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Original paper

Mesenchymal stem cells attenuate amiodarone-induced pulmonary fibrosis in rats via blockade of inflammation and TGF-β1/Smad3/S100A4 signaling

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Abstract

Aim: This study aimed to clarify the anti-fibrotic potential of bone marrow-derived mesenchymal stem cells (BM-MSCs), compared to conditioned media (CM), in amiodarone (AD)-induced lung fibrosis. **Methods:** A total of 64 Wistar rats were categorized into eight groups: negative control group, positive control group, 3 AD-challenged-BM-MSCs-treated groups (1, 2 and 4 months) and 3 AD-challenged-CM-treated groups (1, 2 and 4 months). Serum macrophage inflammatory protein 2 (MIP-2) levels were measured. Gene expression levels of TGF-β1, SMAD3 and S100A4, were estimated in the lung tissues. **Results:** Treatment with BM-MSCs/CM mediated a significant reduction in serum MIP-2 concentrations, while downregulating AD-induced up-regulation of lung TGF-β1, SMAD3 and S100A4 gene expression levels. BM-MSCs transplantation revealed better effect than CM in mitigating lung fibrosis. **Conclusion:** BM-MSCs advance anti-fibrotic effect on lung fibrosis by targeting inflammatory response and TGF-β1 signaling.

Keywords

Amiodarone; conditioned media; inflammatory response; lung fibrosis; mesenchymal stem cells; TGF-β1 signaling

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Introduction

Stem cells are accounted as cells with unique biological nature owing to their capability to self-renewing and differentiation into multiple cell types. Stem cell-based therapy is emerging as a potential therapeutic opportunity for treatment of numerous disorders. Mesenchymal stem cells (MSCs) are multipotent adult stem cells that are derived from different tissues and can be utilized as an alternative to embryonic stem cells (Kang et al, 2019). Mesenchymal stem cells have emerged as promising therapeutic options in regenerative medicine (Motavaf et al*,* 2016) for the treatment of various diseases (Bianco et al*,* 2013; Farini et al*,* 2014), including lung fibrosis particularly on the experimental level (Gotts and Matthay, 2011; Akram et al*,* 2013). Mesenchymal stem cells transplantation has been reported to induce repair of the damaged lung tissues through suppressing inflammation and collagen deposition (Ortiz et al, 2003; Rojas et al*,* 2005). Since studies in animal models and patients indicated that low number of transplanted MSCs localized to the target tissue and transdifferentiate to appropriate cell lineage and the regenerative potential of MSCs has been found at least in part to be mediated *via* their paracrine actions, the use of MSC conditioned media has been suggested to improve tissue regeneration following injury (Ionescu et al, 2012).

Lung fibrosis is an aggressive and lethal form of interstitial lung diseases (Noble et al*,* 2012). This devastating disease is often fatal within 3-5 years after diagnosis (Ley et al, 2011), and the currently available treatment options are of unproven benefit with lung transplantation being the only definitive therapy (Aslam et al*,* 2009), emphasizing the urgent need for development of novel therapeutic strategies.

It is well known that the recruitment and activation of inflammatory cells leads to the release of inflammatory mediators that have an essential role in the stimulation and proliferation of cells involved in fibrotic processes (Noble et al*,* 2012). Further, it has been previously demonstrated that the angiogenic CXC chemokine, macrophage inflammatory protein 2 (MIP-2), is an important factor that regulates angiogenesis during the development of PF (Keane et al, 1999).

Transforming growth factor beta 1 (TGF-β1), a pro-fibrogenic cytokine, has long been believed to be a central mediator of the lung fibrotic response (Allen and Spiteri, 2002). TGF-β1 signals are transduced by transmembrane serine/threonine kinase receptors. The intracellular signaling pathway downstream of TGF-β1 receptors has been found to be mediated by a family of transcription factors, known as the SMAD proteins, and among these, mothers against decapentaplegic homolog 2 (SMAD2) and SMAD3 are direct substrates of the TGF-β type I receptor (Flanders, 2004). The contribution of TGF-β1 to fibrosis has been observed to be mediated mainly through the Smad-dependent signaling axis (Heldin and Moustakas, 2012). Of note, calcium-binding protein A4 (S100A4), a regulator of numerous cellular processes crucial to fibrotic progression (Donato et al*,* 2013), has been found to be a target of TGF-β1 signaling (Matsuura et al, 2010).

