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Original Article

Bioactivity of CD34+ cells in patients with acute-on-chronic liver failure

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Abstract: Liver failure is a life-threatened serious disease with many complications and high mortality rate. Stem cells have been applied to replacement therapy, gene therapy and tissue engineering for its capacity of self-renewal and multi-lineage differentiation. To investigate the bioactivity of the peripheral blood hematopoietic stem cells (PBHSC) in patients with acute-on-chronic liver failure, we isolated CD34+ cells from peripheral blood of patients with acute-on-chronic liver failure and healthy controls. After cultured it in serum-free medium (SFEM), we studied the bioactivity of CD34+ cells by observing the morphology, recording growth curve, detecting cell cycle and cell apoptosis. CD34+ cells and culture solution were collected at the time points of 3, 5, 7, 10, 12 and 14 days, and the levels of hepatocyte growth factor (HGF), matrix metalloproteinase-9 (MMP-9), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in culture solution were detected by ELISA. Also, the expressions of pyruvate kinase muscle isoenzyme 2 (PKM2), integrin-β1 and liver-type pyruvate kinase (LPK) were detected by RT-PCR and immunofluorescence. Our results showed the bioactivity of CD34+ cells from patients with acute-on-chronic liver failure was identified to be similar with that from healthy controls. HGF, MMP-9, TNF-α and IL-6 were found in cell culture medium. RT-PCR and immunofluorescence results indicated that PKM2, Integrin-β1 expressed on CD34+ cells from patients with acute-on-chronic liver failure. In conclusion, bioactivity of CD34+ cells of patients with acute-on-chronic liver failure was demonstrated to be normal, which could secrete HGF, MMP-9, TNF-α and IL-6, promote the growth of hepatocytes, and differentiate along a direction to hepatocyte lineage.

Keywords: CD34+ cells, peripheral blood hematopoietic stem cells, liver failure, bioactivity

Introduction

Liver cirrhosis (LC) is the end stage of chronic liver disease and is very difficult to treat. Currently, liver transplantation is one of the only effective therapies available to such patients. However, serious problems are associated with liver transplantation: lack of donors, surgical complications, rejection, and high cost. Regenerative therapies have the potential to provide minimally invasive procedures with few complications. The potential for stem cells in bone marrow (BM) to differentiate into hepatocytes and intestinal cells was recently confirmed through detection of Y chromosome-containing cells in samples from female recipients of BM cells (BMCs) from male donors. BMC transplantation has been performed to treat hematological diseases, and several clinical studies have applied BMC injection to induce regeneration of myocardium and blood vessels. Taken together, these findings suggest that BMCs are effective sources for regenerative liver therapy [1-3].

The hematopoietic stem cell (HSC) is classically defined as a cell which, when transplanted into a recipient animal, can regenerate all elements of blood. There are specific cell-surface markers which define subsets of cells enriched with HSCs (e.g. CD34+CD38- cells in humans, and Lineage-Kit+Sca+CD48-CD150+ cells in mice) [4]. Despite hematopoietic stem cells were able to undergo either self-renewal for the life-long maintenance of HSC pools or lineage differentiation for blood cell production, evidence of single cell-mediated asymmetric self-renewal and differentiation has not been formally prov-

en, which will be used as our objective and be focused on our study.

Materials and methods

Patients

Between March 2013 and October 2014, 26 patients with acute-on-chronic liver hepatic failure and 20 healthy volunteer entered this phase I/II study. Eligibility criteria included age between 18 and 55 years and acute-on-chronic liver failure; abnormal serum albumin and/or bilirubin and/or prothrombin time; unsuitable for liver transplantation; World Health Organization performance status < 2; women of childbearing potential using reliable and appropriate contraception; life expectancy of at least 3 months; and ability to provide informed consent. Exclusion criteria were: patients aged below 20 years or over 65 years; liver tumors present, or history of other cancer; pregnant or lactating women; recurrent gastrointestinal bleeding or spontaneous bacterial peritonitis; evidence of active infection including human immunodeficiency virus; and inability to provide informed consent. This study was approved by the Ethical Committee of 302 Hospital of PLA (2014221D).

Mobilization protocol and harvesting of CD34+ cells of patients

Patients included in the study were admitted to the Department of Hepatobiliary Surgery. Each received a subcutaneous injection of 300 μg of granulocyte colony-stimulating factor (G-CSF, Chugai Pharmaceuticals, Tokyo, Japan) daily for 5 days to increase the number of circulating CD34+ cells in their systems. Any symptoms or signs of adverse reaction were appraised and recorded. Leukophoresis (Cobe Spectra IV, Cobe-BCT Inc., Lakewood, CO, USA) was performed in the Department of Hematology on day 5. The leukophoresis product was transferred to the Stem Cell Laboratory where CD34+ cells were immunoselected immediately using the CliniMACS device (Miltenyi Biotech, Bergisch-Gladbach, Germany). Leucocytes obtained were diluted in 1:4 in Hanks' buffered saline solution (HBSS, Gibco, Paisley, UK), before the mononuclear cells (MNC) were separated, by centrifugation over a Lymphoprep (Axis-Shield, Kimbolten, UK) density gradient at 750 g for 30 min (Heraeus, Herts, UK). The MNC fraction was collected and was washed first in HBSS, then with MACS buffer (phosphate-buffered saline supplemented with 0.5% bovine serum albumin and 5 mM EDTA, pH 7.2). CD34+ cells were isolated from the MNC using the CD34+ cell isolation kit (CliniMACS, Miltenyi Biotech).

