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RESEARCH ARTICLE

Murine bone marrow stromal cells pulsed with homologous tumor-derived exosomes inhibit proliferation of liver cancer cells

Bo Ma · Hanfang Jiang · Jun Jia · Lijun Di · Guohong Song · Jing Yu · Yulin Zhu · Zhihao Lu · Xiaoli Wang · Xinna Zhou · Jun Ren

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Abstract
Background Increasing evidence shows that bone marrow stromal cells (BMSCs) have antitumor activities both in vitro and in animal models. Further studies fleshed out the supportive data that the antitumor activity of BMSCs could be markedly enhanced by cytokines such as IL-2 and IFN-β (interferon). However, powerful strategies to activate BMSCs other than by genetically engineering interventions are still required.
Methods In this study, new methods of generating antitumor activities of murine marrow-originated MSCs pulsed with homologous tumor-derived exosomes (TEX) were explored to yield potent immune effectors against hepatocellular carcinoma cells in vitro.
Results The results showed that BMSCs pulsed with exosomes and IFN-γ exhibited increased migration ability with a result of 163.22 ± 26.90 versus 129.89 ± 29.28 cells/HP by transwell determination ($p < 0.05$). The inhibition of homologous hepatocellular carcinoma cells line H_{22} cells by exosomes pulsed BMSCs was significantly increased by 41.9 % compared with control ($p < 0.05$), and flow cytometry analysis showed that the cell cycle of H_{22} cells was arrested in G_0/G_1 phase. Meanwhile, western blot analysis showed that PCNA protein expression in the supernatant of H_{22} cells was significantly decreased.
Conclusions This study demonstrated that BMSCs pulsed with TEX could enhance its antitumor activities, which might be regarded as a novel promising antitumor treatment.

Keywords Exosome · Hepatocellular carcinoma · Bone marrow stromal cells

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and causes more than 600,000 deaths annually worldwide. The incidence and mortality of HCC is especially high in China, where the annual mortality rate of HCC is 54.7 per 100,000 according to recent statistical data [1, 2]. Surgery, transarterial chemo embolization (TACE) and liver transplantation are the major effective therapeutic approaches of this disease. However, most HCC patients are not eligible due to the advanced stage at diagnosis or insufficient liver function in the setting of cirrhosis [3, 4]. Therefore, innovative treatments of HCC are desperately needed, among which cellular therapy is opening a new promising field [5].

Bone marrow stromal cells (BMSCs) are derived from bone marrow pluripotent stem cells with homologous properties of stem cells: self-renewal and the capacity to develop into multiple lineages [6–9]. BMSCs are well suited for the vehicle of gene therapy because they are

B. Ma · H. Jiang · J. Jia · L. Di · G. Song · J. Yu · Y. Zhu · Z. Lu · X. Wang · X. Zhou · J. Ren
Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Medical Oncology, Peking University Cancer Hospital, Beijing Cancer Hospital and Institute, Beijing, China

J. Ren
Duke-PKU Cancer Program, Department of Medical Oncology and Immunotherapy, Peking University Cancer Hospital/Beijing Cancer Hospital and Institute, 52 Fucheng Rd, Beijing 100142, China

Present Address:
J. Ren (✉)
Capital Medical University Cancer Center, Beijing Shijitan Hospital, 10 Tieyi Road, Beijing 100038, China
e-mail: renjun@bjcancer.org; renjun9688@yahoo.com

isolated easily and can be expanded in culture and genetically manipulated [10–13]. It has been reported that BMSCs have considerable therapeutic potentials in several disease processes, including cardiovascular disease, cellular replacement therapy, and tissue engineering [14–17]. In addition to these progresses, more and more evidence shows that BMSCs possess potential antitumor ability. Khakoo et al. [18] demonstrated that human BMSCs significantly delayed tumor growth by inhibiting the activity of Akt protein kinase in a model of Kaposi's sarcoma. Our previous studies have also shown that murine BMSCs could induce coagulative necrosis in tumor cells in a mouse model of orthotopic hepatocellular carcinoma and prolonged the survival of tumor-bearing mice [19]. However, these antitumor activities of BMSCs were weak and insufficient. Some pilot studies indicate that BMSCs modified by cytokine such as IL (interleukin)-2 or INF (interferon)-β exhibited much stronger antitumor activities [11, 20].

Other methods to enhance the antitumor activities of BMSCs directly were explored. Stagg [21] suggested that antigen-pulsed BMSCs could induce an effective antigen-specific cellular immune response by means of ovalbumin-pulsed INF-stimulated BMSCs. In autologous conditions, IFN-γ treated BMSCs could process exogenous antigens and be efficiently activated in vitro, which induced antigen-specific immune responses in vivo. This property of BMSCs provides such a possibility to stimulate antitumor immune responses by using antigen-pulsed BMSCs instead of genetically modified BMSCs.

Exosomes are small (50–90 nm in diameter), membrane-bound vesicles of the endocytic pathway that are externalized by a variety of cell types [22–25]. Dendritic cell-derived exosomes (DEX) have been demonstrated to be capable of inducing strong antitumor immunity in both experimental studies and clinical trials [26–29]. Phase II/III trials of DEX in non-small cell lung cancers (NSCLC) have been carried out due to the encouraging results of a phase I clinical trials [29]. Tumor-derived exosomes (TEX), which express tumor-associated antigens, MHC-I molecules and heat shock protein (HSP), are an ideal tumor antigen delivery system that induces tumor-specific cytotoxic lymphocytes (CTL) both in vitro and in vivo [30, 31]. Our previous studies have shown that TEX can activate the dendritic cells to induce tumor-specific immune responses. The possibility and the safety of exosomes-based vaccination assessed in phase I clinical trials are encouraging the further development of exosome-based vaccines in cancer immunotherapy [32].

