

The conditioned medium of human mesenchymal stromal cells reduces irradiation-induced damage in cardiac fibroblast cells

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ABSTRACT

Recently, multipotent mesenchymal stromal cell (MSC) treatment has attracted special attention as a new alternative strategy for stimulating regeneration. Irradiation myocardial fibrosis (IMF) is a major complication associated with total body irradiation for hematopoietic stem cell transplantation, nuclear accidents, and thoracic radiotherapy for lung cancer, esophageal cancer, proximal gastric cancer, breast cancer, thymoma, and lymphoma. The aim of the present study was to assess the therapeutic paracrine effects of human umbilical cord-derived mesenchymal stromal cells (UC-MSCs) in the cell model of IMF. For this purpose, primary human cardiac fibroblasts (HCF) cells were irradiated and cultured with the conditioned medium of UC-MSCs (MSCCM). MSCCM promoted cell viability, reduced collagen deposition as measured by Sircol assay and qPCR (*Col1A1* and *Col1A2*), prevented oxidative stress and increased antioxidant status (as measured by malondialdehyde content and the activities and mRNA levels of antioxidant enzymes), and reduced pro-fibrotic TGF- β 1, IL-6 and IL-8 levels (as examined by ELISA kit and qPCR). Pretreatment with inhibitor of NF- κ B led to a decrease in the levels of TGF- β 1 in cell lysate of HCF cells by ELISA kit. Furthermore, we also found that MSCCM prevented NF- κ B signaling pathway activation for its proinflammatory actions induced by irradiation. Taken together, our data suggest that MSCCM could reduce irradiation-induced TGF- β 1 production through inhibition of the NF- κ B signaling pathway. These data provide new insights into the functional actions of MSCCM on irradiation myocardial fibrosis.

Keywords: irradiation myocardial fibrosis; mesenchymal stromal cell; oxidative damage; NF- κ B signaling pathway

INTRODUCTION

Irradiation myocardial fibrosis (IMF) is a major complication associated with total body irradiation for hematopoietic stem cell transplantation, nuclear accidents, and thoracic radiotherapy for lung cancer, esophageal cancer, proximal gastric cancer, breast cancer, thymoma, and lymphoma [1]. IMF, which is characterized by inflammatory cell infiltration, fibroblast proliferation, and excessive deposition of extracellular matrix (ECM) proteins in heart parenchyma, is a chronic, progressive and fatal interstitial heart disease with a poor prognosis and ineffective response to available medical therapies.

This complication, when it occurs, usually develops several years after radiation exposure in humans and ~6 months post-irradiation in the rat model [1]. Recent clinical data have demonstrated that the incidence of irradiation-related heart disease among cancer survivors who have received radiotherapy is at 10% and 30% 5 and 10 years after treatment [2]. The current clinical treatment for IMF primarily involves drugs such as steroids or non-steroidal anti-inflammatory agents and immunosuppressive agents. These drugs cannot effectively mitigate fibrosis. In addition, immunosuppressive agents can cause serious side effects, including death. Hence, many

innovative strategies for repair of the heart have been developed and tested in the past decades.

Recently, multipotent mesenchymal stromal cell (MSC) treatment has attracted special attention as a new alternative strategy for stimulating regeneration. MSCs are primitive cells originating from the mesodermal germ layer and are classically described as giving rise to connective tissues, skeletal muscle cells, and cells of the vascular system [3]. MSCs may exhibit immunosuppressive properties, and it has been suggested that they are ‘immune-privileged’, i.e. protected from rejection, potentially permitting their use in allogeneic transplantation. It appears that MSCs have the capacity to localize to injured tissue, where they can differentiate into specific cell types [4]. Some reports suggest that the therapeutic effects of MSCs in injured tissue are mainly mediated by paracrine activity, including the stimulation of endogenous repair, angiogenesis and arteriogenesis; attenuation of remodeling; and reduction of apoptosis [5]. Direct evidence for a paracrine role of MSCs has been obtained and confirmed in studies utilizing animal models of lung [6], heart [7], and liver [8] injury, which showed that conditioned medium (CM) generated from MSCs significantly reduced damage and stimulated regeneration *in vivo*. Downregulation of pro-inflammatory cytokines and upregulation of anti-inflammatory cytokines have been proposed as paracrine effects of MSCs [8]. In addition, injected murine MSCs, but not human MSCs, differentiated into osteosarcomas in injured lungs. Thus, human MSCs appear to be more feasible and safer for use than murine MSCs [9].

The aim of the present study was to assess the therapeutic paracrine effects of human umbilical cord-derived mesenchymal stromal cells (UC-MSCs) in the cell model of IMF. In the present study, we showed that the conditioned medium of UC-MSCs (MSCCM) counteracts irradiation-induced oxidative damage in primary human cardiac fibroblast (HCF) cells. Analysis of mRNA and activity levels of antioxidant enzymes, collagen generation, pro-fibrotic and anti-fibrotic cytokines, and the NF- κ B signaling pathway allow us to propose that such an effect is rendered via modulation of the NF- κ B signaling pathway. To the best of our knowledge, this is the first clinically based translational study to assess and highlight human MSCCM therapy for the treatment of IMF.

MATERIALS AND METHODS

Primary human cardiac fibroblasts cells culture

Primary HCF cells were purchased from ScienCell Research Laboratories (San Diego, CA) and were maintained in a humidified incubator at 37°C with 5% CO₂ in Dulbecco’s Modified Eagle’s Medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA) and 1% penicillin-streptomycin (Beyotime Biotechnology, Nantong, China). At 70–80% confluence, the complete medium was replaced with medium containing 1% FBS.

