Identification of cytokines involved in hepatic differentiation of mBM-MSCs under liver-injury conditions

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Abstract

AIM: To identify the key cytokines involved in hepatic differentiation of mouse bone marrow mesenchymal stem cells (mBM-MSCs) under liver-injury conditions.

METHODS: Abdominal injection of CCl4 was adopted to duplicate a mouse acute liver injury model. Global gene expression analysis was performed to evaluate the potential genes involved in hepatic commitment under liver-injury conditions. The cytokines involved in hepatic differentiation of mBM-MSCs was functionally examined by depletion experiment using specific antibodies, followed by rescue experiment and direct inducing assay. The hepatic differentiation was characterized by the expression of hepatic lineage genes and proteins, as well as functional features.

RESULTS: Cytokines potentially participating in hepatic fate commitment under liver-injury conditions were initially measured by microarray. Among the up-regulated genes determined, 18 cytokines known to closely relate to liver growth, repair and development, were selected for further identification. The fibroblast growth factor-4 (FGF-4), hepatocyte growth factor (HGF) and oncostatin M (OSM) were finally found to be involved in hepatic differentiation of mBM-MSCs under liver-injury conditions. Hepatic differentiation could be dramatically decreased after removing FGF-4, HGF and OSM from the liver-injury conditioned medium, and could be rescued by supplementing these cytokines. The FGF-4, HGF and OSM play different roles in the hepatic differentiation of mBM-MSCs, in which FGF-4 and HGF are essential for the initiation of hepatic differentiation, while OSM is critical for the maturation of hepatocytes.

CONCLUSION: FGF-4, HGF and OSM are the key cytokines involved in the liver-injury conditioned medium for the hepatic differentiation of mBM-MSCs.

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Key words: Hepatic differentiation; Mouse bone marrow mesenchymal stem cells; Inducing cytokines; Fibroblast growth factor-4; Hepatocyte growth factor; Oncostatin M

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INTRODUCTION

It is generally believed that the bone marrow mesenchymal stem cells (BM-MSCs) provide an appropriate hematopoietic microenvironment that exerts regulatory effects on the self-renewal and differentiation of hematopoietic stem/progenitor cells. They are capable of differentiating into mesoderm cell lineages, including osteoblasts, chondrocytes and adipocytes[1-3]. However, emerging new findings suggest that BM-MSCs are able to give rise to a more broad range of cells, including hepatocytes, neurons, epithelial cells and keratinocytes[4-6]. This plasticity of BM-MSCs has attracted much attention to their in vivo new functions under either metabolic or pathologic conditions, and their clinical therapy for tissue repair. In fact, several studies in animal models have suggested that endogenous MSCs may “naturally” be involved in wound healing and tissue regeneration, and the engrafted exogenous MSCs have beneficial effects in tissue repair, including that of bone, myocardial tissue, skin, kidney and liver[5,7]. These may encourage further studies on the mechanisms underlying MSCs differentiation, which are still poorly understood at present.

Recently, by an in vivo tracing technology, we have demonstrated that BM-MSCs could be recruited from the bone marrow into peripheral blood, and toward into the wounded sites in response to the injured-liver signals, which indicated a close relationship between BM-MSCs and liver repair[8]. Moreover, we have also found that the engrafted exogenous BM-MSCs could be recruited to the injured liver, and were able to differentiate into multiple hepatic-lineage cells, which greatly improved the wound healing, providing further insight into the relationship between BM-MSCs and injured liver[9]. Our previous reports also support the idea that the liver-injury conditioned culture medium can induce the differentiation of BM-MSCs into functional hepatic cells in an in vitro experiment[10]. These observations indicated that the hepatic differentiation of BM-MSCs may be induced by the cytokines secreted from the injured liver cells, since no cellular interactions existed in such cell-free culture medium. However, which cytokines direct hepatic fate specification of BM-MSCs still remains unclear. In the present study, we identified the key cytokines that play a crucial role in the differentiation of BM-MSCs in the liver-injury conditioned medium. We hope our finding will benefit the better understanding of the novel mechanisms underlying BM-MSCs involved liver repair and regeneration, and help improve the cytokine-based hepatic inducing strategy and provide a rich cellular resource from BM-MSCs for cytotherapy of acute liver diseases.