In this study, we investigated the antitumor activities of BMSCs pulsed with TEX in homologous hepatocellular carcinoma in vitro in order to establish a novel experimental therapeutic method against HCC.

Materials and methods

Isolation and expansion of murine BMSCs

The femurs and tibias of 4- to 6-week-old male BALB/c mice were dissected away from attached muscle and connective tissue. The ends of the bones were removed and marrow was extruded by inserting a 21-gauge needle into the shaft of the bone and flushing it with 2 mL of α-MEM supplemented with 2 % fetal bovine serum (FBS, Hyclone Lab, Inc.; Logan, UT). The marrow was filtered through a 75-μm filter. Cells were plated at a density of 1×10^6 cells/cm^2. The non-adherent cell population was removed after 72 h. Cells were cultured an additional 7–10 days by incubation in α-MEM containing 10 % FBS at 37 °C in 5 % CO_2. Medium was changed every 3 days. Then cells were harvested by gentle scraping after 5 min incubation in 0.25 % trypsin with 1 mM EDTA and filtered through a 30-μm filter to remove cell clumps. The cell pellet was resuspended in 80 μL buffer per 10^7 cells and magnetic beads conjugated to anti-CD11b (10 μL) and anti-CD45 antibodies (10 μL, Miltenyi Biotec; Bergisch, Germany) were added. MSCs were incubated for 15 min at 4 °C and immunodepleted cells were plated into 6-well plates and cultured with media changes every 3 days.

Flow cytometry analysis

Adherent cells were retrieved by trypsin digestion and aliquots of 1×10^6 cells were labeled with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against mouse CD29 and CD106, stem cell antigen-1 (Sca-1), CD31, phycoerythrin (PE)-conjugated monoclonal antibodies against mouse CD73, CD90, CD11b, or phycoerythrin-cy5 (PE-cy5)-conjugated monoclonal antibodies against mouse CD44, CD45 (all products from BD Pharmgin Co., CA, USA) for 30 min at 4 °C in the dark. After washing twice, events were acquired by FACSCalibur (BD Company, USA) and data analysis was conducted with FCS Express V2 software after gating for the designated population.

Multipotent differentiation assay

To induce adipocytic differentiation, cells were cultured as monolayers, allowed to become nearly confluent, and then maintained in adipogenic induction medium (α-MEM supplemented with 10 % FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 10 μg/mL insulin, 100 μg/mL indomethacin, 100 U/mL penicillin, 100 μg/mL streptomycin) (Sigma-Aldrich; CA, USA) for 3 weeks with a medium change every 3 days. Then, cells were assessed using Oil red-O staining as an indicator of intracellular lipid accumulation.

Osteogenic differentiation was induced by culturing cells with osteoinductive medium (α-MEM supplemented with 10 % FBS, 50 mg/L vitamin C, 10^{-7} dexamethasone, 2 g/L β-glycerol phosphate, 100 U/mL penicillin, 100 μg/mL streptomycin). The medium was changed every 3 days until 21 days. A histochemical kit (Sigma-Aldrich; CA, USA) was used to assess alkaline phosphatase (ALP) (Sigma-Aldrich; CA, USA) activity according to the manufacturer's protocols.

Isolation, purification of TEX

1×10^6 H_{22} cells, a hepatocellular carcinoma cell line (American Type Culture Collection, Manassas, VA, USA) were injected into the abdominal cavity of BALB/c mice. Ascitic fluid was gently aspirated after 1 week and used immediately for exosomes isolation. Ascitic fluid was centrifuged successively at 300×*g* for 10 min, 2,000×*g* for 20 min, and 10,000×*g* for 30 min to pellet cells and debris. The clarified supernatant was concentrated by centrifugation for 30 min at 1,000×*g* in a 100 kDa MWCO Amicon Ultra centrifugal filter (Millipore; USA) to a volume of 5 mL. The exosomes were underlayed with a 30 % sucrose/D_2O (Sigma-Aldrich; CA, USA) density cushion, followed by ultracentrifugation at 100,000×*g* for 60 min. The cushion was collected from the bottom and diluted in PBS. The exosomes were concentrated by centrifuging for 30–60 min at 1,000×*g* in a 100 kDa MWCO Amicon Ultra centrifugal filter and stored at −80 °C [33].

Identification of TEX

Electron microscopy analysis

The 100,000×*g* pellets were fixed with 2 % w/v paraformaldehyde for 4 h. 5 mL of the suspended exosomes were dropped onto a formvar–carbon coated grid and left to dry at room temperature for 20 min. Then exosomes were fixed in 1 % w/v glutaraldehyde and stained with saturated aqueous uranyl acetate for 10 min. Samples were then embedded in 0.4 % w/v uranyl acetate, 1.8 % w/v methylcellulose on ice. The excess liquid was removed and the grid dried at room temperature then viewed in a transmission electron microscope (TEM).

