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Original Article

Therapeutic effects of bone marrow mesenchymal stem cells expressing interleukin-12 in mice bearing malignant ascites tumor

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Abstract: This study is to investigate the therapeutic effects of bone marrow mesenchymal stem cells (BMSCs) expressing interleukin (IL)-12 on malignant ascites tumor-bearing mice and the related mechanisms. Malignant ascites tumor mouse model was established by the intraperitoneal inoculation with MethA or H_{22} tumor cells. Mouse BMSCs were transfected with lentiviral vector containing IL-12, and then transplanted into these mouse models via intraperitoneal injection. The peritoneal permeability in these mice was evaluated and compared. The contents of INF-γ and VEGF in ascites were determined by ELISA. Mouse models receiving IL-12-expressing BMSCs were rechallenged with tumor cells, and the animal survival was observed and analyzed. In both MethA and H_{22} tumor cell-induced malignant ascites tumor mouse models, there were no significant differences in the peritoneal permeability between the normal saline (NS), BMSC-control, and BMSC-null groups. However, compared with NS control group, the peritoneal permeability was significantly decreased by IL-12-expressing BMSCs. Moreover, ELISA showed that, in both the MethA and H_{22} tumor cell-induced mouse models, compared with the NS control group, the contents of INF-γ in ascites were significantly elevated, while the contents of VEGF in ascites were significantly decreased, in the BMSC-IL-12 groups. In addition, IL-12-expressing BMSCs significantly elongated the survival of mouse models after rechallenging with tumor cells. IL-12-expressing BMSCs exert protective effects against malignant ascites tumor, and the anti-tumor effects might be associated with the enhanced anti-tumor immunity. Our findings might bring new insights into the treatment of tumors with immunotherapy.

Keywords: Bone marrow mesenchymal stem cells (BMSCs), interleukin (IL)-12, malignant ascites tumor, immunotherapy

Introduction

In recent years, biotherapy has gradually become an important modality for the treatment of tumors, in addition to surgery, chemotherapy, and radiotherapy. The biological behavior of tumor cells could be directly regulated by the action of biologically active substance, or by the regulation of autoimmune system. Nowadays, immunotherapy and gene therapy for tumors have been rapidly developing, and the targeting therapy is currently one of the most promising therapeutic strategies [1, 2].

It has been shown that interleukin (IL)-12 not only prevents the tumor occurrence, but also inhibits the tumor growth and metastasis [3]. IL-12 is one of the suitable candidates for genetic immunization, which could exert anti-tumor effects in various animal models. However, to achieve satisfactory therapeutic effects, repeated high-dose injection of IL-12 is needed, which might induce systemic toxicity [4, 5]. Therefore, investigators have been trying to develop a novel treatment modality to confine the effects of IL-12 within the tumor lesions, with enhanced efficacy and reduced toxicity [6].

Recent studies have found that bone marrow mesenchymal stem cells (BMSCs) can specifically migrate to the tumor site [7-11]. Therefore, BMSCs could be used as delivery vehicles for the therapeutic genes to directly inhibit and/or

kill the tumor cells. In this study, mouse BMSCs were transfected with lentiviral vector expressing IL-12, and the therapeutic effects of these modified BMSCs on malignant ascites tumor and the related mechanisms were investigated.

Materials and methods

BMSC isolation and culture

Male BALB/c mice, 6-8 weeks old, were provided by the Experimental Animal Center of Sichuan University. The isolation and culture of BMSCs were performed as our previously published protocols [12]. Briefly, bone marrow was obtained from femurs and tibias taken from these mice, and the tissue was suspended with H-DMEM containing 10% fetal bovine serum (FBS) as well as penicillin and streptomycin. The bone marrow suspension was mixed with the same volume of PBS supplemented with 2% FBS and 0.6% sodium citrate, followed by centrifugation at 900×g for 10 min. The cells were re-suspended with PBS, and spread on the Ficoll-Hypaque solution (1.088 g/mL). After centrifuged at 800×g for 20 min, the cells within the intermediate zone were collected, and cultured with DMEM complete medium in a 37°C, 5% CO_2 incubator. 48 h later, non-adherent cells were removed by washing with serum-free DMEM. The culture medium was changed every 3-4 d. For purification, the primary cells were cultured from 2 w before the first passage.

