

Comparison of biological characteristics of marrow mesenchymal stem cells in hepatitis B patients and normal adults

Liang Peng, Hua Li, Lin Gu, Xiao-Mou Peng, Yang-Su Huang, Zhi-Liang Gao

Liang Peng, Hua Li, Lin Gu, Xiao-Mou Peng, Yang-Su Huang, Zhi-Liang Gao, Department of Infectious Diseases, The Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510630, Guangdong Province, China

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Correspondence to: Zhi-Liang Gao, MD, PhD, Department of Infectious Diseases, The Third Affiliated Hospital of Sun Yat-Sen University, 600# Tianhe Road, Guangzhou 510630, Guangdong Province, China. gaozl@21cn.com

Telephone: +86-20-85516867-2034 Fax: +86-20-87584302

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Abstract

AIM: To establish a culture system of marrow mesenchymal stem cells (MSCs) from hepatitis B patients and normal adults and to compare their biological characteristics.

METHODS: MSCs were isolated from bone marrow in 34 male hepatitis B patients and 15 male normal adults and cultivated *in vitro*. Their biological characteristics including surface markers, shapes and appearances, growth curves, first passage time and passage generations were compared.

RESULTS: Cultivation achievement ratio of hepatitis B patients was lower than that of normal adults, no statistical significance (82.35% *vs* 100%, $P > 0.05$). Compared with MSCs of normal adults, MSCs of hepatitis B patients presented a statistical lower growth curve, longer first passage time (13.0 ± 1.6 d *vs* 11.4 ± 1.5 d, $P < 0.05$), fewer passaging generation numbers (10.5 ± 1.4 generations *vs* 12.3 ± 1.7 generations, $P < 0.05$), though both shared same appearances, shapes and surface markers. MSCs in hepatitis B patients would expand, spread out and age more easily and there were more refractive particles in the cytoplasm.

CONCLUSION: MSCs from hepatitis B patients can be cultured *in vitro*. Although their appearance, shape and surface marker are similar to those of MSCs from normal adults, there are differences in their biological characteristics.

INTRODUCTION

Marrow mesenchymal stem cells (MSCs) are characterized by self-renewal and multi-directional differentiation^[1-6]. MSCs have the following merits: ease of isolation and cultivation, expansion potential, stable phenotype, compatibility by different delivery methods, and slight adverse reaction after implantation *in vivo*, and playing an important role in cellular therapeutics and tissue engineering^[7-10].

Autogenetic MSCs do not cause immunological rejections and have been used in France, Germany, Japan, Italy and China^[11,12]. We have used autogenetic MSCs in autoplasmic transplantation therapy for severe liver failure and gained satisfactory therapeutic effects (in press).

However, there is only one MSC among 10^4 - 10^5 mononuclear cells in the bone marrow, which limits its clinical application. Many researches have proved that MSCs can be incubated and amplified *in vitro*^[13-15]. Up to now, no studies on the biological characteristics, cultivation and amplification *in vitro* of MSCs in hepatitis B patients are available.

We tried to establish a cultivation system of *in vitro* MSCs from hepatitis B patients to compare their biological characteristics with those from normal humans.

MATERIALS AND METHODS

Preparation of bone marrow-derived MSCs

MSCs from bone marrow of 34 hepatitis B patients and 15 normal adults were prepared. All the 34 patients at the age of 35-57 years were inpatients visiting our department from September 2005 to June 2006. Of the 15 normal adults at the age of 30-40 years, 5 were volunteers and 10 were patients with external injury after plastic operation in Department of Orthopaedics.

