Biotechnol. Bioprocess Eng. **1998**, 3, 51-54

Enhancement of BDNF Production by Co-cultivation of Human Neuroblastoma and Fibroblast Cells

Jong-Soo Hong[1], Se-Jong Oh[1], Sun-Hee Kim[1], Kwon-Tae Park[2], Jin-Sang Cho[2], Kyung-You Park[2], Jin-Ha Lee[1], and Hyeon-Yong Lee[1]*

[1]Division of Food and Biotechnology, Kangwon National University, Chunchon 200-701, Korea
[2]Dept. of Medicine, Hanllym University, Chunchon 200-802, Korea

It has been proved that co-cultivation of human neuroblastoma cells and human fibroblast cells can enhance nerve cell growth and the production of BDNF in perfusion cultivation. In batch co-cultivation, maximum cell density was increased up to 1.76×10^6 viable cells/mL from 9×10^5 viable cells/mL of only neuroblastoma cell culture. The growth of neuroblastoma cells was greatly improved by culturing both nerve and fibroblast cells in a perfusion process, maintaining 1.5×10^6 viable cells/mL, which was much higher than that from fed-batch cultivation. The nerve cell growth was greatly enhanced in both fed-batch and perfusion cultivations while the growth of fibroblast cells was not. It strongly implies that the factors secreted from human fibroblast cells and/or the environments of co-culture system can enhance both cell growth and BDNF secretion. Specific BDNF production rate was not enhanced in co-cultures; however, the production period was increased as the cell growth was lengthened in the co-culture case. Competitive growth between nerve cells and fibroblast cells was not observed in all cases, showing no changes of fibroblast cell growth and only enhancement of the neuroblastoma cell growth and overall BDNF production. It was also found that the perfusion cultivation was the most appropriate process for cultivating two cell lines simultaneously in a bioreactor.

Key words: Human neuroblastoma cells, BDNF, co-cultivation, nerve cells, fibroblast cells

INTRODUCTION

There have been many limitations in cultivating nerve cells due to low proliferation and differentiation compared to other cell lines [1-2] even though great advances have been made to efficiently produce biopharmaceuticals from many kinds of animal cells. To overcome these bottlenecks, so far, biological and physiological understandings of the nerve systems have been obtained in many areas [3-5]. However, there have not been much improved in developing processes of *in vitro* cultivating nerve cells to economically secrete bioactive molecules such as Brain-derived Neurotrophic Factor (BDNF), Nerve Growth Factor (NGF) and Neurotrophin-3 (NT-3) from them. Especially, BDNF secreted from nerve cells is known to be effective in treating Alzheimer's and Parkinson's diseases and studies on its applications are just beginning [6-7]. The development of mass production system is very important and useful in industrial stand points. Biological and physical parameters should be seriously considered to *in vitro* cultivate nerve cells for secreting BDNF in a relatively large scale bioreactor. The addition of biochemicals such as biological response modifiers can possibly enhance the differentiation and/or proliferation of nerve cells; however, only one application of the chemicals would not be much effective in improving the productivity due to the cost of the chemicals and the addition of purification steps. The physical considerations of the culture system such as reactor configuration, controls of perfusion rate, etc. have also limitations because of the characteristics of growing nerve cells *in vitro* [8]. To faciliate these two parameters co-culture of both nerve and other cells which can improve the growth of the nerve cells would be an alternative of scaling-up the culture process for industrial production of BDNF. It is reported that adult epithelia could influence connective tissue formation and play an important role in morphogenesis of diffusing stromal signals [9-11]. Therefore, in this work, a co-cultivation system with fibroblast cells is to be introduced for efficient production of BDNF from nerve cells for the long-term process.

