Biological effects of bone marrow mesenchymal stem cells on hepatitis B virus *in vitro*

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Abstract. The aim of the present study was to explore the effects of co-culturing bone marrow-derived mesenchymal stem cells (BM-MSCs) cultured with hepatitis B virus (HBV)-infected lymphocytes *in vitro*. BM-MSCs and lymphocytes from Brown Norway rats were obtained from the bone marrow and spleen, respectively. Rats were divided into the following five experimental groups: Group 1, splenic lymphocytes (SLCs); group 2, HepG2.2.15 cells; group 3, BM-MSCs + HepG2.2.15 cells; group 4, SLCs + HepG2.2.15 cells; and group 5, SLCs + BM-MSCs + HepG2.2.15 cells. The viability of lymphocytes and HepG2.2.15 cells was assessed using the MTT assay at 24, 48 and 72 h, respectively. Levels of supernatant HBV DNA and intracellular HBV covalently closed circular DNA (cccDNA) were measured using quantitative polymerase chain reaction. Supernatant cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA). T cell subsets were quantified by flow cytometry using fluorescence-labeled antibodies. In addition, the HBV genome sequence was analyzed by direct gene sequencing. Levels of HBV DNA and cccDNA in group 5 were lower when compared with those in group 3 or group 4, with a significant difference observed at 48 h. The secretion of interferon-γ was negatively correlated with the level of HBV DNA, whereas secretion of interleukin (IL)-10 and IL-22 were positively correlated with the level of HBV DNA. Flow cytometry demonstrated that the percentage of $CD3^+CD8^+$ T cells was positively correlated with the levels of HBV DNA, and the $CD3^+CD4^+/CD3^+CD8^+$ ratio was negatively correlated with the level of HBV DNA. Almost no mutations in the HBV DNA sequence were detected in HepG2.2.15 cells co-cultured with BM-MSCs, SLCs, or in the two types of cells combined. BM-MSCs inhibited the expression of HBV DNA and enhanced the clearance of HBV, which may have been mediated by the regulation of the Tc1/Tc2 cell balance and the mode of cytokine secretion to modulate cytokine expression.

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Abbreviations: BM-MSCs, bone marrow-derived mesenchymal stem cells; SLCs, splenic lymphocytes; HBV, Hepatitis B virus; BN, Brown Norway; UC-MSCs, umbilical cord-derived mesenchymal stem cells; DMEM, Dulbecco's modified Eagle's medium; MTT, methylthiazolyl tetrazolium; EGF, epidermal growth factor; HGF, hepatocyte growth factor

Key words: hepatitis B virus, bone marrow mesenchymal stem cells, lymphocytes, cytokines, gene sequence

Introduction

In China, the yearly mortality rate for end-stage liver disease is >300,000 patients (1). Of the >30 million patients with chronic liver disease in China, ~80% are infected with the hepatitis B virus (HBV) (2). The most effective treatment for HBV-associated end-stage liver disease is liver transplantation. However, without effective prophylaxis, the risk of HBV re-infection following transplantation may reach >80% (3,4). The current treatment protocol of nucleos(t)ide analogues combined with hepatitis B immunoglobulin (HBIG) following liver transplantation, greatly reduces the hepatitis B recurrence rate (2,5,6). However, the high cost remains a heavy burden for patients (7,8), and the long-term use of nucleos(t)ide analogues may lead to HBV resistance (9,10). Application of the HBV vaccine following liver transplantation may potentially lead to the withdrawal of nucleoside analogues and HBIG therapy, however the vaccine is less effective due to the use of immunosuppressants following transplantation (11,12). Therefore, it is important to identify novel methods to prevent hepatitis B recurrence following liver transplantation.

Bone marrow-derived mesenchymal stem cells (BM-MSCs) have demonstrated anti-inflammatory (13,14) and angiogenesis-enhancing effects (15,16) with low immunogenicity (17,18). In addition, BM-MSCs exhibit immunomodulatory capabilities in animal models of rejection following transplantation (19-21), which may represent a promising method for inducing immune tolerance. Transfusions of umbilical cord-derived MSCs for patients with HBV-associated acute-on-chronic liver failure resulted

in improved liver function and alleviated liver damage (22). However, the biological effects of BM-MSCs on HBV have not yet been reported. In the present study, the effect of BM-MSCs on HBV replication and genome mutation *in vitro* was investigated, as well as its associated mechanisms. The results of the current study may provide innovative strategies for the prevention of hepatitis B recurrence following liver transplantation.

Materials and methods

Animals and cell lines. A total of 12 specific pathogen-free Brown Norway (BN) male rats (age, 4-5 weeks; body weight, 200-220 g) were used for the isolation and identification of BM-MSCs. Inbred male BN rats were kept 2 rats per cage at 24˚C, with 50% humidity and a 12 h light and dark cycle, with free access to water and food. An additional 6 specific pathogen-free BN male rats (age, 4-5 weeks; body weight, 200-220 g) were used for the extraction of splenic lymphocytes (SLCs), and were kept under the same conditions as described above. All animals were purchased from the Chinese Academy of Military Medical Sciences (Beijing, China). The use of animals and the animal experimental procedures employed for the purposes of this study were approved by the Ethics Committee of Tianjin First Central Hospital (Tianjin, China). The human hepatocellular carcinoma cell line HepG2.2.15 was donated by Professor Wei Lai (Hepatology Institute of Peking University Affiliated Hospital, Beijing, China), and contained the complete HBV genome, as well as expressed HBV-associated antigens and secreted whole Dane particles (23,24).

