Condition medium of HepG-2 cells induces the transdifferentiation of human umbilical cord mesenchymal stem cells into cancerous mesenchymal stem cells

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Abstract: This study aimed to investigate the transdifferentiation of human umbilical cord mesenchymal stem cells (hUCMSCs) into cancer-associated mesenchymal stem cells (CA-MSCs) after incubation with condition medium (CM) from liver cancer HepG-2 cells, and the biobehaviors (proliferation and migration) of these CA-MSCs were further evaluated. The supernatant of HepG-2 cells was collected and mixed with equal volume of low glucose DMEM. The resultant medium was used to treat hUCMSCs for 48 h. The expression of CA-MSCs related proteins and miR-221 was detected in cells. The supernatant of induced hUCMSCs was mixed with equal volume of high glucose DMEM, and the resultant medium was used treat HepG-2 cells for 48 h and the proliferation and migration of HepG-2 cells were evaluated. Moreover, HepG-2 cells were co-cultured with hUCMSCs and then the proliferation and migration of HepG-2 cells were assessed. After incubation with the supernatant from HepG-2 cells, hUCMSCs showed significantly elevated expression of vimentin, fibroblast activation protein (FAP) and miR-221. The supernatant of induced hUCMSCs was able to significantly increase the proliferation and migration of HepG-2 cells. Following co-culture, the proliferation and migration of HepG-2 cells increased dramatically. These findings suggest that the supernatant of HepG-2 cells is able to induce the phenotype of CA-MSCs and the supernatant of CA-MSCs may promote the proliferation and migration of HepG-2 cells. These findings provide experimental evidence for the cellular remodeling in tumor microenvironment and the safety of clinical use of hUCMSCs.

Keywords: Stem cells, umbilical cord mesenchymal stem cells, hepatocellular carcinoma, transdifferentiation, cell migration

Introduction

Primary liver cancer is a common malignancy. In recent years, the incidence of primary liver cancer increases significantly with the alterations of living style and environment pollution in China. To date, primary liver cancer has been the second most common malignancy of the digestive system and significantly threatens the human health [1, 2]. Tumor microenvironment is a new concept proposed in recent years. It refers to the microenvironment composed of extracellular matrix (ECM) and cells and has been found to be able to facilitate the proliferation and migration of cancer cells. Cells in the microenvironment include vascular endothelial cells, immune and carcinoma-associated fibroblasts (CAFs) [3, 4]. Tumor microenvironment has been a focus in recent studies on cancers. There is evidence showing that tumor microenvironment can induce the proliferation, differentiation, epithelial-mesenchymal transition (EMT) and migration of cancer cells. The proliferation and differentiation of cancer cells are closely related to multiple factors, in which tumor microenvironment may facilitate the angiogenesis, induce the production of cytokines and increase the synthesis of ECM, which may directly or indirectly affect the proliferation, migration and EMT of cancer cells [5, 6].
HepG-2 cells induces the transdifferentiation of hUCMSCs

recent years, studies have confirmed that there are stem cells in the liver cancer, which are also known as liver cancer-derived mesenchymal stem cells (LC-MSCs). As compared to MSCs of other origins (such as human umbilical cord mesenchymal stem cells [hUCMSCs]), adipose derived mesenchymal stem cells, and bone marrow mesenchymal stem cells, the proportion of CAF positive cells increases significantly in LC-MSCs [7, 8]. Studies also confirm that CAF is an important factor that can induce the proliferation and migration of cancer cells. The tumor microenvironment may also induce the recruitment of MSCs of different origins into the cancer, affecting the progression of the cancer [9-12]. The source of MSCs is wide, the collection of MSCs is relatively easy, the method used for the expansion of MSCs is mature and MSCs have a low immunogenicity. Thus, they have been widely applied in the therapy of tissue injury. In specific conditions, MSCs can differentiate into blood vessels, lung, hear, pancreas, muscle and neurons. MSCs are also an important component of the tumor microenvironment. Thus, to safely and efficiently use MSCs has been a focus in clinical studies. In the present study, the supernatant of liver cancer HepG-2 cells was used to treat hUCMSCs and the transdifferentiation of hUCMSCs into cancer associated MSCs (CA-MSCs) was investigated, and the influence of supernatant from CA-MSCs on the proliferation and migration of HepG-2 cells was further explored.

