Acknowledgments

This work was supported by a research grant for Human Genome Tissue Engineering 009 from the Ministry of Health, Labour and Welfare, and by Japan Vascular Disease Research Foundation.

References

1. Rosamond W, Flegal K, Friday G, et al, for the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics—2007 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation.* 2007;115:e69-e171.
2. Fox KF, Cowie MR, Wood DA, et al. Coronary artery disease as the cause of incident heart failure in the population. *Eur Heart J.* 2001;22:228-236.
3. He J, Ogden LG, Bazzano LA, Vupputuri S, Loria C, Whelton PK. Risk factors for congestive heart failure in US men and women: NHANES I epidemiologic follow-up study. *Arch Intern Med.* 2001;161:996-1002.
4. Fraser JK, Schreiber RE, Zuk PA, Hedrick MH. Adult stem cell therapy for the heart. *Int J Biochem Cell Biol.* 2004;36:658-666.
5. Tiyyagura SR, Pinney SP. Left ventricular remodeling after myocardial infarction: past, present, and future. *Mt Sinai J Med.* 2006;73:840-851.
6. Sharpe N. Cardiac remodeling in coronary artery disease. *Am J Cardiol.* 2004;93:17B-20B.
7. Hunt SA, Abraham WT, Chin MH, et al. ACC/AHA 2005 Guideline Update for the Diagnosis and Management of Chronic Heart Failure in the Adult: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Update the 2001 Guidelines for the Evaluation and Management of Heart Failure): developed in collaboration with the American College of Chest Physicians and the International Society for Heart and Lung Transplantation: endorsed by the Heart Rhythm Society. *Circulation.* 2005;112:e154-e235.
8. Bukharovich IF, Kukin M. Optimal medical therapy for heart failure. *Prog Cardiovasc Dis.* 2006;48:372-385.
9. Adorisio R, De Luca L, Rossi J, Gheorghiade M. Pharmacological treatment of chronic heart failure. *Heart Fail Rev.* 2006;11:109-123.
10. Tateishi-Yuyama E, Matsubara H, Murohara T, et al. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet.* 2002;360:427-435.
11. Tse HF, Kwong YL, Chan JK, Lo G, Ho CL, Lau CP. Angiogenesis in ischaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. *Lancet.* 2003;361:47-49.
12. Iwami Y, Masuda H, Asahara T. Endothelial progenitor cells: past, state of the art, and future. *J Cell Mol Med.* 2004;8:488-497.
13. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science.* 1999;284:143-147.
14. Friedenstein AJ, Gorskaja JF, Kulagina NN. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol.* 1976;4:267-274.
15. Gimble J, Guilak F. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. *Cytotherapy.* 2003;5:362-369.
16. De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP. Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum.* 2001;44:1928-1942.
17. Nakahara H, Bruder SP, Goldberg VM, Caplan AI. In vivo osteochondrogenic potential of cultured cells derived from the periosteum. *Clin Orthop Relat Res.* October 1990:223-232.
18. Asakura A, Komaki M, Rudnicki M. Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. *Differentiation.* 2001;68:245-253.
19. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci U S A.* 2000;97:13625-13630.
20. Seo BM, Miura M, Gronthos S, et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet.* 2004;364:149-155.
21. Fukuchi Y, Nakajima H, Sugiyama D, Hirose I, Kitamura T, Tsuji K. Human placenta-derived cells have mesenchymal stem/progenitor cell potential. *Stem Cells.* 2004;22:649-658.
22. Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol.* 2000;109:235-242.
23. da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci.* 2006;119:2204-2213.
24. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science.* 1997;276:71-74.
25. Kawada H, Fujita J, Kinjo K, et al. Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction. *Blood.* 2004;104:3581-3587.
26. Nagaya N, Fujii T, Iwase T, et al. Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis. *Am J Physiol Heart Circ Physiol.* 2004;287:H2670-H2676.
27. Makino S, Fukuda K, Miyoshi S, et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. *J Clin Invest.* 1999;103:697-705.
28. Wakitani S, Saito T, Caplan AI. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. *Muscle Nerve.* 1995;18:1417-1426.
29. Xu W, Zhang X, Qian H, et al. Mesenchymal stem cells from adult human bone marrow differentiate into a cardiomyocyte phenotype in vitro. *Exp Biol Med (Maywood).* 2004;229:623-631.
30. Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM. Origin of endothelial progenitors in human postnatal bone marrow. *J Clin Invest.* 2002;109:337-346.
31. Fukuhara S, Tomita S, Yamashiro S, et al. Direct cell-cell interaction of cardiomyocytes is key for bone marrow stromal cells to go into cardiac lineage in vitro. *J Thorac Cardiovasc Surg.* 2003;125:1470-1480.
32. Rangappa S, Entwistle JW, Wechsler AS, Kresh JY. Cardiomyocyte-mediated contact programs human mesenchymal stem cells to express cardiogenic phenotype. *J Thorac Cardiovasc Surg.* 2003;126:124-132.
33. Xaymardan M, Tang L, Zagreda L, et al. Platelet-derived growth factor-AB promotes the generation of adult bone marrow-derived cardiac myocytes. *Circ Res.* 2004;94:E39-E45.
34. Shim WS, Jiang S, Wong P, et al. Ex vivo differentiation of human adult bone marrow stem cells into cardiomyocyte-like cells. *Biochem Biophys Res Commun.* 2004;324:481-488.
35. Yoon YS, Wecker A, Heyd L, et al. Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. *J Clin Invest.* 2005;115:326-338.
36. Wang T, Xu Z, Jiang W, Ma A. Cell-to-cell contact induces mesenchymal stem cell to differentiate into cardiomyocyte and smooth muscle cell. *Int J Cardiol.* 2006;109:74-81.
37. Kadivar M, Khatami S, Mortazavi Y, Shokrgozar MA, Taghikhani M, Soleimani M. In vitro cardiomyogenic potential of human umbilical vein-derived mesenchymal stem cells. *Biochem Biophys Res Commun.* 2006;340:639-647.
38. Li H, Yu B, Zhang Y, Pan Z, Xu W, Li H. Jagged1 protein enhances the differentiation of mesenchymal stem cells into cardiomyocytes. *Biochem Biophys Res Commun.* 2006;341:320-325.
39. Belema Bedada F, Technau A, Ebelt H, Schulze M, Braun T. Activation of myogenic differentiation pathways in adult bone marrow-derived stem cells. *Mol Cell Biol.* 2005;25:9509-9519.
40. Shake JG, Gruber PJ, Baumgartner WA, et al. Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. *Ann Thorac Surg.* 2002;73:1919-1925, discussion 1926.
41. Liu J, Hu Q, Wang Z, et al. Autologous stem cell transplantation for myocardial repair. *Am J Physiol Heart Circ Physiol.* 2004;287:H501-H511.
42. Davani S, Marandin A, Mersin N, et al. Mesenchymal progenitor

cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a rat cellular cardiomyoplasty model. *Circulation.* 2003;108(suppl 1):II253-II258.

43. Kinnaird T, Stabile E, Burnett MS, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ Res.* 2004;94:678-685.
44. Nagaya N, Kangawa K, Itoh T, et al. Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. *Circulation.* 2005;112:1128-1135.
45. Gnecchi M, He H, Liang OD, et al. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. *Nat Med.* 2005;11:367-368.
46. Ohnishi S, Yanagawa B, Tanaka K, et al. Transplantation of mesenchymal stem cells attenuates myocardial injury and dysfunction in a rat model of acute myocarditis. *J Mol Cell Cardiol.* 2007;42:88-97.
47. Ohnishi S, Yasuda T, Kitamura S, Nagaya N. Effect of hypoxia on gene expression of bone marrow-derived mesenchymal stem cells and mononuclear cells. *Stem Cells.* 2007;25:1166-1177.
48. Tateno K, Minamino T, Toko H, et al. Critical roles of muscle-secreted angiogenic factors in therapeutic neovascularization. *Circ Res.* 2006;98:1194-1202.
49. Saito T, Kuang JQ, Bittira B, Al-Khaldi A, Chiu RC. Xenotransplant cardiac chimera: immune tolerance of adult stem cells. *Ann Thorac Surg.* 2002;74:19-24, discussion 24.
50. Cooper LT Jr, Gersh BJ. Viral infection, inflammation, and the risk of idiopathic dilated cardiomyopathy: can the fire be extinguished? *Am J Cardiol.* 2002;90:751-754.
51. Hein S, Schaper J. Pathogenesis of dilated cardiomyopathy and heart failure: insights from cell morphology and biology. *Curr Opin Cardiol.* 1996;11:293-301.
52. Miyahara Y, Nagaya N, Kataoka M, et al. Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. *Nat Med.* 2006;12:459-465.
53. Chen SL, Fang WW, Ye F, et al. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. *Am J Cardiol.* 2004;94:92-95.
54. Chen S, Liu Z, Tian N, et al. Intracoronary transplantation of autologous bone marrow mesenchymal stem cells for ischemic cardiomyopathy due to isolated chronic occluded left anterior descending artery. *J Invasive Cardiol.* 2006;18:552-556.
55. Katritsis DG, Sotiropoulou PA, Karvouni E, et al. Transcoronary transplantation of autologous mesenchymal stem cells and endothelial progenitors into infarcted human myocardium. *Catheter Cardiovasc Interv.* 2005;65:321-329.
56. Katritsis DG, Sotiropoulou P, Giazitzoglou E, Karvouni E, Papamichail M. Electrophysiological effects of intracoronary transplantation of autologous mesenchymal and endothelial progenitor cells. *Europace.* 2007;9:167-171.

FEBS Letters 581 (2007) 3961–3966

Mesenchymal stem cells attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions

Shunsuke Ohnishi[a,*], Hideaki Sumiyoshi[b], Soichiro Kitamura[c], Noritoshi Nagaya[a,*]

[a] *Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Osaka 565-8565, Japan*
[b] *Department of Anatomy, Biology and Medicine, Faculty of Medicine, Oita University, Oita, Japan*
[c] *Department of Cardiovascular Surgery, National Cardiovascular Center, Osaka, Japan*

Received 2 July 2007; accepted 12 July 2007

Edited by Veli-Pekka Lehto

Abstract Mesenchymal stem cells (MSC) transplantation has been shown to decrease fibrosis in the heart; however, whether MSC directly influence the function of cardiac fibroblasts (CFB) remains unknown. MSC-conditioned medium significantly attenuated proliferation of CFB compared with CFB-conditioned medium. MSC-conditioned medium upregulated antiproliferation-related genes such as elastin, myocardin and DNA-damage inducible transcript 3, whereas CFB-conditioned medium upregulated proliferation-related genes such as alpha-2-macrogrobulin and v-kit Hardy–Zuckerman 4 feline sarcoma viral oncogene homolog. MSC-conditioned medium significantly downregulated type I and III collagen expression, and significantly suppressed type III collagen promoter activity. MSC may exert paracrine anti-fibrotic effects at least in part through regulation of CFB proliferation and collagen synthesis.

Keywords: Mesenchymal stem cell; Cardiac fibroblast; Cell proliferation; Collagen; Paracrine effect; Collagenase activity

1. Introduction

Mesenchymal stem cells (MSC) can differentiate into a variety of cell types including cardiomyocytes and vascular endothelial cells, and can be easily isolated from bone marrow and expanded in culture [1]. These features make MSC an attractive therapeutic tool for cardiovascular disease. We and others have previously demonstrated that MSC transplantation caused significant improvement in hindlimb ischemia [2], myocardial infarction [3,4], dilated cardiomyopathy [5] and acute myocarditis [6]. MSC transplantation has been shown to result in cardiac repair and protection at least in part through paracrine actions such as angiogenic, anti-apoptotic, and anti-inflammatory effects [2,3,5–10]. In addition, it has been demonstrated in animal models that MSC transplantation decreases fibrosis in the heart [5] and other organs such as lung [11,12], liver [13,14] and kidney [15]. However, whether transplanted MSC directly influence the function of cardiac fibroblasts (CFB) remains unknown.

Deposition of collagen fibers in the myocardial interstitium occurs in the remodeling process seen in a variety of cardiovascular diseases, and CFB are predominantly involved in the maintenance of extracellular matrix such as types I and III collagen by cell proliferation, collagen synthesis and degradation [16]. Collagen synthesis is regulated by fibrogenic factors, and collagen degradation is mediated by members of the matrix metalloproteinases (MMPs), which are also regulated by tissue inhibitors of metalloproteinases [17,18].