The current study mainly focused on assessing the anti-fibrotic outcome of bone marrow mesenchymal stem cells (BM-MSCs) versus bone marrow mesenchymal stem cells conditioned media (BM-MSCs-CM) against amiodarone-induced lung fibrosis in rats, exploring their effect on inflammatory response and TGF-β1/Smad3/S100A4 signaling.

Materials and methods

BM-MSCs isolation, propagation, characterization, and labelling

Bone marrow cells were collected from decapitated adult male Wistar rats by flushing the medullary cavity of the excised femur and tibia with Gibco Dulbecco's Modified Eagle's medium (DMEM)-high glucose (Biowest, France). Mononuclear cells were purified by density gradient centrifugation at 400×g for 30 minutes using GE Healthcare Ficoll-Paque Premium (Sigma-Aldrich, St. Louis, MO, USA). After three washes with phosphate-buffered saline (PBS) (Biowest, France), purified cells were plated into 25 cm^2 cell culture flasks in a complete culture medium (DMEM-high glucose supplemented with 30% fetal bovine serum [FBS], 1% non-essential amino acids and 1% penicillin/streptomycin (Biowest, France), and maintained at 37°C in a humidified incubator with 5% CO_2. After 48 hours, the culture medium was replaced with a fresh medium and non-adherent cells were discarded. The adherent cells (BM-MSCs) were grown in the complete culture medium to 80–90% confluency, defined as passage zero (P0) cells. The P0 cells were washed with PBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and harvested by incubation with 0.25 % trypsin-EDTA solution (Biowest, France) for 5 minutes at 37°C. The detached cells were centrifuged at 200×g for 10 minutes, resuspended in complete culture medium, counted, and plated as P1 in cell culture flasks at a density of 1×10^6 cells/flask. The culture medium was replaced every third day over a 10–14-day period. For each passage, the cells were seeded and grown similarly. As cells reached 80-90% confluency, cells were harvested and passaged by trypsinization (Alhadlaq and Mao, 2004). BM-MSCs were morphologically identified by their characteristic fibroblast-like spindle appearance. At the 3rd passage,

cells were immunephenotyped to determine the expression of various cell surface antigens. In brief, cells were harvested by trypsinization, washed by PBS, aliquoted at a concentration of 0.5×10^6 cells/mL, and stained for 30 minutes at room temperature in the dark with a set of monoclonal antibodies, including phycoerythrin-conjugated CD14, CD34 and CD44 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Stained cells were washed twice with PBS, resuspended in PBS, and analyzed by the COULTER EPICS XL flow cytometer, using the SYSTEM II software (Beckman Coulter, Brea, CA, USA), according to the manufacturer's protocol (Dominici et al*,* 2006; Abdel Halim et al*,* 2020). Immunophenotyping demonstrated that the cells were positive for the typical MSC markers, CD44+ (90.4%), and negative for the hematopoietic lineage markers, CD14− (5.80%) and CD34− (4.59 %). The third passage BM-MSCs were incubated with Ferumoxides injectable solution (Feridex IV, Berlex Laboratories, Cedar Knolls, NJ, USA), a sterile aqueous colloid of dextran-coated superparamagnetic iron oxide nanoparticles, at a final concentration of 25 µg/mL, and poly-L-lysine (PLL, Sigma-Aldrich, St. Louis, MO, USA), at a final concentration of 375 ng/mL to boost the incorporation of the nanoparticles, for 24 hours. Feridex was mixed with PLL and shaken for 30 minutes at room temperature before being added to the cells in supplemented DMEM. After incubation with FePLL mixture, prussian blue staining was conducted to visualize the iron particles in Ferumoxides-labeled BM-MSCs. Briefly, BM-MSCs were harvested by trypsinization, washed twice with PBS, transferred to cytospin slides, fixed with 4% glutaraldehyde, washed with PBS, incubated for 30 minutes with 2% potassium ferric-ferrocyanide (Perl's reagent, Sigma-Aldrich, St. Louis, MO, USA) in 3.7% hydrochloric acid, washed 3 times with PBS, counterstained with nuclear fast red, and assessed for labelling efficiency using light microscopy (Balakumaran et al*,* 2010).

Generation of mesenchymal stem cells conditioned medium (MSCs-CM)

Passage 3 MSCs at 80–90% confluence was washed twice with PBS and maintained in supplemented FBS-free DMEM for 24 hours. The medium from equal numbers of cells in each culture (3×10^6 cells) was collected, centrifuged at 400×g for 20 minutes, and concentrated 10-fold using an Amicon Ultra Centrifugal Filter (Sigma-Aldrich, St. Louis, MO, USA) with a molecular weight cut-off of 10 kDa. Concentrated CM was filter-sterilized and stored at −80°C for later use (Ionescu et al, 2012).

