で確認するようにとのPMDA における薬事戦略相談の結果も影響している可能性も考えられた。医薬品と異なる、医療機器は治療手技の一部を補完、またはより簡便な手技に変換する目的で使用されることがほとんどである。我々の開発している細胞分離デバイスも、CPCで行われる細胞分離手技をより簡便な手技に変換することを目的としている。従って、分離された生産物の臨床的有用性の検討のレベルも、自ずから医薬品とは異なってしかるべきと考える。今後、薬事法の改正によって医薬品と医療機器の相違点が明確になる可能性もあるため、来年度以降も、医療機器の有用性を検討しつつ、過剰なコストはかけずに採算性を担保するという課題を検討し続けたい。

E. 結論

本年度は開発中の医療機器のクラス分類が明らかになった。また、現行薬事法および関連法規制では、当該デバイスの最終産物である分離細胞の臨床的有用性の検討までが求められることも判明した。来年度以降、薬事法改正の動向を注視しつつ、当該機器の臨床開発の道筋について検討を続ける予定である。

F. 健康危険情報

なし。

G. 研究発表

1. 論文発表

なし

（発表誌名巻号・頁・発行年等も記入）

2. 学会発表

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

なし

2. 実用新案登録

なし

3. その他

なし

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分担研究報告書

幹細胞分離デバイスの事業化に向けた研究

研究分担者　赤川　英毅　国立循環器病研究センター知的資産部　室長
妙中　義之　国立循環器病研究センター　研究開発基盤センター長

構造がシンプルで大規模な設備を必要とせずに細胞治療を実施可能とする幹細胞分離デバイスは，製品化によって医療の均てん化に貢献するであろうと期待できる．一方で，このような新しい治療コンセプトのデバイスは，既存あるいは後発となる医療機器に比して製品化までのロードマップを描くのが難しい．さらに，大学や研究機関などから創出された医療機器へとつながるシーズが市場に流通するためには，医療機器製造販売業を営む企業からの製品化が不可欠であるが，このような企業とのマッチングには特許など知的財産として価値の高いものを保有していることが重要となる．しかしながら，知的財産の汎用的な評価の指標はあるものの医療機器などに特化して評価する指標としての明確なものはこれまでのところ見当たらない．本研究分担者らは他の有識者も交えて医療機器に関する知的財産の評価指標の策定に取り組んできたところであり，このデバイスについても適用することによって知的財産としての価値・特徴を把握するとともに，指標そのもののブラッシュアップにもつなげた．

共同研究者
大屋知子　国立循環器病研究センター知的資産部　研究員

A. 研究目的

新しい治療コンセプトに基づいて発案された医療機器が実際に患者への治療に用いられるようにするためには，対象となる前例が無い状況下において薬事のプロセスを考慮しつつ，いかに製品化に至らせるかという課題を解決しなければならない．そこでは，製品化を担うべき企業とのマッチング・合意形成など，優れた技術のみではなく事業性や社会性など俯瞰的・包括的な立場からの価値の認識が重要である．特に企業とのマッチングにおいては，特許など知的財産の権利化が進められていることが，その後の事業化戦略に大きく影響を及ぼす．

他方，本分担研究者らは，これまで医療機器を対象とした知的財産の評価指標の策定に取り組んできた．この指標は従来の特許権としての取得のしやすさや事業としての可能性のみでなく，治療・診断での効果やオーファン性など社会への貢献についても考慮しており，特許性4項目，事業性4項目，社会性10項目から構成されたものである．また，それぞれの項目においては，提示された幾つかの条件の中で合致するものの数をポイント化するなど，できる限り定量的な評価に近づけるように配慮した．なお，この指標を策定するに当たっては，医療機器開発のクラスターとして著名なミネソタ大学をはじめとする海外の産学連携の専門家の知見も反映させ，国際的にも展開可能なものとなるように考慮した．

そこで，本研究では上述の確立しつつあった医療機器に関する知的財産を評価する指標に本幹細胞分離デバイスを適用することによって，このデバイスについて知的財産の観点から価値・特徴を把握するとともに，当該指標のブラッシュアップにつなげることを目的とした．

B. 研究方法

本分担研究者・研究協力者をはじめとする産学連携に携わる当研究センターの関係者らによって，開発を進めている本デバイスがどの状況に想定されるかをこの指標に適用した．この適用においては本研究代表者らにヒアリングして得られていた情報をもとに実施した．

C. 研究結果

特許性については，進歩性，発明の技術的性格において高い評価を得た．また，社会性については，治療効果，有用性，均てん化などについて評価が高いものとなった．

D. 考察

一般的に新しい治療コンセプトのデバイスを製品化するためには，将来的に収益を見込んだ製品とすべく事業計画を策定することができなければ企業にとってインセンティブのあるものとはいい難く，治験が必要か否かあるいは製品化までのロードマップをどのように描くかなど顧慮すべきところは多い．本研究においてはそのような論点について踏み込んではいないものの，この指標については社会性の観点を取り入れたところにその意義の一端があり，上記の課題解決への導入となるポテンシャルを具備しているのか今後その可能性を見極めるべきところである．

一方で，本研究で用いた医療機器に関する知的財産の指標は全米屈指の医療機器開発クラスターであるミネソタにおける最新の知見をも反映させたものであり，このデバイスの将来的・潜在的な市場として世界を対象とすべきことに鑑みても，その条件下で高い評価が得られたことは意義深い．この幹細胞分離デバイスの due diligence につながるところについて確かめられたことは，今後の事業化への取り組みに向けて大きな進捗であった．

なお，実際にこの指標がどの程度有効であるかについては今後さらにその妥当性などの検証を必要とするものであり，本デバイス以外にも他のものを適用して検証データを蓄積すべく，引き続き進捗を図っているところである．

E. 結論

この幹細胞分離デバイスについて，医療機器に関する知的財産の指標に適用してその価値・特徴の把握とともに，当該指標のブラッシュアップにもつながり，極めて有用な成果が得られた．

F. 研究発表

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Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
	該当なし						

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Ⅳ. 研究成果の刊行物・別刷

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Original article

Systemic transplantation of allogenic fetal membrane-derived mesenchymal stem cells suppresses Th1 and Th17 T cell responses in experimental autoimmune myocarditis

Makiko Ohshima [a], Kenichi Yamahara [a,*], Shin Ishikane [a], Kazuhiko Harada [c], Hidetoshi Tsuda [a], Kentaro Otani [a], Akihiko Taguchi [a,e], Mikiya Miyazato [e], Shinji Katsuragi [b], Jun Yoshimatsu [b], Makoto Kodama [f], Kenji Kangawa [d], Tomoaki Ikeda [a,b,g]

[a] Department of Regenerative Medicine and Tissue Engineering, National Cerebral and Cardiovascular Center, Osaka, Japan
[b] Department of Perinatology, National Cerebral and Cardiovascular Center, Osaka, Japan
[c] Department of Pharmacy, National Cerebral and Cardiovascular Center, Osaka, Japan
[d] Department of Biochemistry, National Cerebral and Cardiovascular Center, Osaka, Japan
[e] Department of Regenerative Medicine Research, Biomedical Research Innovation, Hyogo, Japan
[f] Division of Cardiology, Niigata University Graduate School of Medical and Dental Science, Niigata, Japan
[g] Department of Obstetrics and Gynecology, Mie University School of Medicine, Mie, Japan

