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The Changes of Growth Patterns and the Production of Brain-Derived Neurotrophic Factors (BDNFs) in Perfusion Cultivation of Human Neuroblastoma Cells

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Abstract It was shown that brain-derived neurotrophic factors (BDNFs) secreted from human neuroblastoma cells can significantly improve the growth of the neurites of PC12 nerve cells. The addition of purified BDNFs elongated the neurites of PC12 nerve cells two to three times more than the case where the addition was not made. The perfusion rate strongly affected the change of the size of human neuroblastoma cells because the cell size decreased as the perfusion rate increased. This could also influence the productivity of BDNF from the cells. It is also important to note that the BDNF production was decreased when the cell size was reduced. BDNF production rate also decreased at a fast perfusion rate in a smaller cell size. At the relatively fast perfusion rate of 18 ml/h, the ratio of apoptotic to necrotic cells dramatically decreased, which possibly caused the decrease of BDNF production. It has been proven that the secretion of BDNF from human neuroblastoma cells was a partially growth-related process by yielding 6.2×10^{-8} g of BDNF/cell of growth related parameter and 0.48×10^{-9} g of BDNF/cell/h of nongrowth-related parameter in a growth kinetic model. In addition, it was also found that the perfusion rate played a very important role in controlling the cell death mechanism.

Key words: Perfusion cultivation, human neuroblastoma cells, BDNF production, apoptosis, necrosis

For the past twenty years, great improvements have been made to produce biopharmaceuticals economically from mammalian cell cultures by using various techniques of genetic manipulation due to advancement of the process engineering. So far, perfusion technology has been proven to be the most economical and reliable process for *in vitro* cultivation of animal cells [4, 9, 10]. Physical factors in the perfusion process, such as shear stress, dissolved oxygen concentration, pH, and bead concentrations, etc. are most often considered to be key parameters for enhancing both cell growth and protein production [1, 14]. Biological factors such as the selection of mutated cells, the control of medium components, the osmotic pressure, and finally, the effective removal of the wastes in the medium have also been seriously considered in developing cell culture processes [5, 8]. Recently, there has been wide interest regarding the mechanisms and characteristics of death patterns in cultivating animal cells in perfusion cultivation. The protein productivity from the cultured cells must depend on maintaining high density of viable cells for long-term cultivations. However, there have been no reports on the correlation between the cell death pattern and process parameters such as the perfusion rate which is a key variable in perfusion cultivation. Changes in the cell size during the cultivation can also be used as an indicator to effectively control the process, especially in perfusion cultivations. The environment around the cells within the reactor is continuously changing in perfusion cultivation due to the constant flow of fresh medium as well as shear stress. Therefore, it is necessary to correlate between the cell death pattern and the cell size distribution as a function of perfusion rate for the economic production of Brain-Derived Neurotrophic Factor (BDNF) from human neuroblastoma cells [14]. BDNF is a protein secreted from nerve cells. Since this protein has been reported to have the potentials for treating Alzheimer's disease and Parkinson's disease [2, 12, 15], it is not only interesting but fascinating as well.

Materials and Methods

Cell Line and Growth Conditions

Human neuroblastoma cells were generously donated by Dr.. Thiele in NIH, USA, and grown in DMEM/F12 (1:1

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w/w) basal medium enriched with 10% (v/v) FBS (Gibco, Gaithersburg, U.S.A.). 3×10^4 viable cells/ml grown from a 75-cm^2 T-flask were inoculated into a bioreactor (Celligen, NBS, New Brunswick, U.S.A.) for perfusion cultivation. The working volume of the reactor was 1.5 l. The pH and dissolved oxygen concentration were automatically adjusted by the use of a microprocessor to 7.1 and 20% air saturation, respectively. 2.5 g/l of pretreated porous microcarriers (Cultisper G, Madison, U.S.A.) were added into the reactor before inoculating the cells. Perfusion rate was altered by a peristaltic pump and a level probe according to the cell density. In perfusing fresh medium into the reactor, 5% of the FBS (v/v) was added and the effluent was immediately centrifuged and harvested at -20°C until it was used [7]. The perfusion rate was changed when the system was stabilized based on the cell growth and BDNF production.

