Human umbilical cord mesenchymal stem cells inhibit proliferation of hepatic stellate cells in vitro

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Abstract. The effect of human umbilical cord mesenchymal stem cells (hUC-MSCs) on the proliferation of hepatic stellate cells (HSCs) is largely unknown. The purpose of this study was to explore the mechanism of action of hUC-MSCs on the proliferation of HSCs in vitro. The upper and lower double-cell co-culture system was established between hUC-MSCs and HSCs in the experimental group. HSCs were cultured alone as a negative control group. Cell proliferation and apoptosis were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry, respectively. Cell supernatants were harvested to determine the concentration of transforming growth factor-β1 (TGF-β1) by ELISA. mRNA and protein of TGF-β1, Smad3 and Smad7 in HSCs were determined by reverse transcription-polymerase chain reaction and western blotting, respectively. In the co-culture group, the proliferation of HSCs was significantly inhibited compared with the negative control group at 24 and 48 h (p<0.05). Apoptosis of HSCs in the co-culture group increased compared with that in the negative control group, which was more obvious at 48 h (p<0.05). The concentration of TGF-β1 in the co-culture group was significantly lower than in the HSCs cultured alone (p<0.05). After HSCs were co-cultured with hUC-MSCs for 48 h, expression of TGF-β1 and Smad3 mRNA and protein was reduced and expression of Smad7 mRNA and protein was increased compared with the negative control group (p<0.05). hUC-MSCs inhibited proliferation of HSCs, possibly through inhibiting TGF-β1 and Smad3 expression and increasing Smad7 protein expression.

Introduction

Viral hepatitis affects millions of people worldwide, and is now the leading cause of cirrhosis in China. Cirrhosis is a pathological manifestation of end-stage liver disease that contributes significantly to the high mortality of liver diseases. The most important part in the formation of hepatic fibrosis is extracellular matrix (ECM) deposition from hepatic stellate cells (HSCs) (1). HSCs are the final target cells in all types of liver fibrosis. HSCs are activated and transformed into muscle fibroblast-like cells, which causes increased synthesis and degradation of collagen (2).

At present, orthotropic liver transplantation is the last resort for the treatment of cirrhosis. However, due to the shortage of donor organs, orthotropic liver transplantation is restricted clinically (3), and alternative treatment strategies are urgently needed.

Stem cell therapy as a potential therapeutic method has attracted increased attention. Mesenchymal stem cells (MSCs) are important members of the stem cell family. They have the characteristics of multi-differentiation potential, hematopoietic support and promotion of stem cell transplantation, immune regulation and self replication (4). Human umbilical cord MSCs (hUC-MSCs) are derived from umbilical cord tissue. Aspiration of MSCs does not require invasive procedures, which differs from bone marrow MSCs (BM-MSCs). hUC-MSCs are routinely discarded after delivery, without ethical consideration. There have been few studies on the treatment of liver fibrosis with tissue-derived MSCs (umbilical cord, placenta, or adipose tissue), and their role in the
treatment of liver fibrosis has rarely been compared with that of hUC-MSCs and BM-MSCs. MSCs can secrete various cytokines in a paracrine manner, which can promote liver repair, such as hepatocyte growth factor (5), while others can inhibit the occurrence of liver cirrhosis (6).

A recent investigation found that hUC-MSCs can accelerate the resolution of acute liver injury without any differentiation and manipulation (7). Additionally, it has been confirmed histologically that transplantation of hUC-MSCs into rats with CCl₄-induced liver fibrosis results in significant reduction of liver fibrosis (8). However, to the best of our knowledge, there are few studies concerning the mechanism of action of hUC-MSCs on HSCs, and what type of signal transduction pathways are used in HSCs.

Transforming growth factor-β1 (TGF-β1) is a key member of the TGF-β superfamily and plays a critical role in the development of hepatic fibrosis. The Smads protein family is located on HSCs and is divided into receptor activation and inhibitory Smads, such as Smad3 and Smad7. TGF-β1/Smads is an important signaling pathway in hepatic fibrosis (9). In this study, we confirmed that hUC-MSCs could inhibit proliferation of HSCs, and clarified the effect on the TGF-β1/Smads pathway when hUC-MSCs were co-cultured with HSCs. This will provide a theoretical basis for the therapeutic use of MSCs in the treatment of liver fibrosis.