Instruments and reagents. The following instruments and reagents were used: Dulbecco's modified Eagle's medium (DMEM) and DMEM/F12 media (1:1; Hyclone, Logan, UT, USA), G418 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), fetal bovine serum (FBS; Biowest, Nuaille, France), transwell plates (Corning, Inc., Corning, NY, USA), MTT reagent (Beijing Dingguo Changsheng Biotechnology, Co., Ltd., Beijing, China), dimethyl sulfoxide (DMSO; Amresco, Solon, OH, USA), lymphocyte separation medium (Beijing Dingguo Changsheng Biotechnology, Co., Ltd.), TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), antibodies directed against CD29 (cat. no. 102207), CD90 (cat. no. 202503), RT1A (cat. no. 205208), CD45 (cat. no. 202207) and RT1B (cat. no. 205305) for the identification of BM-MSCs (Biolegend, Inc., San Diego, CA, USA), CD34 (cat. no. sc-7324; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), CD3-APC mAb (cat. no. 11-0040-82), CD8a-PE-Cy7 (cat. no. 12-0084-82), and CD4-FITC mAb (cat. no. 11-0040-82; eBiosciences, Inc., San Diego, CA, USA), a cell genomic DNA extraction kit (Beijing Kangwei Century Biotech Co. Ltd., Beijing, China) and enzyme-linked immunosorbent assay (ELISA) kits for measuring IL-10 (cat. no. R1000), IL-22 (cat. no. M2200), and IFN-γ (cat. no. RIF00; R&D Systems, Inc., Minneapolis, MN, USA). Primer sequences used for quantitative polymerase chain reaction (PCR) assay analysis for the detection of HBV covalently closed circular DNA (cccDNA) were as follows: cccDNA, forward, 5'-GTG TGC ACT TCG CTT CAC-3', and reverse, 5'-GGG TCA ATG TCC ATG CC-3' (designed by Shanghai Jikang Biotechnology Company, Co., Ltd., Shanghai, China). The TaqMan probe (5'-FAM-ATG TCC TAC TGT TCA AGC CTC CAA-BHQ-3') was designed by Takara Bio, Inc. (Otsu, Japan). Instruments included the CO_2 incubator (Sheldon Manufacturing, Inc., Cornelius, OR, USA), an inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan), the FACSCalibur flow cytometer (BD Biosciences), the ABI PRISM® 3700 DNA Analyzer and the fluorescence-based 7500 Fast Real-Time PCR system (Applied Biosystems™; Thermo Fisher Scientific, Inc.), the automatic fluorescence quantitative flow cytometer (PerkinElmer, Inc., Waltham, MA, USA), and the RT-6000 automatic microplate reader (Omega Bio-Tek, Inc., Norcross, GA, USA). Serum levels of alanine transaminase (ALT) and aspartate aminotransferase (AST) were determined using a 7180 clinical chemistry analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan).

Isolation and identification of BM-MSCs. BM-MSCs were aseptically isolated from the femur and tibia of 12 male BN rats. Red blood cells were lysed using 0.1 mol/l NH_4Cl, and the remaining cells were washed, resuspended and cultured in DMEM/F12 (1:1) media containing 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.), and 15% FBS. BM-MSCs were cultured in an incubator at 37˚C and 5% CO_2 with saturating humidity. The medium was refreshed every 48 h. When cells at passage 3 had reached 80% confluence, cells were trypsinized, washed, centrifuged at 300 x *g* for 5 min at room temperature, and resuspended at $1x10^7$ cells/ml in phosphate-buffered saline (PBS). BM-MSCs (100 µl) were incubated with the following fluorescence-labeled antibodies at 4˚C for 30 min in the dark: CD29-PE (1:80), CD34-FITC (1:20), CD45-PE (1:80), CD90-FITC (1:200), RT1A-PE (1:80) and RT1B-FITC (1:200). Cells were then washed with PBS and analyzed by flow cytometry (FACSCalibur; BD Biosciences) to determine the phenotype and purity of BM-MSCs.

Harvesting of rat SLCs. Spleens of 6 rats were extracted following sacrifice by cervical dislocation under aseptic conditions, disassociated by grinding, and then filtered through a 200-µm nylon mesh. Cell suspensions were transferred to a centrifuge tube containing Percoll lymphocyte separation medium (1.083 g/ml; Beijing Dingguo Changsheng Biotechnology, Co., Ltd., Beijing, China). Following centrifugation at 670 x *g* for 20 min at room temperature, the white middle layer was extracted and centrifuged at 330 x *g* for 8 min at room temperature, before the supernatant was discarded. After washing with PBS, the lymphocytes were counted and cultured in RPIM 1640 media (Gibco; Thermo Fisher Scientific, Inc.) containing 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mmol/l glutamine, and 10% FBS ($5x10^5$ cells/ml).