Western blot analysis

The exosomes were separated on 10 % SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore; USA), and incubated with antibodies against HSP-70 and MHC-I (Santa Cruz; California, USA) followed by HRP conjugated secondary antibodies. Signals were detected using an enhanced chemiluminescence ECL kit (Amersham Biosciences, USA).

Migration assay of BMSCs

BMSCs (5×10^4) treated with IFN-γ (50 ng/mL) and TEX (10 μg) for 20 h were placed in the upper well of 24-mm tissue culture transwell plates (Corning Incorporated; Corning, NY). H_{22} cells (1×10^5) were placed in the lower well of the transwell plates and incubated for 24 h at 37 °C. Then, the transwell inserts were fixed and stained and the number of cells that crossed the transwell member was determined by directly counting six high-power fields of light microscopy. All experiments were done in triplicate. MSCs (5×10^4) without IFN-γ and exosomes were used as controls.

Cell proliferation assay

The mouse hepatoma cell line H22 cell (strain BALB/c) was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum at 37 °C in a humidified CO_2 (5 %) incubator (Hyclone Lab, Inc.; Logan, UT).

Six groups (H_{22} alone, H_{22} + IFN-γ, H_{22} + BMSCs, H_{22} + exosomes, H_{22} + IFN-γ + BMSCs, and H_{22} + IFN-γ + BMSCs + exosomes) were set up to evaluate the proliferation of H_{22} cells in co-culture experiment. The concentration of IFN-γ was 50 ng/mL. The exosomes was 10 μg and plated in complete media (α-MEM with 10 % FBS) for 20 h before co-cultured with H_{22} cells. The BMSCs cells (5×10^4) were plated in 12-well plates alone, or pre-treated with IFN-γ, IFN-γ pulsed with exosomes in complete media (α-MEM with 10 % FBS) for 20 h. Then, H_{22} cells (2×10^5) were added to each plate and the number of H_{22} cells was counted using a hemocytometer after co-cultured for 72 h. Each measurement was done in triplicate and the mean value was calculated. After 72 h, the supernatants were collected and PCNA protein expression was detected by western blot to determine the proliferation of H_{22} cells in this co-culture system. Quantitative values for the PCNA proteins were obtained by densitometric analysis and analyzed by AlphaEaseFC (ChemiImager 5500) software (Alpha Innotech, San Leandro, CA). This provided PCNA/β-actin integrated density values.

Cell cycle analysis

H_{22} cells were treated with BMSCs pulsed with exosomes (10 μg) and IFN-γ (50 ng/mL) or cultured alone for 72 h. Cells were harvested, washed in PBS, fixed with −20 °C

absolute ethanol and stored at 4 °C over night. Cells were stained with 50 μg/mL propidium iodide. DNA content was analyzed on a cytofluorimeter by fluorescence-activated cell sorting analysis (FACScan, Becton-Dickinson and Company, San Jose, CA) using ModFit software (Verity Software House, Inc.)

Statistical analysis

Data were presented as the mean ± standard deviation and analyzed with independent samples *t* test. Data from the cell proliferation assays were analyzed with analysis of variance. A *p* value less than 0.05 was considered statistically significant.

Results

Morphology of murine BMSC

Bone marrow cells attached to the culture dish at 24 h and the non-adherent cells were removed at 72 h. The initial adherent cells appeared as individual cells or clusters of only a few cells. Between day 4 and day 10, the number of MSCs increased and a few large colonies could be observed. Cellular appearance was highly heterogeneous, including round, spindle-shaped and flattened cells (Fig. 1a, b). On 12–14 days, colonies grew to confluence and adherent cells were harvested to deplete hematopoietic cells by immunodepletion using specific antibodies against anti-CD11b and CD45. By the end of the sixth passage, normal cultures had very low frequencies of round cells and mostly consisted of bipolar fibroblastic cells (Fig. 1c, d).

Phenotypic characteristics of BMSCs

BMSC were analyzed for the expression of a panel of antigens. As showed in Fig. 2, more than 90 % of cells were strongly stained by antibodies directed against Sca-1, CD44 and CD29. Additionally, cells expressed low levels of CD73, CD90 and CD106. They were homogenously negative for CD45, CD11b and CD31 (Fig. 2).

Multilineage differentiation of BMSCs

To test the multilineage differentiation ability, BMSCs were maintained in matched inductive medium and cells cultured in BMSCs medium served as controls. BMSCs revealed specific differentiation into osteocytes and adipocytes after induction as assessed by ALP and oil red-O staining, while none of the controls displayed characteristics of differentiation.