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T cell response

ABSTRACT

We have reported that systemic administration of autologous bone marrow or allogenic fetal membrane (FM)-derived mesenchymal stem cells (MSCs) similarly attenuated myocardial injury in rats with experimental autoimmune myocarditis (EAM). Since rat EAM is a T-helper (Th) cell-mediated autoimmune disease, and recent evidence has indicated that both autologous and allogenic MSCs exert an immunosuppressive effect on Th cell activity, we focused on Th cell differentiation in allogenic FM-MSC administered EAM rats. EAM was induced in Lewis rats by injecting porcine cardiac myosin (day 0). Allogenic FM-MSCs, obtained from major histocompatibility complex mismatched ACI rats, were intravenously injected (5×10 cells/rat) on days 7, 10, or 14 (MSCd7, MSCd10, or MSCd14 groups, respectively). At day 21, echocardiography confirmed that reduced ejection fraction in the untreated EAM group ($63\pm2\%$) was significantly improved in the MSCd10 and MSCd14 groups (74 ± 1 and $75\pm2\%$, respectively, $P<0.01$). CD68 immunostaining revealed that prominent macrophage infiltration in the myocardium of the EAM group (1466 ± 93 cells/mm^2) was significantly decreased in the MSCd10 group (958 ± 139 cells/mm^2, $P<0.05$). To evaluate Th cell differentiation, we used flow cytometry to determine the percentage of interferon (IFN)-γ positive Th1 and interleukin (IL)-17 positive Th17 cells in peripheral CD4-positive Th cells. The percentage of Th1 cells at day 16 was significantly lower in the MSCd10 ($1.3\pm0.2\%$) and MSCd14 ($1.6\pm0.3\%$) groups compared to the EAM group ($2.4\pm0.3\%$, $P<0.05$), as was the percentage of Th17 cells in the MSCd10 group ($1.9\pm0.5\%$) compared to the EAM group ($2.2\pm0.9\%$, $P<0.05$). At day 21, infiltrating Th17 cells in myocardium were significantly decreased in the MSCd10 group (501 ± 132 cells/mm^2, $P<0.05$) compared to EAM (921 ± 109 cells/mm^2). In addition, human CD4+ Th cells co-cultured with human FM-MSCs exhibited reduced Th1 and Th17 cell-differentiation and proliferation, with increased expression of immunosuppressive molecules including indoleamine 2,3-dioxygenase 2 and IL-6 in co-cultured FM-MSCs. These results suggest that intravenous administration of allogenic FM-MSCs ameliorates EAM via the suppression of Th1/Th17 immunity.

1. Introduction

Experimental autoimmune myocarditis (EAM) induced by immunizing with cardiac myosin is a T cell-mediated autoimmune disease. We previously reported that CD8+ T cells are not required to induce myocarditis in rat EAM and that CD4+ helper T (Th) cells regulate its disease severity [1–3]. Activated Th cells secrete various cytokines which recruit and activate other inflammatory cells, such as macrophages, neutrophils, and mast cells [4]. Histological examination of hearts with EAM demonstrates the infiltration of inflammatory cells two weeks after immunization. An excess amount of cytokines and chemokines secreted from recruited inflammatory cells contributes to myocardial damage, and myocarditis peaks around the third week in EAM rats [5,6].

* Corresponding author at: Department of Regenerative Medicine and Tissue Engineering, National Cerebral and Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. Tel.: +81 6 6833 5012; fax: +81 6 6833 9865.
E-mail address: yamahara@ri.ncvc.go.jp (K. Yamahara).

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During the initiation of EAM, naïve CD4+ Th cells are traditionally thought to differentiate into interferon (IFN) γ-producing Th1 [7] and interleukin (IL)-4-producing Th2 cell subsets [8], and it has been assumed that the Th1/Th2 cytokine balance is important in the pathogenesis of EAM [8,9]. Recently, several reports have noted that IL-17-producing Th17 cells are involved in the development of EAM [10], because the severity of EAM is associated with the myocardial expression of IL-17 [11]. Therefore, in addition to the Th1/Th2 cytokine balance, the examination of Th17 cytokine is necessary to determine their pathological roles in EAM.

We have reported that intravenous injection of autologous bone-marrow (BM)-derived mesenchymal stem cells (BM-MSCs) and allogenic fetal membrane (FM)-derived MSC (FM-MSCs) ameliorates myocardial injury in rats with EAM [12,13]. Although we focused on the angiogenic effect and the paracrine action of transplanted MSCs on EAM in our previous study, we showed that allogenic FM-MSCs significantly reduced the proliferation and activity of T lymphocytes [12,13]. Accumulated evidence has indicated that MSCs modify CD4+ Th cell function [14]. MSC-induced inhibition of T cell proliferation leads to decreased IFN-γ production by Th1 cells and increased IL-4 production by Th2 cells, indicating a shift in T cells from an IFN-γ-dominant pro-inflammatory state to an IL-4-dominant anti-inflammatory state. In addition, recent reports indicate that MSC may inhibit Th17 differentiation [16]. Therefore, we speculated that administered MSCs in EAM rats would attenuate myocardial inflammation by altering Th1/Th2/Th17 immunity.

In this study, we investigated the mechanism by which allogenic FM-MSC administration ameliorates myocarditis in EAM rats, focusing on CD4+ Th cell function. We also examined the effect of allogenic FM-MSC on the differentiation of Th1/Th17 cells.

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-1Al; Japan SLC, Hamamatsu, Japan) and August–Copenhagen–Irish (ACI) rats (MHC haplotype: RT-1Aa; Japan SLC). The experimental protocols were approved by the Animal Care Committee of the National Cerebral and Cardiovascular Center.

2.2. Preparation of rat and human FM-MSCs

To obtain rat FMs, pregnant ACI rats (15 days postconception) were sacrificed and their uteri were harvested and placed in PBS (Invitrogen, Carlsbad, CA) [12]. Human FMs were obtained following caesarean section of healthy donor mothers. Human amnion membranes (AMs) were separated from human FMs by mechanical peeling. All experiments using human FMs were approved by the Ethics Committee of the National Cerebral and Cardiovascular Center.

Rat FMs and human AMs were minced and digested with type II collagenase (300 U/ml; Worthington Biochemicals, Lakewood, NJ) for 1 h at 37 °C. After filtration through a mesh filter (100 μm; BD Biosciences, Bedford, MA) and centrifugation at 300×g for 5 min, the dissociated FM-derived cells were suspended in α-MEM supplemented with 10% FBS (Thermo Fisher Scientific Inc., Waltham, MA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) and incubated at 37 °C in 5% CO_2. The population of adherent, spindle-shaped MSCs was expanded. In all experiments, FM-MSCs were used at passages 5–7.

2.3. Acute myocarditis model

Purified cardiac myosin was prepared from pig hearts according to the previously described procedure [17]. 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 6 mg/ml *Mycobacterium tuberculosis* (Difco Laboratories, Sparks, MD). Eight-week-old Lewis rats were injected with 0.1 ml of the antigen–adjuvant emulsion into each footpad and randomly divided into four groups: no transplantation, or FM-MSC administration on day 7, 10, or 14 after myosin injection (EAM, MSCd7, MSCd10, or MSCd14 groups, respectively). For FM-MSC transplantation, we intravenously injected 5×10^5 cells in 0.2 ml of PBS. Sham-operated rats and EAM rats (without FM-MSC administration) were injected with 0.2 ml of PBS on day 7.

2.4. Echocardiographic studies

Echocardiography was performed on day 21 after myosin injection [12]. Rats were anesthetized with isoflurane and a 12-MHz probe was used for M-mode imaging of two-dimensional echocardiography (Sonos 5500, Philips, Bothell, WA). The left ventricular systolic dimension (LVDs), left ventricular diastolic dimension (LVDd), anterior wall thickness (AWT), posterior wall thickness (PWT), fractional shortening (FS) and ejection fraction (EF) were measured. The echocardiography studies were performed with the investigators blinded to exposure and outcome.

2.5. Flow cytometric analysis of Th cell subsets

The presence of Th1, Th2, Th17, or regulatory T (Treg) cells was determined by measuring IFN-γ-, IL-4-, IL-17, or Foxp3-expressing CD4+ cells using flow cytometry [18]. Blood samples diluted 1:1 with RPMI 1640 medium (Invitrogen) were cultured with 60 ng/ml phorbol myristate acetate (PMA), 1.2 μg/ml ionomycin and 12 μg/ml brefeldin A (all from Sigma-Aldrich, St Louis, MO) for 4 h at 37 °C. After incubation with APC-conjugated anti-rat CD4 antibodies (BioLegend, San Diego, CA), cells were fixed and permeabilized, and further incubated with FITC-conjugated anti-rat IFN-γ (BioLegend), R-phycoerythrin (PE)-conjugated anti-rat IL-4 (BioLegend), PE-conjugated anti-rat IL-17A (BD Bioscience), or Alexa Fluor 488-conjugated anti-rat Foxp3 (eBioscience, San Diego, CA) antibody. Cells were then resuspended in 1% paraformaldehyde (PFA) and subjected to flow cytometric analysis (FACSCanto; BD Bioscience).