MATERIALS AND METHODS

Experimental animals

Eight to ten-week-old male ICR mice obtained from the Laboratory Animal Unit of Zhejiang Academy of Medical Sciences (Hangzhou, China) were used in the experiments. Animals were housed under specified pathogen-free conditions. All animal experiments were done in accordance with a legal regulation, which includes approval by a local ethical committee.

Isolation and culture of bone marrow MSCs

The mouse bone marrow MSCs (mBM-MSCs) were prepared as described previously[20]. Briefly, the bone marrow was extruded by clipping of the epiphysial ends of the bones and flushing with IMDM (Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (Hyclone, Rockville, MD), 1% penicillin/streptomycin (Medium A). After 3 d, non-adherent cells and debris were removed, and the adherent cells were cultured continuously. At near confluence, the cells were replated at 5 × 10⁴ cells/cm². Osteogenic, chondrogenic and adipogenic differentiations were examined for functional identification[21].

Preparation of acute liver-injury mouse model

The acute liver-injury mouse model was prepared according to the method described previously[22]. Briefly, the mice were treated with CCl₄ (1.0 mL/kg body weight of a 10% solution in mineral oil injected intraperitoneally) twice a day and then sacrificed by cervical vertebrae luxation on the 24th h after the last injection.

Hepatocyte isolation and preparation of conditioned medium

The hepatocytes were isolated by the two-step collagenase perfusion from healthy mice (as control) or liver-injury mouse model prepared by the method described above. Briefly, donor animals received 25 U heparin (Sigma) prior to cell isolation. After cannulation of the portal vein, the liver was perfused with a calcium-free buffer solution, 3 mL/min at 37°C for 10 min. Then, the liver was perfused with 0.025% collagenase IV (Invitrogen, Carlsbad, CA), 2 mL/min at 37°C for 15 min. The perfused liver was resected, and the cells were released by gentle shaking and collected in 20 mL IMDM. The supernatant cell suspension was filtered using a 200 mm nylon mesh and the filtrate was washed twice with PBS by centrifugation at 50 × g for 45 s to remove cell debris, damaged cells, and non-parenchymal cells. After washing, the hepatocytes were cultured in medium A at 5 × 10⁵ cells/cm². Forty-eight hours later, the supernatant was collected and passed through a 0.25 mm filter. The filtrate was finally defined as hepatocyte-injury conditioned medium and stored in aliquots at -20°C for future use.
**Induction of hepatogenic differentiation of mBM-MSCs in conditioned medium**

The mBM-MSCs of passage 3 were inoculated in differentiation medium at $5 \times 10^4$ cells/cm$^2$ on culture flasks. The differentiation medium consisted of 50% fresh IMDM medium (Medium A) and 50% conditioned medium. As a negative control, mBM-MSCs were cultured in medium A only: Cells were cultured in a humidified atmosphere of 5% CO$_2$ and 95% air at 37°C. Cultures were maintained by medium exchange every 3 d. The cell morphology was observed under a confocal laser-scanning microscope (LSM 510; Carl Zeiss Inc., Jena, Germany).

**Gene expression analysis by real-time polymerase chain reaction**

The expressions of hepatic lineage genes [α-fetoprotein (AFP), albumin (ALB), hepatocyte nuclear factor 3β (HNF3β), tyrosine aminotransferase (TAT)] and cytokine genes [Fibroblast growth factor-4 (FGF-4), hepatocyte growth factor (HGF) and oncostatin M (OSM)] involved in hepatic differentiation and commitment were analyzed by real-time polymerase chain reaction (PCR). For this, total RNA was extracted from undifferentiated control or differentiated cells, normal or injured liver cells, respectively, using NucleoSpin® RNAIIKits, and then 5 μg of which was reversely transcribed into cDNA with SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The transcripts normalized to β-actin were measured by real-time PCR using Mastercycler ep realplex system and Real-Time Detection software (Eppendorf, Germany), in which the standard double-stranded DNA dye (SYBR Green 1) was used. Gene-specific primers were designed using the Primer Premier software (Table 1).

