Attenuating Effect of Long-term Culture of Umbilical Cord Vein Mesenchymal Stromal Cells on Pulmonary Fibrosis in C57BL/6 Mice

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Received: 7 March 2017; Received in revised form: 15 August 2017; Accepted: 23 August 2017

ABSTRACT

In recent studies, mesenchymal stromal cells (MSCs) have been increasingly employed to treat various diseases like pulmonary fibrosis (PF). There are very few MSCs in tissues so in order to obtain their sufficient numbers for therapeutic applications, their in vitro expansion is necessary. The aim of this study was to investigate the effects of long-term culture of the human umbilical cord vein MSCs (hUCV-MSCs) on pulmonary fibrosis in mice.

MSCs were first isolated from human umbilical cord vein and cultured up to 18 passages. In C57BL/6 mice, 15 min after belomycin instillation, UCV-MSCs at passages (P) 0, 4, 8, 12, and 18 (long-term culture) were transplanted intratracheally. Mice were weighted every 5 days and were euthanized on day 21. For histopathological examination, the lung sections were stained with hematoxylin-eosin (HE) and Masson’s trichrome. The mRNA expression of TGF-β1, alpha-smooth muscle actin (α-SMA), and collagen type I alpha 1 (COL1A1) in lung tissues were assessed using RT-PCR. For cell tracking, human cytochrome B DNA was detected in mice lung tissues by PCR.

The weight of mice receiving long-term culture of UCV-MSCs increased compared to other mice (p<0.056). Also, transplantation of UCV-MSCs at P18 led to increased alveolar space and decreased connective tissue and collagen deposition of the lung tissues. The mRNA expression of TGF-β1, α-SMA, and COL1A1 also decreased in this group.

The results showed that intratracheally transplanted long-term culture of the UCV-MSCs attenuated lung fibrosis in mice.

Keywords: Bleomycin; Long-term culture; Mesenchymal stromal cell (MSC); Pulmonary fibrosis
INTRODUCTION

Fibrosis is accompanied by the accumulation of fibroblasts, deposition of extracellular matrix proteins like collagen, and remodeling of tissues. Increased extracellular matrix and impaired healing causes that chronic inflammation lead to tissue fibrosis. As a result of cellular damage and oxidative stress, proinflammatory cytokines such as TNF-α, IL-1β, and IL-8 recruit and activate neutrophils. Then, activated neutrophils intensify tissue damage by producing reactive oxygen species (ROS). TGF-β1 is a primary fibrogenic cytokine secreted by infiltrated cells which leads to the differentiation of fibroblasts to myofibroblasts. These cells express alpha-smooth muscle actin (α-SMA) and produce a considerable amount of collagen which deposits in extracellular matrix.

Pulmonary fibrosis (PF) has a high mortality rate and the current treatments are not considerably effective. Antifibrotic agents such as colchicine, penicillamine, pirfenidone, anti-TGF-β1, anti-TNF-α, and corticosteroids have not been able to make significant improvements in patients suffering from PF. Recently, cell therapy is a new approach to treating PF using mesenchymal stromal cells (MSCs). MSCs have immunomodulatory properties and can be effective in treating PF.

As a result of tissue damage and inflammation, MSCs migrate to lungs through CXCR4/SDF-1 axis and are differentiated to a lung cells phenotype. In an experiment, human umbilical cord MSCs reduced the mRNA level of TGF-β1, TNF-α, IL-1, IL-8 and collagen deposition, and therefore decreased the severity of bleomycin-induced PF in mice. In another study, the transplantation of rat bone marrow MSCs reduced TGF-β, PDGF-A, and IGF-1, and then diminished PF, which is characterized by reduced hydroxyproline and hyaluronate in the lung tissues, and bronchoalveolar lavage fluid (BALF), respectively. In another report, the administration of chorionic MSCs decreased neutrophil infiltration, inflammation, and collagen deposition, and also improved tissue repair in bleomycin-induced PF of murine.