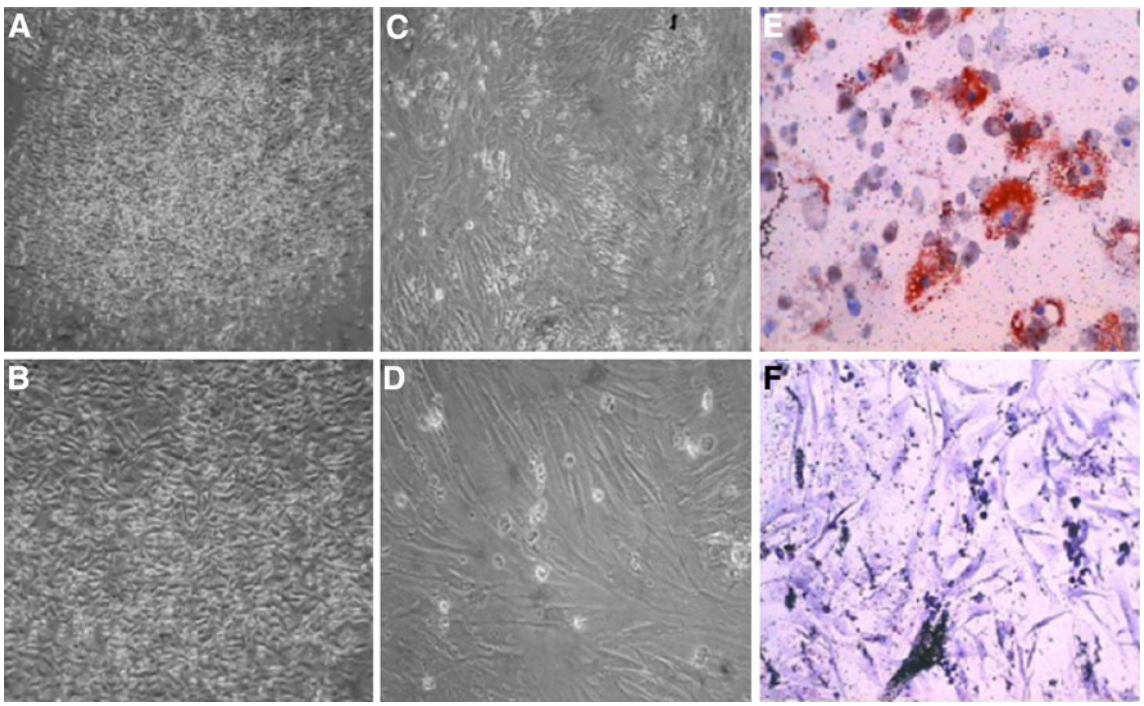

Fig. 1 Morphological features and in vitro differentiation of murine bone marrow mesenchymal stem cells. **a**, **b** Phase contrast image of MSC showing an adherent colony of spindle-shaped cells that appeared on day 14 of primary passage (**a** magnification ×100, **b** magnification ×200). **c**, **b** By the end of the sixth passage, most cells appeared as bipolar fibroblast-like cells (**c** magnification ×100, **d** magnification ×200). **e**, **f** Differentiation of murine MSCs. Cells were incubated in lineage-specific induction media and then analyzed by cytological staining: Oil red-O staining (**e**) and ALP staining (**f**)