UC-MSCs and conditioned medium of UC-MSCs preparation

UC-MSCs were collected from the human umbilical cord with informed consent of the mother. The collection was performed in accordance with the ethical standards of the local ethics committee.

UC-MSCs were successfully isolated from the Wharton’s jelly (WJ) of umbilical cords according to methods described in previous reports [10–12]. The umbilical cords were rinsed in phosphate-buffered saline (PBS) until the cord blood was cleared, and the blood vessels were removed. The remaining WJ tissue was cut into 1–2 mm³ pieces and placed in six-well plates in the presence of 0.1% collagenase type II (Sigma, USA) in PBS at 37°C for 1 h. Ten percent FBS (Invitrogen, USA) was then added to stop the digestion. The dissociated mesenchymal cells were dispersed in 10% FBS-DMEM and further cultured until well-developed colonies of the fibroblast-like cells reached 80% confluence. Then, the cultures were trypsinized with 0.25% trypsin-EDTA (Invitrogen, USA) and passaged into new flasks for further expansion. The multipotent differentiation capacity of the UC-MSCs was confirmed by their differentiation into osteoblasts and adipocytes using alkaline phosphatase (osteoblasts) and Oil Red O staining (adipocytes), respectively. UC-MSCs cells were maintained in DMEM containing 10% FBS in uncoated 25 cm² culture flasks (Corning, New York, USA) at 37°C in 5% CO₂. When the cell culture reached about 70–80% confluence, the MSCCM was replaced with medium containing 1% FBS and cultured for 2–3 days, then collected, filtered through a membrane of 0.2 mm pore, aliquoted and stored at -20°C [13]. The same method was used to generate the conditioned medium from MRC-5 cells as a control, called MRCCM.

HCF cells irradiation and MSCCM treatment

HCF cells at 70–80% confluence were irradiated with 8-Gy gamma rays using a ⁶⁰Co source (Model GWXJ80, NPIC, Chengdu, China) at a dose rate of ~130 cGy/min. After irradiation, the culture medium was replaced and cultured with MSCCM for 7 days. Cells cultured without any treatment were used as controls. In some experiments, HCF cells were pre-incubated either with NF- κ B inhibitor MG-132 (5 μM; Calbiochem, San Diego, CA), or TGF- β 1 type I receptor (T β RI) inhibitor SB 431 542 (10 μM; Sigma-Aldrich, St Louis, MO) for 60 min before irradiation.

CCK8 assay

Cell viability was measured by CCK8 assay (cell counting kit-8, Dojindo Molecular Technologies, Tokyo, Japan). HCF cells were seeded at 1 × 10⁴ cells per well in 100 μl of complete growth culture media and treated as described above. Finally, CCK-8 solution (10 μl/well) was added to the wells. After 2 h incubation at 37°C, the absorbance of each well was determined at 450 nm using a microplate reader.

Analysis of lipid peroxidation

HCF cells were treated as described above, after which we analyzed cell lipid peroxidation. Polyunsaturated lipids are susceptible to an oxidative attack, typically by reactive oxygen species (ROS), resulting in a chain reaction, with the production of end products such as malondialdehyde (MDA). We determined lipid peroxidation by quantifying the amount of cellular MDA via the measurement of a red complex produced during the reaction of thiobarbituric acid (TBA) with MDA. A microplate reader (UV-7504, Shanghai, China) was used to measure the absorbance of cellular MDA at

532 nm, and the MDA content was calculated according to the detailed instructions of the MDA assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Enzyme activity assays

HCF cells were treated as described above, after which we assayed the enzyme activity. The activities of three enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), were determined using commercial kits according to the manufacturer's protocols (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Enzyme activity assays were carried out using a UV-visible spectrophotometer (UV-7504, Shanghai, China).

Measurement of collagen content

HCF cells were treated as described above, and collected and examined for the amount of collagen using a Sircol soluble collagen assay kit (Biocolor, Belfast, North Ireland).

Cytokine expression in HCF cells

The levels of transforming growth factor- β 1 (TGF- β 1), interleukin-6 (IL-6), interleukin-8 (IL-8) and interferon- γ (IFN- γ) in cell lysate of HCF cells were determined using commercially available ELISA kits according to the manufacturer's protocols (Boster Biological Technology, Wuhan, China). The optical density (OD) value was determined by an ELISA reader at a wavelength of 450 nm and calculated in the linear part of the curve.

Measurement of NF- κ B p65

HCF cells were treated and collected as described above, nuclear extracts and cytoplasmic extracts were prepared by Nuclear Extract Kit (Active Motif, Carlsbad, CA), and NF- κ B p65 were detected by ELISA kit (BioSource and Active Motif, respectively).

Real-time fluorescence polymerase chain reaction

HCF cells were treated and collected as described above. Total RNA was extracted from HCF cells with Trizol reagent (TaKaRa, Tokyo, Japan). First strand complementary DNA (cDNA) was synthesized using the Reverse Transcription Kit (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. For quantitative PCR (q-PCR), a 10 μ l reaction system including 5 μ l 2×SYBR Green (TaKaRa, Tokyo, Japan), 0.8 μ l cDNA templates, and a 0.8 μ l q-PCR primers set were used. The samples were run and analyzed in triplicate by CFX Connect Real-Time System (Bio-Rad, Hercules, USA). The q-PCR conditions were as follows: an initial 3 min denaturation step at 95°C, followed by 40 cycles of 95°C for 5 s, 58°C for 30 s, and 72°C for 30 s. Melting curve analysis showed a single amplification peak for each reaction. Cycle threshold values for targets were expressed as relative expression compared with the average for housekeeping genes (GAPDH). The expression of each mRNA was calculated as $2^{-\Delta\Delta Ct}$.