Because there is a small number of MSCs in different tissues, their expansion prior to clinical use is necessary. Some properties of bone marrow-derived MSCs including proliferation rate, differentiation potential and self-renewing capacity could decrease as a result of long-term in vitro expansion. In long-term culture, small spindle-shaped MSCs change to large polygonal-shaped MSCs, and also become heterogeneous. In addition, an inclusion body emerges in the cytoplasm and cell debris is observed in cell culture media. Overall, these changes are indicative of MSCs senescence and is also associated with a decrease in the telomeres length. On the other hand, no significant difference was observed in the expression of CD13, CD29, CD44, CD166, and CD14, CD34 and CD45 in different passages of MSCs. In long-term cultures of MSCs, the proliferation capability, chromosomal stability, and anti-inflammatory functions of MSCS did not differ either. In addition to lower immunogenic properties, the long-term cultures of MSCs showed more immunosuppressive effects and the population doubling time of these cells decreased.

Considering the effects of long-term culture on the properties of MSCs, this study aimed to investigate the effects of different passages of the umbilical cord vein mesenchymal stromal cells (hUCV-MSCs) on the PF in C57BL/6 mice.

MATERIALS AND METHODS

Isolation and Expansion of hUCV-MSCs

After having the parents’ written agreements, 38 to 40-week healthy term umbilical cords (n=23) were taken to the lab in Hanks balanced salt solution (HBSS) buffer containing 300 μg/mL streptomycin and 300 U/mL penicillin (Biosera, France) at 2-8°C, and the isolation process was started within one hour. The umbilical cords vein were washed twice with HBSS, filled with 0.1% (w/v) collagenase IV (Gibco, UK), and incubated for 20 min at 37°C and 5% CO2. Then, the suspended cells were centrifuged in 600 g for 10 min. The isolated cells were cultured in Dulbecco’s modified Eagle’s medium-low glucose (DMEM-LG) containing 15% FBS (Gibco, USA), 10 ng/mL fibroblast growth factor (FGF) (Royan Institute, Iran), 100 μg/mL streptomycin, and 100 U/mL penicillin in a 6-well plate at 37°C and 5% CO2. The first medium replacement was performed after 24 hours and the next replacements were performed every 72 hours, then the cells were passaged at cell confluence of 70-80%. The isolated cells were expanded until passage (P) 18. This research was carried out in compliance with the Helsinki Declaration. This work has been approved by
Mesenchymal Stromal Cells Attenuate Pulmonary Fibrosis

the Ethics Committee of Kurdistan University of Medical Sciences (No. MUK.REC.1392.148).

Flow Cytometry Analysis
The cells at P4 and P18 were resuspended in the DMEM-LG and were washed with PBS, then MSCs-related surface markers were examined by BD FACSCalibur™ flow cytometer and analyzed using FlowJo software. 1x10^5 of the hUCV-MSCs in 100 µL were stained for 45 min at 4°C with one of the following anti-human monoclonal antibodies: PE-conjugated CD105, CD73 and CD34, FITC-conjugated CD45, mouse isotypic antibodies, PE-IgG1κ, and FITC-IgG1κ (Biolegend, USA).

Animals
6 to 8-week male C57BL/6 mice (20-30 g) were purchased from Tehran Pasteur Institute and kept at animal house at 25°C in light and dark standard periods. The mice were fed with pellets and had access to sufficient amounts of water and food. This work has been approved by ethical review committee by the ethic code MUK.REC.1392.148. Mice were allocated to 7 groups (n=7); in the mice receiving the hUCV-MSCs, 15 min after the bleomycin instillation, the hUCV-MSCs at passages 0 (cells after seeding in day 7), 4, 8, 12, and 18 were administered intratracheally in 50 µL sterile PBS. In the positive control mice, the sterile PBS was injected 15 min after the bleomycin instillation, and in the negative control, only the sterile PBS was administered at both times. Then all of the mice were kept in standard conditions for 21 days according to the Ethics Committee of Kurdistan University of Medical Sciences. There were 7 mice in each independent expriment.