Measurement of the Cell Size, Density, and Death Pattern, and BDNF Concentration

Cell density in the reactor was measured by a trypan blue dye exclusion method [6] every day after trypsinizing the attached cells from beads by 0.25% trypsin-EDTA. The cell death pattern was observed by a fluorescent dye method as follows: Collected beads from the reactor were trypsinized by 0.25% trypsin to detach the cells from the beads. Then, 100 µl of the cells were mixed with 4 µl of a dye solution which contains acridine orange and ethidium bromide (1:1 v/v), and finally, the cells were counted through a fluorescent microscope (Olympus, Tokyo Japan). The size and numbers of the stained cells were counted and from pictures were compared to those of normal cells. Detail methods are described elsewhere [6]. The size distribution of cultured cells was observed by a Flow cytometer (Becton Dickisin FACscan, St. Louis, U.S.A.). Ten milliliter of the medium containing the cells were collected from the reactor, then trysinized and treated with 1% formalin, at different perfusion rates when the cells reached the steady state based on the cell density and O.D. concentration along with the BDNF production. It was designed to understand the effect of the change of the perfusion rate on the size of the cells and BDNF production. The concentration of BDNF in the effluent medium was measured by the ELISA method using the BDNF IgG goat antibody (Genzyme, Cambridge, U.S.A.). The crude medium was consecutively purified by S-Sepharose and Sephadex G75 in order to measure the activity of BDNFs secreted from the cells. The biological activity of the BDNF was estimated by the growth of the neurite in PC12 cells, monitored by an inverted microscope with a graticule [13]. Specific growth rate, μ (1/day), and specific BDNF production rate, q_p (ng/cell/day), were calculated by the following equations using the data collected from perfusion cultivations.

$$\mu=(1/X)(dX/dt) \quad (1)$$

$$q_p=(1/X)(dP/dt) \quad (2)$$

where X is the cell concentration (viable cells/ml) and P is the BDNF concentration (ng/ml) during cultivation time, t (day). The relationship between cell growth and production rate can be explained by the following equation:

$$q_p=\alpha\mu+\beta \quad (3)$$

where α (µg/cell) is a maximum concentration of BDNFs secreted from a cell and β (µg/cell/h) is a specific BDNF production rate.

Results and Discussion

Figure 1 is the result of growing human neuroblastoma cells, feeding 5% FBS-containing medium under perfusion cultivation. The cell growth and BDNF production was correlated to the change of perfusion rates. 5.6×10^6 viable cells/ml of maximum cell density was obtained at 18.5 ml/h of perfusion rate. The cell growth was gradually increased as the perfusion rate increased up to 18.5 ml/h. After that, the cell density dropped, due to the fast perfusion rate. It is important to state that this reaction is a typical pattern of the cell growth in the perfusion process. For an overall process, BDNF production was closely related to cell growth, except for the latter period of the cultivation when the cell density dropped drastically. This also means that the BDNF production is partially growth related for this process. The maximum secretion level of BDNF was

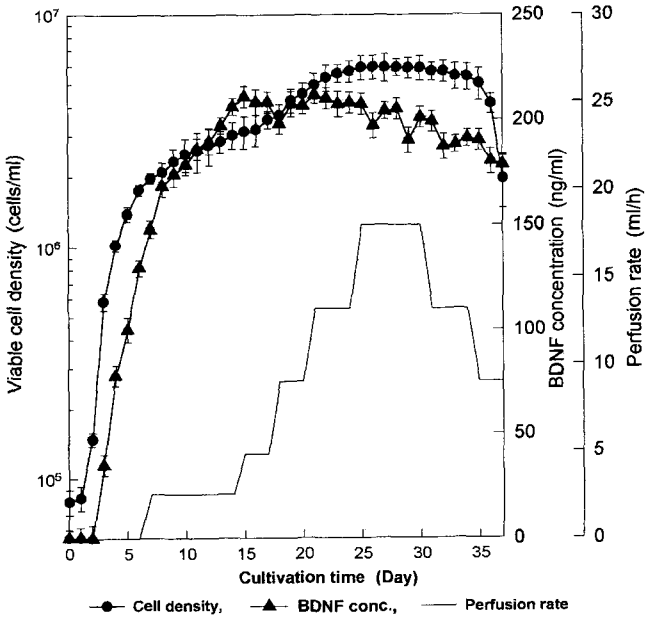

Fig. 1. The kinetics of cell growth and BDNF production for the perfusion cultivation of human neuroblastoma cells.

