Secretion of nerve growth factor, brain-derived neurotrophic factor, and glial cell-line derived neurotrophic factor in co-culture of four cell types in cerebrospinal fluid-containing medium★

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Abstract

The present study co-cultured human embryonic olfactory ensheathing cells, human Schwann cells, human amniotic epithelial cells and human vascular endothelial cells in complete culture medium-containing cerebrospinal fluid. Enzyme linked immunosorbent assay was used to detect nerve growth factor, brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor secretion in the supernatant of co-cultured cells. Results showed that the number of all cell types reached a peak at 7–10 days, and the expression of nerve growth factor, brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor peaked at 9 days. Levels of secreted nerve growth factor were four-fold higher than brain-derived neurotrophic factor, which was three-fold higher than glial cell line-derived neurotrophic factor. Increasing concentrations of cerebrospinal fluid (10%, 20% and 30%) in the growth medium caused a decrease of neurotrophic factor secretion. Results indicated co-culture of human embryonic olfactory ensheathing cells, human Schwann cells, human amniotic epithelial cells and human vascular endothelial cells improved the expression of nerve growth factor, brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor. The reduction of cerebrospinal fluid extravasation at the transplant site after spinal cord injury is beneficial for the survival and secretion of neurotrophic factors from transplanted cells.

Key Words

olfactory ensheathing cells; Schwann cells; amniotic epithelial cells; vascular endothelial cells; nerve growth factor; brain-derived neurotrophic factor; glial cell line-derived neurotrophic factor; cerebrospinal fluid; regeneration; neural regeneration

Research Highlights

(1) During spinal cord injury, reduced extravasation of cerebrospinal fluid at the site of cell transplantation after spinal cord injury is beneficial for the survival and secretion of neurotrophic factors from transplanted cells.
(2) Co-culture of human embryonic olfactory ensheathing cells, human Schwann cells, human amniotic epithelial cells and human vascular endothelial cells in medium containing the cerebrospinal fluid improved the expression of nerve growth factor, brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor.

Abbreviations

OECs, olfactory ensheathing cells; SCs, Schwann cells; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor

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INTRODUCTION

Transplantation of cells such as olfactory ensheathing cells (OECs) and Schwann cells (SCs) has emerged as a promising therapy for spinal cord repair. Injured neurons and damaged axons can survive and regenerate to a certain extent, with some axons even extending their severed processes to their original neuronal targets. The transplantation of myelin-forming cells, such as SCs or OECs, is capable of bridging the repair site by establishing an environment permissive for axonal regeneration[1], but which requires the support of neurotrophic factors[2]. As a unique type of glial cell, OECs not only support the continual regeneration of olfactory sensory neurons throughout life in adult mammals, but also promote neuronal survival and neurite outgrowth in the central nervous system[3-7]. Recent studies suggest that the mechanism of this reparative effect involves a variety of growth factors and cytokines, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF)[8-13]. SCs are glial cells that exist in the peripheral nervous system, which secrete NGF, BDNF, ciliary neurotrophic factor, basic fibroblast growth factor, and other neurotrophic factors[14]. They promote axonal sprouting and regrowth of axons to their original destinations, after axotomy[15]. After transplantation into an injured spinal cord, SCs can enhance neural induction and remyelination to improve functional recovery[16]. In addition, the transplanted SCs can provide a growth-supportive substrate for axons in the central nervous system to regenerate and mature[17]. Amniotic epithelial cells (AECs) are the major cellular components of the amnion, which is directly associated with the neural epithelium in the early stage of neurogenesis. Thus, AECs play an important role in the process of neural development and by releasing neurotransmitter and neurotrophic factors to the amniotic fluid. AECs can synthesize and secrete NGF, BDNF, ciliary neurotrophic factor, neurotrophin 3 and cytokines[18], and can also promote neuronal survival and axonal regeneration[19]. Vascular endothelial growth factor and basic fibroblast growth factor, secreted by vascular endothelial cells (VECs), are important nutritional factors in neuroprotection and angiogenesis[20]. They stimulate angiogenesis and promote proliferation, migration, and differentiation of neural stem cells. These cells secrete different types of neurotrophic factors, which promote different aspects of neural regeneration. Thus, transplantation of co-cultured mammalian cells could improve the disproportion of neurotrophic factors secreted by a single cell. This may also allow for synergistic effects