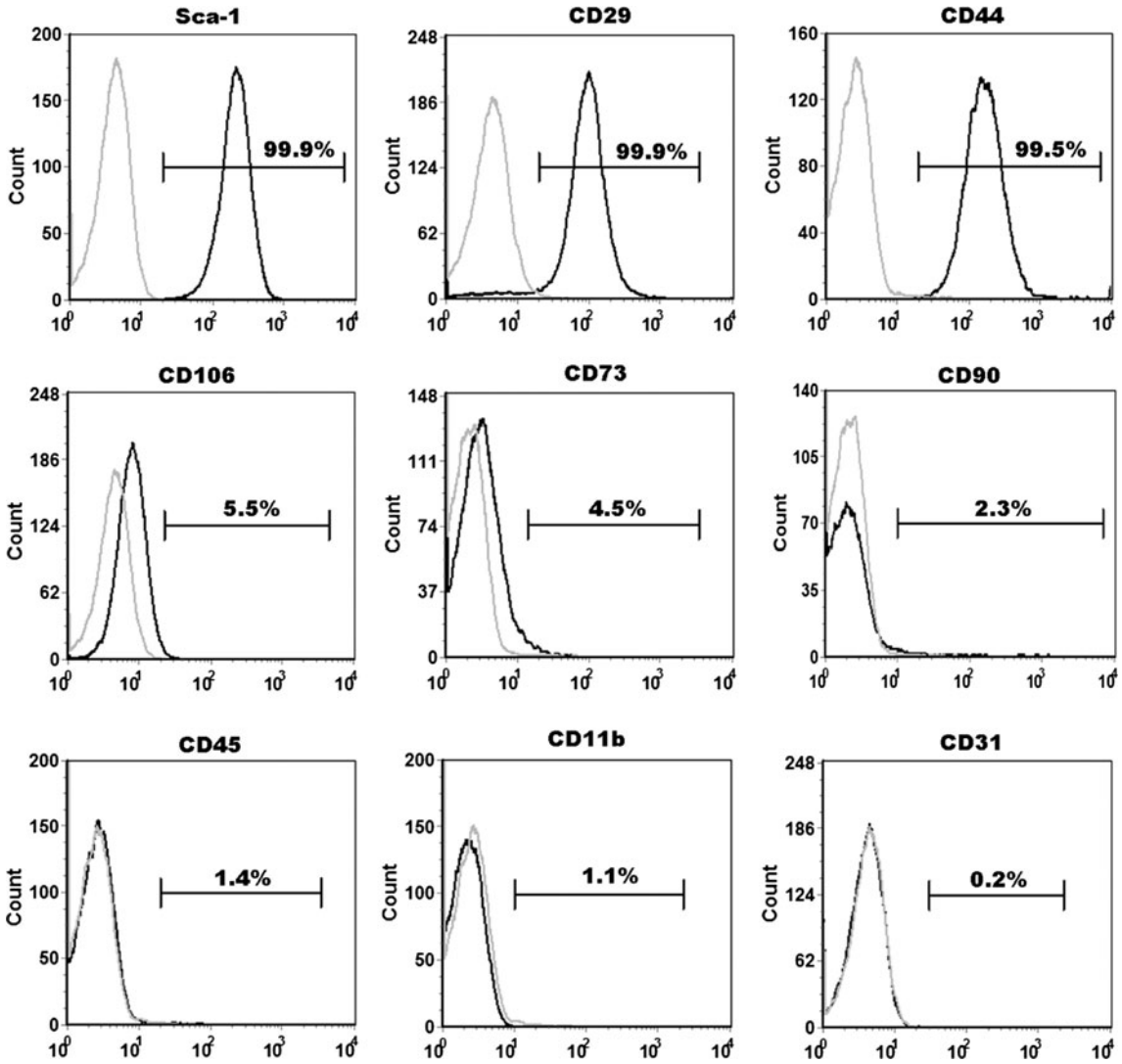

Fig. 2 Flow cytometric analysis of MSCs cell-surface antigens. Cells were stained with the assigned monoclonal antibodies conjugated to FITC, PE or PE-cy5 and thereafter analyzed with FACSCalibur cytometer and FCS Express V2 software

When cultured in adipogenic medium, little lipid droplets were seen in some cells at 24 h, mainly surrounding the cellular nucleus. Oil red stain showed these lipid droplets become salmon pink after 21 days induction (Fig. 1e).

Osteoblastic differentiation could be induced in vitro in the presence of osteoinductive medium. After 3 or 4 days, the BMSCs became polygonic and fusocellular cells were reduced. ALP stain showed black mineralized nodus granula in cells on day 21 (Fig. 1f).

Characterization of tumor-derived exosomes

These membrane vesicles contained in ascetic fluid were round and 50–100 nm diameter in electron microscopy. Western blotting assay showed that exosomes expressed MHC class I molecules and HSP-70 (Fig. 3).

Migratory capacity of BMSCs pulsed with tumor-derived exosomes in transwell experiment

We evaluated the migratory capacity of BMSCs towards hepatocellular carcinoma cells in vitro when BMSCs were pulsed with tumor-derived exosomes. As shown in Fig. 4, the migratory capacity of BMSCs was enhanced to a significantly greater extent by pulsing with TEX (163.22 ± 26.90 cells/HP vs. 129.89 ± 29.28 cells/HP, $p < 0.05$).

BMSCs pulsed with TEX inhibited the proliferation of hepatocellular carcinoma cells

The effect of BMSCs pulsed with TEX on H_{22} hepatocellular carcinoma cells (target cells) were evaluated by cell proliferation assay. This study was able to directly calculate the change in target cell number by cell hemocytometer

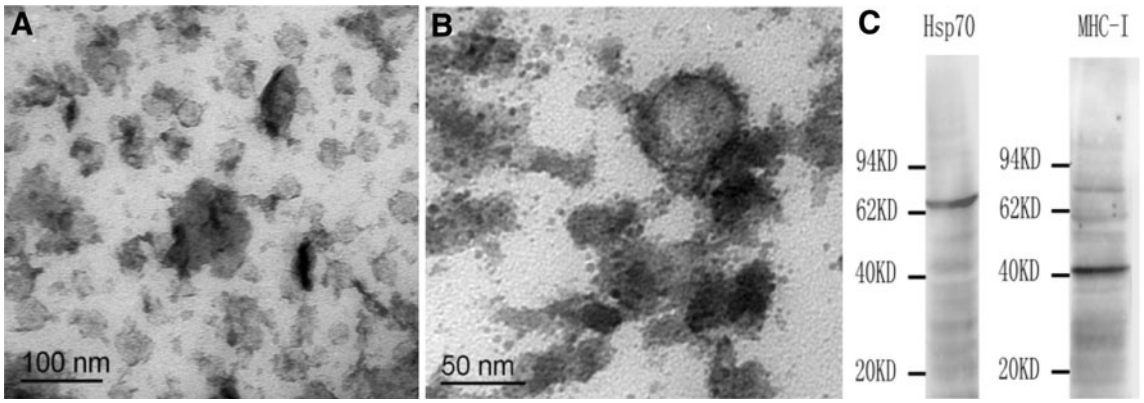

Fig. 3 Identification of TEX isolated from ascites of BALB/c mice *Left* electron microscopy images of TEX. Exosomes were defined as round shaped membrane vesicles in size 50–100 nm. *Right* western blot analysis of TEX with antibodies directed against MHC class I molecules, and heat-shock protein 70 (Hsp 70)