Statistical analysis

All experiments were conducted at least in triplicate, and representative data are expressed as the mean \pm SD. The comparisons were

evaluated by one-way analysis of variance and for those significant, *post-hoc* multiple comparisons between means were realized using the Tukey test. All statistical analyses were performed using SPSS statistics software, and values of $P < 0.05$ were considered to be significant.

RESULTS

Characterization of UC-MSCs and MSCCM rescued HCFs from irradiation-induced cell death

The UC-MSCs exhibited similar spindle- and fibroblast-like shapes (Fig. 1A). The multipotent differentiation capacity of the UC-MSCs was confirmed by their differentiation into adipocytes, osteoblasts and chondroblasts, as shown by the staining of the *in vitro* differentiation cultures with Oil Red O (Fig. 1B, adipocytes), alkaline phosphatase (Fig. 1C, osteoblasts), and alcian blue (Fig. 1D, chondroblasts).

Our preliminary data showed that no obvious damage was observed in HCF cells that had undergone 2 Gy or 4 Gy radiation, but nearly all HCF cells died with 16 Gy radiation (data not shown). Thus, we used 8 Gy to induce cell damage in the present study. Whereas cell viability for irradiation-treated cells was significantly lower than that of control cells (CTRLs), MSCCM significantly reduced irradiation-induced cell death compared with irradiation only-treated cells (Fig. 1E). Cell death in irradiation-induced HCF cells was not affected by inhibitors of NF- κ B or T β R1 (Fig. 1E). No beneficial potential was observed in MRCCM (Fig. 1E).

MSCCM modulates the redox state in HCFs

We also asked whether treatment could restore antioxidant status, by determining the enzymatic activities and gene expression of SOD, CAT and GPx. Exposure to irradiation resulted in significantly lower levels of total SOD, CAT and GPx enzymatic activities, compared with non-treated cells. These activities significantly increased in irradiation + MSCCM-treated HCF cells (Fig. 2A). After irradiation, the gene expression level ($2^{-\Delta\Delta Ct}$) of SOD1, SOD2, CAT and GPx suffered a significant reduction. Such expression levels were partially restored by MSCCM, with a significant increase in irradiation + MSCCM-treated HCF cells compared with the levels in irradiation-only-treated cells (Fig. 2B).

Because oxidative stress has been shown to trigger and sustain the pathogenesis of irradiation-induced cell toxicity, we examined whether MSCCM treatment decreased the oxidative stress induced by irradiation. For this purpose, we analyzed lipid peroxidation by determining the MDA level. There was a significant increase in MDA levels in HCF cells treated with irradiation, compared with those in non-treated cells. These levels returned to control conditions in irradiation + MSCCM-treated HCF cells (Fig. 2C).

MSCCM reduced collagen generation in HCFs

We next examined the impact of irradiation on collagen synthesis in HCF cells. As shown in Fig. 3A, in the Sircol assay, increased synthesis of collagen by irradiation was suppressed by MSCCM. We also examined the mRNA levels of Col1A1 and Col1A2, which reflect collagen synthesis, in HCF cells. The mRNA levels of Col1A1 (Fig. 3B) and Col1A2 (Fig. 3C) in irradiated HCF cells

