Hepatocyte growth factor modified human umbilical cord mesenchymal stem cells accelerate the recovery of mouse hepatic injury

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Abstract

Aim: MSC-based therapies have been accepted as a promising approach for treating liver injury. Here we aimed to investigate the effect of hepatocyte growth factor (HGF) modified human umbilical cord MSCs (HGF-hucMSCs) on mouse hepatic injury. Methods: HGF transduction to hucMSCs by using adenovirus-HGF was prepared. HGF-hucMSCs were transplanted into carbon tetrachloride (CCl4)-injured mice. Reverse transcriptase-polymerase chain reaction for the human 17α gene and fluorescence examination of green fluorescent protein positive cell were used to locate exogenous hUCMSCs in mouse livers. For evaluating the recovery of injured liver tissues, the pathological state of the hepatocytes was assessed, and survival rate of the mice was measured.

Results: When transplanted into liver injured mouse, hucMSCs could located in the area of liver injury. Pathological examination revealed that the mice in HGF-hucMSCs group showed less inflammation and denaturation than those in GFP-hucMSCs group. Kaplan-Meier survival curves showed that the survival rate in HGF-hucMSCs group was higher than which in GFP-hucMSCs and PBS group. Moreover, the least hepatocyte apoptosis were found in HGF-hucMSCs group.

Conclusions: Our results suggest that transplantation of HGF-hucMSCs can accelerate the recovery of mice hepatic injury.

Keywords: HGF, hepatic injury, mesenchymal stem cells, umbilical cord

Introduction

Hepatocyte transplantation have been accepted as an effective approach for patients with liver disease[1~3]. However, application of this technique is limited by the lack of organ availability[4]. It is urgent that new therapeutic approaches are developed. Mesenchymal stem cells (MSCs) provide an therapeutic alternative to hepatocyte because of their multidifferentiation capacity[5,6]. Compared with MSCs from other sources such as bone
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marrow and umbilical blood, the use of human umbilical cord MSCs (hucMSCs) has some advantages: no appreciable ethical problems, more primitive and be frozen and thawed efficiently [7,8]. In addition, human umbilical cords can easily get from delivery rooms in hospital. Recently, several studies have provided encouraging results regarding the utility of hucMSCs in the treatment for various hepatic injuries [9–11]. In our previously study, we also found that transplantation of hucMSCs can ameliorate mouse hepatic injury by differentiate into hepatocyte-like cells [12]. Thus hucMSCs treatment may be a potential approach for liver injury.

In addition to the regenerative potential, MSCs can be used as a platform for targeted delivery of therapeutic agents. It has been shown that MSCs have high transfection efficiency with exogenous genes. MSC-based targeted gene therapy can enhance the efficacy of therapeutic molecules by virtue of their delivery at higher concentrations [13,14]. Our previous study showed that MSCs can be efficiently transduced with target genes by viral vectors such as adenovirus and lentivirus without changing the MSCs properties, which makes it a very useful tool for cell-based gene therapy [14,15].

Hepatocyte growth factor (HGF), a multifunctional growth factor known to affect apoptosis, inflammation, and tissue regeneration processes, was identified as the most potent growth factor to hepatocytes [16–18]. Furthermore, HGF can mobilizes and recruits hematopoietic progenitor cells from bone marrow into the liver through SCF-mediated mechanism under circumstances of severe liver injury, which will promote the recovery of liver function [19]. Recently it has been demonstrated that MSCs derived from bone marrow, cord blood and fetal blood are capable of transduced with HGF and increase the therapeutic potential for cerebral ischemia, myocardial infarction and diseases of the nervous system [20–23].

Previously we demonstrated that hucMSCs transplantation is an effective strategy to promote liver regeneration [12]. HGF-hucMSCs had been established by adenovirus [15]. However, few studies focused on the role of HGF transduced hucMSCs (HGF-hucMSCs) in acute liver injury. Here we aimed to investigate whether HGF modified hucMSCs can accelerate the recovery of mice hepatic injury.