Induction of Pulmonary Fibrosis
The mice were anesthetized using intraperitoneal injection of 4 µg/mouse xylazine (Sigma, USA) and 160 µg/mouse ketamine (Rotexmedica, Germany). Then, 50 µL of bleomycin (Celon, India) with the concentration of 2 mg/kg in sterile PBS was instilled intratracheally and the mice were kept in standard conditions for 21 days.25

Cell Injection
The hUCV-MSCs at P0, 4, 8, 12 and 18 at cell confluency of 70-80% were trypsinized and resuspended in DMEM-LG containing 15% FBS, and centrifuged at 600 g for 5 min. Then the cell pellet was resuspended in sterile PBS and was washed with PBS twice. The hUCV-MSCs at different passages were counted with neubauer and were suspended in sterile PBS for administration to the mice in each group. In the mice receiving the hUCV-MSCs, 15 min after the bleomycin instillation, the hUCV-MSCs at passages 0 (cells after seeding in day 7), 4, 8, 12, and 18 with a concentration of 5x10^5 cell/mouse were administered intratracheally in 50 µL sterile PBS. In the positive control mice, the sterile PBS was injected 15 min after the bleomycin instillation, and in the negative control, only the sterile PBS was administered at both times. Then all of the mice were kept in standard conditions for 21 days according to the Ethics Committee of Kurdistan University of Medical Sciences. There were 7 mice in each independent expriment.

Histopathology
The animals were euthanized on day 21 and their lungs were removed and fixed at Bouin’s solution. After dehydration in a graded ethanol series, the lung tissues were embedded in paraffin, and were then cut into 5-µm sections. The sections were stained with hematoxylin-eosin to evaluate the alveolar space and the connective tissue. Masson’s trichrome was used to assess collagen deposition. In order to evaluate the changes of the lung, the percentages of the alveolar space and connective tissue were calculated using morphometric analysis and the graticule checkerboard 18×kpl-w12.5. The stained tissue sections were analyzed using light microscopy at ×400 magnification.

RT-PCR
On the day 21, the lung tissues of the euthanized animals were used to extract the total RNA using phenol chloroform method according to the manufacturer’s protocol (Pars-tous, Iran). cDNA were reverse transcribed from 1µg of RNA in the final volume of 20 µL using random hexamers and MMLV reverse transcriptase according to the manufacturer (Pars-tous, Iran). The synthesized cDNA and the specific designed primers of TGF-β1, α-SMA, COL1A1, and housekeeping gene (β-actin) were used to amplify the targeted genes.25 The PCR profile consisted of an initial denaturation for 5 min at 94°C, followed by 40 cycles at 95°C for 30 seconds and an annealing temperature for each gene25 for 30 seconds and the final step at 72°C for 45 seconds. The PCR products were electrophoresed on the 2% (w/v) agarose gel using a safe stain. GelQuant.NET software
(biochemlabsolutions.com) was used for the densitometry analysis of the gel bands.

Cell Tracking
The xenogeneic hUCV-MSCs were tracked in the mice lungs using the specific primers for human cytochrome B gene. The DNA of the lung tissues was extracted using the enzymatic method according to the kit recommendations (Pars-tous, Iran). Then, using the specific primers for cytochrome B (Forward: 5´-AGCCACTTTCCACACAGAC-3´ and Reverse: 5´-AGTAGTATGGGAGTGAGG-3´), the extracted DNA was amplified by a thermocycler, and the PCR products were analyzed using agarose gel electrophoresis.

Statistical Analysis
The results were presented as mean±SD (7 mice in each group). Using Mann-Whitney U, the means from the data were compared. p values<0.05 were considered significant. The data were analyzed with SPSS Version16 (SPSS Inc., Chicago, IL, USA).

RESULTS

Cell Characterization
The hUCV-MSCs were adherent, fibroblast-like, elongated cells. The cells at early passages were smaller and less elongated than the later ones (Figure 1A). The cells at P4 and P18 were positive for CD105 and CD73, and negative for CD45 and CD34, which are consistent with MSCs markers (Figure 1B).

Mice Weight
The mice weights increased over the 21 days in all of the groups. However, the mice receiving the hUCV-MSCs at P18 after bleomycin instillation had better weight gain compared to the mice receiving only bleomycin (positive control) (p=0.056). There was no significant difference in weight gain of the mice receiving the hUCV-MSCs at passages 0, 4, 8, and 12 compared to the positive control (p>0.05) (Figure 2).