Materials and methods

Ethics statement

All the procedures were conducted according to the Ethical Issues in Animal Experimentation in 2009. Umbilical cord tissues were collected from discarded placenta. The informed consent was obtained from pregnant women before study, and the whole study protocol was approved by the Ethics Committee of the Third People's Hospital of Yunnan Province.

Sample collection and cell sources

The umbilical cord tissues were collected in the Department of Obstetrics from the healthy pregnant women with a good nutritional status. Informed consent was obtained from the pregnant women or their relatives. The Ethics Committee approved this study. Human liver cancer HepG-2 cells were stored in the Key Laboratory of Molecular Medicine in Kunming Medical University.

Instruments and reagents

Fetal bovine serum (FBS; Santa Cruz, USA), high glucose (HG) DMEM (Santa cruz, USA), EDTA (Santa cruz, USA), β-Glycerophosphate sodium (santa cruz, USA); trypsin (China Gino Biotechnology Co., Ltd.), protease inhibitor (PMSF) (Beyotime Biotech Co., Ltd), RIPA protein lysis buffer (Beyotime Biotech Co., Ltd), Tris base (Mbchem, USA), Glycine (Fluka, USA), sodium dodecyl sulfate (SDS) (Sigma, USA), acrylamide (Fluka, USA), N, N-methylene bis propanamide (Fluka, USA), Tetramethyl-die-thylamine (TEMED) (Sigma, USA), bovine serum albumin (BSA) (Sigma, USA), polyvinylidene-fluoride (PVDF) membrane (Millipore, USA), Protein Marker (Fermentas), Taq polymerase (Shanghai Jingke Chemical Technology Co., Ltd.), dNTP (Shanghai TaKaRa Biotech Co., Ltd), DEPC (Shanghai TaKaRa Biotech Co., Ltd), Trizol (Shanghai TaKaRa Biotech Co., Ltd), Marker (Shanghai TaKaRa Biotech Co., Ltd), Donkey anti-rabbit IgG (cy3), Goat anti-mouse IgG (Cy3) (Beijing Kangwei Shijii Biotech Co., Ltd), rabbit anti-vimentin antibody (Bioworld), rabbit anti-FAP antibody (Abcam), Hoechst dye (Sigma), clean beach (Suzhou Purification Equipment Factory), inverted microscope, confocal fluorescence microscopy (Olympus), nucleic acid and protein analyzer and chemiluminescent gel image system (GE) were used in this study.

Separation, culture and identification of hUCMSCs

Tissue explants adherent method was used to separate hUCMSCs which were then maintained in complete α-MEM containing 10% FBS. When the cell confluence reached 80-90%, cells were passaged at a ratio of 1:3. Under an inverted phase contrast microscope, the cell morphology and adhesion were observed. Cells of passage 5 were subjected to the detection of cell surface markers (CD19, CD29, CD90 and CD105) by flow cytometry.

Treatment of hUCMSCs with supernatant from HepG-2 cells

HepG-2 cells were maintained in HG-DMEM containing 10% FBS. HepG-2 cells in logarithmic growth phase were digested with trypsin and then seeded into 10-cm dish (5×10⁵ cells /
HepG-2 cells induces the transdifferentiation of hUCMSCs

dish). Cells were cultured over night and the supernatant was removed. Cells were washed with PBS thrice (2 min for each). Cells were grown in HG-DMEM for 48 h. The supernatant was harvested and centrifuged at 1000 r/min for 5 min. Supernatant was collected and filtered through a 0.22-μm filter. The filtrate was stored at -70°C. hUCMSCs in logarithmic growth phase were digested with trypsin and seeded into 50-ml dish (2×10^5 cells/dish). After over night culture, low glucose (LG)-DMEM was mixed with equal volume of supernatant from HepG-2 cells. The resultant medium was used to incubate hUCMSCs. In control group, LG-DMEM was mixed with equal volume of HG-DMEM.