Lentiviral preparation and BMSC transfection

The lentivirus-expressing pLenti6/V5-mIL-12-Dest plasmid (Invitrogen, Carlsbad, CA, USA) and the ViraPowerTM Packaging Mix (Invitrogen) were co-transfected into 293FT cells, with lipofectamine 2000 (Invitrogen). 72 h later, the cultured supernatant was collected by centrifugation. The lentivirus of Lenti-GFP and Lenti-null were then obtained with the pLenti6/V5-GFP-Dest and pLenti6/V5-null-Dest plasmids, respectively.

For BMSC transfection, BMSCs were incubated with 800 mL DMEM (containing 10% FBS), 200 mL previously described lentivirus, and 20 mL 6 mg/mL polybrene. 24 h later, the culture medium was changed with 2 mL DMEM containing 10% FBS. After another 24 h, these BMSCs were screened with 5 mg/mL blasticidin to obtain Lenti-mIL-12-BMSCs. The expression of IL-12 was confirmed with real-time PCR and ELISA. With the same procedures, Lenti-GFP-BMSCs and Lenti-null-BMSCs were also obtained and used as controls.

Animal modeling and grouping

Mouse model of malignant ascites tumor was established according to a previously published protocol [13]. Male BALB/c mice, 6-8 weeks old, were subjected to intraperitoneal inoculation of 1×10^6 MethA tumor cells [American Type Culture Collection (ATCC; Rockville, MD, USA)]. These mice were then randomly divided into the following groups (n=10 per group): (1) the normal saline (NS) control group, in which the model mice were intraperitoneally injected with 200 mL normal saline at days 2 and 7, respectively, after modeling; (2) the BMSC-control group, in which model mice were inoculated with 200 mL non-transfected BMSC suspension (approximately 2×10^6 cells); (3) the BMSC-null group, in which model mice were inoculated with 200 mL Lenti-null-BMSC suspension (approximately 2×10^6 cells); and (4) the BMSC-IL-12 group, in which the model mice were inoculated with 200 mL Lenti-mIL-12-BMSC suspension (approximately 2×10^6 cells). The BMSCs were transplanted into these mouse models through the intraperitoneal injection. The ascites volume was measured every 2 d after treatment, and the survival of these mouse models was observed over the next 40 d. On the other hand, the procedures for the model establishment with H_{22} tumor cells (ATCC) and the animal grouping were the same as the above described method. All the experimental procedures were approved by the local Animal Care Committee.

Peritoneal permeability measurement

Mice were subjected to the intravenous injection of 0.25 mL Evans blue via the tail vein at 10 d after modeling. After another 2 h, the mice were sacrificed, and the ascites was collected and centrifuged. The concentration of Evans blue was determined with a microplate reader (at the wavelength of 540 nm), and the peritoneal permeability was analyzed accordingly.

Elisa

The ascites was collected at 12 d after modeling, and the ascites volume was recorded. The

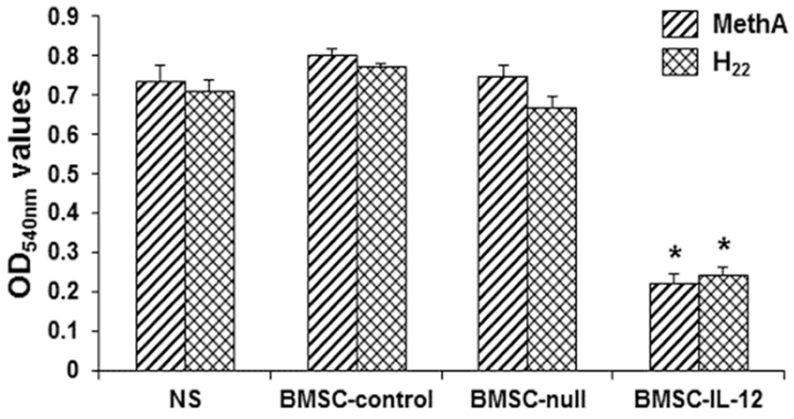

Figure 1. IL-12-expressing BMSCs decreased the peritoneal permeability in mice bearing malignant ascites tumor. The peritoneal permeability was indicated as the concentration of Evans blue (OD_{540nm} values) in ascites, for the normal saline (NS), BMSC-control, BMSC-null, and BMSC-IL-12 groups, from mice inoculated with MethA or H_{22} tumor cells. Compared with the NS control group, $^{*}P < 0.05$.

contents of INF-γ and VEGF in ascites were measured with commercially available ELISA kits (Dakewe, Beijing, China), according to the manufacturer's instructions [14].

Tumor cell rechallenge

After modeling and treatment, the surviving mice in the BMSC-IL-12 group were rechallenged with 5×10^6 MethA or H_{22} tumor cells, and then the survival of these mice was observed. The BALB/c mice without any treatment were rechallenged with the same amount of MethA or H_{22} tumor cells as the control group.

