厚生労働科学研究研究費補助金

臨床研究基盤整備推進研究事業

心筋微小血管造影装置の開発による糖尿病性心筋微小循環障害の可視化

（若手医師・協力者活用に要する研究）

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主任研究者　竹下　聡　国立循環器病センター　心臓血管内科医長

微小血管の可視化を目的とし、病院設置型微小血管造影装置の開発とその臨床応用を行った。ファントムを用いた検討では、理論上直径50μmまでの血管を描出することが可能であり、被検者の被曝量も、臨床上許容範囲にあることが判明した。血管新生療法の施行患者を対象として、治療前後における微小側副血管網の変化を比較検討した。

A. 研究目的

従来型の血管造影装置では描出できないような微小血管を観察することによって、様々な疾患の病態把握が可能となり得る。例えば、難知性の重症末梢動脈閉塞症に対する血管新生療法の臨床応用が進められているが、従来型血管造影検査では、新生血管の描出は不可能である。また、糖尿病性微小血管障害の病態把握や治療効果の判定に有効な検査はない。

本研究の目的は、病院設置型微小血管造影装置を開発、臨床応用することによって、微小循環障害の病態把握法や血管新生の新しい評価法を確立することである。

B. 研究方法

新エネルギー産業技術総合開発機構(NEDO)の支援のもと、浜松ホトニクス(株)を中心に、NHK エンジニアリングサービス、国立循環器病センター研究所、東海大学医学部等が協力して、病院設置型の微小血管造影装置を開発した。装置は、高出力の CT 用 X 線源とハイビジョンの高感度撮像系により構成されている。チャートを用いて、解像度を測定し、犬冠動脈のファントムで中核枝の評価およびウサギの虚血肢モデルでの再生血管の評価を行った。また、吸収線量および散乱線の測定を行い、安全性の検討を行った。臨床応用では、末梢動脈閉塞症に対する血管新生療法前後に微小血管造影を施行し、虚血下肢の微小血管を評価した。

(倫理面への配慮)

倫理委員会の審議・承認を得、本検査の合併症・効能・不利益・利益を説明し、本人及び家族の同意の元に施行した。

C. 研究結果

一般の血管造影では250μmが解像度の限界であるが、病院設置型微小血管造影装置では50μmまで観察可能であった。ヒトに対する臨床応用として、血管新生療法を行う下肢末梢動脈閉塞症の患者を対象に、合計8回の微小血管造影を施行した。造影に伴う被曝線量は通常の血管造影と同レベルであることが判明した。微小血管造影によって通常の造影では描出困難な100μm以下の微小血管が鮮明に描出された。DSA に比較して少なくとも1−2分枝末梢側の血管が描出可能であった。1ヶ月から1年の間隔を置いて施行したフォローアップ造影における微小血管の再現性は良好であった。血管新生療法前後で微小血管網の発達を検討すると、治療後に明らかな血管新生が認められたのは3割の症例に過ぎなかった。

D. 考察

病院設置型微小血管造影装置の1号機は、通常の血管造影と同等の安全性を有している。また、その微小血管描出能は通常装置に比し優れていた。造影を繰り返し施行し得た症例における微小血管の再現性は良好であった。血管新生療法前後において必ずしも微小血管数の増加が認められないことから、本療法の作用機序における血管新生以外の因子の関与が示唆された。

E. 結論

本研究で開発された病院設置型微小循環造影装置によって 50μm レベルの微小血管が観察可能であり、その安全性や再現性に問題はなかった。微小循環障害を伴う疾患の評価や、末梢動脈閉塞症に対する血管新生療法の作用機序の解明、治療効果判定に有用な検査法と思われた。

F. 健康危険情報

特になし。

G. 研究発表

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H. 知的財産権の出願・登録状況

特になし。

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別紙４

研究成果の刊行に関する一覧表

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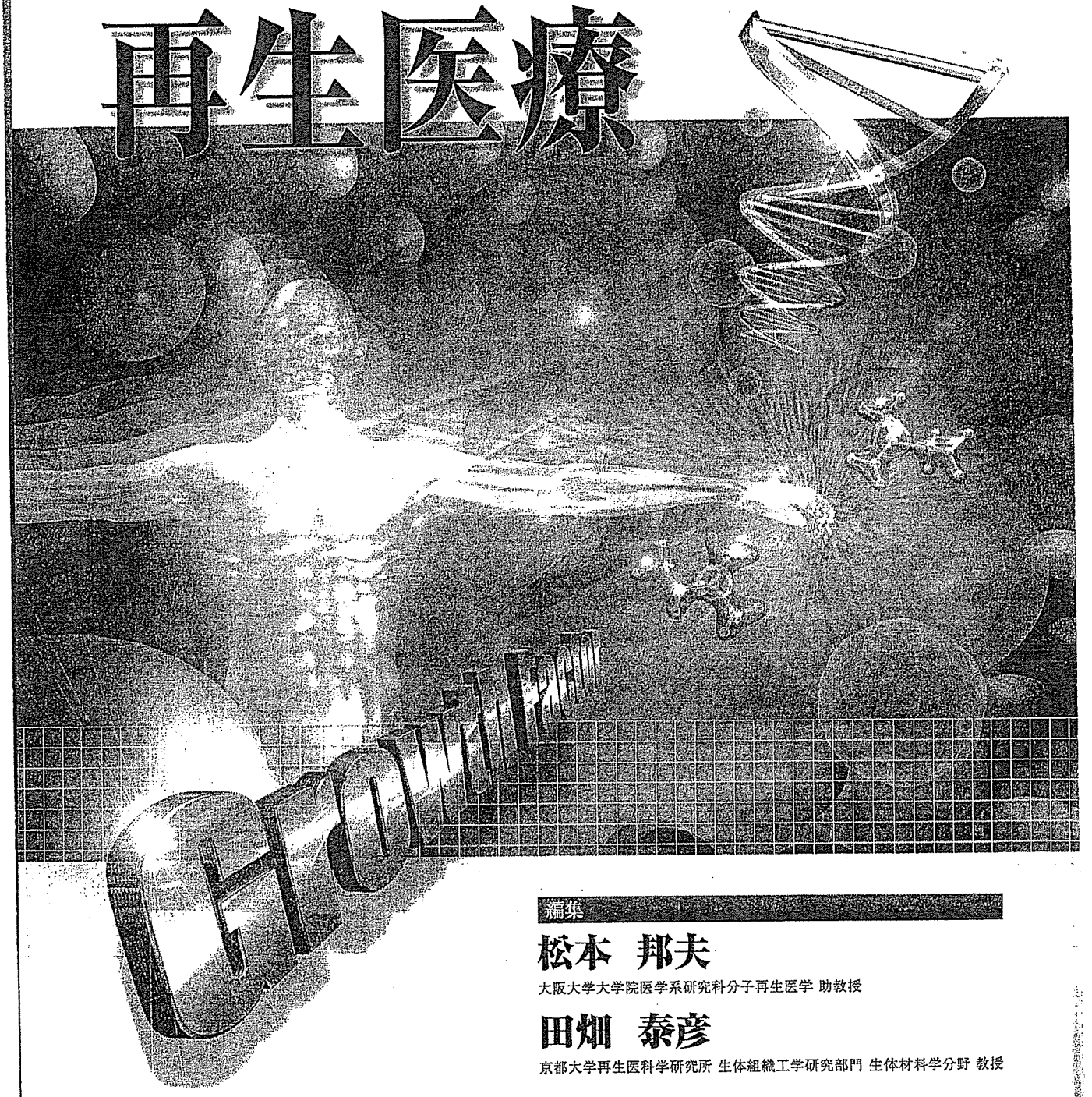
細胞増殖因子と再生医療