Figure 1. Morphology and the surface markers of the hUCV-MSCs in different in vitro passages. (A) At early passages, these cells were smaller and less elongated than the later passages. (B) Immunophenotyping of the cells at P4 and P18 were analyzed by flow cytometry, and no considerable difference was observed at both passages. The cells were positive for CD105 and CD73, and negative for CD45 and CD34.

hUCV-MSC: human umbilical cord vein mesenchymal stromal cells; P: passage. Magnification 400×
Mesenchymal Stromal Cells Attenuate Pulmonary Fibrosis

Figure 2. Weight gain of the mice over the 21 days. Statistical analysis of results showed weight gain of the mice receiving the UCV-MSCs at P18 after bleomycin instillation were higher than the mice receiving only bleomycin (p=0.056). The data are the mean of seven independent experiments (7 mice in each group). These data are normalized.

Neg ctrl: mice intratracheally instilled with sterile PBS; Pos ctrl: mice receiving sterile PBS after intratracheal instillation of the bleomycin; hUCV-MSC: human umbilical cord vein mesenchymal stromal cells; P: passage.

Lung Histopathology

At day 21, the alveolar space of the mice receiving bleomycin (positive control) decreased (35.33 ± 0.178) and their connective tissue increased (64.66 ± 0.179) compared to the mice receiving only PBS (negative control) (p<0.05). The mean of alveolar space and connective tissue of the mice receiving the hUCV-MSCs at P18 following the bleomycin instillation were 36.42±0.202 and 63.56±0.2, respectively. In this group, the alveolar space increased significantly and the connective tissue decreased significantly compared to the positive control (p=0.02). Following the bleomycin instillation, in the mice receiving the hUCV-MSCs at P12, there was a slight improvement in the histological changes (p>0.05), but no significant histological change was observed after the administration of the hUCV-MSCs at passages 0, 4, and 8 on the bleomycin-treated mice (p>0.05) (Figure 3).

At the day 21, Masson’s trichrome staining in the lung sections of the mice receiving bleomycin (positive control) showed increased collagen deposition compared to the mice receiving only PBS. The transplantation of the hUCV-MSCs at P18 considerably decreased collagen deposition after 21 days, compared to the positive control. This histological change was not observed in the mice treated with the hUCV-MSCs at passages 0, 4, 8, and 12 (Figure 4).

Expression Profiles of the Fibrosis-Associated Genes

On day 21, the mRNA expressions of the TGF-β1, α-SMA, and COL1A1 in the lung tissues were assessed using RT-PCR. The mRNA expressions of all these genes increased significantly in the bleomycin-receiving mice compared to the PBS-receiving mice (p<0.05). The administration of the hUCV-MSCs at P18 following the bleomycin instillation significantly decreased the mRNA synthesis of the TGF-β1 and COL1A1 in the lung tissues compared to the positive control (p<0.05). Although the administration of the hUCV-MSCs at P18 decreased the mRNA level of the α-SMA, this change was not significant compared to the positive control (p=0.076). The mRNA level of these genes did not change significantly in the mice treated with the hUCV-MSCs at passages 0, 4, 8, and 12 compared to the positive control (p>0.05) (Figure 5).

Xenogeneic hUCV-MSCs Detection in the Mice Lung Tissues

On day 21, human cytochrome B genome was assessed for the presence of the xenogenic hUCV-MSCs in the mice lung tissues using PCR. 21 days after the transplantation of the hUCV-MSCs at P8, P12, and P18, cytochrome B genome was detected in the lung tissues of the mice. However, it’s PCR product was stronger in the hUCV-MSCs at P18. This experiment was performed on 7 mice and the xenogeneic human DNA was observed in two of them. The xenogeneic human DNA was not observed in the lung tissues of the mice receiving the hUCV-MSCs at P0 and P4 (Figure 6).
Figure 3. Hematoxylin and eosin staining of the lung sections. (A) The lung tissues of the mice receiving PBS were normal. 21 days after bleomycin instillation, the alvelolar space of the lung tissue diminished, and their connective tissue increased. (B) These histological changes were significantly attenuated by post-bleomycin administration of the hUCV-MSCs at P18 (p=0.02). No significant improvement was observed in the lung sections following the administration of the hUCV-MSCs from P0, P4, P8, and P12 (p>0.05). The data are the mean±SD of seven independent experiments (7 mice in each group). The star shows a significant statistical difference (p≤0.05).

