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Neurotrophic Effect of Bone Marrow Stromal Cells on Proliferation and Committed Differentiation of Ventral Mesencephalic Precursors

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OBJECTIVE To explore the potential neurotrophic effect of bone marrow stromal cells (BMSCs) on cell proliferation and committed neuronal differentiation of ventral mesencephalic precursors (VMPs) *in vitro*.

METHODS Ventral mesencephalic precursors from E11 inbred rat embryos and BMSCs from adult rats were cultured both separately and in co-culture. After a 7-day incubation in vitro, three conditioned culture media were obtained, termed VMP or common medium, BMSC medium, and BMSC+VMP medium. Ventral mesencephalic precursors cells were cultured in each of these media and the effects on proliferation and VMP differentiation were assessed. The relative yield of TH+ cells was calculated and compared by immunocytochemical staining.

RESULTS After a 7-day culture and induction of VMPs, the total cell counts were increased by (44.13$\pm$4.75)-fold (common), (60.63$\pm$5.25)-fold (BMSC), and (64.00$\pm$7.63)-fold (BMSC+VMP). The proportions of TH+ cells were (18.76$\pm$5.20)%, (23.49$\pm$4.10)%, and (28.08$\pm$5.42)%, respectively, with statistically significant differences among the treatment groups.

CONCLUSION BMSCs release factors that promote the proliferation of VMPs and facilitate the committed differentiation of VMPs into dopaminergic neurons.

KEY WORDS: mesencephalic precursor, bone marrow stromal cell, proliferation, differentiation.

Introduction

Bone marrow stromal cells (BMSC) are a multipotential stem cell (MPSC) with the ability to differentiate into many functional cell types. Recent studies revealed that BMSC could differentiate into neuron-like cells under appropriated induction conditions in vivo and in vitro, and can secrete several neurotrophic factors (NTFs), including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF)[1,2]. Kwasaki et al.[3,4] found that BMSC could significantly enhance the neuronal differentiation of co-cultured embryonic stem cells (ESCs). In this experiment, we investigated whether BMSC could promote *in vitro* proliferation and committed neuronal differentiation of ventral mesencephalic precursor (VMP) cells.

Materials and Methods

Materials

Experimental animals

Pregnant inbred Wistar rats (F > 36th generation; BW: 300 g) were provided by Shanghai Experimental Animal Center, Chinese Academy of Science.

Reagents

Lymphocyte separating medium was purchased from Shanghai Jinghua BioTech Co. (Shanghai, China). Dulbecco's modified Eagle medium (DMEM), F12, N2, B27, L-glutamine, and Neurobasal culture media were purchased from Invitrogen–Gibco (Grand Island, NY, USA). L-Ascorbic acid-2-phosphate sesqui-magnesium salt (AA-2P), fibroblast growth factor (bFGF), and poly-L-ornithin were from Sigma Co. (USA). The primary antibodies used for immunofluorescent staining were mouse anti-TH (Sigma Co., USA) and rabbit anti- CD44 (Wuhan Boster Bioengineering Co., China). The secondary antibodies were goat anti-rat IgG/Cy3 (Dianova Co.) and goat anti-rabbit IgG/Alexa Fluor 488 (Molecular Probes Co.). Cell nuclei were counterstained with 4′,6-diamidino-2- phenylindole (DAPI, Sigma Co., USA). Fetal bovine serum (FBS) and goat serum were purchase from Hangzhou Ever Green Organism Engineering Materials Co. (China) and PAA Co. (place), respectively.

Instrumentation

The instrumentation used included a stereomicroscope (TaiKe XTL20), CO_2 cell culture incubator (ESPEC BNA-311), inverted phase-contrast microscope (XDS-1B), and laser confocal microscope (Ziess LSM410).