GROWTH FACTOR & REGENERATIVE MEDICINE



編集

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8. VEGF/VEGF-E

末梢動脈疾患

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I 末梢動脈疾患とは

末梢動脈疾患（peripheral artery disease：PAD）とは，末梢動脈の狭窄や閉塞によって，四肢をはじめとする末梢組織に虚血をきたすような疾患を指す。末梢動脈疾患には，閉塞性動脈硬化症，閉塞性血栓血管炎（Buerger病），膠原病に伴う血管炎などが含まれるが，その中でも最も多いのは閉塞性動脈硬化症である。閉塞性動脈硬化症では，末梢動脈の粥状動脈硬化により血管内腔の狭窄が進行し下肢に虚血が生じる。これに伴い，しびれ，冷感，間歇性跛行（後述），疼痛，潰瘍，壊疽などのさまざまな症状が出現する。欧米における罹患率は人口の数%程度とされているが，わが国における確立された疫学データは残念ながら存在しない。自覚症状による病期分類としてFontaine分類が代表的である（表）。Fontaine Ⅰ度の軽症患者に対しては，禁煙指導や糖尿病・高血圧など動脈硬化の危険因子をコントロールしながら経過観察するのが通常である。病状が進行してくると，Fontaine Ⅱ度にみられるような間歇性跛行が出現する。間歇性跛行とは，一定距離の歩行後に下肢の疼痛が出現するが，休息により痛みは消失し，再び歩行可能

表　Fontaine分類

グレード	症　状
Ⅰ	なし（しびれ，冷感）
Ⅱ	間歇性跛行
Ⅲ	安静時疼痛
Ⅳ	皮膚潰瘍，壊疽

となるような状態を指す。末梢動脈疾患の症状で最も多いのは，この間歇性跛行である。間歇性跛行が軽度の場合，運動療法や抗血小板剤などによる薬物療法を行うが，重症例では狭窄した血管をカテーテルによって拡張する経皮的血管形成術（percutaneous transluminal angioplasty：PTA）や外科手術（バイパス手術）が必要となる。Fontaine Ⅲ～Ⅳ度を重症下肢虚血（critical limb ischemia：CLI）と呼ぶが，このような状態にまで進行すると，安静時にも下肢疼痛が出現し，皮膚の潰瘍や壊疽もみられるようになる。重症下肢虚血を呈する患者では，痛みや壊疽のために運動療法を施行するのは困難で，薬物治療も無効のことが少なくない。また，重症下肢虚血をきたすような血管は動脈硬化性変化が強く，血管形成術やバイパス手術もしばしば困難である。このような重症例に対する治療法として考えられたのが血管新生療法（therapeutic angiogenesis）[1]である。

Ⅱ 血管新生療法とは

血管新生療法は，血管増殖因子やその遺伝子あるいは骨髄や末梢血細胞を用いて血管新生を促進させ，組織虚血の改善を図る治療法で，1994年，米国のIsnerらにより初めて臨床応用された[2]。Isnerらが行ったのは，vascular endothelial growth factor（VEGF）[3]遺伝子を用いた血管新生療法であり，循環器領域における初の遺伝子治療としても知られている。以後，今日までに10年以上が経過し，遺伝子以外にも増殖因子蛋白，骨髄細胞，末梢血細胞などを用いたさまざ

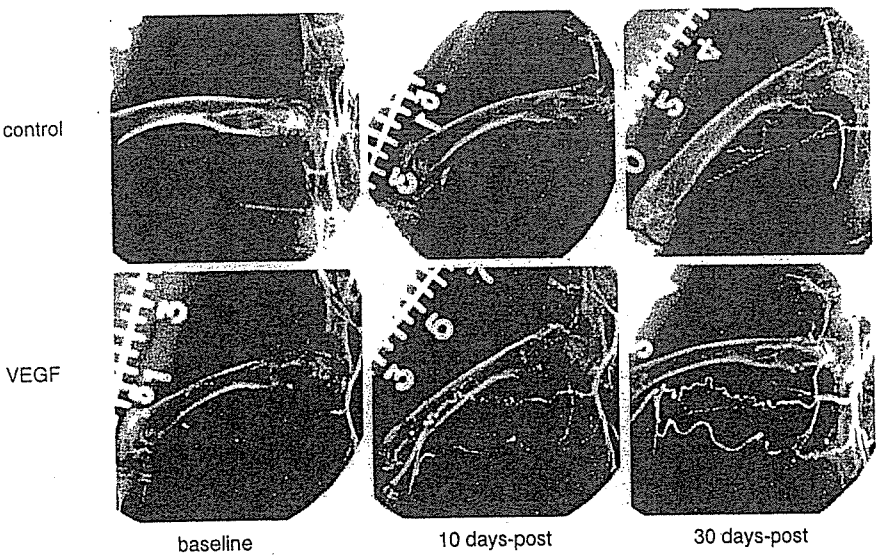

図1　VEGF蛋白投与後の血管造影所見
生理食塩水または組み換えVEGF蛋白を家兎虚血肢モデルの内腸骨動脈内へ選択的に投与し，側副路の発達を比較した。上段の対照群では，治療後30日間において側副血行路に大きな変化は認められない。これに対し，下段のVEGF治療群においては治療から10日間で側副路の著明な改善が認められる。
（文献1より引用）

まな血管新生療法が開発されてきた。各々の治療法の有効性が臨床試験により明らかにされつつある中，その対象疾患も，末梢動脈疾患から虚血性心疾患や虚血性脳疾患などへと拡大されてきている。

Ⅲ 血管新生療法の臨床応用まで

血管新生療法のコンセプトは決して新しいものではない。1980年代後半には，ネコの虚血肢モデルに対して大網の脂肪分画を投与し，虚血を改善させる試みが行われている。大網や脂肪細胞の再生医療への応用は最近のトピックであり，このような研究がすでに20年以上前に存在したことは興味に値する。これらの血管新生療法とIsnerらが行ったそれとの違いは，後者がVEGFという血管内皮細胞に特異的な増殖因子を用いた点にある。1990年代初頭，Isnerらは家兎の虚血肢モデルにVEGF蛋白を投与することにより下肢の側副血行発達を促進できないか検討を行った（図1）[1]。VEGF蛋白の動脈投与，静脈投与，繰り返し静脈投与，ヘパリン併用などのさまざまな投与法が検討されたが，投与法の如何にかかわらず，側副血行の促進には100～1,000 μgのVEGF蛋白が必要なことが明らかとなった。しかしながら，大量のVEGF蛋白を投与すると，投与した蛋白が全身を循環し，非目的部位へと到達するのは避け難い。血管増殖因子の全身への拡散は，糖尿病患者においては網膜症を悪化させ，癌患者では腫瘍血管の発達を促進させ得る。また，一部の血管増殖因子は一酸化窒素（NO）を介した血管拡張作用を有しており，遷延性低血圧を惹起させ得る。事実，VEGF蛋白を用いた血管新生療法の臨床試験では，低血圧を避けるためにその投与量が制限された。