Materials and methods

Materials. Human HSC cell line LX2 was kindly gifted by Scott L. Friedman, Mount Sinai School of Medicine, New York, NY, USA. We used Dulbecco’s modified Eagle’s medium (DMEM)–low glucose (LG) culture medium and fetal bovine serum (FBS; HyClone, Logan, UT, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent cartridge (Sigma, St. Louis, MO, USA), TGF-β1 enzyme-linked immunosorbent assay (ELISA) reagent cartridge (R&D Systems, Minneapolis, MN, USA), human TGF-β1 and β-actin antibody (Bioworld, Minneapolis, MN, USA), human Smad7 antibody (Abcam, Hong Kong, China), horseradish-peroxidase-labeled goat anti-rabbit IgG (Bioworld), RNA extraction reagent RNAiao Plus, reverse transcription kit (both from Takara Biotechnology, Dalian, Japan), ECL light kit (Thermo Fisher Scientific, Inc., Waltham, USA), polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), semipermeable Transwell insert film (Corning Costar, Acton, MA, USA), Hoechst 33342 dye (Sigma), BCA protein concentration assay kit (Beijing Soledad Bao Biological Technology Co., Ltd., Beijing, China), and protein lysis (Biotechnology Research Institute, Haimen, China) in our experiment.

Culture of hUC-MSCs. hUC-MSC cultures were established from the umbilical cords of healthy donors using the direct plastic adherence method after informed consent had been obtained. The study was approved by the Ethics Committee of the School of Life Science and Biopharmaceutics of Lanzhou University (Lanzhou, China) and performed in accordance with the Helsinki Declaration. The cord tissue pieces were minced into 3-5-mm long fragments, plated separately in 100-mm diameter polystyrene tissue culture dishes and maintained in Dulbecco’s modified Eagle’s medium-low glucose (DMEM-LG) medium with 10% FBS at 37˚C in a humidified atmosphere with 5% CO₂. The culture medium was changed on day 7 and then every 3–4 days. Approximately 3 weeks later, when well-developed colonies of fibroblast-like cells were 80-90% confluent, the cord tissue pieces were removed and the cultures were washed and harvested with 0.25% trypsin. The cells were then seeded in new 100-mm diameter flasks for further expansion (6).

Culture of HSCs. LX2 cells were grown in DMEM-LG medium with 10% FBS in 5% CO₂ at 37˚C. For all of the experiments, subconfluent cells (80%) were incubated in 25-cm² culture bottles for different time periods (24 and 48 h).

Establishment of co-culture system. For indirect co-culture, hUC-MSCs and LX2 cells were seeded at a 1:1 ratio in each well of a 6-well plate, using Transwell membranes (24 mm diameter, 0.4 μm pore size; Corning Costar). Approximately 10⁵ LX2 cells were placed in the lower chamber with 10³ hUC-MSCs placed on the membrane insert. Co-cultures were maintained in DMEM-LG with 10% FBS for 24 or 48 h. The upper and lower double-cell co-culture system was established between hUC-MSCs and HSCs as the experimental group. HSCs were cultured alone as a negative control group.

Growth curve. hU-MSC and LX2 cell suspensions were inoculated in 96-well plates for 9 days. Living cells from three wells were harvested and counted serially at 24-h intervals. Then a growth curve was plotted.

MTT assay. Cell proliferation inhibition rate of each group was evaluated by MTT assay. Cells were seeded into the aforementioned co-culture system in 2 ml of medium in each well and cultured for 24 or 48 h. The Transwell membranes were removed and MTT solution [5 mg/ml in phosphate-buffered saline (PBS)] was added to each well and plates were incubated for an additional 4 h at 37˚C. Dimethyl sulfoxide (DMSO) was added to each well, followed by incubation on a shaker at 10 min at 37˚C. The liquid was transferred into 96-well plates, 150 μl/hole, 10 wells/group. Absorbance was measured on a microplate reader (Bio-Rad, Hercules, CA, USA) at 490 nm. Cell growth inhibition rate = (1-A value of experimental group/control group A value) x100% were then calculated.

Hoechst. The co-culture and control groups were cultured for 24 or 48 h. Cells were fixed with 3.7% paraformaldehyde for 30 min at room temperature, washed with PBS, and stained with Hoechst 33342 at a final concentration of 5 μg/ml at 37°C for 6-9 min. Cells were observed under a fluorescence microscope equipped with a UV filter. The images were recorded on a computer with a digital camera (Olympus, Takachiho, Japan) attached to the microscope, and the images were processed by computer. The Hoechst reagent was taken up by the nuclei of the cells, and apoptotic cells exhibited a bright blue fluorescence.