Methods

Sampling and in vitro cell culture

Pregnant rats at E11 were anesthetized with diethyl ether, and the embryos removed from the uterus under aseptic conditions. Under a stereomicroscope, 0.6 mm × 0.8 mm × 0.3 mm tissue blocks were dissected away from the median line of the ventral mesencephalic region of the embryos, and a single cell suspension was prepared by repeated trituration. The suspension was seeded into 24-well plates coated with poly-L-ornithin at 7×10^3 cells/cm^2, and 250 μL of amplified culture medium was added into each well (DMEM/F12, 2:1, 2 g/L $NaHCO_3$, 2% B27, 1% N2, 20 ng/mL bFGF, 1% FBS), and cells cultured in a 5% CO_2 incubator at 37°C.

To obtain BMSCs, inbred Wister rats were sacrificed by cervical dislocation, and their femurs were removed under aseptic conditions. The medullary cavity was rinsed with DMEM and a medullary cell suspension was obtained. Lymphocyte separating medium was used to segregate and imbibe the karyocytes, then the cells were resuspended in DMEM with serum. These cells were seeded into 24-well plates at 5×10^4 cells/cm^2 with 500 μL DMEM containing 20% FBS per well. The cells were cultured in the 5% CO_2 incubator at 37°C. The cells were passaged three times with media changes to purify BMSCs.

Identification of BMSC

After the 4th passage, some BMSC cultures were fixed in 4% paraformaldehyde and immunostained for the BMSC marker CD44. A rabbit anti-CD44 antibody (1:25) and goat anti-rabbit Alexa Fluor 488 secondary antibody (1:150) were used and all cells were counterstained with DAPI. The cells were observed under laser confocal microscopy, and the proportion of CD44 positive cells in the total cell population was determined.

VMP induction before culturing in differentiation medium

After a 7-day in vitro culture, VMPs were harvested by trypsinization, resuspended, and seeded in plates containing fourth generation BMSCs at a 1:1 cell ratio. Ventral mesencephalic precursor differentiation medium was then added (Neurobasal, 2% B27, 1% L-glutamine, 1% FBS and 100 μM AA-2P). The whole media was changed after three days, and replaced with fresh medium. After 24 h, these conditioned media was collected. Centrifugal separation of cellular debris was conducted at 1500 r/min for 10 min, and supernatant (conditioned media) was retained and stored at 4°C. The conditioned medium from the co-cultures was named BMSC+VMP differentiation medium. Similarly, VMP culture medium was added to the 4th generation BMSCs or to VMP monocultures for 24 h to obtain the BMSC medium and the common medium.

VMP differentiation in conditioned media and phenotyping

After routine sampling and a seven day cell proliferation period, VMP cells were randomly divided into two experimental groups and 1 control group. In the experimental groups, the BMSC + VMP or BMSC differentiation medium were added and VMP cells cultured for another 7 days. In the control group, the common (VMP-conditioned) differentiation medium was added for cell culture. After 7 days, the cells were counted. At the same time, cells in 4 wells from each group were fixed in 4% formalin and immunostained with mouse anti-TH (1:10000) and then fluorescence labeled with goat anti-mouse-Cy3 (1:200). Fixed and immunostained cells were counterstained with DAPI (1:5000) to label the nuclei. Fluorescence staining was observed using laser confocal microscopy and cell counting conducted to determine the proportion of the TH-positive (neuronal) cells derived under each culture condition.

Statistical analysis

Statistical analysis was performed using the SPSS10.0 statistical package. The data each treatment group are expressed as mean ± standard deviation, and t-tests were performed for pair-wise mean comparisons. A $P < 0.05$

indicated that the differences among the groups were significant.

Results

Purification and identification of BMSC

Many free-floating blood cells were mingled in with the BMSC after initial cell harvesting from bone. Adherent cells were observed on culture plates with short rod-like projections. With time in culture, the non-adherent blood cells in the culture medium died out slowly, while the adherent cells actively proliferated, forming cell colonies. Most of the (non-adherent) blood cells were removed during the first passage and media change, and adherent cells actively proliferated and presented multiple cell morphologies. After the 2nd, 3rd, and 4th passage, cell morphology gradually became uniform, and most cells were long and spindle-shaped. These cells proliferated at a relatively constant rate. Immunofluorescent staining at the 4th passage revealed CD44-positive cells (green) under the laser confocal microscopy that accounted for $(94.2 \pm 2.78)\%$ of the total cell population (Fig.1).