Thus, we investigated the paracrine effects of MSC on (1) CFB proliferation, (2) collagen synthesis and (3) collagen degradation in vitro.

2. Materials and methods

2.1. Cell culture and collection of conditioned medium

Isolation and expansion of MSC were performed as described previously [2]. Briefly, bone marrow cells were isolated from male Lewis rats weighing 220–250 g by flushing out the femoral and tibial cavities with phosphate-buffered saline, and cultured in standard medium: α-minimal essential medium, 10% 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 4–5 passages. These cells were previously demonstrated to be positive for CD29 and CD90 surface markers, and negative for CD34 and CD45 [5]. The Animal Care Committee of the National Cardiovascular Center approved the experimental protocol.

Primary CFB were obtained as described previously with modification [19]. Briefly, after heparinization by intraperitoneal injection of 1000 U/kg heparin sodium, the heart was rapidly excised, and pulmonary, connective and other non-cardiac 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), 0.5 mg/ml hyaluronidase (Sigma–Aldrich, St. Louis, MO) and 1% bovine serum albumin (fraction V, ICN, Aurora, OH), in a recirculating fashion for 3 h. After perfusion, the heart was removed from the perfusion apparatus, and the atrium was removed and gently minced. CFB were gravitationally separated from cardiomyocytes, and cultured in standard medium.

Conditioned medium was collected from MSC and CFB after the second passage of 3×10^5 cells cultured in standard medium for 48 h, and filtered through a 0.22 μm-filtration unit (Millipore, Bedford, MA).

---

*Corresponding authors. Fax: +81 6 6833 9865.
E-mail addresses: sonishi@ri.ncvc.go.jp (S. Ohnishi), nnagaya@ri.ncvc.go.jp (N. Nagaya).

Abbreviations: MSC, mesenchymal stem cell; CFB, cardiac fibroblast; MMP, matrix metalloproteinase; qRT-PCR, quantitative real-time reverse-transcription polymerase chain reaction; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; A2m, alpha-2-macroglobulin; Kit, v-kit Hardy–Zuckerman 4 feline sarcoma viral oncogene homolog; Catna1, catenin alpha 1; Rarb, retinoic acid receptor beta; Eln, elastin; Myocd, myocardin; Ddit3, DNA-damage inducible transcript 3

0014-5793/$32.00 © 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2007.07.028

2.2. *MTS assay*

We investigated the paracrine effects of MSC on fibroblast proliferation in vitro. Experiments were carried out using cells derived from five passages. CFB were plated on 96-well plates (4×10^3 cells/well). After 24 h, the medium was changed to conditioned medium obtained from MSC or CFB culture for 48 h. After 48 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 of living cells and cell viability, was measured with a CellTiter96 AQueous One Solution Kit (Promega, Madison, WI) and a Microplate Reader (490 nm, Bio-Rad, Hercules, CA).

2.3. *Microarray analysis*

Total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was quantified by spectrometry and the quality confirmed by gel electrophoresis. Microarray analysis was performed as described previously [20]. In brief, double-stranded cDNA was synthesized from 4 μg total RNA, and in vitro transcription was performed to produce biotin-labeled cRNA using GeneChip One-Cycle Target Labeling and Control Reagents (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. After fragmentation, 10 μg cRNA was hybridized with GeneChip Rat Genome 230 2.0 Array (Affymetrix) containing 31 099 genes. GeneChips were then scanned in a GeneChip Scanner 3000 (Affymetrix). Normalization, filtering, and Gene Ontology analysis of the data were performed with GeneSpring GX 7.3.1 software (Agilent Technologies, Palo Alto, CA). The raw data from each array were normalized as follows; each CEL file was preprocessed with Robust Multichip Average (RMA), and each measurement for each gene was divided by the 80th percentile of all measurements. Genes with an at least 1.8-fold change were then selected.

2.4. *Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)*

One microgram of total RNA was reverse-transcribed into cDNA using a Quantitect Reverse Transcription Kit (Qiagen). PCR amplification was performed in 50 μl containing 1 μl cDNA and 25 μl Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) mRNA amplified from the same samples served as an internal control. Primers used in qRT-PCR analysis were as follows: Col1a1: forward, 5′-TCAAGATGGTGGCCGTTAC-3′, reverse, 5′-CTGCGGATGTTC-TCAATCTG-3′, Col3a1: forward, 5′-CGAGATTAAAGCAAGAGGAA-3′, reverse, 5′-GAGGCTTCTTTACATACCAC-3′, Gapdh: forward, 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′, reverse, 5′-CATGTAGGCCATGAGGTCCACCAC-3′. After an initial denaturation at 95°C for 10 min, a two-step cycle procedure was used (denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min) for 40 cycles in a 7700 sequence detector (Applied Biosystems). Gene expression levels were normalized according to that of Gapdh. The data were analyzed with Sequence Detection Systems software (Applied Biosystems).

2.5. *Transient transfection and reporter gene assay*

Transient transfection and subsequent reporter gene assay were performed as described previously with modification [21]. The Col1a1 clones containing the promoter fragments −1685/+68Luc and −96/+68Luc were provided by Dr. H. Yoshioka of Oita University, Japan. CFB were plated at a density of 1×10^4 cells in 96-well plates with 100 μl culture medium. After incubation for 24 h at 37 °C, cells were transfected with 200 ng luciferase plasmid DNA plus 10 ng Renilla phRL-TK vector (Promega, Madison, WI) as an internal control, using lipofectamine2000 (Invitrogen). Six hours after transfection, cells were rinsed with PBS, fed with conditioned medium obtained from MSC or CFB culture, and then further cultured for 48 h. Reporter gene assay was performed using the Dual-Glo Luciferase reporter assay system (Promega), and the luminescence intensity was measured using a microplate reader (Dia-Iatron, Tokyo, Japan), according to the manufacturer's protocol. The transcription activity was normalized according to Renilla luciferase activity.

2.6. *Collagenase activity*

Collagenase activity assay was performed using a collagenase assay kit (Chondrex, Redmond, WA) following the manufacturer's instructions. CFB were cultured in CFB- or MSC-conditioned medium with fluorescein isothiocyanate-labeled type I collagen for 48 h, and degraded collagen was extracted by denaturation, proteinase treatment and centrifugation. Fluorescence intensity was measured using a fluorometer (Tecan, Salzburg, Austria) at excitation/emission of 485/535 nm.

2.7. *Statistical analysis*

Data were expressed as means ± 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. *Effect of MSC-conditioned medium on proliferation of CFB*

To investigate the effect of MSC-conditioned medium on CFB proliferation, we cultured CFB in standard medium, CFB- or MSC-conditioned medium for 48 h, and MTS assay was performed. Viable cell number was significantly larger when CFB were cultured in CFB-conditioned medium compared with standard medium, whereas this increase was not observed when CFB were cultured in MSC-conditioned medium (Fig. 1A, B). These results suggest that MSC attenuates proliferation of CFB through paracrine actions.

3.2. *Effect of MSC-conditioned medium on expression of genes involved in cell proliferation in CFB*

We next performed microarray analysis to examine the effect of MSC-conditioned medium on the expression of genes involved in the regulation of cell proliferation in CFB. Highly expressed genes in CFB cultured in CFB-conditioned medium (>1.8-fold) included positive regulators for cell proliferation such as alpha-2-macroglobulin (A2m) and v-kit Hardy–Zuckerman 4 feline sarcoma viral oncogene homolog (Kit), as well as negative regulators for cell proliferation such as catenin alpha 1 (Catna1) and retinoic acid receptor beta (Rarb). On the other hand, CFB cultured in MSC-conditioned medium highly expressed negative regulators for cell proliferation such as elastin (Eln), myocardin (Myocd) and DNA-damage inducible transcript 3 (Ddit3) (Table 1).

3.3. *Effect of MSC-conditioned medium on collagen gene expression*

To investigate the effect of MSC-conditioned medium on collagen gene expression, we performed qRT-PCR on types I and III collagen genes (Col1a1 and Col3a1, respectively) in CFB. Expression of Col1a1 and Col3a1 genes was significantly upregulated when CFB were cultured in CFB-conditioned medium in comparison to standard medium. However, this increase was significantly attenuated when CFB were cultured in MSC-conditioned medium (Fig. 2A, B).

3.4. *Effect of MSC-conditioned medium on collagen gene promoter activity*

To investigate the effect of MSC-conditioned medium on Col3a1 gene promoter activity, we performed reporter gene assay in CFB. We prepared −1685/+68Luc and −96/+68Luc constructs (Fig. 3A), as the −96 to −34 region has been reported to be fundamental for Col3a1 gene transcription [21]. The activity of −1685/+68Luc was significantly higher in

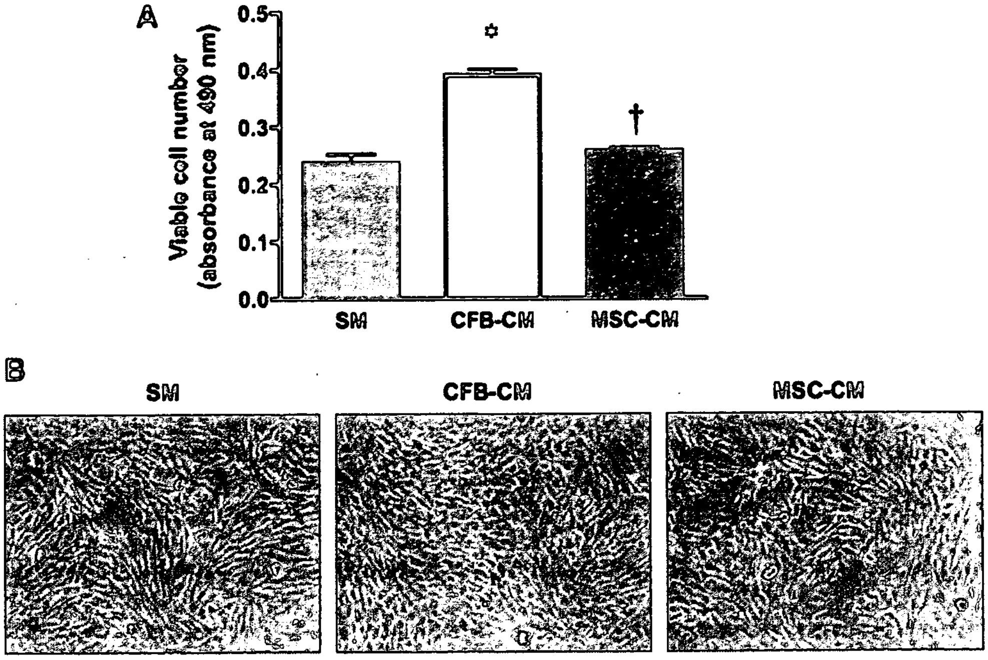

Fig. 1. Effect of MSC-conditioned medium on CFB proliferation MTS assay (A) and representative photographs (B) of CFB after 48 h of culture in the indicated medium. SM, standard medium; CM, conditioned medium. Values are means ± S.E. *$P < 0.05$ vs SM, †$P < 0.05$ vs CFB-CM.

Table 1
Expression of genes involved in regulation of cell proliferation (>1.8-fold)

Gene name	Action on cell proliferation	Fold change
Genes highly expressed in CFB-conditioned medium		
catenin, alpha 1 (Catna1)	Neg	2.3
retinoic acid receptor, beta (Rarb)	Neg	2.1
alpha-2-macroglobulin (A2m)	Pos	2.0
v-kit Hardy–Zuckerman 4 feline sarcoma viral oncogene homolog (Kit)	Pos	1.8
Genes highly expressed in MSC-conditioned medium		
elastin (Eln)	Neg	3.8
myocardin (Myocd)	Neg	1.9
DNA-damage inducible transcript 3 (Ddit3)	Neg	1.8

CFB-conditioned medium as compared to standard medium, whereas it was markedly decreased in MSC-conditioned medium (Fig. 3B). However, the activity of −96/+68Luc was not affected in either CFB- or MSC-conditioned medium. These results suggest that MSC-conditioned medium inhibits Col3a1 gene transcription through regulation of the −1685 to −96 promoter region.

3.5. Effect of MSC-conditioned medium on collagenase activity

We finally investigated the effect of MSC-conditioned medium on collagen degradation. Type I collagenase activity was markedly higher in CFB-conditioned medium than in standard medium; however, MSC-conditioned medium had as high collagenase activity as CFB-conditioned medium (Fig. 4).