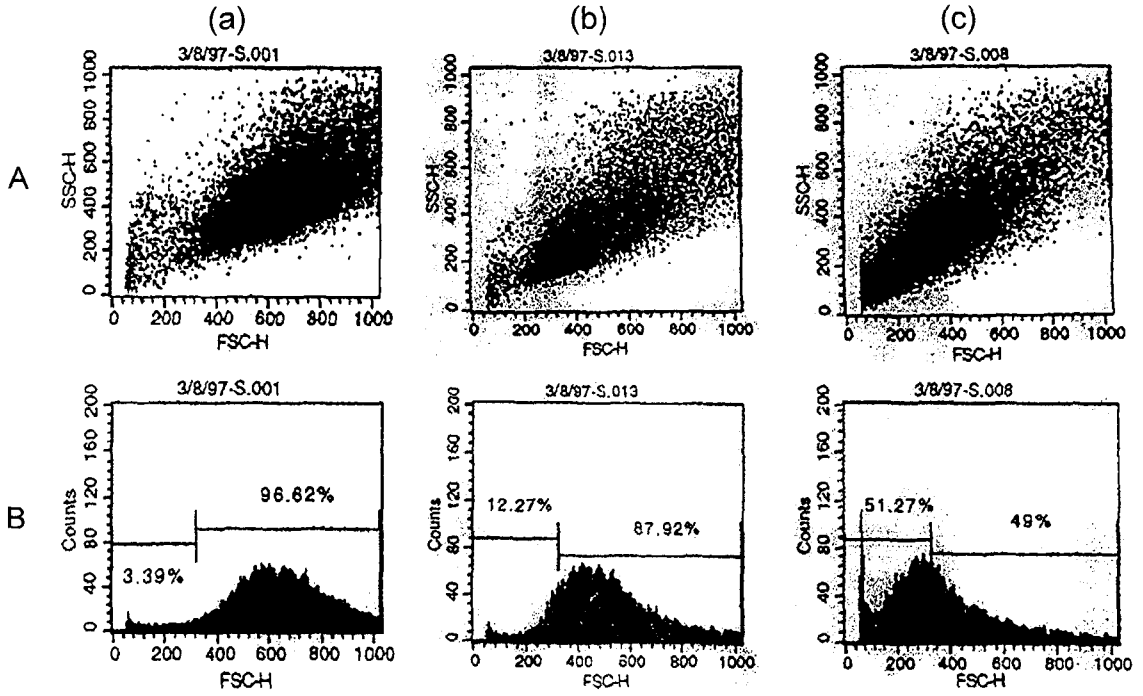

Fig. 2. The pattern of changing the cell size according to cultivation time in perfusion cultivation.
(a) at the begining of the cultivation; (b) at the 12th day of the cultivation; (c) at the 32nd day of the cultivation.

measured as 200 ng/ml at 5 ml/h of perfusion rate. Since the maximum BDNF production was observed at a relatively slow perfusion rate, it implies that not only is perfusion rate considered for maintaining maximum protein production, but it also counts for the change of the cell characteristics during the perfusion cultivation. Both cell growth and BDNF production are strongly correlated to and should be controlled by the perfusion rate which is an important and easily controlled parameter in perfusion processes.

Figure 2 illustrates the cell size distribution according to the cultivation time. It was apparent that an overall cell size was decreased from 96% to 49% as the perfusion rate increased, as shown in the row B. The size reduction was accelerated at a high perfusion rate which was in the middle periods of the cultivation, as showen in (b)-(c) in Fig. 2, compared to that of the low perfusion rate which was in the early periods of the cultivation, as in Fig. 2 (a)-(b). Its relationship was quantitatively illustrated in Fig. 3 as a function of the perfusion rate. The relative cell size continuously decreased as the perfusion rate increased except for the periods of low perfusion rate at the end of the exponential growth phase. While at a low perfusion rate, a relative cell size was increased and BDNF production also maintained as a maximum value, and it was found that the cell size gradually decreased. The reason for this could be caused by a long-term exposure to an environmental stress such as shear stress and culture time, etc. While the cell density remained relatively constant,