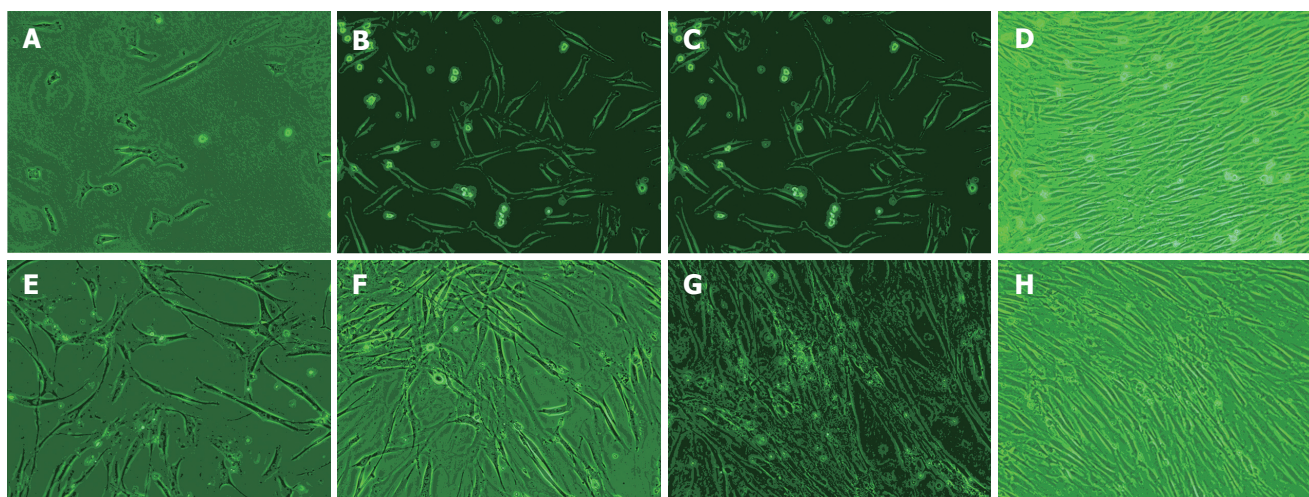


Figure 1 Morphology of MSCs from hepatitis B patients and normal humans after incubation for 4 d (A, B) and 8 d (C, D), generations 5 d and 10 d (E-H).

Table 1 Comparison of cultivation ratio of MSCs from hepatitis B patients and normal humans *n* (%)

	Success	Failure	Total
MSCs from hepatitis B patients	28 (82.35)	6 (17.65)	34
MSCs from normal human	15 (100)	0 (0)	15
Total	43 (87.76)	6 (12.24)	49

Cultivation of MSCs in vitro

Five mL bone marrow was aspirated from anterosuperior iliac spine, mixed with 5 mL 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 Percoll separating medium (Sigma Company, USA) and centrifuged at 2500 r/min for 30 min. Nucleated cells in the middle layer were drawn off and washed with 5 mL PBS, centrifuged at 1500 r/min for 5 min. The supernatant was removed. Dispersed by Cult-M culture solution (An-Pu Biochemistry Company, Beijing, China), the cell sediments were inoculated in a 25 cm² culture flask and incubated at 37°C in an atmosphere containing 5% CO₂. The culture medium was changed after 3 d and then every 2 d. MSCs were digested by 0.25% Trypsin, 0.1% EDTA and passaged (1:2) when they grew to 70%-80%.

Comparison of MSCs from hepatitis B patients and normal adults

MSCs from bone marrow of 34 hepatitis B patients and 15 normal adults were inoculated and cultivated. The cultivation ratios were compared.

The appearance of MSCs was observed under an inverted phase contrast microscope (M20-35DX, Olympus Company, Japan) and their differences were compared on d 4 and 8 after inoculation and at generations 5 and 10, respectively. The 4th passage MSCs from hepatitis B patients were digested, washed with PBS and adjusted to the density of 1.0×10^6 cells/mL. After addition of FITC-CD44 antibody, PerCP-CD45 antibody, PE-CD34 antibody (BD Biosciences Company, USA), they were detected by flow cytometry using mouse IgG₁ as the

isotypism contrast. Results were compared with MSCs from normal adults. P3 MSCs from hepatitis B patients and normal adults were inoculated in a 24-shadow mask at the density of 10^3 cells/hole. The number of cells in two holes was counted everyday for 10 d, and the growth curve was plotted. The primary passage time of MSCs from hepatitis B patients and normal humans was compared after inoculation at the density of 1.0×10^6 cells/cm². The number of passage generations of MSCs from 28 hepatitis B patients and 15 normal human was also compared.

Statistical analysis

Data were expressed as mean \pm SD and analyzed by SPSS s13.0 software using Chi square test. $P < 0.05$ was considered statistically significant.

RESULTS

MSCs from bone marrows of hepatitis B patients and normal adults were cultivated, the ratio was 82.35% and 100%, respectively, without statistical significance (Table 1, $P > 0.05$). All MSCs had a fusiform shape with a high karyoplasmic ratio and were integrated into steady colonies, like collagenoblasts. After observation on differences of appearance between hepatitis B patients and normal adults on the 4th and 8th d after inoculation and at the 5th and 10th generations, MSCs grew slower and were much easier to expand, spread out and age in hepatitis B patients than in normal adults, and more refractive particles were found in cytoplasm (Figure 1). CD34⁺CD44⁺ and CD44⁺CD45⁻ cells accounted for 97.83% and 99.38% separately of P4 MSCs from hepatitis B patients, demonstrating a high purity. CD34⁺CD44⁺CD45⁻ was expressed on its surface molecules as on MSCs^[1] in the normal adults (Figure 2). The growth curve of MSCs from hepatitis B patients was lower than that from normal humans (Figure 3). The primary passage time of MSCs from hepatitis B patients and normal human was 13.0 ± 1.6 d and 11.4 ± 1.5 d, respectively ($P < 0.05$). The MSCs passage amount of MSCs from hepatitis B patients and normal humans were 10.5 ± 1.4 and 12.3 ± 1.7 generations, respectively ($P < 0.05$).