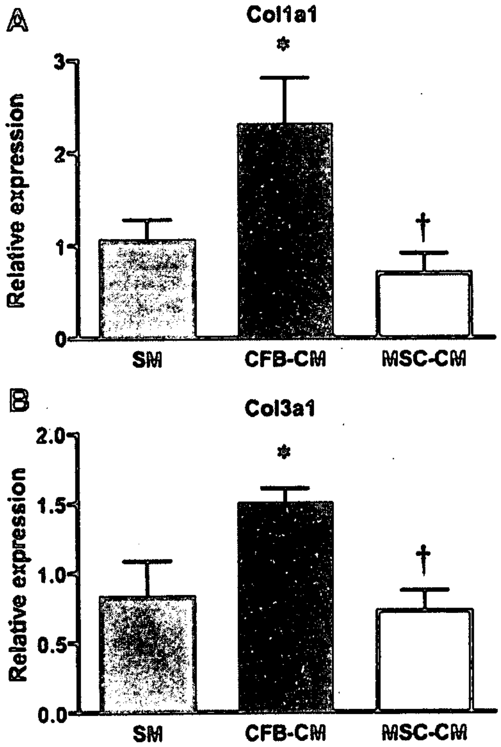

Fig. 2. Effect of MSC-conditioned medium on collagen gene expression (A) Quantitative RT-PCR for Col1a1 expression in CFB after 48 h of culture in the indicated medium. (B) Quantitative RT-PCR for Col3a1 expression in CFB after 48 h of culture in the indicated medium. SM, standard medium; CM, conditioned medium. Values are means ± S.E. *$P < 0.05$ vs SM, †$P < 0.05$ vs CFB-CM.

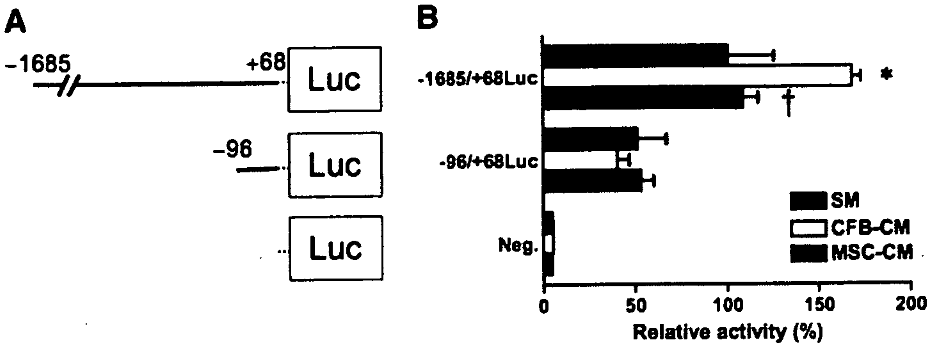

Fig. 3. Effect of MSC-conditioned medium on type III collagen gene promoter activity (A) Schematic illustration of 5′-deletion constructs of the Col3a1 promoter. (B) Luficerase activity in CFB after 48 hours of transfection of reporter plasmids and culture in the indicated medium. All constructs were co-transfected with the phRL-TK vector as an internal control for transfection efficiency. SM, standard medium; CM, conditioned medium. Values are means ± S.E. $^{*}P < 0.05$ vs SM, $^{\dagger}P < 0.05$ vs CFB-CM.

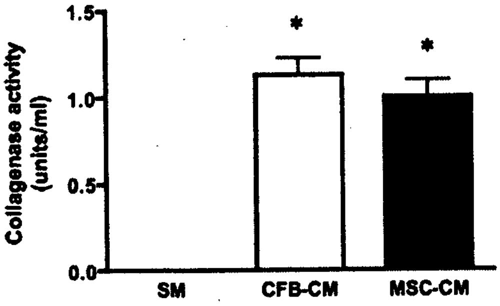

Fig. 4. Effect of MSC-conditioned medium on collagenase activity Type I collagenase activity of the indicated medium. SM, standard medium; CM, conditioned medium. Values are means ± S.E. $^{*}P < 0.05$ vs SM.

4. Discussion

In this study, we focused on the paracrine effects of MSC on CFB in vitro, and demonstrated that MSC-conditioned medium: (1) attenuated CFB proliferation, (2) regulated expression of several genes involved in CFB proliferation, (3) transcriptionally inhibited type I and III collagen gene expression in CFB, and (4) had comparable collagenase activity to that of CFB-conditioned medium.

We and others have previously demonstrated that MSC mediate pleiotropic effects by secreting a large number of growth factors, anti-apoptotic factors and cytokines [2,3,5–10]. In addition, we have recently reported that MSC transplantation improved cardiac function at least in part through an anti-fibrotic effect in a rat model of dilated cardiomyopathy and acute myocarditis [5,6], and also demonstrated that the highly expressed genes in cultured MSC included a number of molecules involved in biogenesis of extracellular matrix such as collagens, MMPs, serine proteases and serine protease inhibitors [20]. These results suggest that transplanted MSC may inhibit the fibrogenic process through paracrine actions.

In the present study, CFB proliferation was slower when they were cultured in MSC-conditioned medium than in CFB-conditioned medium, and microarray analysis demonstrated that the expression levels of several genes involved in cell proliferation were differently regulated. Out of four highly expressed genes in CFB cultured in CFB-conditioned medium, two genes (A2m and Kit) are known to positively regulate cell proliferation, whereas the other two genes (Catna1 and Rarb) are known to be negative regulators. Catna1 encodes α-catenin which interacts with cadherin, a cell adhesion molecule, and targeted deletion of Catna1 in either the skin or in neuronal progenitor cells leads to hyperproliferation [22]. Rarb encodes a member of retinoic acid receptors, and regulated cell growth and differentiation in a variety of cells [23]. A2m encodes a plasma proteinase inhibitor [24], and induces macrophage proliferation through cAMP-dependent signaling [25]. Kit encodes c-kit protein, a tyrosine kinase receptor for stem cell factor, and ectopic expression of c-kit in fibroblasts induces tumorigenesis [26]. On the other hand, three negative regulators of cell proliferation were upregulated in CFB cultured in MSC-conditioned medium. Eln encodes a polymer of a precursor protein (tropoelastin), and impaired elastogenesis coincides with increased cell proliferation [27]. Mycd encodes a transcription factor important for smooth muscle and cardiac muscle development, and inactivation of Mycd in fibroblasts increases their proliferative potential [28]. Ddit3 belongs to the CCAAT/enhancer binding protein family of transcription factors, and exogenous Ddit3 is capable of inducing growth arrest and apoptosis [29]. Taking these findings together, MSC may negatively regulate CFB proliferation by controlling these factors, although the precise mechanism remains to be elucidated.

Types I and III collagen are the major fibrillar collagen produced by CFB, and the expression of collagen genes is regulated at the transcriptional and post-transcriptional levels [30]. It has been suggested that an initial mesh of type III collagen forms the scaffold for subsequent deposition of large, highly aligned type I collagen fibers at the fibrotic phase after myocardial infarction [31]. In the present study, the expression of Col1a1 as well as Col3a1 was downregulated when CFB were cultured in MSC-conditioned medium. This result is consistent with a recent study by Guo et al., which demonstrated that MSC transplantation in a rat model of myocardial infarction inhibited deposition of types I and III collagen [32]. Although the transcriptional mechanism of the Col3a1 gene is not entirely characterized, our in vitro experiments suggest that, in comparison to CFB-conditioned medium, MSC-conditioned medium may be rich in humoral factors that can inactivate transcription, or poor in humoral factors that can activate transcription, of Col3a1.

Collagenase (MMP-1) and gelatinase (MMP-2 and -9) activity are known to be elevated during the necrotic phase of infarct healing, and are involved in disruption of the collagen network [33]. In the present study, type I collagenase activity of MSC-conditioned medium was as high as that of CFB-conditioned medium. Type I collagen is a substrate for MMP-1, -2, -8 and -13, and the mechanisms involved in the differential regulation of the various collagen types during cardiac fibrosis appear to be complex and diverse [34]. However, our results imply that MSC have equivalent paracrine effects on type I collagenase activity to those of CFB.

In conclusion, MSC exerted paracrine anti-fibrotic effects at least in part through regulation of CFB proliferation and transcriptional downregulation of types I and III collagen syntheses. These features of MSC may be beneficial for the treatment of heart failure in which fibrotic changes are involved.

Acknowledgements: This work was supported by research grants for Cardiovascular Disease (18C-1 and 19C-7) and Human Genome Tissue Engineering 009 from the Ministry of Health, Labour and Welfare, and by the Japan Vascular Disease Research Foundation.

References

[1] Pittenger, M.F. and Martin, B.J. (2004) Mesenchymal stem cells and their potential as cardiac therapeutics. Circ. Res. 95, 9–20.

[2] Iwase, T., Nagaya, N., Fujii, T., Itoh, T., Murakami, S., Matsumoto, T., Kangawa, K. and Kitamura, S. (2005) Comparison of angiogenic potency between mesenchymal stem cells and mononuclear cells in a rat model of hindlimb ischemia. Cardiovasc. Res. 66, 543–551.

[3] Miyahara, Y., Nagaya, N., Kataoka, M., Yanagawa, B., Tanaka, K., Hao, H., Ishino, K., Ishida, H., Shimizu, T., Kangawa, K., Sano, S., Okano, T., Kitamura, S. and Mori, H. (2006) Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. Nat. Med. 12, 459–465.

[4] Abdel-Latif, A., Bolli, R., Tleyjeh, I.M., Montori, V.M., Perin, E.C., Hornung, C.A., Zuba-Surma, E.K., Al-Mallah, M. and Dawn, B. (2007) Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis. Arch. Intern. Med. 167, 989–997.

[5] Nagaya, N., Kangawa, K., Itoh, T., Iwase, T., Murakami, S., Miyahara, Y., Fujii, T., Uematsu, M., Ohgushi, H., Yamagishi, M., Tokudome, T., Mori, H., Miyatake, K. and Kitamura, S. (2005) Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. Circulation 112, 1128–1135.

[6] Ohnishi, S., Yanagawa, B., Tanaka, K., Miyahara, Y., Obata, H., Kataoka, M., Kodama, M., Ishibashi-Ueda, H., Kangawa, K., Kitamura, S. and Nagaya, N. (2007) Transplantation of mesenchymal stem cells attenuates myocardial injury and dysfunction in a rat model of acute myocarditis. J. Mol. Cell Cardiol. 42, 88–97.

[7] Kinnaird, T., Stabile, E., Burnett, M.S., Lee, C.W., Barr, S., Fuchs, S. and Epstein, S.E. (2004) Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ. Res. 94, 678–685.

[8] Kinnaird, T., Stabile, E., Burnett, M.S., Shou, M., Lee, C.W., Barr, S., Fuchs, S. and Epstein, S.E. (2004) Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. Circulation 109, 1543–1549.

[9] Gnecchi, M., He, H., Liang, O.D., Melo, L.G., Morello, F., Mu, H., Noiseux, N., Zhang, L., Pratt, R.E., Ingwall, J.S. and Dzau, V.J. (2005) Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat. Med. 11, 367–368.

[10] Gnecchi, M., He, H., Noiseux, N., Liang, O.D., Zhang, L., Morello, F., Mu, H., Melo, L.G., Pratt, R.E., Ingwall, J.S. and Dzau, V.J. (2006) Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J. 20, 661–669.

[11] Ortiz, L.A., Gambelli, F., McBride, C., Gaupp, D., Baddoo, M., Kaminski, N. and Phinney, D.G. (2003) Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. Proc. Natl. Acad. Sci. USA 100, 8407–8411.

[12] Rojas, M., Xu, J., Woods, C.R., Mora, A.L., Spears, W., Roman, J. and Brigham, K.L. (2005) Bone marrow-derived mesenchymal stem cells in repair of the injured lung. Am. J. Respir. Cell Mol. Biol. 33, 145–152.

[13] Oyagi, S., Hirose, M., Kojima, M., Okuyama, M., Kawase, M., Nakamura, T., Ohgushi, H. and Yagi, K. (2006) Therapeutic effect of transplanting HGF-treated bone marrow mesenchymal cells into CCl4-injured rats. J. Hepatol. 44, 742–748.

