Bone marrow mesenchymal stem cell from chronic hepatitis B patients differentiation into hepatocyte-like cells

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The aim of this study was to observe the biological characteristics and hepatic differentiation potential of bone marrow mesenchymal stem cells (BM-MSCs) from chronic hepatitis B patients in vitro. MSCs were isolated from bone marrow of 15 chronic hepatitis B patients and 11 normal donors by density gradient centrifugation. The adipogenic and osteogenic differentiation potential, immunophenotyping and DNA contents were detected. Chronic hepatitis B patients BM-MSCs were induced to hepatocytes. Hepatic markers were detected by immunohistochemical analysis. Periodic acid-schiff staining was used to detect for glycogen production. The results showed that both MSCs had adipogenic and osteogenic differentiation potential, nearly all the BM-MSCs expressed CD105, CD166, CD44 and CD29. Compared with normal donors, the primary passage time of BM-MSCs from chronic hepatitis B patients were much longer, and the S-phase nuclei were lower. Chronic hepatitis B patients BM-MSCs were induced to hepatocyte-like cell, which express the hepatic-specific markers and have the glycogen production function. Though it was difficult to cultivate BM-MSCs from chronic hepatitis patients in vitro and they had deficient proliferation, they could differentiate into functional hepatocyte-like cells.

Key words: Hepatitis B, mesenchymal stem cell, bone marrow, hepatocyte-like cell, glycogen.

INTRODUCTION

Bone marrow isolates contain several adult stem cell population which are well characterized; the most used and best understood are hematopoietic stem cells (HSCs), bone marrow-derived mesenchymal stem cells (BM-MSCs) and endothelial precursor cells. In the last years another cell populations have been identified such as side population (SP), with mainly myogenic potential; multipotent adult progenitor cells (MACP), that have been described as pluripotent (Valerie et al., 2011). MSCs with similar biological properties have also been isolated from other tissue including adipose tissue, skeletal muscles, fetal liver and pancreas, cord blood and amniotic fluid (Campagnoli et al., 2001; Hu et al., 2003; Romanov et al., 2003). MSCs have self-renewal and multi-directional differentiation potential characteristics when cultured in appropriate conditions, such as: osteoblasts, cardiomyocytes, adipocytes, myocytes, oligodendrocytes and neurons (Pettenger et al., 1999; Woodbury et al., 2000; Mezey et al., 2000; Krause et al., 2001). BM-MSCs possess substantial differentiation, replication potential, anti-proliferative and anti-inflammatory activity (Tyndall et al., 2006; Uccelli et al., 2006), which, together with their privileged immunological properties (cause low immune reaction) (Bartholomew et al., 2002; Le Blanc et al., 2003), make them safe for allogeneic and atologous use. They are also easy to transfect and tolerate cryopreservation well (Robb et al., 2007). MSCs play an important role in cellular and tissue engineering. Bone marrow-derived MSCs have attracted great interest in both basic research and clinical practice in recent years. Hepatitis B is one of the most common infectious diseases worldwide. There are approximately 350 million
chronic hepatitis B virus (HBV) carriers all over the world (Yue-Si et al., 2010). The therapy for hepatitis B is very limited. HBV infection may cause liver cirrhosis and hepatocellular carcinoma. Following chronic liver damage, the regenerative ability of hepatocyte is lost, which leaves the liver unable to maintain its functional mass. This is clinically so-called “liver failure”. Currently, orthotopic liver transplantation (OLT) is considered to be the most suitable therapeutic method for patients with hepatic failure. Cell-based therapy has been proposed as a potential alternative to OLT (Strom et al., 1997; Bahir et al., 2000). Stem cell transplantation may promote liver regeneration and self-repair, it provides a theoretical approach for liver regeneration (Abdel et al., 2007). MSCs are easily obtained and extensively expanded in vitro without ethical complication and immunologic rejection and MSCs-based therapy for liver disease is more suitable than other kind of stem cells (Aurich et al., 2007). Some researchers conclude that MSCs represent a promising candidate for liver stem cell therapy (Sato et al., 2005; Chou et al., 2006). Several studies have demonstrated that MSCs can be differentiated along hepatogenic lineage in vitro (Banas et al., 2009; Cho et al., 2009). Studies on animal models have shown that MSCs transplanted by either intrasplenic or intravenous route, can be engrafted into the recipient liver and differentiated into functional hepatocytes (Abdel et al., 2007; Kuo et al., 2008). To date, some clinical trials of autologous bone marrow-derived MSCs transplantation have been conducted, and the preliminary result seems feasible and safe for treating patients with liver diseases with MSCs (Kharazia et al., 2009; Peng et al., 2011).