Flow cytometry analysis

The phenotype of circulating cells was evaluated by conventional dual color immunofluorescence using PE-conjugated anti-CD34, FITC-conjugated anti-CD45 or FITC-conjugated anti-CD14 (Becton-Dickinson, Mountain View, CA). Briefly, small aliquots of heparinized peripheral blood (100 μL) were incubated with 8 μl PE-conjugated anti-CD34 antibody and FITC-conjugated anti-CD45 or FITC-conjugated anti-CD14 for 20 min in the dark at room temperature. Erythrocytes were lysed with lysis buffer (0.155 M NH_4Cl, 0.012 M $NaHCO_3$ and 0.1 mM EDTA, pH 7.2) for 5 min at room temperature. After washing with PBS, the cells were fixed with 1% paraformaldehyde and then analyzed by flow cytometry (BD FACS CaliburTM, Mississauga, ON, Canada). The data were analyzed by FlowJo software (TreeStar Inc., Ashland, OR). Live cells were identified by forward and side scatter. Gating was based upon isotype controls. Peripheral blood mononuclear cells (PBMCs), monocytes and HSCs were identified as CD45, CD14 and CD34 positive, respectively. The percentage of positive cells was calculated by subtracting the value of the isotype control. The absolute numbers of positive cells per ml were calculated as percentage of positive cells ×PBMC count.

For purification of circulating CD34+ cells, additional 20 ml blood samples were collected from five healthy controls, five patients, respectively. Mononuclear cells were separated by lymphocyte separation medium (Haoyang Biological Manufacture, Tianjin, China). Then CD34+ cells were isolated by using CD34+ MiniMacs high-gradient magnetic separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) following manufacturer's instruction. An aliquot of CD34+ cell fraction was re-stained with PE-conjugated anti-CD34 antibody (8G12, Becton-Dickinson) which recognized an epitope of CD34 antigen that was different from the antibody used in the magnetic-separation system (QBEND/10, Miltenyi Biotec). The purity of iso-

Table 1. Primers (sense and antisense) used for gene characterization

Gene	Primers	bp	Annealing temperature
PKM2	F: 5'-CCATTACCAGCGACCCCACAG-3'	138 bp	53°C
	R: 5'-GGGCACGTGGGCGGTATCT-3'		
Integrin-β1	F: 5'-CATCCCTGAAAGTCCC-3'	227 bp	52°C
	R: 5'-CATTTGTATTATCCCTCTTC-3'		
LPK	F: 5'-TGCCTGATACCCTGCTTG-3'	272 bp	53°C
	R: 5'-CACCCTCACTCTGCCTGTC-3'		

lated CD34+ cells was analyzed by flow cytometry as described above.

In vitro colony growth assay

Circulating CD34+ cells were evaluated daily, peripheral blood mononuclear low density (LD) cells were separated on a Ficoll-Hypaque gradient (density 1.077, Nygard, Oslo, Norway), then re-suspended in Iscove's modified Dulbecco's medium (IMDM). LD separated cells were cultured at different concentrations in triplicate dishes containing 1 mL of methylcellulose-based medium (HCC-4100; Stem Cell Technologies, Vancouver, Canada) and human recombinant cytokines (rhSCF, rhGM-CSF, rhIL3, rhG-CSF)+/- erythropoietin (Stem Cell, Vancouver, BC, cult_ GFH 4534). Cultures were incubated at 37°C in a fully humidified 5% CO_2 atmosphere and evaluated after 14-18 days. The number of CD34+ cells is expressed in milliliters of peripheral blood cells. Fresh CD34+ cells and expanded cells at 10th day of culture (1-2×10^3) were seeded in semi-solid culture (Metho Cult GF H4434, Stem Cell Technology) following the manufacturer's instruction. Cells were mixed with Methylcellulose-based media and purred in 35-mm Petri dishes and incubated at 37°C, 5% CO_2 in a humidified incubator. After the 14th day of culture, the number of colony was counted under the inverted microscope.

Cell cycle distribution analysis by flow cytometry

Cell cycle distribution was evaluated at 10th day of culture by flow cytometry. Prior to staining, 200 μl of 1×10^6 cells/ml were washed by phosphate buffered saline (PBS) and re-suspended in 200 μl of PBS. Cells were treated with 50 μl of RNase (1 mg/ml) and 100 μl propidium iodide (PI, 400 μg/ml) (Sigma-Aldrich, Spain) for 30 min at 37°C in the darkness. The fluorescence of stained cells was analyzed by flow cytometry and relative gated cells in each cell cycle phase were determined. Data acquisition and analysis were performed using flowmacs 2.0 software.

