Therapeutic Effects of CXCL9-overexpressing Human Umbilical Cord Mesenchymal Stem Cells on Liver Fibrosis in Rats

Yang Li

Hebei Medical University

Xueshuai Ye

Hebei Medical University

Xueqian Zhang

Hebei General Hospital

Ziqi Cai

Hebei Engineering Technology Research Center for Cell Therapy

Li Shen

Hebei Engineering Technology Research Center for Cell Therapy

Mengya Zhang

Hebei Engineering Technology Research Center for Cell Therapy

Wentao Zhang

Hebei Medical University

Jianhui Cai (✉ jianhuicai2001@163.com)

Hebei General Hospital <https://orcid.org/0000-0001-8744-3565>

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Abstract

Background: Umbilical cord mesenchymal stem cells (UC-MSCs) transplantation have become a promising treatment for liver fibrosis. However, UC-MSCs have limited anti-fibrosis ability, and their homing ability of UC-MSCs to the injured liver sites appears to be poor. In this study, we aimed to determine if overexpression of CXCL9 could have the synergistic anti-fibrosis effect with UC-MSCs, and whether it can promote the homing ability of UC-MSCs.

Methods: Overexpression of CXCL9 in UC-MSCs (CXCL9-UC-MSCs) was attained by transfection of naive UC-MSCs with the lenti-CXCL9-mCherry. The impact of transplanted CXCL9-UC-MSCs on both repairing of liver fibrosis and homing was evaluated and compared with lenti-mCherry empty vector transfected UC-MSCs (control UC-MSCs).

Results: After puromycin screening, UC-MSCs could stably express CXCL9 without affecting the stem and differentiation ability of UC-MSCs. In addition, biochemical analysis showed that the liver function of CXCL9-UC-MSCs was significantly increased in comparison with that of control UC-MSCs ($P < 0.05$). Furthermore, histopathology after 4 weeks of cell therapy demonstrated that the content of collagen fibers decreased obviously, the pseudo-lobules almost disappeared, and the morphology of hepatic lobules was basically normal. Frozen sections were performed 24 hours and 4 weeks after the cell injection. It can be seen that the fluorescence expression of the CXCL9-UC-MSCs group was significantly higher than that of the control UC-MSCs group, which proved that CXCL9-UC-MSCs have a stronger chemotactic ability, and can stay longer than control UC-MSCs in the injured liver.

Conclusion: Overexpression of CXCL9 improves the efficacy of UC-MSC therapy for liver fibrosis repair, thereby promoting the homing and staying of UC-MSCs to injured hepatic sites in a rat model of liver fibrosis.

Background

Liver fibrosis is a intersection of the end-stage chronic liver disease caused by various reasons. With the progress of the disease, it will progressively worsen to cirrhosis, decompensation of cirrhosis or even liver cancer, which is a serious threat to human's health and life. Orthotopic liver transplantation (OLT) is an important treatment for end-stage liver disease. However, it is difficult to widely carry out OLT due to few donors, high risk, immune rejection and complications. Therefore, seeking new effective treatment methods has become the focus of end-stage liver disease, such as cell therapy and gene therapy. Umbilical cord mesenchymal stem cells (UC-MSCs) are pluripotent stem cells, which have a wide range of sources and low immunogenicity. In addition to their differentiation activity and self-renewal ability, they also have paracrine effects improving the disease microenvironment [1–4]. Under the induction of specific conditions, these cells can also differentiate into osteoblasts, adipocytes and hepatocytes [5]. Many studies have applied Umbilical cord mesenchymal stem cells can be treatment to liver fibrosis and have shown effectiveness of anti-fibrosis. Unfortunately, there is still uncertainty about the effect. One of

the reasons is that it is difficult to accurately homing to the target organ after intravenous transplantation, which affects the therapeutic effect of UC-MSCs [6]. Therefore, using gene technology to modify mesenchymal stem cells to further improve their chemotaxis and repair ability may provide a more effective treatment for chronic liver diseases such as liver fibrosis.