Experimental model of lung fibrosis induced by amiodarone

Amiodarone hydrochloride (Cordarone) was obtained from Sanofi pharmaceutical company (Paris, France) as 200 mg tablets. A fresh solution was prepared by dissolving AD in Saline, 0.85% with 0.05% Tween 80 (Hardy Diagnostics, Santa Maria, CA, USA) before each administration. To induce PF, rats were given a daily dose of 30 mg/kg body weight AD by oral gavage for 3 months (Kolettis et al*,* 2007).

Study design

A total of 64 adult male Wistar rats, with a body weight of 100–120 g, procured from the holding company for biological products and vaccines, Giza, Egypt, were maintained in ventilated polypropylene cages in a specified pathogen-free air-conditioned (25°C and 50% humidity) room, with 12 hours-light/dark cycles and free access to a commercial standard pellet diet (PMI Nutrition, Shoreview, MN, USA) and fresh drinking water throughout the study period, in the animal care facility at the National Research Centre (NRC), Giza, Egypt. Rats were allowed to adapt in the animal care facility for one week before any experimentation. All procedures were done with proper approval of medical Research Ethics committee of the NRC, Giza, Egypt (Approval ID: 17-096), and in compliance with the guidelines of the National Institutes of Health (NIH) for the care and use of laboratory animals, 8th edition, 2011.

Rats were randomly allocated into 8 groups (8 rats/group), including a negative control (vehicle, saline) group, a positive control (AD) group, 3 BM-MSCs-treated groups (for post-treatment durations of 1, 2 and 4 months) and 3 CM-treated groups (for post-treatment durations of 1, 2 and 4 months).

The negative control rats were given 0.5 mL of Saline, 0.85% with 0.05% Tween 80/day by oral gavage for 3 months, whereas positive control rats as well as BM-MSCs- and CM-treated rats were orally given 0.5 mL of 30 mg/kg body weight AD once daily for 3 months. Following the 3-months AD administration, rats of the BM-MSCs- and CM-treated groups were given a single intravenous injection with 3×10^6 BM-MSCs in 0.5 mL PBS and 0.5 mL CM (Yu et al, 2015), respectively, whereas the positive control rats were given a single intravenous injection with 0.5 mL PBS, through the tail vein.

Blood collection, dissection, and tissues preparation

At the end of the experimental interval of 7 months, negative and positive control rats were sacrificed by decapitation, whereas rats of the 3 BM-MSCs- and 3 CM-treated groups were sacrificed by decapitation at 1, 2 and 4 months follow-

ing BM-MSCs or -CM injection. Blood samples were collected from the orbital sinus and serum samples were separated by centrifugation, snap-frozen in liquid nitrogen, and stored at −80 °C for subsequent analysis. Both lungs were immediately excised and divided into four separate lobes. The largest lobe was fixed in 10% neutral buffered formalin for 24 hours, dehydrated through a graded alcohol, and embedded in paraffin wax for histopathological analysis. The remaining three lobes were snap-frozen in liquid nitrogen and stored at −80 °C for later gene expression analysis.

Histopathological procedures

Paraffin-embedded lung tissues were cut into as 5 μm thick slices by using microtome, mounted on glass slides, and stained with hematoxylin and eosin (H&E) (Sigma-Aldrich, St. Louis, MO, USA) according to the standard method (Mohamed et al, 2014) with little modification to visualize morphological deformation of the lung tissues, with Masson's trichrome (Sigma-Aldrich, St. Louis, MO, USA) to assess collagen fibers accumulation, and with prussian blue to track the Ferumoxides-labeled BM-MSCs, using light microscopy (Olympus BX51 microscope, Shinjuku, Tokyo, Japan).

Biochemical assay

Serum macrophage inflammatory protein 2 (MIP-2) levels were determined by a commercial "Sandwich" enzyme-linked immunosorbent assay (ELISA; Elabscience, Houston, TX, USA), according to the standard protocol provided by the manufacturer.

Quantitative genes expression analyses

Transforming growth factor beta 1 (TGF-β1), mothers against decapentaplegic homolog 3 (SMAD3) and S100 calcium-binding protein A4 (S100A4) gene expression levels were determined in lung tissues using quantitative real-time polymerase chain reaction (qPCR). The sequences of forward and reverse primers used for qPCRs are listed in Table 1.