Materials and Methods

HucMSC isolation and expansion

HucMSCs were isolated as previously described [12]. Briefly, human umbilical cords were obtained with donor consent and umbilical cord collection was approved by the Research Ethics Committee (Jiangsu University, Jiangsu, People’s Republic of China) regarding the use of umbilical cord tissue for research purposes. Fresh umbilical cord samples (n=16) and cut into 1 mm3-sized pieces, the cords were cultured in low glucose Dulbecco’s modified Eagle’s medium (LG-DMEM) (containing 10% fetal bovine serum (FBS, Gibco, USA), penicillin, and streptomycin) at 37°C with 5% CO2. Medium was replaced 3 days after initial plating and changed in 3-day intervals. when cells reached 70–80% confluence, cultures were trypsinized and replated for subsequent expansion.
Adenoviral transfection of hucMSCs

Adenoviral transfection was performed as described previously[15]. In brief, Homologous recombination was carried out between pAd-Track-CMV-HGF plasmid and pAd-Easy plasmid (Vector Gene Technology Co., China) in BJ5183 bacteria. Adenovirus-HGF(Ad-HGF) was generated by transfecting 293A cell with recombinant linearized plasmid. Then hucMSCs of passage 3 were transfected with recombinant Ad-HGF to generate HGF-hucMSCs. The transduction of adenovirus green fluorescent protein (Ad-GFP) were used as vector control. GFP positive cell were evaluated on the automated CytoVision system from Applied Imaging (San Jose, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)

HucMSCs were plated in a flask at 1.0×104 cells/cm2 and transfected with Ad-GFP and Ad-HGF. Culture supernatants were collected at 3rd day. HGF secreted by HGF-hucMSCs in the medium was assayed by human enzyme-linked immunosorbent assay kit (Senxiong Technology Limit Co., China) according to the manufacturer’s protocol.

Animal model and cell transplantation

All experimental procedures were in accordance with Chinese legislation regarding experimental animals. BALB/c-nu/nu mice (Laboratory Animal Center, Yangzhou University, China) with age of 4 to 5 weeks were randomized into 3 groups: CCl4/PBS (n=6), CCl4/GFP-hucMSCs (n=6) and CCl4/HGF-hucMSCs (n=6) group. Normal mice served as a negative control. Mice were intraperitoneal administrated with CCl4(Nanjing Chemical Company, China) in a dose of 0.3ml/kg in vegetable oil to establish acute hepatic injury. After eighteen hours, hucMSCs (3×106 cells) were injected into livers of CCl4-treated mice. Then the mice were treated with 10% CCl4 every three days for 28 days. At day 7 post huMSCs transplantation, liver samples were removed, fixed and prepared routinely for further analysis.

RT-PCR

Total RNA was extracted from hucMSCs using Trizol Reagent (Invitrogen) and and cDNA was synthesized according to the manufacturer's instructions (MBI). RT-PCR was performed to determine the expression of MSC-specific genes (Bmi-1, Nanog, Sox-2) and HGF by a PCR thermal cycler (PCR Express, ThermoHybaid, USA). GAPDH was used as an internal control. All of the primers were gene specific (Table 1) and were produced by Shanghai Bio-Engineering Company (Shanghai, China).

Table 1. Specific primers for target and control genes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer Sequence (5’-3’)</th>
<th>Annealing Temperature(°C)</th>
<th>Product size (bp)</th>
</tr>
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<tbody>
<tr>
<td>HGF</td>
<td>F: ATGATGTCCACCGGAAGAGGAG R: GCTGCCAATGGCTCTAATG</td>
<td>60 61</td>
<td>435 530</td>
</tr>
<tr>
<td>Bmi-1</td>
<td>F: ACTGCAATGGCTCTAATG R: ATGCCTCACACGGAGAGGAG</td>
<td>60 61</td>
<td>530 435</td>
</tr>
<tr>
<td>Sox-2</td>
<td>F: ACACCAATCCATCCACACT R: GCAAACTTCCTGCAAAGCTC</td>
<td>60 61</td>
<td>224 530</td>
</tr>
<tr>
<td>Nanog</td>
<td>F: ATGCCTCACACGGAGAGGAG</td>
<td>60 61</td>
<td>369 435</td>
</tr>
</tbody>
</table>
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<table>
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<tr>
<th><strong>H17a</strong></th>
<th>R: CTGCCTACACCATTTGCTA</th>
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<tbody>
<tr>
<td></td>
<td>F: ACACCTTTTTTGAGGATCTA</td>
</tr>
<tr>
<td></td>
<td>R: AGCAATGTGAAAACCTCGGGA</td>
</tr>
<tr>
<td></td>
<td>F: GGATTTGGTCGTATTGGG</td>
</tr>
<tr>
<td></td>
<td>R: GGAAGATGGGTGATGGGATT</td>
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<tr>
<td>GAPDH</td>
<td>64 1171</td>
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<td></td>
<td>55 205</td>
</tr>
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TdT mediated dUTP nick end labeling