Chemokines are a class of chemotactic molecules with cytokine function, which play a role in regulating inflammatory response in many tissues. In acute and chronic liver diseases, chemokines usually indirectly participate in the injury response by regulating the infiltration of immune cells (monocytes, T cells) into the liver. Many studies have shown that chemokines can act directly on hepatocytes and stellate cells, and participate in the response to inflammation and fibrosis damage. In liver fibrosis, a direct inhibitory effect of CXCL9 on collagen secretion of hepatic stellate cells (HSC) was identified, and CXCL9 was also a strong counter-regulatory molecule of VEGF-driven aberrant liver vascularization and perfusion in vitro and in vivo [7]. Interestingly, CXCL9 also have been shown to influence the adhesion, crawling and spreading between MSCs and aortic endothelial cells [8].

In this study, we aim to establish the CXCL9-UC-MSCs by overexpression of CXCL9, and to determine whether CXCL9-UC-MSCs could improve their capability for ameliorating liver fibrosis, thereby promoting homing of UC-MSCs to rat livers and provide experimental basis for UC-MSCs in the treatment of liver fibrosis and other chronic liver diseases in the future.

Methods

Isolation culture of UC-MSCs

The UC-MSCs were isolated and cultivated with the tissue explants adherent method in vitro. Human umbilical cord were obtained from Hebei General Hospital with patients' informed consent, and in compliance with national legislation. All experimental protocols were approved by the Ethics Committee of Hebei General Hospital. The cord is washed with cold phosphate-buffered saline (PBS) after removal of umbilical arteries and veins. Then, cut the cord into small pieces (2-5mm) and cultured in a 10cm² culture dish supplemented with Clin-SFM®-Human MSC (Beijing Clin-Biotechnology, Beijing, China). Cells are incubated at 37°C at an atmosphere of 5% CO₂ with saturated humidity. The UC-MSCs from the third passage were selected and then a total of 10⁶/100uL cells were labeled with different antibodies, including CD45, CD34, CD73 and CD90 (BD Biosciences, CA, USA). Subsequently, the solution was stored in the dark for 30 min. After washing with PBS, the cells were subjected to analysis and validation of specific markers by flow cytometry.

Adipogenic and osteogenic differentiation

UC-MSCs were plated at a density of 1 x 10⁵ cells in 6 well plates and incubated at 37°C in a humidified atmosphere of 5 % CO₂. At 100 % confluence, we performed adipogenic differentiation by treating UC-MSCs with Human Umbilical Cord Mesenchymal Stem Cell Adipogenic Differentiation Kit (Cyagen

Biosciences, Guangzhou, China) for 3 weeks according to protocol, followed by staining with oil red O to detect lipids.

To investigate the osteogenic differentiation capacity of UC-MSCs, cells in 6 well plates were treated with Mesenchymal Stem Cell Osteogenic Differentiation Kit (Cyagen Biosciences, Guangzhou, China) at 60%-70% confluence. The medium was changed every 3–4 days. After 3 to 4 weeks, the cells were stained with Alizarin Red S, rinsed with water and examined by microscopy.

Peraperation of CXCL9-UC-MSCs and control UC-MSCs

To establish CXCL9-UC-MSCs and control UC-MSCs, the lenti-CXCL9-mCherry or lenti-mCherry empty vector was used to transfect naive UC-MSCs at passage 3 respectively. The lenti-CXCL9-mCherry was purchased from GenePharma (Suzhou, Jiangsu, China). In preparatory experiment, we transfected UC-MSCs with MOI = 1, 10, 20, 50, 80, 100 in 96 well plates, polybrene with concentration of 5ug/ml was added to enhance the infection efficiency. After 72–96 hours of infection, the number of infection with the best cell condition and the highest expression of mCherry was selected as the amount of virus used in the formal infection experiment.

We conducted the formal test in 6 well plates. UC-MSCs were plated in a 6 well plates at 1×10^5 cells/well. After 24 hours of transfection (MOI = 50), the cells were cultured in complete medium continuously. 72 hours after infection, CXCL9-UC-MSCs and control UC-MSCs were cultured in cell culture medium containing 0.4μg/mL puromycin for 7 days to generate a stable cell lines. The screening effect was observed under the inverted fluorescence microscope. When the fluorescence positive rate was close to or higher than 99%, the stable expression of CXCL9 gene of umbilical cord mesenchymal stem cell line was obtained.