In brief, lung tissues total RNA was purified using Invitrogen PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using SensiFAST cDNA Synthesis Kit (Bioline, London, UK) according to the manufacturer's instructions. Quantitative measurement of gene expression levels was conducted using QuantiNova SYBR Green PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. Stratagen Mx3000P Real-Time PCR System (Agilent, Santa Clara, CA, USA) was used for quantitative real-time analysis. Relative gene expression was analyzed by the comparative Ct method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001), using β-actin (ACTB) as the endogenous control. For AD-treated rats, data were expressed as the fold change in gene expression in the AD-treated rats normalized to the expression levels of the endogenous control and relative to the saline-treated rats, whereas for the BM-MSCs- or CM-treated rats, data were expressed as the fold change in gene expression in the BM-MSCs- or CM-treated rats normalized to the expression levels of the endogenous control and relative to the AD-treated rats.

Statistics

The statistical package for the social sciences (SPSS Statistics for Windows, Version 23.0; IBM Corp., Armonk, NY, USA) was used for statistical analysis of data. Variables were expressed as mean ± standard deviation (SD) if normally distributed and compared using the independent Student's t-test or one-way analysis of variance (ANOVA) as appropriate. On contrary, variables were expressed as median (interquartile range, IQR: 25th quartile to 75th quartile) if not normally distributed and compared using the non-parametric Mann–Whitney U test or Kruskal-Wallis test as appropriate. *P* values were two-sided, and a *P* value of less than 0.05 was considered statistically significant.

Results

Histological alteration of lung tissue

Our previously published manuscript (Abdel Halim et al, 2020) reported that hematoxylin and eosin (H&E) stained

Table 1. The sequence of forward and reverse primers used for qPCR

Gene	Gene ID	Primer type	Primer sequence (5`→ 3`)
ACTB	81822	FP	CCCATCTATGAGGGTTACGC
		RP	TTTAATGTCACGCACGATTTC
TGFB1	59086	FP	GGAGCCACTGCCCATCGTCTACTAC
		RP	GGAGCGCACGATCATGTTGGAC
SMAD3	25631	FP	AGGGCTTTGAGGCTGTCTACC
		RP	ACCCGATCCCTTTACTCCCA
S100A4	24615	FP	AGCTACTGACCAGGGAGCTG
		RP	TGCAGGACAGGAAGACACAG

FP, forward primer; RP, reverse primer.

lung tissue sections from rats given vehicle showed normal lung structure, while rats given AD exhibited the characteristic histological changes of AD-induced PF model, as indicated by notable pathological alterations of lung structure. BM-MSCs/CM treatment markedly improved the lung histology, with BM-MSCs/CM-treated rats possess less pathological alterations compared with AD-treated rats, demonstrating that the injection of BM-MSCs/CM exerted a considerable anti-fibrotic influence. Noteworthy, the alleviation of AD-induced histopathological changes was more apparent in BM-MSCs-treated rats than those treated with CM. At 1-, 2-, and 4-months following treatment with BM-MSCs/CM, fibrosis-associated histological alterations were ameliorated with differing degrees according to the time interval.

Collagen fibers deposition in lung tissue

In our previously published manuscript (Abdel Halim et al, 2020), vehicle-administered rats stained with Masson's trichrome showed normal collagen fibers distribution surrounding the bronchioles and blood vessels as well as in between the air alveoli. On the other hand, lung tissue sections from AD-administered rats revealed intense collagen fibers accumulation, demonstrating that AD resulted in a significant increase in collagen deposition. The AD-induced collagen accumulation was significantly reduced by treatment of the AD-administered rats with BM-MSCs/CM. Clearly, the collagen accumulation-reducing ability of BM-MSCs was more apparent than that of CM. At 2 and 4 months following BM-MSCs/CM treatment, the collagen amount determined in the lung tissue sections were remarkably decreased; however, at 1 month following BM-MSCs/CM treatment, the BM-MSCs/CM did not exhibit the same ability to decrease the collagen accumulation. These findings underscore the differing collagen accumulation-reducing influences of BM-MSCs and CM kept treating AD-administered rats for various time intervals.