HepG2.2.15 cell culture. HepG2.2.15 cells were cultured in high glucose-DMEM (Hyclone; GE Healthcare Life Sciences), which contained 10% heat-inactivated FBS, 200 mg/l G418, 6 mmol/l glutamine, 100 U/ml penicillin and 100 mg/l streptomycin, in an incubator at 37˚C and 5% CO_2 with saturating humidity. The medium was refreshed every 48 h, and healthy cells were selected for downstream experiments.

Co-culture of different cell types. The following experimental groups were studied: Group 1, SLCs; group 2, HepG2.2.15

cells; group 3, BM-MSCs + HepG2.2.15 cells; group 4, SLCs + HepG2.2.15 cells; and group 5, SLCs + BM-MSCs + HepG2.2.15 cells. HepG2.2.15 cells were plated in the lower chamber of a 6-well transwell dish (pore size, 0.4 μm; Corning, Incorporated) at $1x10^5$ cells/well, and SLCs and BM-MSCs were inoculated in the upper chamber of the transwell plate at $5x10^5$ cells/well. Plates were cultured at 37˚C and 5% CO_2 with saturating humidity in an incubator for 24, 48 or 72 h. Each group was plated in triplicate wells for each time point. At each time point, supernatants and cells were collected for further analysis.

MTT cell viability assay. Cell suspensions (200 μl) from each experimental group were added to each well of a 96-well plate (SLCs, $2x10^4$ cells/well; BM-MSCs, $2x10^4$ cells/well; HepG2.2.15 cells, $4x10^3$ cells/well), which was incubated at 37˚C with 5% CO_2. Cells were cultured for 24, 48 or 72 h. MTT solution (15 μl at 5 g/l) was added to each well and incubated for 3 h. The medium was subsequently aspirated and DMSO (100 μl) was added to each well before the plates were placed on a shaker for 10 min to fully dissolve the formazan crystals. The absorbance (A) at 490 nm was measured using an automated microplate reader, and the cell survival rate was calculated using the following formula: Survival rate = $(A_{test\ well}-A_{blank\ well}) / (A_{control\ well}-A_{blank\ well}) \times 100\%$.

Detection of supernatant HBV DNA and intracellular cccDNA of HepG2.2.15 cells and BM-MSCs. The supernatant HBV DNA levels were measured using a real-time PCR kit according to the manufacturer's instructions (Shanghai Kehua Bioengineering Co., Ltd.), using an ABI 7500 Real-Time PCR system. Genomic DNA was extracted from HepG2.2.15 cells ($2x10^6$ cells) or BM-MSCs ($5x10^6$ cells) using a UniversalGen DNA kit (CWBio, Co., Ltd., Beijing, China), and 2 μg HBV DNA or cccDNA was subjected to quantitative PCR analysis using an optimized quantitative PCR method described previously (25).

HBV genomic DNA extraction and sequencing analysis. HBV genomic DNA was extracted from the supernatants of co-cultured HepG2.2.15 cells using a Viral DNA Isolation kit (DAAN Gene, Co., Ltd., of Sun Yat-sen University, Guangzhou, China) according to the manufacturer's instructions. Briefly, cell supernatants were added to virus lysis buffer, and lysates were loaded onto a spin column. After viral DNA was bound to the membrane, each column was washed and the viral DNA was eluted.

PCR was performed using HBV genomic DNA as a template to amplify the P, S, X and C regions using the primer sequences listed in Table I. The PCR conditions were as follows: Initial denaturation at 94˚C for 2 min, followed by 35 cycles of 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 1 min and a final extension at 72˚C for 10 min. PCR products were resolved by 2% agarose gel electrophoresis, and the bands were visualized under ultraviolet light following ethidium bromide staining. The DNA was recovered from the agarose gel using a MiniBEST Agarose Gel DNA Extraction kit (Takara Bio, Inc.) according to the manufacturer's protocol, and the amplified DNA was subjected to sequencing analysis by Sangon Biotech (Shanghai, China).

Table I. Sequences of the primers used for polymerase chain reaction in the present study.

Primer name	Sequence (5'-3')	Length (bp)
HBV-F1	GGGTCACCATATTCTTGGGAAC	22
HBV-R1	ATTGAGAGAAGTCCACCACGAGT	23
HBV-F2	TAGGACCCCTGCTCGTGTTACAG	18
HBV-R2	GAACCACTGAACAAATGGCACTAG	24
HBV-F3	GAACCTCTATGTTTCCCTCT	20
HBV-R3	TGCGTCAGCAAACACTT	17
HBV-F4	CCTATTGATTGGAAAGTATG	20
HBV-R4	ATGAGAAGGCACAGACG	17
HBV-F5	CCGATCCATACTGCGGAACTCC	22
HBV-R5	GCTTGGAGGCTTGAACAGTAGGACA	25
HBV-F6	TACTAGGAGGCTGTAGGCATAA	22
HBV-R6	GTGTTGATAAGATAGGGGCATTT	23
HBV-F7	GGTGTCTTTTGGAGTGTGGA	20
HBV-R7	TTGTTCCCAAGAATATGGTGA	21
HBV-F8	AGAACTCCCTCGCCTCG	17
HBV-R8	TTGAAGTCCCAATCTGGATT	20

HBV, hepatitis B virus; F, forward; R, reverse.