[14] Abdel Aziz, M.T., Atta, H.M., Mahfouz, S., Fouad, H.H., Roshdy, N.K., Ahmed, H.H., Rashed, L.A., Sabry, D., Hassouna, A.A. and Hasan, N.M. (2007) Therapeutic potential of bone marrow-derived mesenchymal stem cells on experimental liver fibrosis. Clin. Biochem. 40, 893–899.

[15] Ninichuk, V., Gross, O., Segerer, S., Hoffmann, R., Radomska, E., Buchstaller, A., Huss, R., Akis, N., Schlondorff, D. and Anders, H.J. (2006) Multipotent mesenchymal stem cells reduce interstitial fibrosis but do not delay progression of chronic kidney disease in collagen4A3-deficient mice. Kidney Int. 70, 121–129.

[16] Brown, R.D., Ambler, S.K., Mitchell, M.D. and Long, C.S. (2005) The cardiac fibroblast: therapeutic target in myocardial remodeling and failure. Annu. Rev. Pharmacol. Toxicol. 45, 657–687.

[17] Jugdutt, B.I. (2003) Remodeling of the myocardium and potential targets in the collagen degradation and synthesis pathways. Curr. Drug Targets Cardiovasc. Haematol. Disord. 3, 1–30.

[18] Lijnen, P.J. and Petrov, V.V. (2003) Role of intracardiac renin–angiotensin–aldosterone system in extracellular matrix remodeling. Methods Find. Exp. Clin. Pharmacol. 25, 541–564.

[19] Tanaka, K., Honda, M. and Takabatake, T. (2001) Redox regulation of MAPK pathways and cardiac hypertrophy in adult rat cardiac myocyte. J. Am. Coll. Cardiol. 37, 676–685.

[20] Ohnishi, S., Yasuda, T., Kitamura, S. and Nagaya, N. (2007) Effect of hypoxia on gene expression of bone marrow-derived mesenchymal stem cells and mononuclear cells. Stem Cells 25, 1166–1177.

[21] Yoshino, T., Sumiyoshi, H., Shin, T., Matsuo, N., Inagaki, Y., Ninomiya, Y. and Yoshioka, H. (2005) Multiple proteins are involved in the protein-DNA complex in the proximal promoter of the human alpha1(III) collagen gene (COL3A1). Biochim. Biophys. Acta 1729, 94–104.

[22] Vasioukhin, V., Bauer, C., Degenstein, L., Wise, B. and Fuchs, E. (2001) Hyperproliferation and defects in epithelial polarity upon conditional ablation of alpha-catenin in skin. Cell 104, 605–617.

[23] Chambon, P. (1994) The retinoid signaling pathway: molecular and genetic analyses. Semin. Cell Biol. 5, 115–125.

[24] Baker, A.H., Edwards, D.R. and Murphy, G. (2002) Metalloproteinase inhibitors: biological actions and therapeutic opportunities. J. Cell Sci. 115, 3719–3727.

[25] Misra, U.K., Akabani, G. and Pizzo, S.V. (2002) The role of cAMP-dependent signaling in receptor-recognized forms of alpha 2-macroglobulin-induced cellular proliferation. J. Biol. Chem. 277, 36509–36520.

[26] Caruana, G., Cambareri, A.C., Gonda, T.J. and Ashman, L.K. (1998) Transformation of NIH3T3 fibroblasts by the c-Kit receptor tyrosine kinase: effect of receptor density and ligand-requirement. Oncogene 16, 179–190.

[27] Urban, Z., Riazi, S., Seidl, T.L., Katahira, J., Smoot, L.B., Chitayat, D., Boyd, C.D. and Hinek, A. (2002) Connection between elastin haploinsufficiency and increased cell proliferation in patients with supravalvular aortic stenosis and Williams-Beuren syndrome. Am. J. Hum. Genet. 71, 30–44.

[28] Milyavsky, M., Shats, I., Cholostoy, A., Brosh, R., Buganim, Y., Weisz, L., Kogan, I., Cohen, M., Shatz, M., Madar, S., Kalo, E., Goldfinger, N., Yuan, J., Ron, S., MacKenzie, K., Eden, A. and Rotter, V. (2007) Inactivation of myocardin and p16 during malignant transformation contributes to a differentiation defect. Cancer Cell 11, 133–146.

[29] Maytin, E.V., Ubeda, M., Lin, J.C. and Habener, J.F. (2001) Stress-inducible transcription factor CHOP/gadd153 induces apoptosis in mammalian cells via p38 kinase-dependent and -independent mechanisms. Exp. Cell Res. 267, 193–204.
[30] Ghosh, A.K. (2002) Factors involved in the regulation of type I collagen gene expression: implication in fibrosis. Exp. Biol. Med. (Maywood) 227, 301–314.
[31] Whittaker, P., Boughner, D.R. and Kloner, R.A. (1989) Analysis of healing after myocardial infarction using polarized light microscopy. Am. J. Pathol. 134, 879–893.
[32] Guo, J., Lin, G.S., Bao, C.Y., Hu, Z.M. and Hu, M.Y. (2007) Anti-inflammation role for mesenchymal stem cells transplantation in myocardial infarction. Inflammation 30, 97–104.
[33] Cleutjens, J.P., Kandala, J.C., Guarda, E., Guntaka, R.V. and Weber, K.T. (1995) Regulation of collagen degradation in the rat myocardium after infarction. J. Mol. Cell Cardiol. 27, 1281–1292.
[34] Bishop, J.E. and Lindahl, G. (1999) Regulation of cardiovascular collagen synthesis by mechanical load. Cardiovasc. Res. 42, 27–44.

ELSEVIER

Cardiovascular Research 76 (2007) 110–118

Cardiovascular Research

www.elsevier.com/locate/cardiores

Infusion of adrenomedullin improves acute myocarditis *via* attenuation of myocardial inflammation and edema

Bobby Yanagawa [a,1], Masaharu Kataoka [a,1], Shunsuke Ohnishi [a,*,1], Makoto Kodama [b], Koichi Tanaka [a], Yoshinori Miyahara [a], Hatsue Ishibashi-Ueda [c], Yoshifusa Aizawa [b], Kenji Kangawa [d], Noritoshi Nagaya [a,*]

[a] *Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, Osaka, Japan*
[b] *Division of Cardiology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan*
[c] *Department of Pathology, National Cardiovascular Center, Osaka, Japan*
[d] *Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka, Japan*

Received 23 January 2006; received in revised form 7 May 2007; accepted 24 May 2007
Available online 6 June 2007
Time for primary review 23 days

Abstract

Objective: Our aim was to assess whether adrenomedullin (AM), a potent vasodilator peptide with a variety of cardioprotective effects, has a therapeutic potential for the treatment of acute myocarditis in a rat model.
Methods: One week after myosin injection, rats received a continuous infusion of AM or vehicle for 2 weeks, and pathological and physiological investigations were performed.
Results: AM treatment significantly reduced the infiltration of inflammatory cells in myocarditic hearts, and decreased the expressions of macrophage chemoattractant protein-1, matrix metalloproteinase-2 and transforming growth factor-β. Myocardial edema indicated by increased heart weight to body weight ratio and wall thickness was attenuated by AM infusion (5.7±0.5 *vs.* 6.5±0.4 g/kg, and 1.9±0.3 *vs.* 2.8±0.5 mm, respectively). Infusion of AM significantly improved left ventricular maximum dP/dt and fractional shortening of myocarditic hearts (4203±640 *vs.* 3450±607 mm Hg/s, and 21.3±4.1 *vs.* 14.7±5.1%, respectively).
Conclusion: Infusion of AM improved cardiac function and pathological findings in a rat model of acute myocarditis. Thus, infusion of AM may be a potent therapeutic strategy for acute myocarditis.

Keywords: Autoimmune myocarditis; Adrenomedullin; Angiogenesis; Inflammation

---

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]. Although early evidence for efficacy of immunoglobulin and interferon therapy appears promising, these results have yet to be demonstrated in randomized or controlled clinical trials [2]. Current therapeutic options are restricted to supportive care for heart failure or arrhythmias [3]. The lack of specific treatment and the potential severity of the illness emphasize the importance of novel and effective therapeutic strategies for myocarditis.

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma [4]. Earlier studies have shown that AM has beneficial hemodynamic effects on failing hearts *via* its vasodilatory action and diuretic effects [5,6]. Furthermore, AM has direct cardioprotective effects such as anti-inflammatory effects [7], inhibition

---

* Corresponding authors. Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. Tel.: +81 6 6833 5012; fax: +81 6 6835 5496.
E-mail addresses: sonishi@ri.ncvc.go.jp (S. Ohnishi), nnagaya@ri.ncvc.go.jp (N. Nagaya).

[1] These authors contributed equally to this work.

0008-6363/$ - see front matter © 2007 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.cardiores.2007.05.027

of apoptosis [8], induction of angiogenesis [9] and attenuation of myocardial hypertrophy [10]. Interestingly, AM has also been shown to decrease endothelial hyperpermeability in the heart [11]. These findings raise the possibility that infusion of AM may attenuate myocardial inflammation and edema in acute myocarditis. Although previous findings have demonstrated that infusion of AM is effective for heart failure, its therapeutic effects in acute myocarditis are still unknown.

Experimental autoimmune myocarditis can be induced in rats by immunizing them with cardiac myosin, providing a model that resembles human giant cell myocarditis [12,13]. 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 autoantigens such as cardiac myosin to the immune system [14,15].

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

2. Methods

2.1. Experimental autoimmune myocarditis

Purified cardiac myosin from the ventricular muscle of pig hearts was prepared according to a procedure described previously [16]. 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 (CFA) containing 11 mg/ml of *Mycobacterium tuberculosis* (Difco Laboratories, Sparks, MD, USA).

Male 10-week-old Lewis rats were used in the present study. Rats were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg) and were given an injection of either 0.2 ml of antigen–adjuvant emulsion or saline mixed with CFA into the footpad. One week after myosin injection, an osmotic pump (Alzet, Cupertino, CA, USA) was filled with either AM (0.05 μg/kg/min) or PBS for 2 weeks, and implanted subcutaneously between the scapulae. This protocol resulted in the creation of 3 groups ($n=11$ in each group): sham rats given PBS (sham group), myosin-treated rats given PBS (control group), and myosin-treated rats given AM (AM group). The dose of AM used in this study has anti-apoptotic effects without significant hypotension [8]. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.2. Histopathology

After completion of hemodynamic measurements on day 21 post-myosin injection, the heart was excised above the origin of the great vessels, and ventricular weight was recorded. Midventricular portions of the heart were formalin-fixed and embedded in paraffin, and 4 μm-sections were cut and stained with hematoxylin and eosin (H&E). H&E-stained sections were graded by a cardiovascular pathologist (H.I-U.) as described previously [17]. Briefly, coagulation necrosis, granulation, inflammation and edema were evaluated without knowledge of the experimental groups on the following scale: 0, no or questionable presence; 1, limited focal distribution less than 25% area of the section; 2, intermediate severity covering less than 50% area of the section; 3, intermediate severity covering greater than 50% and less than 75% area of the section; and 4, coalescent and extensive foci more than 75% area to the entirety of the transversely sectioned ventricular tissue (5 fields per rat, $n=8$ in each group).

2.3. Picrosirius red staining

Paraffin-embedded sections were submitted for picrosirius staining for total collagen distribution. Slides were hydrated, placed in Weigert's iron hematoxylin and in Bouin's fluid (70% saturated aqueous picric acid, 5% acetic acid, 25% formalin) for 10 min. The slides were rinsed in distilled water and placed in 0.025% picrosirius red solution overnight. The sections were rinsed, dehydrated, cleared, and mounted. Amount of collagen stain was quantitated using image analysis software on high-powered (×200) cross-sectional images (10 fields per rat, $n=5$ in each group).

2.4. Immunohistochemistry

Paraffin-embedded heart sections were washed in increasing concentrations of ethanol and then in PBS. Immunohistochemical staining of the sections was performed with antibodies raised against macrophage chemoattractant protein-1 (MCP-1) (BD Bioscience Pharmingen, San Jose, CA, USA) or CD68 (DakoCytomation, Glostrup, Denmark), a marker of monocytes and macrophages. The number of CD68-positive cells was counted with a light microscope (×200, 10 fields per rat, $n = 6$ in each group). To detect capillary endothelial cells, immunohistochemical staining of the sections was performed with a rabbit polyclonal antibody raised against von Willebrand factor (vWF, DakoCytomation). The number of capillary vessels was counted using a light microscope (×200, 10 fields per rat, $n=6$ in each group).