2.6. Immunohistochemical studies

For immunostaining of IL-17 and CD68, cardiac tissues were fixed with 4% PFA, embedded in paraffin blocks, and cut into 2 μm sections. For IFN-γ and IL-23 immunostaining, hearts were quickly frozen, cut into 5 μm sections, and fixed with 2% PFA for 30 s and acetone for 30 s [19]. After treatment with Protein Block (Dako Cytomation, Glostrup, Denmark), sections were incubated with rabbit anti-rat IL-17 (clone H-132; Santa Cruz Biotechnology, CA,), rabbit anti-rat IFN-γ (clone DB-1; BioLegend), mouse anti-rat CD68 (clone ED-1; Millipore, Bedford, MA), or rabbit anti-rat IL-23 (clone H-113; Santa Cruz) in diluent (DAKO Cytomation) overnight at 4 °C, followed by anti-mouse LSAB/HRP (Dako Cytomation) or anti-rabbit Envision+ system-HRP Labeled Polymer (Dako Cytomation) for 30 min. HRP-labeled sections were visualized with 0.5% diaminobenzidine (Dako Cytomation) and 0.03% H_2O_2, and counterstained with hematoxylin. For immunofluorescent staining, Alexa Fluor dye-conjugated anti-mouse or anti-rabbit IgG antibodies (1:1000, Invitrogen) were used as the secondary antibody and nuclei were stained with SYBR green I (1:10,000, Invitrogen). Sections were photographed using a digital microscope (BIOREVO BZ-9000; KEYENCE, Osaka, Japan), and the number of IL-17-, IFN-γ-, and CD68-positive cells was counted in 20 randomly selected fields per section using image processing software (WinROOF, Mitani Co. Ltd., Tokyo, Japan).

Table 1
Echocardiographic findings in all groups at day 21 after myosin injection.

		Sham	EAM	MSCd7	MSCd10	MSCd14
HR	(bpm)	427±12	370±15	378±7	386±14	378±16
AWT diastole	(mm)	1.5±0.0	2.1±0.1**	2.0±0.1**	1.8±0.0*†	1.9±0.1**
PWT diastole	(mm)	1.5±0.0	2.2±0.1**	2.1±0.1**	1.9±0.0**††§	2.0±0.0**
LVDs	(mm)	3.2±0.1	5.5±0.2**	5.3±0.2**	4.6±0.1**††§	4.5±0.2**††§
LVDd	(mm)	6.7±0.1	7.7±0.2**	7.9±0.2**	7.4±0.2	7.3±0.2

Sham: sham-operated rats given vehicle on day 7; EAM: myosin-treated rats given vehicle on day 7; MSCd7, MSCd10, MSCd14: myosin-treated rats given FM-MSCs (5×10^5 cells/animal) on days 7, 10, and 14 after myosin injection; HR: heart rate; AWT: anterior wall thickness; PWT: posterior wall thickness; LVDs: left ventricular systolic dimension; LVDd: left ventricular diastolic dimension. *$P<0.05$ and **$P<0.01$ vs. sham group, †$P<0.05$, ††$P<0.01$ vs. EAM group, §$P<0.05$ vs. MSCd7.

2.7. In vitro Th1/Th17 differentiation assay

Human peripheral blood CD4+ T cells (5×10^5 cells/well; Lonza, Walkersville, MD) were cultured with X-VIVO medium (Lonza) containing 2% FBS in anti-CD3- and anti-CD28-coated 24-well plates (clone OKT3 and CD28.2, respectively, BioLegend). To induce Th1 differentiation, medium was supplemented with recombinant IL-2 (10 ng/ml; HumanZyme, Chicago, IL), IL-12 (1 μg/ml; HumanZyme), and anti-human IL-4 antibody (1 μg/ml; clone 8D4-8; BioLegend) [15]. To induce Th17 differentiation, medium was supplemented with recombinant TGF-β, IL-1β, IL-6, and IL-23 (10 ng/ml; HumanZyme) [20]. During in vitro differentiation of CD4+ T cells, human BM-MSCs (Lonza) or FM-MSCs were co-cultured at 5×10^4 cells/well. After 5 days of co-culturing, T cells were separated from the MSC monolayer and counted with an automated cell counter (Countess, Invitrogen). T cells were stimulated with 60 ng/ml PMA, 1.2 μg/ml ionomycin, and 12 μg/ml brefeldin A (all from Sigma-Aldrich) for 4 h at 37 °C. Stimulated cells were labeled with APC-Cy7-conjugated anti-human CD4 antibodies (BioLegend), followed by fixation and permeabilization with BD FACS Lysing Solution and Permeabilizing Solution (BD Bioscience). Cells were then incubated with FITC- or PE-conjugated anti-human CD90 (BD Bioscience) and FITC-conjugated anti-human IFN-γ or PE-conjugated anti-human IL-17 (BioLegend) antibodies for a further 30 min, resuspended in 1% PFA, and subjected to flow cytometric analysis (FACSCanto, BD Bioscience).

2.8. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from isolated rat spleens or co-cultured human FM-MSCs using an RNeasy mini kit (Qiagen, Hilden, Germany). RNA was reverse-transcribed into cDNA using a Quantitect Reverse Transcription kit (Qiagen). PCR amplification was performed on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using Power SYBR Green PCR Master Mix (Applied Biosystems). β-actin (for rat spleens) or human GAPDH (for FM-MSCs) was used as an internal control. Primer sequences are listed in Table 2.

2.9. Statistical analysis

All data are expressed as mean±SEM. Comparisons between two parameters were analyzed by using the unpaired Student's t-test. Comparisons of parameters among the groups were made by one-way analysis of variance (ANOVA) followed by Tukey's test. FACS data were analyzed by two-way repeated measures ANOVA. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Improvement in cardiac function by allogenic administration of FM-MSCs

All rats with myocarditis survived during the observation period. Echocardiographic analysis on day 21 post myosin injection is summarized in Fig. 1 and Table 1. Allogenic FM-MSC transplantation improved FS and EF, as we reported previously (Fig. 1) [12]. Among the groups, the MSCd10 and MSCd14 (FM-MSC transplanted on days 10 and 14, respectively) groups showed significant improvement in FS (MSCd10: 38.5±1.0, MSCd14: 39.1±1.5, vs. EAM: 29.3±1.2%, $P<0.01$) and EF (MSCd10: 74.4±1.3, MSCd14: 74.9±2.2, vs. EAM: 62.6±2.1%, $P<0.01$). In addition, FM-MSC administration significantly decreased PWT at diastole (MSCd10: 1.9±0.0, vs. EAM: 2.2±0.1 mm, $P<0.05$) and LVDs (MSCd10: 4.6±0.1 vs. EAM: 5.5±0.2 mm, $P<0.05$), although AWT) at diastole and LVDd did not significantly differ between the groups (Table 1).

3.2. Reduced macrophage infiltration of myocardium after FM-MSC treatment

Immunohistochemical analysis of myocardial tissues on day 21 demonstrated that, compared to the untreated EAM group, the infiltration of CD68-positive macrophages was significantly attenuated in the MSCd10 group (958±139 cells/mm², n=11; $P<0.05$ vs. 1466±93 cells/mm², n=13) (Fig. 2). In the other cell transplanted groups, systemic injection of FM-MSC tended to suppress the myocardial infiltration of macrophages compared to the EAM group. (MSCd7: 1279±179 cells/mm², n=6; MSCd14: 1040±195 cells/mm², n=6).

3.3. Suppression of Th1/Th17 cells after FM-MSC treatment in EAM rats

In the FACS analysis, peripheral CD4+ Th cells were further subdivided based on the expression of IFN-γ (Th1) and IL-17 (Th17) [18] (Figs. 3 and 4). In FM-MSC-untreated EAM rats (the EAM group),

Table 2
Primer sequences used for qRT-PCR.

Gene	species	Forward	Reverse
IL-17	Rat	5′-TGTCCAAACGCCGAGGCCAA-3′	5′-AGGGCCTTCTGGAGCTCGCTT-3′
β-actin	Rat	5′-GCCCTAGACTTCGAGC-3′	5′-CTTTACGGATGTCAACGT-3′
IDO2	Human	5′-CCACAGACCGAATGTGAAGAC-3′	5′-TGTTGGCAATTTCCATCCAAGG-3′
HLA-G	Human	5′-CACGCACAGACTGACAGAATG-3′	5′-GCCATCGTAGGCATACTGTTCA-3′
IL-6	Human	5′-TTGGAAGGTTCAGGTTGTTTTCT-3′	5′-AATTCGGTACATCCTCGACGG-3′
GAPDH	Human	5′-GAGGCAGGGATGATGTTCTGGA-3′	5′-CAATGCCTCCTGCACCACCAA-3′