*Corresponding author
Tel: 0361-250-6455 Fax: 0361-56-4819
e-mail: Hyeonl@cc.kangwon.ac.kr

MATERIALS AND METHODS

Cell Line and Culture Conditions

Both human neuroblastoma cells (IMR 32, ATCC) and human fibroblast cells (Hs68, ECACC, London,

UK) were grown in DMEM/F12 mixture (1:1 v/v) enriched with 10% FBS (Gibco, NJ, USA). The 40 μg/mL of gentamycin was also added to all media. They were first grown in a 75cm^2 T-flask at a 37℃ CO_2 incubator supplying 5% CO_2 in air. When the cell density reached to 1×10^4 viable cells/mL, the c ells were separately inoculated into a modified bioreactor (1.5L working volume, Celligen, NBS, NJ, USA) shown in Fig. 1. The reactor was prefilled with 300 mL of warm medium containing 0.5g/L of porous beads (Cultisper-G, Cellex, Amherst, USA) for attaching the cells. Then, DMEM/F12 mixture with 5% FBS medium was fed into the system, up to 1.5 L for batch cultivation. For fed-batch cultivation the fresh medium implemented with 5% FBS was step-wisely added into the bioreactor up to 1.5 L, proportional to the cell growth.

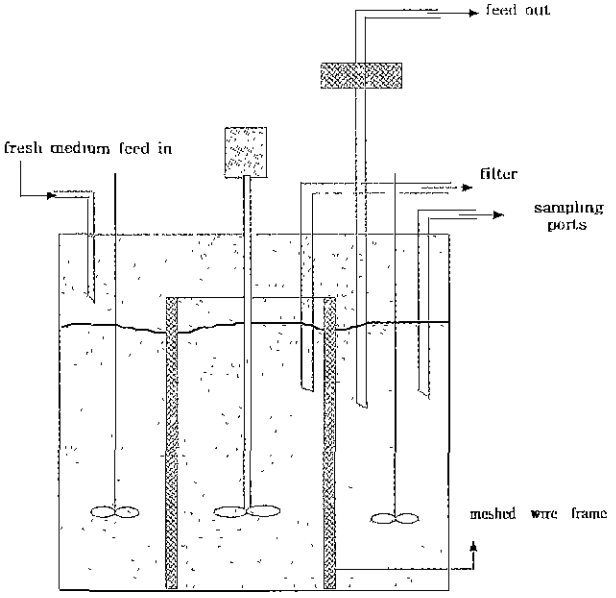

Fig. 1. A schematic diagram of the bioreactor system for cultivations of two different animal cells. A: human neuroblastoma cells ; B: human fibroblast cells

Configuration of Co-culture Perfusion System

The inside of the reactor was modified by fixing a meshed wire frame (pore size, 0.22 μm) which can permeate the medium and the beads can not be passed. It allows to culture two different anchorage-dependent cells separately inside (working volume 0.7 L) and outside (working volume 0.8 L) of the wire frame as shown in Fig. 1 (total working volume 1.5L). Two mixing systems were used to effectively agitate the cells. Inside of the frame was mixed by a magnetic stirrer at the bottom of the reactor, and outside was mixed by a top-driven motor to slowly agitate the medium. First, each 0.5 g/L of porous microcarriers (Culitsper-G, USA) was added into the inside and outside of the wire frame with 37℃ pre-warmed medium. 1×10^4 viable cells/mL of neuroblastoma and fibroblast cells were inoculated into each side of the frame, then remained for 4 hours to let the cells attach onto the beads (human neuroblastoma cells were inoculated into the inside of the frame). Both mixing systems were started slowly, and increased up to 45 rpm. The pH and temperature of the medium were automatically controlled to 7.0 and 37℃, respectively by adding CO_2, O_2, and N_2 gases into the system Fresh medium containing 5% FBS was fed into the system by a level controller, and fed out of the bioreactor by a peristaltic pump with 0.24 μm pore size meshed screen(NBS, NJ, USA). The 10 mL samples were collected from two different sample ports to count cell densities.