**Protein expression analysis by ELISA**

The expressions of hepatic lineage proteins (AFP and ALB) in differentiated hepatocytes and cytokine proteins (FGF-4, HGF and OSM) in injured liver cells were further analyzed by ELISA. The total proteins from differentiated cells or undifferentiated control cells, normal or injured liver cells were extracted using T-PER (a protein extraction reagent) (Qiagen, Germany) according to the manufacturer’s manual, and were coated on a 96-well polystyrene plate at 4°C overnight. The wells were washed with PBST (500 μL. Tween 20/L PBS) and then blocked with 0.5% BSA for 1 h at 37°C. The hepatic lineage proteins (AFP and ALB) or cytokines (FGF-4, HGF and OSM) were detected using the polyconal antibodies, including rabbit anti-mouse HGF and FGF4, goat anti-mouse OSM (abcam, UK) rabbit anti-AFP and goat anti-ALB (Biosdesign, Saco, Maine, USA) after washing with PBST for 3 times. The secondary antibodies, including goat anti-rabbit and rabbit anti-goat (Santa Cruz Biotechnology, Santa Cruz, CA, USA), labeled with horseradish peroxidase (HRP) were added at a proper dilution. Finally, the TMB was added, and the observation density was detected by a microplate reader at 450 nm after 2 mol/L sulphuric acid was added to stop the reaction.

**Periodic acid-Schiff stain for glycogen**

The hepatic differentiation was functionally determined by glycogen storage. For this, the culture dishes containing differentiated cells were fixed in 95% alcohol for 10 min. Samples were then oxidized in 1% periodic acid for 5 min, rinsed three times in deionized (d) H$_2$O, treated with Schiff’s reagent for 15 min, and rinsed in dH$_2$O for 5 min. Finally, the preparations were assessed under light microscope, and the positive rate of differentiated cells was counted as previously described.$^{[9]}$

**Evaluation of urea synthesis**

The hepatic differentiation was functionally determined by urea synthesis. The mBM-MSCs were plated at $5 \times 10^4$ cells/cm$^2$ on collagen coated six-well plates in differentiation medium or control medium. After washing extensively with PBS, cells differentiated at days 0, 4, 8, 16, 20 and 21 were incubated in 2 mL of serum-free Hanks’ buffered salt solution containing 5 mmol/L NH$_4$Cl for 2 h at 37°C. After incubation, the urea concentrations in the supernatant were measured according to the method described previously.$^{[10]}$

**Global evaluation of cytokines potentially involved in injured liver commitment**

A global gene expression analysis was performed by microarray to identify the potential cytokines responsible for hepatic commitment. Total RNA and complementary DNA were prepared from CCl$_4$-treated and untreated mouse livers. An Illumina Mouse WG-6 v2.0 BeadChip was used. Gene-specific primers were designed using the Primer Premier software (Table 1).

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**Table 1 Primers and annealing temperatures used for PCR**

| Gene     | Sequence (5’-3’) | Annealing (°C) | Product (bp) |
|----------|------------------|----------------|--------------|
| AFP-F    | CACCTGGCTGAACTCTTCGTA | 52             | 300          |
| AFP-R    | CTGAGCACTTTCCTTCGTA  | 51             | 551          |
| HNF3β-F  | GGCTTCTGTTTCTTACCC   | 55             | 475          |
| HNF3β-R  | TGGAAAGCCAGATGCCGTC  | 55             | 619          |
| ALB-F    | TCTTGGCTTCGCCCTGTC   | 62             | 483          |
| ALB-R    | CTGGCAACTTCATGCAAT   | 62             | 399          |
| FGF4-F   | CTGGTGGCTCACAGGACATTAAGAT | 62             | 123          |
| FGF4-R   | GCTGGCCTGAAGAAGACGTAATAGT  | 62             | 200          |
| HGF-F    | GTGCCAACAGGTGTACG    | 62             | 399          |
| HGF-R    | TGTCACAGACTGCTGACG   | 62             | 123          |
| OSM-F    | CTCAACGCTACACACACAC  | 55             | 200          |
| OSM-R    | GAGCCATGCTCCATGTC    | 55             | 200          |