Western blot analysis of CXCL9 protein

Western blot (WB) analysis was performed to examine levels of CXCL9 protein, in which the same number of CXCL9-UC-MSCs and control UC-MSCs (transfected with lenti-mCherry empty vector) were used to extract total protein samples. The concentration of protein was determined by BCA method (Thermo Fisher Scientific, MA, USA) following the manufacturer's instructions. Proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked in 5% skim milk and then incubated with the primary antibody (1:5000, Abcam) at 4°C overnights. After washing the PVDF membrane by TBST, it was incubated with the secondary antibody which is a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:3000, Abcam) for 50 minutes at room temperature. The resulting blots were detected by enhanced chemiluminescence (ECL) and visualized under an imaging system(Thermo Fisher Scientific).

Cell counting kit (CCK8) and flow cytometry assay of CXCL9-UC-MSCs and control UC-MSCs

CXCL9-UC-MSCs and control UC-MSCs cells were cultured in 96 wells at a density of 1×10^4 cells per well respectively, and each group has 5 multiple wells. On the 1st-7th day after inoculation, 20 μ l CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well and incubated at 37°C in a humidified atmosphere of 5 % CO₂ for 4 hours. Then, the optical density (OD) value of each well was measured and recorded by an enzyme-labeled microplate spectrophotometer at 450 nm. The Flow cytometry assay of CXCL9-UC-MSCs was performed as described above.

Hepatogenic differentiation

To further investigate whether overexpression of CXCL9 affects the differentiation of UC-MSCs into hepatocyte like cells, we induced CXCL9-UC-MSCs and control UC-MSCs into hepatocytes in vitro. The two kinds of cells were plated in the 6 well plates which had been coated with gelatin in advance, and the complete medium was added until about 70% – 80% confluence. After that, the complete medium was replaced by hepatocyte induction medium. First, cells were cultured with IMDM (Sigma Chemical, MO, USA) containing EGF (20ng/ml, Novoprotein, Shanghai, China) and bFGF(10ng/ml, Novoprotein) for 2 days. Next, the IMDM containing HGF(20ng/ml, Novoprotein), bFGF(10ng/ml, Novoprotein) and nicotinamide (0.61g/L, Sigma Chemical, MO, USA) was used for 7 days' culture. During that time we exchanged the medium once. IMDM which was changed every 3–4 days containing OSM (20ng/ml, Novoprotein), dexamethasone (1 μ mol/L, Beijing Solarbio Science & Technology, Beijing,China) and ITS (50g/L, Sigma Chemical) was used for culture at last. At the fourth week of culture, the induced cells were stained with immunofluorescence respectively, and the fluorescent expression levels of hepatocyte specific proteins ALB(1:100, Abcam), AFP(1:100, Abcam) and CK18(1:200, Abcam) were observed under inverted fluorescence microscope (Tokoy, Japan, Nikon).

Animal model of liver fibrosis

We purchased 55 male Sprague-Dawley rats aged 6 to 8 weeks from Beijing SiPeiFu Biotechnology Co., Ltd. All animals were raised and treated in specific pathogen-free (SPF)-grade cages in the Animal Center of the Fourth Hospital of Hebei Medical University. All animal experiments were approved by the Hebei Medical University Animal Care and Use Committee. Animal care and related procedures were approved by the Animal Care and Management Committee of the Fourth Hospital of Hebei Medical University. Liver fibrosis was induced by intraperitoneally (i.p.) injecting rats with CCL4 (3ml/kg; RHAWN, Shanghai, China) dissolved in olive oil (Macklin, Shanghai, China), twice a week for 6 weeks (12 dose). We randomly selected 45 rats for modeling, and the remaining 10 were used as normal controls.

Transplantation of CXCL9-UC-MSCs and control UC-MSCs

In the remaining 30 rats of liver fibrosis group, ten rats were injected CXCL9-UC-MSCs (1×10^6) via the tail vein after 3 days of the last dose of CCL4. In parallel with the treated group, twenty CCL4- induced rats were injected with control UC-MSCs (1×10^6) and PBS (200 μ l) instead of CXCL9-UC-MSCs as positive control group and negative control group. Moreover, another 10 rats were treated as normal controls without any treatment. The survival of rats in the four groups were observed and recorded daily.

Assessment of liver function

The treatment effect was quantified by assessing serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (T-Bil). In addition, hematoxylin-eosin (HE) and masson 's trichrome (MT) staining were used to observe the pathological changes of liver in each group. The serum of rats were determined in an automated biochemical analyser (Selectra 2, Merck, Darmstadt, Germany) 4 weeks after the initiation of the cell-based therapies. Animal liver tissues of each group were obtained after cardiac perfusion with ice saline. Next, tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sliced to a thickness of 4 μ m. Slices were subsequently stained by HE and MT.

