Exogenous VEGF-C Augments the Efficacy of Therapeutic Lymphangiogenesis Induced by Allogenic Bone Marrow Stromal Cells in a Rabbit Model of Limb Secondary Lymphedema

Hua Zhou, Mo Wang, Chuanqiang Hou, Xing Jin* and Xuejun Wu

Department of Vascular Surgery, Shandong Provincial Hospital, Shandong University, Jinan, Shandong Province, China

*For reprints and all correspondence: Xing Jin, Department of Vascular Surgery, Shandong Provincial Hospital, Shandong University, 324 Jingwu Road, Jinan 250021, Shandong Province, China. E-mail: drjinxing@sina.com

Received December 2, 2010; accepted March 25, 2011

Objective: To determine the effect of bone marrow stromal cells transplantation and vascular endothelial growth factor C administration as a treatment for secondary lymphedema.

Methods: Bone marrow stromal cells and/or vascular endothelial growth factor C protein were injected into a rabbit model of limb lymphedema. Water displacement volumetry was performed to measure limb volume changes. Immunohistochemistry was performed to detect VEGFR-3 and to count lymph vessel. Western blot analysis was performed to detect vascular endothelial growth factor C.

Results: Before treatment, rabbits had an average volume of edema in the limb of 61.25 ± 5.28, 62.37 ± 4.97, 60.58 ± 7.18 and 61.79 ± 4.33 ml (P = 0.753), respectively, in the BMSC + VEGF-C, bone marrow stromal cell, vascular endothelial growth factor C and control groups. With therapy, this was reduced to an average volume of 7.60 ± 3.02, 12.78 ± 3.41, 31.55 ± 3.51 and 62.33 ± 6.59 ml, respectively, in the four groups 6 months after treatment. Quantitative analysis showed that the vessel numbers were significantly increased in the BMSC + VEGF-C, bone marrow stromal cell and vascular endothelial growth factor C groups compared with the control group at 28 days after the operation (P < 0.05). Western blot analysis demonstrated that expression of vascular endothelial growth factor C was higher in the BMSC + VEGF-C and BMSC groups.

Conclusions: The combined treatment with bone marrow stromal cell transplantation and vascular endothelial growth factor C administration is superior to bone marrow stromal cell transplantation alone in the treatment of limb lymphedema. Bone marrow stromal cell transplantation and vascular endothelial growth factor C administration could enhance the therapeutic effect of each other.

Key words: bone marrow stromal cells – VEGF-C – transplantation – lymphedema

INTRODUCTION

As estimated by the International Institute of Lymphatic, there are ~140 million people among the whole world who suffer from different kinds of lymphedema. Among them, 45 million suffer from limb lymphedema, which still cannot be treated in an effective way. From the perspective of pathophysiology, it should be the best choice to repair and re-establish the lymphatic pipeline. So, how to repair and re-establish the damaged, deficient lymphatic system has become the heated topic in the biomedical field in recent years, and the treatment by transplantation of bone marrow stromal cells (BMSCs) is most striking. Recently, the research on the basis of BMSC and clinical study has been
developed rapidly. Fang (1) has reported that BMSCs induced by vascular endothelial growth factor C (VEGF-C) can form lymphatic vessels in vitro, and then provided experimental basis to rebuild lymphatic vessels in vivo. The results of the research done by Karkkainen et al. (2) indicated that VEGF-C partly ameliorated the lymphedema.

As it is said above, it is theoretically feasible to treat lymphedema by the way of transplantation of BMSCs, and our clinical practice has proved to support this theory (3). This topic is to observe whether combination therapy with BMSC transplantation and VEGF-C infusion could have additional or synergetic effects on in the treatment of lymphedema.

**PATIENTS AND METHODS**

**ISOLATION, CULTIVATION AND IDENTIFICATION OF BMSCS**

Isolation and culture of BMSCs were performed as reported previously (4,5). Two New Zealand white rabbits were randomly selected for bone marrow extraction. Briefly, bone marrow was extracted from the distal femur with an injector of 10 ml heparin saline which has been extracted 1 ml, 25 U/ml concentration, from which BMSCs were isolated by density gradient centrifugation and then incubated in DMEM (Hyclone) supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in a 5% CO₂ atmosphere. The culture medium was exchanged every 3 days to discard floating cells. At 80–90% confluence, the cells were harvested for passage with 0.25 trypsin containing 0.02% EDTA. BMSCs were cultured for additional 14 days with at least two passes at 70–80% confluence. Flow cytometry was performed to identify the BMSCs as described previously (5). Detect the surface antigen expression of cells CD11b, CD29, CD44 and CD45 with flow cytometry. All experiments were performed using cells from the second passage.