Apoptosis analysis by Annexin V

Apoptosis rate were evaluated at 10th day of culture by Apoptosis kit (Bioscience, USA). 1×10^6 cells were washed by phosphate buffered saline (PBS) and re-suspended in 1× binding buffer. The cells were treated with 5 μl of fluorochrome conjugated Annexin V for 15 min at room temperature. These cells were washed and re-suspended in 1× binding buffer and then 50 μl of propidium iodide solution was added. The fluorescence of stained cells was analyzed by flow cytometry after 4 hours.

Cytokine measurements

All serum samples were stored at -80°C until analysis was performed. The serum levels of cytokines were measured by high-sensitivity enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions. Hepatocyte growth factor (HGF), matrix metalloproteinase-9 (MMP-9), tumor necrosis factor α (TNF-α), and interleukin-6 (IL-6) kits were from R&D Systems (R&D system, Minneapolis, MN, USA).

Reverse transcription-polymerase chain reaction

Total RNA of CD34+ cells were extracted using Trizol reagent and following the manufacturer's instructions (Invitrogen). Reverse transcription was carried out using Moloney murine leukemia virus reverse transcriptase, and the resulting cDNA fragments were amplified using Taq polymerase (both from Invitrogen). Primer sequences were obtained from GenBank (Los Alamos, NM) and are listed in **Table 1**. Thermocycling was performed with an Eppendorf Mastercycler personal thermocycler (Westbury, NY), and the PCR products were electrophoresed on a 2% agarose (Invitrogen) gel containing ethidium bromide (Sigma-Aldrich). Gels were visualized under UV light and photographed using a Kodak DC120 digital camera (Eastman Kodak, Rochester, NY).

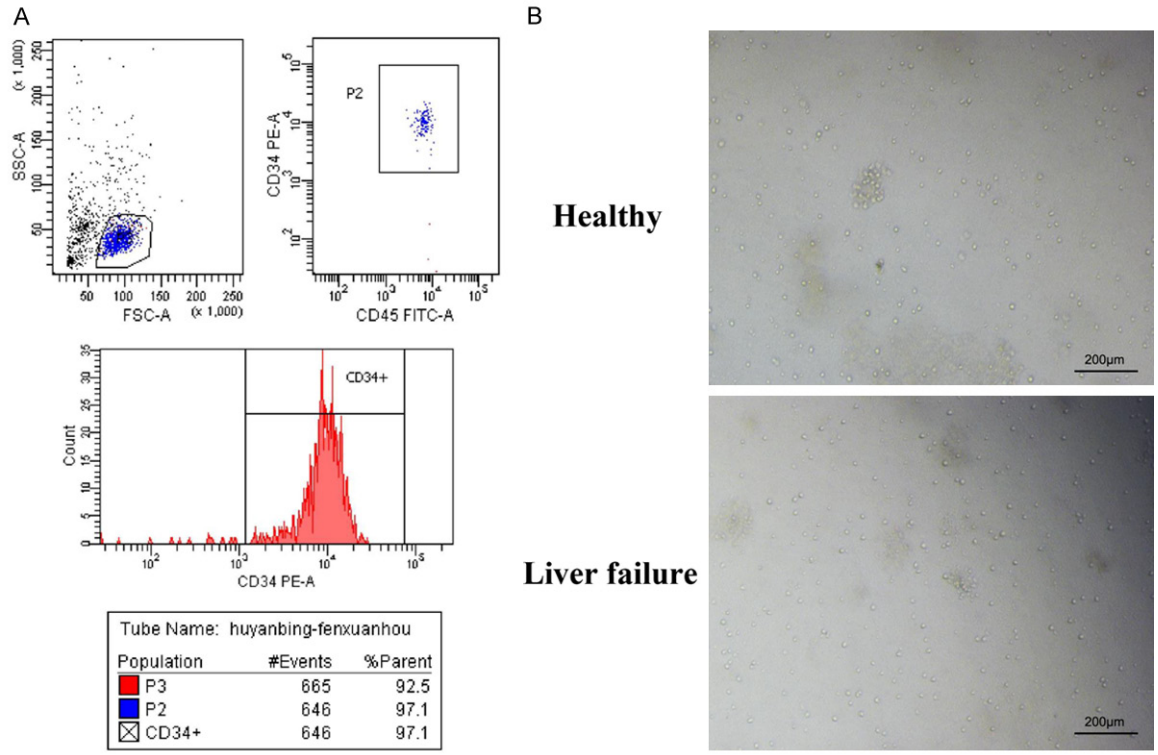

Figure 1. Identification of CD34+ cells isolated from liver failure patients. A. Cells were stained with CD34+ antibody then were analyzed by FACS. B. CD34+ cells isolated from healthy volunteers and acute-on-chronic liver failure patients were cultured in vitro. Cell morphology was overserved by microscopy (100×).