PCR: Polymerase chain reaction; AFP: α-fetoprotein; HNF: Hepatocyte nuclear factor; ALB: Albumin; TAT: Tyrosine aminotransferase; FGF-4: Fibroblast growth factor-4; HGF: Hepatocyte growth factor; OSM: Oncostatin M.
To further investigate the role of FGF-4, HGF and OSM in hepatic differentiation, an in vitro hepatic induction assay was conducted using different combinations of FGF-4, HGF and OSM. The mBM-MSCs were inoculated in medium A with different combinations of 10 ng/mL FGF-4, 20 ng/mL HGF and 10 ng/mL OSM at 5 × 10^5 cells/cm^2 on culture flasks. As a negative control, the mBM-MSCs were cultured in medium A without FGF-4, HGF and OSM. Cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. After 72 h, non-adherent cells and debris were removed and the adherent cells were cultured continuously. Cultures were maintained by medium exchange every 3 days. The hepatic differentiation was examined based on the hepatic lineage gene and protein expressions and functional characterizations as described above.

**RESULTS**

**Evaluation of cytokines in response to liver injury**

To evaluate the possible cytokines that participate in the hepatic differentiation of BM-MSCs under liver-injury conditions, the gene expression levels of cytokines/proteins in injured liver were initially examined at different injury time-points (12, 24 and 48 h) by microarray analysis. The results showed that more than 1200 genes were significantly up- or down-regulated during these time periods, and most of them were closely related to hepatocyte detoxification and metabolisms (data not shown). Totally, 40 cytokines/chemokines or their corresponding receptors closely associated with cellular growth, differentiation and migration were found to be significantly up-regulated (2-62 folds), among which 18 cytokines, including FGF-3, FGF-4, FGF-10, FGF-12, FGF-13, FGF-14, FGF-15, FGF-17, FGF-18, FGF-20, FGF-21, HGF, OSM, b-NGF, IGF-2, TGF-β1, TGF-β2 and TGF-β3, were largely contributed to the hepatic growth and development. Therefore, these cytokines were considered to be potentially involved in the hepatic differentiation of mBM-MSCs under liver-injury conditions, and to be the candidates for further identification.

**Cytokine identification in depletion experiment**

The cytokines involved in hepatic specification in liver-injury conditioned medium were initially identified in a depletion experiment. The liver-injury conditioned medium was incubated with a number of different cytokine antibodies (anti-HGF, -FGF-4, -OSM, -FGF-3, -FGF-10, -FGF-12, -FGF-13, -FGF-14, -FGF-15, -FGF-17, -FGF-18, -FGF-20, -FGF-21, -bNGF, -IGF-1, -TGF-β1, -TGF-β2 and -TGF-β3, abcam and Biodesign) at different concentrations (1:100, 1:200 and 1:500) overnight at 4°C under agitation. Then, the cytokines were removed by affinity co-immunoprecipitation. The protein A-coupled sepharose beads were prepared according to the manufacturer’s instructions (Abcam), and were added into the antibody pretreated liver-injury conditioned medium. After being incubated at 4°C for 4 h, the samples were centrifuged at 10000 × g for 5 min. Supernatants were collected for hepatic differentiation induction assay. The hepatic differentiation was examined based on the hepatic lineage gene and protein expressions and functional characterizations as described above. In parallel, a non-specific rabbit antibody (IgG isotype) was used in control groups.
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Effects of FGF-4, HGF and OSM in hepatic differentiation