following the transplantation of cocultured cells[21]. However, a synergistic effect in human cells has not been verified. The quantity of neurotrophic factors secreted by cocultured human cells remains unknown. In the present study, a "niche" was designed with the purpose of developing a model of co-cultured cells and validating their functions. The "niche" was defined as the cell co-culture environment (containing OECs, SCs, AECs and VECs). The present study simulated the spinal cord environment in the "niche" by co-culturing cells in medium containing cerebrospinal fluid, then collected culture supernatant at different time points and quantified the neurotrophic factors secreted into the surrounding environment. Moreover, the conditions of co-culture and the secretory actions were analyzed in an attempt to prove whether the transplantation of co-cultured cells (niche) could support proportionate secretion of neurotrophic factors in the repair of spinal cord injury.

RESULTS

Cell morphology after co-culture in medium containing cerebrospinal fluid

Cell morphology of the whole culture was observed by an inverted microscope (all cells were cultured in 6-cm dishes at 5×10^4 cells/mL with Dulbecco's modified Eagle's medium/F12). OECs in the healthy culture displayed oval or spindle-shaped cell morphology. The cells began to adhere to the bottom of the dish at 6 hours after inoculation. Most were adherent by 16 hours. When cultured for 6 days, bipolar or tripolar cells began to emerge as well as cells displaying a "fried egg"-shaped cell body (Figure 1A). Cell processes became longer and thinner, and were intertwined into a grid. The cells were highly refractive and were 95% confluent after 6–8 days of culture. Most SCs were adherent 6 hours after inoculation. They were spindle- or oval-shaped, with intertwined cell processes (Figure 1B). SCs were stacked at the bottom of the dish and were 95% confluent after 4–6 days, at which point cell aggregates emerged, adhering to cells that had already attached to the bottom of the plate. The cell aggregates were loosely adherent. AECs took approximately 16–24 hours to attach the dish after inoculation. AEC cell bodies were flat and displayed irregular polygonal morphology. AECs had a clear outline and were tightly arranged (Figure 1C). Some particulates emerged between the cells during culture. AECs grew into agglomerate and had a low growth rate. They entered a period of rapid proliferation at 3–4 days, and covered the bottom of the dish by 8–10 days of culture. VECs showed typical cobblestone

morphology. They were tightly adherent displaying little cytoplasm. Multilayer cells could stack and grow together (Figure 1D). VECs completely attached to the dish after 16–18 hours of culture and extended pseudopodia. The cells were completely confluent after 6 days of culture and the intercellular gaps were difficult to distinguish.
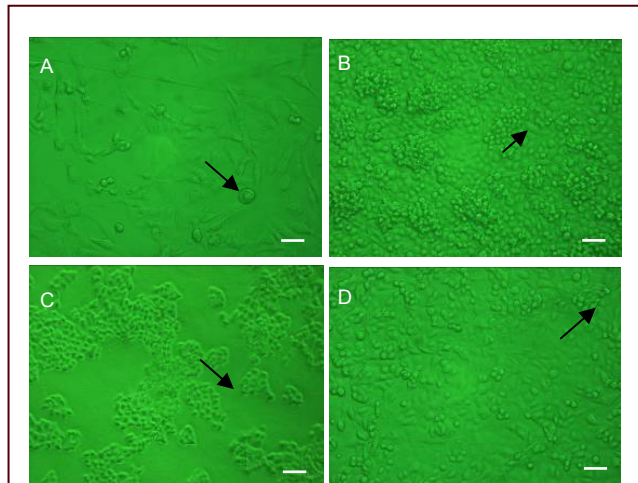

Figure 1   Morphology of cells cultured for 6 days with Dulbecco's modified eagle's medium/F12 containing cerebrospinal fluid (invert microscope). Scale bars: 50 μm.

Arrows represent newly generated cells.