Cell apoptosis were evaluated using TdT mediated dUTP nick end labeling (TUNEL) assay. Liver tissues were fixed in 1% paraformaldehyde and the TUNEL assay was performed following the manufacturer’s instructions (Boster Bioengineering Company Limited). Positive cells were counted in 10 consecutive fields at 20× and 40× magnification in the tissue slides and expressed as percentage.

Histology

Paraffin sections were made from the harvested liver tissue and stained with hematoxylin and eosin for general histological evaluation. For immunohistochemistry, liver slides were incubated with diluted primary antibody against Bcl-2 (1:500) (Santacruz, CA, USA) according to the manufacturer’s instructions (Boster Bioengineering Company Ltd, Wuhan, China). Then sections were examined under the inverted microscope (Nikon, Japan, Ti-S).

Western blot

Western blot was performed to analyze the expression changes of Bcl-2 (1:500) (Santacruz, CA, USA) along with GAPDH (1:3000; Kang Cheng, Shanghai, China) in different groups. Liver tissues were harvested and lysed in RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, and 1 μg/ml leupeptin). Aliquots containing identical amounts of protein were fractionated by SDS-PAGE and transferred to methanol pre-activated PVDF membranes. After blocking with 5% (w/v) milk, membranes were sequentially incubated with primary and secondary antibodies. Blots were developed and detected by ECL kit (Applygen Gene Technology Corp).

Statistical analysis

All data were expressed as mean ± SD. Statistical significance was determined by Student’s t-test using SPSS software (SPSS, Chicago, IL, USA). Statistical significance was considered as P-values less than 0.05.

Results

Efficient transduction of hucMSCs by Ad-HGF

After transduction with adenovirus-GFP (Ad-GFP) and adenovirus-HGF (Ad-HGF), GFP- and HGF-hucMSCs emitted green fluorescence when excited with ultraviolet illumination. 48 hours post adenoviral transduction, almost 80% GFP expressing hucMSCs were observed by fluorescent microscopy (Figure 1A). We investigated the expression of human HGF and MSC-specific genes such as Bmi-1, Sox-2, Nanog. RT-PCR results revealed that HGF expression was only detectable in HGF-hucMSCs group, which was not found in hucMSCs and GFP-hucMSCs group (Figure 1B). However, Bmi-1, Sox-2, Nanog were expressed in...
GFP/HGF-hucMSCs and there is no difference between them (Figure 1B). Furthermore, the supernatants HGF concentration were approximately 100 ng/mL in HGF-hucMSCs group at day 6 post Ad-HGF transduction, whereas it was 0.6 and 1.0 ng/mL approximately in hucMSCs and GFP-hucMSCs group (Figure 1C).

**Fig. 1.** Adenoviral transduction of hucMSCs A HucMSCs transduced with Ad-GFP and Ad-HGF. a,d: hucMSCs; b, e: Ad-GFP transduced hucMSCs; c,f: Ad-HGF transduced hucMSCs. a,b,c: fluorescent field; d,e,f: bright field. (×200, Scale bars=40 μm) B RT-PCR for HGF and MSC-specific genes in hucMSCs. C ELISA for HGF level in the culture supernatant of HGF-hucMSCs at 3 days post-transduction.