To investigate the effects of each cytokine in the hepatic differentiation, a number of depletion experiments were conducted using different combinations of antibodies. The results showed that after treatment with anti-FGF-4 and anti-HGF independently, the hepatic differentiation induced by the conditioned medium was significantly (P < 0.01) down-regulated with the increased use of antibody (E1: 1:500, E2: 1:200, E3: 1:100) as determined by the expression of both the early liver-specific marker (HNF3β) and AFP) and the late liver-specific marker (ALB and TAT) at mRNA or protein levels, and by the functional [Periodic acid-Schiff (PAS) and urea production] analyses (Figure 4A-H). However, after treatment with anti-OSM alone, the expressions of HNF3β and AFP in cells were not significantly decreased (Figure 4A and B), while the expressions of ALB and TAT were dramatically (P < 0.01)

Expression analysis of FGF-4, HGF and OSM during liver injury

To obtain a deep insight into the role of FGF-4, HGF and OSM in liver injury, a kinetic expression analysis of these cytokines during liver injury was performed. The results showed that the expression of HGF mRNA was significantly up-regulated at 6 h after injury (P < 0.05), peaked at 12 h (P < 0.01) and kept in high level up to 72 h (Figure 2A). Similarly, the expressions of FGF-4 and OSM mRNAs were importantly up-regulated at 12 h (P < 0.01) after injury, peaked in 24-48 h (P < 0.01) and also kept in high levels up to 72 h (Figure 2B and C). Accordingly, the expression changes of HGF, FGF-4 and OSM proteins were generally identical with their mRNA expressions (Figure 2D-F).

Detection of FGF-4, HGF and OSM in culture medium

To further investigate the existence and occurrence of FGF-4, HGF and OSM in liver-injury conditioned medium, the kinetic secretion of FGF-4, HGF and OSM into the cultured medium was determined. The results showed that the concentrations of HGF and OSM proteins were dramatically up-regulated at 12 h after culture, peaked at 24 or 48 h and kept in high levels up to 72 h (Figure 4A and B); while the FGF-4 was significantly up-regulated at 24 h and peaked at 48 h (Figure 3B). Notably, it showed that FGF-4, HGF and OSM protein expressions could also be detected in the cell culture without undergoing liver injury (Figure 3), possibly due to the fact that isolation of cells from liver itself is a tissue “anatomy” which may result in the “injured signal” to stimulate the cells to secrete cytokines.

Expression analysis of FGF-4, HGF and OSM during hepatic differentiation of mBM-MSCs

MSC group: Control MSCs cultured in medium A with PBS; C group: MSCs induced with liver-injury conditioned medium and different combinations of specific antibodies (E1: Anti-FGF-3 + anti-FGF-4; E2: Anti-FGF-10 + anti-FGF-12 + anti-FGF13; E3: Anti-FGF-14 + anti-FGF-15 + anti-FGF-17; E4: Anti-FGF-18 + anti-FGF-20 + anti-FGF-21; E5: Anti-HGF + anti-OSM + anti-bNGF; E6: Anti-IGF-1 + anti-TGF-β1 + anti-TGF-β2 + anti-TGF-β3); (B) E1-E5 groups: MSCs induced with liver-injury conditioned medium and different specific antibody alone (E1: Anti-FGF-4; E2: Anti-FGF-3; E3: Anti-bNGF; E4: Anti-HGF; E5: Anti-OSM). Triplicate samples were determined. The results showed that the expression of HGF mRNA was significantly up-regulated at 6 h after injury (P < 0.01) and kept in high level up to 72 h (Figure 2B and C); while the FGF-4 was significantly up-regulated at 12 h after culture, peaked at 24 or 48 h and kept in high levels up to 72 h (Figure 3A and B). Accordingly, the expression changes of HGF, FGF-4 and OSM proteins were generally identical with their mRNA expressions (Figure 2D-F).