Immunofluorescence detection of Integrin-β1

CD34+ cells of were fixed with 4.0% paraformaldehyde (PFA) (Pierce, Rockford, IL) for 20 min at room temperature with gentle shaking and permeabilized with 0.2% Triton-X-100 (Sigma) for 5 min at room temperature with gentle shaking. Coverslips were incubated with 1.0% BSA for 30 min at room temperature with gentle shaking. Coverslips were incubated with appropriate primary antibodies overnight at 4°C, washed with 1× PBS and incubated with appropriate secondary antibodies for 1 h at 37°C. Primary and secondary antibodies were used at optimized dilutions recommended (1:200 or 1:100 for bovine anti-goat conjugated with Alexa 488, TRITC, Alexa 594, or 1:50 for bovine anti-goat conjugated to Alexa 647). Phalloidin staining used a dilution 1:200. Coverslips were washed with 1× PBS and mounted on Mowiol (Calbiochem, Billeritca, MA) to minimize photobleaching. Secondary antibody-only controls were performed to rule out non-specific staining (data not shown).

Fluorescent labels were visualized using the Zeiss© Laser Scanning Microscope (LSM) 510 Meta confocal microscope with a 63 X oil-immersion lens at room temperature. Stress fiber images in the CD34+ cells were taken with a Nikon C2+ confocal microscope, 60 X oil-immersion lens. Gain intensity and amplifier settings for each channel were set at a level below saturation and appropriate to provide a dynamic range of intensity for Integrin-β1 and DUSP1 using control slides from initial experiments. Gain intensity and amplifier settings for Alexa488-phalloidin were set by scanning to approximately the same Z-plane in control cells where both intensity and resolution of stress fibers was optimal. All settings were saved and used to image each sample slide within an experiment as well as for slides in all subsequent repeats of experiments. Z-stacks were taken for all slides; all images of DUSP1 and actin stress fibers represent approximately the same Z-plane of each cell.

Statistical analysis

The results were expressed as mean ± SD. Statistical significance was determined by a two-tailed Student's t test, with a p value less than 0.05 considered statistically significant.

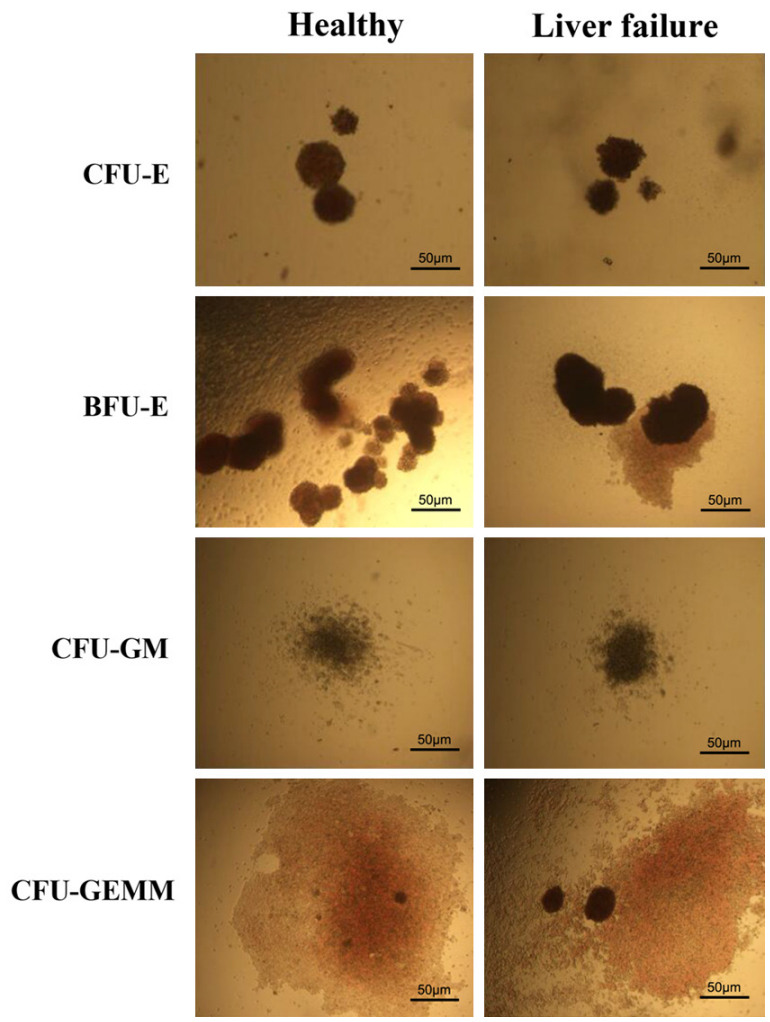

Figure 2. Colony formation analysis of CD34+ cells from healthy volunteer and liver failure patients. Cells were cultured in vitro for two weeks, then the colony formation was observed using microscopy (40×).

Data processing was carried out with SPSS 21.0 (IBM Corporation, USA).

Results

Characteristics of CD34+ cells

Followed by lysis and isolation of PBMC from patients with acute-on-chronic liver failure, we obtained 5.29 ± 1.9×10^5 CD34+ cells in patients. These cells were identified to be > 90% purity using flow cytometry (**Figure 1A**). Additionally, cell morphologies of the cultured CD34+ cells were observed by phase contrast microscopy at 2 days (**Figure 1B**) at later stages of differentiation, there was no significant difference between two groups, showing normal rule, well-stacked, uniform cells with shape of spheroid, no particle, high transparency and refraction.