Measurement of Cell Density and BDNF Concentration

The collected samples from the bioreactor every day were first trypsinized by using 0.25% trypsin-EDTA to detach the cells from the beads. Then, total and viable cell concentrations were measured by using tryphan blue dye exclusion and nuclei count methods [12]. The amount of BDNF in the medium was measured by using ELISA method at 530 nm of absorbance [12] with anti-BDNF and goat IgG(Promega, Cambridge, USA). Specific BDNF production rate, q_p was calculated by a following equation:

$$q_p = (1/X)(dP/dt) \quad (1)$$

where X is cell density (viable cells/mL), t is cultivation time(day) and P is BDNF concentration in the medium(ng/mL).

RESULTS AND DISCUSSION

Fig. 2 shows the result of cultivating only human neuroblastoma cells in a bioreactor in a batch mode. Fig. 3 also shows the data of growing only human fibroblast cells in medium containing 5% FBS. Comparing with the data in Fig. 2, both cells seemed to have similar growth patterns, maintaining maximum cell concentration at 10-12 days of batch cultivations; however, human neuroblastoma cells showed relatively lower cell density than human fibroblast cells. Specific BDNF production rate was closely related to cell growth, having 2.5×10^{-5} ng/cell/day of maximum value at 9.1×10^5 viable cells/mL of maximum cell density. Fibroblast cells grew faster than neuroblastoma cells, and pH of the medium was also dropped at the end of the cultivation. The

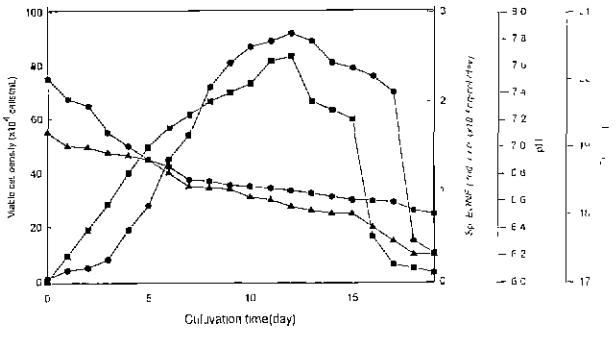

Fig. 2. Profiles of cell growth and specific BDNF production rate for batch cultivation of human neuroblastoma cells in medium containing 5% FBS. :
● : Cell density ▲ : pH
■ : Sp. BDNF prod. rate ◆ : Dissolved oxygen

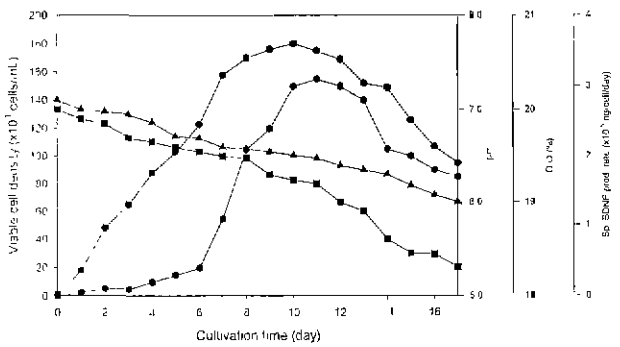

Fig. 3. The growth of human fibroblast cell for batch cultivation in medium containing 5% FBS.
-●- : Cell density -▲- : pH
-■- : Dissolved oxygen -◆- : Sp. BDNF prod. rate

level of dissolved oxygen concentration was similar in cultivating both cell lines as ca.18% of air saturation, which implies that there was no limitation of oxygen supply and competitive growth between two cell lines. It should be carefully considered in cultivating two cell lines together, not to negatively affect both cell growth and BDNF production.

Fig. 4 shows the cell growth and BDNF production in co-cultivating human neuroblastoma and fibrolast cells in a bioreactor for batch cultivation. Maximum cell density of human neuroblastoma cells was increased up to 1.65×10^6 viable cells/mL, while the growth of fibroblast cells was not affected, compared to the data in Figs. 2 and 3. The 2×10^{-5}(ng/cell/day) of maximum specific BDNF production rate from the co-cultivation process seemed to be lower than that in growing only neuroblastoma cells. However, overall specific BDNF production rate was not decreased even though the cell growth was slowly decreased at the latter period of the cultivation, which was definitely different from the case of cultivating only neuroblastoma cells. On the contrary, the growth of fibroblast cells was not affected by the growth of neuroblastoma cells, showing similar growth pattern and maintaining maximum cell density in Fig. 3. The pH of the medium was dropped in the latter periods of the cultivation. It was interesting to note that relatively stable growth of neuroblastoma cells could be maintained until the end of the cultivation, when the neuroblastoma cell growth was sharply decreased as shown in Fig. 2, while fibroblast cell growth was gradually decreased as shown in Figs. 3 and 4.