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restrained at both mRNA and protein levels (Figure 4C, D and F). Accordingly, the PAS and urea production were also dramatically ($P < 0.01$) decreased in anti-OSM treatment groups (Figure 4G and H). These results suggested that FGF-4, HGF and OSM may play different roles in the hepatic differentiation of mBM-MSCs. Among these factors, FGF-4 and HGF may be essential for the initiation of early hepatic differentiation, while OSM may be critical for the maturation of hepatocytes.

**Functional evaluation of FGF-4, HGF and OSM in rescue experiment**

In order to find further evidence on the role of FGF-4, HGF and OSM in the conditioned medium in hepatic differentiation, we performed a rescue experiment in which three recombinant cytokines were added back to the cytokine-removed medium treated with anti-FGF-4 (1:100), anti-HGF (1:100) and anti-OSM (1:100). The results showed that administration of FGF-4, HGF and OSM into this cytokine-removed medium could significantly rescue the hepatic differentiation with the increased concentrations of the cytokines as determined by the expression of ALB protein and urea synthesis (E1: 0 ng/mL FGF-4 + 0 ng/mL HGF + 0 ng/mL OSM; E2: 1.25 ng/mL FGF-4 + 2.5 ng/mL HGF + 1.25 ng/mL OSM; E3: 2.5 ng/mL FGF-4 + 5 ng/mL HGF + 2.5 ng/mL OSM;
Mesenchymal stem cells (MSCs) have emerged as a promising resource of functional hepatocytes for treatment of liver diseases because of its plasticity of multiple cell lineages. To date, many inducing systems for hepatic differentiation from MSCs have been developed\[^{[22,27,28]}\]. However, the rate of hepatocyte-like cells differentiated from MSCs is still very low and the mechanisms are also not well known. It is undoubted that exposition of MSCs to the inducing systems, resembling the conditions in liver development, injury and regeneration, could acquire a more efficient differentiation\[^{[29]}\]. Our previous studies have shown that mBM-MSCs could be induced to differentiate into hepatic cells by conditioned culture medium of hepatocytes\[^{[30]}\]. Thus identification of the exact cytokines involved in liver-injury conditions for the mBM-MSCs differentiation needed further studies. This study provides such investigation. Eighteen cytokines closely related to liver growth, repair and development were chosen as candidates from numerous up-regulated cytokine genes by microarray. It was found that three cytokines (FGF-4, HGF and OSM) may play a crucial role in the conditioned medium-induced hepatic differentiation, since hepatic differentiation was dramatically decreased after removing FGF-4, HGF and OSM from the conditioned medium.

**Hepatic differentiation induced directly by FGF-4, HGF and OSM**

Based on the observations from the above experiments that FGF-4, HGF and OSM are crucial for hepatic differentiation in the conditioned medium, we performed a further hepatic differentiation experiment induced directly by FGF-4, HGF and OSM in different combinations. The results showed that FGF-4, HGF and OSM may have synergistic effects on the hepatic differentiation of mBM-MSCs, indicating that the three cytokines may play different roles in the induction of hepatic differentiation. As shown in Figure 6, after induced with a factor alone or combination of the two, the hepatic differentiation of mBM-MSCs was slowly detectable as determined by the synthesis of both AFP (early hepatic differentiation marker) and ALB (late hepatic differentiation marker) in differentiated cells. In the group induced with FGF-4 (10 ng/mL) and HGF (20 ng/mL, E4), the concentration of AFP was highly detectable, while ALB was slowly detectable, suggesting that FGF-4 and HGF have a synergistic effect on the initiation of early hepatic differentiation. In contrast, both of the AFP and ALB proteins could be highly detected in group E5 in which the mBM-MSCs were induced by the combination of these three factors. This indicated that FGF-4, HGF and OSM had a synergistic effect in the differentiation of functional hepatocytes from mBM-MSCs. Furthermore, FGF-4 and HGF exhibited a cooperative effect on the early hepatic differentiation of mBM-MSCs, while OSM is essential to the maturation of hepatocytes in the late hepatic differentiation.