CD34+ cells from patients with acute-on-chronic liver failure display normal colony formation

To compare the behaviors of CD34+ cells from liver failure patients and that from healthy volunteers, we performed the colony formation assay. The highest CFU fold change was observed in CD34+ cells at 10th day (90 ± 24) and the highest BFU-E, CFU-GM and CFU-GEMM in CD34+ cells were observed in 14th day. Results in **Figure 2** demonstrated the under the microscopy, the CFU-E (later-stage erythroid progenitor cells), BFU-E (primitive erythroid progenitor cells), CFU-GM (granulocyte-macrophage progenitor) and CFU-GEMM (granulocyte, erythrocyte, monocyte, megakaryocyte) of CD34+ cells from healthy volunteers and liver failure patients did not display significant difference (**Figure 2**).

CD34+ cells from patients with acute-on-chronic liver failure display normal proliferation rate and cell cycle phases

We next measured the cell proliferation rates of CD34+ cells from liver failure patients and normal volunteers. Cells were cultured in vitro for 14 days and the proliferating cells were counted at day 3, 5, 7, 10, 12 and 14. As we expected, at day 3, CD34+ cells from both groups displayed exponential phase and at day 10, cell growth were slow down (**Figure 3A**). The CD34+ cell growth rates were not significantly different between healthy and liver failure groups.

We further analyzed the cell cycles of CD34+ cells between healthy and liver failure groups. In CD34+ cells of patients with 70.09 percent of expanded cells were at G0/G1 and 29.91% were at S phase of cell cycle and in controls,

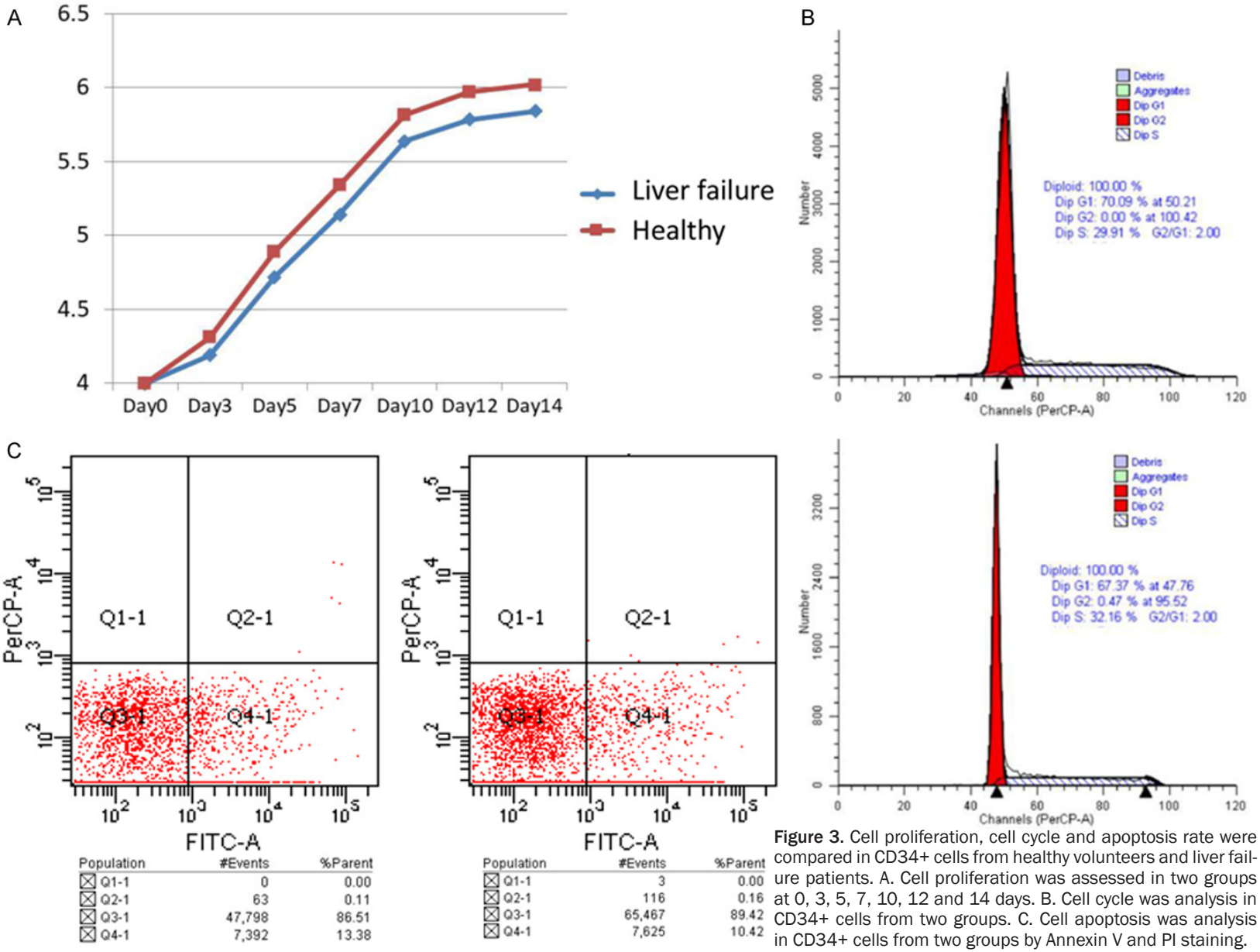

Figure 3. Cell proliferation, cell cycle and apoptosis rate were compared in CD34+ cells from healthy volunteers and liver failure patients. A. Cell proliferation was assessed in two groups at 0, 3, 5, 7, 10, 12 and 14 days. B. Cell cycle was analysis in CD34+ cells from two groups. C. Cell apoptosis was analysis in CD34+ cells from two groups by Annexin V and PI staining.