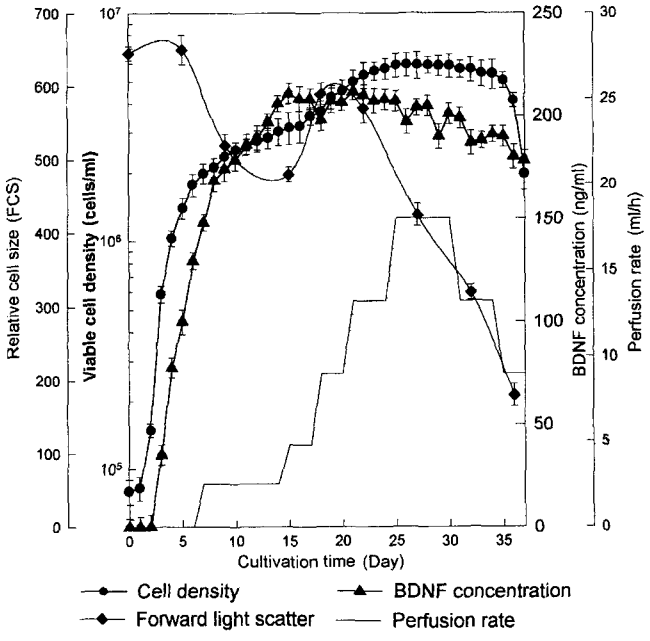

Fig. 3. The changes of relative cell size of human neuroblastoma cells according to cultivation time.

the BDNF secretion fluctuated. It showed that the change in the cell size could have a much greater effect on BDNF production than the cell growth.

There must be relationship between the cell death pattern and BDNF production as well as the perfusion rate

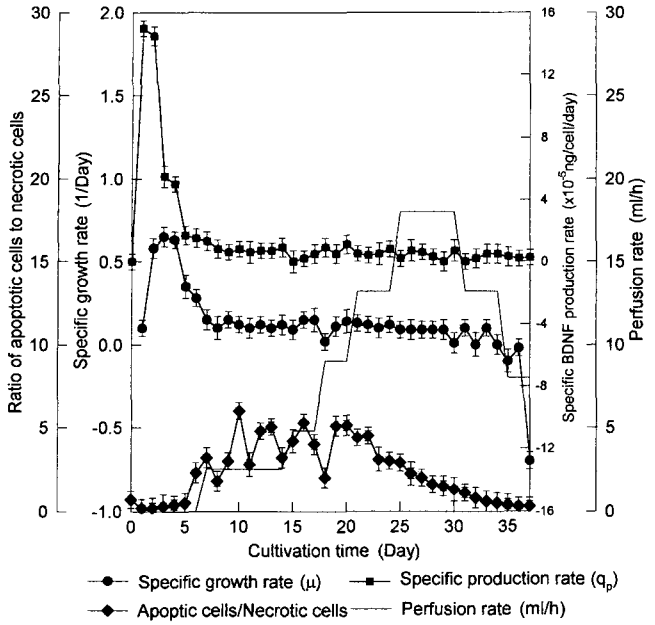

Fig. 4. The change of the ratio of apoptic to necrotic cells in perfusion cultivation of human neuroblastoma cells.

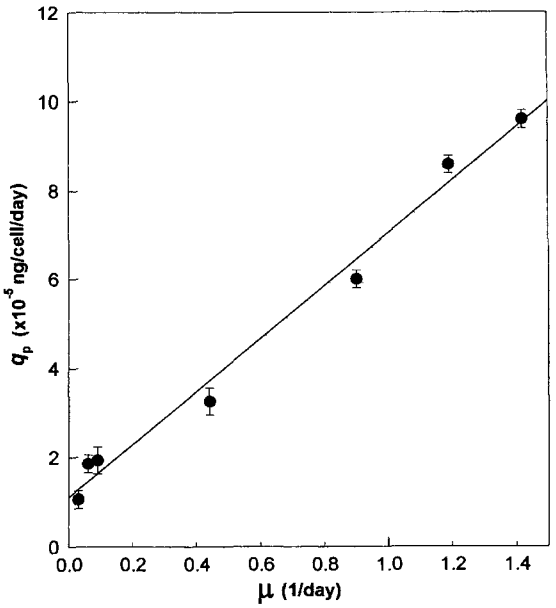

Fig. 5. The correlation between specific growth rate and specific BDNF production rate for the growth of human neuroblastoma cells.

which is one of the controllable parameters for perfusion cultivation, as shown in Fig. 4. The proportion of necrotic cells in the reactor showed an increase from 5% to 60% at 18 ml/h of high perfusion rates, while the ratio of apoptotic cells remained relatively constant until the latter periods of the cultivation. It is quite obvious that the number of necrotic cells become dominant in a long-term perfusion cultivation. It is also apparent that the ratio of the necrotic cells has greatly increased in a batch cultivation. Fast perfusion rate can also cause the climb of the necrotic cell numbers during the perfusion cultivation, possibly due to the increase of shear stress and the fast change of the environment surrounding the cells [11]. The necrotic cells never return to apoptotic cells when the cells get into the necrotic death phase, which is definitely harmful for cell growth. However, BDNF production was not much affected by the patterns of cell death, compared to the cell growth shown in Fig. 4.