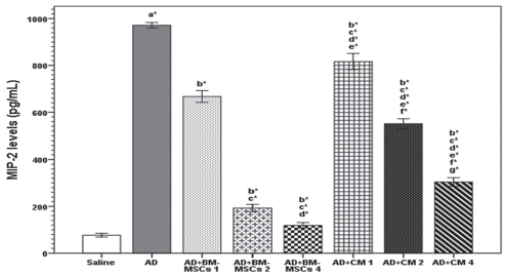

Figure 1. Serum concentrations of MIP-2 in vehicle- and AD-administered rats as well as at 1, 2 and 4 months post-BM-MSCs and -CM treatments. AD, amiodarone; BM-MSCs, bone marrow derived-mesenchymal stem cells; CM, conditioned media; MIP-2, macrophage inflammatory protein 2.
Data are represented as mean ± SD.
a: statistical difference compared to the vehicle group.
b: statistical difference compared to the AD group.
c: statistical difference compared to the AD+BM-MSCs at 1 month group.
d: statistical difference compared to the AD+BM-MSCs at 2 months group.
e: statistical difference compared to the AD+BM-MSCs at 4 months group.
f: statistical difference compared to the AD+CM at 1 month group.
g: statistical difference between the AD+CM at 2 and 4 months groups.
*, P<0.05.

Ferumoxides-labeled BM-MSCs tracking

As evidenced in our prior manuscript (Abdel Halim et al, 2020), at 1, 2 and 4 months post-BM-MSCs treatment, Prussian blue staining displayed slight, moderate, and extensive BM-MSCs homing, respectively, as manifested by mild, moderate, and highly positive blue staining, respectively. In fact, BM-MSCs homing at 4 months post-BM-MSCs treatment was significantly superior to that at 2 months' post-treatment, which was considerably higher than that at 1-month post-treatment.

Impact of BM-MSCs/CM treatment on inflammatory response

To assess the effect of BM-MSCs/CM on inflammatory response, serum level of MIP2 was determined. As shown in Figure 1, AD administration resulted in a significant enhancement ($p<0.05$) in the serum concentration of MIP2 versus the saline-administered rats, as documented by marked increase in MIP2 concentration by 1165% in AD-administered rats. BM-MSCs or CM treatment attenuated AD-induced elevation in the serum concentration of MIP2. This finding is manifested by a significant reduction ($p<0.05$) in MIP2 concentration by 31%, 80% and 88% at 1, 2 and 4 months post-treatment with BM-MSCs respectively. Also, CM injection significantly suppressed ($p<0.05$) MIP2 concentrations by 16%, 43% and 69% at 1, 2 and 4 months post-treatment, respectively, when compared to AD group. Following AD administration, 4 months post-BM-MSCs or -CM treatment performed significantly better than 1 and 2 months post-treatment in reducing MIP-2 levels. Also, 2 months post-BM-MSCs or -CM treatment displayed significantly better than 1 month post-treatment in reducing MIP-2 concentrations. In general, BM-MSCs treatments were more efficacious than CM treatments in reducing MIP-2 concentrations.

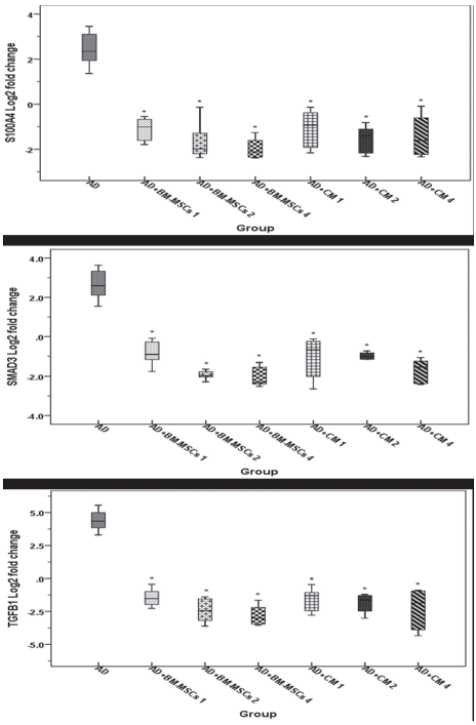

Figure 2. Lung tissue mRNA levels of S100A4 (A), SMAD3 (B) and TGF-β1 (C) in AD-administered rats as well as at 1, 2 and 4 months post-BM-MSCs and -CM treatments. Data are expressed as median (interquartile range, IQR: 25th quartile to 75th quartile) of Log_2 fold change. For AD-treated rats, the fold change is the gene expression in the AD-treated rats normalized to the expression levels of β-actin and relative to the saline-treated rats, whereas for the BM-MSCs- or BM-MSCs-CM-treated rats, the fold change is the gene expression in the BM-MSCs- or BM-MSCs-CM -treated rats normalized to the expression levels of β-actin and relative to the AD-treated rats. AD, amiodarone; BM-MSCs, bone marrow derived-mesenchymal stem cells; BM-MSCs-CM, conditioned media; S100A4, S100 calcium-binding protein A4; SMAD3, mothers against decapentaplegic homolog 3; TGF-β1, transforming growth factor beta 1. *: statistical significance when compared to the AD group (P<0.05).