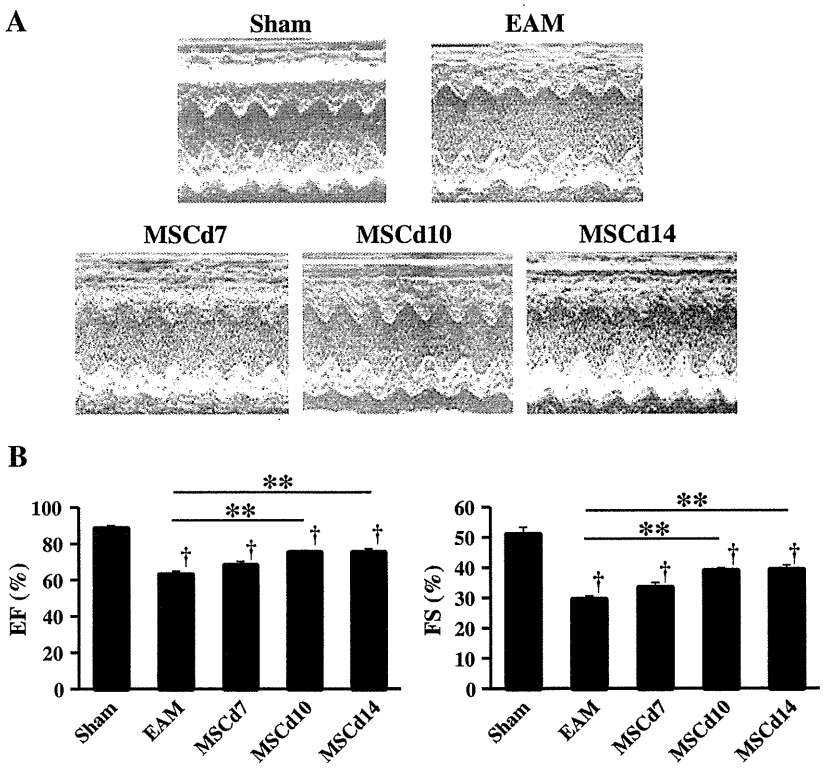

Fig. 1. Effects of allogenic FM-MSC transplantation on echocardiographic parameters in EAM. (A) Representative echocardiographic images showing poor movement in the EAM group compared to the sham group. Improved cardiac contractility was observed in the FM-MSC-administered groups at day 10 (MSCd10) and 14 (MSCd14) after the induction of myocarditis. (B, C) FM-MSC administration significantly improved EF and FS in the MSCd10 and MSCd14 groups. $^{**}P<0.01$ vs. EAM, †$P<0.05$ vs. MSCd7, n = 11–13.

there was an increase in Th1 cells until day 16 after myosin injection (2.4 ± 0.3% of CD4+ cells on day 16), which subsequently decreased gradually (1.1 ± 0.2% on day 36) (Fig. 3B). In the FM-MSC-treated groups, a decrease in the percentage of Th1 cells was observed. On day 16, the FM-MSC-treated MSCd10 and MSCd14 groups showed a significant decrease in the percentage of Th1 cells (MSCd10: 1.3 ± 0.2%, MSCd14: 1.6 ± 0.3% of CD4+ cells, $P<0.05$ vs. EAM), but no difference was seen between the EAM and MSCd7 groups.

As shown in Fig. 4B, the percentage of Th17 cells was substantially increased in the EAM group until day 16 (2.2 ± 0.9% of CD4+ cells on day 16) and decreased thereafter (1.0 ± 0.1% on day 21). Similar to what was found for Th1 cells, a significant decrease in Th17 cells was found in the FM-MSC-treated MSCd10 group (1.9 ± 0.5% on day 16, $P<0.05$ vs. EAM). On day 16, IL-17 mRNA expression in the spleen was markedly decreased in the MSCd10 group compared with the untreated EAM group ($P<0.001$ vs. EAM, n = 7 in each group).

Although we also examined other Th subsets in the peripheral blood of myocarditis rats, no significant changes in the Th2 or Treg were observed between the groups (data not shown).

3.4. Decreased infiltration of IFN-γ- and IL-17-positive cells in the myocardium following FM-MSC treatment

Immunohistochemical staining of IL-17 in EAM hearts on day 21 demonstrated that FM-MSC administration significantly decreased the infiltration of IL-17-postive cells in the MSCd10 group compared to the EAM group (MSCd10: 501 ± 132 vs. EAM: 921 ± 109 cells/mm², $P<0.05$; n = 9 in each group). (Figs. 5C, D) A similar tendency toward decreased infiltration of IL-17-positive cells was found in the other FM-MSC-transplanted groups (MSCd7: 534 ± 96 cells/mm², n = 11; MSCd14: 508 ± 77 cells/mm², n = 6). IFN-γ staining also revealed that there was a tendency for suppressed infiltration of IFN-γ+ cells following FM-MSC administration (MSCd10: 85 ± 20 vs. EAM: 196 ± 75 cells/mm², $P=0.17$, n = 10 in each group) (Figs. 5A, B).

3.5. Inhibition of Th1/Th17 differentiation in CD4+ T cells when co-cultured with MSCs

The effect of human FM-MSCs on Th1/Th17 differentiation was analyzed using human peripheral blood CD4+ T cells. After 5 days of culturing of CD4+ T cells under Th1-polarizing conditions, the number of CD4+ cells markedly increased ($7.1 \pm 0.4 \times 10^4$ cells/ml) and a significant number of Th1 cells appeared (IFN-γ+ cells/CD4+ cells: 13.9 ± 1.2%) (Fig. 6A). When co-cultured with FM-MSCs or BM-MSCs, the number of CD4+ T cells and the differentiation into Th1 cells were significantly suppressed (CD4+ cells: $1.8 \pm 0.4 \times 10^5$ cells/ml in FM-MSC, $3.1 \pm 0.3 \times 10^5$ cells/ml in BM-MSCs; Th1 cells: 1.8 ± 0.2% in FM-MSCs, 10.1 ± 1.0% in BM-MSCs, $P<0.01$ vs. control) (Figs. 6C, D). Compared to BM-MSCs, FM-MSCs significantly suppressed Th1 differentiation ($P<0.01$ vs. BM-MSCs) (Fig. 6C).

When human CD4+ T cells were stimulated for 5 days under Th17-differentiating conditions, the number of CD4+ cells and IL-17-expressing Th17 cells also increased (CD4+ cells: $3.8 \pm 1.6 \times 10^5$ cells/ml, IL-17+ cells/CD4+ cells: 6.8 ± 0.4%) (Figs. 6A, B). The presence of FM-MSCs as well as BM-MSCs significantly suppressed the number of CD4+ cells (FM-MSCs: $0.9 \pm 0.6 \times 10^5$ cells/ml, BM-MSCs: $1.3 \pm 1.5 \times 10^5$ cells/ml, $P<0.01$ vs. control) (Fig. 6F) and differentiation into Th17 cells (FM-MSCs: 1.5 ± 0.1%, BM-MSCs: 2.3 ± 0.3%, $P<0.01$ vs. control) (Fig. 6E).

Under Th17-polarizing culture conditions with CD4+ T cells, we also quantified the gene expression of immunosuppressive molecules including indoleamine 2,3-dioxygenase (IDO) 2, IL-6, and HLA-G in co-cultured FM-MSCs. The expression of IDO2 and IL-6 was significantly increased in co-cultured FM-MSCs (IDO2: 27.7 ± 11.0 fold,