(A) Olfactory ensheathing cells were spindle- or oval-shaped. Bipolar or tripolar cells began to emerge and cell processes became intertwined. There were also clustered "fried egg"-shaped cells.

(B) Schwann cells displayed a typical bipolar spindle, cell processes were intertwined, and cells became stacked upon overgrowth.

(C) Amniotic epithelial cells were flat with an irregular polygonal shape. Cells possessed a clear outline and were tightly arranged. Some particulates were distributed between cells.

(D) Vascular endothelial cells formed a characteristic cobblestone shape. Cells were adherent to the dish and multilayer cells stacked and grew.

Statistically significant differences in cell numbers were observed when they were grown in different concentrations of cerebrospinal fluid added to fresh complete Dulbecco's modified eagle's medium/F12. There was no difference in cell number when the concentration of cerebrospinal fluid was 30% or less ($P > 0.05$). However, cell growth arrest occurred when the concentration was greater than 30% ($P < 0.05$). The cells underwent apoptosis when the concentration was greater than 60% ($P < 0.01$).

Cell growth after cultured for 10 days
All cells were cultured with fresh complete Dulbecco's modified Eagle's medium/F12 without cerebrospinal fluid.

Results showed that the number of SCs peaked at 7–8 days at a density of 1.27×10^6 cells/mL. Similarly, ECs and OECs peaked at a density of 6.1×10^5 cells/mL and 4.72×10^5 cells/mL, respectively. The number of AECs reached a peak at 9–10 days at a density of 7.35×10^5 cells/mL (Figure 2). Both SCs and ECs entered a logarithmic growth phase after being cultured for 3–4 days, while OECs and AECs had lower proliferation rates, without displaying a significant logarithmic growth phase (Figure 2).
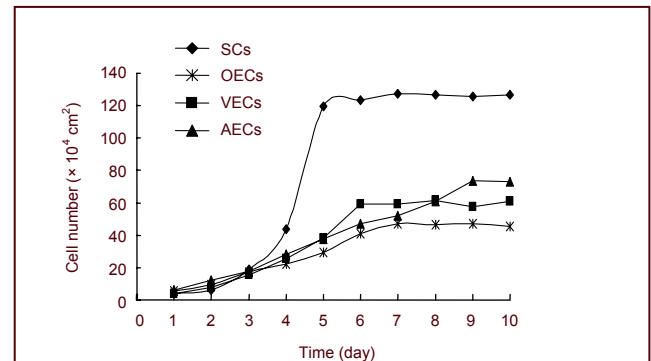

Figure 2   Cell growth curves of four cells after co-cultured for 10 days.

Results showed that SCs, ECs, and OECs reached maximum growth at 7–8 days. AECs reached a maximum at 9–10 days.

SCs: Human Schwann cells; VECs: human vascular endothelial cells; OECs: human embryonic olfactory ensheathing cells; AECs: human amniotic epithelial cells.

NGF, BDNF and GDNF expression in cell supernatants
OECs, SCs, AECs, and ECs were co-cultured at a constant proportion of 10:10:1:1. The NGF, BDNF and GDNF in the culture supernatant gradually increased until the day 9 of culture (Table 1). The levels of NGF ranged from 1 208–1 388 pg/mL, while BDNF ranged from 326–384 pg/mL and GDNF ranged from 115–138 pg/mL (Table 1). Differences in expression of BDNF at all time points were statistically significant ($P < 0.01$; Table 1). The Student-Newman-Keuls test demonstrated a significant difference ($P < 0.05$; Table 1) between day 3 and day 6, day 3 and day 9, day 6 and day 15, day 9 and day 12, and day 9 and day 15. There were no significant differences at the other time points ($P > 0.05$; Table 1). The differences in expression of GDNF at all time points were statistically significant ($P < 0.05$; Table 1). The Student-Newman-Keuls test showed there were significant differences ($P < 0.05$; Table 1) between the day 3 and day 9, day 9 and day 12, and day 9 and day 15. There were no significant differences at the other time points ($P > 0.05$; Table 1). The differences in NGF expression at all time points were not statistically