Effect of BM-MSCs/CM treatment on TGF-β1/ Smad3/S100A4 signaling

Gene expression levels of TGF-β1, SMAD3 and S100A4 were determined in lung tissues to assess the effect of BM-MSCs/CM treatment on TGF-β1/Smad3/S100A4 signaling pathway. As shown in Figure 2, after induction of lung fibrosis by AD, there was a significant upregulation (P<0.05) in the lung expression level of genes encoding TGF-β1, SMAD3 and S100A4 in comparison to vehicle-administered group. Following infusion of BM-MSCs or BM-MSCs-CM, the lung expression level of TGF-β1, SMAD3 and S100A4 genes were significantly downregulated at 1, 2 and 4 months post-treatments, versus the AD-administered group.

Noteworthy, 4 months post-BM-MSCs treatment produced significantly (P<0.05) better performance than 1 month post-treatment in downregulating the expression level of TGF-β1, SMAD3 and S100A4 in the lung, underscoring the various influences of BM-MSCs kept treating the AD-administered rats for various time intervals. However, the transcript levels of lung TGF-β1, SMAD3 and S100A4 genes at 4 months post-BM-MSCs treatment were lower than those at the 2 months post-BM-MSCs treatment, but this difference did not meet the criteria for statistical significance (P>0.05), demonstrating that both 2 and 4 months post-BM-MSCs treatments were equally effective in downregulating the mRNA level of TGF-β1, SMAD3 and S100A4 genes. Except for SMAD3, the gene expression levels of TGF-β1 and S100A4 at the 2 months post-BM-MSCs treatment did not differ significantly from those at the 1 month post-BM-MSCs treatment, indicating that both 1 and 2 months post-BM-MSCs treatments were of equal effect in downregulating the mRNA level of TGF-β1 and S100A4 genes but not of SMAD3.

Concerning the infusion of BM-MSCs-CM, there was no significant difference (P>0.05) in the transcript level of lung TGF-β1, SMAD3 and S100A4 genes among 1, 2 and 4 months post BM-MSCs-CM treatment, suggesting that BM-MSCs-CM treatment at all time points were equally effective in downregulating the transcript level of TGF-β1, SMAD3 and S100A4 genes.

Overall, BM-MSCs treatment displayed significantly (P<0.05) better effect than BM-MSCs-CM treatment in downregulating AD-induced upregulation in the gene expression levels of TGF-β1, SMAD3 and S100A4 in the lung.

Discussion

Current treatment options for PF are limited (Fernandez and Eickelberg, 2012) and there is no effective therapy capable of improving or at least suppressing the progressive course of the disease (King et al, 2011). MSCs have gained increasing interest as a promising cell-based therapeutic approach in several disease, including PF (Parekkadan and Milwid, 2010).

In the current research, BM-MSCs/ BM-MSCs-CM infusion evoked an obvious improvement in the architectural integrity of lung tissue. Moreover, treatment with BM-MSCs/ BM-MSCs-CM significantly blunted collagen deposition in the lung of AD-administered rats as evidenced by reduced

collagen deposition in Masson's trichrome-stained lung sections. At 1, 2 and 4 months post-treatment, BM-MSCs have been detected in damaged lung, as manifested by positive Prussian blue staining, emphasizing the concept that allogeneic or xenogeneic MSCs could accommodate in hosts after infusion (Javazon et al, 2004), thus, indicating the potentiality of BM-MSCs to relocate to the inflammatory sites and migrate into the lung tissue following the intravenous injection, to trigger repair and regeneration of lung tissue.

The production of humoral mediators by damaged lung that induce stem cell proliferation could cause local proliferation of stem cells mobilized from the bone marrow (or delivered as a stem cell transplant), or could be a signaling mechanism to the bone marrow to expand the pool of progenitor cells in response to tissue damage. The generation of substances chemotactic for stem cells by damaged lung may aid in explaining the selective accommodation of these progenitor cells to sites of tissue damage. In acute lung injury, a pro-inflammatory immune response has been initiated within the injured lung, and pro-inflammatory cytokines have been secreted, which can induce the homing of MSCs to the damaged lung (Yagi et al, 2010). Importantly, it has been proposed that most of the exogenously administered MSCs had disappeared shortly following administration, irrespective of the way of administration utilized; but these cells were able to stimulate longer-term paracrine effects that persisted long after they had been vanished, asserting that MSCs might still be able to alleviate lung damage despite gradually declining in numbers post-administration (De Becker and Riet, 2016). Another critical characteristic of MSCs linked to their retention in the lung following systemic administration. A previous study has mentioned that intravenous infusion of MSCs results in their homogeneous distribution into the lung parenchyma (Gao et al, 2001), ascribable to a well-defined cell-trapping phenomenon happening in the lung microvasculature (Schrepfer et al, 2007).