The relationship between cell growth and BDNF production rate is shown in Fig. 5. The specific BDNF production rate can be linearly correlated to the specific growth rate, having a high correlation coefficient rate of 0.95. This linearity confirms that the pattern of BDNF secretion from the cells is partially a growth related process, as already shown in Figs. 1 and 2 under perfusion cultivations. The production parameters such as in Eq. (3) were calculated as 6.02×10^{-9} g/cell and 0.48×10^{-9} g/cell/h, respectively from, Fig. 5. These values are higher than those obtained from recombinant CHO cells for tPA production [7, 10]. In particular, the level of BDNF secretion per unit cell was much greater than 0.35×10^{-9} g/cell from CHO cells. What this means is that maintaining a large number of viable cells can enhance the BDNF production in a long-term cultivation.

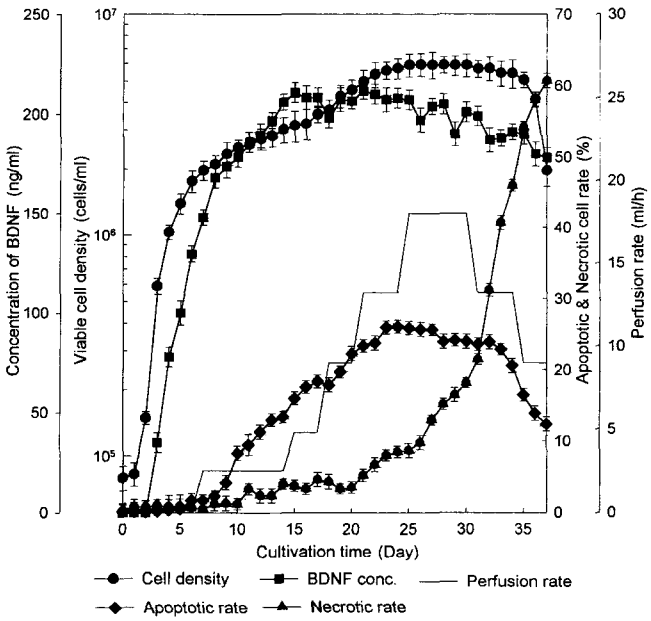

Fig. 6. The kinetics of cell growth and specific BDNF production rate and the changes of ratio of apoptotic and necrotic cells according to cultivation time in perfusion cultivation.

Outcomes of the perfusion rate on the cell growth and BDNF production rates are shown in Fig. 6. The kinetics of changing the numbers of apoptotic and necrotic cells are also analyzed during the cultivation for comparison to the

results of Fig. 4. Specific BDNF production rate remained relatively constant for overall ranges of perfusion rates, except for the first period of the cultivation when the batch cultivation was maintained. The cell growth rate was also constant when the process reached a steady state, however, the rate dropped sharply at the end of the cultivation period due to the fast perfusion rate. Once neuroblastoma cells began to secrete the BDNFs, they were secreted in a continual pace until the the cells were in Crisis. This was caused by the fact that the specific BDNF production rate was not affected by the decrease in the cell growth rate. This is a typical phenomenon of producing proteins from mammalian cells, as well as supporting an argument that BDNF production from human neuroblastoma cells is a partially growth related production system. The numbers of apoptotic and necrotic cells were very much fluctuated until the system reached its steady state, possibly due to the rapid change of perfusion rate. This could have a negative effect on both cell growth and BDNF production rate, mostly on BDNF production, showing a sudden drop of the production rate to between 3.4 and 8.5 ml/h of the perfusion rate. When the system was in a steady state, the ratio of apoptotic to necrotic cells gradually decreased despite that the cell growth rate was not altered by the changing perfusion rates. The perfusion rate that affected the most on the cell death mechanism can eventually result in the BDNF production rate. What this means is that the cell death pattern should be seriously considered in maintaining a high production rate for a long-term culture system with fine adjustment of perfusion rates.

Acknowledgments

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