2.5. Western blot analysis

Western blot was performed as previously described [18]. Briefly, LV tissues were homogenized in 0.1% Tween-20 with a protease inhibitor, loaded (40 μg) on a 7.5% sodium dodecyl sulfate-polyacrylamide gel, and blotted onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After blocking for 2 h, membranes were incubated with MMP-2 (Laboratory Vision, Fremont, CA, USA) or MMP-9

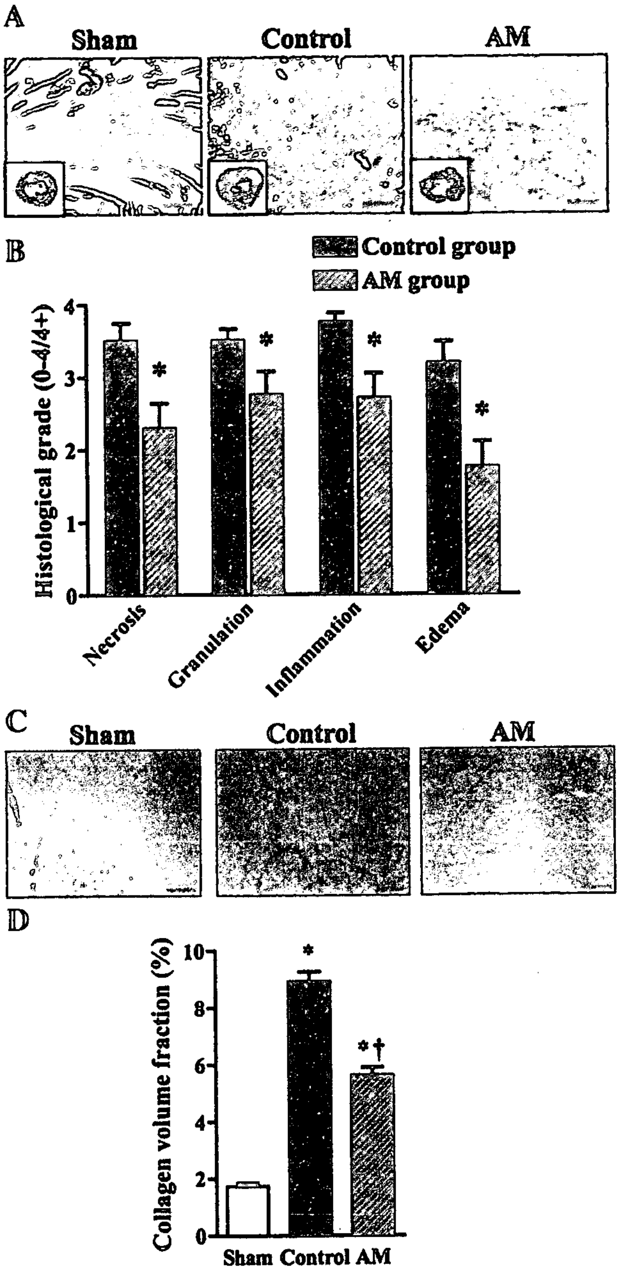

Fig. 1. Pathological findings in acute myocarditis after AM infusion. A: Representative H&E staining of myocardial sections showed markedly decreased inflammation and tissue necrosis in AM-treated hearts as compared to control hearts. Insets are transverse sections of myocardial freewall. B: Semi-quantitative histological grades for necrosis and tissue granulation as well as for inflammation and edema were significantly lower in AM-treated hearts as compared to control hearts ($n=8$ in each group). Sham tissues exhibited no measurable pathological changes. Data are mean±S.E. *, $P<0.05$ *vs.* control. C: Representative picrosirius staining showed decreased collagen deposition in AM-treated hearts as compared to control hearts. D: Collagen volume fraction in 10 random representative fields (×200) confirmed a significant decrease in AM-treated hearts *vs.* control hearts ($n=5$ in each group). Scale bars: 50 μm. Data are mean±S.E. *, $P<0.05$ *vs.* sham; †, $P<0.05$ *vs.* control.

(Chemicon, Temecula, CA, USA) rabbit polyclonal antibodies (1:200), then incubated with peroxidase labeled with secondary antibody (1:1000). Positive protein bands were visualized with an ECL kit (GE Healthcare, Piscataway, NJ, USA) and measured by densitometry. A mouse polyclonal antibody against β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a control ($n=5$ in each group).

2.6. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Heart tissues ($n=5$ in each group) were homogenized with TissueLyser (Qiagen, Hilden, Germany). Total RNA was extracted using RNeasy Mini Kit (Qiagen), followed by reverse transcription into cDNA using the avian myeloblastosis virus transcriptase (Ambion, Austin, TX, USA), according to the manufacturers' protocol. PCR amplification was performed in 50 μl containing 1 μl of cDNA and 25 μl of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The following sequence-specific primers were used for TGF-β, as described previously [19]: forward, 5′-GTTCTTCAATACGTCAGACATTCG-3′; reverse, 5′-CATTATCTTTGCTGTCACAAGAGC-3′. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA amplified from the same samples was served as an internal control: forward, 5′-GAACATCATCCCTGCATCCA-3′; reverse, 5′-CCAGTGAGCTTCCCGTTCA-3′. After an initial denaturation at 95 °C for 10 min, a 2-step cycle procedure was used (denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min) for 40 cycles in a 7700 sequence detector (Applied Biosystems). The data were analyzed with Sequence Detection Systems software.

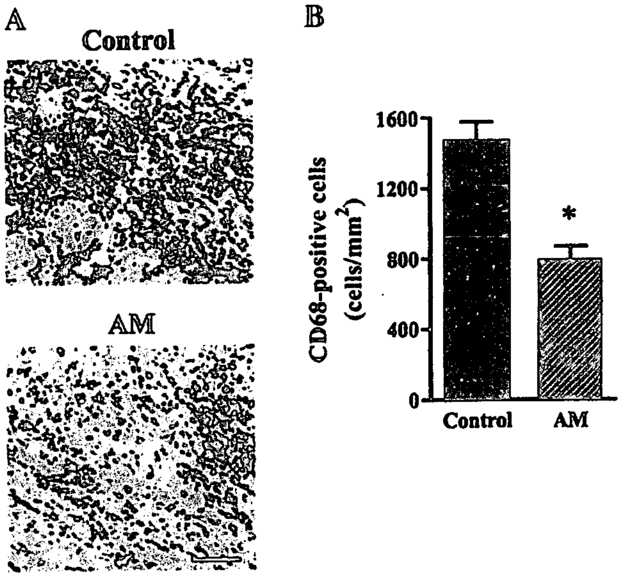

Fig. 2. Infiltration of inflammatory cells in myocardium. A: Immunohistochemical analysis of CD68-positive cell infiltration in myocardium. AM infusion markedly attenuated the increase in CD68-positive cells in myocarditic hearts. Scale bars: 50 μm. B: Semi-quantitative analysis of CD68-positive cell infiltration. CD68-positive cells in 10 random representative high-power fields (×200) confirmed a significant decrease in AM-treated hearts *vs.* control hearts ($n=6$ in each group). Data are mean±S.E. *, $P<0.05$ *vs.* control.

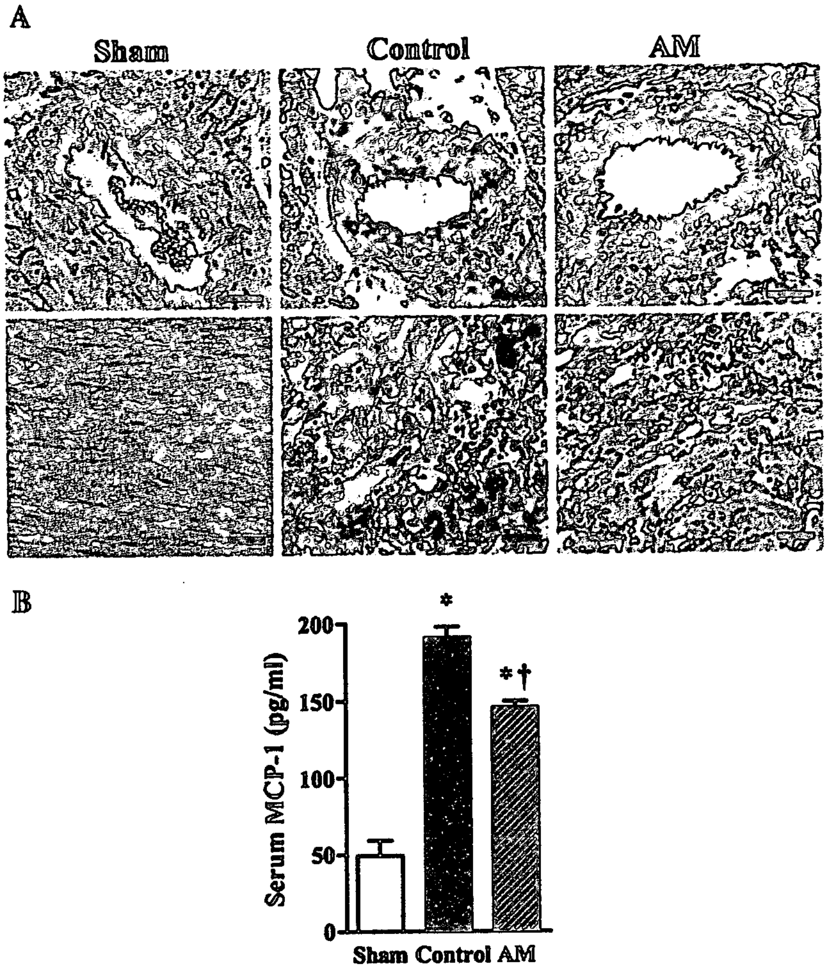

Fig. 3. Effects of AM infusion on MCP-1 expression. A: Representative myocardial sections immunohistochemically stained with anti-MCP-1 antibody showed increased vascular endothelial and myocyte staining of MCP-1 (arrows) and the presence of giant cells (arrowheads) in control hearts as compared to AM-treated hearts. Sham hearts showed subtle endothelial staining. Scale bars: 20 μm. B: Serum MCP-1 level was greatly increased in myocarditic rats. However, the increase in serum MCP-1 was significantly attenuated by AM infusion ($n=6$ in each group). Data are mean±S.E. *, $P<0.05$ vs. sham; †, $P<0.05$ vs. control.

2.7. Enzyme-linked immunosorbent assay (ELISA)

To investigate the effect of AM infusion on serum MCP-1 level, blood was drawn from the heart before excision ($n=6$ in each group). Blood was centrifuged and serum samples were frozen and stored at −80 °C. Serum MCP-1 level was measured by ELISA according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

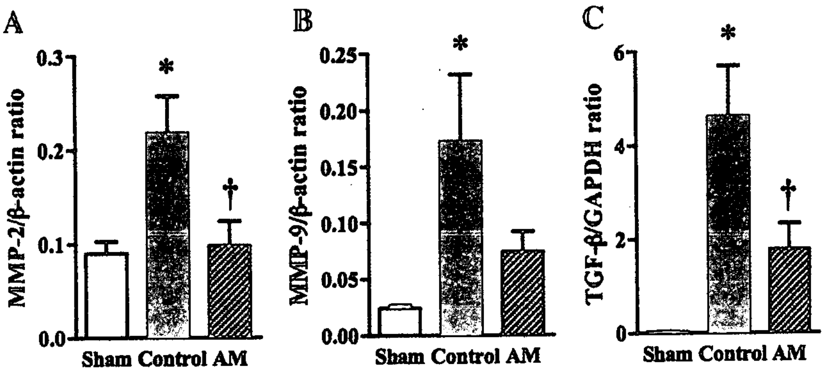

Fig. 4. Effects of AM infusion on MMP and TGF-β expression. A and B: Western blot analysis for MMP-2 (A) and -9 (B) expression. Levels of MMP-2 and -9 were significantly increased in control hearts. MMP-2 expression was markedly decreased by AM infusion, and MMP-9 expression tended to be decreased after AM infusion ($n=5$ in each group). C: Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) for TGF-β expression. Expression of TGF-β was increased in myocarditis and significantly decreased by AM treatment ($n=5$ in each group). Data are mean±S.E. *, $P<0.05$ vs. sham; †, $P<0.05$ vs. control.

