The enhancement of growth and differentiation of rat adrenal nerve cells by the addition of conditioned medium from human fibroblast cultures

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Abstract

The growth of rat adrenal nerve cells was remarkably enhanced by supplementing the cultured medium from the human fibroblast cell line, Hs 68. Maximum specific growth rate and length of the neurites were observed as 0.076 (1/hr) and 0.026 mm, respectively in 20% supplement of five day old medium. In adding more than 20% of the cultured medium both cell and neurite growth was severely decreased. It was interesting that the cultured medium from Hs 68 cells could play a role in the extension of the neurites rather than in the growth of neurite cells. It was also found that molecules lower than 50,000 daltons in the conditioned medium could improve the growth of neurite bearing cells and the extension of the neurites than larger molecules. The efficacy of the proteins (<50,000 MW) was similar to that of human nerve growth factor and much better than that of basic fibroblast growth factor which was mainly secreted from human fibroblast cells.

Introduction

Recently *in vitro* cultivation of nerve cells has greatly been increased since the spectacular advances in the understanding of mechanisms of neural connectivity (Brown, 1981; Willis and Coggeshall, 1991) which increases the possibility of treating nerve related diseases, such as Alzheimer's and Parkinson's (Schorderet, 1995; Barinaga, 1994). However, *in vivo* and *in vitro* growth mechanisms and differentiation of nerve cells are not clearly understood yet as well as the relation between neurites and neurotransmit systems. Functions of several neurotrophic factors like Brain-derived Neurotrophic Factor (BDNF), Nerve Growth Factor (NGF) and Neurotrophins-3 (NT3) have only been revealed recently (Hokfelt, 1991; Patterson and Nawa, 1993) and the studies on their applications are just beginning (Barde *et al.*, 1982; Fantl *et al.*, 1993; Faden and Salzman, 1992).

However, there have been many limitations in cultivating mammalian nerve cells due to low proliferation and differentiation of the neurites compared to other

cell lines. Generally nerve cells are known to require additional growth factors, such as NGF, BDNF and retinoic acid, etc for *in vitro* cultivation (Frank and Westgerfield, 1983; Silos and Snider, 1992; Roush, 1996; Kaplan *et al.*, 1993). To manage these problems, the biochemical pathway of nerve systems and signal transmitting molecules have been intensively investigated (Merlio *et al.*, 1993; Kumar *et al.*, 1993). Some tumor cells display more differentiated features and contain cells resembling Schwann cells of the glial lineage, or secretory cells expressing markers of neuroendocrine differentiation (Copper *et al.*, 1990). Neuroblastoma cells have also been proved to have the ability of undergoing differentiation (Kumon *et al.*, 1993). Similarly, adult epithelia remain responsive to inductive influences from connective tissue and can play a role in morphogenesis of diffusing stomal signals (Cunha *et al.*, 1985; Wang *et al.*, 1990). Moreover, many chemicals and biological response modifiers are known to promote *in vitro* morphological, biochemical, and ultrastructural changes to well-differentiated neuroectodermal phenotypes (Abemayor and Sidell,

Figure 1. The growth of neurites of PC12 cells on adding 20% fibroblast cell culture medium: A, 0 days; B, three days; C, five days; D, seven days. Arrows are the extended neurites.

1989). Therefore, in this work, a methodology for efficiently cultivating nerve cells is developed by supplementing the conditioned medium from human fibroblast cultures.

Materials and methods

Cell culture

Rat adrenal nerve cell, PC12 generously donated by Dr. Thiele (NCI, USA) was grown in DMEM/F12 basal medium containing 365 mg/L of L-glutamine (GIBCO, USA) and enriched with 20% FBS (GIBCO) in a 37 °C CO₂ incubator supplying 5% CO₂ in air. Human fibroblast cells (Hs 68, ECACC 89051701) were grown in

RPMI 1640 with 20% FBS. 40 µg/ml of gentamycin was also added to all media. The conditioned medium from Hs 68 cells was collected in 75 T-flasks everyday. It was stored at -20 °C, thawed as required and mixed with fresh DMEM/F12 medium (v/v). Proteins of greater than 50,000 MW were separated from dialyzed conditioned medium by ultrafiltration (Amicon, USA) and mixed with fresh DMEM/F12. The growth of nerve cells and the neurites was also observed after adding 100 ng/ml of human nerve growth factor (NGF) or basic fibroblast growth factor (bFGF) (GGIBCO). The effect of serum on proliferation of PC12 cells and differentiation of neurites was examined by gradually reducing serum concentrations from 20% to 1% with supplementing the conditioned medium.