Analysis of UC-MSCs homing ability to rat liver

To compare the homing efficiency of CXCL9-UC-MSCs and control UC-MSCs to the injured rat liver, the frozen section technique were used. At the 24 hours and 4 weeks after cell therapies. We took out the rats' liver of each group, frozen the tissues in refrigerator at -80°C immediately, embedded in optimal cutting temperature compound (OTC), frozen sections (8 μ m thick) were prepared to observe the accumulation and metabolism of mCherry in liver tissue under inverted fluorescence microscope (Tokoy, Japan, Nikon).

Statistical analysis

Statistical analysis used a commercially available statistical software package IBM SPSS Statistics, version 23.0 for Windows (SPSS, Chicago, IL, USA). Statistical differences were analysed for significance by applying one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Characterization and differentiation ability of UC-MSCs

About 7 days after umbilical cord tissue was isolated, some cells migrated from tissue fragment. Then, continued to culture and passage. UC-MSCs were confirmed successfully by their morphology under the inverted microscope (Fig.1a). Next, we characterized and validated the UC-MSCs at passage 3 by flow cytometry using specific cell surface markers for UC-MSCs. As shown in the figure, the positive expression rates of CD34 and CD45 were both 0.5%, 98.2% of the cells were CD73-positive and 99.7% were CD90-positive (Fig.1b), indicating that the surface markers of UC-MSCs were consistent with expression characteristics. Meanwhile, after 3 weeks of adipogenic differentiation, the shape of the MSCs gradually became round or irregular, and bright red lipid droplets could be seen after Oil red O staining. Subsequently, UC-MSCs were induced for 4 weeks in the osteogenic differentiation medium, the morphology of cells were obviously changed from long fusiform to polygonal, and Alizarin red staining showed the calcium nodules (Fig.1c). This indicates that MSCs have multiple differentiation potentials.

Preparation of CXCL9-UC-MSCs and Control UC-MSCs

The screening results of pre-experiment confirm that MOI=50 is the best transfection efficiency. Then, naive UC-MSCs at passage 4 were transfected with the lenti-CXCL9-mCherry and lenti-mCherry empty vector, and puromycin selection was completed after 7 days. Under the invert fluorescence microscopy, the UC-MSCs were tested positive for red fluorescence at 99% approximately (Fig.2a), indicating successful genetic modification of the UC-MSCs. Western blot analysis has shown that CXCL9 protein was overexpressed markedly in CXCL9-UC-MSCs compared with control UC-MSCs (Fig.2b), demonstrating the successful establishment of the CXCL9-UC-MSCs cell line with stable CXCL9 overexpression.

Growth trend and phenotype identification of CXCL9-UC-MSCs

The result of CCK-8 analysis demonstrated that in the process of UC-MSCs cell proliferation, the 1st to the 3rd days is the incubation period of cell growth. Next, it entered logarithmic growth period at the 3rd to the 6th day. After that, due to the excessive number of cells, the cell growth entered the plateau phase, and the growth curve was S-shaped which was consistent with the regularity of cell growth. However, compared with the control UC-MSCs, the proliferation ability of the CXCL9-UC-MSCs was significantly decreased (Fig.2c). Afterwards, we identified CXCL9-UC-MSCs by flow cytometry, the results showed that the CD34-positive and CD45-positive rats were both 0.2%, and the positive expression rates of CD73 and CD90 was 99.8% and 99.9%, respectively (Fig.2d). This suggested that the overexpression of CXCL9 didn't affect the characterization of UC-MSCs, but it has a significant inhibitory effect on the proliferation of UC-MSCs.

Comparison of the ability to differentiate into hepatocytes

We used the same induction method to induce CXCL9-UC-MSCs and UC-MSCs into hepatocytes. About 12 days after induction, the morphology of the cells changed from long spindle to round or irregular polygon gradually, the nucleus was more obvious than before, which was similar to the morphological characteristics of hepatocytes, and the granules in the cytoplasm increased gradually (Fig.3). After 4 weeks of induction, we stained the cells with immunofluorescence. The results showed that the green fluorescence of CK18 was expressed in the cell membrane, AFP and ALB were expressed in the cytoplasm, and there was no significant difference between CXCL9-UC-MSCs and control UC-MSCs (Fig.4). Illustrating that the UC-MSCs overexpressing CXCL9 still had the ability to differentiate into hepatocyte like cells.