The studies advocated in the past two decades suggested that MSCs mediate their actions *via* engraftment in the lung and differentiation into alveolar cells. The notion of differentiation is supported by studies in which transplanted MSCs adopted lung cell phenotypes in lung injury models (Ortiz et al, 2003; Rojas et al, 2005). The anti-inflammatory activity of MSCs has also been suggested as a potential therapeutic mechanism in lung damage. It has been demonstrated that the inhibition of the prolonged local inflammatory response may furnish an environment more suitable to the normal repair process. Additionally, the decline in inflammatory cytokine expression supports the documentation that MSCs suppress the inflammatory reaction through the activation/inhibition of critical cytokines, resulting in alterations in the local cytokine environment in favor of tissue repair (Matthay et al, 2010).

Few studies reported that the use of MSCs may carry some risks to the patient (Lepperdinger et al, 2008), therefore, the administration of MSCs-CM may confer an alternative therapeutic option for lung injury. The intratracheal administration of BM-MSCs-CM has shown to protect against PF in terms of lung inflammation and collagen accumulation in a lung fibrosis model induced by bleomycin. Furthermore, it has been stated that BM-MSCs-CM has the potential to reduce apoptosis of alveolar epithelial cells (AECs), stimulate AECs regeneration and suppress development of lung fibrosis. It has been suggested that the therapeutic impact of BM-MSCs-CM on AECs may be linked with the epithelial specific growth factors released by BM-MSCs and the synergistic effects of these factors (Shen et al, 2015). BM-MSCs-CM has shown to have dynamic influences relating to fibrotic therapy, stimulating both the migration and proliferation of AECs while inhibiting the activation and proliferation of pulmonary fibroblasts, supporting the potential role of BM-MSCs-CM in tissue repair (Akram et al*,* 2013). It has been mentioned that MSCs-CM contain multiple factors that may confer therapeutic benefit, remarkably resolving lipopolysaccharide (LPS)-induced lung injury by ameliorating lung inflammation. MSCs-CM have been found to mostly contribute to the reprograming modulation of monocytes–macrophages, from a proinflammatory M1 phenotype to an anti-inflammatory M2 phenotype (Ionescu et al, 2012).

A growing body of evidence indicates that excessive cytokine-mediated inflammation plays a key role in the initiation of PF. In the present study, there was a significant elevation in the serum concentrations of MIP2 in AD-treated rats as compared to control rats. Treatment with BM-MSCs or CM produced a remarkable drop in serum MIP2 concentrations. In a mouse model of bleomycin-induced PF, the MIP-2 protein levels in lung tissue homogenates of bleomycin-treated mice have been found to be significantly increased, in comparison to saline-treated control mice (Keane et al*,* 1999). Additionally, in a model of endotoxin-induced lung injury in mice, the intrapulmonary administration of BM-MSCs has been shown to mediate a reduction of MIP-2 levels in the bronchoalveolar lavage (BAL) fluid and plasma, hence, mitigating lung injury, a beneficial effect that has been shown to be mediated by a shift from a proinflammatory to an anti-inflammatory response (Gupta et al, 2007). Furthermore, it has been documented that BM-MSCs have a profound anti-inflammatory effect in endotoxin-induced lung injury in mice by markedly blunting the BAL fluid MIP-2 levels (Hao et al, 2015).