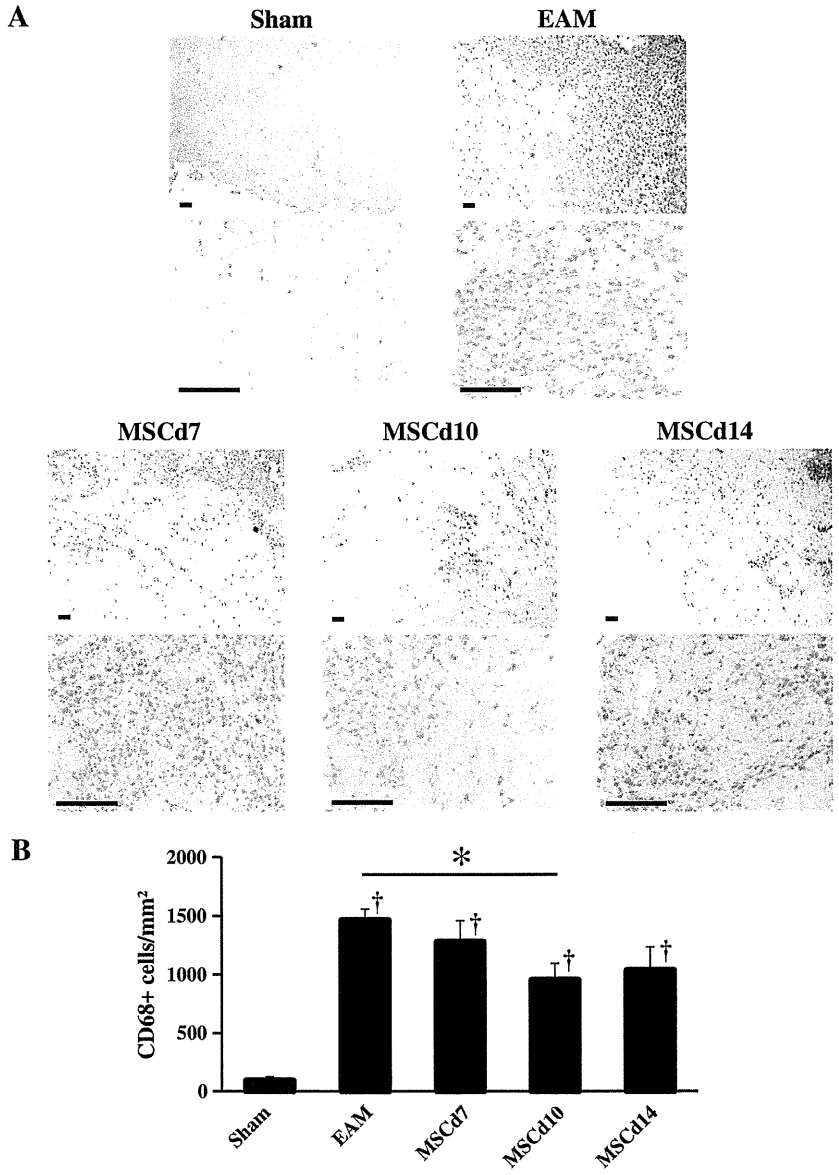

Fig. 2. Effects of allogenic FM-MSC administration on myocardial macrophage infiltration in EAM. (A) Representative myocardial sections of CD68 immunostaining demonstrate a decrease in CD68-positive cells after FM-MSC transplantation. (B) Quantitative counts of CD68-positive macrophages show a significant reduction in the MSCd10 group compared to the EAM group. Scale bars: 100 μm. *$P<0.05$ vs. EAM, †$P<0.05$ vs. sham. n = 6-13.

IL-6: 23.7 ± 8.9 fold, $P<0.05$ vs. control mono-cultured FM-MSCs). Co-cultured FM-MSCs also showed a tendency for increased gene expression of HLA-G (HLA-G: 3.8 ± 1.5 fold, $P=0.096$ vs. control FM-MSCs).

4. Discussion

In this study, we elucidated the mechanisms involved in the therapeutic effects of FM-MSC transplantation in EAM, particularly focusing on the regulatory effect of FM-MSCs on Th cells. We demonstrated that 1) intravenous administration of allogenic FM-MSCs on the tenth day after myosin injection significantly improved both cardiac function and myocardial inflammation, 2) administered FM-MSCs decreased Th1 and Th17 responses in EAM, 3) cultured FM-MSCs as well as BM-MSCs suppressed Th1 and Th17 differentiation from naïve CD4+ Th cells and 4) the expression of immunosuppressive molecules including IDO, IL-6, and HLA-G was increased in FM-MSC when they were co-cultured with Th17-polarized CD4+ T cells.

Myosin-induced EAM provides a model that resembles human giant cell myocarditis, consisting of an antigen priming phase from days 0 to 14, an autoimmune response phase from days 14 to 21, followed by a phase characterized by repair and fibrosis [21]. In rat EAM, Th immunity is thought to be responsible for disease pathogenesis induced by cardiac myosin immunization [8,22,23]. Published data suggest that the induction of EAM might be associated with Th1 dominance and recovery is related to Th2 polarity [8,22]. Recently, Th17 was reported to participate in cardiac inflammation in EAM [24]. Daniels et al. reported that Th17 as well as Th1 cells increase in number during the acute phase of EAM in A/J mice [24]. In addition, in mice lacking the T box transcription factor T-bet, required for Th1 differentiation, myocardial inflammation was more severe [25]. This knockout mouse showed a marked increase in production of

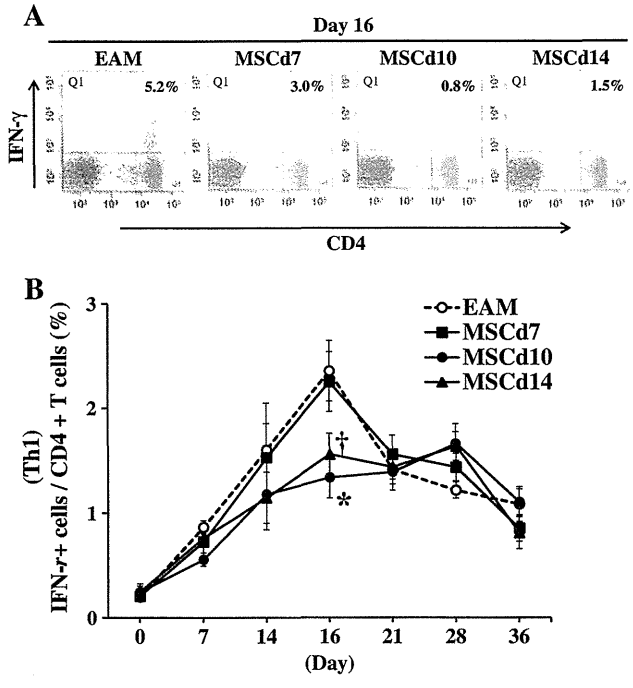

Fig. 3. Reduced induction of Th1 cells by allogenic FM-MSC administration in EAM rats. (A) Representative flow cytometric analysis of IFN-γ+/CD4+ T cells obtained from peripheral blood on day 16. (B) Time course of Th1 cell in myocarditis rats. The number of Th1 cells within CD4+ T cells increased after myosin administration, peaked at the stage of EAM (day 16), then gradually decreased. On day 16, FM-MSC administration significantly reduced Th1 cell induction in the MSCd10 and MSCd14 groups, compared to the untreated EAM group. *$P<0.05$ and **$P<0.01$ vs. EAM, n=12–14.

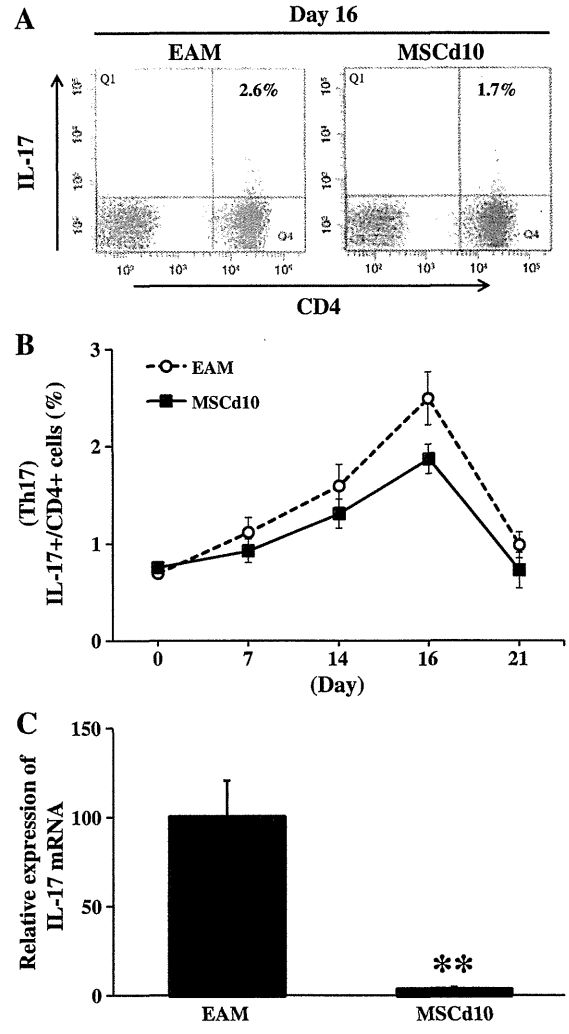

Fig. 4. Suppressed induction of Th17 cells by allogenic FM-MSC administration in EAM rats. (A) Representative flow cytometric analysis of peripheral IL-17+/CD4+ T cells on day 16. (B) Time course of Th17 cells in myocarditis rats. Th17 cells within CD4+ T cells increased after myosin administration, peaked at the stage of EAM (day 16), and decreased on day 21. FM-MSC administration significantly reduced Th17 cell induction in the MSCd10 group compared to the untreated EAM group. *$P<0.05$ vs. EAM, analyzed by two-way repeated measures ANOVA, n=7. (C) IL-17 mRNA expression in the spleen of myocarditis rats on day 16. Quantitative RT-PCR analysis demonstrated that FM-MSC administration significantly decreased IL-17 gene expression. **$P<0.01$ vs. EAM, n=7.

the IL-23-dependent cytokine IL-17 by heart-infiltrating lymphocytes. In vivo IL-17 depletion markedly reduced EAM severity in T-bet knockout mice. Since Th17 is credited with causing cardiac inflammation in EAM, we elected to examine the role of Th17 cells in the pathogenesis of EAM.