大量の蛋白投与に伴う副作用を回避するために行き着いた結論が，遺伝子を用いたローカルドラッグデリバリーであった。Isnerらはカテーテルを用いてVEGF遺伝子を経皮的に血管細胞へと導入し，それらの細胞からVEGF蛋白を分泌させることに成功した。ここでは，表面が親水性ゲルでコーティングされた冠動脈形成術用バルーンカテーテル（ハイドロゲル・バルーンカテーテル）を用いて下肢血管への遺伝子導入が行われた。ハイドロゲルは，狭窄部位におけるバルーンの通過性を改善するために施されたコーティングであるが，Isnerらはこのゲルにプラスミド DNAの水溶液をしみ込ませ，遺伝子キャリアとして使用したのである。通常のPTAテクニックを用いてバルーンを目的部位へと進め，4～8気圧で1分間バルーンを拡張させることで遺伝子を血管壁へと導入する。その遺伝子導入効率はリポゾームによる遺伝子導入に比し100倍以上の高効率ではあったが，βガラクトシダーゼ遺伝子を用いた組織所見の検討では，導入部位のわずか0.1%以下の細胞にしか遺伝子発現が認められなかった[4]。このわずかな細胞によって血管新生を促進することが可能なのか疑問なわけだが，遺伝子の導入効率（transfection efficiency）と治療効率（therapeutic efficiency）とは同義ではない。遺伝子産物である増殖因子が細胞外へと分泌されれば，たとえ導入効率は低くとも，パラクリン効果が期待できる[5]。この仮説は動物実験によって検証された。すなわち，ハイドロゲル・バルーンカテーテルを用いて家兎虚血肢モデル

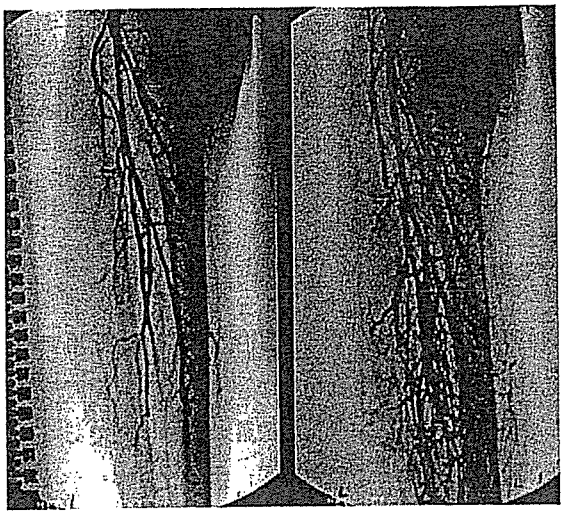

図2　遺伝子治療前後におけるDSA（digital subtraction angiography）所見
左：遺伝子治療前，右：遺伝子治療1ヵ月後
VEGFによる遺伝子治療の1ヵ月後，下肢側副血行の著明な発達を認める。
（文献2より引用）

にVEGF遺伝子の導入を行うと，約3週間にわたりその発現が認められ，VEGF蛋白の動脈内投与と同等以上の側副路発達効果が得られたのである。一方，末梢血中のVEGF蛋白の濃度はELISAによる測定限界付近にあり極めて低値であった。つまり，遺伝子の導入効率は低くとも，局所では治療効果を得るに十分な組織濃度が維持され，逆に血中濃度は希釈効果によって低く抑えられるわけである。ここで忘れてならないのは，本法がプラスミドDNA以外には何のベクターも用いない遺伝子導入法であった点である（naked DNAアプローチ）。この研究によって，臨床応用における本法の高い安全性が裏づけられた。

Ⅳ　VEGFを用いた血管新生療法の臨床応用

1994年，Isnerらは血管新生療法の臨床試験を開始した[2]。前述のように，この試験は循環器領域における初の遺伝子治療としても知られており，内科治療や外科治療が無効な重症末梢動脈疾患患者を対象に行われた。遺伝子治療から1～2ヵ月で，血管造影上，新生血管の出現が認められ，これに伴い下肢疼痛や難治性潰瘍が消失した（図2）。副作用は下腿浮腫や良性血管腫など，一過性の軽微なものだけであった。しかしながら，バルーンカテーテルを用いた遺伝子導入は，動脈穿刺が不可能な例，動脈硬化が高度でカテーテルの標的血管へのアクセスが困難な例，遺伝子導入に際し解離などの血管損傷リスクが高い例には施行できない。そこで考案されたのが，虚血筋への遺伝子導入である。Baumgartnerらは，VEGFプラスミドの虚血下肢への筋注を行い，7～8割の症例において血管造影上の側副路発達や臨床症状改善を得ることに成功した[6]。筋注

法の導入は，遺伝子治療の手技を単純化させるだけでなく，それまでカテーテルのアクセスが困難であった症例さえも治療可能とし，その適応症例を大きく拡大させることにつながった。また，筋注法は，心筋への遺伝子導入にも応用可能であり，虚血性心疾患に対する血管新生療法の臨床応用への契機ともなった。

Ⅴ 血管新生療法の問題点

末梢動脈疾患に対する血管新生療法は，今から約10年前，VEGFを用いた遺伝子治療として幕を開けた。重症下肢虚血に対する本法の治療成績は良好である。安静時疼痛や難治性潰瘍を有する患者の少なくとも6～7割において，臨床所見の改善が期待可能である。しかしながら，本法のメカニズムに関しては不明な点が少なくない。臨床症状の改善にもかかわらず血管造影での改善が明らかでないことも多く，はたして血管新生療法によって血管新生が本当に促進されるのか，その治療メカニズムの基本的な部分でさえ，解明されていないのが実情である。また，遺伝子のパテント問題，遺伝子を用いることの倫理的問題など，一般臨床の場に普及するに至るまでに解決されるべき問題も決して少なくない。

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Original article

Transplantation of mesenchymal stem cells attenuates myocardial injury and dysfunction in a rat model of acute myocarditis

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Abstract

Acute myocarditis is a non-ischemic inflammatory disease of the myocardium for which there is currently no specific treatment. We have previously shown that mesenchymal stem cells (MSC) can ameliorate heart injury during acute ischemia and in dilated cardiomyopathy; however, the therapeutic potential in acute myocarditis is unclear. In this study, we investigated the ability of MSC to attenuate myocardial injury and dysfunction during the acute phase of experimental myocarditis. Ten-week-old male Lewis rats were injected with porcine myosin to induce myocarditis. Cultured MSC (3×10^6 cells/rat) were injected intravenously 7 days after myosin injection. At 3 weeks, myosin injection resulted in severe inflammation and significant deterioration of cardiac function. MSC transplantation attenuated increases in CD68-positive inflammatory cells and monocyte chemoattractant protein-1 (MCP-1) expression in myocardium, and improved cardiac function in this model. Furthermore, myocardial capillary density was higher in myocarditis tissue, and was further increased by MSC transplantation. *In vitro*, cultured adult rat cardiomyocytes were injured in response to MCP-1, whereas this effect was attenuated by MSC-derived conditioned medium, suggesting cardioprotective effects of MSC acting in a paracrine manner. MSC transplantation attenuated myocardial injury and dysfunction in a rat model of acute myocarditis, at least in part through paracrine effects of MSC.

Keywords: Acute myocarditis; Mesenchymal stem cell; Paracrine effect; Cytokine; Cell death

1. Introduction

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

Mesenchymal stem cells (MSC) are multipotent stem cells present in adult tissues, and have the ability to differentiate into a variety of lineages, including vascular smooth muscle cells, endothelial cells and cardiomyocytes [3,4]. We have previously reported that bone marrow-derived MSC engrafted in experimental myocardial infarction expressed both cardiac and endothelial phenotypes in the heart, and further increased capillary density and decreased the infarct size [5]. Moreover, we have recently demonstrated that monolayered MSC derived from adipose tissue reversed wall thinning in the scar area and improved cardiac function in rats with myocardial infarction [6]. The cardioprotective effects of MSC are known to be mediated

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not only by their differentiation into vascular cells and cardiomyocytes, but also by their ability to supply large amounts of angiogenic, anti-apoptotic and mitogenic factors [5–7]. These findings suggest the therapeutic potential of MSC for heart failure. However, whether intravenously transplanted MSC attenuate myocardial inflammation and cardiac dysfunction in acute myocarditis remains unknown.