However, there are many different views on the efficacy of autologous MSCs transplantation in patients with chronic HBV infection and cirrhosis of the liver and whether HBV infection can affect the function of MSCs for transplantation remains unknown. Up to now, it is not completely clear about the biological characteristics and hepatic differentiation potential in vitro of MSCs in chronic hepatitis B patients bone marrow. In this study, we tried to establish a cultivation system of BM-MSCs from chronic hepatitis B patients in vitro, compared their biological characteristics with those from normal donors, and investigated whether it would be able to differentiate into hepatocyte-like cells in vitro.

MATERIALS AND METHODS

Participants

BM-MSCs were isolated from 11 normal donors and 15 patients with chronic hepatitis B. The normal donors were aged between 29 and 48. Eight were males and three were females. Of the 11 normal donors, six were volunteers and five were patients with external injury after plastic operation in the First Clinical Hospital of Jilin University. The chronic hepatitis B patients were aged 28 to 53 and comprised of eleven males and four females. They were recruited from the Hepatobiliary Hospital of Jilin Province. The study was approved by the Ethics Committee of the Hospital and informed consent was obtained from each donor.

MSCs isolation and culture

Mononuclear cells were isolated from bone marrow of both normal donors and chronic hepatitis B patients, as previously described (Pettenger et al., 1999). Briefly, 5 mL of bone marrow which was aspirated from anterosuperior iliac spine was mixed with 5 mL of phosphate buffered saline (PBS), and centrifuged at 1500 r/min for 5 min. The supernatant was removed and dispersed in 5 mL PBS. Cell suspension was added to 5 mL Ficoll (density: 1.077 g/mL, Sigma) and centrifuged for 20 min at 2000 r/min. The top layer of mononuclear cells were collected and washed three times with low glucose DMEM (Invitrogen). The isolated cells were resuspended in DMEM supplemented with 15% fetal bovine serum (FBS; Hyclone), and then seeded into 50 cm2 flasks at 1×10⁵ cells/cm². The cells were cultured at 37°C in a 95% humidified incubator with 5%(v/v) CO₂. After 72 h of incubation, non-adherent cells were removed by replacing the medium. When these primary BM-MSCs reached 80% confluence, the cells were harvested with 0.25% trypsin (Sigma), and then subcultured at a ratio of 1:3. Cell density and morphology were monitored under an inverted microscope (Olympus, Japan).

Comparison of MSCs from chronic hepatitis B patients and normal donors

BM-MSCs from 15 chronic hepatitis B patients and 11 normal donors were inoculated and cultivated. The cultivation ratios were compared. The primary passage time of BM-MSCs from them was compared after inoculation at the density of 1.0×10⁶ cells/cm². The morphology of BM-MSCs were observed under an inverted microscope and their difference were compared on 4 and 7 days after inoculation at the 5th and 7th generation, respectively. BM-MSCs of 5th generation from both were inoculated at 24−shadow mask at the density of 10⁵ cells/hole. The number of cells in two holes was counted everyday for 10 days, and the growth curve was plotted.

Adipogenic differentiation was induced by culturing the above two group BM-MSCs of 5th generation in adipogenic medium (10%FBS, Ig/L dexamethasone, 0.5 mg/mL of 3-isobutyl-1-methylxanthine, 10 mg/L insulin, and 100 mmol/L indo-methacin in H-DMEM, Gibco) and assessed using an oil red O (Sigma) staining as an indicator of intracellular lipid accumulation at days 14 and 21. Osteogenic differentiation was induced by culturing it in osteogenic medium (10% FBS, 10−2 mol/L dexamethasone, 10 mmol/L b-glycerophosphate, and 50 lg/ascorbic acid in L-DMEM, Gibco) and examined by alkaline phosphatase staining (Mai Xi Biology Technology Company, Fuzhou, China) at 14 and 21 days.