VMP growth after induction

Compared with the control group, the growth of VMP cells was more vigorous in the BMSC-conditioned medium and the BMSC+VMP-conditioned medium. Cells exhibited a more mature morphology, with larger cell bodies and longer processes. At the end of the in vitro cell culture period (seven days), the average cell count per well in the three treatment groups was $(3.53 \pm 0.38) \times 10^5$ in the common media, $(4.85 \pm 0.42) \times 10^5$ in BMSC-media, and $(5.12 \pm 0.61) \times 10^5$ in BMSC+VMP media. Compared with the values measured at initial plating, cell numbers were (44.13 ± 4.75)-fold, (60.63 ± 5.25)-fold, and (64.00 ± 7.63)-fold higher. There were significant differences in the cell count and cell proliferation rate between the experimental groups and the control group ($P < 0.05$), but there were no statistical differences between the two experimental groups.

Comparison of VMP differentiation

Immunofluorescent staining was performed to assess cell differentiation in the presence of the three conditioned media. After formalin fixation, the percentage of TH-positive cells was as follows: $(18.76 \pm 5.20)\%$ in the common media control group, $(23.49 \pm 4.10)\%$ in BMSC-conditioned media group, and $(28.08 \pm 5.42)\%$ in the BMSC+VMP conditioned media group. The fraction of TH-positive cells was significantly higher in the BMSC and in the BMSC+VMP culture groups than that in the control group ($P < 0.05$), and the differences in the fraction of cells expressing TH was also significantly different between the two experimental groups ($P < 0.05$) (Fig.2).

Discussion

Transplantation of dopaminergic neurons (DN) for the treatment of Parkinson's disease (PD) requires a supply of TH-positive neurons, as can be obtained by the in vitro culture of VMP cells. In the past, many studies simulated the microenvironment needed for VMP neuronal differentiation by adding various growth factors and nutrients. Carvey et al.[5] used IL-1, IL-11, LIF, and GDNF in combination to induce VMP differentiation, and obtained 20% to 25% TH-positive neurons that expressed Nurrl. Furthermore, there was a satisfactory therapeutic effect on the PD-rats following DN transplantation. However, the induction of mature functional neurons is a complex multistage process requiring precisely time dynamic changes in the microenvironment and the interaction of extrinsic with intrinsic factors. In the case of neurons, environmental signals can be affected by associated support cells through both proximal secretion and distal paracrine action. Many studies have utilized normal supporting cells, such as astrocytes, olfactory