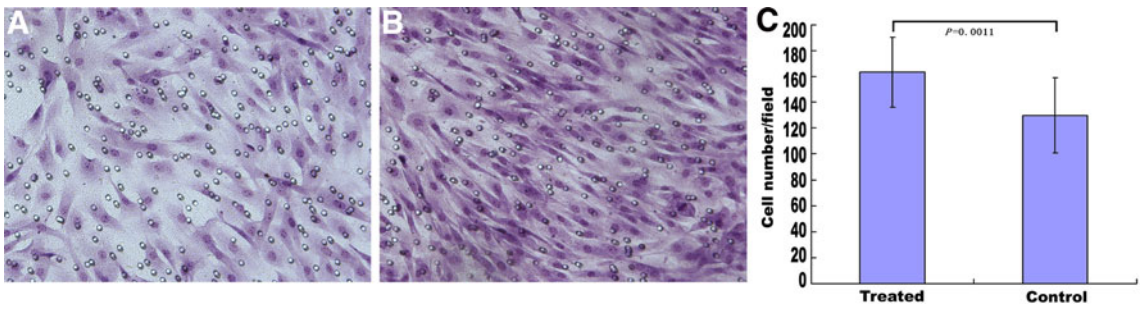

Fig. 4 Tumor-derived exosomes enhance the migration of MSCs in vitro MSCs pulsed with tumor-derived exosomes or MSCs alone (5×10^4) were placed in the upper chamber of the transwell isolated with 8 μm pores, and H_{22} cells were placed in the lower chamber. After 24 h, migrated cells across the membrane were counted. The number of migratory cells of MSCs pulsed with TEX (**b**) was significantly greater (163.22 ± 26.90 cells/HP) than MSCs alone (**a**) (129.89 ± 29.28 cells/HP, $p < 0.05$)

because H_{22} cells are suspension cells while BMSCs are adherent. Compared with H_{22} cells alone, the proliferation of H_{22} cells co-cultured with BMSCs pulsed with exosomes and IFN-γ was significantly inhibited ($3.32 \pm 0.41 \times 10^5$ vs. $5.71 \pm 0.56 \times 10^5$, $p < 0.05$). However, H_{22} cells co-cultured with only BMSCs, exosomes, IFN-γ or IFN-γ treated BMSCs, showed relatively faint growth inhibition ($5.28 \pm 0.68 \times 10^5$, $5.50 \pm 0.94 \times 10^5$, $5.57 \pm 0.50 \times 10^5$, and $5.15 \pm 0.48 \times 10^5$, $p > 0.05$). These results indicated that MSCs pulsed with exosomes and IFN-γ could significantly inhibit the proliferation of H_{22} cells (Fig. 5a, b).

At the same time, we collected the supernatant of H_{22} hepatocellular carcinoma cells and evaluated the PCNA protein expression by western blotting, and calculated the PCNA/β-actin ratio using integrated density values. The results showed that the PCNA protein expression of MSCs pulsed with exosomes and IFN-γ was the lowest compared with other groups, the PCNA integrated density values decreased 44 % compared with control (0.88 ± 0.09 vs 1.56 ± 0.04, $p < 0.05$) These results indicated that MSCs pulsed with exosomes and IFN-γ might possess a direct antitumor effect against H_{22} cells (Fig. 5c).

BMSCs pulsed with TEX induced the cell cycle arrest of hepatocellular carcinoma cells

To explore the potential mechanism of inhibition of H_{22} cells treated with BMSCs pulsed with exosomes and IFN-γ, the cell cycle progression was analyzed by fluorescence-activated cell sorting analysis (Fig. 6). H_{22} cells treated with BMSCs pulsed with IFN-γ and exosomes showed a decreased percentage of cells in G_2/M and S phases and an increased number of cells in the G_0/G_1 population, and there was no appearance of hypodiploid cells. It implied that there was a cell cycle blockage in the G_1-to-S transition and the inhibitory effect of BMSCs pulsed with exosomes and IFN-γ on the proliferation of H22 cells might be induced by cell cycle arrest rather than apoptosis.