MSCs immunophenotyping

The above two group BM-MSCs of 5th generation were tested for surface marker expression by flow cytometry and immunohistochemical method. BM-MSCs were both digested with 0.25% trypsin, then the digested cells were washed once with PBS and resuspended in PBS. Cells (1×10⁶ per sample) were treated at room temperature for 30 min with the following specific anti-human antibodies: anti-CD34-FITC, -CD29-PE, -CD44-FITC (Becton Dickinson). After two PBS washes, the cells were fixed with FIX solution (Becton Dickinson), as recommended by the manufacturer. Fluorescent labeling was analyzed with a FACScalibur flow cytometer, using Cell Quest software. BM-MSCs sections were fixed with 4% paraformaldehyde (Sigma) and immunohistochemical methods (Immunohistochemical staining Kit, Mai Xi Biology Technology Company, Fuzhou, China) (Shuang-zhi Huo et al., 2007). Some researchers conclude that MSCs represent more suitable than other kind of stem cells (Aurich et al., 2007).
Figure 1. The morphology characteristics of MSCs from bone marrow (×40). (A) MSCs from normal donors (5 days), (B) MSCs from normal donors (14 days), (C) MSCs from chronic hepatitis B patients (5 days), (D) MSCs chronic hepatitis B patients (14 days).

2010) were performed to detect the expression of surface markers CD105 (1:500, Dako) and CD166 (1:500, Dako).

Analysis of MSCs DNA content

The DNA content of the above two group BM-MSCs of 5th generation were determined using a BD CycleTest Plus DNA Reagent Kit (BD Biosciences) according to the manufacturer’s instructions. Briefly, the logarithmic phase cell suspension of BM-MSCs was centrifuged at 1500 r/min for 5 min. The cell density was then adjusted to 1.0 × 10^6 cells/mL. The cells were then immediately stained according to the manufacturer’s instruction for flow cytometric analysis. The data were analyzed using Modifit software.

Hepatic differentiation potential

As previously described (Chan et al., 2009), BM-MSCs of 5th generation reaching 60% confluence were used for differentiation assays. Cells were cultured in differentiation media of Heptozyme-SFM (Gibco) supplemented with 20 ng/ml EGF (Sigma) and 20 ng/ml FGF-4 (Sigma). Two days later, 20 ng/ml HGF (Sigma) and 2% FBS were added to the media. Media were refreshed twice weekly and hepatic differentiation was assessed at different time-points. The protocol was applied to BM-MSCs from 6 chronic hepatitis B patients and 6 normal donors.

Immunohistochemical analysis of hepatic markers

Cell sections were fixed with 4% paraformaldehyde (Sigma) for 10 min. Immunohistochemical was performed as previously described (Shuang-zhi et al., 2010). The cell sections were incubated with rabbit monoclonal anti-ALB (1:200, Dako), anti-CK-18 (1:200, Dako) and mouse monoclonal anti-AFP (1:200, Dako). The positive cells were examined and mounted under microscope.

Periodic acid-schiff (PAS) staining for glycogen

Cell sections were fixed with 4% paraformaldehyde for 10 min. The PAS staining system (Sigma) was used according to the manufacturer’s instruction.

Statistical analysis

Experimental data were presented as mean±SD. Statistical calculations were performed using SPSS13.0 (SPSS Inc., USA). For statistical analyses, we used the Student’s t-test to compare data from normal donors with that obtained from chronic hepatitis B patients and used a non-parametric test to compare the expression of hepatic-specific markers. Statistical significance was defined as p < 0.05.

RESULTS

MSC proliferation is impaired in chronic hepatitis patients

First, it was investigated whether HBV infection affect the proliferation of BM-MSCs. BM-MSCs from chronic hepatitis B patients and normal donors were cultivated and expanded in vitro and the ratio was 73.33% (11/15) and 100% (11/11), respectively. The primary passage time of MSCs from the two groups was (16.0 ± 1.8) days and (12.0 ± 1.5) days, respectively (P<0.05). All BM-MSCs appeared similar to fibroblasts, with a characteristic spindle shaped fusiform morphology (Figure 1). After observation on difference of appearance between chronic hepatitis B patients and normal donors on days 4 and 8 after inoculation at 5th and 7th generation, we found that BM-MSCs grew slower and were easier to expand, spread out and age in chronic hepatitis B patients than in normal donors.

The growth of 5th generation BM-MSCs from chronic hepatitis B patients was lower than that of normal donors. Furthermore, DNA content of BM-MSCs during the log phase were measured by flow cytometry. The percentage of S-phase nuclei in MSCs from chronic hepatitis grown was (4.58±0.96)%, which compared with (7.58±1.24)%, the S-phase of the normal donors MSCs nuclei, the proportion of S-phase MSCs from chronic hepatitis B was significantly lower than that of normal donors (P<0.05).