Measurement of cell and neurite growth

The specific growth rate of PC12 cell on adding various volumes of conditioned medium was estimated by the trypan blue dye exclusion method everyday (Freshney, 1983). The neurite growth of PC12 cell was monitored using an inverted microscope with a graticule (Rekenstein and Greene, 1983) as a measure of differentiation of nerve cells. The ratio of neurite bearing cells to total cells was also calculated by counting total and neurite bearing cells in a hemocytometer.

Results and discussion

Figure 1 illustrates the neurite growth of PC12 cells grown in 5% serum with 20% supplement of the conditioned medium. There was no visible growth of the neurites on the first day, but by the third day, the neurite had grown to about 0.015 mm and there were many initiations of the neurites within the cells. Fully developed neurons were observed on the fifth day. Then, at least five or six neurites were maintained within a single cell after six day of cultivation. It is reported that more than 10 days will be required to develop a similar morphology (Figure 1) in growing PC12 cells without supplementing the conditioned medium from fibroblast cells under normal conditions (Greene and Tischler, 1976; deSouza *et al.*, 1995).

Figure 2 shows the effect of collection time and addition volume of the conditioned medium from fibroblast cells on the growth of PC12 cells in 5% serum containing basal medium. The best cell and neurite growth was observed by adding 20% (v/v) of supplemented medium. It was found that better cell growth was observed with 5 day old supplemented medium than one day old medium. The cell growth was greatly reduced on supplementing more than 20%, possibly due to low pH and high accumulations of lactate and other toxicants in the conditioned medium. Noticable improvement of cell growth occurred on adding 10% of one day old, compared to no supplement. Then, cell growth was gradually enhanced by increasing the amount of supplemented medium up to 20% of total working volume. It implies that unknown growth factors are released from fibroblast cells and can improve the growth of nerve cells. To confirm this hypothesis, the effect of known growth factors such as NGF and bFGF on the growth of PC12 cells was compared later (Figures 5 and 6).

Figure 2. The growth of PC12 cells on adding various concentrations of conditioned medium collected from human fibroblast cells on the fifth day of cultivation.

Figure 3. The effect of serum concentration on the growth of nerve cells on adding 20% cultured medium.

Figure 3 shows the effect of serum concentration on the growth of PC12 cells in 20% supplemented medium. The worst cell growth was observed in 1% serum containing medium with 20% supplement. Cell growth gradually improved with increasing serum concentrations up to 10% (v/v). Low concentration of serum

Figure 4. Kinetics of growing neurites and neurite cells in 20% of cultured medium or no addition of the medium in 5% serum containing basal medium.

in the supplemented medium cannot dilute enough the conditioned medium. However, the cell growth rate was similar in 10% and 20% addition of serum in the medium (data not shown). It is obvious that at least 5% serum should be added to grow PC12 cells in supplemented medium.

Figure 4 shows the effect of five day old medium on the growth of neurites within PC12 by 20% supplement. The number of neurite bearing cells was higher in supplemented medium than in non-supplemented medium (90% vs 50%). Both growth of cells and neurites had similar patterns in supplemented medium, showing that it was closely related to cell growth as shown in Figure 2. It is interesting that the supplemented medium enhances the growth of neurites by more than three times compared to non-supplemented medium (0.026 mm vs 0.008 mm of maximum length of the neurites). Based on this result, cells that already contain neurites are more likely to make additional neurites than cells without. Table 1 compares the cell growth, number of neurite bearing cells and the growth of neurites in adding various concentrations of five day old medium. As shown in previous Figures, the best growth of cells and neurites was observed in 20% supplement of cultured medium. It was also found that the supplemented medium can well improve neurite growth rather than cell growth. It possibly implies that the signal for generating neurites within the cell can be possibly induced by adding certain levels of supplemented medium from fibroblast cells cultured medium.

Figures 5 and 6 are to understand which portions in the supplemented medium play a role in cell growth

Figure 5. The effect of molecular size in cultured medium on the growth of neurite cells in 5% serum containing medium.