In the present study, we used porcine myosin-induced acute myocarditis in Lewis rats. This model closely resembles human giant cell myocarditis, a frequently fatal disorder characterized by multinucleated giant cells in the myocardium [8]. To examine the therapeutic potential of MSC in the acute phase of myocarditis, MSC were intravenously injected into rats 7 days after myosin injection.

Thus, the purposes of this study were 1) to investigate whether intravenous transplantation of MSC improves cardiac function and pathological findings including myocardial inflammation in rats with myosin-induced myocarditis, and 2) to investigate the underlying mechanisms responsible for the effects of MSC.

2. Materials and methods

2.1. Animals

Ten-week-old male Lewis rats (Japan SLC, Hamamatsu, Japan) were used in all experiments, and were maintained in our animal facilities. The experimental protocols were approved by The Animal Care Committee of the National Cardiovascular Center.

2.2. Preparation of cardiac myosin

Purified cardiac myosin from the ventricular muscle of pig hearts was prepared according to a procedure described previously [8]. The antigen was dissolved at a concentration of 20 mg/ml in phosphate-buffered saline (PBS) containing 0.3 M KCl, mixed with an equal volume of complete Freund's adjuvant containing 11 mg/ml *Mycobacterium tuberculosis* (Difco Laboratories, Sparks, MD, USA). 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 the each footpad.

2.3. Acute myocarditis model

Forty-five rats were randomly divided into three groups and received the following treatment: 1) 0.2 ml saline and sham surgery (Sham group, $n=15$), 2) 0.2 ml cardiac myosin antigen and sham surgery (MyoC group, $n=15$), and 3) 0.2 ml cardiac myosin followed by MSC transplantation 7 days post-myosin injection (MyoC+MSC group, $n=15$). Rats were weighed and observed daily for signs of morbidity and for death.

2.4. Preparation and transplantation of bone marrow-derived MSC

MSC were prepared as described previously [5]. Briefly, bone marrow cells were isolated by flushing out the femoral and tibial cavities with PBS, and plated onto 10-cm dishes in complete culture medium: Dulbecco's Modified Eagle's Medium (DMEM), 15% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Five days after plating, non-adherent cells were removed, and adherent cells were further propagated for 4 to 5 passages.

Seven days after myosin injection, MSC (3×10^6 cells) or vehicle (0.9% saline) was intravenously administered via the jugular vein. Sham rats also received saline administration but without myosin injection.

2.5. Hemodynamic studies

Hemodynamic studies were performed on day 21 post-myosin injection. Anesthesia was maintained with an intraperitoneal injection of 20 mg/kg sodium pentobarbital, and a 1.5 Fr micromanometer-tipped catheter was placed in the left ventricle through the right carotid artery (Millar Instruments, Houston, TX, USA). Heart rate (HR) was also monitored by electrocardiography. HR, mean arterial pressure (MAP), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), maximum dP/dt (Max dP/dt) and minimum dP/dt (Min dP/dt) were used as indices of hemodynamics, and recorded simultaneously during ventilation after a minimum equilibration period of 20 min.

2.6. Echocardiographic studies

Echocardiography was performed on day 21 post-myosin injection. Rats were anesthetized with an intraperitoneal injection of 20 mg/kg sodium pentobarbital. A 12 MHz probe was placed at the left 4th intercostal space for M-mode imaging using 2D echocardiography (Sonos 5500, Philips, Bothell, WA, USA). Left ventricular systolic dimension (LVDs), left ventricular diastolic dimension (LVDd), anterior wall thickness (AWT), posterior wall thickness (PWT) and ejection fraction (EF) were measured, and taken as an average of three beats. Fractional shortening (%FS) was calculated as $(\mathrm{LVDd}-\mathrm{LVDs})/\mathrm{LVDd}\times100$.

2.7. Histological examination

The heart was excised above the origin of the great vessels, and heart weight and body weight were recorded on day 21 post-myosin injection. Portions of the midventricular heart, spleen, pancreas, kidney and liver were fixed with 4% paraformaldehyde, embedded in paraffin, sectioned at 4-μm thickness, stained with either hematoxylin and eosin (H & E) or Masson's trichrome, and subjected to immunohistochemical staining. H & E-stained sections were evaluated by a cardiovascular pathologist (H.I.-U.) for the characterization of myocardial injury and inflammation without knowledge of the experimental groups, on 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.

2.8. Immunohistochemical study

Paraffin-embedded heart sections were washed in increasing concentrations of ethanol and then with PBS. Sections were incubated with Protein Block (DakoCytomation, Glostrup, Denmark), then with mouse anti-rat von Willebrand Factor (vWF) (DakoCytomation), CD68 (DakoCytomation) or monocyte chemoattractant protein-1 (MCP-1) (BD Biosciences Pharmingen, San Jose, CA, USA) antibody in diluent for 40 min, followed by incubation with horseradish peroxidase (HRP)-linked rabbit anti-mouse IgG (DakoCytomation) for 30 min. Sections were visualized using 0.5% diaminobenzidine and 0.03% hydrogen peroxide, and counterstained with hematoxylin. The numbers of CD68-stained cells and vWF-stained capillaries were determined in 10 randomly selected fields (×200).

2.9. Enzyme-linked immunosorbent assay (ELISA)

Serum MCP-1 level of rats on day 21 post-myosin injection was measured using a Rat MCP-1 ELISA kit (Biosource International, Camarillo, CA, USA). Vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) levels in the supernatant of MSC culture (2.3×10^5 cells in 10-cm dish cultured for 48 h) were measured using ELISA kits, according to the manufacturers' protocols (HGF, Institute of Immunology, Tokyo, Japan; VEGF, R&D Systems, Minneapolis, MN, USA).

2.10. Isolation of cardiomyocytes

Ventricular cardiomyocytes were obtained as described previously with modification [9]. Briefly, after heparinization by intraperitoneal injection of 1000 U/kg heparin sodium, the heart was rapidly excised, and pulmonary, connective and other noncardiac tissues were removed. The heart was then mounted on the cannula of a modified Langendorff apparatus and perfused with buffer containing 0.75 mg/ml collagenase type I (Worthington, Lakewood, NJ, USA), 0.5 mg/ml hyaluronidase (Sigma) and 1% bovine serum albumin (fraction V, ICN, Aurora, OH, USA), in a recirculating fashion for 3 h. After the perfusion sequence, the heart was removed from the perfusion apparatus, the atrium was removed, and gently minced. The enzyme-containing buffer was harvested and the cardiomyocytes resuspended in fresh buffer. The calcium concentration in the suspension was raised stepwise to 1.2 mM. Quiescent, calcium-tolerant cardiomyocytes were gravitationally separated from any nonventricular cells and resuspended in complete culture medium. The culture medium was exchanged for fresh medium to remove the damaged myocytes that failed to attach 3 h after plating. After this procedure, 80% to 90% myocytes were viable and showed rod-shape.

2.11. Cardiomyocyte stimulation and MTS assay

To assess cardioprotective effects of MSC acting in a paracrine manner, we investigated whether conditioned medium obtained from MSC culture attenuated MCP-1-induced cardiomyocyte injury. Cardiomyocytes were plated on 96-well plates (1×10^3 viable cells/well) precoated with laminin (BD Biosciences Pharmingen). After 3 h, the medium was changed to fresh DMEM containing 15% FBS or conditioned medium obtained from MSC culture, with or without 50 ng/ml MCP-1 (R&D Systems, Minneapolis, MN, USA). After 24 h, the cellular level of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), indicative of the mitochondrial function in living cells and cell viability, was measured ($n=6$) with a CellTiter96 AQueous One Kit (Promega, Madison, WI, USA) and a Microplate Reader (490 nm, Bio-Rad, Hercules, CA, USA).