significant ($P > 0.05$; Table 1). When the cells were co-cultured at a constant proportion for 9 days, a constant ratio of NGF, BDNF, and GDNF expression levels was measured in the culture supernatant. NGF levels were four-fold higher than BDNF, which was three-fold higher than GDNF (Figure 3). The differences in expression of BDNF and NGF between the 20% or 30% cerebrospinal fluid group and the 0% cerebrospinal fluid group were statistically significant ($P < 0.05$; Figure 3). The differences in expression of BDNF and NGF between the 30% and the 20% cerebrospinal fluid groups were not statistically significant ($P > 0.05$; Figure 3). The differences in GDNF expression among the three groups were not statistically significant ($P > 0.05$; Figure 3).

Table 1 Comparison of GDNF, BDNF and NGF expression levels (pg/mL) in cell supernatant at various time points

Group	GDNF	BDNF	NGF
3 days	112.2±8.2[b]	305.1±25.8[ab]	1 162.7±85.6
6 days	119.8±10.1	353.8±27.8[d]	1 228.5±103.7
9 days	126.9±11.5[cd]	354.9±28.8[cd]	1 298.0±89.6
12 days	117.3±9.6	328.2±27.8	1 269.5±118.0
15 days	113.8±5.4	325.5±17.7	1 257.3±115.8
Mean value	118.0±10.2	333.5±31.1	1 243.2±108.5
P	0.025	0.002	0.119

[a]$P < 0.05$, *vs.* day 6 group; [b]$P < 0.05$, *vs.* day 9 group; [c]$P < 0.05$, *vs.* day 12 group; [d]$P < 0.05$, *vs.* day 15 group. Results were expressed as mean ± SD, *n* = 8 (one-way analysis of variance, Student-Newman-Keuls test).

NGF: Nerve growth factor; BDNF: brain-derived neurotrophic factor; GDNF: glial cell line-derived neurotrophic factor.
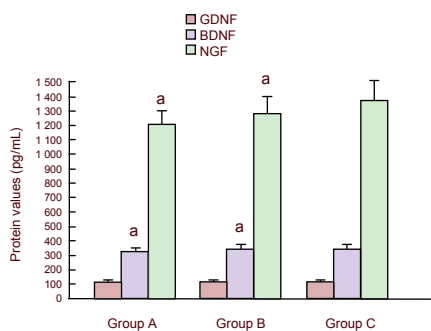

Figure 3 Mean concentrations of NGF, BDNF and GDNF secreted in the culture supernatant of different medium groups on day 9 by enzyme-linked immunosorbent assay.

Groups A, B and C represent 30%, 20%, 0% CSF groups, respectively. [a]$P < 0.05$ *vs.* group C. Results were expressed as mean ± SD, *n* = 8 (independent-samples *t*-test).

NGF: Nerve growth factor; BDNF: brain-derived neurotrophic factor; GDNF: glial cell line-derived neurotrophic factor; CSF: cerebrospinal fluid.

DISCUSSION

Growth factors are necessary for the development and maintenance of the peripheral and central nervous systems. In addition, they prevent neuronal death, and promote neuronal differentiation and survival and neurite outgrowth in the central nervous system. However, because of the presence of neuronal growth inhibitory factors and the lack of neurotrophic factors after spinal cord injury, the reparative capacity of transplanted cells is greatly limited. Studies have shown that peripheral target tissues and damaged neurons can produce limited or trace amounts of neurotrophic factors after spinal cord injury[22]. Surviving neurons compete for neurotrophic factors that lead to some neurons undergoing apoptosis, and the inhibition of axonal regeneration. In addition, due to the presence of inhibitory molecules and a shortage of neurotrophic factors, transplanted cells differentiate into astrocytes rather than neurons in the damaged environment. The effects of exogenous neurotrophic factor treatment are difficult to sustain. A single type of cell transplanted into an injured spinal cord cannot completely improve this inhibitive environment. Multicellular transplantation provides a better method to improve this inhibitive environment[21]. Li *et al*[23] first used OEC transplantation in the treatment of spinal cord injury. The upper cervical corticospinal tract was transected on one side in adult rats. In this acute spinal cord injury model, a suspension of OECs, cultured from the adult rat olfactory bulb, was injected into the lesion site. Results showed that axons in the transected corticospinal tract grew through the transplant, continued to regenerate into the denervated caudal host tract after 3–10 weeks and formed functional synapses. Ruitenberg *et al*[24] confirmed that OECs transduction by *ex vivo* adenoviral vector-based gene therapy producing neurotrophins (such as BDNF) was better at promoting rubrospinal tract axonal regeneration and functional recovery. Ma *et al*[25] implanted engineered NT-3-OECs into a contused T_9 spinal cord of adult rats. Results showed that genetically modified OECs significantly improved recovery after spinal cord injury. Other studies showed that OECs enhanced the repair of peripheral nerve fibers by direct contact and secretion of soluble molecules[26], for example promoting the recovery of vibrissae motor performance when transplanted into a transected facial nerve[27].