Verification of CCl4-induced liver fibrosis

We dissected the rats that received CCL4, we found that there is a severe adhesion between liver and other abdominal organs, the edge of the rat's liver was round and blunt, and the texture was harder than that of the normal liver. Haematoxylin-eosin (H&E) staining and Masson's trichrome (MT) staining of the paraffin sections demonstrated that rat liver tissue has obvious necrosis, significant steatosis, and fibrous structure disorder, fibrous tissue intersperses to separate liver lobules, and pseudo-lobules have

formed. These observations are in accordance with the histological characteristics of liver fibrosis, and the fibrosis scored as grade 4 based on Knodell's scoring system (Table.1).

Recovery of the liver in the CCl₄-induced fibrosis rat model after transplantation CXCL9-UC-MSCs

H&E staining and MT staining showed the morphological changes and level of hepatic damage in the livers of rats with liver fibrosis after transplantation. Histologically the degeneration and necrosis of hepatocyte in the CXCL9-UC-MSCs and UC-MSCs livers were less severe than in the PBS-treated pathologic control group. However, there was less degeneration and necrosis in the CXCL9-UC-MSCs group. Moreover, in the CXCL9-UC-MSCs group, we observed that the content of collagen fibers around and inside the hepatic lobules decreased obviously, the pseudo-lobules almost disappeared, and the morphology of hepatic lobules was basically normal, the ballooning degeneration of hepatocytes was also greatly improved (Fig.5a). Both H&E and MT staining indicated that the infusion of CXCL9-UC-MSCs into rats with CCL4-induced fibrosis improved the histological structure of the liver compared with the control treatment. Meanwhile, the activities of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (T-Bil) were measured with automatic biochemistry analyser. The ALT, AST and T-Bil has highly increased in the PBS group relative to the normal group. However, the levels were significantly relieve in the CXCL9-UC-MSCs and UC-MSCs groups 4 weeks after initiation of cell transplantation (Fig.5b).

Enhanced ability of homing in CXCL9-UC-MSCs to the liver of rats with liver fibrosis

The liver of rats in each group were collected after 24 hours and 4 weeks of cell transplantation for frozen sections. Under the fluorescent inverted microscope, 24 hours after the injection of cells, the mCherry-labeled cells in the livers of CXCL9-UC-MSCs and control UC-MSCs groups were scattered in the liver lobules, mainly concentrated around the blood vessels, and the fluorescence of CXCL9-UC-MSCs group was significantly more than that of control UC-MSCs group. Four weeks later, the CXCL9-UC-MSCs group still had more mCherry fluorescence expression, but the control UC-MSCs fluorescence expression was very weak (Fig.6). These results indicate that CXCL9-UC-MSCs have stronger chemotaxis than control UC-MSCs, and can stay longer in the fibrotic liver tissue and play a stronger anti-fibrotic role.

Discussion

The effectiveness of CXCL9-expressing UC-MSCs in suppressing liver fibrosis and stimulating liver regeneration in vitro and in vivo shows that CXCL9-UC-MSCs have a powerful therapeutic effect in rats with CCl₄-induced liver fibrosis. The major findings are as follows:(1) The expression of CXCL9 did not affect the characterization of UC-MSCs, nor does it affect the ability of UC-MSCs to differentiate into hepatocyte like cells, but had an effect on the growth and proliferation of UC-MSCs; (2) transplantation of CXCL9-UC-MSCs greatly ameliorated liver injury, and the effects of CXCL9-UC-MSCs exceeded those of control UC-MSCs; (3) treatment with CXCL9-UC-MSCs reversed liver damage and decreased toxicity, seen as reduction of ALT, AST and T-Bil back to normal levels; and (4) CXCL9-UC-MSCs homed into fibrosis-affected rat liver more efficiently than control UC-MSCs.

Transplantation of liver progenitor cells (LPCs), mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs) has become a promising therapy to treat various liver disease mainly due to the ability of these cell types to promote liver regeneration [9,10]. Among the that, MSCs have the characteristics of multi-directional differentiation potential, low immunogenicity, and non-induction of tumorigenesis even after multiple passages in vitro [11-13]. Human MSCs, can be derived from bone marrow (BM-MSCs), adipose tissue (AT-MSCs), amniotic fluid (AF-MSCs), dental pulp (DP-MSCs), umbilical cord (UC-MSCs) and fetal lung (FL-MSCs) [14]. UC-MSCs and BM-MSCs are widely used in liver injured diseases, but UC-MSCs have the advantages of convenient source, lower immunogenicity and less ethical restrictions compare to BM-MSCs. Therefore, UC-MSCs are the cell source in this study.