**DISCUSSION**

Mesenchymal stem cells (MSCs) have emerged as a promising resource of functional hepatocytes for treatment of liver diseases because of its plasticity of multiple cell lineages. To date, many inducing systems for hepatic differentiation from MSCs have been developed\[^{[22,27,28]}\]. However, the rate of hepatocyte-like cells differentiated from MSCs is still very low and the mechanisms are also not well known. It is undoubted that exposition of MSCs to the inducing systems, resembling the conditions in liver development, injury and regeneration, could acquire a more efficient differentiation\[^{[29]}\]. Our previous studies have shown that mBM-MSCs could be induced to differentiate into hepatic cells by conditioned culture medium of hepatocytes\[^{[30]}\]. Thus identification of the exact cytokines involved in liver-injury conditions for the mBM-MSCs differentiation needed further studies. This study provides such investigation. Eighteen cytokines closely related to liver growth, repair and development were chosen as candidates from numerous up-regulated cytokine genes by microarray. It was found that three cytokines (FGF-4, HGF and OSM) may play a crucial role in the conditioned medium-induced hepatic differentiation, since hepatic differentiation was dramatically decreased after removing FGF-4, HGF and OSM from the conditioned medium. Therefore, the present study provided a direct basis on the selection of cytokines for hepatic differentiation. However, besides these three key cytokines, some other factors involved in the liver injury need to be identified for improving the cytokine-based inducing system.

FGF-4, HGF and OSM play important roles in liver regeneration, healing, initiation and development. FGF-4 was considered to be one of the most important fibroblast growth factor family members that can irritate the proliferation of mesodermal and endodermal cells and improve development of fetal liver\[^{[31]}\]. HGF was found to be essential for the development of several epithelial organs and was one of the most well characterized cytokine for the stimulation of DNA synthesis in primary hepatocyte cultures, and for liver development\[^{[32]}\]. The OSM, however, is a member of the interleukin-6 family produced by hematopoietic cells and induces differentiation of fetal hepatic cells, conferring various metabolic activities of

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**Figure 3** Detection of kinetic secretions of HGF (A), FGF-4 (B) and OSM (C) proteins in liver-injury conditioned medium. Experiment: Hepatocyte-injury conditioned medium; Control: Normal-hepatocyte conditioned medium.
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Relative expression level of HNF3β

- A: Anti-HGF
- B: Anti-FGF4
- C: Anti-OSM

Relative expression level of AFP

- A: Anti-HGF
- B: Anti-FGF4
- C: Anti-OSM

Relative expression level of ALB

- A: Anti-HGF
- B: Anti-FGF4
- C: Anti-OSM

Relative expression level of TAT

- A: Anti-HGF
- B: Anti-FGF4
- C: Anti-OSM

a, b denote significant differences at P < 0.05.
Figure 4  Effects of FGF-4, HGF and OSM in hepatic differentiation. A number of depletion experiments using different combination of antibodies were conducted, and the expression of both liver-specific markers and function was analyzed. A-D: The expression of both the early liver-specific markers (HNF3β and AFP) 10 d after induction and the late liver-specific markers 20 d after induction (ALB and TAT) at mRNA levels detected by real-time PCR; E, F: The expression of AFP 10 d after induction and ALB 20 d after induction at protein levels detected by ELISA; G: Analysis of intracellular glycogen accumulation by PAS staining on day 20 after induction; H: Urea production by differentiated cells on day 20 after induction. MSC group: Control MSCs cultured in medium A with PBS; C group: MSCs induced with liver-injury conditioned medium with non-specific IgG; E1-E3 groups: MSCs induced with liver-injury conditioned medium and different concentrations of specific antibodies (E1: 1:500; E2: 1:200; E3:1:100). *P < 0.05, **P < 0.01 vs C group.
adult liver[29]. These three factors participate in different liver developmental stages. Thus, we further examined the exact roles of FGF-4, HGF and OSM in the hepatic differentiation from mBM-MSCs. It clearly showed that after removing FGF-4 and HGF from the conditioned medium by their antibodies, either the early or the late hepatic differentiation induced by the conditioned medium could be significantly down-regulated, while after removing OSM from the conditioned medium, only the late hepatic differentiation was down-regulated. It suggested that FGF-4, HGF and OSM also play different roles in the hepatic differentiation of mBM-MSCs, and FGF-4 and HGF are essential for the initiation of hepatic differentiation, while OSM is critical for the maturation of hepatocytes.