Detection of lymphocyte surface markers CD4 and CD8 in the $CD3^+$ cell by flow cytometry. SLCs were harvested and centrifuged at 300 x *g* for 5 min at 4˚C following culture for 24, 48 or 72 h. Then SLCs ($1x10^6$ cells) were resuspended in 100 μl PBS for detection, and the fluorescence-labeled antibodies anti-CD3-APC (1:80), anti-CD4-FITC (1:200), and anti-CD8a-PE-Cy7 (1:160) were added for incubation at 4˚C for 30 min in the dark, to detect the expression intensity of each cell surface marker by flow cytometry.

Detection of supernatant cytokines. Concentrations of IFN-γ, IL-10, and IL-22 in the cell supernatants were determined using an ELISA kit (R&D Systems, Inc.) according to the manufacturer's protocol. The absorbance at 450 nm was measured using an automated microplate reader.

Statistical analysis. SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Normally distributed data were presented as the mean ± standard deviation. Additional data sets were compared by analysis of variance, and Dunnett's method was used when the variance was not homogenous. Linear correlation analysis was used to test the interdependence of the variables. $P<0.05$ was considered to indicate a statistically significant difference. GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to plot data for presentation.

Results

Morphology and phenotypic analysis of HepG2.2.15 cells and BM-MSCs. HepG2.2.15 cells were confirmed to be plastic-adherent cells with a spindle-shaped morphology

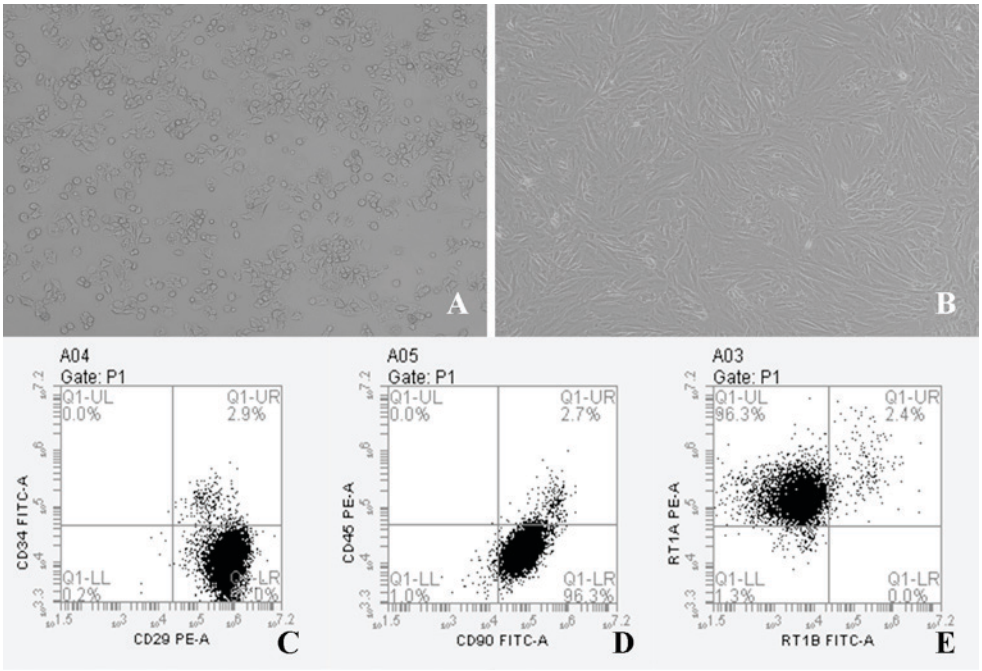

Figure 1. Morphological and flow cytometry analysis of BM-MSCs derived from Brown Norway rats. Microscope images of (A) HepG2.2.15 cells and (B) BM-MSCs at passage 3 (magnification, x100). Flow cytometry analysis of the expression of (C) CD29 and CD34, (D) CD45 and CD90, and (E) RT1A and RT1B. BM-MSCs, bone marrow-derived mesenchymal stem cells; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

(Fig. 1A). Rat BM-MSCs were successfully established in culture and proliferated *in vitro*. Morphological and phenotypic examination revealed that BM-MSCs were confirmed to be plastic-adherent cells with a spindle-shaped morphology under standard culture conditions, as determined by microscopy, and some of the cells exhibited a whirlpool or chrysanthemum pattern (Fig. 1B). BM-MSCs were incubated with antibodies against CD29, CD90, RT1A, CD34, RT1B and CD45, and were analyzed by flow cytometry. Phenotypic examination of BM-MSCs at passage 3 demonstrated that 97.0% of cells expressed CD29, 96.3% of cells expressed CD90, and 96.3% of cells expressed RT1A (Fig. 1C-E). By contrast, >95% of BM-MSCs were negative for CD34, CD45 and RT1B (Fig. 1C-E), which was in accordance with the results of a previous study (26).