Location of transplanted HGF-hucMSCs in liver injury area

Expression of the h17α, human HGF, gene and GFP protein were detected to localize the cells in the engrafted livers at 7 days after the hucMSCs were transplanted into CCl4-injured mice. In compare with normal liver (Figure 2Aa) and PBS treated liver (Figure 2Ab), we found that GFP expressing hucMSCs were observed in the sections from GFP- (Figure 2Ac) and HGF-hucMSCs groups(Figure 2Ad). RT-PCR results showed that human 17α was expressed in GFP- and HGF-hucMSCs groups (Figure 2B). HGF expression was only found in HGF-hucMSCs group(Figure 2B).
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Fig. 2. Expression of GFP, human HGF and h17α in liver injury area A Fluorescent microscopy of transplanted hucMSCs in mouse liver. a: Normal mouse liver; b: CCl4/PBS group; c: CCl4/GFP-hucMSCs; d: CCl4/HGF-hucMSCs. (×100, Scale bars=80μm); B RT-PCR of human HGF(hHGF) and h17α in mouse liver.

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Having undergone CCl4 injection to induce liver injury, the livers were examined macroscopically. Normal liver was reddish-brown, soft texture, with a smooth surface(Figure 3Aa) and injured liver appeared brownish, granular(Figure 3Ab). After GFP- and HGF-hucMSCs transplantation, the liver surface was more smoothly and less granular(Figure 3Ac–d). HE staining also revealed large areas of inflammation and hepatocyte denaturation in CCl4 treated liver(Figure 3Bb) compared with normal liver (Figure 3Ba). Decreased necrosis, lower denaturation were found in HGF-hucMSCs(Figure 3Bd) than in GFP-hucMSCs (Figure 3Bc). We further investigated whether transplantation of HGF-hucMSCs can extend the
survival of liver injured mice in our model. As shown is Figure 3C, the survival rates of the HGF-hucMSCs groups were improved compared to the PBS and the GFP/MSCs groups after 3 days. At week 3, the survival rates of the PBS, GFP-hucMSCs, and HGF-hucMSCs were 40%, 66.7%, and 100%, respectively (Figure 3C).

![Fig. 3. HGF-hucMSCs promote the recovery of hepatic injured mouse](image)

A Macroscopically examination of the mouse liver surface. a: Normal mouse liver; b: CCl4/PBS group; c: CCl4/GFP-hucMSCs; d: CCl4/HGF-hucMSCs. B HE staining of the mice received hucMSCs transplantation. (×200, Scale bars=40μm) C Kaplan-Meier plot of recipients survival rates following various hucMSCs transplantation in CCl4 injured mice.

HGF-hucMSCs suppress hepatocytes apoptosis by elevating the Bcl-2 expression
HGF have been shown to contribute to inhibit apoptosis in response to liver injury. To determine the extent of this contribution, TUNEL staining were used to evaluate apoptosis in the livers. Compare with the normal liver section(Figure 4A), wide-spread hepatocyte apoptosis was found in the liver of PBS group (Figure 4B). The level of apoptosis was decreased on GFP- and HGF-hucMSCs group (Figure 4C-D), and there are less apoptosis in HGF-hucMSCs group than in GFP-hucMSCs group (Figure 4I). Immunohistochemistry results also showed that the expression of Bcl-2 decreased in PBS group (Figure 5b). In compared with GFP-hucMSCs group(Figure 5Ac), the percentage of positively stained hepatocytes was increased in HGF-hucMSCs group (Figure 5Ad). Similar to immunohistochemistry results, western blot results demonstrated that HGF-hucMSCs could improve the expression of Bcl-2(Figure 5B).
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Fig. 4. HGF-hucMSCs suppress hepatocytes apoptosis TUNEL staining of mice liver after huMSCs transplantation. A, E: normal liver section; B, F: CCl4/PBS group. C, G: CCl4/GFP-hucMSCs group; D, H: CCl4/HGF-hucMSCs group. I: Quantitative analysis of hepatocyte apoptosis. * P<0.05: compared with GFP-hucMSCs group. Scale bars=20 μm. (A–D, ×100, Scale bars=80 μm; E–H, ×400, Scale bars=20 μm)

Fig. 5. Expression of Bcl-2 in mouse liver. A Bcl-2 expression in mouse liver at day 7 post transplantation were determined by immunohistochemistry.a: Normal mouse;b: CCl4/PBS group;c: CCl4/GFP-hucMSCs; d: CCl4/HGF-hucMSCs. (×100, Scale bars=80 μm). B Bcl-2 expression in mouse liver at day 7 post
transplantation were determined by western blot. (×200, Scale bars=40 μm).