Differentiation potential and surface markers of MSCs from chronic hepatitis patients

Our results are consistent with those reported by Pettenger et al. (1999); Dominici et al. (2006). Indicating that both MSCs from chronic hepatitis B and normal donors have the potential of adipogenic and osteogenic differentiation (Figure 2). The surface marker of the 5th generation BM-MSCs were analyzed, nearly all the cells expressed CD4, CD29 and CD105, CD166, which are the surface marker characteristics of MSCs. The absence of contaminating hematopoietic cells in the MSCs population was verified by the lack of surface antigen defining hematopoietic progenitor cells (CD34). Thus, the bone
Figure 2. The immunophenotyping of BM-MSCs from chronic hepatitis B. A: FITC-isotype, B: Expression of CD44, C: Expression of CD34, D: PE-isotype, E: Expression of CD29, F: Expression of CD105 (×100), G: Expression of CD166 (×100).
marrow after the 5th generation was of high purity, and expressed CD44 and CD29, which are markers of MSCs (Figure 3).

**Differentiated MSCs expressed hepatocyte-specific proteins**

In order to determine whether BM-MSCs from chronic hepatitis B can be induced into hepatocyte-like cells, we cultured the BM-MSC in serum-free media supplemented with EGF (20 ng/mL) and FGF-4 (20 ng/mL) for 2 days. The cells were treated with HGF (20 ng/mL) to induce hepatic differentiation. By day 5, the fibroblastic morphology of MSCs developed a broad, flattened shape (Figures 4A and B).

And in order to determine whether differentiated cells showed the characteristic expression of hepatic-specific markers, we confirmed the expression of AFP, ALB and CK18 and in differentiated cell populations by immunohistochemical methods at days 7, 14 and 21. The results showed that undifferentiated cells stained negative for ALB, CK-18 and AFP. BM-MSCs were exposed to growth factors at days 7, 14, 21 and the positive rates of AFP were (55.6±9.3)%, (20.8±5.2)%, and (7.5±1.2)% respectively (Figures 4C, D and E); the positive rate of ALB were (12.8±4.2)%, (40.5±7.6)% and (70.5±10.9)% respectively (Figures 4F, G and H); the positive rate of CK-18 were (10.6±3.8)%, (50.4±9.3)% and (81.2±12.3)% respectively (Figures 4I, J and K); Compared with un-induced group, the difference are all statistically significant (p < 0.05). The expression of ALB and CK-18 significantly increased in a time-dependant manner, with the highest expression level at day 21. However, AFP was at its highest expression level at day 7. Its expression gradually decreased with increasing induction time, indicating that the hepatocytes derived from BM-MSCs matured in the differentiation protocol. The results demonstrated that the levels of hepatic protein markers increased in BM-MSCs in response to the differentiation protocol.

**MSCs from chronic hepatitis patients were induced into functional hepatocytes**

To detect whether the differentiated cells possessed liver cell function, we measured liver functions of the differentiated cells. Glycogen production is a unique characteristics of hepatocytes. We assayed glycogen storage of the differentiated cells using PAS staining. BM-MSCs showed no activity of glycogen production in their undifferentiated stage. Glycogen staining was present in BM-MSCs after they were exposed to growth factors and at days 7, 14 and 21 the positive rate were (11.3±3.9)%, (41.2±8.5)% and (86.9±11.3)%, respectively (Figure 5). Compared with un-induced group, the difference are all statistically significant (P < 0.05). The glycogen production significantly increased in a time-dependant manner, with the highest expression level at day 21. The results suggest that MSCs from chronic hepatitis B patients induced by growth factors are functional in vitro.
Figure 4. The expression of AFP, Alb and CK-18 in MSCs from chronic hepatitis B patients cultured in differentiation media of Heptozyme-SFM. A: MSCs in induced group cultivated for 7 days (×40), B: MSCs in induced group cultivated for 14 days (×40), C: AFP expression in MSCs in control group cultivated for 7 days (×100), D: AFP expression in MSCs in induced group cultivated for 7 days (×100), E: AFP expression in MSCs in induced group cultivated for 14 days (×100), F: Alb expression in MSCs in control group cultivated for 7 days (×100).