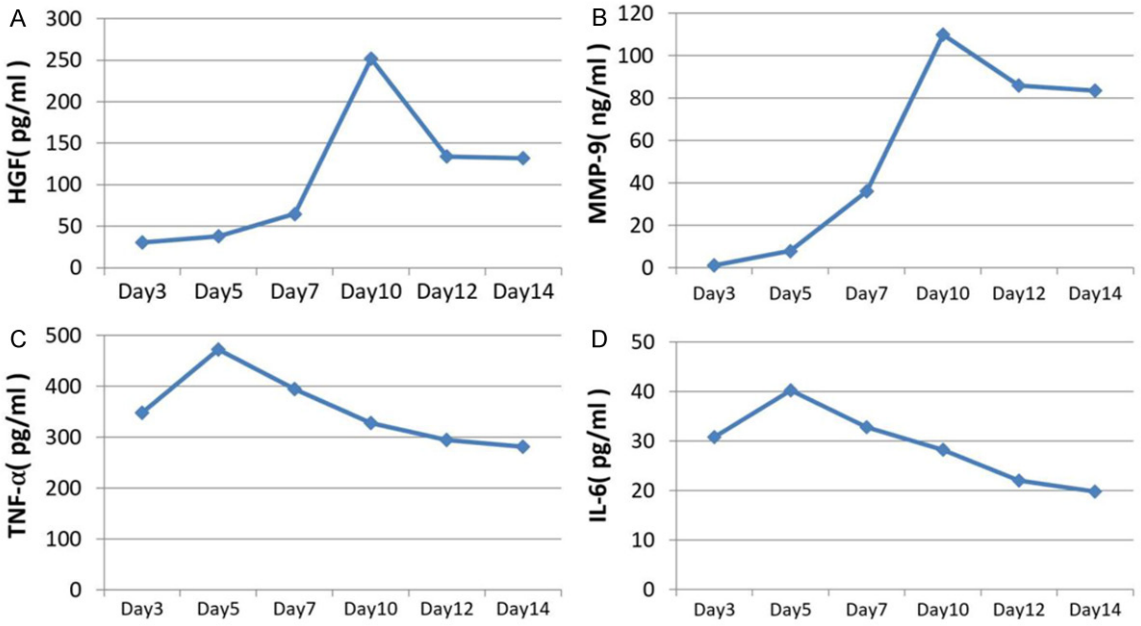

Figure 4. Analysis of levels of cytokines in CD34+ cells from patients with acute-on-chronic liver failure. The amounts of (A) HGF, (B) MMP9, (C) TNF-α and (D) IL-6 from CD34+ cells of liver failure patients were measured.

distribution in percentage of expanded cells was 67.37% at G0/G1 and 32.16% at S phase of cell cycle (**Figure 3B**). In every experiment there were some apoptotic cells wherein the percentages were not included. Our results showed that the populations of cells in G0/G1 and S phase in CD34+ cells of patients were not significantly changed compared with that of controls.

Normal apoptosis rates were detected in CD34+ cells from patients with acute-on-chronic liver failure and healthy volunteers

Apoptosis analysis was performed by Annexin V and PI staining on expanded CD34+ cells from patients with acute-on-chronic liver failure and healthy volunteers at day 10th of culture. The percentage of apoptotic cells was about 10-15% percent in CD34+ cells from both groups. Our results showed that the apoptosis rates in CD34+ cells were similar in both groups ($P > 0.005$) (**Figure 3C**).

The levels of cytokines in CD34+ cells from patients with acute-on-chronic liver failure

Followed by culture of CD34+ cells in vitro, and collection of cultures at days 3rd, 5th, 7th, 12th, 14th, and the levels of HGF, MMP-9, TNF-α and IL-6 were analyzed. At day 10th of culture of CD34+ cells of patients in vitro, the levels of HGF and MMP-9 have reached to peak, and then began to decrease (**Figure 4A** and **4B**). Also, at day 5th of culture of CD34+ cells of patients in vitro, the levels of TNF-α and IL-6 have reached to peak, and then began to decrease (**Figure 4C** and **4D**). In contrast, the levels of HGF, MMP-9, TNF-α and IL-6 have not been detected in culture of CD34+ cells of controls (data not shown).