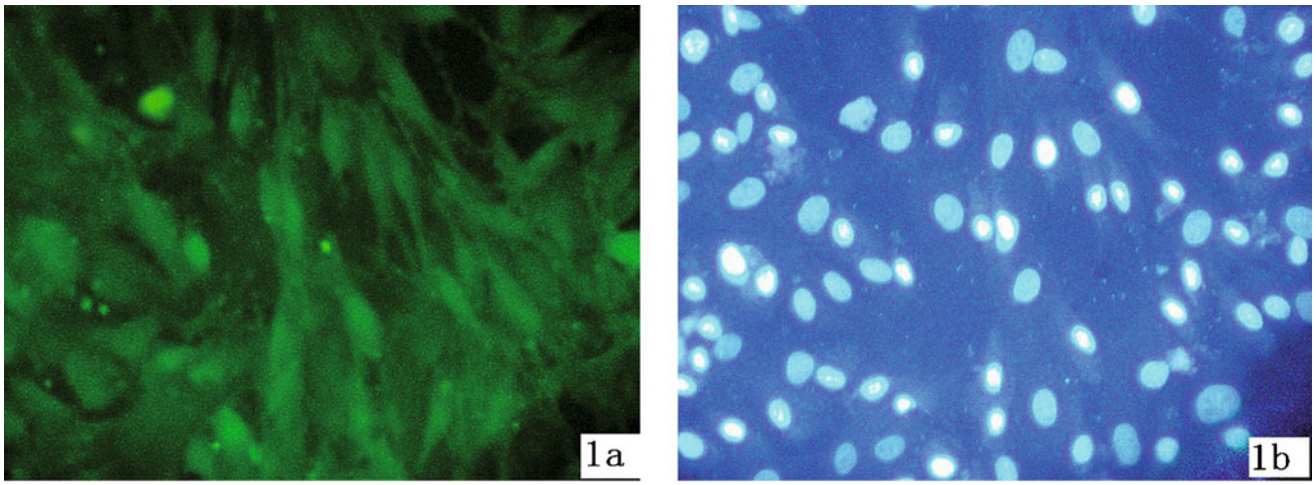

Fig.1. Immunocytochemical staining of bone marrow stromal cells; 1a: CD44 positive cells; 1b: DAPI-labled cell nucleus, ×400

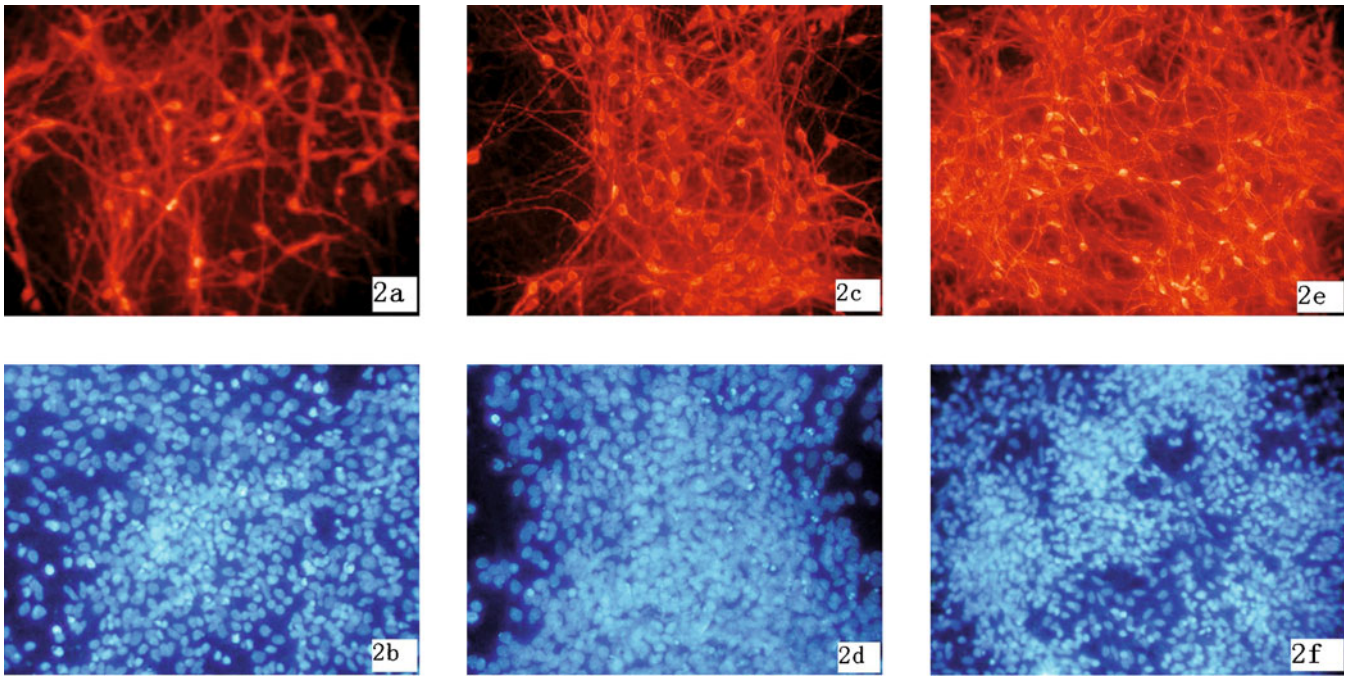

Fig.2. Immunocytochemical staining of ventral mesencephalic precursors ×200; 2a and 2b were the control group; 2c and 2d were BMSC group; 2e and 2f were BMSC+VMP coculture group.