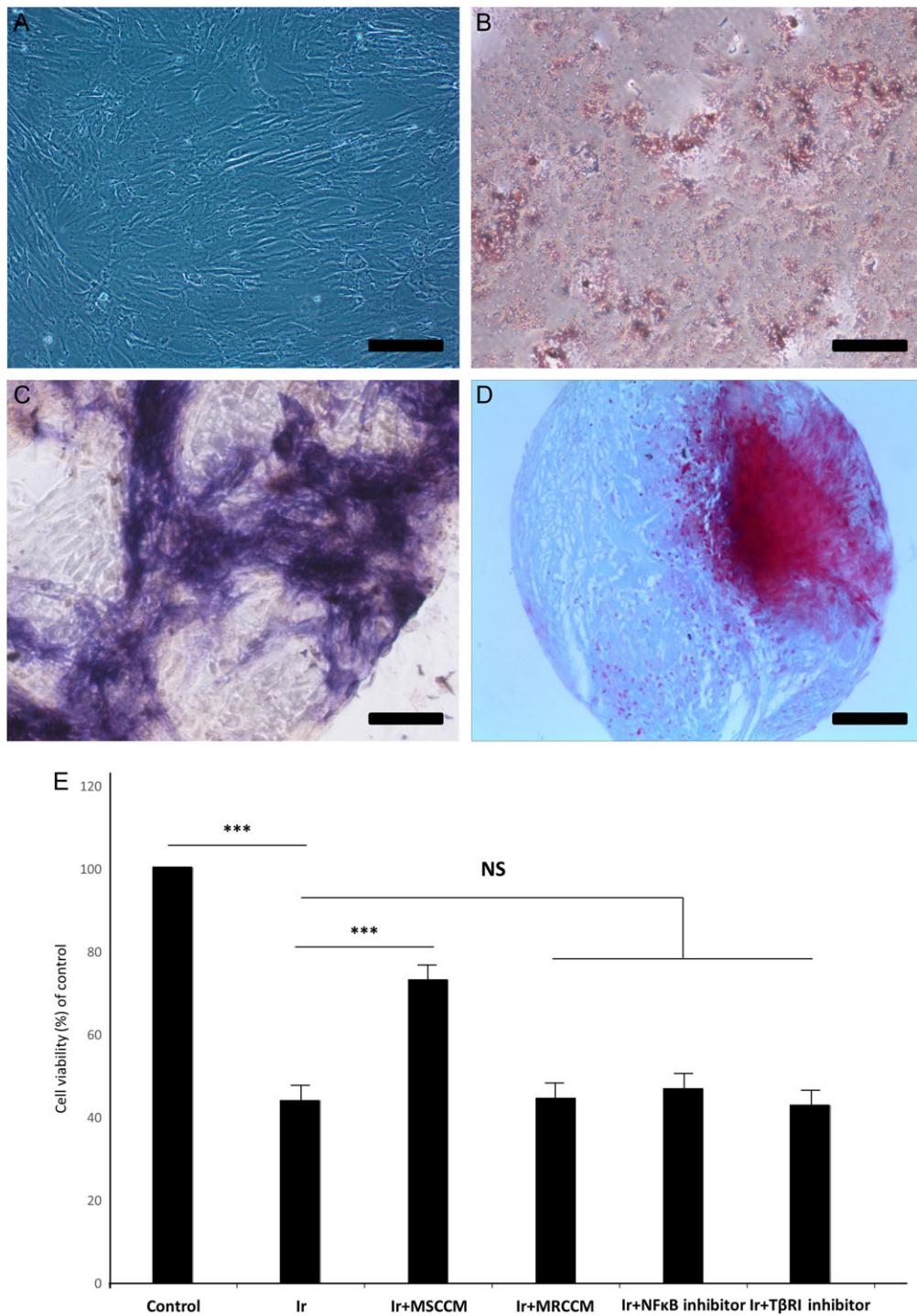


Fig. 1. Identification of UC-MSCs, and MSCCM rescuing HCFs from irradiation-induced cell death. (A) UC-MSCs exhibited a spindle- and fibroblast-like shape. (B–D) Multipotential differentiation of UC-MSCs. UC-MSC differentiation into adipocytes, osteoblasts and chondroblasts, as shown by Oil Red O (B), alkaline phosphatase (C), and alcian blue (D) staining, respectively, of *in vitro* differentiation cultures. (E) Cell viability was analysed by CCK8. The results were compared between control cells (Control), single-irradiated HCF cells (Ir), irradiation + MSCCM-treated HCF cells (Ir+MSCCM), irradiation + MRCCM-treated HCF cells (Ir+MRCCM), irradiation + NF- κ B inhibitor-treated HCF cells (Ir + NF- κ B inhibitor), and irradiation + T β RI inhibitor-treated HCF cells (Ir + T β RI inhibitor). Data are expressed as the mean \pm SD ($n = 5$). *** $P < 0.001$; no significance is indicated as 'NS'; scale bar: 100 μ m.