The expressions of surface markers on CD34+ cells from patients with acute-on-chronic liver failure

By gel-based RT-PCR, we detected expressions of specific markers, PKM2, Integrin-β1, and LPK in CD34+ cells of patients at the days 3rd, 5th, 7th, 12th, 14th. Interestingly, KM2 and Integrin-β1 were consistently expressed in CD34+ cells of patients at the days 3rd, 5th, 7th, 12th, 14th, but no any expression of LPK in CD34+ cells of patients, suggesting an immature phenotype on CD34+ cells of liver failure patients and they had potential to be differentiated into hepatocytes (**Figure 5**). Using immunofluorescence analysis, Integrin-β1 was stained in Hepatic failure groups and it was mainly expressed in cell membrane and cytoplasm, only little expressed at endoplasmic reticulum of CD34+ cells of

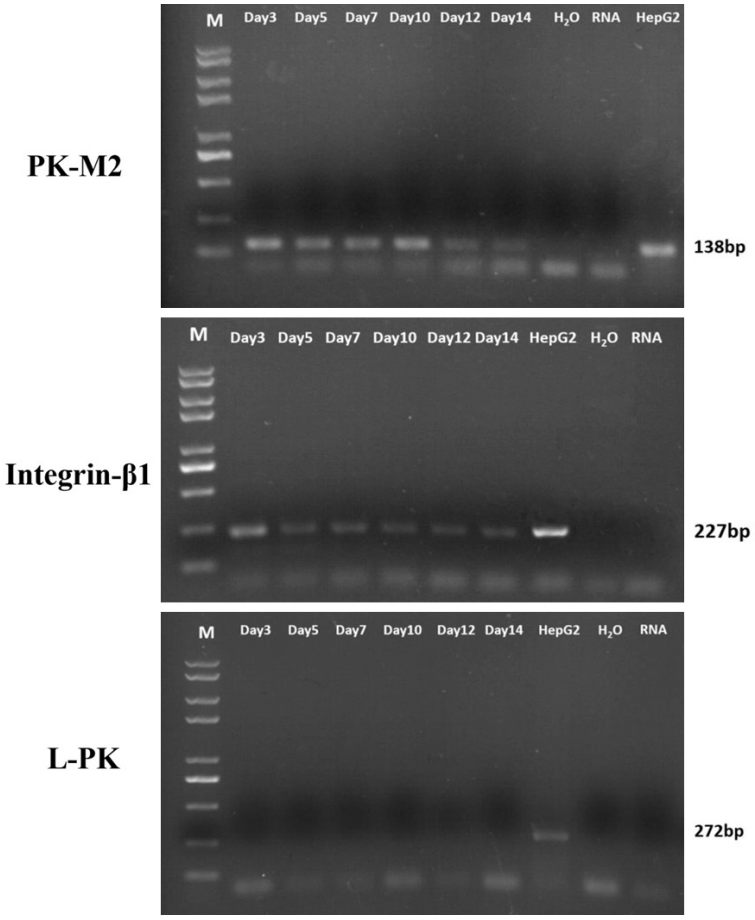

Figure 5. The expressions of surface markers on CD34+ cells from patients with acute-on-chronic liver failure. mRNAs of PKM-2, Integrin β1 and LPK were measured by RT-PCR in day 3, 5, 7, 10, 12 and 14. HepG2 cells were used as control. H_2O was negative control.

Hepatic failure groups, through confocal laser scanning microscope (CLSM) (**Figure 6**).

Discussion

In patients suffering from cirrhosis due to distortion of the hepatic architecture and the formation of regenerative nodules, liver transplantation is the main modality of treatment [5, 6]. However, a serious shortage of organ donors, surgical complications, rejection and the high cost of this procedure has sparked tremendous interest in research to find new treatment modalities for this disease [7-13].

Several sources of stem cells have been proposed for cell therapy. Bone marrow is the most accessible and interesting since it contains different stem cells that can generate a variety of cell types found in other tissues. Orthotropic liver transplantation is the only definitive therapeutic option available for patients with chronic end-stage liver disease. However, a shortage of suitable donor organs and requirement for immunosuppression restrict its usage, highlighting the need to find suitable alternatives. In the past few years, multiple studies have demonstrated that adult stem cell plasticity is far greater and complex than previously thought, raising expectations that it could lay the foundations for new cellular therapies in regenerative medicine. Haematopoietic stem cells (HSCs) are most widely studied example of adult sources-they sustain formation of blood and immune systems cells throughout normal life. These cells are capable of differentiating into many types of other tissues, including skeletal and cardiac muscle, endothelium, and a variety of epithelia including neuronal cells, pneumocytes and hepatocytes [14-18].

Many clinical trials in humans have also shown the potential for bone marrow stem cells (BMSCs) to treat liver fibrosis. Transplantation of CD34+ cells, MSCs, CD133+, and mononuclear cells (MNCs) have been used in clinical practice for the treatment of chronic liver disease in humans. CD133 expression is believed to represent a more stem cell-enriched subpopulation of the CD34+ cells. Importantly, another rationale for using purified CD133+ cells has been to avoid injection of large numbers of leukocytes and their progenitors, which have limited plasticity and the presence of which, in large numbers, may give rise to an unwanted inflammatory response at the graft site [19-21]. So, in this study, it was carried out to study the safety, feasibility and clinical outcome of this approach. As results, it has been demonstrated that the cultured CD34+ cells in vitro showed similar traits with healthy volunteers,