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was performed with the SPSS 19.0 software. Student's *t* test was used for the group comparison, and the Kaplan-Meier survival curve was analyzed with the log-rank test. $P < 0.05$ was considered statistically significant.

Results

IL-12-expressing BMSCs decrease the peritoneal permeability in mice bearing malignant ascites tumor

To assess the effects of BMSCs expressing IL-12 on malignant ascites tumor, the peritoneal permeability was analyzed in these model mice. The peritoneal permeability was indicated as the concentration of Evans blue (OD_{540nm} values) in ascites. Our results showed that, in MethA tumor cell-induced mouse models, there were no significant differences between the normal saline (NS), BMSC-control, and BMSC-null groups ($P > 0.05$) (**Figure 1**). However, compared with NS control group, the peritoneal permeability was significantly decreased by the IL-12-expressing BMSCs ($P < 0.05$) (**Figure 1**). Similar results were observed for the H_{22} tumor cell-induced mouse models bearing malignant ascites tumor (**Figure 1**). These results suggest that IL-12-expressing BMSCs could decrease the peritoneal permeability in mice bearing malignant ascites tumor.

IL-12-expressing BMSCs increase INF-γ and decrease VEGF contents in ascites

To investigate the mechanism through which IL-12-expressing BMSCs protect against malignant ascites tumor, the contents of INF-γ and VEGF in ascites was determined with ELISA. Our results showed that, in both the MethA and H_{22} tumor cell-induced mouse models, no significant differences were observed between the NS, BMSC-control, and BMSC-null groups ($P > 0.05$) (**Figure 2**). On the other hand, the contents of INF-γ in ascites were significantly elevated ($P < 0.05$) (**Figure 2A**), while the contents of VEGF in ascites were significantly decreased ($P < 0.05$) (**Figure 2B**), in the BMSC-IL-12 group, compared with the NS control group. These results suggest that IL-12-expressing BMSCs could significantly increase INF-γ and decrease VEGF contents in ascites, which might contribute to the therapeutic effects of these BMSCs.

IL-12-expressing BMSCs prolong the survival of mouse models after rechallenging with tumor cells

To further investigate the effects of IL-12-expressing BMSCs on the survival of ascites mice, these models were rechallenged with

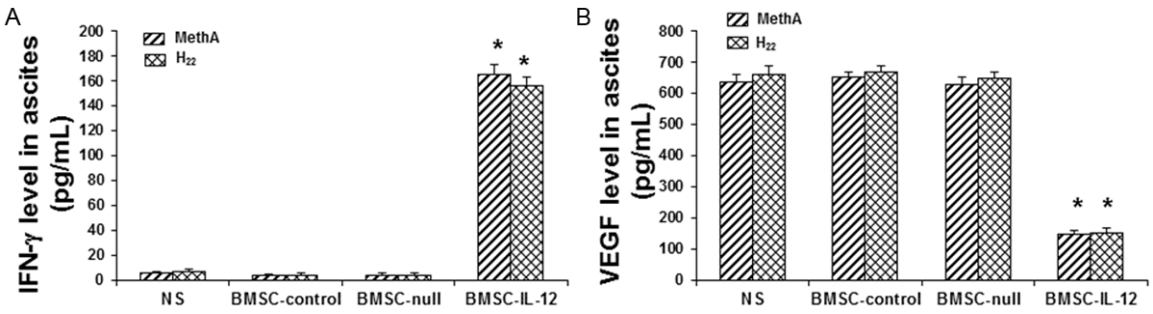

Figure 2. IL-12-expressing BMSCs altered cytokine levels in ascites. The contents of INF-γ (A) and VEGF (B) in ascites from mice inoculated with MethA or H_{22} tumor cells were investigated with ELISA. Compared with the NS control group, $^{*}P < 0.05$.