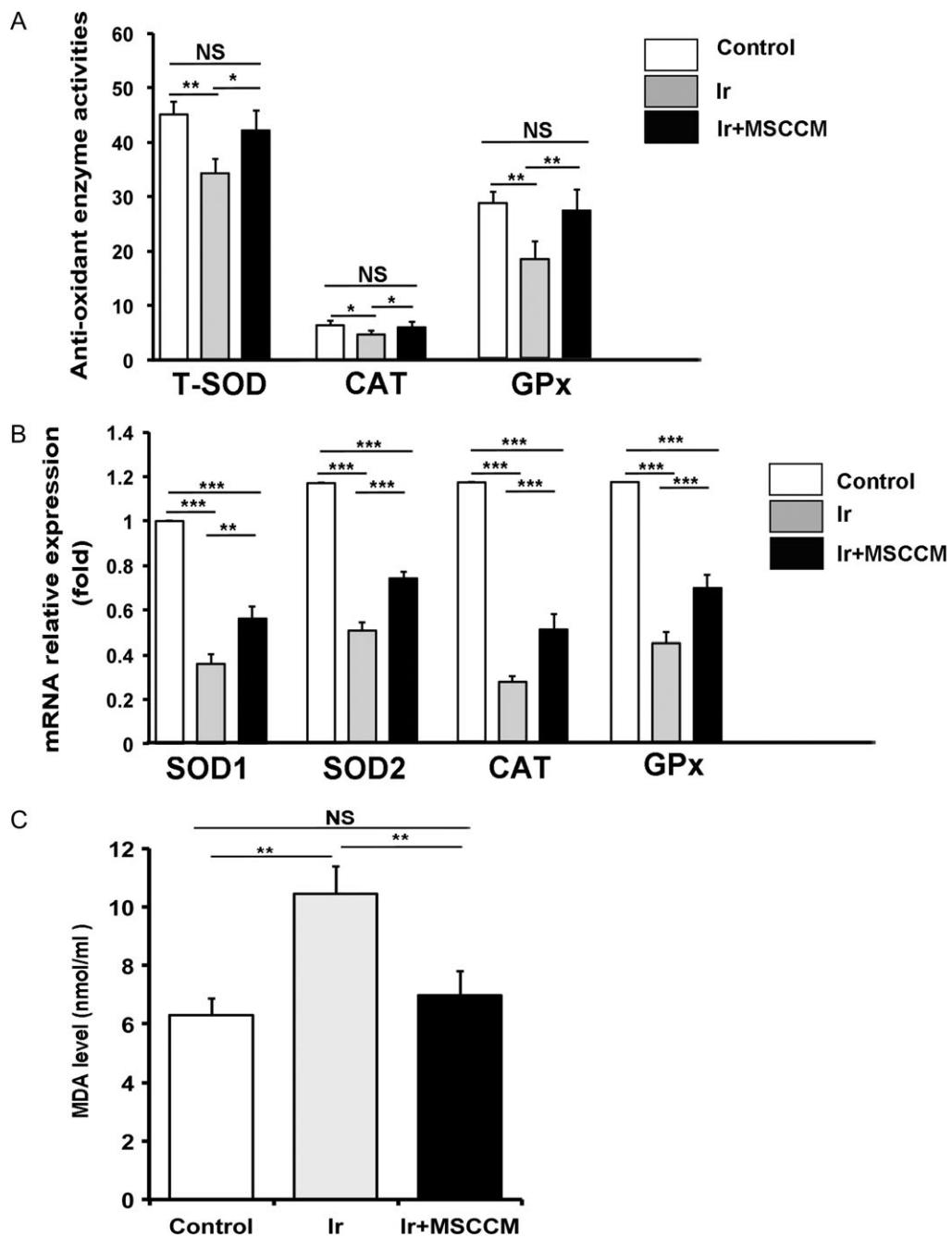


Fig. 2. MSCCM modulated the redox state of HCF cells exposed to irradiation. (A) Enzymatic activities of total SOD (T-SOD), CAT and GPx in control cells (Control), single-irradiated HCF cells (Ir), and irradiation + MSCCM-treated HCF cells (Ir+MSCCM). (B) mRNA levels of endogenous antioxidant enzyme genes were detected by q-PCR. The expression of each mRNA was calculated as $2^{-\Delta\Delta Ct}$ and the mRNA levels of SOD1 and SOD2, CAT and GPx were normalized with the mRNA levels of GAPDH. (C) The levels of malondialdehyde (MDA) in the cell supernatant were measured by a UV-visible spectrophotometer ($n = 5$). Data are expressed as mean \pm SD ($n = 3$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; no significance is indicated as 'NS'.

were highly elevated compared with the levels in control cells without irradiation and MSCCM. Notably, the mRNA levels of Col1A1 (Fig. 3B) and Col1A2 (Fig. 3C) in irradiated HCF cells treated with MSCCM were markedly decreased.

MSCCM improved the inflammation status in HCFs
As several key pro-fibrotic cytokines involved in the pathogenesis of fibrosis, TGF- β 1, IL-6 and IL-8 are crucial in promoting proliferation of fibroblasts, driving differentiation of fibroblasts to

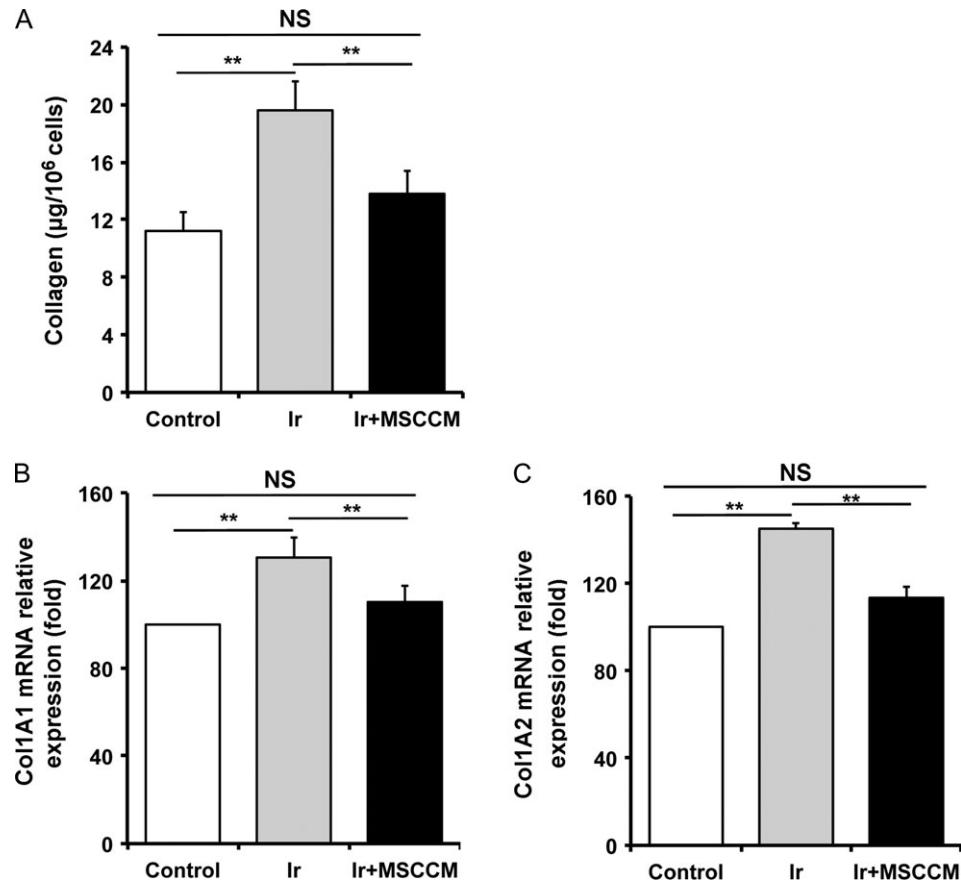


Fig. 3. MSCCM reduced the collagen generation in HCF cells. (A) The amount of collagen was examined using the Sircol soluble collagen assay in control cells (Control), single-irradiated HCF cells (Ir), and irradiation + MSCCM-treated HCF cells (Ir+MSCCM). (B–C) mRNA levels of Col1A1 (B) and Col1A2 (C) were detected by q-PCR. The expression of each mRNA was calculated as $2^{-\Delta\Delta Ct}$ and the mRNA levels of Col1A1 (B) and Col1A2 (C) were normalized with the mRNA levels of GAPDH. Data are expressed as the mean \pm SD ($n = 5$). ** $P < 0.01$; no significance is indicated as ‘NS’.