Neg ctrl: mice intratracheally instilled with sterile PBS; Pos ctrl: mice receiving sterile PBS after intratracheal instillation of the bleomycin; hUCV-MSC: human umbilical cord vein mesenchymal stromal cells; P: passage. Magnification 400×

Figure 4. Masson’s trichrome staining of the lung sections. At day 21, the mice were euthanized and the collagen deposition was assessed using Masson’s trichrome staining. In the lung sections of the mice receiving only PBS, the collagen deposition (blue) was normal, while it considerably increased in the mice receiving bleomycin (positive control). Post-bleomycin instillation of the UCV-MSCs at P18 considerably decreased the collagen deposition compared to the positive control. In the mice receiving the UCV-MSCs at passages 0, 4, 8, and 12, the collagen deposition showed no significant change.

Neg ctrl: mice intratracheally instilled with sterile PBS; Pos ctrl: mice receiving sterile PBS after intratracheal instillation of the bleomycin; hUCV-MSC: human umbilical cord vein mesenchymal stromal cells; P: passage. Magnification 400×
Mesenchymal Stromal Cells Attenuate Pulmonary Fibrosis

Figure 5. The expression of the fibrosis-associated genes in the lung tissues. (A) Representative gel of the PCR products of the \( \beta \)-actin, TGF-\( \beta \)1, \( \alpha \)-SMA, and COL1A in the Pos and Neg ctrl, P0, P4, P8, P12, and P18 groups. (B) The densitometry analysis of the PCR product bands were used to compare the data. At day 21, the expressions of TGF-\( \beta \)1, \( \alpha \)-SMA, and COL1A1 were up-regulated in the lung tissues of the bleomycin-treated mice (Pos ctrl). The subsequent administration of the UCV-MSCs at P18 significantly down-regulated the mRNA level of the TGF-\( \beta \)1 and COL1A1 \((p<0.05)\). The administration of the cells in this passage down-regulated the mRNA level of the \( \alpha \)-SMA but was not significant compared to the mice only receiving bleomycin (Pos ctrl). The UCV-MSCs at passages 0, 4, 8, and 12 had no significant impact on the expressions of these genes. The data are the mean ± SD of seven independent experiments (7 mice in each group). The star shows a significant statistical difference \( (p \leq 0.05) \).

Neg ctrl: mice intratracheally instilled with sterile PBS; Pos ctrl: mice receiving sterile PBS after intratracheal instillation of the bleomycin; hUCV-MSC: human umbilical cord vein mesenchymal stromal cells; P: passage.

Figure 6. The detection of human cytochrome B genome in the lung tissues. Using PCR analysis, the human cytochrome B genome was observed in the lung tissues of the bleomycin-treated mice receiving the hUCV-MSCs at P8, P12, and P18. The xenogeneic human DNA was not observed in the lung tissues of the mice receiving the hUCV-MSCs at P0 and P4.

Neg ctrl: mice intratracheally instilled with sterile PBS; Pos ctrl: mice receiving sterile PBS after intratracheal instillation of the bleomycin; hUCV-MSC: human umbilical cord vein mesenchymal stromal cells; P: passage.
DISCUSSION

PF is a chronic lethal disease in which the normal parenchyma of the lung is gradually replaced by connective tissue. Considering the fact that there is no effective cure for PF, it seems that MSCs therapy could be an alternative treatment to PF. We found that the transplantation of the hUCV-MSCs at the last passage down-regulates the mRNA expression of the fibrosis-associated genes in the mice with PF. In addition, the hUCV-MSCs transplantation improved the alveolar space of the bleomycin-treated mice lungs and decreased the collagen deposition and the connective tissue of the lungs.