Treatment of HepG-2 cells with supernatant from induced hUCMSCs

After treatment with supernatant from HepG-2 cells, hUCMSCs were harvested, washed with PBS thrice (2 min for each) and then maintained in LG-DMEM containing 10% FBS for 48 h. The supernatant was collected. Then, HepG-2 cells in logarithmic growth phase were washed in PBS thrice (2 min for each), digested with trypsin and counted. These cells were seeded into 10-cm dish (5×10^5 cells/dish). After incubation over night, the supernatant was removed and cells were washed in PBS thrice (2 min for each). The supernatant of induced hUCMSCs was mixed with equal volume of HG-DMEM, and the resultant medium was used to incubate HepG-2 cells. In control group, the supernatant from untreated hUCMSCs was used. In blank control group, only culture medium was used.

Protein extraction

1×10^6 cells were collected and lysed in 50 μl of lysis buffer (PIRA: PMSF=1:250). After vortexing for 1 min, the cells were allowed to stay on ice for 10 min. These procedures were repeated thrice. Then, centrifugation was done at 10000 rpm/min for 30 min at 4°C, which repeated thrice. The supernatant was harvested after each centrifugation. The supernatant was mixed and alloquoted, followed by centrifugation at 13000 rpm/min for 30 min at 4°C. The above supernatants were filtered with a 0.22-μm needle, and the filtrate was transferred into a 15-ml centrifuge tube and stored at -70°C. The protein quantification was performed in 10-μl supernatant.

Western blotting

The total protein concentration was determined with Bradford method. Then, proteins denaturized in loading buffer at 100°C for 5 min, and 50 μg of proteins was loaded for separation in 10% separating gel and 5% stacking gel at 20 mA until the bromophenol blue reached the bottom of the gel. The proteins were electronically transferred onto PVDF membrane at 100 V for 1 h. The membrane was incubated with 5% non-fat milk at room temperature for 1 h, followed by incubation with primary antibody at 4°C over night. Following washing in TBST thrice, the membrane was treated with secondary antibody at room temperature for 1 h, followed by washing in TBST thrice. The membrane was visualized with enhanced chemiluminescent substrate (Pierce Biotechnology, USA) for 5 min, and the protein bands were scanned and analyzed with image analysis system. The optical density (OD) of each band was determined and normalized to that of GAPDH as an internal reference. Experiment was done in triplicate at least three times.

Immunofluorescence staining

hUCMSCs in logarithmic growth phase were washed in PBS thrice (2 min for each), digested with trypsin and counted. Then, these cells were seeded into 24-well plates (2×10^4 cells / well), followed by incubation at 37°C in an environment with 5% CO₂. hUCMSCs were treated for 48 h as described in 1.4.2, and then washed in PBS twice (2 min for each). These cells were treated with 3% H₂O₂ for 10 min to inhibit the endogenous peroxidase activity. Following washing in PBS, cells were treated with 0.5% Triton-100 for 10 min, and washed in PBS thrice (2 min for each). These cells were treated with secondary antibody IgGHRP (Zhongshan Goldenbridge, China) at 37°C for 30 min and then washed in PBS twice (2 min for each). These cells were treated with 3% H₂O₂ for 10 min to inhibit the endogenous peroxidase activity. Following washing in PBS, cells were treated with 0.5% Triton-100 for 10 min, and washed in PBS thrice (2 min for each). These cells were treated with secondary antibody IgGHRP (Zhongshan Goldenbridge, China) at 37°C for 30 min and then washed in PBS twice (2 min for each). These cells were treated with 3% H₂O₂ for 10 min to inhibit the endogenous peroxidase activity. Following washing in PBS, cells were treated with Hoechst 33342 at 1:1000 (Sigma-Aldrich) in dark at room temperature for 15 min. These cells were subsequently observed under a fluorescence microscope.
HepG-2 cells induces the transdifferentiation of hUCMSCs

Cell co-culture

HepG-2 cells in logarithmic growth phase were washed in PBS thrice (2 min for each), digested with trypsin and counted. These cells were seeded into the lower chambers of 6-well plates at a density of 1×10⁵/well. hUCMSCs in logarithmic growth phase were washed in PBS thrice (2 min for each), digested with trypsin and counted. Then, hUCMSCs were seeded into the upper chambers of 6-well plates (1×10⁵/well). In the lower chamber, HG-DMEM containing 10% FBS (2.5 ml) was added; in the upper chamber, LG-DMEM containing 10% FBS (1.5 ml) was added. In control group, DMEM was not added to the lower chamber, and LG-DMEM containing 10% FBS was added to the upper chamber (1.5 ml). Incubation was done at 37°C in an environment with 5% CO₂ for 2 d.