Fouad *et al*[28] transplanted a combination of SCs, OECs, and chondroitinase ABC into rats with spinal cord injury. Results showed that function had been markedly restored and the effects of axon myelination were significant. It was concluded that the results might be due to chondroitinase

ABC reducing inhibitory cues in the glial scar, SCs providing a growth-supportive substrate for axonal regeneration in the SCs bridge and OECs enabling regenerated axons to exit the bridge to re-enter the spinal cord to achieve functional complementation. Recent studies demonstrated that NGF was critical for the survival, differentiation, and maintenance of peripheral sympathetic and sensory neurons and basal forebrain cholinergic neurons, and could promote neurite growth in cultured dorsal root ganglia[29]. Studies involving a variety of rodent models have also confirmed that BDNF is an important neuroprotective factor, which can promote the regeneration of neurons and axons and support the survival of sensory neurons and motor, serine, GABAergic and cortical spinal neurons in several parts of the brain[30-31].

Human-derived cells were used in the present study. Co-culture of four types of cells replaced a single cell type that was cultured and purified in previous studies. Cerebrospinal fluid is present in the central nervous system environment after spinal cord injury. Cerebrospinal fluid-containing medium in the present study replaced pure complete medium used in previous experiments, and was closer to the growth environment corresponding to cell transplantation *in vivo*. Moreover, we detected three secreted proteins, including NGF, BDNF, and GDNF, compared to a single factor in previous studies. The standard growth curves showed that the four cell types cultured individually did not reach a growth peak until days 7–10 of culture, while the co-cultured cells became confluent in 3 days. The fastest growing SCs required 6–9 days of growth before reaching their growth peak. The growth rate of the co-cultured cells was faster than cells cultured individually, indicating there may be synergy between cells to promote growth. This may be related to the neurotrophic factors and proteins that were secreted in the co-cultured cells.

In the present study, the expression of NGF, BDNF and GDNF all reached peak levels following 9 days of co-culture. The differences in expression of both BDNF and GDNF at all time points were statistically significant. Results of standard curves showed that cells from the coculture all entered the plateau period between 7–10 days when the number of cells reached a steady state, consistent with the results of the enzyme-linked immunosorbent assay (ELISA). The proteins in the culture supernatant increased parallel to total amount of the cells co-cultured, and reached peak expression concomitant with cell numbers, and then decreased. OECs secrete NGF, BDNF and GDNF[8-13], while SCs

and AECs secrete only NGF and BDNF[14, 18]. VECs secrete none of these three factors but secrete vascular endothelial growth factor and basic fibroblast growth factor[20], which could promote cellular proliferation. The differences in expression of NGF at all time points were not statistically significant in the present study, but expression levels had reached 1 162.7 ± 85.6 pg/mL by 3 days culture, similar to the mean value. The expression levels after 3 days of culture did not decrease below this value. In addition, the expression of NGF was about four times greater than BDNF, which was about three times greater than GDNF, in contrast to the study by Woodhall *et al* [10]: NGF and BDNF were secreted into OECs-conditioned medium, in which NGF levels were seven times higher than BDNF levels. This may be due to a number of reasons as follows: the additive effects of secretion from a variety of cells; species differences of cells (human-derived cells used in the present study and rat cells used previously); and differences in culture conditions, as cerebrospinal fluid-containing medium was used here and complete medium was used previously. As demonstrated by cell morphology, there was significant growth retardation when the ratio of cerebrospinal fluid was greater than 30%. The cells underwent apoptosis when the cerebrospinal fluid levels were greater than 60%. None of the cells survived in 100% cerebrospinal fluid. The reduced secretion of neurotrophic factors correlated with the increase of cerebrospinal fluid proportions in the growth medium in the present study. As cerebrospinal fluid surrounds transplanted cells after spinal cord injury, we believe neuronal growth inhibitory factors present in the cerebrospinal fluid inhibit neuronal differentiation and survival but also influence the secretion of neurotrophic factors. Therefore, reducing the extravasation of cerebrospinal fluid at the transplant site in the "niche" would be beneficial for the survival and neurotrophic factor secretion of the transplanted cells.