2.12. In vitro apoptosis assay

Terminal dUTP nick end labeling (TUNEL) assay (ApopTag Fluorescein In Situ Apoptosis Detection Kit, Chemicon International, Temecula, CA, USA) was performed to evaluate apoptosis of cultured cardiomyocytes. After incubation for 24 h, cardiomyocytes were fixed in 1% paraformaldehyde, and TUNEL staining was performed for detection of apoptotic nuclei according to the manufacturer's

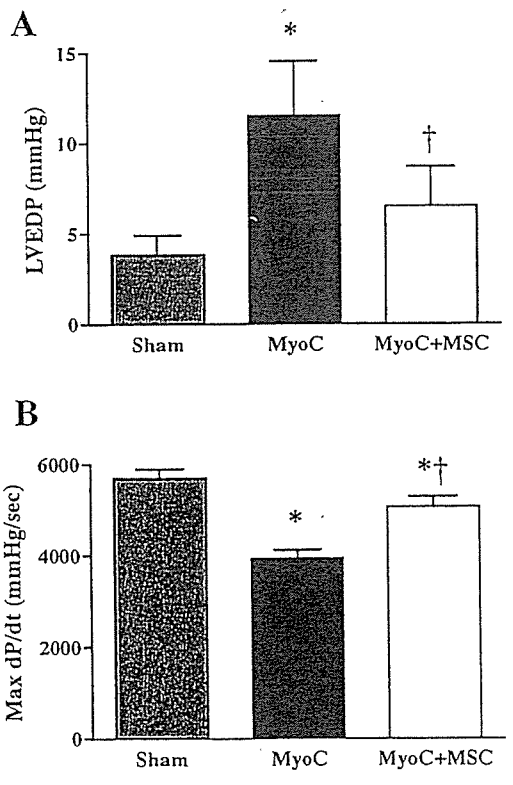

Fig. 1. Effects of MSC transplantation on hemodynamic parameters in acute myocarditis. (A) Left ventricular end-diastolic pressure (LVEDP) and (B) maximum dP/dt (Max dP/dt) were measured in sham-operated rats given vehicle (Sham group), myosin-treated rats given vehicle (MyoC group), and myosin-treated rats given MSC (MyoC+MSC group). Values are mean±S.E. *$P<0.05$ *vs* Sham, †$P<0.05$ *vs* MyoC group.

Table 1
Physiological parameters in three experimental groups

	Sham	MyoC	MyoC+MSC
HW/BW (g/kg)	2.9±0.3	6.4±0.3*	4.7±0.3*,†
HR (bpm)	446±11	363±14*	442±12*,†
MAP (mm Hg)	108±3	87±3*	108±4†
LVSP (mm Hg)	130±2	105±4*	125±4†
Min d*P*/d*t* (mm Hg/s)	−5440±199	−3097±183*	−4617±171*,†

Sham, sham-operated rats given vehicle; MyoC, myosin-treated rats given vehicle; MyoC+MSC, myosin-treated rats given MSC (3×10^6 cells); HW/BW, heart weight to body weight ratio; HR, heart rate; MAP, mean arterial pressure; LVSP, left ventricular systolic pressure; Mim d*P*/d*t*, minimum d*P*/d*t*. Data are mean±S.E. *$P<0.05$ *vs* Sham, †$P<0.05$ *vs* MyoC group.

instructions. The cells were then mounted in medium containing DAPI. Randomly selected microscopic fields ($n=5$) were evaluated to calculate the ratio of TUNEL-positive cells to total cells.

Table 2
Echocardiographic findings in three experimental groups

	Sham	MyoC	MyoC+MSC
LVDs (mm)	3.1±0.1	5.0±0.4*	3.8±0.2†
EF (%)	74.9±1.2	56.6±3.4*	71.2±3.5†
AWT diastole (mm)	1.9±0.1	3.0±0.2*	3.0±0.3*
PWT diastole (mm)	1.9±0.1	3.4±0.1*	2.7±0.2*,†

Sham, sham-operated rats given vehicle; MyoC, myosin-treated rats given vehicle; MyoC+MSC, myosin-treated rats given MSC (3×10^6 cells); LVDs, left ventricular systolic dimension; EF, ejection fraction; AWT, anterior wall thickness; PWT, posterior wall thickness. Data are mean±S.E. *$P<0.05$ *vs* Sham, †$P<0.05$ *vs* MyoC group.

2.13. Creatine kinase (CK) activity assay

CK activity in culture media was measured after incubation of cardiomyocytes for 24 h ($n=5$), using the enzyme measurement kit (Kanto Chemical, Tokyo, Japan).

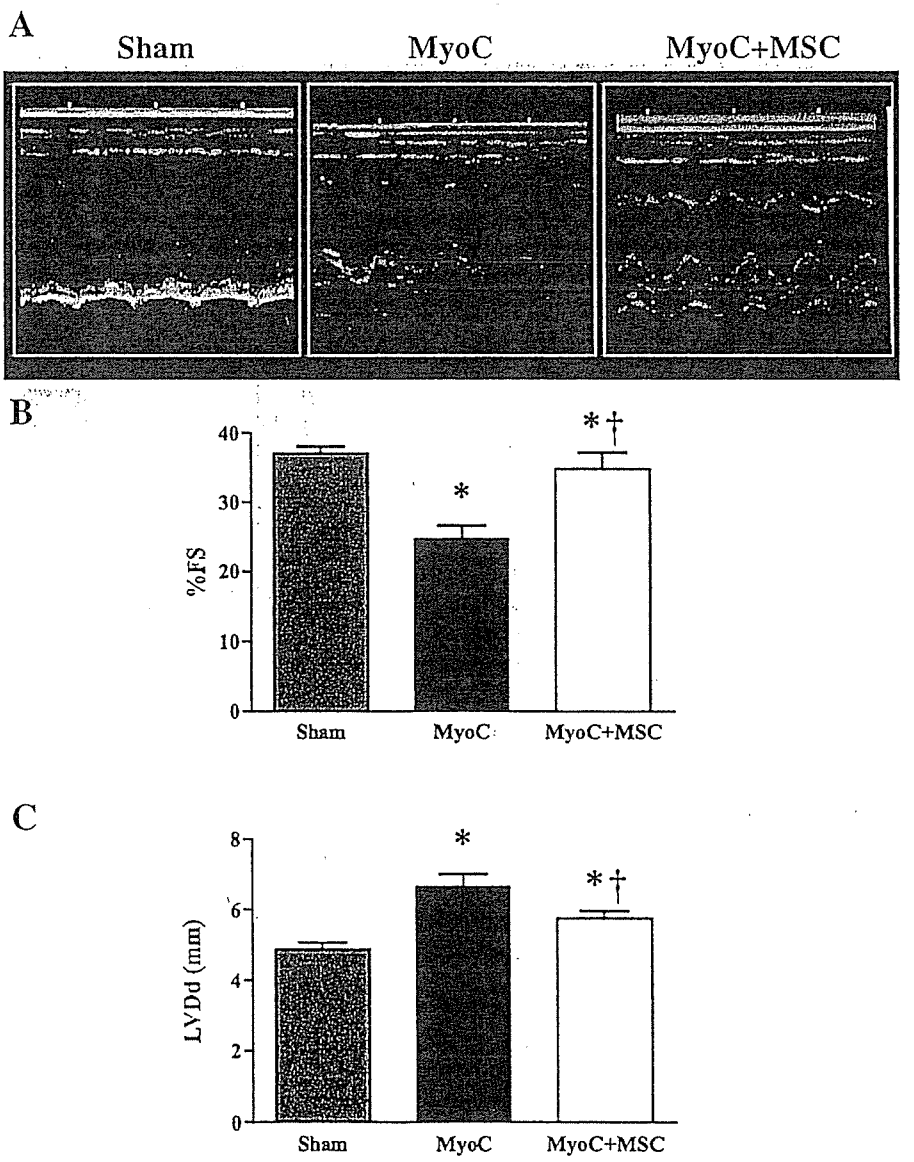

Fig. 2. Effects of MSC transplantation on echocardiographic parameters in acute myocarditis. (A) Representative echocardiographic images showing wall thickening and poor movement in the MyoC group, and improvement of cardiac contractility in the MyoC+MSC group. (B and C) MSC transplantation significantly improved fractional shortening (%FS) and left ventricular diastolic dimension (LVDd). Values are mean±S.E. *$P<0.05$ *vs* Sham, †$P<0.05$ *vs* MyoC group.