myofibroblasts, increasing synthesis of extracellular matrix, and stimulating protease inhibitor expression. IFN- γ , an anti-fibrotic cytokine, plays pivotal roles in modulating immune responses, inhibiting fibroblast proliferation and reducing extracellular matrix deposition [14]. We measured the levels of TGF- β 1, IL-6, IL-8 and IFN- γ in cell lysate of HCF cells using ELISA kits. Consistent with the previous reports, we found that the TGF- β 1, IL-6 and IL-8 levels in HCF cells significantly increased post-irradiation compared with in normal controls. The TGF- β 1, IL-6 and IL-8 levels in MSCCM-treated HCF cells were significantly lower than those in radiation-only HCF cells (Fig. 4A). However, there was no significant difference in the IFN- γ level between the three groups (Fig. 4C). We also measured the mRNA levels of TGF- β 1, IL-6, IL-8 and IFN- γ in HCF cells by q-PCR and obtained similar results (Fig. 4B and D).

MSCCM prevented NF-κB activation in HCF cells

We next examined whether irradiation utilized the NF-κB signaling pathway for its proinflammatory actions and whether MSCCM prevented NF-κB signaling pathway activation. Pretreatment with

inhibitor of NF-κB led to a decrease in the levels of TGF- β 1 in cell lysate of HCF cells as determined by ELISA kit (Fig. 5A). We further investigated NF-κB p65 amounts in HCF cells by ELISA. The activated form of NF-κB p65, which was detected in the nuclear fraction, increased after irradiation compared with in controls. MSCCM reduced the level of the activated form of NF-κB p65 (Fig. 5B).

DISCUSSION

In the present study, we aimed to investigate the therapeutic paracrine potential of UC-MSCs in a cell model of IMF. We demonstrated in a cell model of IMF that administration of MSCCM rescued HCFs from irradiation-induced cell death, reduced collagen deposition as measured by Sircol assay and qPCR (Col1A1 and Col1A2), prevented oxidative stress, and increased antioxidant status (as measured by MDA content and the activities and mRNA levels of antioxidant enzymes), and reduced pro-fibrotic TGF- β 1, IL-6 and IL-8 levels (as examined by ELISA kit and qPCR). Pretreatment with inhibitor of NF-κB led to a decrease in the levels of TGF- β 1 in cell lysate of HCF cells as determined by ELISA kit.