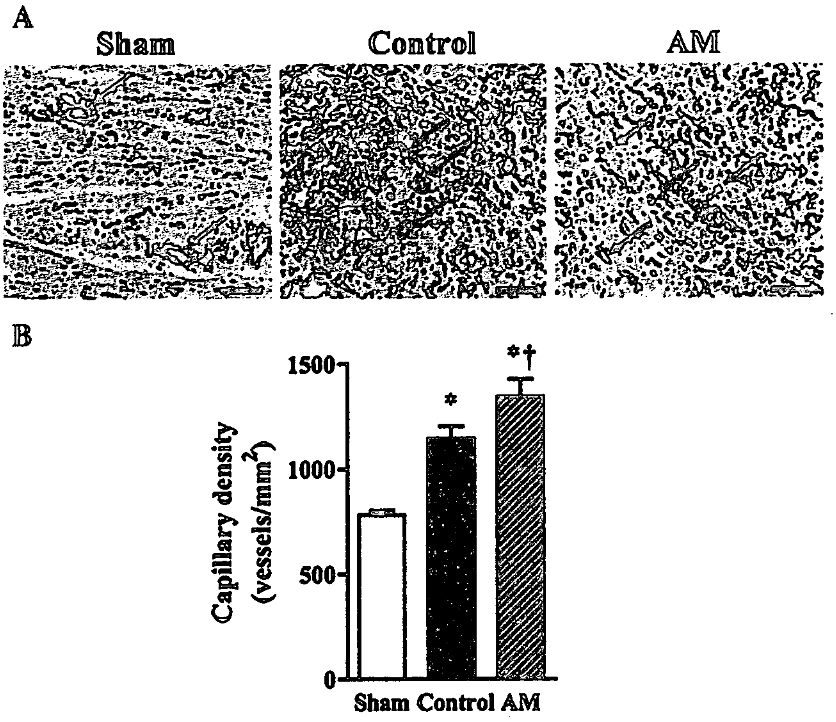

Fig. 5. Increased endothelial regeneration with AM infusion. A: Immunohistochemical demonstration of von Willebrand factor in myocardium. Arrows indicate microvasculature. Scale bars: 20 µm. B: Capillary density measured in 10 random representative high-power fields (×200) showed a significant increase in control hearts and a further increase in AM-treated hearts *vs.* sham hearts ($n=6$ in each group). Data are mean±S.E. *, $P<0.05$ *vs.* sham; †, $P<0.05$ *vs.* control.

2.8. Hemodynamic study

Hemodynamic measurements were taken on day 21 post-myosin injection ($n=7$ in each group). Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg) as a supplement to maintain mild anesthesia. A 1.5 Fr micromanometer-tipped catheter (Millar Instruments, Houston, TX, USA) was advanced into the left ventricle through the right carotid artery, and a polyethylene catheter (PE-50) was advanced into the right ventricle through the right jugular vein to measure right ventricular pressure. Heart rate was also monitored by electrocardiography. As hemodynamic indices, heart rate, mean arterial pressure, LV end-diastolic pressure, maximum dP/dt, and minimum dP/dt were used.

2.9. Echocardiography

Echocardiography was performed on day 21 post-myosin injection. A 12-MHz probe was placed in the left 4th intercostal space for M-mode imaging using 2D echocardiography (Sonos 5500, Philips, Bothell, WA, USA). M-mode tracings were obtained at the level of the papillary muscles. Anterior and posterior end-diastolic wall thickness, left ventricular (LV) end-diastolic and end-systolic dimension, LV fractional shortening (FS), and LV ejection fraction (EF) were measured in three consecutive cardiac cycles by the American Society for Echocardiology leading-edge method ($n=10$ in each group). EF and FS were calculated from the following formula, respectively:

$$\text{EF} = (\text{end-diastolic volume} - \text{end-systolic volume})/\text{end-diastolic volume}$$

$$\text{FS} = (\text{end-diastolic diameter} - \text{end-systolic diameter})/\text{end-diastolic diameter}$$

2.10. Statistical analysis

All data were expressed as mean±S.E. Comparisons of parameters among the groups were made by one-way

Table 1
Physiological profiles of three experimental groups

	Sham	Control	AM
Body weight, g	236±2	197±2*	199±2*
Ventricular weight, g	0.70±0.01	1.28±0.02*	1.15±0.03*†
Lung/body weight	4.9±0.4	4.9±0.5	5.0±0.8
Heart rate, bpm	432±10	373±11	393±6
MAP, mm Hg	103±3	77±5*	93±3†
LVSP, mm Hg	127±3	103±5*	117±3†
LVEDP, mm Hg	4±1	21±5*	14±3

Sham, sham rats given vehicle; Control, myosin-treated rats given vehicle; AM, myosin-treated rats given AM; MAP, mean arterial pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure. Data are mean±S.E. *$P<0.05$ *vs.* sham; †$P<0.05$ *vs.* control. $n=7$ in each group.

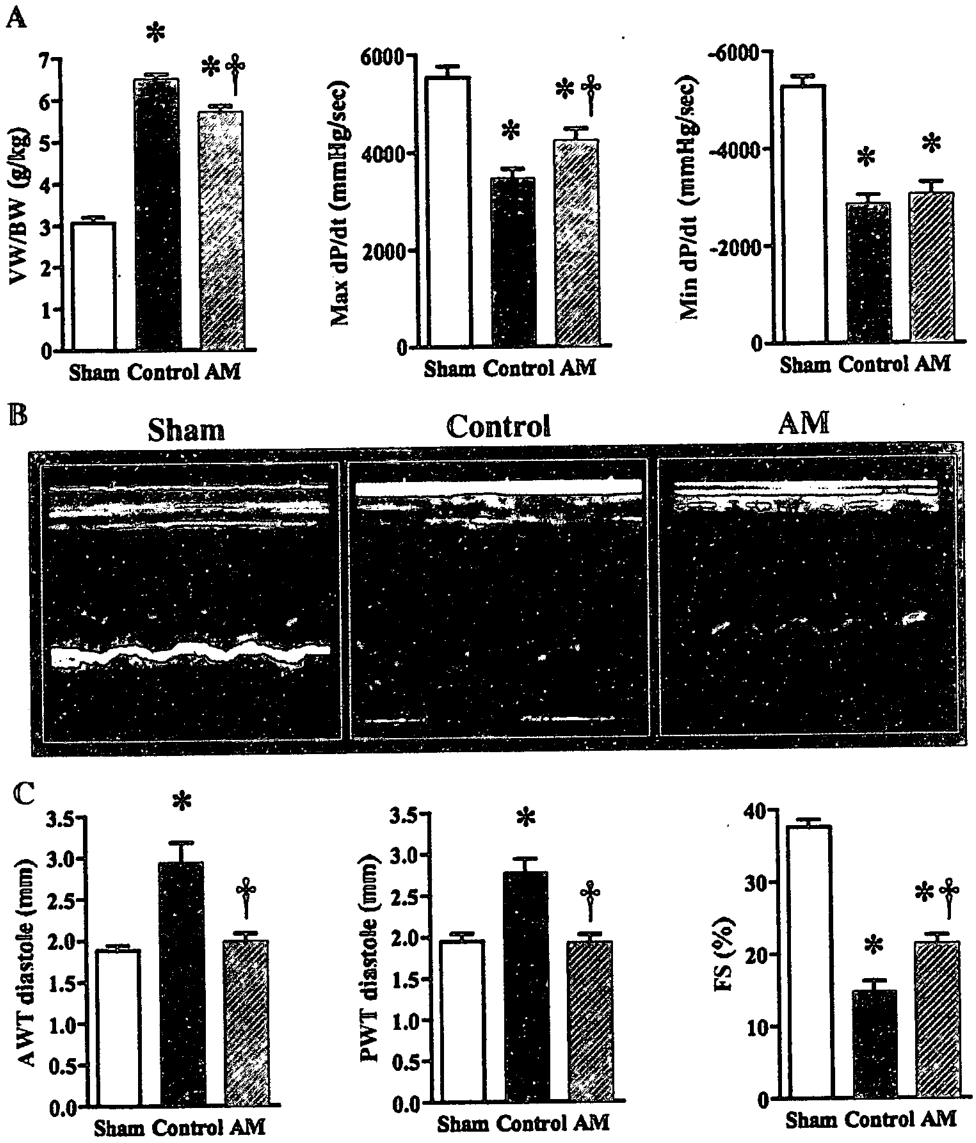

Fig. 6. Effects of AM infusion on physiologic properties and hemodynamic parameters. A: Effects of AM infusion on physiologic properties ($n=7$ in each group). B: Representative echocardiographic images show wall thickening and poor movement in myocarditis and improvement with AM treatment. C: Effects of AM infusion on echocardiographic findings ($n=10$ in each group). VW/BW, ventricular weight/body weight ratio; Max dP/dt, maximum dP/dt; Min dP/dt, minimum dP/dt; AWT, anterior wall thickness; PWT, posterior wall thickness; %FS, %fractional shortening. Data are mean±S.E. *, $P<0.05$ vs. sham; †, $P<0.05$ vs. control.

ANOVA, followed by Newman–Keuls' test. Comparisons of parameters between two groups were made by Student's t-test. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Histopathological improvement after AM infusion

Sections of left ventricular tissue demonstrated substantial myocardial necrosis, infiltration of inflammatory cells and edema in the control group, which was significantly limited primarily to areas directly adjacent to arterial vessels with AM treatment (Fig. 1, panel A). Blinded histological grading confirmed decreased myocyte necrosis, granulation, inflammation and tissue edema in the AM group as compared in the control group (Fig. 1, panel B). Picrosirius red staining revealed increased collagen deposition in the control group (Fig. 1, panel C). However, AM infusion attenuated collagen deposition in the myocardium (Fig. 1, panel D).

Table 2
Echocardiographic findings

	Sham	Control	AM
LVDd, mm	4.9±0.1	6.5±0.2*	7.3±0.3*
LVDs, mm	3.1±0.1	5.7±0.2*	5.7±0.3*
EF, %	75±1	35±3*	52±2*†

Sham, sham rats given vehicle; Control, myosin-treated rats given vehicle; AM, myosin-treated rats given AM; LVDd, left ventricular diastolic dimension; LVDs, left ventricular systolic dimension; EF, ejection fraction. Data are mean± S.E. *$P<0.05$ vs. sham; †$P<0.05$ vs. control. $n=10$ in each group.

3.2. Infiltration of CD68-positive cells in myocardium

A significant decrease in infiltration of CD68-positive inflammatory cells was observed in the AM group as compared to the control group (790±80 *vs.* 1468±109 cells/mm^2; Fig. 2, panel A and B). Sham tissues showed little or no myocardial CD68 positivity (data not shown).

3.3. Expression of MCP-1 after AM infusion

The expression of MCP-1 was increased in myocarditis; it was localized to the vascular endothelium and also in myocytes surrounding and adjacent to the areas of inflammation (Fig. 3, panel A). Heart sections in the AM group showed a partial decrease in MCP-1 expression. Serum MCP-1 level was greatly increased in the control group, whereas a significant decrease was observed in the AM group (Fig. 3, panel B).

3.4. Effects of AM infusion on MMPs and TGF-β expression

Western blotting analysis revealed that myocardial levels of MMP-2 and -9 were significantly increased in the control group. MMP-2 expression was markedly decreased by AM infusion, and MMP-9 expression tended to be decreased after AM infusion (Fig. 4, panel A). Quantitative real-time RT-PCR analysis demonstrated increased expression of TGF-β in the heart of the control group which was significantly attenuated by AM treatment (Fig. 4, panel B). AM infusion did not significantly influence cardiac expression of IL-1β and TNF-α (data not shown).

3.5. Angiogenesis induced by AM infusion

To determine the effect of AM treatment on angiogenesis, vWF-stained heart sections were subjected to capillary density counting. Capillary density was increased in the control group, particularly in areas directly adjacent to tissue necrosis (1146±57 *vs.* 782±21 cells/mm^2, Fig. 5, panel A and B). However, in AM-treated tissues, capillary density was further significantly increased not only in the peri-necrotic areas but also in apparently healthy myocardium (1347±82 *vs.* 1146±57cells/mm^2), suggesting that stimulation of angiogenesis was further augmented by AM treatment.

3.6. Heart weight and hemodynamics after AM infusion

The physiological and catheter-derived functional properties on day 21 post-myosin injection are summarized in Table 1 and Fig. 6, panel A. Myocarditic hearts showed significantly increased heart weight to body weight ratio, which was decreased by AM treatment. AM treatment also significantly improved maximum d*P*/d*t*. For both minimum d*P*/d*t* and LVEDP, we did not find significant differences. On echocardiography, AM administration significantly attenuated increased wall thickness after acute myocarditis. AM significantly improved LV fractional shortening and ejection fraction, although LVDd did not significantly differ between control and AM groups (Table 2 and Fig. 6, panel B and C).