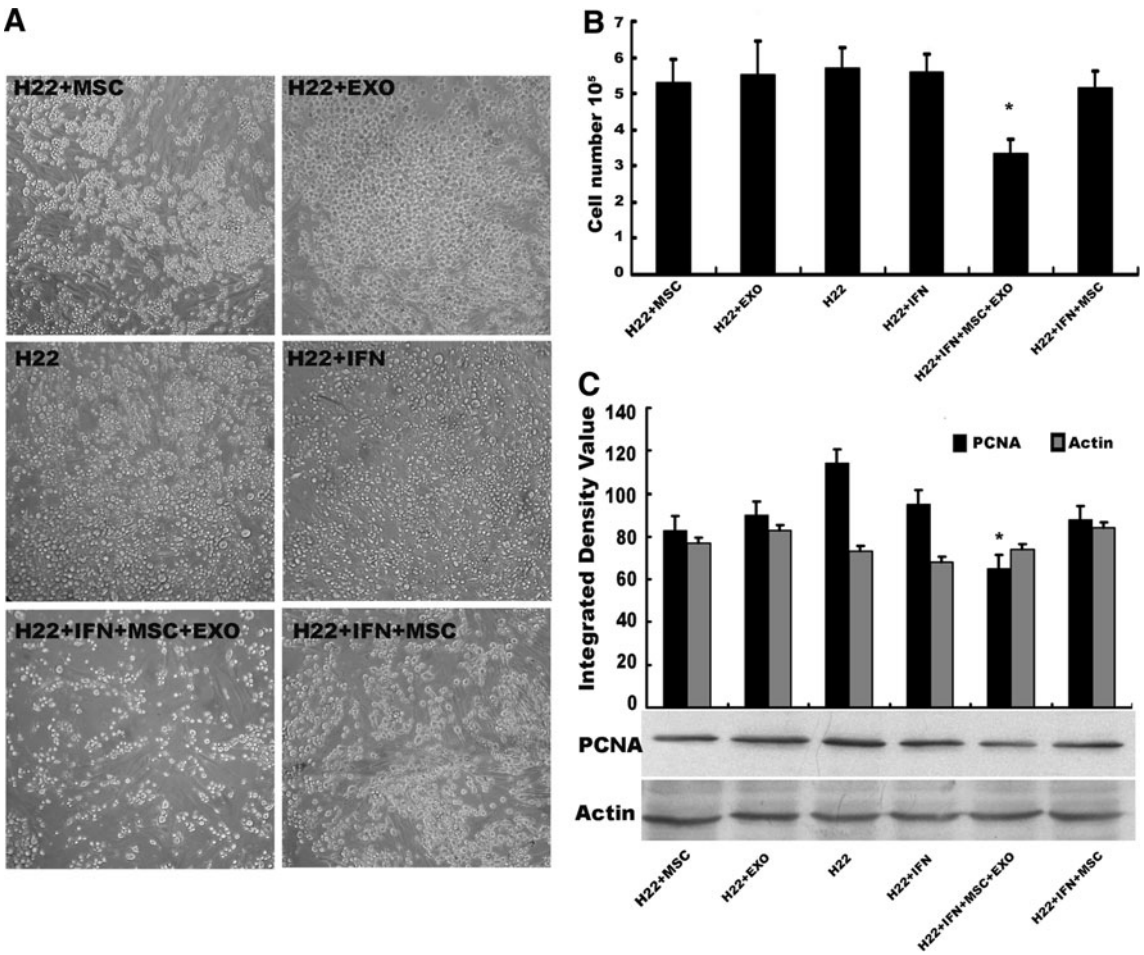

Fig. 5 MSCs pulsed with tumor-derived exosomes inhibit hepatocellular carcinoma cell proliferation in vitro MSCs (5×10^4) treated with 50 ng/mL IFN-γ and 10 μg TEX, MSCs treated with 50 ng/mL IFN-γ or IFN-γ alone, TEX alone, MSCs alone were co-cultured with H_{22} cells (2×10^5) for 72 h. **a**, **b** After 72 h, the number of H_{22} cells co-cultured with MSCs pulsed with TEX was significantly lower than other groups ($p < 0.05$). **c** Western blot analysis of proteins extracted from H_{22} hepatocellular carcinoma cells after co-culture, with antibodies directed against PCNA. The PCNA integrated density values analyzed using AlphaEaseFC (ChemiImager 5500) software. The result showed the PCNA protein expression of MSCs pulsed with exosomes decreased than other groups ($p < 0.05$)

Discussion

Tumor-derived exosomes (TEX) originate from culture supernatants of tumor cells or biological fluids in carcinomatoses. Malignant effusions are more abundant sources of TEX and MHC-I/peptide complexes than tumor cell culture supernatants [34]. Therefore, malignant effusion-derived exosomes showed much stronger immune stimulatory activities. In this study, we successfully isolated and purified exosomes contained in malignant ascites using the classical serial ultracentrifugation process and ultrafiltration. This kind of TEX was verified with typical morphology by electron microscopy, and western blotting showed the presence of MHC class I molecules and HSP 70 (Fig. 3). Extracellular localized HSP 70 stimulates the immune system and plays key roles in the activation of the immune system. Many preclinical studies have shown that immune cells stimulated with HSPs could inhibit different kinds of cancer cells [35–38]. The enrichment of tumor antigens and immune molecules in TEX yields stronger antitumor activities than simple tumor antigens. Therefore, TEX could be a good therapeutic vaccine for cancer immunotherapy.

Migration and tumor tropism is an intrinsic property of mesenchymal stem cells. Nakamura et al. [11] showed that when BMSCs were inoculated into the contralateral hemisphere in a rat glioma model, they migrated towards 9L glioma cells through the corpus callosum. Our previous studies showed that regardless of the injected sites of tumor lesion or normal liver tissue, BMSCs from Rosa 26 transgenic mice could migrate to the border zone between tumor and normal liver tissue in a BALB/c mouse model of orthotopic hepatocellular carcinoma [19]. In this study, we confirmed the migratory capacity of BMSCs by transwell

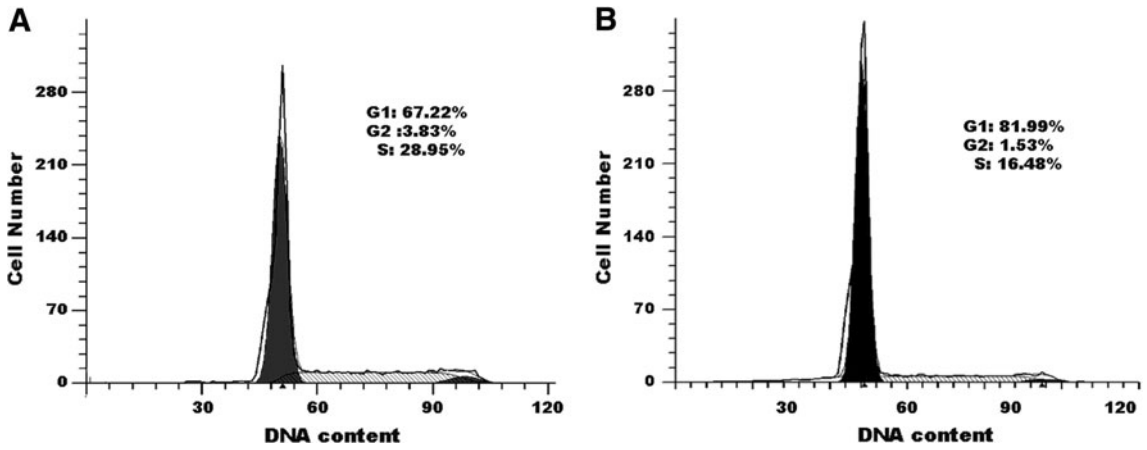

Fig. 6 MSCs pulsed with exosomes induces cell cycle arrest in H_{22} cell lines H_{22} cell lines were incubated with and without BMSCs pulsed with exosomes for 72 h. Cell cycle was arrested at G_0/G_1 phase with propidium iodide staining and fluorescence-activated cell sorting analysis. **a** H_{22} cell lines without BMSCs pulsed with exosomes group. **b** H_{22} cell lines treated with BMSCs pulsed with exosomes group