In conclusion, the present study analyzed the potential factors in injured liver for hepatic differentiation from mBM-MSCs. It was found that FGF-4, HGF and OSM might be the key cytokines. They played different roles during hepatic differentiation, which is similar to their functions in liver development. Hopefully, our study would not only provide evidence of cytokine selection for hepatic differentiation, but also benefit the exploration of the molecular mechanisms underlying the differentiation of BM-MSCs into hepatocytes.

**COMMENTS**

**Background**

Acute liver failure is a severe liver disease with a mortality of 60%-90%. The only therapeutic option, orthotopic liver transplantation, is limited because of the shortage of suitable donor organs. Mesenchymal stem cells (MSCs), known for their capacity to proliferate indefinitely and differentiate into almost all types of cells, including hepatocytes, have provided the hope of cellular replacement therapy for liver failure.

**Research frontiers**

Mouse liver-injury conditioned culture medium dramatically facilitated the differentiation of mouse bone marrow MSCs (mBM-MSCs) into functional hepatic cells. However, which cytokines direct hepatic fate specification of mBM-MSCs still remains unclear. In this study, the authors demonstrate that fibroblast growth factor-4 (FGF-4), hepatocyte growth factor (HGF) and oncostatin M (OSM) may play crucial roles in the differentiation of mBM-MSCs in the liver-injury conditioned medium.
Innovations and breakthroughs
In the present study, the authors reported the identification of cytokines involved in hepatic fate specification of mBM-MSCs in the liver-injury conditioned medium. By removing cytokines from conditioned medium and adding back cytokines into the “cytokine-removed” conditioned medium, it was demonstrated that FGF-4, HGF and OSM may play crucial roles in the conditioned medium-induced hepatic differentiation. Furthermore, different combinations of FGF-4, HGF and OSM were used to induce hepatic differentiation, and the result showed that FGF-4 and HGF had a cooperative effect on the early hepatic differentiation of mBM-MSCs, while OSM was essential to the maturation of hepatocytes in the late hepatic differentiation. This is the first study to report that FGF-4, HGF and OSM FGF-4, HGF and OSM not only play crucial roles in the hepatic differentiation of mBM-MSCs, but also have profound synergistic effect on the hepatic differentiation of mBM-MSCs and pro and con. This in vitro study would contribute to the improvement of hepatic cell resource for cell-based therapies for acute and chronic end-stage liver diseases and provide a model for more detailed characterization on the molecular mechanisms underlying the differentiation of BM-MSCs into hepatocytes.

Applications
This study may benefit not only the better understanding of novel mechanisms underlying BM-MSCs involved in liver repair and regeneration, but also the improvement of cytokine-based hepatic inducing strategy, in which a rich cellular resource for cytotherapy of acute liver diseases with BM-MSCs would be provided.

Terminology
FGF-4 is one of the most important fibroblast growth factor family members that can stimulate the proliferation of mesodermal and endodermal cells and improve development of fetal liver; HGF is one of the most well characterized cytokines for the stimulation of DNA synthesis in primary hepatocyte cultures and liver development; OSM is a member of the interleukin-6 family which is produced by hematopoietic cells and induces differentiation of fetal hepatic cells, conferring various metabolic activities of adult liver.

Peer review
The authors corroborate that 3 cytokines (HGF, FGF4 and OSM) are fundamental for directing the differentiation of BM-MSCs towards hepatocytes. The work is interesting and could be helpful for developing effective inducing systems of hepatic differentiation from BM-MSCs.

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