4. Discussion

In the present study, AM treatment showed the following effects in acute myocarditis: 1) reduced necrosis, inflammation and edema in the myocardium; 2) attenuated expression of MCP-1, MMP-2 and TGF-β; 3) increased capillary density suggestive of angiogenesis; and 4) improved cardiac function.

This experimental autoimmune myocarditis model is triphasic, consisting of an antigen priming phase from days 0 to 14, an autoimmune response phase from days 14 to 21, and a reparative phase thereafter, associated chronically with a dilated cardiomyopathy phenotype [20]. MCP-1 expression is increased in the heart from days 15 to 27 post-myosin injection, and serum MCP-1 level is elevated from days 15 to 24 [21]. We treated rats with AM at 1 week after myosin injection, corresponding to an early time point in the disease process. Pathological examination demonstrated that infusion of AM attenuated myocyte necrosis and inflammation in acute myocarditis. This observation was supported by a decrease in infiltration of CD68-positive inflammatory cells in the myocardium. Interestingly, both MCP-1 expressions in the myocardium and serum MCP-1 level were decreased after AM infusion. MCP-1 is a member of the C–C subfamily of chemokines with chemoattractant activity for major inflammatory cells such as monocytes and T lymphocytes [22], and this model of acute myocarditis has previously been shown to be associated with MCP-1 [21]. Thus, the decrease in CD68-positive cell infiltration in the myocardium following this treatment may be attributable to inhibition of MCP-1 production by AM. The inhibitory effect of AM on MCP-1 expression is consistent with a previous *in vitro* study showing that AM inhibited pressure-induced MCP-1 expression in mesangial cells [23]. Recently, it has been demonstrated that AM has anti-inflammatory effects through modulation of macrophage migration inhibitory factor secretion [24]. Importantly, overexpression of MCP-1 induces myocarditis and subsequent development of heart failure [25]. These findings suggest that the inhibitory effect on MCP-1 expression and subsequent anti-inflammatory effect of AM are possible mechanisms of the improvement in acute myocarditis.

We found a significant increase in heart weight to body weight ratio and wall thickness 3 weeks after myosin injection. These results indicate exaggerated edematous changes in myocarditic hearts. Infusion of AM reduced overall heart weight to body weight ratio and wall thickness in myocarditic hearts and attenuated histological edematous changes. Earlier studies have demonstrated that AM decreases vascular congestion and endothelial hyperpermeability in the heart [11], reduces hyperpermeability of cultured endothelial cells and inhibits pulmonary edema [26]. Thus, it is interesting to speculate that the attenuation of edematous changes in the heart may be attributable to reduction of endothelial hyperpermeability by AM.

In the present study, AM infusion significantly increased the capillary density in myocarditic hearts. In fact, earlier studies have demonstrated angiogenic properties of AM *in vitro* and *in vivo* [27–29]. Importantly, improvement in myocardial vascular supply has been shown to decrease necrosis and inflammation in viral myocarditis [30,31]. These results suggest that AM-induced angiogenesis in the myocardium may be responsible for the improvement in acute myocarditis, which was indicated by reduced necrosis and inflammation in myocarditic hearts.

As previously mentioned, experimental autoimmune myocarditis chronically develops into a dilated cardiomyopathy phenotype [20]. MMPs have been associated with left ventricular remodeling [32] and here we showed increased expression of MMP-2 and -9 as well as increased collagen deposition in myocarditic hearts. In the present study, AM treatment significantly reduced both MMP-2 expression and collagen deposition. In addition, our observation demonstrated that the expression of TGF-β, a profibrogenic factor, was also attenuated by AM treatment. It has been demonstrated that AM decreases the expression of TGF-β in experimental mesangioproliferative glomerulonephritis [33]. These results suggest that AM may have beneficial effects on myocardium, possibly through regulation of factors involved in LV remodeling. In the present study, LVDd did not significantly differ between the control and AM groups. However, it should be noted that AM significantly reduced wall thickness possibly due to reduction of myocardial edema, leading to a slight increase in the inner diameter of the LV and a significant increase in ejection fraction. The major effect of AM was to reduce myocardial edema but not remodeling, despite reducing biochemical markers of remodeling.

Earlier studies have shown that short-term infusion of AM decreases arterial pressure and increases cardiac output in patients with acute heart failure [5]. These findings suggest that the improvement in cardiac function after acute myocarditis may be mediated partly by the hemodynamic effects of AM. However, despite the well-characterized vasorelaxant properties of AM [4], there was a significant increase in mean arterial pressure after AM treatment in our model. These findings suggest that AM induced limited direct hemodynamic action. Taking these findings together, the improvement of cardiac function after AM treatment may have been mediated by the improvement of pathological findings including necrosis, inflammation and edema in the myocardium rather than by AM-induced hemodynamic effects.

In conclusion, infusion of AM improved cardiac function and pathological findings including inflammatory infiltration and edema in a rat model of acute myocarditis. The beneficial effects of AM may occur at least in part by inhibitory effects on MCP-1, MMP-2 and TGF-β, and by enhancement of angiogenesis after acute myocarditis. Thus, infusion of AM may be a potent therapeutic strategy for acute myocarditis.

Acknowledgements

This work was supported by research grants for Cardiovascular Disease (16C-6 and 17A-1) and Comprehensive Research on Aging and Health from the Ministry of Health, Labour and Welfare, the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO); and Health and Labor Sciences Research Grants (Human Genome Tissue Engineering 009).

References

[1] Levi D, Alejos J. Diagnosis and treatment of pediatric viral myocarditis. Curr Opin Cardiol 2001;16:77–83.

[2] Liu Z, Yuan J, Yanagawa B, Qiu D, McManus BM, Yang D. Coxsackievirus-induced myocarditis: new trends in treatment. Expert Rev Anti Infect Ther 2005;3:641–50.

[3] Feldman AM, McNamara D. Myocarditis. N Engl J Med 2000;343: 1388–98.

[4] Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun 1993;192:553–60.

[5] Nagaya N, Satoh T, Nishikimi T, Uematsu M, Furuichi S, Sakamaki F, et al. Hemodynamic renal and hormonal effects of adrenomedullin infusion in patients with congestive heart failure. Circulation 2000;101:498–503.

[6] Oya H, Nagaya N, Furuichi S, Nishikimi T, Ueno K, Nakanishi N, et al. Comparison of intravenous adrenomedullin with atrial natriuretic peptide in patients with congestive heart failure. Am J Cardiol 2000;86:94–8.

[7] Clementi G, Caruso A, Cutuli VM, Prato A, Mangano NG, Amico-Roxas M. Antiinflammatory activity of adrenomedullin in the acetic acid peritonitis in rats. Life Sci 1999;65:PL203–8.

[8] Okumura H, Nagaya N, Itoh T, Okano I, Hino J, Mori K, et al. Adrenomedullin infusion attenuates myocardial ischemia/reperfusion injury through the phosphatidylinositol 3-kinase/Akt-dependent pathway. Circulation 2004;109:242–8.

[9] Iwase T, Nagaya N, Fujii T, Itoh T, Ishibashi-Ueda H, Yamagishi M, et al. Adrenomedullin enhances angiogenic potency of bone marrow transplantation in a rat model of hindlimb ischemia. Circulation 2005;111: 356–62.

[10] Tsuruda T, Kato J, Kitamura K, Kuwasako K, Imamura T, Koiwaya Y, et al. Adrenomedullin: a possible autocrine or paracrine inhibitor of hypertrophy of cardiomyocytes. Hypertension 1998;31:505–10.

[11] Chu DQ, Smith DM, Brain SD. Studies of the microvascular effects of adrenomedullin and related peptides. Peptides 2001;22:1881–6.

[12] Kodama M, Matsumoto Y, Fujiwara M, Zhang SS, Hanawa H, Itoh E, et al. Characteristics of giant cells and factors related to the formation of giant cells in myocarditis. Circ Res 1991;69:1042–50.

[13] Kodama M, Matsumoto Y, Fujiwara M, Masani F, Izumi T, Shibata A. A novel experimental model of giant cell myocarditis induced in rats by immunization with cardiac myosin fraction. Clin Immunol Immunopathol 1990;57:250–62.

[14] Fairweather D, Kaya Z, Shellam GR, Lawson CM, Rose NR. From infection to autoimmunity. J Autoimmun 2001;16:175–86.

[15] Cunningham MW. T cell mimicry in inflammatory heart disease. Mol Immunol 2004;40:1121–7.

[16] O'Connell JBRD. Myocarditis and specific myocardial diseases. New York: McGraw-Hill; 1994. Pages.

[17] Yanagawa B, Spiller OB, Choy J, Luo H, Cheung P, Zhang HM, et al. Coxsackievirus B3-associated myocardial pathology and viral load reduced by recombinant soluble human decay-accelerating factor in mice. Lab Invest 2003;83:75–85.

[18] Nagaya N, Kangawa K, Itoh T, Iwase T, Murakami S, Miyahara Y, et al. Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. Circulation 2005;112:1128–35.

[19] Hanawa H, Abe S, Hayashi M, Yoshida T, Yoshida K, Shiono T, et al. Time course of gene expression in rat experimental autoimmune myocarditis. Clin Sci (Lond) 2002;103:623–32.

[20] Kodama M, Hanawa H, Saeki M, Hosono H, Inomata T, Suzuki K, et al. Rat dilated cardiomyopathy after autoimmune giant cell myocarditis. Circ Res 1994;75:278–84.

[21] Fuse K, Kodama M, Hanawa H, Okura Y, Ito M, Shiono T, et al. Enhanced expression and production of monocyte chemoattractant protein-1 in myocarditis. Clin Exp Immunol 2001;124:346–52.

[22] Rollins BJ. Chemokines. Blood 1997;90:909–28.

[23] Iwamoto M, Osajima A, Tamura M, Suda T, Ota T, Kanegae K, et al. Adrenomedullin inhibits pressure-induced mesangial MCP-1 expression through activation of protein kinase A. J Nephrol 2003;16:673–81.

[24] Wong LY, Cheung BM, Li YY, Tang F. Adrenomedullin is both proinflammatory and antiinflammatory: its effects on gene expression and secretion of cytokines and macrophage migration inhibitory factor in NR8383 macrophage cell line. Endocrinology 2005;146:1321–7.

[25] Kolattukudy PE, Quach T, Bergese S, Breckenridge S, Hensley J, Altschuld R, et al. Myocarditis induced by targeted expression of the MCP-1 gene in murine cardiac muscle. Am J Pathol 1998;152:101–11.

[26] Hippenstiel S, Witzenrath M, Schmeck B, Hocke A, Krisp M, Krull M, et al. Adrenomedullin reduces endothelial hyperpermeability. Circ Res 2002;91:618–25.

[27] Kim W, Moon SO, Sung MJ, Kim SH, Lee S, Kim HJ, et al. Protective effect of adrenomedullin in mannitol-induced apoptosis. Apoptosis 2002;7:527–36.

[28] Tokunaga N, Nagaya N, Shirai M, Tanaka E, Ishibashi-Ueda H, Harada-Shiba M, et al. Adrenomedullin gene transfer induces therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia: benefits of a novel nonviral vector gelatin. Circulation 2004;109:526–31.

[29] Nagaya N, Mori H, Murakami S, Kangawa K, Kitamura S. Adrenomedullin: angiogenesis and gene therapy. Am J Physiol Regul Integr Comp Physiol 2005;288:R1432–7.

[30] Lee JK, Zaidi SH, Liu P, Dawood F, Cheah AY, Wen WH, et al. A serine elastase inhibitor reduces inflammation and fibrosis and preserves cardiac function after experimentally-induced murine myocarditis. Nat Med 1998;4:1383–91.

[31] Ono K, Matsumori A, Shioi T, Furukawa Y, Sasayama S. Contribution of endothelin-1 to myocardial injury in a murine model of myocarditis: acute effects of bosentan an endothelin receptor antagonist. Circulation 1999;100:1823–9.

[32] Tsuruda T, Costello-Boerrigter LC, Burnett JC. Matrix metalloproteinases: pathways of induction by bioactive molecules. Heart Fail Rev 2004;9:53–61.