Detection of liver enzymes in supernatants. When co-cultured with xenogeneic SLCs or BM-MSCs, no significant difference in liver enzyme levels in HepG2.2.15 cell supernatants was observed (Table II). This suggested that neither BM-MSCs nor SLCs induced rejection of the human hepatocellular carcinoma cell line, HepG2.2.15.

Effects of BM-MSCs on the activity of SLCs and HepG2.2.15 cells. The viability of SLCs in group 5 was significantly lower when compared with that of group 4 at each time point (24 h, $P<0.05$; 48 h, $P<0.01$; 72 h, $P<0.01$; Fig. 2A), which suggested that BM-MSCs may reduce the viability of SLCs.

The viability of adherent cells in group 3 was significantly lower when compared to that of groups 2 at 48 and 72 h, respectively ($P<0.01$ at 48 and 72 h; Fig. 2B). These results suggested that BM-MSCs may inhibit the viability of HepG2.2.15 cells. In contrast, the viability of adherent cells in group 5 was significantly higher when compared to that of groups 3 at 24, 48 and 72 h, respectively ($P<0.01$ at 24, 48 and 72 h; Fig. 2B). These results suggested that BM-MSCs exhibited stimulatory effects on HepG2.2.15 cell viability when co-cultured with SLCs.

Effects of BM-MSCs on the supernatant levels of HBV DNA in HepG2.2.15 cells. The quantity of supernatant HBV DNA in group 5 was significantly lower when compared to that of groups 2, 3 and 4 at 24, 48 and 72 h, respectively (Fig. 3A).

When co-cultured with BM-MSCs and SLCs (group 5), the intracellular quantity of HBV cccDNA in HepG2.2.15 cells was lower than that of groups 2 and 4 at 24 h, however this did not reach statistical significance. The intracellular quantity of HBV cccDNA in group 5 was statistically higher than that of group 2 at 72 h ($P<0.01$; Fig. 3B). The intracellular quantity HBV cccDNA in group 5 was significantly lower than that of groups 2, 3 and 4 at 48 h (Fig. 3B). These findings suggested that BM-MSCs and SLCs may inhibit HBV replication in HepG2.2.15 cells, and that the inhibitory effect was more significant when HepG2.2.15 cells were co-cultured with BM-MSCs and SLCs.

Detection of intracellular HBV cccDNA in BM-MSCs. No intracellular HBV cccDNA was detected in the BM-MSCs in any of the groups (data not shown), which suggested that BM-MSCs co-cultured with HepG2.2.15 cells were not be infected by HBV.

HBV gene sequencing. No mutations in the C or X regions of the HBV genome were detected in HepG2.2.15 cells co-cultured with BM-MSCs, SLCs, or both types of cells (Table III). However, a T45 N mutation in the S region, and an rtR192S mutation in the P region was identified in the supernatants of BM-MSCs + HepG2.2.15 and SLCs + HepC2.2.15 groups, respectively (Table III).

Table II. Supernatant ALT and AST levels in different groups at different time points.

Group	24 h		48 h		72 h	
	ALT (IU/l)	AST (IU/l)	ALT (IU/l)	AST (IU/l)	ALT (IU/l)	AST (IU/l)
HepG2.2.15	1.17±0.41	9.53±1.63	1.50±0.38	13.25±2.65	1.47±0.27	19.82±1.64
BM-MSCs+HepG2.2.15	1.20±0.36	11.30±0.40	1.40±0.33	15.65±1.02	1.77±0.59	23.12±2.22
SLCs+HepG2.2.15	1.45±0.37	11.02±2.95	1.72±0.20	17.62±3.26	1.83±0.43	23.42±3.49
SLCs+BM-MSCs+HepG2.2.15	1.17±0.40	11.70±3.37	1.43±0.14	15.93±0.68	1.73±0.19	21.27±0.74
P-value	0.442	0.099	0.862	0.447	0.056	0.145

All values are presented as the mean ± standard deviation (n=3). ALT, alanine transaminase; AST, aspartate transaminase; BM-MSC, bone marrow-derived mesenchymal stem cells; SLC, splenic lymphocytes; IU, intentional unit.