Most chronic liver diseases involving inflammation have a fibrotic component which involves remodeling and excess accumulation of extracellular matrix components (EMC), and fibrosis leads to liver failure and death eventually. In this study, the liver fibrosis rat model was generated by intraperitoneal injection of CCL4 as previously reported [15]. In recent years, MSCs transplantation has been considered as a promising approach for the treatment of liver fibrosis [16-18]. In vitro and in vivo experiments demonstrated that MSCs mainly exert antifibrotic effects by paracrine mechanisms [16]. MSCs can produce a range of cytokines and growth factors, such as insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), hepatocyte growth factor (HGF), and milk fat globule-EGF factor-8 (MFG8) which have been shown to promote hepatocyte regeneration in situ, inhibit apoptosis of hepatocyte, suppress inflammatory responses, affect HSC activation, and promote the degradation of extracellular matrix [19-22]. Moreover, MSCs can differentiate into hepatocyte-like cells and are suitable for transfection with exogenous genes [23]. However, the treatment effect of MSCs transplantation is not very satisfactory, the main reason is lacking of enough cells to function in the injured sites of liver after injected into the body.

The CXCL9 is a famous member of the CXC chemokine family and a potent chemoattractant for activated T lymphocytes, natural killer cells and monocytes, participating recruitment of Th1 regulatory T cells[24]. In addition to immune responses of Th1, CXCL9 and its receptor, CXCR3 has also been reported to be involved in the occurrence of chronic diseases in lung and liver [7,25,26-29]. As we known, the occurrence of liver fibrosis is closely related to the activation and proliferation of HSC and the up-regulation of the expression of vascular endothelial growth factor (VEGF). Activated HSCs are the major source of collagen in the liver, which can secrete a large amount of ECM protein, tissue inhibitors of metalloproteinases and matrix metalloproteinases (MMPs), causing the remodeling of liver structure subsequently[30,31]. Interestingly, increasing concentrations of CXCL9 lead to dose-dependent down-regulation of transforming growth factor β 1 (TGF- β 1) and collagen 1 α 1mRNA, which are playing an important role in the process of liver fibrosis. Furthermore, collagen protein expression of stellate cells can be strongly suppressed by CXCL9 [7]. VEGF is a central angiogenic factor during chronic liver injury. In the process of fibrosis formation, VEGF promotes the formation of new blood vessels and accelerates the remodeling process of liver lobules[32]. However, CXCL9 can be used as an anti-fibrotic cytokine together with metalloproteinase 13 (MMP13) to achieve an anti-fibrotic effect by regulating the expression of VEGF[33]. Recently, some studies have shown that the CXCL9/CXCR3 signaling pathway can promote cell migration, and proposed that MSCs could interact with aortic endothelial cells showing firm adhesion,

and crawling and spreading behaviour, which were all enhanced by CXCL9 [34,35]. But some researchers suggested that the antifibrotic action of CXCL9 might not be strong enough to ultimately cause a reduction in fibrosis in human diseases[7]. Therefore, we hypothesized that overexpression of CXCL9 could improve the therapeutic efficacy of UC-MSCs transplantation for the treatment of liver fibrosis, and consequently promote the homing capabilities of UC-MSCs to the rat livers affected by fibrosis.

The purpose of this study is to combine UC-MSCs and chemokines with genetic engineering technology, so that they can play a stronger role in anti-fibrosis. Extensive studies have suggested that gene therapy is likely to become an option for the treatment of many forms of liver diseases, and may ameliorate patient care, survival, and prognosis[36,37]. Lentiviral vector is one of the most widely used gene delivery system, the vector can effectively deliver foreign genes into host chromosomes, so as to achieve stable expression. In terms of infection ability, it can highly efficient infect neurons, hepatocytes, cardiomyocytes, tumor cells, endothelial cells, stem cells and other types of cells, so as to achieve good gene therapy effect[38]. Considering the advantages of the lentivirus as a highly efficient gene transfection system for gene therapy, the lentivirus vector was selected in this study to deliver the CXCL9 gene into UC-MSCs. Our results validated that after puromycin screening, the transfected UC-MSCs could persistently expressing CXCL9.