oligodendrocytes, or Schwann cells in co-culture with the target cells to create a physical and chemical microenvironment close to that in vivo. These support cells provide nutrients and trophic factors to facilitate the growth and differentiation of the target cells. Bone marrow-derived stromal cells are multifunctional cells that can also provide these nutrients and neurotrophins, and so are of major interest to the field of neuroscience.

Over the past few years, BMSCs have been used for the treatment of PD, cerebrospinal injury, or cerbral infarction in animal models, and these treatments have demonstrated reasonable therapeutic effects[6]. It is still unclear how intracephalic transplantation of BMSCs ameliorates these different central nervous system pathologies. It is unlikely that transplanted cells simply replace necrotic neurons. Rather, transplanted BMSC can migrate and grow in the brain. These surviving BMSCs may secrete NTFs that activate endogenous repair mechanisms, including angiogenesis, synaptogenesis, and neural reorganization, at the site of brain damage thus facilitating functional recovery[7]. Zhong[8] et al. co-cultured BMSC and hippocampal tissue from neonatal rats and exposed the cultures to in vitro ischemia. They found that BMSCs reduced cell death and promoted the growth of post-ischemic neurons, suggesting that BMSC may exert neuroprotective effects by secreting NTF. Chen et al.[9] demonstrated that BMSC could significantly enhance the survival of injured cholinergic neurons in rats.

In our experiments, we found that VMP cell growth was vigorous in both BMSC- and BMSC+VMP-conditioned media, and that the number of dead cells decreased under both conditions. It is clear that BMSC promoted the growth and proliferation of VMP cells, and also had a definite cytoprotective action; indeed, the number of VPMs and TH-positive cells was much higher after culture in the BMSC-conditioned media compared to the common media.

We have provided evidence that BMSC can facilitate the survival and proliferation of VMP cells, and the differentiation of VMP towards a mature DN phenotype. We suggest these effects are due to the secretion of nutritive or trophic factors from BMSCs into the conditioned media. The VMP cells were taken from the ventral mesencephalic tissue of rat embryos since this region has the greatest number of dopaminergic precursors. During in vitro culture, these cells were easier to injure and kill than the glial cells, but the neuroprotective effect of BMSC may have reduced the death rate of dopaminergic precursors, thus increasing the proportion of DNs compared to that in the control group. After the in vitro culture and 7-day induction, there were still many more precursor cells than in the control cultures. Thus, factors released from BMSCs promoted proliferation in addition to differentiation. The total cell count in the BMSC+VMP group was similar to that in the BMSC group, while the proportion of DNs was higher in BMSC+VPM-conditioned media, demonstrating that the co-culture of VMP and BMSC can further promote BMSCs to produce factors that facilitate DN differentiation. Whether BMSC-VPM interactions are mediated through chemical signaling or other unknown mechanisms requires further investigation.

Our experiment demonstrated that BMSCs are favorable supporting cells that can increase the yield of DNs from VMP precursors. Co-transplantation of VMPs together with BMSCs (if possible) might further enhance the therapeutic effects of VMP transplantation in PD patients. Alternatively, BMSC could be transplanted into the brain of PD patients to improve the local

neurotrophic microenvironment to enhance the survival of transplanted DNs. In addition, a relatively healthy intracephalic microenvironment would promote the long-term survival of the transplanted DN and allow fully functional expression.

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Conflict of interest statement

No potential conflicts of interest were disclosed.

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