Figure 5. The glycogen storage in MSCs from chronic hepatitis B patients cultured in differentiation media of Heptozyme-SFM (×100). A: Control group, B: MSCs in induced group cultivated for 7 days, C: MSCs in induced group cultivated for 14 days, D: MSCs in induced group cultivated for 21 days.
DISCUSSION

Recent advances in cell biology have led to the therapeutic potential of stem cells (SCs). Different types of SCs are theoretically eligible for liver cell replacement. These include embryonic and fetal SCs, MSCs, induced pluripotent cells, annex SCs, endogenous liver SCs, and extrahepatic adult SCs (Trebol Lopez et al., 2011). Due to the advantages of rich resource, anti-rejection, low-cost and less risk, BM-MSC transplantation became an alternative treatment for OLT. Oyagi et al. (2006) implanted hepatic phenotype of MSCs to liver damage, with results indicating that the liver fibrosis was reduced and the albumin (ALB) lever were increased. Kuo et al. (2008), also have shown that a single systemic transplantation of as few as $1.4 \times 10^6$ MSCs/Kg Wt, effectively rescued the recipient mice from Fulminant Hepatic Failure (FHF). However, there are also some contrary results. Carvalho et al. (2008) implanted MSCs and placebo to liver cirrhosis rats and the results indicated that the liver function of two groups were not significantly different after two months. Some clinical trials have been performed in patients with end-stage liver disease and the results of these studies have shown that MSCs injection can gain satisfactory tolerability and clinically relevant effects (Kharazia et al., 2009). For patients with liver failure caused by hepatitis B, who received transplantation with analogous BM-MSCs, the results showed a success rate of transplantation was of 100%, without serious side effects or complications and the liver function and levels of MELD score of patients in the transplantation group were markedly improved after transplantation (Peng et al., 2011). However, these studies have not provided definitive evidence that the injected MSCs differentiated to functional hepatocytes (Alison et al., 2009). Some studies also showed that HBV infection may result in systemic disorders, such as an dysfunction of the peripheral blood dendritic cells and bile ducts (Van der Molen et al., 2004; Hsing et al., 2008). Therefore, in the present study we attempt to investigate whether the chronic HBV infection could effect the biological characteristics of MSCs and its differential potential to hepatocyte-like cells.

First, we cultured MSCs from chronic hepatitis B patients, observed their biological characteristics in vitro and compared them with those from normal donors. MSCs have the potential of adipogenic and osteogenic differentiation and do not express the hematopoietic surface markers CD34 and CD45, but staining positive for CD44, CD29, CD105, CD73, and CD166 (Pettenger et al., 1999; Dominici et al., 2006). We isolated MSCs from normal donors and patients which highly express surface marker CD29, CD44 and CD105, CD166, and hematopoietic surface markers CD34 were negative. They all have the potential of adipogenic and osteogenic differentiation. The results are consistent with the last report (Pettenger et al., 1999). In addition, the result suggested that the immunophenotypes and differentiation of MSCs from two group people were intact. However, our results also show that there are evident difference in culture ratio, primary passages time, growth curve, the percentage of S-phase nuclei in MSCs and appearance change after several passage time between them. Also the result suggested that MSCs from chronic hepatitis are more difficult to be cultured and grow more slowly in vitro, which is demonstrated by the longer time to reach confluence and lower percentage of S-phase nuclei. The result is consistent with the report of Yue-Si et al. (2010).

To identify whether HBV infection could affect the differentiation of MSCs to hepatocyte-like cells, we used heptozyme-SFM medium that was specifically intended for hepatocyte culture. It contains much of the basic nutrition that is suitable for hepatocyte growth, and provided a proper microenvironment for the differentiation of MSCs into hepatocyte-like cells. First the hepatic-specific markers were detected, the results indicated that differentiated cells expressed hepatic-specific markers such as ALB, CK-18 and AFP. Furthermore, we assessed that the MSCs-derived hepatocyte-like cells can function like liver cells by glycogen staining, the result showed glycogen staining was present in MSCs from chronic hepatitis B after 7 days of differentiation. The glycogen production significantly increased in a time-depandant manner. The results suggest that MSCs from chronic hepatitis B patients induced by growth factors may differentiate to functional hepatocyte-like cells in vitro.

Although HBV can infect many organs, including the bone marrow cells, Chan et al. (2009) isolated bone marrow MSCs from early and late stage hepatitis B patients, analyzed the presence of HBV antigens and HBV DNA in MSCs and culture media of the 3rd, 4th and 5th generation. Their results demonstrated that the MSCs were not susceptible to infection by HBV. Our results also showed that BM-MSCs from chronic hepatitis patients could differentiate into hepatocyte-like cells in vitro, we consider that it is easy and safe for autologous bone marrow-derived MSCs transplantation for HBV infection patients, without considering whether the patients were infected with HBV.

In conclusion, we successfully isolated the bone marrow MSCs from chronic hepatitis B patients, though it was difficult to cultivate in vitro and it had deficient proliferation. Our study demonstrates that MSCs from chronic hepatitis patients could differentiate into functional hepatocyte-like cells suggesting that the therapeutic use of autologous MSCs from patients with chronic hepatitis B is feasible and should be further studied.

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