ELISA. TGF-β1 protein was measured in the LX2-conditioned medium after co-culture with the hUC-MSCs for 24 or 48 h. Serum-deprived LX2 cells served as a positive control. We used
a commercial ELISA kit (R&D Systems). Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from the hUC-MSCs and LX2 cells using the RNA Plus kit. TGF-β1, primers forward, 5'-CCACACAACGAAATCTATGAC-3' and reverse, 5'- GTATTCTGGTGAGCTCCA-3' Smad3, primers forward, 5'-CTGCTGCTGATGATTCTCG-3' and reverse, 5'-TGTTAAGCGTGAGATGCTTCC-3'; Smad7, primers forward, 5'-TCTGGTGAACGTAGTCTCCC-3' and reverse, 5'-ACGCACCAGTGTGACCGATC-3' were used for PCR. Reverse transcription was carried out using the Takara PrimeScript reagent kit with 1 µg total RNA as a template and oligo(dT) as a primer. All semi-quantitative PCR experiments were performed using the same serially diluted cDNA batches as templates. Amplification was performed at 95˚C for 30 sec and 95˚C for 5 sec followed by 40 cycles of 60˚C for 30 sec and analyzed by fluorescence quantitative thermal cycling PCR (Bio-Rad). The PCR of human β-actin was performed as a control. TGF-β1, Smad7 mRNA expression levels were detected. The data were calculated by the 2-ΔΔCT method.

Apoptosis analysis. To detect early apoptotic changes, LX2 cells were cultured alone or co-cultured with hUC-MSCs for 24 or 48 h, as described previously. Apoptotic cell death was detected by Annexin V/propidium iodide (PI) staining using the MEBCyTO apoptosis kit (MBL, Nagoya, Japan). LX2 cells from the various cultures were digested with 2.5 g/l trypsin, harvested, washed and resuspended in 300 µl binding buffer, followed by incubation with 5 µl Annexin V-FITC and 5 µl PI at room temperature for 15 min in the dark. Following incubation, 200 µl binding buffer was added and the cell samples were measured using flow cytometry (BD Biosciences, New Jersey, NY, USA).

Western blotting. Cells were harvested in 0.15 ml radioimmunoprecipitation assay (RIPA) lysis buffer with protease inhibitors and centrifuged at 12,000 rpm for 5 min. The supernatants were assayed for protein concentration. Protein content was measured by BCA protein concentration determination kit. The sample size was 30 µg. Protein samples were heated at 100˚C for 10 min before loading and the samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Membranes were blocked with 5% skimmed milk powder in TBST buffer (20 mmol/l Tris, 500 mmol/l NaCl, and 0.1% Tween-20) for 2 h at 37˚C with gentle shaking. Membranes were incubated overnight at 4˚C with various primary antibodies. The following primary antibodies were used: 1:1,000 rabbit polyclonal anti-TGF-β1, 1:1,000 rabbit polyclonal anti-Smad7, and 1:1,000 rabbit polyclonal anti-Smad3. The membranes were washed with TBST buffer and incubated in the appropriate peroxidase-conjugated secondary antibody solution at a 1:5,000 dilution before they were finally developed with enhanced chemiluminescence. The density of the individual bands was quantified using a densitometric scanner with Gel-pro Analyzer (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. All statistical calculations were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). The data are presented as the mean ± SD. When applicable, Student’s unpaired t-test, one-way ANOVA and Holm-Sidak test were used to determine significance. p<0.05 was considered statistically significant.

Results

Morphological changes and growth of cells. Umbilical cords tissue block attaching to the wall was observed after 1-2 days, and some cells crawled out of the tissue block. The cultured hUC-MSCs became spindle-shaped on day 6. The cells had a long fusiform or flat shape after ~10 days. The fused cells were elongated and similar to fibroblasts that were in parallel or spiral-like growth after 21 days and cell fusion was 80% (Fig. 1A). The third passage cells were for transplant spare.

LX2 cells reached >80% cell fusion after 5-8 days. The growth of cells retained its original status without change of culture medium for 1-2 days. After 3 days, cell growth entered the logarithmic phase, and reached a plateau after 5 days (Fig. 1B). LX2 showed obvious growth and proliferation when spared 3-4 generations were used in the experiment.
The upper and lower double-cell co-culture system was established between hUC-MSCs and HSCs as the experimental group. HSCs were cultured alone as a negative control group.