In our previous study, we demonstrated that intravenous administration of autologous BM-MSCs significantly improved cardiac function and myocardial inflammation in rats with EAM [13]. Since, in a clinical setting, the BM aspiration procedure is invasive, and a long period of expansion is required to obtain adequate numbers of BM-MSC, we focused on FMs, which are generally discarded as medical waste and possess great advantages due to their abundance and easy accessibility, as an alternative source of MSCs. Although FM-MSC administration implies allogenic transplantation, injection of allogenic rat FM-MSCs effectively attenuated myocardial injury in EAM rats [26]. Moreover, we confirmed that the allogenic human FM-MSCs as well as BM-MSCs similarly reduced human Th1 and Th17 cell-differentiation and proliferation.

Several groups have reported therapeutic effects of MSCs in various autoimmune disease models, including experimental autoimmune encephalomyelitis [27], collagen-induced arthritis [28], and EAM [12,13]. Previous reports, including ours, have focused on the differentiation potential or the paracrine activity of MSCs. We had thought that the therapeutic effects of MSCs in EAM were due to their differentiation into cardiovascular cells, or due to some angiogenic/proliferative property [13,29]. Although we previously reported that BM-MSC administration increased the number of capillaries in the cardiac tissue of EAM rats [13], no significant change in capillary density was observed in FM-MSC administered EAM rats [12], which might have been the result of differences in the angiogenic properties of BM-MSCs and FM-MSCs [12,13]. More importantly, the immune suppressive properties of MSCs have recently attracted increasing attention and seem to constitute a major mechanism through which MSCs provide therapeutic benefits [14,30,31]. In our recent study, we found that splenic T lymphocytes derived from FM-MSC-treated EAM rats demonstrated reduced proliferative activity compared to those from untreated EAM rats [26]. In addition, activated T cell proliferation was suppressed by co-culture with allogenic FM-MSC. These results indicate that allogenic FM-MSCs ameliorate myocardial damage in EAM by inhibiting T cell activation and proliferation, but the effects of transplanted MSCs on individual T cell effector pathways in the pathogenesis of EAM remain unclear.

To gain insight into the mechanism of disease improvement by FM-MSC administration, we examined the population of CD4+ Th cells [7]. Flow cytometric analysis demonstrated that circulating Th1/Th17 cells dominated during the inflammatory phase of disease, similar to previous reports [8,24]. Administration of allogenic FM-MSCs in EAM rats significantly reduced the numbers of circulating Th1 and Th17 cells in the inflammatory phase. Immunostaining of

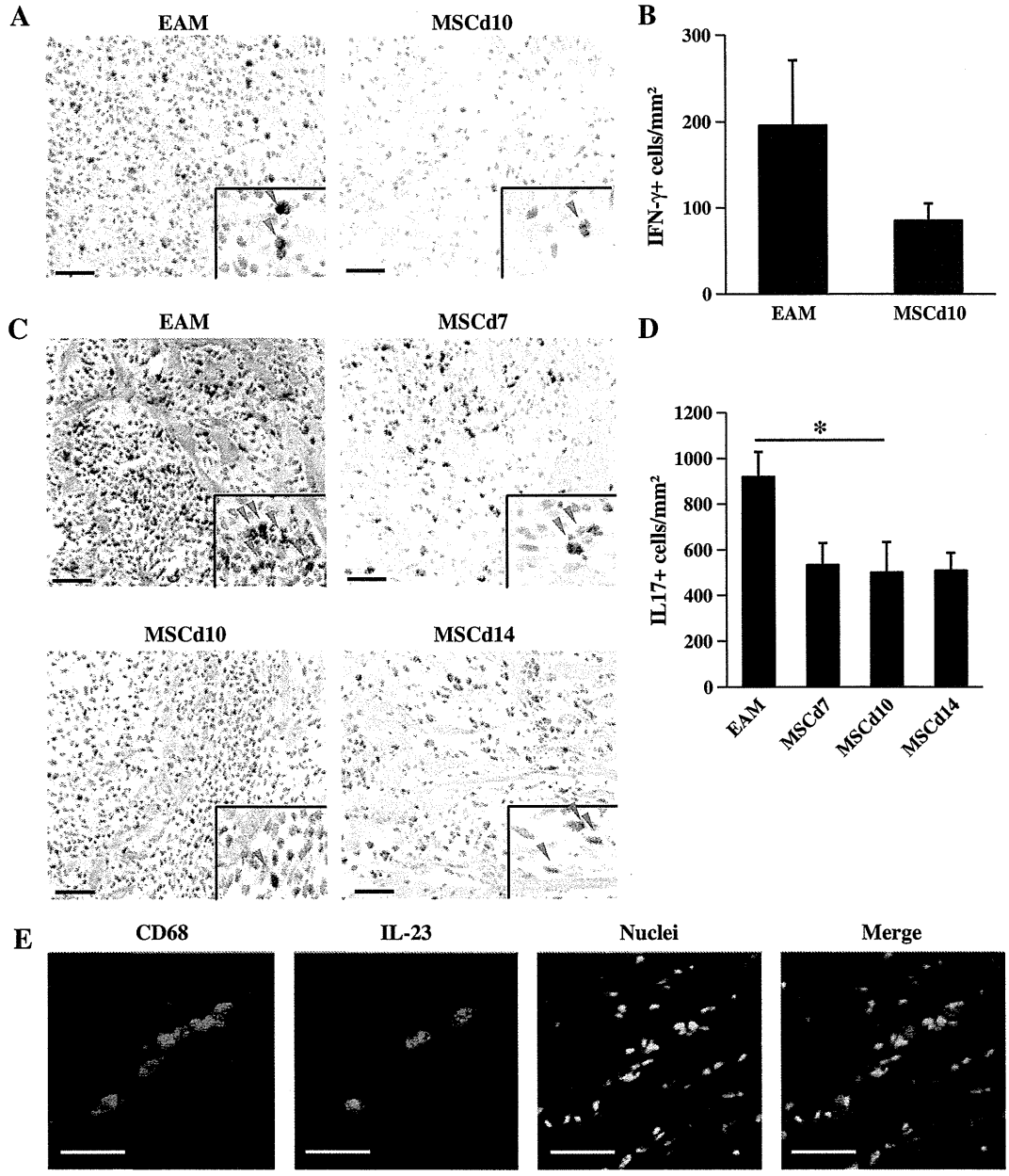

Fig. 5. Effects of FM-MSC administration on myocardial infiltration of IFN-γ+ and IL-17+ cells in EAM. (A, C) Representative immunohistological staining of IFN-γ+ cells (A, red arrow) and IL-17+ cells (B, red arrow). Scale bars: 50 μm. (B, D) Quantitative counts of IFN-γ+ (B) and IL-17+ (D) cells demonstrated that, compared to untreated EAM rats, FM-MSC administration significantly suppressed the infiltration of IL-17+ cells in the MSCd10 group (D), and a similar tendency was found for IFN-γ+ cells (B). *$P<0.05$ vs. EAM group, n=9–10. (E) Representative immunohistological staining of IL-23+ (blue) CD68+ (red) cells. Nuclei were stained with SYBR Green (green). In myocarditis tissue, all IL-23+ cells were entirely merged with CD68+ macrophages (merge). Scale bars: 50 μm.

heart tissues in the myocarditis phase revealed marked infiltration of IFN-γ- and IL-17-positive cells in EAM rats, and FM-MSC administration attenuated the number of IFN-γ/IL-17-positive cells. We also examined IFN-γ and IL-17 gene expression in the spleen and confirmed that FM-MSC administration significantly reduced the expression of IL-17, but not that of IFN-γ (data not shown). We also examined other Th subsets in EAM rats, but no significant changes in circulating Th2 or Treg cells were observed in FM-MSC-treated rats (data not shown). These results suggest that allogenic FM-MSCs ameliorate myocardial inflammation by suppressing the Th1/Th17 response.