In this study, the cell therapy and gene therapy were combined to achieve a better anti-fibrosis effect. And this combination will not affect the characteristics of UC-MSCs, such as the ability to differentiate into hepatocytes. As expected, when the CXCL9-UC-MSCs and control UC-MSCs transplantation groups were compared, we found that liver fibrosis rats in the CXCL9-UC-MSCs transplantation group experienced better therapeutic effects as assessed by markers for liver function and liver pathology. The liver function of the CXCL9-UC-MSCs group was significantly improved, and the fibrosis was also obviously different than that of the control UC-MSCs group. Besides, we detected the levels of liver enzymes such as ALT, AST and T-Bil in serum 4 weeks after the initiation of cell therapy, and found that their levels were notably decreased in both the CXCL9-UC-MSCs and control UC-MSCs groups. The AST, AST and T-Bil levels decreased to normal levels in the CXCL9-UC-MSCs-treated group, whereas they were slightly higher than normal in the control UC-MSCs-treated group. Moreover, from the results of frozen sections, the homing ability of CXCL9-UC-MSCs and the residence time in the liver are indeed stronger and longer than those of the control UC-MSCs group. These results clearly indicated that the transplantation of UC-MSCs have a therapeutic effect, and the transplantation of CXCL9-UC-MSCs has a synergistic regenerative effect on fibrotic livers. Regeneration may be related to the promotion of UC-MSCs migration and incorporation into liver tissue.

This study still has several limitations. First, CXCL9 has undermined the growth and proliferation of UC-MSCs, further studies are underway in our laboratory to characterize CXCL9-UC-MSCs-secreted cytokines in cell culture. Second, we have only observed that the CXCL9-expressing UC-MSCs can improve liver fibrosis better, but we have not discussed the exact mechanism yet. At last, the microenvironment of liver fibrosis cannot be fully replicated in vitro, so whether CXCL9 affects the differentiation of UC-MSCs into hepatocyte like cells in vivo needs to be further explored.

Conclusion

In conclusion, our results demonstrated that overexpression of CXCL9 improves the efficacy of UC-MSCs therapy for the repair of liver fibrosis, thereby promoting homing of UC-MSCs to the injured liver of rats with liver fibrosis.

Abbreviations

UC-MSCs: Umbilical cord mesenchymal stem cells; MSCs: mesenchymal stem cells; OLT: Orthotopic liver transplantation; HSC: hepatic stellate cells; VEGF: vascular endothelial growth factor; PBS: phosphate-buffered saline; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF: polyvinylidene fluoride; HRP: horseradish peroxidase; ECL: enhanced chemiluminescence; CCK8: Cell counting kit; OD: optical density; bFGF: basic fibroblast growth factor; IMDM: Iscove's Modified Dulbecco's Medium; HGF: hepatocyte growth factor; OSM: oncostatin M; ALB: albumin; AFP: alpha fetoprotein; CK18: Cytokeratin 18; SPF: specific pathogen-free; i.p.: intraperitoneally; ALT: alanine aminotransferase; AST: aspartate aminotransferase; T-Bil: total bilirubin; HE: hematoxylin-eosin; MT: masson 's trichrome; OTC: optimal cutting temperature compound; SPSS: Statistical Package for the Social Sciences; LPCs: liver progenitor cells; HSCs: hematopoietic stem cells; BM-MSCs: bone marrow mesenchymal stem cells; AT-MSCs: adipose tissue mesenchymal stem cells; DP-MSCs: dental pulp mesenchymal stem cells; FL-MSCs: fetal lung mesenchymal stem cells; EMC: extracellular matrix components; IGF-1: insulin-like growth factor-1; EGF: epidermal growth factor; HGF: hepatocyte growth factor; MFGE8: milk fat globule-EGF factor-8; MMPs: matrix metalloproteinases.

Declarations

Ethics approval and consent to participate

Human umbilical cord were obtained from Hebei General Hospital with patients' informed consent, and in compliance with national legislation. All experimental protocols were approved by the Ethics Committee of Hebei General Hospital. (protocol number: 2019224).

All animal experiments were approved by the Animal Care and Use Committee of the Fourth Hospital of Hebei Medical University. (protocol number:W2021001).