Inhibition of HSC proliferation by hUC-MSCs. We detected the absorbance of hUC-MSCs on HSCs by MTT assay after being cultured for 24 and 48 h. Cell growth inhibition rates (average absorbance of each inhibited group/non-inhibited group) were then calculated. The inhibition rate of hUC-MSCs on HSCs at 24 and 48 h was 2.21±0.02 and 6.37±0.06%, respectively. A significant difference was observed between the co-culture experimental group (2.21±0.02 and 6.37±0.06%) and negative control group (1.66±0.02 and 4.82±0.05%) at 24 h (p<0.05) and 48 h (p<0.05) (Fig. 2). hUC-MSCs significantly inhibited the proliferation of HSCs in a time-dependent manner at 24 and 48 h.

Apoptosis of HSCs induced by hUC-MSCs. After hUC-MSCs co-cultured with LX2 for 24 and 48 h, the cells were double stained with Annexin V-FITC and PI to detect apoptosis rate of LX2 using flow cytometry. Apoptosis of LX2 cells in the co-culture system was significantly increased compared with the control group (Figs. 3 and 4). The effect of hU-MSCs on LX2 cell apoptosis was evaluated by Hoechst 33342 staining. Apoptotic cells demonstrating nuclear condensation were detected by Hoechst 33342 staining and fluorescence microscopy. As illustrated in Fig. 5, co-culture of LX2 cells and hU-MSCs for 24 h showed more cells with condensed and fragmented nuclei than in the negative control group. Similar results were obtained at 48 h. The number of apoptotic bodies in the experimental group was significantly increased compared with the control group.

Decreased level of TGF-β1 caused by hUC-MSCs. TGF-β1 is a cytokine that plays a central role in fibrosis. To investigate whether hU-MSCs affected TGF-β1 production in the co-culture system, the cells were subjected to ELISA to measure production of the profibrotic cytokine TGF-β1 by LX2. TGF-β1 protein in LX2 was significantly decreased in the co-culture group compared with the negative control group at 24 h, with similar results at 48 h (p<0.05) (Fig. 6).

The expression of TGF-β1, Smad3 and Smad7 mRNA. TGF-β1, Smad3 and Smad7 mRNA expression in LX2 cells was determined by RT-PCR. After 24 h co-culture, TGF-β1
Figure 4. Apoptosis of LX2. (A) Apoptotic cell level in the co-culture system was significantly increased than compared with that in the control group at 24 h. (B) The results had significant difference at 24 h. (C) Apoptotic cell level in the co-culture system was significantly increased than compared with that in the control group at 48 h. (D) The results had significant difference at 48 h.

Figure 5. Hoechst results. (A) Control group at 24 h. (B) Experimental group at 24 h. (C) Control group at 48 h. (D) Experimental group at 48 h. (Magnification, x100).
and Smad3 mRNA expression in the experimental group was significantly lower than that in the negative control group (p<0.05), but Smad7 mRNA expression increased compared with that in the negative control group (p<0.05) (Fig. 7). LX2 cells synthesized and secreted less TGF-β1 and Smad3 and more Smad7 after being co-cultured with hUC-MSCs.

Figure 6. Decreased level of transforming growth factor-β1 (TGF-β1) caused by human umbilical cord mesenchymal stem cells (hUC-MSCs). TGF-β1 protein in hepatic stellate cells (HSCs) was significantly decreased in co-culture groups compared with control group at 24 h, and with similar results at 48 h.

Figure 7. The expression of transforming growth factor-β1 (TGF-β1), Smad3 and Smad7 mRNA.

Figure 8. Expression of transforming growth factor-β1 (TGF-β1), Smad3 and Smad7 protein in control group and experimental group. (A) From left to right: control group at 24 h, experimental group at 24 h, control group at 48 h, the experimental group at 48 h. (B) The expression of TGF-β1, Smad3 and Smad7 protein at 24 h. (C) The expression of Smad3 and Smad7 protein at 48 h.
Protein expression of TGF-β1, Smad3 and Smad7 by hUC-MSCs. After the hUC-MSCs were co-cultured with LX2 cells for 24 h, TGF-β1 and Smad3 protein began to decrease, and their expression (1.51±0.06 and 1.06±0.07) was significantly lower than that in the control group (2.20±0.04 and 1.37±0.07) (p<0.05). Similar results were found at 48 h, their expression (1.13±0.03 and 0.97±0.06) was significantly lower than that in the control group (1.29±0.04 and 1.18±0.04) (p<0.05). After 24 and 48 h co-culture, Smad7 protein expression in the experimental group (2.58±0.07 and 2.35±0.18) increased significantly compared with that in the control group (2.09±0.13 and 1.74±0.59) (p<0.05) (Fig. 8).