Chang et al reported that IT injection of the human umbilical cord blood-derived MSCs resulted in improved hyperoxia-induced lung injury through a reduction in the expressions of IL-6, TNF-α, TGF-β, and α-SMA, and collagen deposition. The IV administration of the human umbilical cord-derived MSCs of P6 led to decreased expressions of TGF-β1, IFN-γ, MIF, and TNF-α in the lung tissues of the bleomycin-induced PF. It also reduced the inflammation and collagen deposition in the lung tissues of the rats.

Due to the low frequency of MSCs in various tissues, the expansion of these cells is essential prior to clinical use. In long-term cultures, the telomere length, the proliferation rate, the differentiation potency, and the self-renewing capacity of the MSCs diminished during their in vitro expansion. Increased proinflammatory cytokines production is another effect of long-term culture on the MSCs, which could impact their immunomodulatory properties. Despite these changes, long-term culture had no effect on the karyotype modification and the surface markers expression of the MSCs, which is indicative of consistency in the basic nature of these aged cells.

The colony formation rate and the differentiation potential of the BM-MSCs reduced as the passages increased. Nevertheless, these cells suppressed the nitrite oxide (NO) production from the IFN-γ stimulated microglial cells, which is suggestive of augmented anti-inflammatory properties of the MSCs.

We found that the hUCV-MSCs at later passages attenuated PF in the bleomycin-treated mice, which could be associated with the higher antioxidant capacity of these aged cells. In PF, the resulting oxidative stress could intensify the injury of the epithelial cells of the lungs. Zhuang, et al demonstrated that the production of the immunosuppressive mediators and the anti-oxidative molecules increased in long-term culture of the MSCs while the proinflammatory cytokines expression decreased. Aged MSCs are more immunosuppressive and have lower immunogenicity, which might be another reason for the PF improvement by the senescent MSCs transplantation in our study.

Moreover, our results show that the hUCV-MSCs at P18 decreased the mRNA synthesis of TGF-β1, α-SMA, and COL1A1 in the lung tissues of the mice with PF. TGF-β1 is a primary factor in fibrosis development whose level increases in PF. Also, it results in excessive production of the extracellular matrix, especially collagen. This cytokine leads to the differentiation of fibroblasts to myofibroblasts, which is accompanied by the production of α-SMA, and causes considerable collagen deposition in the extracellular matrix. Therefore, lower expression of the TGF-β1 may lead to reduced differentiation of fibroblasts to myofibroblasts and decreased collagen deposition in the PF-healing process.

21 days after the hUCV-MSCs transplantation, the xenogeneic genome was detected in the hUCV-MSCs at P8, P12, and P18 in the mice lung tissues, but it was detected stronger in the P18. Previous studies reported that the immunogenicity of the MSCs decreased during long-term culture. Therefore, the persistence of the hUCV-MSCs of P18 in the lung tissues could be associated with lower immunogenicity of these aged cells, which might lead to reduced risk of immunological rejection. Cargnoni et al observed higher copy of the xenogeneic DNA than the allogeneic DNA in the lung tissues of the bleomycin-treated mice on day 14. We demonstrated that in the bleomycin-treated mice, the transplantation of the hUCV-MSCs at P18 could alleviate PF than the hUCV-MSCs at the earlier passages. It seems that in vitro expansion of the hUCV-MSCs does not affect the anti-fibrotic behaviors of these cells. Therefore, in vitro expansion of the hUCV-MSCs could be beneficial in achieving a sufficient source of mesenchymal stromal cells for antifibrotic therapy.

ACKNOWLEDGEMENTS

This paper is the result of a thesis for master degree in immunology, funded by the Research Council of the
Mesenchymal Stromal Cells Attenuate Pulmonary Fibrosis

Kurdistan University of Medical Sciences [grant number 1392.148].

REFERENCES

24. Hesami S, Mohammad M, Rezaee MA, Jalili A, Rahmani MR. The effects of hyperthermia on the
immunomodulatory properties of human umbilical cord vein mesenchymal stem cells (MSCs). Int J Hyperthermia 2017; 1-8.