Discussion

Transplantation of mesenchymal stem cells have being considered as a more acceptable way than whole hepatocyte or organ transplantation to cure patients suffering from liver disease[5,6]. HucMSCs are a subset of more primitive stem cell and yields a higher number of MSCs compared with other sources of bone marrow and peripheral blood ex vivo. But more importantly, hucMSCs can be obtained without ethical disagreements and had weak immunogenicity[7,8]. Our previous work and several studies have reported the presence of transplanted MSCs in injured liver and promote the recovery of liver function[6,10,12]. Recently, MSCs were considered to be delivery vehicles for high-efficiency gene transfer. HGF, the most potent growth factor to hepatocytes, can not only stimulates the liver regeneration but also recruit MSCs from bone marrow to the injured liver[24]. Although there have been a few studies have investigated the role of HGF transduced bone marrow MSCs in various models[21~23], there have not been any previous reports on the effect of HGF-hucMSCs on acute liver injury. HGF modified hucMSCs by adenovirus transduction have been established. We therefore focused on demonstrating that HGF-hucMSCs can promote the recovery of CCl4-induced mice hepatic injury.

After 48 hours, most of hucMSCs were successfully transduced with adenovirus-HGF and the HGF concentration increased to 100 ng/mL in the supernatant of HGF-hucMSCs. At the same time, the expression of MSC-specific genes were not changed during HGF transduction. These results indicate HGF can be delivered into hucMSCs in vitro while still maintaining MSCs-specific gene expression profile, consistent with previous reports[15,23]. Furhermore, localization of HGF-hucMSCs in liver injury area were determined by the expression of GFP, HGF, and h17α gene after transplanted into liver injured mice. Our results revealed that transplanted GFP- and HGF-hucMSCs could localized in the site of injured liver.

And the HGF expression were only found in HGF-hucMSCs groups.

We next observe the recovery of hepatic injured mouse after HGF-hucMSCs transplantation. HE staining also revealed heptocyte necrosis and degeneration were most reduced in GFP- and HGF-hucMSCs group. In addition, GFP- and HGF-hucMSCs transplantation significantly increased the survival of CCl4 injured mice compared with PBS group. However, least cell apoptosis and largest survival rate were found in HGF-hucMSCs group. The results demonstrated a modest therapeutic effect of GFP-hucMSCs, consistent with our previous finding[12]. Transplantation of human umbilical cord-derived MSCs with and without HGF transduction into CCl4-injured nude mice resulted in the improvement of liver function in vivo. Based on our observation, we suggested HGF modification could promote the amelioration of hucMSCs on mouse hepatic injury.

HGF has been reported to involved in anti-apoptotic processes during tissue repair, specifically in liver injury[25]. To address the problem, TUNEL assay were performed to detect the cell apoptosis in PBS, GFP- and HGF-hucMSCs treated mice. Our results indicated that GFP- and HGF-hucMSCs transplantation significantly reduced hepatocyte apoptosis. And the number of apoptotic cell is lower in HGF-hucMSCs group than which in GFP-hucMSCs group. Moreover, we found the antiapoptotic protein, Bcl-2, was increased mostly in HGF-hucMSCs Romanian Biotechnological Letters, Vol. 17, No. 5, 2012
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group. HGF-hucMSCs could inhibit cell apoptosis by elevating Bcl-2 expression.

In conclusion, the present study showed that HGF-hucMSCs transplantation can prevent hepatocyte apoptosis in injured liver, resulting in significant improvement of survival during CCl4-induced hepatic injury. Furthermore, our studies also suggested that the inhibitory effect mediated by HGF-hucMSCs on hepatic injury is associated with MSCs and HGF, which act synergistically.

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