Discussion

Our study is believed to be the first to show that hUC-MSCs inhibit the proliferation of HSCs by affecting the TGF-β/Smads pathway, and the formation of liver fibrosis. It is well known that HSCs are the main source of ECM, and the proliferation and activation of HSCs may promote the occurrence of hepatic fibrosis. It has been shown previously that the MSCs may be affected by the signal transduction pathway during activation of HSCs (10), but the effect of hUC-MSCs on HSCs is not clear. This study confirmed that using an in vitro model, hUC-MSCs could inhibit proliferation of HSCs by inhibiting the TGF-β/Smads pathway.

Prior to this study, we used BM-MSCs to inhibit liver fibrosis (11). However, compared with BM-MSCs, hUC-MSCs are more primitive, have greater differentiation ability and low immunogenicity, are not subject to ethical constraints, and are easy to culture through a noninvasive procedure. Röslund et al (12) reported a 45.8% rate of spontaneous malignant transformation during culture of BM-MSCs. It has been suggested that spontaneous malignant transformation represents a biohazard in long-term ex vivo expansion of BM-MSCs, but hUC-MSCs propagating in continuous culture ultimately enter senescence and are not susceptible to spontaneous malignant transformation (13). Therefore, we chose hUC-MSCs for this study. We separated the hUC-MSCs from the umbilical cord for culture, and verified their ability to differentiate into fat cells in vitro.

The proliferation and activation of HSCs is an important step in the development of hepatic fibrosis (14). We would like to understand further whether hUC-MSCs can inhibit the proliferation of HSCs by regulating their proliferation. We used Transwell migration assay to co-culture hUC-MSCs and HSCs, and confirmed that hUC-MSCs inhibited the proliferation of HSCs. We then used the flow cytometry technique to verify that the increase in HSC inhibition was the result of apoptosis of hUC-MSCs and not the result of death of HSCs. Finally, we directly observed apoptosis of HSCs by Hoechst staining. At the same time, we concluded that these effects were not through direct contact among cells, but rather by cytokines secreted into the culture medium. ELISA showed that hUC-MSCs secreted low levels of TGF-β1, while HSCs secreted a large amount, and the TGF-β1 levels of co-cultured HSCs were significantly decreased. These results are similar to those reported previously in a study of low levels of TGF-β1 in liver cirrhosis (15).

Previous studies have indicated that hUC-MSC therapy results in significant improvement of liver function and hepatic fibrosis, but the specific mechanism is still unclear.

Liver fibrosis is related to gene expression of many cytokines, such as TGF-β, platelet-derived growth factor, endothelin, fibroblast growth factor, connective tissue growth factor and leptin, and there are multiple signaling pathways involved in the formation of liver fibrosis (16-19). TGF-β is a cytokine that causes hepatic fibrosis and plays an important role in the activation of muscle fibroblasts. It has been shown that, in the 6 weeks after CCl4-induced liver fibrosis, TGF-β1 levels in the serum and liver increase (9). The TGF-β/Smads signal transduction pathway is the most important in liver fibrosis, therefore, we are also interested in the effects of hUC-MSCs on the pathway. The Smads protein family is located on HSCs and is divided into receptor activation and inhibitory Smads. The former includes Smad3, which can transfer the signal from the cytoplasm to the nucleus, and promote formation of liver fibrosis. Inhibitory Smads include Smad7, which can inhibit the formation of Smads complexes and the signal transduction process, thereby inhibiting the formation of fibrosis (20). Many studies have shown that activation of the TGF-β/Smads signaling pathway can induce collagen deposition (21). Our experiments found that the levels of TGF-β1 and Smad2 secreted by HSCs decreased after co-culture, while the concentration of Smad7 increased. These data confirmed that hUC-MSCs inhibit the TGF-β1/Smads pathway to inhibit proliferation and promote apoptosis of HSCs, thereby inhibiting the formation of liver fibrosis.

There are still a few issues to resolve. For example, will similar results be obtained in vivo. Previous studies have shown that inhibition of the TGF-β1/Smads pathway may lead to tumor occurrence (22). This needs further research.

In conclusion, this study is believed to be the first to demonstrate hUC-MSCs inhibit proliferation and induce apoptosis of HSCs by paracrine inhibition of the TGF-β1/Smads pathway. Our results indicate the potential of hUC-MSCs as a method for the treatment of liver fibrosis. However, the complexity of the mechanism requires further study.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

LTZ and JFL conceived and designed the experiments. XBP and XQF performed the experiments. XBP, HC and XRM analyzed the data. XBP wrote the paper.
Ethics approval and consent to participate

The study protocol was conducted in accordance with the provisions of the Declaration of Helsinki, 1975 and approved by the Institutional Review Board of the First Hospital of Lanzhou University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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