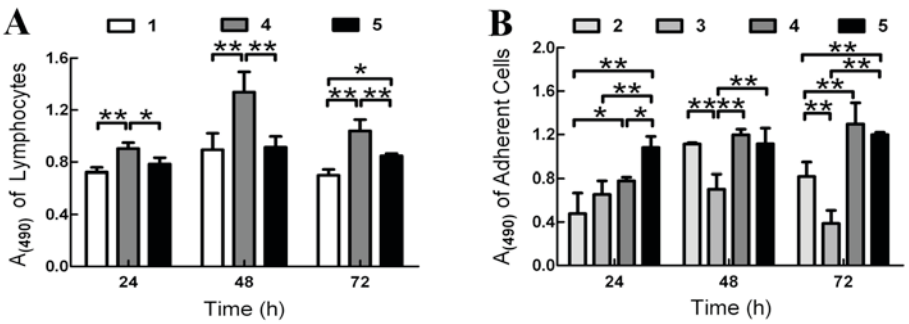

Figure 2. Viability of SLCs and adherent cells as determined using the MTT assay. The viability of (A) SLCs in groups 1, 4 and 5, and (B) adherent cells in groups 2, 3, 4 and 5. *P<0.05 and **P<0.01 as indicated. SLCs, splenic lymphocytes; 1, SLCs alone; 2, HepG2.2.15 cells alone; 3, bone marrow-derived mesenchymal stem cells (BM-MSCs) + HepG2.2.15 cells; 4, SLCs + HepG2.2.15 cells; 5, SLCs + BM-MSCs + HepG2.2.15 cells.

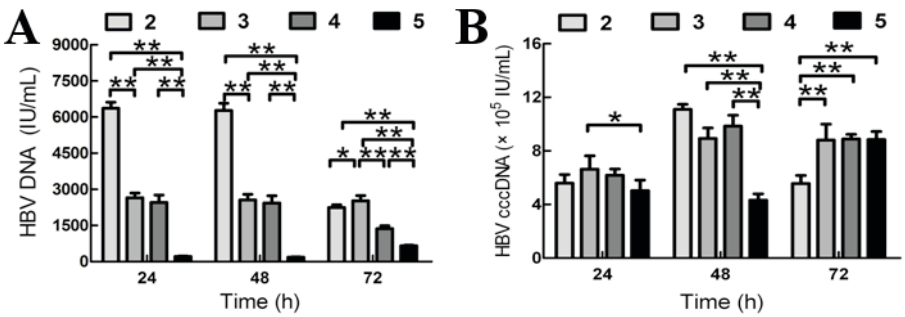

Figure 3. Supernatant HBV DNA quantities and intracellular covalently cccDNA levels. The levels of (A) supernatant HBV DNA and (B) HBV cccDNA in cells from groups 2-5. *P<0.05 and **P<0.01 as indicated. HBV, hepatitis B virus; cccDNA, covalently closed circular DNA; IU, international unit; 2, HepG2.2.15 cells; 3, bone marrow-derived mesenchymal stem cells (BM-MSCs) + HepG2.2.15 cells; 4, splenic lymphocytes (SLCs) + HepG2.2.15 cells; 5, SLCs + BM-MSCs + HepG2.2.15 cells.

Effect of BM-MSCs on lymphocyte subsets. Detection of lymphocyte surface markers by flow cytometry revealed that the percentage of $CD3^+CD4^+$ cells in group 5 was higher than that of group 4 at 24 and 72 h, but was lower at 48 h. These differences did not reach statistical significance (Fig. 4A).

The percentage of $CD3^+CD8^+$ cells in group 5 was significantly lower than that of group 4 at all time points (24 h, P<0.01; 48 h, P<0.05; 72 h, P<0.05; Fig. 4B). When compared with group 4, the $CD3^+CD4^+/CD3^+CD8^+$ ratio in group 5 significantly increased at 24 and 48 h (P<0.01 and P<0.05, respectively; Fig. 4C), but no significant difference was observed at 72 h. The percentage of $CD3^+CD8^+$ cells was positively correlated with HBV DNA levels when co-cultured with BM-MSCs (24 h, r=0.865; 48 h, r=0.766; 72 h, r=0.912; P<0.05).

Effect of BM-MSCs on cytokine levels in co-cultured SLCs and HepG2.2.15 cell supernatants. The supernatant concentrations of IFN-γ in group 5 were higher than those of groups 3 and 4 at 24, 48 and 72 h (Table IV). By contrast, IL-10 and IL-22 levels in group 5 were lower than those of group 3 and group 4 at 24, 48 and 72 h (Table IV). IFN-γ secretion

Table III. Effect of BM-MSCs and SLCs on the HBV gene sequence.

Group	HBV gene sequence			
	Mutation in C region	Mutation in X region	Mutation in S region	Mutation in P region
HepG2.2.15	No mutation	No mutation	No mutation	No mutation
BM-MSCs+HepG2.2.15	No mutation	No mutation	T45N	No mutation
SLCs+HepG2.2.15	No mutation	No mutation	No mutation	rtR192S
SLCs+BM-MSCs+HepG2.2.15	No mutation	No mutation	No mutation	No mutation

BM-MSCS, bone marrow-derived mesenchymal stem cells; SLCs, splenic lymphocytes; HBV, hepatitis B virus.