Transforming growth factor beta 1 (TGF-β1) has been shown to play a pivotal role in the pathogenesis of PF (Allen and Spiteri, 2002). It is widely reported that TGF-β1 mediated fibrotic tissue remodeling by increasing the production and decreasing the degradation of connective tissue (Bartram and Speer, 2004). In a rat model of AD-induced lung fibrosis, TGF-β1 (mRNA and protein) expression in the lung has been found to be upregulated relative to controls (Chung et al, 2001). In addition, in a rat model of bleomycin-induced lung injury, the TGF-β1 mRNA levels have been recorded to be upregulated following intratracheal perfusion of bleomycin. The present results are in complete agreement with the previous report indicating that the engraftment of BM-MSCs attenuates lung injury and fibrosis as evidenced by significant reduction in the TGF-β1 mRNA levels in injured rat lungs (Zhao et al*,* 2008). Moreover, in a double dose bleomycin-induced model of lung injury, intravenous infusion of BM-MSCs has been shown to reduce TGF-β1 protein levels in lung tissue lysates (Moodley et al, 2013). It has been reported that the intratracheal administration of bleomycin causes an elevation in the lung protein levels of TGF-β1. Treatment with BM-MSCs reduced TGF-β1 protein levels, suggesting that the anti-fibrotic action of BM-MSCs might be mediated by down-regulating lung expression of TGF-β1 protein (Yu et al, 2015). Smads comprise a family of structurally similar proteins that are the main signal transducers for receptors of the TGF-β superfamily, and TGF-β1/Smad2/3 signaling is one of the main pathways involved in a variety of pulmonary fibrogenesis processes, including inflammation, epithelial to mesenchymal transition (EMT), and extracellular matrix deposition (Flanders, 2004). It has been demonstrated that the pro-fibrogenic TGF-β1 signaling is mediated predominantly via the Smad-dependent pathways (Heldin and Moustakas, 2012). In a bleomycin-induced PF model in mice, induced pluripotent stem cells (iPSCs) intravenous administration remarkably suppressed bleomycin-mediated activation of TGF-β1/Smad2/3 in lung tissues (Zhou et al, 2016). Calcium-binding protein A4 (S100A4), a calcium binding protein, is a regulator of a number of cellular processes important to the progression of fibrosis (Donato et al, 2013). In a prior genome-wide transcriptional analysis, idiopathic pulmonary fibrosis (IPF) mesenchymal progenitor cells (MPCs) have been shown to express higher levels of S100A4 compared with control MPCs (Xia et al, 2014). The mechanism by which S100A4 imparts fibrogenic properties to IPF MPCs has been proposed to involve high nuclear levels of S100A4, which function to promote p53 proteasomal degradation, stimulating IPF MPC proliferation and expansion of the IPF MPC population (Xia et al, 2017). Previously, it has been documented that in the process of fibrosis, epithelial cells can be transformed into fibroblasts, a process called EMT, suggesting that EMT might be involved in many fibrotic diseases. Additionally, it has been reported that TGF-β1 is a powerful mediator of EMT. Interestingly, S100A4 has been shown to be a target of TGF-β1 signaling, suggesting that S100A4 might be a key factor in TGF-β1-induced EMT (Matsuura et al, 2010). Recently, S100A4 expression has been found to be induced by the TGF-β1 pathway (Ning et al, 2018).

It has been demonstrated that MSCs and MSCs-CM exert observable cytoprotective effects in a human relevant pre-clinical model of chronic obstructive pulmonary disease. These protective influences were mediated *via* MSCs secretion of soluble mediators. Of note, MSCs-CM has been shown to be less effective than MSCs and this is likely ascribed to the capability of MSCs to continually release soluble factors (Kennelly et al, 2016). These findings showed parallelism with our study as the infusion of BM-MSCs revealed more favorable impact than BM-MSCs-CM in attenuating lung fibrosis induced by AD. The therapeutic efficacy of BM-MSCs might be partly attributed to enhanced mobilization of BM-MSCs to the fibrotic lung tissue.

In the present approach, maintaining the rats in AD-induced lung fibrosis group with BM-MSCs for prolonged time intervals showed a better fibrosis-attenuating influence than of short time Likewise, keeping the BM-MSCs-CM-treated rats for extended time intervals revealed a better effect in ameliorating lung fibrosis than in case of limited time.

Conclusions

Taken together, our data provide insight into the molecular mechanisms, namely, down-regulating inflammatory response and TGF-β1 signaling, behind the potent effectiveness of BM-MSCs as anti-fibrotic candidates, and further reinforce the feasibility of BM-MSCs transplantation in the treatment of PF. Additionally, the present findings justify the optimal treatment period of BM-MSCs in PF induced by AD. BM-MSCs-based therapy may represent an innovative effective approach against lung fibrosis. Nevertheless, further studies using more pathologically oriented lung fibrosis models will be required in the advance towards clinical settings.

Acronyms and abbreviations

BM-MSCs- bone marrow-derived mesenchymal stem cells; CM -conditioned media; AD -amiodarone; TGF-β1-transforming growth factor beta 1; SMAD3 -mothers against

decapentaplegic homolog; S100A4 -S100 calcium-binding protein A4; MIP-2 -macrophage inflammatory protein 2.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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