In conclusion, the coculture of various cell types could provide stable NGF, BDNF, and GDNF production, while the existence of multiple neurotrophic factors could establish an environment permissive for the differentiation and survival of neuronal injury. These findings provide a new development for the treatment of spinal cord injury by "niche" transplantation.

MATERIALS AND METHODS

Design
A randomized, comparison study *in vitro*.

Time and setting

The experiments were performed at the Open Laboratory for Tumor Molecular Biology, Shantou University Medical College, Shantou, China from March to November 2010.

Materials

Cells

Human SCs (HSCs-H1700), human AECs (HAECs-7100), human VECs (ECs-CRL1730) and human OECs were all purchased from the ATCC (Manassas, VA, USA).

Human cerebrospinal fluid

Cerebrospinal fluid was provided by the Department of Neurosurgery, First Affiliated Hospital of Shantou University Medical College. Cerebrospinal fluid was harvested through the external ventricular drainage from patients (20–50 years old) with cerebral trauma or cerebral hemorrhage. Viral, purulent, and tuberculous cerebrospinal fluid was excluded. Cerebrospinal fluid was negative for HBV, HCV, HIV, and syphilis. The patients were informed of the risk and process of the experiment. Informed consent was signed before the experiment[32].

Methods

Preparation of cerebrospinal fluid

The cerebrospinal fluid was collected through aseptic operation after surgery and centrifuged at 3 000 × *g* for 15 minutes. The cell supernatant was collected and the tissue sediment was removed. pH was adjusted to 7.2–7.4, and the cerebrospinal fluid was filtrated through a nitrocellulose membrane (0.22 μm pore diameter), followed by heat-inactivation at 56°C for 30 minutes. The cerebrospinal fluid was added to penicillin streptomycin (100 U/mL), followed by aliquoting and storage at 4°C until use.

Cell culture

OECs, SCs, AECs, and VECs were conventionally cultured in Dulbecco's modified Eagle's medium/F12 medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Amresco, Solon, OH, USA) at 37°C in a 5% CO_2 atmosphere, with saturated humidity. The four cell lines were all adherent cells, and were cultured in 6-cm plastic culture dishes with 3 mL complete medium, which was renewed every 2–3 days. The dishes were pretreated with polylysine for adherent cells prior to use. Cells were subcultured when they were 90% confluent. The culture supernatant was discarded, and cells were rinsed with 3 mL phosphate buffered solution (pH 7.2–7.4), then incubated with 1 mL trypsin/ ethylenediamine tetraacetic acid (0.25%; Amresco, Solon, OH, USA) until 80% of cells

had a round morphology (monitored under a microscope, about 1–2 minutes). Trypsin/ethylenediamine tetraacetic acid was discarded and cells were resuspended in 3 mL complete medium with 10% fetal bovine serum. Cells were inoculated into several new dishes according to the density of cells ($5 × 10^4$ cells/mL), and medium was added to a total volume of 3 mL in each dish. The changes in cell morphology and number were observed and recorded daily under the inverted microscope. Cells were cultured in cerebrospinal fluid-containing medium, and a certain proportion of cerebrospinal fluid was added to the complete medium when the cell growth was stable. Cells were respectively cultured in medium with 0%, 20%, 30%, 60% and 100% cerebrospinal fluid. The number and appearance of the four cell types were observed under an inverted microscope. A suitable density of cerebrospinal fluid was selected in the present study.