experiments in vitro (Fig. 4). The migratory capacity of BMSCs pulsed with TEX was enhanced when co-cultured with H_{22} cells, which might promote BMSCs to implant directly into tumor lesions. This tumor-homing property of BMSCs has been proven to be mediated by many soluble factors released from tumor cells, such as vascular endothelial cell growth factors, TGFs, FGFs, platelet-derived growth factors, etc., [39, 40]. The migration-inducing capability of TEX might be partially due to complex membrane molecules. Gastpar et al. [41] had demonstrated that HSP 70 surface-positive TEX could induce specific migration of natural killer cells. In our study, BMSCs pulsed with TEX also showed enhanced migratory capacity. The great migratory ability and tumor tropism of TEX-pulsed BMSCs provide proof of principle for utilizing BMSCs as a vehicle for cellular therapy in the treatment of HCC [11, 20]. However, further investigation should be done to explain this phenomenon.

Studies aimed at investigating the antitumor activities of MSCs in vitro and/or in vivo have yielded conflicting results [11, 18, 42, 43]. Several observations support the argument that MSCs exert their antitumor effects through direct, local effect on tumor cells. A pivotal study demonstrated that intravenous injection of MSCs potently inhibited tumor growth in a Kaposi's sarcoma (KS) model. And the mechanism of this inhibition was found to depend on E-cadherin-mediated cell-to-cell contact and downregulation of Akt activity in tumor cells [18]. These findings are in line with our previous observations that MSCs could induce coagulative necrosis of murine H_{22} tumor cells in a mouse model of orthotopic hepatocellular carcinoma [19]. The tumor microenvironment is likely to influence greatly the intrinsic antitumor activities of BMSCs. The tumor microenvironment is composed of different cell types, including fibroblasts, endothelial cells, pericytes, adipocytes and recruited inflammatory cells of hematopoietic lineage. These cells establish multiple interactions with cancer cells through either cell-to-cell contact or release of soluble molecules such as cytokines, chemokines, proangiogenic factors and MMPs [44, 45].

Stagg [21] suggested that IFN-γ-stimulated BMSCs could behave as a new type of nonhematopoietic antigen presenting cells, and that in autologous conditions, BMSCs pulsed with exogenous antigens could induce effective antigen-specific cellular immune response both in vitro and in vivo. In the present study, the antitumor activities of IFN-γ-pre-treated BMSCs pulsed with TEX were assessed. The results showed that BMSCs pulsed with homologous TEX exhibited much stronger antitumor effects than BMSCs alone. The proliferation of H22 was inhibited significantly and the PCNA expression of H_{22} cell culture supernatants decreased (Fig. 5). Further cell cycle analysis showed that BMSCs pulsed with homologous TEX induced a transient cell cycle arrest in the G0/G1 phase of H22 cells, which resulted in the proliferation inhibition of H22 cells.

Differentiation to potent immunocytes might be another important mechanism for MSCs to exhibit antitumor activity. Kang et al. [46] demonstrated that when MSCs were stimulated by IL-2, IL-15 and granulocyte macrophage colony stimulating factor (GM-CSF) alone or in combination, their differentiation to immune effector cells (CD8+, CD161a+) was accompanied by the secretion of the immune reaction-related proteins IL-4 and INF-γ. These results indicate that BMSCs can undergo immunocyte phenotypic and functional changes under appropriate

stimulation. BMSCs might thus differentiate into both antigen presenting cells and immune effectors which sequentially inhibit tumor growth.

Tumor-derived exosomes, enriched in tumor antigens, MHC-I molecules and heat shock protein (HSPs), play a major role in enhancing the antitumor activities of BMSCs. After stimulated by IFN-γ, BMSCs might present antigens/MHC complexes and HSP of TEX to induce antigen-specific immune responses which results in enhanced antitumor activity. However, the phenotypic changes and the complex antitumor mechanism of BMSCs pulsed with TEX need further investigation.

Despite the multiple combined therapies are involved in the treatment of HCC, the prognosis of patients with HCC in late stage is still extremely poor. The 5-year overall survival rate of stage 3 and stage 4 HCC is less than 20 % though chemotherapy and TACE could result in transiently regression of tumor [47]. Based on the limitations of the currently available options to treat advanced HCC, there remains a continuing need for innovative, alternative therapies for HCC. Increasing evidences showed that MSC possessed excellent migratory ability and exerted inhibitory effects on the proliferation of malignant cells in animal models, which implied that MSC might be as a new reagent for cytotherapy [48]. Our study shows that BMSCs pulsed with TEX possessed enhanced migratory capacity and exhibited more effective antitumor activities against hepatocellular carcinoma cells after stimulation by the proper cytokines. It provides the rationale for further evaluation of a novel promising cytotherapy modality against human HCC.

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