**RABBIT HIND LIMB LYMPHEDEMA MODEL**

Rabbit limb lymphedema models were obtained according to the previous reports (6). Briefly, New Zealand white rabbits were selected (provided by Shandong Provincial Academy of Agricultural Sciences), weighted ~2.5 kg each one, female and male allowed. The right hind limb was chosen to have the experiment, whereas the left one was chosen to be the control one. Significant lymphedema was achieved by a combination of circumferential removal of lymph nodes and deep lymphatic from the groin areas followed by removal of skin and subcutaneous tissue of the thigh and post-operative irradiation. Three days after operation, irradiation (56Co γ-ray, 2000 cGry) was given. Despite these measures, it was not possible to produce lymphedema in every case, possibly because of the presence of compensation mechanisms. Eighty percent of the animals develop stable chronic lymphedema of the lower extremity, with swelling that persists for at least 12 months. All the animal experiments were approved by the institutional ethical committee of Shandong University and were performed to minimize the pain of animals.

**BMSCS TRANSPLANTATION AND VEGF-C INFUSION**

About 3 months after the operation, rabbits in the BMSC + VEGF-C group were injected with 2 ml of DMEM loaded with BMSCs (1 × 10⁷ cells) and VEGF-C (Biovision, 150 ng/kg body weight) intramuscularly into irradiation and surgical trauma area (0.1 ml each point) using a 1 ml needle. The BMSCs group was injected with DMEM loaded with only BMSCs. Likewise, the VEGF-C and control groups were injected with DMEM containing VEGF-C (150 ng/kg body weight) or only DMEM, respectively.

**MEASUREMENT OF LIMB VOLUME CHANGES**

Water displacement volumetry was performed to measure limb volume changes every week as described previously (7). Put the limb into a long and thin measuring cylinder containing a certain amount of water to make the standard line below the water. The difference between the current volume and the original one is the volume of the limb.

**IMMUNOHISTOCHEMISTRY AND COUNTING OF LYMPHATIC VESSELS**

On the 28th day after the operation, tissue samples were obtained from the transplantation areas, fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 μm intervals. Immunohistochemistry was performed with a specific primary antibody against VEGFR-3 (Zymed) to stain the lymphatic endothelial cells as described previously (8). Briefly, slides were incubated at 4°C overnight in a humidity tray with primary antibodies and rinsed thrice in 0.1 mmol/l phosphate-buffered saline for 2 min, and incubated for 30 min at room temperature with horseradish peroxidase (Abcom) to identify the target. 3,3'-Diaminobenzidine (DAB) was used for coloration. The positive stains were shown as deep brown-yellow color with DAB. Sections were first examined at low magnification (×100) to identify areas with most intense staining. The single endothelial cell, endothelial cell clusters and lymphatic vessels were counted using ×400 magnification (9).

**WESTERN BLOT ANALYSIS**

Western blot analysis was performed as described previously (10). Briefly, tissue samples were harvested and lysed in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40 and 0.1% SDS). The protein concentration was determined using BCA protein assay kit. Proteins were separated by SDS–PAGE, transferred to a nitrocellulose membrane and immunoblotted with the primary antibody against VEGF-C (Booster). Membranes were visualized by chemiluminescence luminal reagents (Santa Cruz) according to the manufacturer’s instructions. To normalize protein loading, the primary antibody against β-actin was used.
STATISTICAL ANALYSIS
All data are expressed as means ± standard deviation. Comparisons between groups were analyzed by one-way ANOVA followed by the least significant difference test. Statistical significance was determined at a level of P < 0.05.

RESULTS
IDENTIFICATION OF BMSCs
The surface antibody identified by flow cytometry shows results with positive CD44 and CD29 surface antibody. Negative results were obtained by CD11b and CD45 surface antibody analysis (Fig. 1). The results are in coincidence with references and proved that the isolated cells are mesenchymal stem cells (16).

LIMB VOLUME CHANGES
The volume of the affected limb was calculated as described previously (7). Before treatment, rabbits had an average volume of edema in the limb of 61.25 ± 5.28, 62.37 ± 4.97, 60.58 ± 7.18 and 61.79 ± 4.33 ml (P = 0.753), respectively, in four groups. With therapy, this was reduced to an average volume of 24.96 ± 3.65, 34.30 ± 5.44, 38.40 ± 5.70 and 58.44 ± 7.08 ml, respectively, in the four groups 28 days after treatment. Six months after treatment, the average volume of edema in the limb was reduced to 7.60 ± 3.02, 12.78 ± 3.41, 31.55 ± 3.51 and 62.33 ± 6.59 ml, respectively. There was a significant volume decrease in the BMSC + VEGF-C, BMSC and VEGF-C groups compared with the control group, with a more significant reduction in the BMSC + VEGF-C group than in the other groups (P < 0.05; Fig. 2).