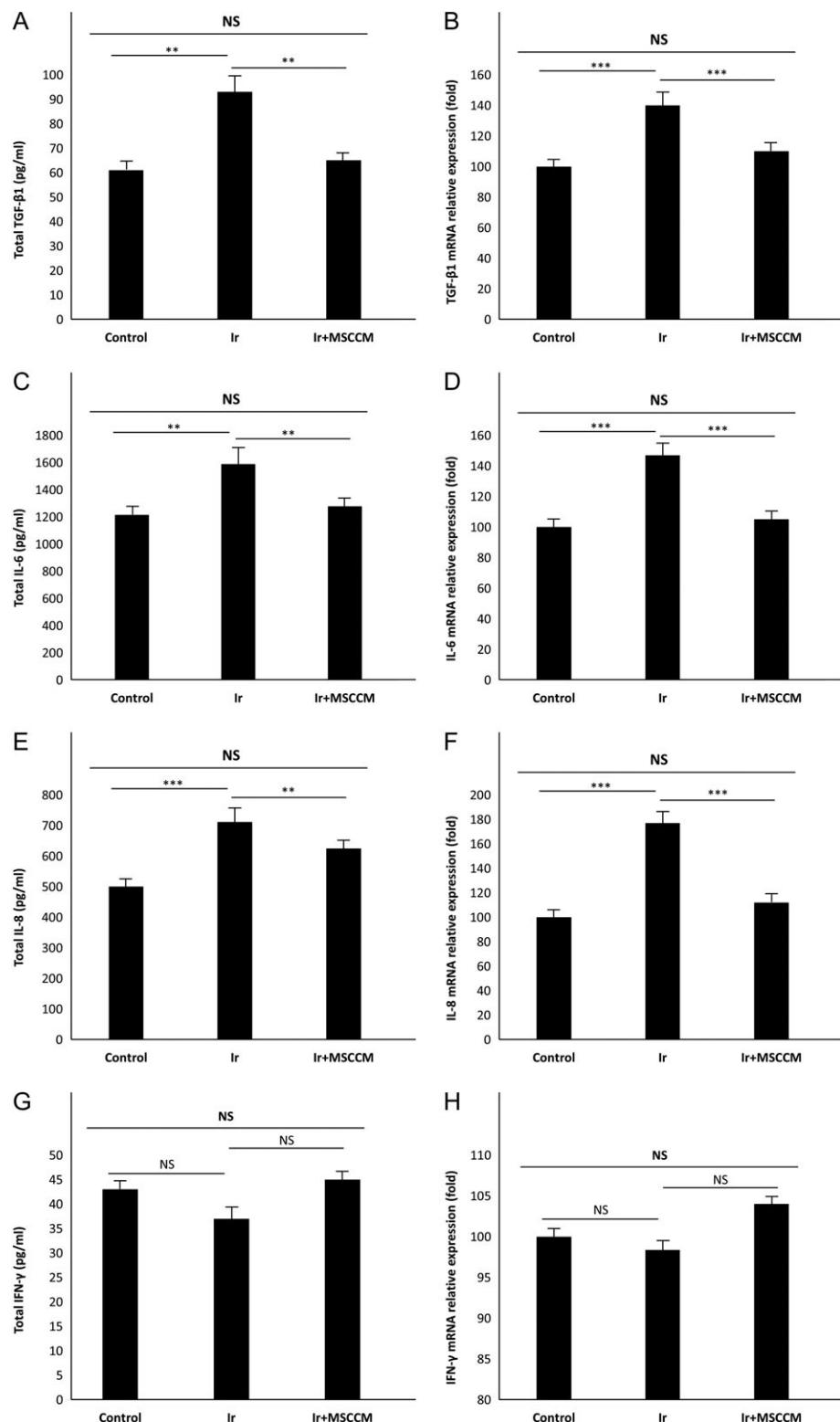


Fig. 4. MSCCM improved the inflammation status in HCF cells. HCF cells were collected, and the levels of TGF- β 1 (A), IL-6 (C), IL-8 (E) and IFN- γ (G) in cell lysate of HCF cells were determined using commercially available ELISA kits in control cells (Control), single-irradiated HCF cells (Ir), and irradiation + MSCCM-treated HCF cells (Ir+MSCCM). The mRNA levels of TGF- β 1, IL-6, IL-8 and IFN- γ were examined by q-PCR. The expression of each mRNA was calculated as $2^{-\Delta\Delta ct}$ and the mRNA levels of TGF- β 1 (B), IL-6 (D), IL-8 (F) and IFN- γ (H) were normalized with the mRNA levels of GAPDH. Data are expressed as the mean \pm SD ($n = 5$). ** $P < 0.01$; *** $P < 0.001$; no significance is indicated as 'NS'.

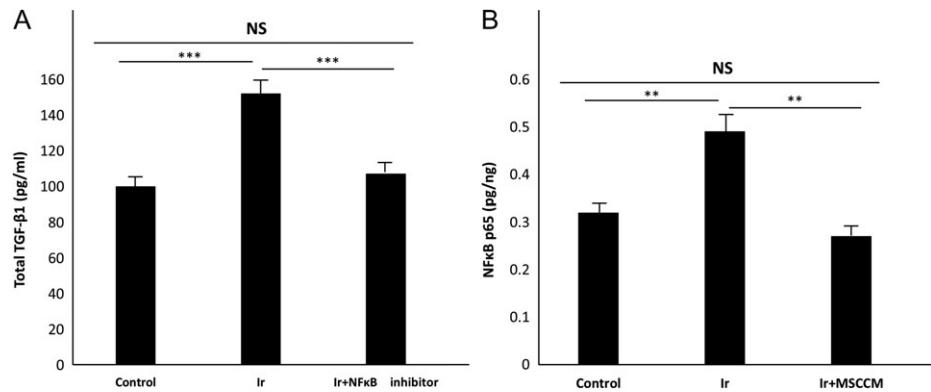


Fig. 5. Irradiation induced TGF- β 1 production via NF- κ B activation, and MSCCM prevented NF- κ B activation in HCF cells. (A) HCF cells were pre-incubated with NF- κ B inhibitor MG-132 (5 μ M) for 60 min before irradiation, and the level of TGF- β 1 in cell lysate of HCF cells was determined using commercially available ELISA kits in control cells (Control), single-irradiated HCF cells (Ir), and irradiation + NF- κ B inhibitor-treated HCF cells (Ir+ NF- κ B inhibitor). (B) HCF cells were collected, and p65 amounts in nuclear protein extracts were analyzed by ELISA kit in control cells (Control), single-irradiated HCF cells (Ir), and irradiation + MSCCM-treated HCF cells (Ir+MSCCM). Data are expressed as the mean \pm SD ($n = 5$). ** $P < 0.01$; *** $P < 0.001$; no significance is indicated as 'NS'.

Furthermore, we also found that MSCCM prevented NF- κ B signaling pathway activation of its proinflammatory actions induced by irradiation. Our data not only defined the potential beneficial roles of treatment with MSCCM, but also provided evidence for paracrine therapeutic effects of UC-MSCs in a cell model of IMF.

MSCs are multipotent progenitor cells derived from a variety of tissues, such as bone marrow, adipose tissue and amniotic membrane, and they have been reported to have therapeutic potential following transplantation as observed in preclinical animal disease models [15]. *In vitro* studies have already shown that MSCs from different origins varied in differentiation potential, proliferation and secretome [16]. However, compared with other MSCs, UC-MSCs are characterized by short amplification times, high proliferation rates, lower immunogenicity, higher safety, greater abundance and being more convenient to manipulate [15]. In addition, UC-MSCs were demonstrated as able to secrete higher concentrations of chemokines, pro-inflammatory proteins, and growth factors compared with other sources of MSCs [16]. In the present study, two human umbilical cords were usually used in every preparation, the collagenase digestion method was applied in order to digest small pieces of UC, and a high proliferation rate was observed for mesenchymal cells. The overall success rate in preparing UC-MSCs exceeded 90% using our methods. It seems that UC-MSCs may be one of the best sources of MSCs for tissue regeneration in preclinical research and ultimately in clinical application.