Colony formation assay

HepG-2 cells were treated as described in 1.4.3/1.4.7. Treated HepG-2 cells and untreated HepG-2 cells were washed in PBS thrice (2 min for each), digested with trypsin, counted and re-suspended. These cells were seeded into 6-well plates (1000 cells/well), followed by incubation at 37°C in an environment with 5% CO₂ for 10 d. The medium was refreshed once daily. Then, the supernatant was removed, and cells were washed in PBS and fixed in 4% parafomaldehyde. After washing in PBS, cells were stained with crystal violet and photographed.

Cell counting

HepG-2 cells were treated as described in 1.4.3/1.4.7. Treated HepG-2 cells and untreated HepG-2 cells were washed in PBS thrice (2 min for each), digested with trypsin, counted and re-suspended. These cells were seeded into 24-well plates (1000 cells/well), followed by incubation at 37°C in an environment with 5% CO₂. The medium was refreshed once every 3 days. Since day 5, cells were washed twice, digested with trypsin and counted at a low magnification.

Cell migration assay

HepG-2 cells were treated as described in 1.4.3/1.4.7. Treated HepG-2 cells and untreated HepG-2 cells were washed in PBS thrice (2 min for each), digested with trypsin, counted and re-suspended. These cells were seeded into 24-well Transwell plates (5×10⁴ cells/well). In the upper chamber, 200 μl of serum free medium was added; in the lower chamber, 600 μl of HG-DMEM containing 10% FBS was added, followed by incubation at 37°C in an environment with 5% CO₂ for 12 h. Then, cells were washed in PBS and fixed in paraformaldehyde. After washing in PBS, cells on the member of upper chamber were removed carefully with a swab, followed by staining with crystal violet. After washing in PBS, cells were observed under a light microscope.

Extraction of total RNA

About 1×10⁶ cells were harvested and added to a 60-mm dish, followed by addition of 1 ml of Trizol reagent. After incubation at room temperature for 5 min, the mixture was transferred into a DEPC-treated EP tube, followed by addition of 0.2 ml of chloroform. Following incubation at room temperature for 5 min, centrifugation was done at 12000 g for 15 min at 4°C. The supernatant was collected and transferred into a new DEPC-treated EP tube, followed by addition of equal volume of isopropanol. Following incubation at room temperature for 10 min, centrifugation was done at 12000 g for 10 min at 4°C. The supernatant was removed, and 1 ml of 75% ethanol was added to the sediment, followed by centrifugation at 12000 g for 5 min at 4°C. The supernatant was removed, and the sediment was dried at room temperature for 2-5 min. The sediment was dissolved in DEPC treated water (30 μl) at 55-60°C for 10 min, and the purity and concentration of extracted RNA were determined by ultraviolet (UV) spectrophotometry. Finally, the extracted RNA was stored at -70°C.

RT-PCR and real time fluorescence quantitative PCR

After PCR, the products (5 μl) were subjected to 1.5% agarose gel electrophoresis and the results were observed. Preparation of agarose gel: 0.3 g of agarose was added to 30 ml of 1×TAE, and the mixture was boiled in a microwave oven to dissolve the agarose. When the temperature returned to 50°C, the agarose was added to the gap between two glasses and a comb was inserted. The comb was removed 20 min later, and electrophoresis was done in 1×TAE. U6 served as a control.
HepG-2 cells induces the transdifferentiation of hUCMSCs

Statistical analysis

Statistical analysis was performed with SPSS version 19.0. Quantitative data are expressed as mean ± standard deviation (Mean ± SD). Comparisons were done with one way analysis of variance among groups and with t test between two groups. A value of $P<0.05$ was considered statistically significant.