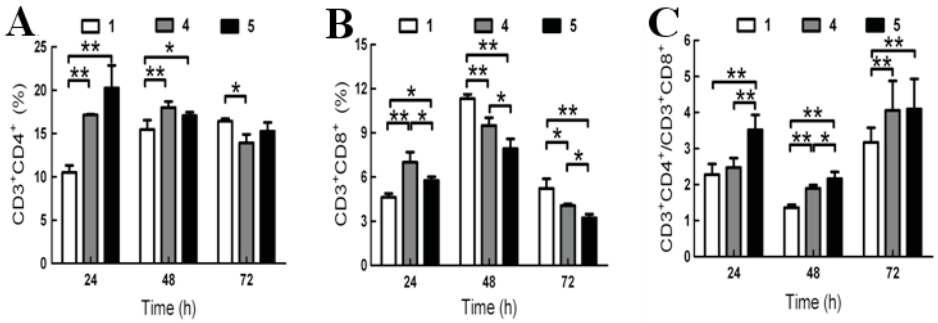

Figure 4. Lymphocyte cell surface markers. The percentage of (A) $CD3^+CD4^+$, (B) $CD3^+CD8^+$, and (C) $CD3^+CD4^+/CD3^+CD8^+$ cells as determined by flow cytometry analysis. $^*P<0.05$ and $^{**}P<0.01$ as indicated. 1, Splenic lymphocytes (SLCs); 4, SLCs + HepG2.2.15 cells; 5, SLCs + bone marrow-derived mesenchymal stem cells (BM-MSCs) + HepG2.2.15 cells.

levels were negatively correlated with HBV DNA levels (24 h, r=-0.900, 48 h, r=-0.982; 72 h, r=-0.968; P<0.05), whereas IL-10 and IL-22 secretion levels were positively correlated with HBV DNA levels (IL-10, 24 h, r=0.860; 48 h, r=0.972; P<0.05; IL-22, 48 h, r=0.858; 72 h, r=0.742; P<0.05). In group 5, the supernatant IFN-γ levels at 48 h were significantly higher than those at 72 h, and the supernatant levels of IL-10 at 48 h were significantly lower than those detected at 24 and 72 h (Table IV). These findings suggested that alterations in IFN-γ and IL-10 levels were most evident at 48 h within the same group.

Discussion

Liver-derived MSCs have been demonstrated to be crucial for the repair of damaged hepatocytes and liver regeneration (27-29). Oh *et al* (30) confirmed that BM-MSCs are potential sources of hepatic oval cells. When the liver is severely damaged, BM-MSCs differentiate into hepatic progenitor-like cells and mediate repair of the liver (31-33). The present study aimed to explore the effects of BM-MSCs on hepatocytes infected with HBV. Previous studies have demonstrated that human MSCs survive and exhibit protective effects on neurological and lung injuries following transplantation into rats (34-36). However, they may also stimulate an allogeneic immune response to increase lymphocyte proliferation in the host (37,38). Therefore, with the lack of stable rat cell lines transfected with HBV, and the strict ethical limits to acquire human stem cells, a xeno-transplantation model was employed in the present study.

The preliminary findings demonstrated that when co-cultured with BM-MSCs, the proliferation of HepG2.2.15 cells was inhibited and HBV DNA levels were decreased. When BM-MSCs were co-cultured with SLCs, HBV DNA levels were markedly reduced. Meanwhile, BM-MSCs induced very few HBV genome sequence mutations and did not cause rejection between xenogeneic cells. To the best of our knowledge the T45N mutation in the S region, and the rtR192S mutation in the P region, are not known to be significant in the clinical treatment of hepatitis B. In addition, the preliminary results of the present study suggested that BM-MSCs may inhibit the replication of HBV cccDNA *in vitro*. It is possible that BM-MSCs may suppress the proliferation of co-cultured T cells *in vitro*, thereby inhibiting immune responses to induce immune tolerance (39-41). Alternatively, BM-MSCs may secrete cytokines, including fibroblast growth factor (42,43), epidermal growth factor (EGF) (44), and hepatocyte growth factor (HGF) (43,45,46) to inhibit HBV replication (47). In addition, intracellular HBV cccDNA in BM-MSCs co-cultured with HepG2.2.15 cells was not detected, which supports the conclusion that HBV is unable to replicate in BM-MSCs (48,49).

BM-MSCs are a cell type that exert immunomodulatory activities (19-21). They inhibit the proliferation and activation of T cells and exhibit immunomodulatory functions mediated by soluble factors (39,41). Prostaglandin E_2 (PGE_2) and indoleamine dioxygenase were observed to be potentially involved in the immunomodulatory function of BM-MSCs (50). The majority of T lymphocytes can be divided into $CD4^+$ T cells

Table IV. Cytokine levels in cell culture supernatants.