Recent studies have indicated that BM-MSCs inhibit the development of a pro-inflammatory Th1 immune response [15]. In addition, BM-MSCs can inhibit Th17 cell differentiation and function [16,20]. To confirm whether FM-MSCs, as well as BM-MSCs, possess an inhibitory effect on Th1/17 cell differentiation, we performed an in vitro co-culture experiment. Similar to previous reports using BM-MSCs, we confirmed that human FM-MSCs markedly suppressed the proliferation of human CD4+ T cells and prevented their differentiation into both Th1 and Th17 cells. Recent studies have demonstrated that MSCs exert their immunosuppressive properties through the release of soluble factors including IDO2, IL-6, and soluble HLA-G5 [14,32–34]. We demonstrated that gene expression of IDO2, IL-6, and HLA-G in FM-MSCs was markedly increased when co-cultured with Th17-polarized CD4+ T cells. Therefore, these immunosuppressive factors secreted from FM-MSCs might suppress Th17 proliferation and differentiation.

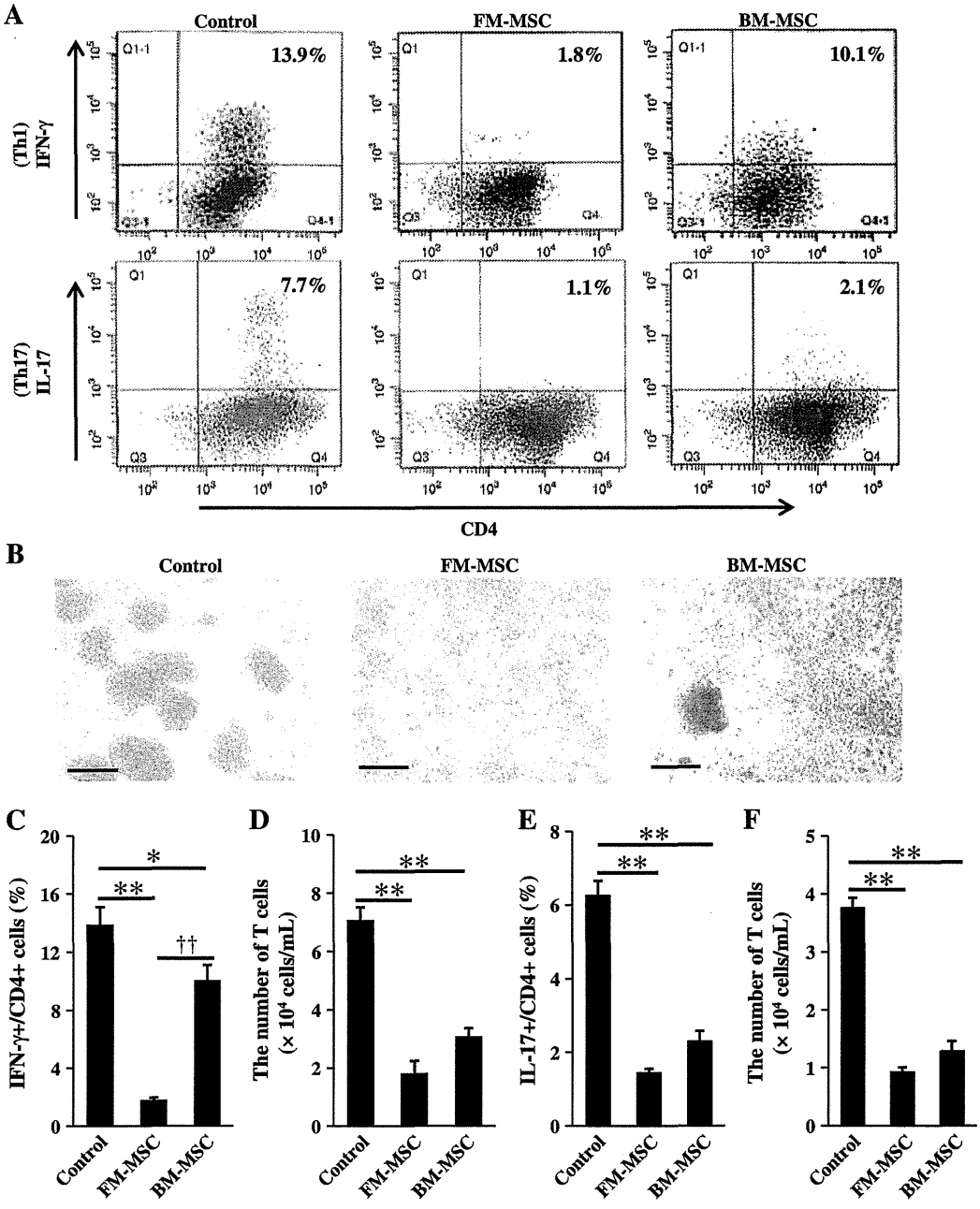

Fig. 6. Inhibition of Th1/Th17 proliferation and differentiation upon co-culture with BM-MSCs and FM-MSCs. (A) Representative flow cytometry plots of IFN-γ+/CD4+ and IL-17+/CD4+ T cells without MSC co-culture (control), with FM-MSC or BM-MSC co-culture. (B) Representative images of cultured CD4+ T cells without MSCs (control), with FM-MSCs or BM-MSCs under the Th17 inducing condition. (C, D) The percentage of Th1 cells within CD4+ T cells (C) and the number of proliferated T cells (D) after five days of co-culture with MSCs. Both FM-MSCs and BM-MSCs significantly inhibited the differentiation of Th1 cells and CD4+ T cell proliferation. FM-MSCs compared to BM-MSC, significantly inhibit the differentiation of Th1 cells. $^{*}P<0.05$ and $^{**}P<0.01$ vs. control, $\dagger\dagger P<0.01$: FM-MSC vs. BM-MSC, n = 5. (E, F) The percentage of Th17 cells within CD4+ T cells (E) and the number of proliferated T cells (F) after five days of co-culture with MSCs. Both FM-MSCs and BM-MSCs significantly suppressed Th17 differentiation and T cell proliferation. $^{**}P<0.01$ vs. control, n = 7.

In this study, we also investigated the optimal timing of FM-MSC transplantation in rats with EAM. We previously reported that a significant number of allogenic FM-MSCs could be detected 24 h after intravenous administration, but only a few FM-MSCs survived after 7 days and no FM-MSC were detected after 21 days [35]. Therefore, we considered that intravenously administered FM-MSCs would not survive for more than a week and speculated that there was an optimal time point to achieve the best therapeutic outcome when using FM-MSC transplantation. FM-MSCs injected intravenously at days 10 and 14 after myosin injection significantly improved cardiac function and suppressed the Th1/Th17 response, while FM-MSCs administered at day 7 showed a reduced therapeutic effect on myocarditis. This result might indicate that, because intravenously administered FM-MSCs survive only for a week, administration of FM-MSCs at day 7 might be too early to suppress the helper T cell response and myocarditis, and timing of FM-MSC administration is an important factor regulating Th1/Th17 immunity. However, Zappia et al. reported that MSC transplantation at day 3 and day 8 before disease onset, as well as at day 15 at the peak of disease, was effective in treating experimental autoimmune encephalomyelitis [27]. The reduced immunosuppressive

potential of FM-MSC with early administration in our EAM model may involve non-Th1/Th17 effects of FM-MSCs.

We have previously demonstrated that both allogenic FM-MSC and autologous BM-MSC transplantation in EAM attenuate heart-infiltrating monocytes/macrophages as well as cardiac monocyte chemoattractant protein-1 (MCP-1) expression [12,13]. Muggini et al. reported that MSCs directly induce activated macrophages to acquire a regulatory profile characterized by a reduced ability to produce inflammatory cytokines [36]. Since the inflammatory cells observed in myocardial lesions consist of 80% monocytes and macrophages [37] and blockade of MCP-1 significantly reduces macrophage infiltration and the severity of EAM [38], FM-MSC administration might directly suppress activated monocytes/macrophages in myocardial tissue. Interestingly, we found that infiltrated CD68-positive macrophages in cardiac tissue secreted IL-23, which is required for full acquisition of the function and maintenance of Th17 cells [39]. Therefore, in EAM, IL-23 secreted from activated macrophages in myocardial tissue might accelerate Th17 cell differentiation and function, which would worsen the disease outcome.

In conclusion, the results described in this study demonstrate the protective effects of intravenously administered FM-MSCs in EAM due to profound suppression of Th1/Th17 cells. Because FM-MSCs are available non-invasively in large numbers, these findings might provide a new perspective for the treatment of acute myocarditis.

Disclosures

None.