Fig. 5 illustrates both cell growth and specific BDNF production rate in fed-batch cultivation with feeding the medium containing 5% FBS. Maximum cell densities of both neuroblastoma and fibroblast cells were increased, compared to those from batch cultivations. Growth rates of both cells were also increased in fed-batch cultivation and human fibroblast cells maintained higher growth rate than neuroblastom cells for batch and fed-batch cultivations. Overall BDNF production was much higher than that obtained from batch cultivations even though maximum BDNF production rate was lower than that from batch process. In fed-batch cultivation the growth of fibroblast cells was greatly enhanced, compared to the data in Fig. 3, possibly due to continuous feeding of fresh medium.

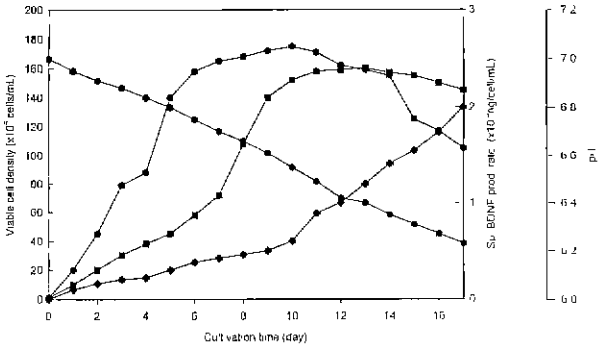

Fig. 4. The cell growth and BDNF specific production rate of co-cultivating human neuroblastoms and fibroblast cells in a batch culture :
-■- : human neuroblastoma cells
-●- : human fibroblast cells
-◆- : Sp. BDNF prod. rate -●- : pH

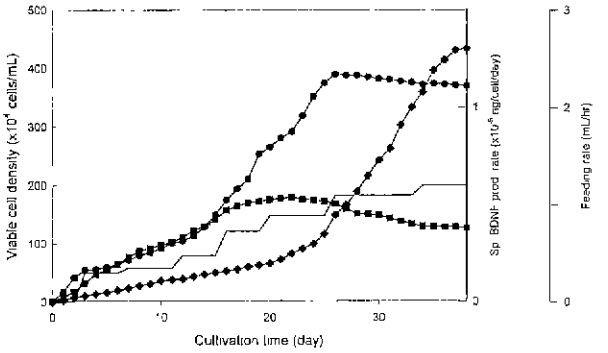

Fig. 5. The cell growth and specific BDNF production rate of human neuroblastoma and fibroblast cells in a fed-batch culture :
-●- : human neuroblastoma cells
-■- : human fibroblast cells
-◆- : Sp. BDNF prod. rate
— . Feeding rate

Dramatic increases in cell growth were observed in perfusion cultivation of both neuroblastoma and fibroblast cells as shown in Fig. 6, while relatively low improvement was made in fed-batch cultivation. The growth of nerve cells was greatly improved, compared to the growth of fibroblast cells. The pattern of producing BDNF was different from that shown in batch cultivation since specific BDNF production rate was increased at the latter period of the cultivation. This is a typical phenomenum in secreting proteins from mammalian cells [13]. At high perfusion rate relatively stable cell density was maintained for neuroblastoma cells while fibroblast cells were sharply dropped. It implies that unknown factors secreted from fibroblast cells can definitely enhance both nerve cell growth and BDNF production from them. There was sudden jumps of BDNF production rate during the co-cultivation, possibly due to the feeding of fresh medium and release of unknown factors from fibroblast cells. Overall BDNF production was also enhanced, compared to the cases of cultivating only neuroblastoma cells.