2.14. Statistical analysis

Data were expressed as mean ± standard error (S.E.). Comparisons of parameters among groups were made by one-way ANOVA, followed by Newman–Keuls' test. Differences were considered significant at $P<0.05$.

3. Results

3.1. Improvement in cardiac function by MSC transplantation

Two of 15 rats in the MyoC group died on day 19 and day 21 post-myosin injection, respectively, whereas the MyoC+MSC group had no mortality. At 3 weeks post-myosin injection, the MyoC group showed increased heart weight/body weight ratio (HW/BW) and LVEDP, and decreased MAP and Max d*P*/d*t* compared with the Sham group, indicating the presence of acute heart failure in this model (Fig. 1 and Table 1). These parameters subsequently returned to baseline with MSC transplantation (MyoC+MSC group). On echocardiography, the MyoC group showed an increase in LVDs and LVDd, and a significant reduction in %FS and EF (Fig. 2 and Table 2). MSC transplantation significantly improved these parameters (MyoC+MSC group).

3.2. Attenuation of myocardial inflammation by MSC transplantation

Myocardial necrosis and tissue granulation as well as giant cell infiltration and edema were markedly increased in our model of acute myocarditis (Fig. 3A). MSC transplantation significantly attenuated these changes observed in the MyoC group. MSC-transplanted hearts exhibited a consistent tendency for a reduction of tissue granulation, inflammation and edema, on blinded histological grading by a cardiovascular pathologist (H.I-U.), as compared to the MyoC group (Fig. 3B). Hearts showed limited fibrosis in the MyoC group, and this observation was not significantly attenuated by MSC

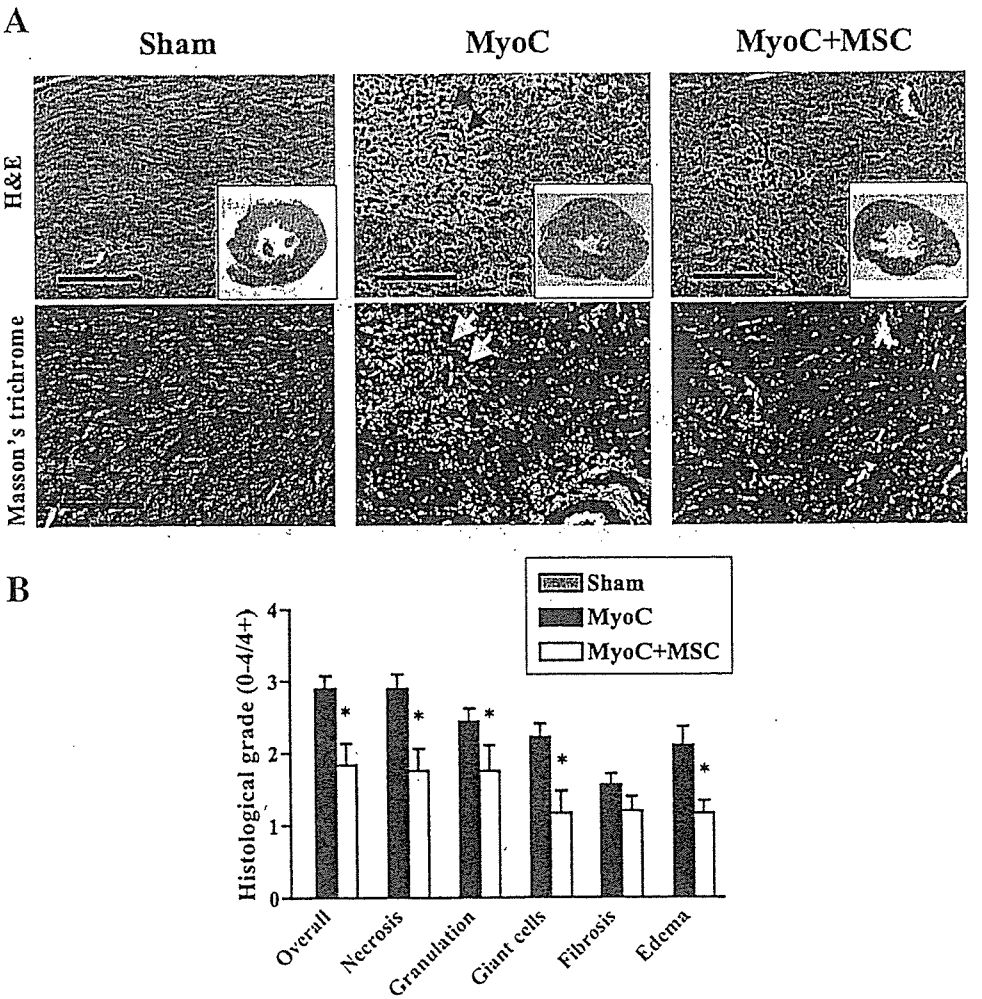

Fig. 3. Effects of MSC transplantation on pathological changes in acute myocarditis. (A) Representative myocardial sections show markedly decreased inflammation and tissue necrosis (H & E) and a comparable degree of early fibrosis (Masson's trichrome) after MSC transplantation (MyoC+MSC) as compared to control (MyoC, arrows). Insets are transverse sections of myocardium. Scale bars: 50 μm. (B) Semi-quantitative histological grades for necrosis and tissue granulation as well as for infiltration of giant cells and edema were significantly lower after MSC transplantation (MyoC+MSC) compared to control (MyoC). Sham tissues exhibited no measurable pathological change. Values are mean ± S.E. *$P<0.05$ *vs* Sham, †$P<0.05$ *vs* MyoC group.

transplantation, possibly because of the acute nature of this experiment (Fig. 3B).

Notably, marked histiocytic infiltration was demonstrated by CD68-positive cells, including multinucleated giant cells, in myocarditis (MyoC group), and this was significantly attenuated by MSC transplantation (Figs. 4A and B). In myocarditis, there was an increase in MCP-1 expression localized to the vascular endothelium and also in cardiomyocytes surrounding areas of inflammation (Fig. 5A). The hearts in the MyoC+MSC group showed a partial decrease in MCP-1 expression. Serum MCP-1 level was greatly increased in the MyoC group, whereas the increase was significantly attenuated in the MyoC+MSC group (Fig. 5B).

3.3. Effect of MSC on angiogenesis

To investigate the angiogenic effect of MSC transplantation in the myocardium, immunohistochemical analysis of vWF was performed. Capillary density was increased in the MyoC group (Figs. 6A and B). Notably, in MSC-transplanted tissues, capillary density was increased compared to that in the MyoC group. The clustering of relatively small vessels seen in MSC-transplanted hearts was indicative of recent neovascularization.