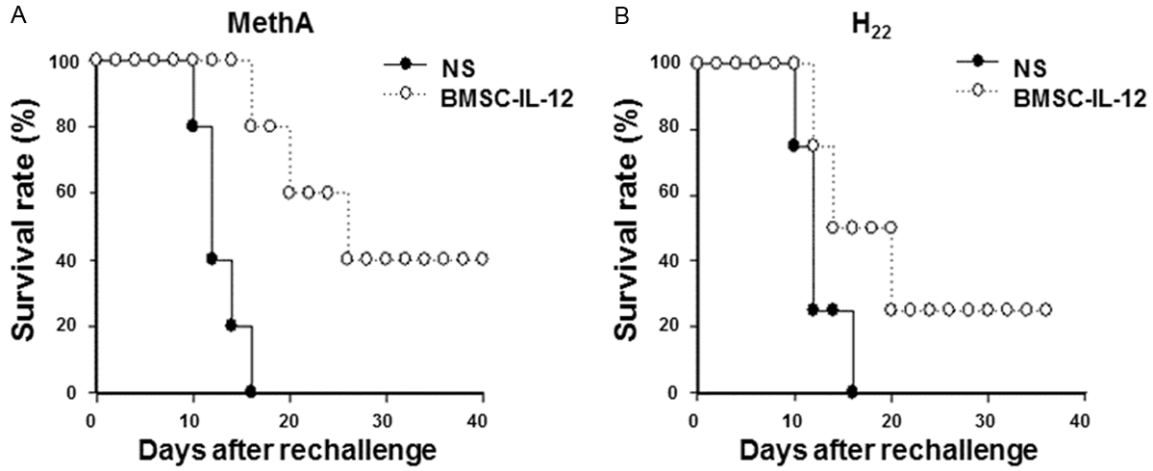

Figure 3. IL-12-expressing BMSCs prolonged the survival of mouse models after rechallenging with tumor cells. The mouse models were rechallenged with MethA or H_{22} tumor cells, and then the survival was recorded and analyzed.

tumor cells, and then the animal survival was recorded and analyzed. Our results showed that, in the MethA tumor cell-induced mouse models, compared with the NS control group, the survival after rechallenging was significantly prolonged in the BMSC-IL-12 group ($P < 0.05$) (**Figure 3**). Similar results were obtained for the H_{22} tumor cell-induced and -rechallenged ascites mouse models (**Figure 3**). These results suggest that IL-12-expressing BMSCs could improve the survival of rechallenged ascites mice, confirming the anti-tumor effects of these BMSCs.

Discussion

Bone marrow mesenchymal stem cells (BMSCs) have been characterized by the extremely low immunogenicity. Bartholomew *et al.* [15, 16] show that, autologous or allogeneic transplantation of BMSCs expressing human EPO would not induce immune rejection in baboon models. Moreover, BMSCs have the multi-directional differentiation potential and the tumor-targeting specificity *in vivo*, making the cells ideal vehicles for gene therapy. Furthermore, BMSCs could modify the pharmacokinetic characteristics of the therapeutic drug molecules, which would achieve the long-term high efficiency with low toxicity and reduced side effects, increasing the treatment safety [17]. It has been shown that BMSCs carrying recombinant adenoviral vectors of IL-12 could exert more significant anti-tumor effects, compared with autologous BMSCs or recombinant plasmids alone [18].

In this study, the lentiviral vector stably expressing IL-12 was constructed and transfected into BMSCs, and then the effects of IL-12-expressing

BMSCs on mice bearing malignant ascites tumor were investigated. Our results showed that, compared with the NS control group, IL-12-expressing BMSCs could significantly decrease the ascites volume and prolong the survival of mouse models. To investigate the anti-tumor immunity in the treatment groups, the mouse models were rechallenged with a large amount of tumor cells, and the long-term survival was recorded. Our results showed that, the survival of mouse models was significantly prolonged by IL-12-expressing BMSCs, suggesting the anti-tumor immunity of these cells. We next investigated the underlying mechanisms for the protective effects of IL-12-expressing BMSCs against malignant ascites tumor. In line with our results, culture supernatant of IL-12-expressing BMSCs has been shown to induce strong chemotaxis and promote maturation of dendritic cells, which would effectively stimulate the anti-tumor immunity [19-21]. Moreover, we found that IL-12-expressing BMSCs could significantly increase INF-γ content, while decrease VEGF content, in ascites. Furthermore, the peritoneal permeability could be significantly decreased by IL-12-expressing BMSCs. Taken together, these results suggest that the protective effects of IL-12-epxressing BMSCs against malignant ascites tumor might be achieved through the enhanced anti-tumor immunity.

In conclusion, our results showed that, IL-12-expressing BMSCs decreased the peritoneal permeability in mice bearing malignant ascites tumor. Moreover, IL-12-expressing BMSCs significantly increased the INF-γ content while decreased the VEGF content in ascites. Furthermore, IL-12-expressing BMSCs could elongate the survival of mouse models after rechallenging with tumor cells. The anti-tumor effects of IL-12-expressing BMSCs might be associated with the enhancement of the anti-tumor immunity. Our findings might bring new insights into the treatment of tumors with immunotherapy.

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Disclosure of conflict of interest

None.

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