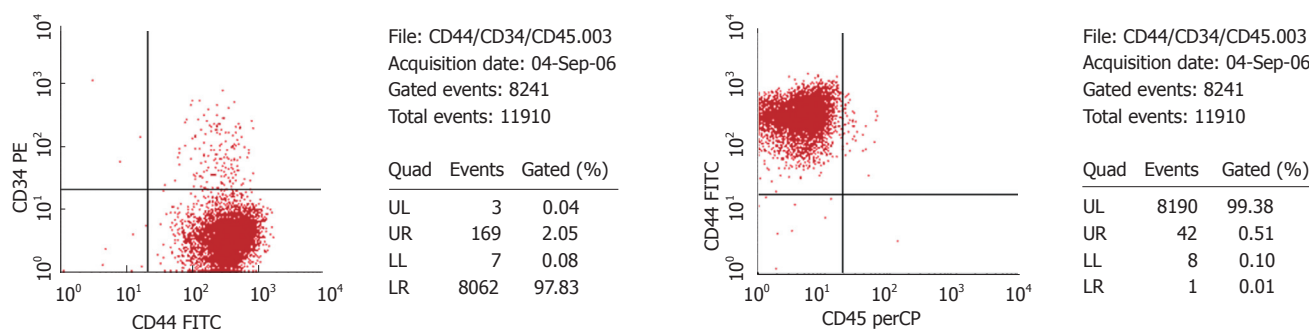


Figure 2 Expression of surface molecules CD34, CD44, CD45 of MSCs in hepatitis B patients.

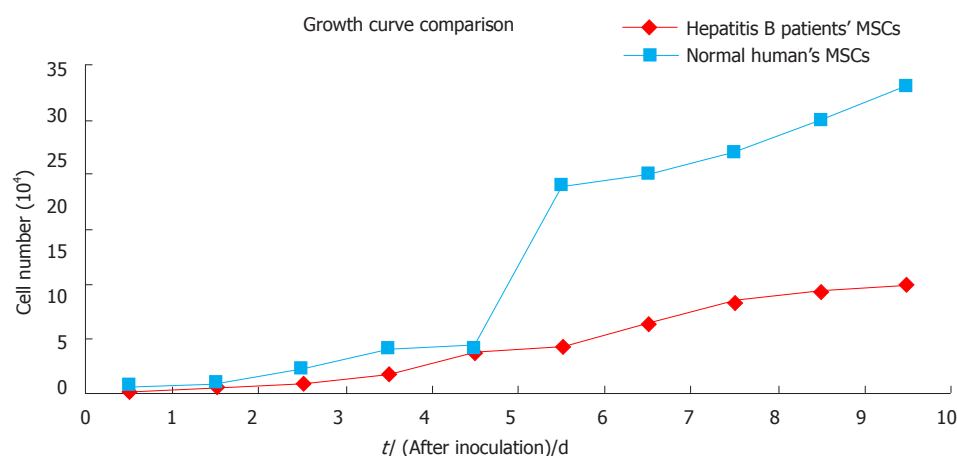


Figure 3 Growth curve of MSCs from hepatitis B patients and normal humans.

DISCUSSION

A total of 130 million Chinese are infected with HBV and every year a half million of patients die of terminal stage liver disease caused by HBV. There are no effective hepatitis B therapies. Internal medical therapy, anti-virus therapy and liver transplantation, cannot recover liver function, improve prognosis and quality of life of such patients. It is urgent to find effective therapies for HBV infection.

MSCs are adult stem cells, which can be differentiated in many tissues like bone, cartilage, muscle, tendon, myocardial cells, liver, lung, kidney, cranial nerve cells and spinal cord^[16]. In recent years, xenogenic and autogenic MSCs have been used in the treatment of hepatitis B patients^[11,12]. Autogenic MSCs in hepatitis B patients can avoid immunological rejection and tumorigenesis caused by cell fusion, although some scholars believe that MSCs have immunological regulation functions and do not lead to immunological rejection because MHC- I antigen is not expressed and MHC- II antigen has a low expression^[17]. However, there is no evidence that MSCs do not express MHC- I and MHC- II antigens and exert immunological regulation after their differentiation into mature hepatocytes.

In the present study, we cultivated *in vitro* MSCs from hepatitis B patients, observed their bionomics and compared them with those from normal adults. Their surface molecules were similar but there were evident differences in culture ratio, growth curve, primary passage

time, number of passage generations and appearance change after several passage times between them, suggesting that MSCs from hepatitis B patients are more difficult to cultivate *in vitro*, grow more slowly with worse activity, faster aging process, and less passage generations. HBV, chemicals and severe liver damage might influence their growth. It was reported that HBV can refrain the growth of stroma cells and hematopoietic stem cells in bone marrow^[18,19]. Though MSCs in hepatitis B patients do not express HBsAg and HBeAg, it is still difficult to prove whether HBV-DNA is integrated into MSCs and affects their bionomics. Otherwise, influence of chemical medication on them cannot be ignored.

In conclusion, it is difficult to cultivate *in vitro* MSCs from hepatitis B patients. We should find a safe, quick and effective transplantation therapy to proliferate MSCs because of their limited number in the bone marrow.

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