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

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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-macroglobulin 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).

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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

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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

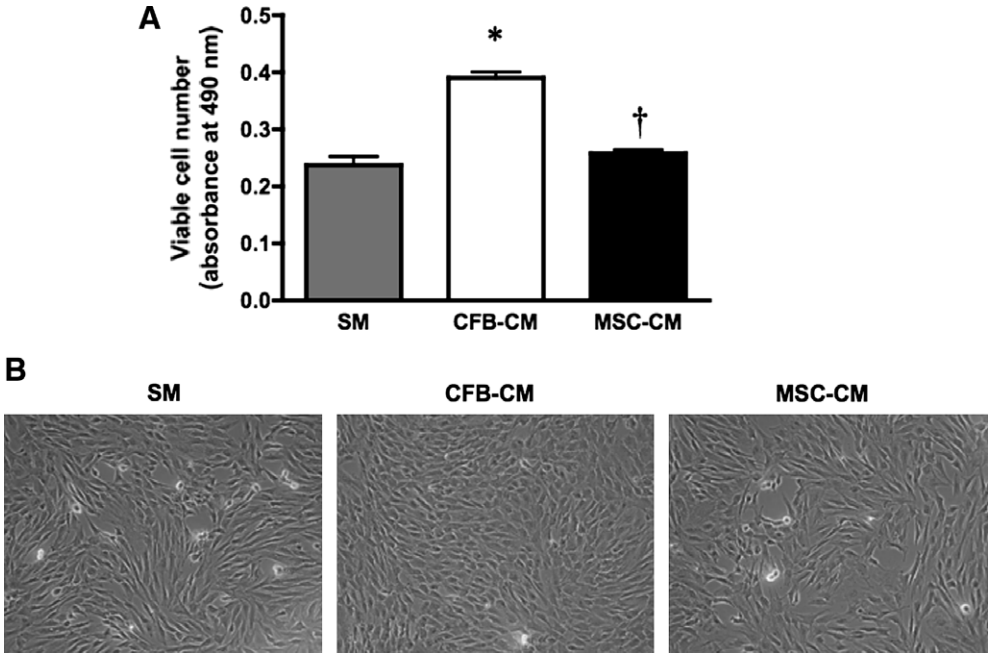

Fig. 1. Effect of MSC-conditioned medium on CFB proliferation MTS assay (A) and representative photographs (B) of CFB after 48 h of culture in the indicated medium. SM, standard medium; CM, conditioned medium. Values are means ± S.E. $^{*}P < 0.05$ vs SM, $^{\dagger}P < 0.05$ vs CFB-CM.

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).

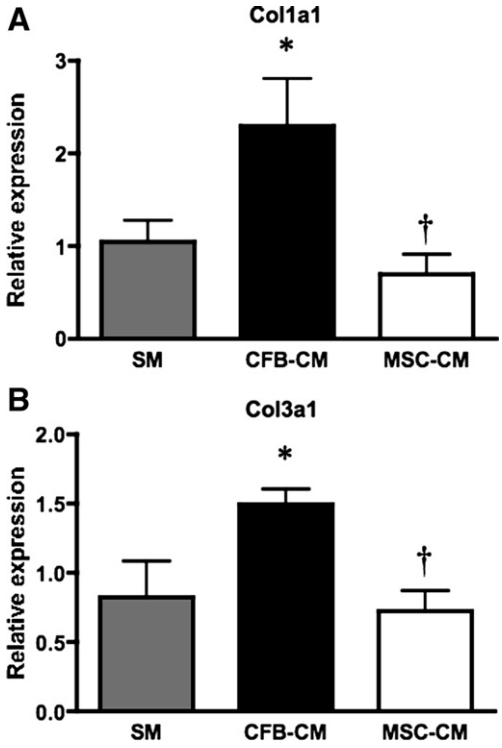

Fig. 2. Effect of MSC-conditioned medium on collagen gene expression (A) Quantitative RT-PCR for Col1a1 expression in CFB after 48 h of culture in the indicated medium. (B) Quantitative RT-PCR for Col3a1 expression in CFB after 48 h of culture in the indicated medium. SM, standard medium; CM, conditioned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

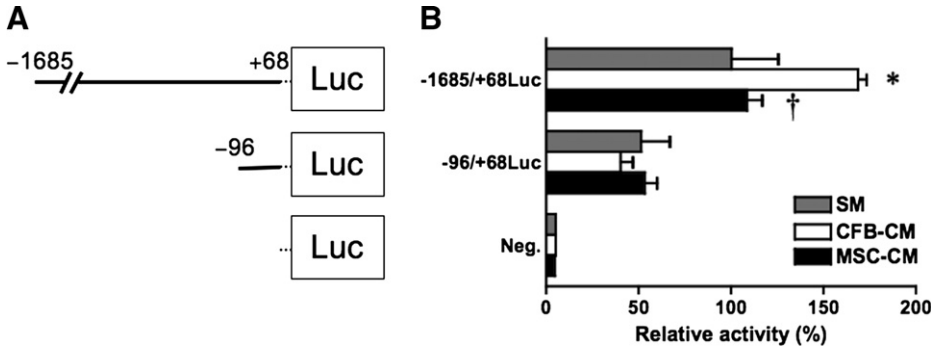

Fig. 3. Effect of MSC-conditioned medium on type III collagen gene promoter activity (A) Schematic illustration of 5′-deletion constructs of the Col3a1 promoter. (B) Luficerase activity in CFB after 48 hours of transfection of reporter plasmids and culture in the indicated medium. All constructs were 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.

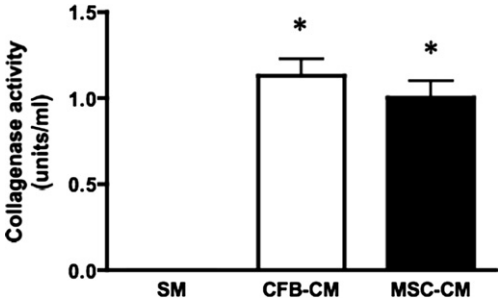

Fig. 4. Effect of MSC-conditioned medium on collagenase activity Type I collagenase activity of the indicated medium. SM, standard medium; CM, conditioned medium. Values are means ± S.E. $^{*}P < 0.05$ vs SM.

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.

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