It was found that co-cultures of nerve and fibroblast cells clearly improved neuroblstoma cell growth and BDNF secretion than single cultivation of

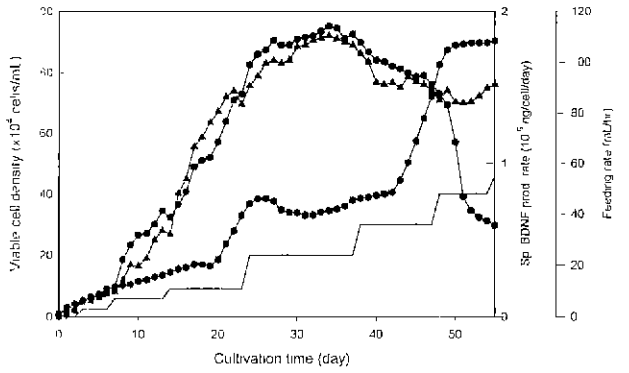

Fig. 6. The cell growth and specific BDNF production rate of human neuroblastoma and fibroblast cells in a perfusion culture :
-■- : human neuroblastoma cells
-▲- : human fibroblast cells
-●- : Sp. BDNF prod. rate
— : Feeding rate

nerve cells. There was no competitive growth between nerve and fibroblast cells because neruoblastoma cell growth was greatly improved and fibroblast cell growth was never reduced and even increased for all three culture processes, such as batch, fed-batch and perfusion cultivations. If the competitive growth occurs, it could be harmful for both cell growth. It should be also noted that the perfusion process has to be employed for co-culture system because batch and/or fed-batch cultivations could not properly control two cell growth due to fast limitation of the nutrients in the medium.

Acknowledgement This study was supported by the academic research fund of Ministry of Education, Republic of Korea.

REFERENCES

[1] Roush, W. (1996) New neurons use outlook to navigate nervous system. *Science* 271:1807-1808.
[2] Silos, S. I. and W. S. Snider (1992) Development of commissural neurons in the embryonic rat spinal cord. *J. Com. Neurol.* 325:514-526.
[3] Kumar, S., J. Huber, L. A. Pena, and J. de Vallis (1990) Characterization of functional nerve growth factor-receptor in a CNS glial cell line. *J. Neurosci. Res. 27:408:417.*
[4] Merlino, J. P., P. Ernfors, Z. Kokaiza, D. S. Middlemas, and H. Persson (1993) Increase production of the trkB protein kinase receptor after brain insults. *Neuron.* 10:151-164.
[5] Greene, L. A. and A. S. Tischler (1976) Establishment of a noradrenergic clonal line of rat pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci.* USA 73: 2424-2428.
[6] Barigana, M. (1994) Neurotrophic factors enter the clinic. *Science* 264:772-774.
[7] Schorderet, M. (1995) Alzheimer's disease: fundamental and therapeutic aspects. *Experientia* 51:99-105.
[8] Frank, E. and P. Westerfield (1983) Development of sensory-motor synapes in the spinal cord of the frog. *J. Physiol.* 343:593-610.
[9] Wang, A. A., G. K. Ojakian and W. J. Nelson (1990) Steps in the morphogenesis of a polarized epithelium. *J. Cell Sci.* 95:137-151.
[10] Spier, R. E., J. R. Griffiths and Berthold, W. eds. (1994) *Animal cell culture technology,* ESACT, Butterworth Pub., London.
[11] Paine, A. J., L. Williams and L. J. Hockins (1989) Potent maintenance by metyrapone. *Biochem. J.* 188:937-942.
[12] Freshney, R. I. (ed.) (1983) *Cultivation of animal cells.* Alan R. Liss, Inc. pp.199, NY.
[13] Hong, J. S., K. H. Woo, K. Y. Park and H. Y. Lee (1997) The Secretion of BDNF from the cultures of human neuroblastoma cells. *Kor. J. Microbiol. Biotechnol.* 25:102-105.