Results

Protein expression in hUCMSCs after treatment with supernatant from HepG-2 cells

The morphology of hUCMSCs was observed before and after treatment with supernatant from HepG-2 cells. The morphology of hUCMSCs remained unchanged after treatment with supernatant from HepG-2 cells. Western blotting was employed to detect the CMSCs related proteins (vimentin and FAP) in hUCMSCs after treatment with supernatant of HepG-2 cells. Results showed the expression of vimentin and FAP increased significantly in hUCMSCs after treatment with supernatant of HepG-2 cells as compared to control group ($P<0.05$) (Figure 1A, 1B). Immunofluorescence staining was employed to detect the expression of vimentin and FAP in hUCMSCs after treatment. Results also indicated the fluorescence intensity of both proteins increased markedly as shown in Western blotting (Figure 1C).

miR-221 expression in hUCMSCs after treatment with supernatant from HepG-2 cells

hUCMSCs were collected after treatment with supernatant from HepG-2 cells, real time fluorescence quantitative PCR was performed to detect miR-221 expression. Results showed the miR-221 expression increased significantly after treatment with the supernatant of HepG-2 cells as compared to control group ($P<0.05$) (Figure 2).

Influence of treated hUCMSCs on the proliferation of HepG-2 cells

hUCMSCs were collected after treatment with supernatant of HepG-2 cells. Then, the supernatant of treated hUCMSCs was harvested and mixed with equal volume of HG-DMEM, and the mixture was used to treat HepG-2 cells for 48 hours.
HepG-2 cells induces the transdifferentiation of hUCMSCs

In control group, the supernatant from untreated hUCMSCs was used to treat HepG-2 cells for 48 h. Results showed the number and size of colonies formed by treated HepG-2 cells increased significantly as compared to control group (P<0.05) (Figure 3A). In addition, the number of treated HepG-2 cells was significantly larger than in control group (P<0.05) (Figure 3C).

Influence of treated hUCMSCs on the migration of HepG-2 cells

Transwell chamber was used to detect the migration of HepG-2 cells after treatment with the supernatant from treated hUCMSCs. Results showed more cells crossed the membrane in HepG-2 cells after treatment with the supernatant from treated hUCMSCs as compared to control group (P<0.05) (Figure 4A, 4B). In addition, the morphology of cells was comparable between two groups.

Proliferation of HepG-2 cells after co-culture

hUCMSCs were co-cultured with HepG-2 cells for 2 d. Results showed the colony formation capability of HepG-2 cells increased significantly, and the number and size of colonies formed by HepG-2 cells were markedly larger in HepG-2 cells co-cultured with hUCMSCs than in un-treated HepG-2 cells (Figure 5A, 5B). Since day 5, the proliferation of HepG-2 cells after co-culture increased significantly as compared to control group (P<0.05) (Figure 5C).

Migration of HepG-2 cells after co-culture with hUCMSCs

After co-culture with hUCMSCs for 2 d, HepG-2 cells were subjected to the detection of cell migration. Results showed the more HepG-2 cells crossed the membrane after co-culture with hUCMSCs as compared to control group (P<0.05) (Figure 6A, 6B).

Discussion

Studies have confirmed that MSCs have high self-renewal and pluripotent potentials. MSCs are easy to collect and expand in vitro. In vivo, MSCs can secret a variety of soluble cytokines and express endogenous ECM to provide an essential microenvironment for the growth, proliferation, migration and differentiation of cancer cells. In addition, MSCs can also specifically migrate into the cancer, which suggests the...
HepG-2 cells induces the transdifferentiation of hUCMSCs

**Figure 4.** A. Migration of HepG-2 cells (scale bar =200 μm); B. Number of HepG-2 cells crossing the membrane in different groups. Treatment with the supernatant from treated hUCMSCs markedly increased the number of HepG-2 cells crossing the membrane (*P<0.05 vs control group).