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed supporting conclusions are included in the current manuscript.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

Yang Li: Design of the study, Data curation, Formal analysis, Writing original draft. Xueshuai Ye: Contributed to the culture of UC-MSCs, Western blot, and flow cytometry. Xueqian Zhang: Data curation, Animal experiments. Ziqi Cai: Data curation, Writing-review & editing. Li Shen and Wentao Zhang: Assisted with the isolation, culture, and characterization of stem cells. Mengya Zhang: Participated in the Western blot experiments. Jianhui Cai: Funding acquisition, Supervision. All authors approved the final version of the manuscript to be published.

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Tables

Table1 Degree of fibrosis based on Knodell’s scoring system

Stage score	Description
1	No fibrosis
2	Fibrous pericentral expansion
3	Bridging fibrosis
4	Fibrosis septa and structural disturbance of hepatic lobule
5	Cirrhosis

Figures

Figure 1

UC-MSCs culture detection and differentiation ability. a The primary umbilical cord mesenchymal stem cells (UC-MSCs) crawled out from the edge of the tissue block, and the morphology of the cells was uniform spindle or fibroblast in the third generation (×100). b Determination of the naive UC-MSCs phenotypes by flow cytometry. c Functional characterization of UC-MSCs. In adipocyte-differentiated cells, the accumulation of oil red O-stained lipid drops was observed (×400). UC-MSCs differentiated into osteocytes and exhibited enhanced calcium deposition by alizarin red S staining (×400).

Figure 2

Overexpression of CXCL9 in UC-MSCs transfected with lenti-CXCL9-mCherry vector. The UC-MSCs were stably transfected with lenti-CXCL9-mCherry or lenti-mCherry empty vector, and then analyzed for characteristics. a Transfection efficiency was detected by invert fluorescence microscopy ($\times 100$) in CXCL9-UC-MSCs and control UC-MSCs. b Western blot analysis of CXCL9 protein expression in CXCL9-UC-MSCs and control UC-MSCs. c Comparing the growth and development of CXCL9-UC-MSCs and

control UC-MSCs, the growth rate of CXCL9-UC-MSCs is significantly limited. d Determination of the CXCL9-UC-MSCs phenotypes by flow cytometry.

Figure 3

Changes of cell morphology during hepatogenic induction of UC-MSCs. With the prolongation of induction time, the shape of cells gradually changed from long spindle to irregular shape, the nucleus gradually became larger and more obvious, and the granules in cytoplasm increased significantly.

Figure 4

Immunofluorescence staining after hepatogenesis induction. Using the same induction method to induce CXCL9-UC-MSCs and control UC-MSCs. Immunofluorescence staining performed 28 days after induction demonstrated that CXCL9-UC-MSCs and control UC-MSCs all expressed hepatocyte-specific protein ALB, AFP and CK18 ($\times 200$).

Figure 5

Effects of CXCL9-UC-MSC transplantation on liver pathology and liver function of rats with liver fibrosis. A total of 30 CCL4-induced (3ml/kg) rats were randomly divided into three groups: CXCL9-UC-MSCs, control UC-MSCs and PBS groups (n=10 in each group). After 3 days of the last dose of CCL4, the CXCL9-UC-MSCs and control UC-MSCs groups were given a transfusion of 1.0×10^6 cells suspended in 500ul of PBS respectively, while the PBS group was given 500ul of PBS. Moreover, another 10 rats were treated as normal controls without any treatment. a 4 weeks later, the liver tissues of each groups were collected and subsequently HE and MT stained ($\times 100$). b The rates in the CXCL9-UC-MSCs, control UC-MSCs, PBS and normal groups, levels of serum alanine transferase (ALT), aspartate aminotransferase (AST), and total bilirubin (T-Bil) in treatment of liver fibrosis rats. Data were expressed as mean $\pm$ SD. $P < 0.05$.

Figure 6

Analysis of cell migration in liver fibrosis rats transplanted with CXCL9-UC-MSCs using the frozen sections. 24 hours after the cell injection, 4 rats were randomly selected from each of the control UC-MSCs, cell CXCL9-UC-MSCs, PBS and normol groups. The rats were sacrificed and the liver was prepared as frozen sections, the fluorescence expression was observed under inverted fluorescence microscope ($\times 100$). After 4 weeks of cell therapy, the remaining rats in each group were dissected for HE and MT staining of liver. At the meanwhile, the frozen sections were performed again to observe the fluorescence expression ($\times 100$) .