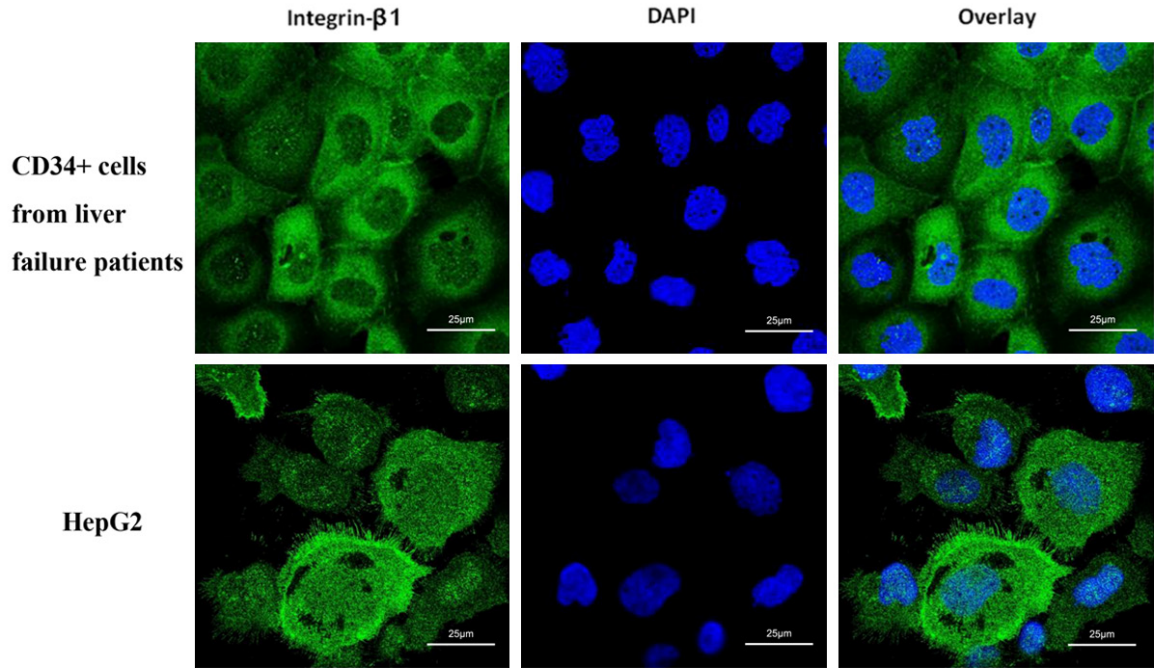

Figure 6. Expression of Integrin β1 in CD34+ cells from patients with acute-on-chronic liver failure. CD34+ cells (upper) and HepG2 (lower) were cultured and fixed by paraformaldehyde (4%) followed by staining of Integrin β1 and DAPI. Expression of Integrin β1 was analyzed by fluorescence microscope.

and no significant differences, which also confirmed the feasibility and safety CD133+ cell infusions into the portal veins of such patients.

Within the bone marrow space multipotent stromal cells, also referred to as mesenchymal stem cells (MSCs) are known to be the precursor cell for stromal tissues that support hematopoiesis. The immunomodulatory function of MSCs was first reported after it was observed that they could evade immunesurveillance after cell transplantation. This ability of MSCs to alter an immune response has been exploited for therapeutic purposes as demonstrated by the case of a patient suffering from steroid-refractory graft-versus-host disease that was successfully treated by the infusion of haploidentical MSCs. In vitro studies have subsequently shown that MSCs actively inhibit the function of several immune cells through secreted cytokines, growth factors and enzymatic action, although controversy exists on the identity of the responsible mediators. The fortification of the soluble microenvironment by MSCs can also affect non-hematopoietic cells as well. MSCs used for cellular cardiomyoplasticity after an ischemic event revealed that MSC-derived soluble molecules inhibited hypoxia-induced apoptosis of cardiomyocytes during the acute phase of injury resolution. Taken together, these studies indicate that MSCs can independently affect immune and tissue cells by paracrine means. Bone marrow derived mesenchymal stem cells (MSCs) are known to naturally support hematopoiesis by secreting a number of trophic molecules, including soluble extracellular matrix glycoproteins, cytokines, and growth factors [22-25]. So, the current study, we have detected the expression of INTβ1 and PKM2 in CD34+ cells of patients, indicating that CD34+ cells from peripheral blood were able to differentiate into hepatocytes. In our further study, we have found that the levels of G-CSF, HGF, and SCF were elevated and thus its expression was positive correlated to the increased CD34+ cells, which expressed the HCG, TNF-α, IL-6 and MMP-9. These results were consistent with some previous studies, which emphasized again that G-CSF, HGF, and SCF were molecules with known proliferative, regeneration inhibition of apoptosis effects on hematopoietic cells [26-30], and thus MMP-9 regulated the recruitment of human CD34+ progenitors with hematopoietic and/or hepatic-like potential to the liver under homing process, an important mechanism for

tissue targeting and repair of injured organs [31, 32].

In conclusion, we have firstly demonstrated that bioactivity of CD34+ cells of patients with acute-on-chronic liver failure was fine and thus CD34+ cells of patients were able to secrete HGF, MMP-9, TFN-α, and IL-6, which can promote the growth of hepatocytes, also can differentiate along a direction to hepatocyte lineage.

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Disclosure of conflict of interest

None.

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