Important role of MSCs in the precise treatment of cancers. MSCs may serve as a drug carrier for the therapy of cancers and specially target the cancer cells [13, 14]. Moreover, MSCs have a low immunogenicity. MSCs in vivo can alter the tumor microenvironment to affect the proliferation and migration of cancer cells. Thus, selective gene modification may be done in these cells to alter the expression of MSCs related miRNAs and/or proteins, which may regulate the capability of MSCs to secrete some cytokines and ECM, exerting a negative regulation on the cancer progression. This may finally delay the proliferation, migration and invasion of cancer cells, exerting the therapeutic effects on cancers [15, 16]. hUCMSCs are easy to collected, can be expanded massively in vitro and have a low immunogenicity. Moreover, they have the transdifferentiation, which displays their prospects in the tissue repair and cancer therapy. hUCMSCs are a group of adult stem cells. In the presence of specific induction, they can differentiate into mesodermal cells (such as osteoblasts, adipocytes, skeletal muscle and tendon cells) and endoderm and ectoderm cells (such as astrocytes, neurons, endothelial cells and cardiomyocytes) [17, 18]. In recent years, studies have revealed that the proliferation and differentiation of cancer cells are closely related to multiple factors in which tumor microenvironment as an important factor is able to promote angiogenesis, induce the production of multiple cytokines and increase ECM, directly or indirectly affecting the proliferation and migration as well as EMT of cancer cells. It has been confirmed that MSCs are a crucial component of tumor microenvironment [19, 20]. To efficiently and safely use MSCs and reduce or avoid the tumorigenic potential of MSCs (especially hUCMSCs) have been a focus in studies on stem cells. Available studies have revealed that MSCs can produce a variety of cytokines and increase ECM to facilitate the growth and metastasis of cancers. Song et al [21] injected both MSCs and cancer cells into immunosuppressive mice, and results showed the cancer size was significantly larger than in mice injected with cancer cells alone. Cuiffo et al [22] confirmed that MSCs could alter the miRNA expression to affect the binding of miRNA to transcriptional factor FOXP2, leading to the migration and invasion of cancer cells.

In our previous study, LC-MSCs were successfully separated from liver cancer tissues. LC-MSCs have the general characteristics of MSCs. In addition, LC-MSCs have high expression of CD13, CD29, CD44 and CD105, and no expression of CD34, CD38 and CD133). However, the in vitro proliferation capability of LC-MSCs is more potent than the normal MSCs. Studies have shown miRNAs are involved in the liver cancer progression and crucial for the proliferation, apoptosis, invasion and metastasis of liver cancer cells. There is evidence showing that LC-MSCs have a high miR-221 expression, and miR-221 can promote the proliferation and migration of liver cancer cells. miRNAs as a...
HepG-2 cells induces the transdifferentiation of hUCMSCs

proto-oncogene or tumor suppressor gene can regulate the expression and function of target genes, further affecting the biological behaviors of cancer cells. Studies have also been conducted to investigate miR-221 in the pathogenesis of liver cancer. As compared to healthy subjects, high miR-221 expression is found in the serum of primary liver cancer patients. In the present study, the supernatant was collected from HepG-2 cells and used to treat hUCMSCs. Then, RT-PCR was done to detect miR-221 expression. Results showed the miR-221 expression increased significantly in hUCMSCs after treatment with the supernatant from HepG-2 cells. This confirms that hUCMSCs are able to express miR-221 after induction with CM from liver cancer cells.

In addition, our results also indicated that the supernatant from HepG-2 cells altered the expression of miRNA and CA-MSCs related proteins and induce the phenotype of CA-MSCs, which promote the tumor microenvironment reconstruction and significantly increase the proliferation and migration of liver cancer cells. Studies [23-25] have shown that liver cancer cells can also induce the differentiation of hUCMSCs into CAFs, which is related to the exocytosis of liver cancer cells. The transforming growth factor (TGF)-β/Smad is an important signaling pathway involved in the regulation of differentiation of hUCMSCs into CAFs. Thus, the cancer cells induced transdifferentiation of hUCMSCs into CA-MSCs should be considered before the clinical application of hUCMSCs, especially in the anti-tumor therapy. Thus, the pluripotent and tumorigenic potentials of MSCs increase the risk in the clinical use of MSCs because it might promote the cancer progression. To reduce the clinical risk of hUCMSCs, it is necessary to modify the hUCMSCs, especially by gene modification, to enhance their therapeutic effects and simultaneously avoid the cancer progression and malignant transformation.

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HepG-2 cells induces the transdifferentiation of hUCMSCs

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Disclosure of conflict of interest

None.

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HepG-2 cells induces the transdifferentiation of hUCMSCs