Determination of cell growth curve

Trypsinized cells were suspended in complete medium containing fetal bovine serum and seeded into two 24-well plates with 1 mL complete medium per well, at a density of $5 × 10^4$ cells/mL. Four wells were selected randomly every 24 hours, and three in four were trypsinized. The cell number of every field of view in the microscope slide was quantified under an inverted microscope (Olympus, Tokyo, Japan). The data were recorded and calculated every day. Medium was renewed every 3 days. Cell growth curves were drawn according to the results.

Co-culture of cells and preparation of culture supernatant

OECs were co-cultured with SCs, AECs, and VECs in 10-cm plastic culture dishes with 12 mL cerebrospinal fluid-containing medium. Each culture dish represented one sample. OECs and SCs were plated at a density of $1 × 10^5$ cells/mL, while AECs and VECs were plated at a density of $1 × 10^4$ cells/mL. Culture dishes were divided into three groups. Groups A, B and C represented 30%, 20%, and 0% cerebrospinal fluid in medium, with each group of eight samples. The supernatant was collected every 3 days followed by the addition of an equal amount of new medium. Supernatant was concentrated using a Millipore Ultrafree-15 centrifugal filter (Millipore, Billerica, MA, USA) with a nominal molecular weight limit of 10 kDa at 3 000 × *g* for 15 minutes at 4°C, and stored in an Eppendorf tube at –80°C for later use.

Detection of NGF, BDNF, and GDNF protein concentration by ELISA

ELISA was performed to detect NGF, BDNF, and GDNF

secreted in the culture supernatant at different days and in different groups. For NGF, a total of 2 000, 1 000, 500, 250, 125, 62.5, 31.3, and 0 pg/mL (an equal volume of sample dilution buffer served as a zero standard) standard preparations (each of 50 μL volume) were added sequentially to the antibody pre-coated wells (anti-NGF-beta, BG, Shanghai, China). Subsequently, 50 μL cell supernatant was added. Samples were thoroughly incubated at 37°C for 120 minutes. Wells were rinsed four times with wash buffer, and allowed to soak for 1 minute each time. The reaction plate was dried with filter paper. The biotinylated antibody working solution (50 μL/well) was added and plates were incubated for 60 minutes at 37°C. The wells were then washed four times with wash buffer. Enzyme-labeled antibody (rabbit anti-human NGF polyclonal antibody; BG, Shanghai, China) working solution (50 μL/well) was added and incubated for 30 minutes at 37°C. Wells were washed. Subsequently. 3,3',5,5'-tetramethyl benzidine solution (50 μL/well) was added for 15 minutes at 37°C in the dark. The reaction was terminated by the addition of 3,3',5,5'-tetramethyl benzidine stopping solution (50 μL/well). Absorbance values were measured at 450 nm with a microplate reader (Labsystems, Helsinki, Finland) 30 minutes after thoroughly mixing the solution in the wells. The mean absorbance value for each standard sample was calculated. The mean value of the zero standard was subtracted from all absorbance values before result interpretation. The standard curve was constructed using the statistical software-Curve Expert 1.3 software (Hixson, TN, USA). The standard curve was generated by plotting the average absorbance value obtained for each of the eight standard concentrations on the vertical (Y) axis the corresponding concentration on the horizontal (X) axis. This standard curve was used to determine the concentration of the unknown sample. The absorbance value was located on the Y-axis and extended a horizontal line to the standard curve. At the point of intersection, a vertical line was drawn to the X-axis and the corresponding concentration was read, *i.e.* the corresponding concentration for the sample. The detection of BDNF and GDNF was also performed following this procedure.

Statistical analysis

All data were presented as mean ± SD. Statistical analysis of the data was performed using SPSS 13.0 (SPSS, Chicago, IL, USA). The mean significant difference between different days was determined using the one-way analysis of variance with a Student-Newman-Keuls test. The mean significant difference for each group was determined using the independent-samples t-test. Differences were considered statistically significant when $P < 0.05$.

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