In the infarcted hearts, MSC stimulated an increase in vascularity (both in the border area of the post-infarction scar, and within the scar), a reduction of the post-infarction scar and fibrosis, and an increase in the left ventricular ejection fraction in a mouse model of myocardial infarction [17]. After intravenous infusion, they are present in the lungs for up to 24 h [18]. MSC retention time in the heart is also short: Four hours after intramyocardial administration, only 10% of injected MSCs are present; 24 h later only 1% are left [19]. Hence, the low levels of MSC engraftment after transplantation

may indicate that their beneficial effects are more likely mediated via their secretion of soluble factors than a long-term presence in repaired tissue [16].

The exact mechanisms of MSC actions are poorly understood, but paracrine actions of MSCs have indicated that MSCs produce bioactive molecules, and exert anti-scarring, anti-inflammatory, anti-apoptotic effects on target cells and surrounding inflammatory cells [20]. As MSC-conditioned media is a source of growth factors, cytokines, chemokines, ECM, and hormones [21], we believed that these positive beneficial biological effects of MSCCM may be mediated by some protein/s in a paracrine manner. This hypothesis has been supported by various CM-related *in vitro* and *in vivo* studies. MSCCM regulated dermal fibroblast proliferation, migration, myofibroblast differentiation and ECM-related gene expression [15]. MSCCM promoted proliferation and migration of alveolar epithelial cells (AECs) and small airway epithelial cells (SAECs) under septic conditions by activating both JNK and p38. Blockage of JNK or p38 resulted in a significant reduction in cell proliferation and migration, suggesting that MSCCM exerted its biological activities on AECs and SAECs in mechanisms that required both the p38 and JNK MAPK pathways [16]. MSCCM suppressed the proteolytic system and ROS generation in muscle atrophied cells [21]. The previous study showed that renal artery administration of MSCCM could reduce inflammation, oxidative stress, and collagen deposition, slow the progress of epithelial-mesenchymal transition (EMT), reduce renal interstitial fibrosis, promote tubular epithelial cell proliferation and inhibit its apoptosis in unilateral ureteral obstruction rats [22]. Pre-conditioning of CM of human periodontal ligament tissue conditioned from relapsing remitting multiple sclerosis (RR-MS) patients protected mouse motor neurons exposed to inflammation, via anti-inflammatory, neurotrophic, and anti-apoptotic properties [23].

Accumulated evidence has supported that oxidative damage induced by irradiation dramatically contributes to the formation of irradiation fibrosis [24]. Hence, the manipulation of own natural

enzyme defense system against oxidative damage has been proposed and tested in recent years. Interestingly, MSCs themselves have anti-oxidative effects [25]. In the present study, irradiation resulted in reduction of mRNA levels of SOD1, SOD2, GPx and CAT, and in activities of total SOD, CAT and GPx in HCF cells. Interestingly, MSCCM restored normal levels in the antioxidant enzymes system. This observation indicated a positive feedback loop in the antioxidant enzymes system of HCF cells, being activated by MSCCM in response to irradiation.

Fibrosis is frequently associated with collagen deposition. We thus investigated whether irradiation generated excessive collagen and whether MSCCM prevented collagen generation in HCF cells. Our data demonstrated that excessive collagen synthesis caused by irradiation was suppressed by MSCCM. This observation was consistent well with the previous studies that injection of MSCs could inhibit fibrosis development *in vitro* and *in vivo*.

Downregulation of pro-fibrotic cytokines and upregulation of anti-fibrotic cytokines have been proposed as one of the paracrine actions of MSCs [26]. TGF- β 1 cytokine potently induced the trans-differentiation of epithelial cells into myofibroblasts through a process termed 'epithelial–mesenchymal transition' (EMT) dependent on the TGF- β /Smad pathway [27], and myofibroblasts produced large amounts of collagens. IL-6 has been shown to be an important mediator of left ventricular (LV) hypertrophy, myocardial fibrosis and LV dysfunction in response to pressure overload. IFN- γ can inhibit TGF- β 1 expression, fibroblast proliferation, differentiation of fibroblasts to myofibroblasts (EMT), and collagen synthesis [28]. Our results demonstrated that the MSCCM significantly reduced the level of TGF- β 1, IL-6 and IL-8. However, we did not detect any difference in the IFN- γ level. Further study is needed to examine more types of cytokines and bioactive factors. In addition, we demonstrated that cell death in irradiation-induced HCF cells was not affected by inhibitors of NF- κ B or T β RI. These results suggest that the radiation-induced cell death was not related to the activation of NF- κ B or the induction of TGF- β 1.

To determine whether irradiation-induced proinflammatory actions involved NF- κ B activation, we used the well-characterized pharmacologic inhibitor for NF- κ B, MG-132. In the present study, we demonstrated the involvement of NF- κ B in the stimulation of TGF- β 1 production. We further demonstrated that MSCCM reduced the activated form of NF- κ B p65 in the nuclear fraction of HCF cells. These results suggest a possible mechanism by which MSCCM could reduce irradiation-induced TGF- β 1 production through inhibition of the NF- κ B signaling pathway.

FUNDING

None

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