3.4. Cardioprotective effects of MSC in paracrine manner

Because MSC transplantation had anti-inflammatory and tissue-protective effects and induced angiogenesis, some paracrine effects were expected. To confirm the paracrine effects of MSC *in vitro*, cardiomycytes were isolated from adult rats, and cultured with MCP-1 in the standard medium or in the conditioned medium obtained from MSC culture. The standard medium containing MCP-1 resulted in a decrease in viable cardiomyocytes; however, MSC-derived conditioned medium containing MCP-1 attenuated the decrease in viable cardiomyocytes (Fig. 7A). TUNEL staining showed that the standard medium containing MCP-1 markedly induced apoptosis of cardiomyocytes (Figs. 7B and C). However, the conditioned medium of MSC significantly attenuated MCP-1-induced cardiomyocyte apoptosis. In addition, CK activity in standard medium containing MCP-1 was significantly increased, whereas the conditioned medium markedly attenuated the CK activity induced by MCP-1 (Fig. 7D).

To investigate whether MSC secreted angiogenic and anti-fibrotic factors, VEGF and HGF levels in MSC culture were measured by ELISA assay. MSC secreted large amounts of VEGF and HGF compared to standard medium, respectively (Fig. 7E).

4. Discussion

In this study, we focused on the therapeutic potential of MSC transplantation in the acute phase of myocarditis. We showed that 1) MSC transplantation 1 week after myosin injection improved cardiac function and attenuated pathological findings including myocardial inflammation, and that 2)

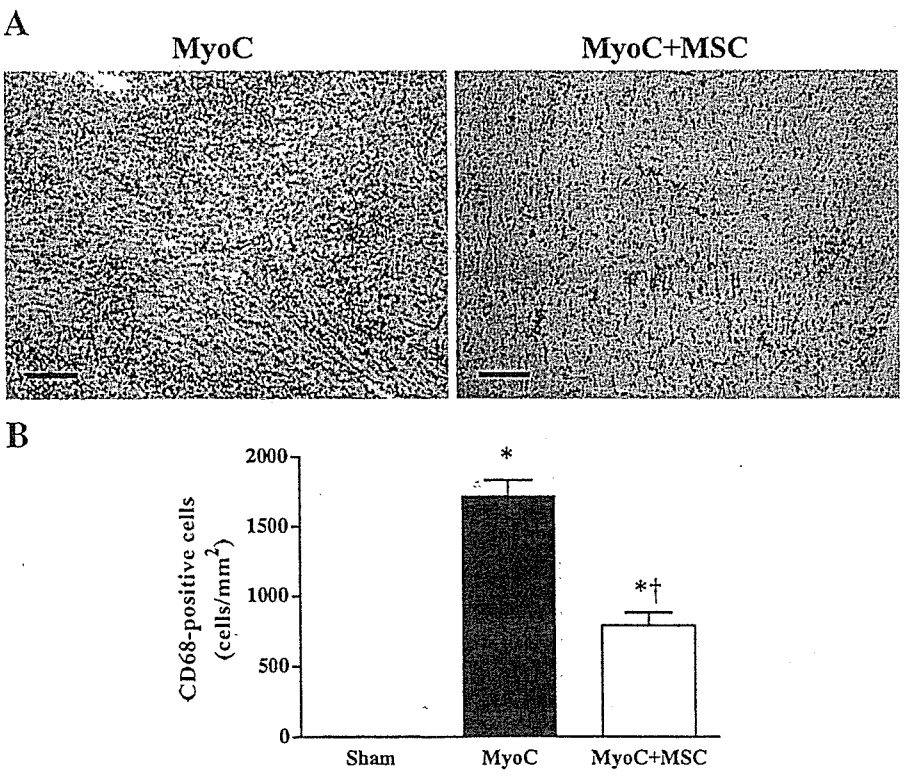

Fig. 4. Effects of MSC transplantation on myocardial CD68 expression in acute myocarditis. (A) Representative myocardial sections immunohistochemically stained for CD68 demonstrate a marked decrease in CD68-positive cells, including giant cells, after MSC transplantation (MyoC+MSC) as compared to control (MyoC). Scale bars: 100 μm. (B) Semi-quantitative counts of CD68-positive cells demonstrate a significant reduction in the MyoC+MSC group. Values are mean±S.E. $^{*}P<0.05$ *vs* Sham, $^{\dagger}P<0.05$ *vs* MyoC group.

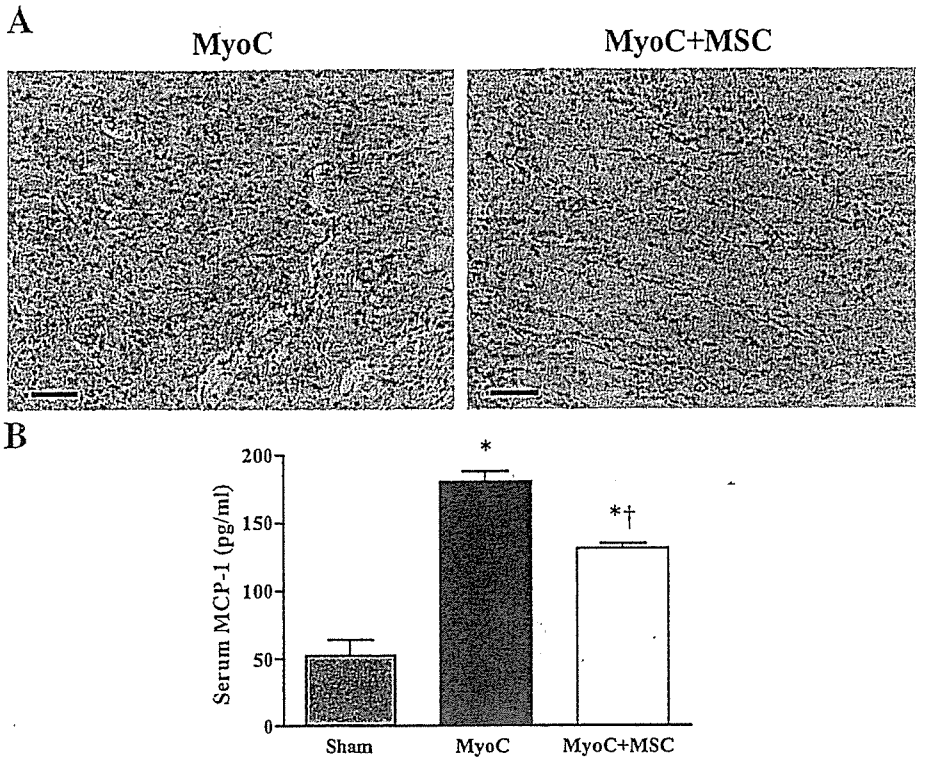

Fig. 5. Effects of MSC transplantation on myocardial MCP-1 expression and serum MCP-1 level. (A) Representative MCP-1-stained myocardial sections from MyoC and MyoC+MSC groups. Scale bars: 50 μm. (B) Serum level of MCP-1 measured by ELISA. Values are mean±S.E. *$P<0.05$ *vs* Sham, †$P<0.05$ *vs* MyoC group.