IMMUNOHISTOCHEMISTRY AND COUNTING OF LYMPHATIC VESSELS
Based on the method of immunohistochemistry, VEGFR-3 was detected, and the lymphatic was stained. The representative images are shown in Fig. 3a. Quantitative analysis showed that the vessel numbers were significantly increased in the BMSC + VEGF-C, BMSC and VEGF-C groups compared with the control group at 28 days after the operation (P < 0.05). Moreover, the BMSC + VEGF-C group had

Figure 1. Identification of BMSCs. Positive surface antibody detected by flow cytometry (CD44 and CD29). Negative surface antibody detected by flow cytometry (CD11b and CD45).
a more significant increase compared with the other groups ($P < 0.05$; Fig. 3).

**Western Blot Analysis**

The expressions of VEGF-C in the transplantation areas were analyzed by western blot analysis. The representative images are shown in Fig. 4. Quantitative analysis demonstrated that expression of VEGF-C was higher in the BMSC + VEGF-C and BMSC groups ($P < 0.05$; Fig. 4).

**DISCUSSION**

Secondary lymphedema is very common in clinic, which can be caused by surgery, radiation, infection or something else. It was reported by WHO that there are 30 million women who had lymphedema in arms following breast cancer treatment. So far, there is no ideal treatment for such kind of common disorders. Therapy commonly used now is the physical therapy, which, to some extent, can alleviate the edema while both expensive and non-radical to the disease. Therefore, more effective treatments are badly in need of.

As the lymphatic-specific growth factors VEGF-C and -D have been found, a ray of hope to the treatment of chronic lymphedema has emerged. VEGF-C is a member of the VEGF family. The present study finds that VEGF-C may be combined with its receptor VEGFR-3 which can also be called Flt-4, specifically promoting the formation of new lymphatic (11). In 2002, Szuba et al. (12) injected VEGF-C protein into the secondary lymphedema of the rabbit ear model. And the results indicated that the one-dose injection
of VEGF-C protein can improve the function of the lymphatic system and promote the proliferation of lymphatic, and can significantly reverse the abnormal morphology caused by chronic lymphedema. In 2006, Cheung et al. (13) applied exogenous recombinant human VEGF-C protein to treat the lymphedema model of rat tail, and the results indicated that edema disappeared significantly earlier in the treatment group than the control group. In 2008, Jin da et al. (14) use a recombinant VEGF-C protein to treat the lymphedema model of rat tail caused by treatment with surgery, and results turned out to be successful.

The present studies demonstrate that BMSCs had the potency to differentiate into lymphatic endothelial cells. Fang (1) applied VEGF-C156s (VEGF-C with point mutation on 156 site, as the result, its homodimer can only activate and bond with VEGFR-3) to induce the bone marrow mesenchymal stem cells, and successfully established new lymphatic vessels in vitro, which indicated that BMSCs have a capacity of differentiating into lymphatic endothelial cells. Conrad et al. (15) found that BMSCs were capable of expressing a lymphatic phenotype when exposed to lymph-inductive media and purified VEGF-C, and the local injection into the lymphedema model of rat tail significantly reduced the edema volume (20%) and symptoms, which had statistical significance (P < 0.01) compared with the control group. By using a rabbit hind limb lymphedema model, we investigated whether combination therapy with BMSCs transplantation and VEGF-C infusion could have additional or synergetic effects on the induction of lymphangiogenesis in the treatment of secondary lymphedema.

In this study, water displacement volumetry showed that the BMSC + VEGF-C, BMSC and VEGF-C groups developed more volume decrease than the control group, and the combination treatment got the most. Likely, lymphatic count showed that BMSCs transplantation or VEGF-C infusion alone could increase the lymphatic vessel density as well. Nevertheless, the combination of BMSCs transplantation and VEGF-C infusion resulted in further increase in the vessel density, which hinted that BMSCs differentiated into lymphatic endothelial cells and VEGF-C promoted the generation of the latter. Immunohistochemistry examination showed that the expression of VEGFR-3 was more significantly increased in the BMSC + VEGF-C group than that in the other groups, which indicated that VEGF-C may be combined with its receptor VEGFR-3, specifically promoting the formation of new lymphatic. Western blot analysis showed that the expression of VEGF-C was higher in the BMSC + VEGF-C and BMSC groups. It hinted that BMSCs can secrete VEGF-C which positively enhance the differentiation of the former. This study also indicated that the topical application of exogenous VEGF-C protein is effective. However, because it is a one-dose injection, as a result of protein degradation, the long-term effect is inferior to the bone marrow stem cell transplantation. In conclusion, the BMSC and VEGF-C monotherapy could induce lymphangiogenesis and ameliorate the lymphedema; however, the combination of BMSC transplantation and VEGF-C infusion could do an even better job in the treatment of secondary lymphedema.

Funding

This study was supported by Shandong Province Young Scientists’ Research Rewarding Foundation (2008bs03011).

Conflict of interest statement

None declared.

References