[33] Plank C, Hartner A, Klanke B, Geissler B, Porst M, Amann K, et al. Adrenomedullin reduces mesangial cell number and glomerular inflammation in experimental mesangioproliferative glomerulonephritis. Kidney Int 2005;68:1086–95.

Mesenchymal stem cells for the treatment of heart disease

Kenichi Yamahara[1] & Noritoshi Nagaya[1†]

[†]Author for correspondence
[1]Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, 5–7-1 Fujishirodai, Suita, Osaka 565–8565, Japan
Tel.: +81 668 335 012;
Fax: +81 668 339 865;
E-mail: nnagaya@ri.ncvc.go.jp

Mesenchymal stem cells are capable of dividing and differentiating into one of several mesenchymal phenotypes, such as osteoblasts, chondrocytes and adipocytes. Recent evidence suggests that mesenchymal stem cells can also express characteristics of endothelial, neural, smooth muscle and skeletal cells and cardiomyocytes. In addition, these mesenchymal stem cells secrete a variety of cytokines and growth factors that have both paracrine and autocrine activities. When transplanted into the infarcted heart, mesenchymal stem cells prevent remodeling and improve cardiac function, although further investigation to seek the desirable method for mesenchymal stem cell transplantation is still needed. Recently, using cell sheet technology, we reported that monolayered mesenchymal stem cells have multipotent and self-propagating properties after transplantation to infarcted heart. In this work, we review evidence and new prospects to support the therapeutic potential of mesenchymal stem cells for the treatment of heart disease.

Keywords: cardiomyoplasty, cell therapy, mesenchymal stem cells, myocardial infarction

Despite recent progress in treatment, coronary heart disease and heart failure are the major causes of morbidity and mortality in the Western world. Therefore, cell therapy is currently emerging as a new potential treatment for these diseases. Recently, various cell types have been explored experimentally and clinically for cardiac repair. With the use of animal models, improvement of ventricular function after acute myocardial infarction (MI) was observed after injection of bone marrow (BM)-derived stem/progenitor cells [1]. However, it was not clear whether the beneficial effect induced by the grafted cells was elicited by hematopoietic stem cells, cardiac progenitor cells, endothelial progenitor cells or mesenchymal stem cells (MSCs). Several recent animal and preclinical data have shown that BM-derived MSCs appear to be a suitable cell source for cardiac regenerative medicine. This article presents an overview of the current experimental evidence on how MSCs may contribute to myocardial repair and their applications in the clinical arena.

Cellular, molecular and preclinical data have demonstrated that BM-derived MSCs represent a suitable cell source for regenerative purposes for treating the failing heart. MSCs are rare in BM, representing approximately 1 in 10,000 nucleated cells. They are isolated from the BM by a simple process using adhering cell culture. The resulting cells have the ability to expand many-fold in culture and retain their growth and multilineage potential [2,3]. However, the clinical use of BM-derived MSCs has presented several problems, including pain and morbidity. Recently, MSCs have been isolated from adipose tissue [4–7], which is typically abundant in individuals with cardiovascular disease and is easy to isolate. Furthermore, we reported the therapeutic potency of adipose tissue-derived MSCs for the treatment of MI [8]. Therefore, adipose tissue may serve as an alternative source of MSCs for the treatment of heart disease.

Under proper stimulation, MSCs can be induced to differentiate into adipocytes, osteoblasts, chondrocytes, cardiomyocytes and hematopoietic-supporting stromal cells [2,9–11]. Furthermore, MSCs may also give rise to other lineages, such as endothelial, renal and neural, revealing a high degree of plasticity [12–14], as well as a high *ex vivo* expansion capacity. This property has been used to initiate studies toward the use of MSCs in clinical strategies [15,16]. Several reports have shown that MSCs, once exposed to a variety of physiologic or nonphysiologic stimuli, can differentiate into cells displaying several features of cardiomyocyte-like cells [17–19]. Furthermore, some researchers have reported that MSCs differentiate not only into cardiomyocytes, but also into vascular smooth muscle cells/pericytes and endothelial cells [20–23]. Thus, these data strongly suggest that MSCs are an ideal donor source of a vast repertoire of cardiovascular cells for

10.2217/17460751.2.2.xxx © 2007 Future Medicine Ltd ISSN 1746-0751

patients with heart failure. Furthermore, the mechanisms underlying the improvement in neovascularization and cardiac function involve the paracrine secretion of growth factors by MSCs [24–26].

We have demonstrated, using cell sheet technology, that monolayered MSCs have multipotent and self-propagating properties, as well as being a paracrine source to trigger angiogenesis after transplantation into infarcted rat hearts [8]. Adipose tissue-derived MSCs were cultured on a temperature-responsive dish. After 4 weeks following coronary ligation, the monolayered MSCs were transplanted onto the scarred myocardium. The engrafted sheet grew gradually *in situ* to become a thick stratum that included newly formed vessels, few cardiomyocytes and undifferentiated cells. The MSC sheet also acted via paracrine pathways to trigger angiogenesis. The monolayered MSCs reversed wall thinning in the scar area and improved cardiac function in rats with MI. Thus, transplantation of monolayered MSCs may be a new therapeutic strategy for cardiac tissue regeneration.

Amado and colleagues reported a randomized study of BM-derived MSC transplantation in pigs after MI [27]. They demonstrated that allogeneic MSCs could be safely and effectively delivered via a percutaneous injection catheter to a region of damaged myocardium. Their MSC transplantation resulted in long-term engraftment, a marked reduction in scar formation and near-normalization of cardiac function. Since MSCs have the ability to evade immunological rejection [28,29], the use of allogenic MSCs might provide a valuable strategy for cardiac regenerative therapy that avoids the need for preparing autologous cells from the recipient.

Recently, Chen and colleagues conducted a randomized clinical study to investigate the effectiveness of intracoronary injection of autologous MSCs in patients with acute MI [30]. They demonstrated a significant and sustained improvement in global left ventricular ejection fraction, in addition to providing evidence that intracoronary infusion of MSC does not produce any cell size-related adverse effect. Now, MSC-based therapy seems to be one of the most promising cellular therapies for treating chronic heart failure, which is often caused by MI.

Conclusion

Although MSCs possess great promise for the repair of damaged heart, many questions concerning MSC transplantation still remain to be answered. Future work is required to seek the desirable method of MSC transplantation for the treatment of cardiac diseases. Determination of optimal cell numbers, route, technique and time course of delivery will have to be made to maximize recovery of cardiac function. However, there is a wealth of preclinical and early clinical data showing safety, feasibility and early efficacy using MSCs. The ongoing clinical trials will contribute greatly to this emerging and exciting new therapeutic approach for diseases of the cardiovascular system.

Executive summary

- Recent preclinical data have demonstrated that mesenchymal stem cells appear to be a suitable cell source for regenerative purpose for treating the failing heart.
- We reported that, using cell sheet technology, monolayered mesenchymal stem cells have multipotent and self-propagating properties after transplantation to infarcted heart.
- However, for clinical application, future work is required to seek the most desirable method of mesenchymal stem cell transplantation for the treatment of cardiac disease.

Bibliography

1. Orlic D, Kajstura J, Chimenti S *et al.*: Bone marrow cells regenerate infarcted myocardium. *Nature* 410(6829), 701–705 (2001).
2. Pittenger MF, Mackay AM, Beck SC *et al.*: Multilineage potential of adult human mesenchymal stem cells. *Science* 284(5411), 143–147 (1999).
3. Muraglia A, Cancedda R, Quarto R: Clonal mesenchymal progenitors from human bone marrow differentiate *in vitro* according to a hierarchical model. *J. Cell Sci.* 113(Pt 7), 1161–1166 (2000).
4. Rangappa S, Fen C, Lee EH, Bongso A, Sim EK, Wei EK: Transformation of adult mesenchymal stem cells isolated from the fatty tissue into cardiomyocytes. *Ann. Thorac. Surg.* 75(3), 775–779 (2003).
5. Zuk PA, Zhu M, Ashjian P *et al.*: Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell* 13(12), 4279–4295 (2002).
6. Gaustad KG, Boquest AC, Anderson BE, Gerdes AM, Collas P: Differentiation of human adipose tissue stem cells using extracts of rat cardiomyocytes. *Biochem. Biophys. Res. Commun.* 314(2), 420–427 (2004).
7. Planat-Benard V, Silvestre JS, Cousin B *et al.*: Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. *Circulation* 109(5), 656–663 (2004).

8. Miyahara Y, Nagaya N, Kataoka M *et al.*: Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. *Nat. Med.* 12(4), 459–465 (2006).
9. Conget PA, Minguell JJ: Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. *J. Cell Physiol.* 181(1), 67–73 (1999).
10. Deans RJ, Moseley AB: Mesenchymal stem cells: biology and potential clinical uses. *Exp. Hematol.* 28(8), 875–884 (2000).
11. Minguell JJ, Erices A, Conget P: Mesenchymal stem cells. *Exp. Biol. Med. (Maywood)* 226(6), 507–520 (2001).
12. Ito T, Suzuki A, Okabe M, Imai E, Hori M: Application of bone marrow-derived stem cells in experimental nephrology. *Exp. Nephrol.* 9(6), 444–450 (2001).
13. Jiang Y, Jahagirdar BN, Reinhardt RL *et al.*: Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 418(6893), 41–49 (2002).
14. Neuhuber B, Gallo G, Howard L, Kostura L, Mackay A, Fischer I: Reevaluation of *in vitro* differentiation protocols for bone marrow stromal cells: disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype. *J. Neurosci. Res.* 77(2), 192–204 (2004).
15. Horwitz EM, Prockop DJ, Fitzpatrick LA *et al.*: Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat. Med.* 5(3), 309–313 (1999).
16. Barry FP, Murphy JM: Mesenchymal stem cells: clinical applications and biological characterization. *Int. J. Biochem. Cell Biol.* 36(4), 568–584 (2004).
17. Wakitani S, Saito T, Caplan AI: Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. *Muscle Nerve* 18(12), 1417–1426 (1995).
18. Makino S, Fukuda K, Miyoshi S *et al.*: Cardiomyocytes can be generated from marrow stromal cells *in vitro*. *J. Clin. Invest.* 103(5), 697–705 (1999).
19. Bittira B, Kuang JQ, Al-Khaldi A, Shum-Tim D, Chiu RC: *In vitro* preprogramming of marrow stromal cells for myocardial regeneration. *Ann. Thorac. Surg.* 74(4), 1154–1159 (2002).
20. Hellström M, Kalén M, Lindahl P, Abramsson A, Betsholtz C: Role of PDGF-B and PDGFR-β in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development* 126(14), 3047–3055 (1999).
21. Gojo S, Gojo N, Takeda Y *et al.*: *In vivo* cardiovasculogenesis by direct injection of isolated adult mesenchymal stem cells. *Exp. Cell Res.* 288(1), 51–59 (2003).
22. Davani S, Marandin A, Mersin N *et al.*: Mesenchymal progenitor cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a rat cellular cardiomyoplasty model. *Circulation* 108(Suppl. 1), II253–II258 (2003).
23. Nagaya N, Kangawa K, Itoh T *et al.*: Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. *Circulation* 112(8), 1128–1135 (2005).
24. Kinnaird T, Stabile E, Burnett MS *et al.*: Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote *in vitro* and *in vivo* arteriogenesis through paracrine mechanisms. *Circ. Res.* 94(5), 678–685 (2004).
25. Tang YL, Zhao Q, Zhang YC *et al.*: Autologous mesenchymal stem cell transplantation induce VEGF and neovascularization in ischemic myocardium. *Regul. Pept.* 117(1), 3–10 (2004).
26. Vandervelde S, van Luyn MJ, Tio RA, Harmsen MC: Signaling factors in stem cell-mediated repair of infarcted myocardium. *J. Mol. Cell Cardiol.* 39(2), 363–376 (2005).
27. Amado LC, Saliaris AP, Schuleri KH *et al.*: Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. *Proc. Natl Acad. Sci. USA* 102(32), 11474–11479 (2005).
28. Bartholomew A, Sturgeon C, Siatskas M *et al.*: Mesenchymal stem cells suppress lymphocyte proliferation *in vitro* and prolong skin graft survival *in vivo*. *Exp. Hematol.* 30(1), 42–48 (2002).
29. Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC: Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation* 75(3), 389–397 (2003).
30. Chen SL, Fang WW, Ye F *et al.*: Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. *Am. J. Cardiol.* 94(1), 92–95 (2004).