Cytokine	Group	24 h (pg/μl)	48 h (pg/μl)	72 h (pg/μl)
IFN-γ	SLCs	848.557±11.409^{aa}	468.347±20.523^{aa}	528.111±15.640^{aa}
	BM-MSCs+HepG2.2.15	636.650±47.047^{aa}	460.953±38.345^{aa}	603.735±26.848^{a}
	SLCs+HepG2.2.15	675.637±19.046^{aa}	621.237±24.709^{aa}	517.170±31.331^{aa}
	SLCs+BM-MSCs+HepG2.2.15	735.030±18.646	780.463±19.879	676.317±34.414^{bb}
IL-10	SLCs	803.930±55.897^{aa}	297.040±32.246^{aa}	183.367±46.742^{aa}
	BM-MSCs+HepG2.2.15	240.747±28.605^{aa}	206.609±13.669^{a}	259.580±30.070^{aa}
	SLCs+HepG2.2.15	511.553±37.490^{a}	413.360±14.133^{aa}	553.133±54.416^{a}
	SLCs+BM-MSCs+HepG2.2.15	420.227±23.235^{bb}	153.087±26.016	447.230±31.192^{bb}
IL-22	SLCs	344.423±36.904^{aa}	180.337±4.672^{aa}	164.537±35.654
	BM-MSCs+HepG2.2.15	183.135±18.123^{a}	166.264±23.206^{aa}	164.722±12.389
	SLCs+HepG2.2.15	166.337±18.651	258.923±23.426^{aa}	305.053±14.766^{aa}
	SLCs+BM-MSCs+HepG2.2.15	146.007±20.407^{bb}	208.537±6.499	210.857±22.527

Data are presented as the mean ± standard deviation (n=3). ^{a}P<0.05, and ^{aa}P<0.01, vs. SLCs + BM-MSCs + HepG2.2.15. ^{b}P<0.05, and ^{bb}P<0.01, vs. the same group at 48 h. IFN-γ, interferon-γ; IL, interleukin; SLCs, splenic lymphocytes; BM-MSCs, bone marrow-derived mesenchymal stem cells.

and $CD8^+$ T cells, and the majority of $CD8^+$ T cells are cytotoxic T lymphocytes (CTL). T cell function is exhausted during chronic HBV infection, and CTLs cannot effectively eliminate the virus. As a result, the virus persists and the proportion of T cell subsets in the peripheral blood is subsequently altered (51-53). The findings of the present study suggested that the percentage of $CD8^+$ cells was positively correlated with HBV DNA levels, which is consistent with a previous study demonstrating that an imbalance of T cell subsets was closely associated with HBV DNA levels (54,55). The $CD4^+/CD8^+$ ratio increased at 24 and 48 h, and then decreased at 72 h. Furthermore, the reduction in the levels of intracellular HBV cccDNA was the most significant at 48 h, which suggested that the increased $CD4^+/CD8^+$ ratio was correlated with inhibitory effects on HBV cccDNA replication. To further confirm these results, the levels of cytokines were measured.

MSCs clearly inhibit the proliferation of allogeneic lymphocytes, and immunosuppression is mediated by $CD8^+$ regulatory cells (56). $CD8^+$ cells are divided into the Tc1 and Tc2 subtypes, and control of the Tc1/Tc2 cell ratio is necessary to maintain normal immune function (57,58). Therefore, IFN-γ and IL-10 cytokine levels were ascertained in the present study, as they are secreted by Tc1 and Tc2 cells, respectively. The results demonstrated that BM-MSCs may influence the expression of IFN-γ and IL-10 by inhibiting $CD8^+$ T cells, as well as inhibit the replication and reduce the levels of HBV DNA.

BM-MSCs secrete various cytokines that affect the function of hematopoietic cells, and release a number of neurotrophic factors, including nerve growth factor, EGF, ciliary neurotrophic factor and IFN-γ (59). The IFN-γ cytokine induces BM-MSCs to constitutively express increased levels of immunosuppressive cytokines, such as PGE_2, HGF, and transforming growth factor (TGF)-β1 (60). Thus, the cytokine expression results obtained in the current study indicate that BM-MSCs may secrete cytokines that affect HBV. However, testing this hypothesis will require further study.

IL-22 was first discovered in the year 2000 (61). As it demonstrates 22% amino acid sequence similarity with IL-10, it was classified as an IL-10 family member (61). However, whether IL-22 exhibits anti- or pro-inflammatory effects on HBV infection remains controversial. Previous studies have demonstrated that intra-hepatic expression of IL-22 was increased in patients with acute and chronic hepatitis B (62). When infected with the virus, T cells mediate antiviral immunity, and cause inflammatory injury to the liver. Meanwhile, inflammation and injury leads to compensatory increases in levels of cytokines (e.g. IL-22) that may protect hepatocytes from inflammation and repair liver damage (63). The results of the present study indicated that IL-22 and IL-10 secretion were reduced significantly when SLCs were co-cultured with BM-MSCs, which suggested that IL-22 exerted anti-inflammatory effects in HBV infection.

HBV-associated end-stage liver disease poses a serious threat to human health, and liver transplantation is currently the only effective treatment. BM-MSC transplantation has been proposed as a novel strategy for the treatment of HBV, and may represent a new method for prophylaxis and the treatment of HBV re-infection following liver transplantation. In addition, studies of the effects of BM-MSCs on HBV cccDNA levels may provide novel strategies to screen for preventative treatments against HBV re-infection. Although HBV does not affect the phenotype or differentiation ability of BM-MSCs, it has been demonstrated to inhibit the proliferation of BM-MSCs *in vitro* (64). Therefore, a number of issues require further investigation before BM-MSCs may be used as a clinical treatment option, and will be a focus of future research.

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