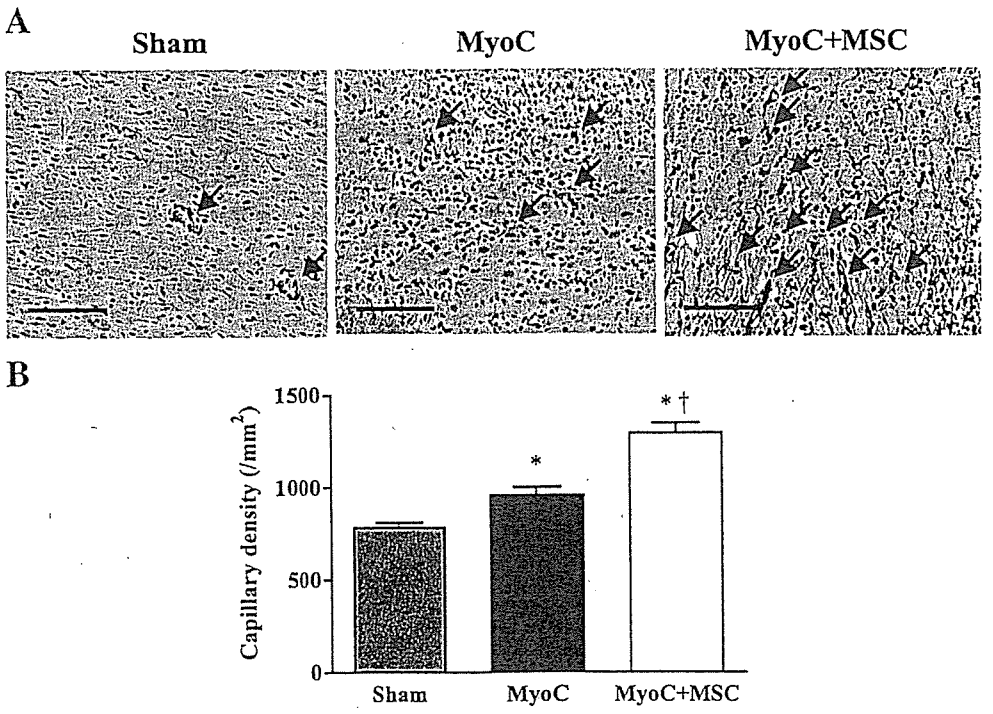

Fig. 6. Effects of MSC on neovascularization. (A) Representative myocardial sections immunohistochemically stained for vWF showing increased microvasculature (arrows) in control hearts (MyoC), which was more marked after MSC transplantation (MyoC+MSC). Scale bars: 50 μm. (B) Capillary density measured in 10 random representative high-power fields showing a significant increase in control (MyoC) and a further increase after MSC transplantation (MyoC+MSC) over the Sham group. Values are mean±S.E. *$P<0.05$ *vs* Sham, †$P<0.05$ *vs* MyoC group.

MSC had cardioprotective effects acting in a paracrine manner.

The rat model of myosin-induced experimental myocarditis provides a model that resembles human giant cell myocarditis [8,10]. Although the majority of acute myocarditis is linked to a viral infection such as coxsackievirus B3, this viral infection can in some cases cause an autoimmune myocarditis with chronic myocardial inflammation without viral persistence, due to the exposure of cardiac autoantigens to the immune system [11,12]. This myocarditis model is triphasic, consisting of an antigen priming phase from days 0–14, an autoimmune response phase from days 14–21, and a reparative phase thereafter, associated chronically with a dilated cardiomyopathy phenotype [13]. In our previous study, MSC were transplanted at the reparative

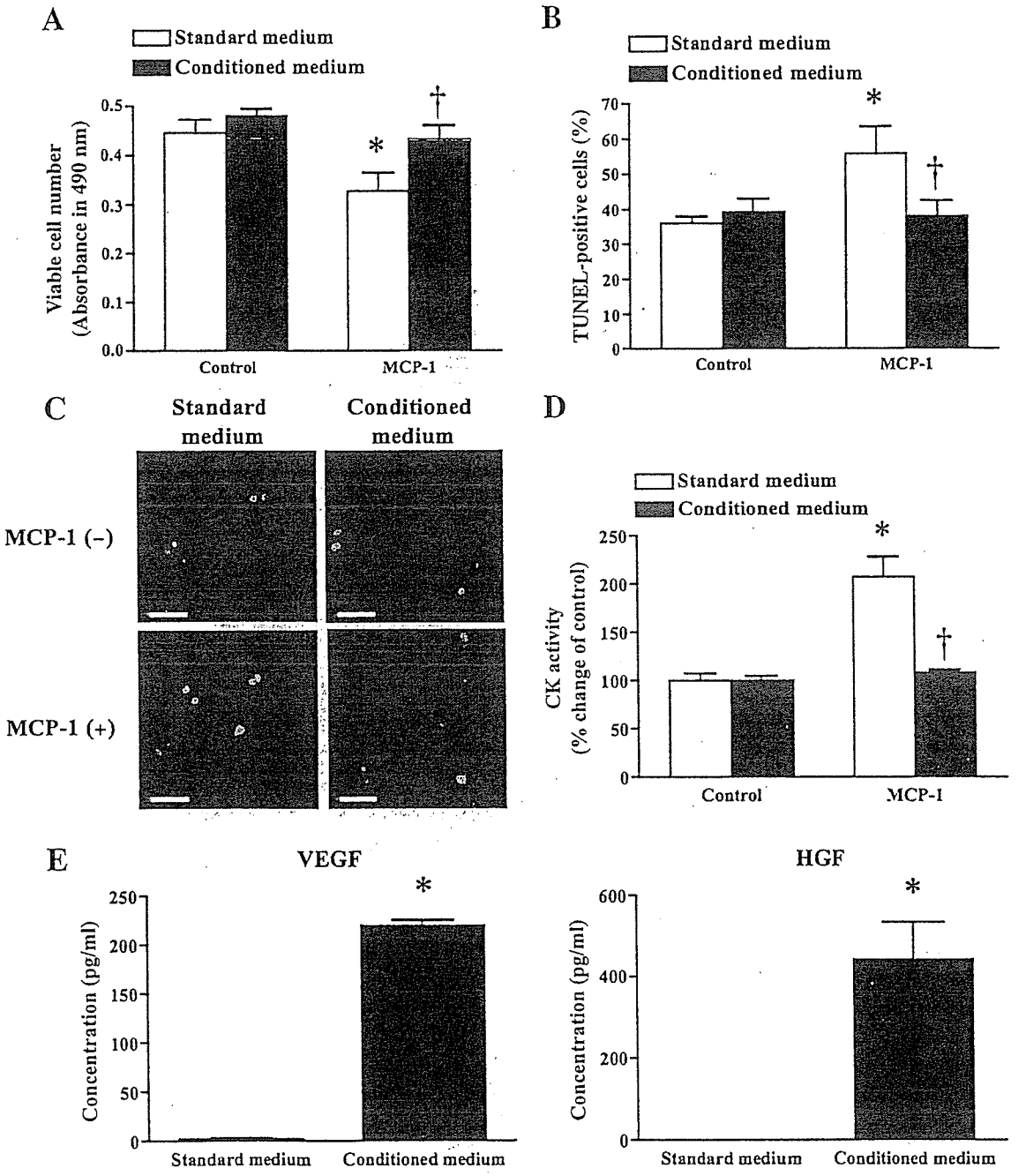

Fig. 7. Effects of MSC on MCP-1-induced cardiomyocyte injury *in vitro*. (A) MTS assay after 24 h of culture with or without MCP-1 in standard medium *vs* MSC conditioned medium. $^{*}P<0.05$ *vs* control in standard medium, $^{\dagger}P<0.05$ *vs* MCP-1 in conditioned medium. (B) Quantitative analysis of TUNEL staining after 24 h of culture with or without MCP-1 in standard medium *vs* MSC conditioned medium. $^{*}P<0.05$ *vs* control in standard medium, $^{\dagger}P<0.05$ *vs* MCP-1 in standard medium. (C) Representative TUNEL staining show increased apoptotic cardiomyocytes (green) cultured with MCP-1 in standard medium, which was attenuated by MSC conditioned medium. Nuclei were counterstained with DAPI (blue). Scale bars: 50 μm. (D) CK activity after 24 h of culture with or without MCP-1 in standard medium *vs* MSC conditioned medium. $^{*}P<0.05$ *vs* control in standard medium, $^{\dagger}P<0.05$ *vs* MCP-1 in standard medium. (E) ELISA for VEGF and HGF secreted from cultured MSC as compared to standard medium. $^{\dagger}P<0.05$ *vs* standard medium.