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Human umbilical cord provides a significant source of unexpanded mesenchymal stromal cells

AKIE KIKUCHI-TAURA[1,2], AKIHIKO TAGUCHI[1], TAKAYOSHI KANDA[3], TAKAYUKI INOUE[2], YUKIKO KASAHARA[1], HARUKA HIROSE[1], IORI SATO[2], TOMOHIRO MATSUYAMA[4], TAKAYUKI NAKAGOMI[4], KENICHI YAMAHARA[1], DAVID STERN[5], HIROYASU OGAWA[2] & TOSHIHIRO SOMA[2]

[1]*Department of Regenerative Medicine, National Cerebral and Cardiovascular Center, Osaka, Japan,* [2]*Division of Hematology, Department of Internal Medicine, Hyogo College of Medicine, Hyogo, Japan,* [3]*Department of Perinatal Medicine, Osaka Minami Medical Center, Osaka, Japan,* [4]*Institute for Advanced Medical Sciences, Hyogo College of Medicine, Hyogo, Japan, and* [5]*Executive Dean's Office, University of Tennessee, Tennessee, USA*

Abstract

Background aims. Human mesenchymal stromal cells (MSC) have considerable potential for cell-based therapies, including applications for regenerative medicine and immune suppression in graft-versus-host disease (GvHD). However, harvesting cells from the human body can cause iatrogenic disorders and *in vitro* expansion of MSC carries a risk of tumorigenesis and/or expansion of unexpected cell populations. *Methods.* Given these problems, we have focused on umbilical cord, a tissue obtained with few ethical problems that contains significant numbers of MSC. We have developed a modified method to isolate MSC from umbilical cord, and investigated their properties using flow cytometry, mRNA analysis and an *in vivo* GvHD model. *Results.* Our study demonstrates that, using umbilical cord, large numbers of MSC can be safely obtained using a simple procedure without *in vitro* expansion, and these non-expanded MSC have the potential to suppress GvHD. *Conclusions.* Our results suggest that the combined banking of umbilical cord-derived MSC and identical cord blood-derived hematopoietic stem cell banking, where strict inspection of the infectious disease status of donors is performed, as well as further benefits of HLA-matched mesenchymal cells, could become one of the main sources of cells for cell-based therapy against various disorders.

Key Words: *cell banking, graft-versus-host disease, mesenchymal stromal cells, umbilical cord*

Introduction

Mesenchymal stromal cells (MSC) can be obtained from various tissues, including bone marrow (1), adipose tissue (2), trabecular bone (3), synovium (4), pancreas (5), lung, liver, spleen (6), peripheral blood (7), cord blood (8), amniotic fluid and umbilical cord (9–11). MSC include multiple cell types that have the capacity to differentiate into neurons, adipocytes, cartilage, skeletal muscle, hepatocytes and cardiomyocytes, under appropriate conditions across embryonic germ layers (2,12). Cell-based therapies using MSC have been initiated in patients with arthrosis (13), corneal disorders (14), stroke (15) and chronic heart failure (16). MSC are also used to suppress graft-versus-host disease (GvHD) in patients after allogeneic hematopoietic stem cell transplantation, and co-transplantation of hematopoietic cells and MSC to enhance engraftment has provided promising results (17). To broaden the indications for use of MSC against multiple disorders, cell banking with HLA-typing would be desirable.

Although MSC can be obtained from a range of organs, the iatrogenic risks associated with harvesting cells from the human body cannot be denied, particularly with harvesting from the large number of individuals required to establish a cell bank. In contrast, harvesting of cells from umbilical cord carries little risk to the donor, and MSC have been identified in placenta (18), amniotic fluid (19), umbilical cord blood (20) and the umbilical cord itself (21). Furthermore, the omission of *in vitro* expansion offers significant benefits for cell banks with large numbers of samples. The present study focused on the umbilical cord, because of the relative ease of cleaning the tissue before harvest and subsequent isolation of the cells. The umbilical cord is covered by

Correspondence: **Akihiko Taguchi**, Department of Regenerative Medicine, National Cerebral and Cardiovascular Center, 5-7-1 Fujishiro-dai, Suita, Osaka, Japan 565–8565. E-mail: taguchi@ri.ncvc.go.jp

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an epithelium derived from the enveloping amnion and contains two arteries and one vein, all of which are surrounded by the mucoid connective tissue of Wharton's jelly. The main role of this jelly-like material is to prevent compression, torsion and bending of the enclosed vessels, which provide bidirectional blood flow between the fetal and maternal circulations. The network of glycoprotein microfibrils and collagen fibrils in Wharton's jelly has already been elucidated (22). The phenotypic stromal cells in Wharton's jelly are fibroblast-like cells (23), morphologically and immunophenotypically similar to MSC isolated from bone marrow (9,24,25). MSC from the umbilical cord have been shown to differentiate into adipocytes, osteocytes, neurons and insulin-producing cells (9,24–30). Carlin *et al.* (31) recently demonstrated embryonic transcription factors, such as octamer-binding transcription factor (Oct)-4, sex determining region Y-box (Sox)-2 and Nanog, in porcine umbilical cord matrix cells. These results indicate that umbilical cord-derived mesenchymal stem (UCMS) cells may represent a major source of cells for cell-based therapy. The present study demonstrates that large numbers of MSC can be obtained from umbilical cord using a simple procedure without *in vitro* expansion. Furthermore, UCMS isolated using this procedure are shown to suppress severe GvHD in a murine model.

Methods

All procedures for the isolation and differentiation of human UCMS cells were approved by the Osaka Minami Medical Center (Osaka, Japan). institutional review board and all volunteer donating mothers provided written informed consent. Animal experiments were carried out in accordance with the guidelines of the Animal Care Committee of Hyogo College of Medicine (Hyogo, Japan). Quantitative analyzes were conducted by an investigator who had been blinded to the experimental protocol, identities of animals and experimental conditions pertaining to the animals under study.

Isolation of UCMS cells

Human umbilical cords were obtained from patients delivered at full-term by Cesarean section ($n = 30$). After collection of cord blood as described previously (32), placenta and umbilical cord were placed into a sterilized bag and the following procedures were performed in a safety cabinet. In this study, we used only umbilical cord tissue for further experiments. Both ends of the umbilical cord (approximately 1 cm from each end) were cut and discarded with the placenta. The remaining umbilical cord was immersed and washed in 80% ethanol for 1 min, rinsed with sterile saline twice and cut into approximately 10-cm lengths. Cord blood and blood clots in the umbilical cord artery were removed by flushing twice, using a 20-G tip cut needle, sterile saline and 20-mL syringe. Then the umbilical cord segments were immersed and washed with 80% ethanol for 1 min, followed by two rinses with sterile saline. Next, the umbilical cord was cut into 2–4-cm lengths and the epithelial tissue was removed using sterilized scissors. The remaining tissue was incubated in an enzyme cocktail solution, containing 1 mg/mL hyaluronidase (Sigma-Aldrich, St Louis, MO, USA), 300 U/mL collagenase (Sigma-Aldrich) and 3 mM $CaCl_2$ (Wako Pure Chemical Industries, Osaka, Japan) in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) for 2 h at 37°C with shaking (50 shakes/min; BR-21UM; Taitec, Saitama, Japan). After incubation, undigested vascular components were removed and the tissue solution was crushed with forceps and passed through 180- and 125-μm diameter stainless steel mesh (Tokyo Screen, Tokyo, Japan), followed by 70-μm diameter mesh (Becton Dickinson, Franklin Lakes, NJ, USA), to remove large pieces of unlysed tissue. The tissue solution was then collected by centrifugation (200 ***g*** for 5 min) and resuspended in phosphate-buffered saline (PBS). The latter washing procedure was performed twice. Next, the tissue solution was incubated with 0.5% trypsin–ethylenediamine tetraacetic acid. (EDTA) (Invitrogen) in PBS for 1 h at 37°C. After trypsinization, the tissue solution was neutralized with 2% fetal bovine serum (FBS) in DMEM and this washing procedure was performed twice. As a control, the conventional method of tissue preparation reported by Weiss *et al.* (29) was used to isolate UCMS cells. That procedure is similar to our own, the major difference being that we did not remove the umbilical artery from the umbilical cord before digestion with hyaluronidase and collagenase.

Flow cytometric analysis of UCMS cells

Antigens expressed by freshly isolated and *in vitro*-expanded and -differentiated umbilical cord-derived cells were investigated by multicolor flow cytometry. The expression of surface markers in 1×10^5 cells was analyzed. The characteristics of each antibody are listed in Table I. As a control, a non-immune isotype control (Beckman Coulter Orange County, CA, USA) was employed.

Expansion and in vitro *differentiation of UCMS cells*

To investigate the properties of isolated UCMS cells as MSC, cells were expanded *in vitro* as described previously (29). Briefly, 1×10^4 cells/cm^2 were plated in a low-serum media, containing 56% low-glucose DMEM (Invitrogen), 37% MCDB201