Figure 6. The effect of molecular size in the cultured medium on the extension of neurites in 5% serum containing medium.

and/or the neurite initiation. Figure 6 illustrates the effect of 20% supplement of 50,000 dalton proteins from cultured medium on the growth of neurite cells by comparison with human NGF and bFGF. As expected, NGF showed the best effect on the initiation of neurites, maintaining almost 100% of neurite bearing cells after five days cultivation. On the contrary, bFGF showed little effect on both cell growth and elongation of the neurites. The growth of neurite cells can be improved by adding proteins lower than 50,000 MW in supplemented medium, whose enhancement is close to that of NGF. Its improvement was close to that of adding crude medium; however, the differentiation of neurite cells was greatly reduced by the addition of large molecules in the medium, showing only 63%

Table 1. The results of estimating specific growth rate and the growth of neurites in adding various concentrations of fibroblast cell cultured medium in 10% serum containing DMEM medium

Mixing conc. (%)	Specific growth rate (1/hr) μ (1/hr)	Ratio of neurite bearing cells (%)	Max. length neurite (mm)
0	0.071	64	0.008
1	0.070	59	0.006
5	0.073	78	0.009
10	0.075	88	0.021
20	0.076	90	0.026
30	0.043	32	0.004

neurite bearing cells (Figure 5). It was also shown that human NGF affected elongation of the neurites more than cell growth. The growth of neurites in adding low molecules was not matched with the result of adding crude medium, which is different from that in Figure 5. It possibly means that unknown factors from fibroblast cultures can play a role in the elongation of the neurites.

Conclusion

It was found that better cell and neurite growth of PC12 cells was observed in supplementing 5 day old cultured medium from human fibroblast cells than one day old medium, assuming that a factor was released from the fibroblast cells to enhance the growth of nerve cells. It was proved that 20% supplement was its optimal addition level for the growth of PC12 cells. In adding more than 20% supplement the cell growth was greatly decreased and less effect on the cell growth was maintained. There was no effect on either cell and neurite growth in the range of 5% to 20% serum concentration by 20% supplement; however, PC12 cells could not grow in lower than 1% serum. It was also found that the supplemented medium enhanced the growth of the neurites rather than the initiation of the neurites. It was shown that the supplement of the cultured medium improved cell growth and the number of neurite bearing cells. Maximum specific growth rates and maximum length of the neurite were estimated as 0.076 (1/hr) and 0.026 mm in 20% supplement, compared to 0.071 (1/hr) and 0.008 mm in non-supplement, respectively. Proteins lower than 50,000 dalton in supplemented medium seemed to be more effective for neurite growth, comparing 0.024 mm to 0.011 mm obtained in more than 50,000 MW cut-off medium. The enhancement of cell and neurite growth of PC12

cells in adding low molecule proteins was close to that of adding human NGF, and much better than in bFGF. It implies that proteins lower than 50,000 daltons in supplemented medium from H6 cells can enhance the initiation and elongation of the neurites, other than the function of fibroblast growth factors. It was observed that 90% of the cultured cells had neurite bearing cells in supplementing below 50,000 MW, compared to 63% in higher than 50,000 MW.

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References

- Abemayor E and Sidell N (1989) Human neuroblastoma cell lines as models for the study of neoplastic and neuronal cell differentiation. *Environ. Health Perspect.* 80: 3–15.
- Barde YA, Edgar D and Thoenen H (1982) Purification of a new neurotrophic factor from mammalian brain. *EMBO J.* 1: 549–553.
- Brinaga M (1994) Neurotrophic factors enter the clinic. *Science* 264: 772–774.
- Brown AG (1981) Organization in the spinal cord., (pp. 1–49). Springer-Verlag, New York.
- Cooper MJ, Hutchins GM and Israel MA (1990) Histogenesis of the human adrenal medulla. *Am. J. Pathol.* 137: 605–615.
- Cunha GR, Bigsby RM, Cooke PS and Sugimura Y (1990) Stromal-epithelial interactions in adult organs. *Cell. Diff.* 17: 137–148.
- deSouza S, Lochner J, Machida CM, Matrisian LM and Ciment G (1995) A novel nerve growth factor-responsive element in the stromelysin-1 gene that is necessary and sufficient for gene expression in PC12 cells. *J. Biol. Chem.* 270: 9106–9114.
- Faden AI and Salzman S (1992) Pharmacological strategies in CNS trauma. *Trends Pharma. Sci.* 13: 29–35.
- Fantl WWJ, Johnson DE and Winkler LT (1993) The invasion of NGF in rat nerve cells. *Ann. Rev. Biochem.* 62: 453–481.
- Frank E and Westerfield P (1983) Development of sensory-motor synapses in the spinal cord of the frog. *J. Physiol.* 343: 593–610.

- Frehney RI (1983) Cultivation of animal cells. pp. 199–215, Alan R. Liss, Inc., N.Y.
- Greene LA and Tischler AS (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA* 73: 2424–2428.
- Hokfelt T (1991) Neuropeptides in perspective: The last ten years. *Neuron* 7: 867–879.
- Kaplan DR, Matimoto K, Lucarelli E and Thiele CJ (1993). Induction of TrkBb by retinoic acid mediates biologic responsiveness to differentiation of human neuroblastom cells. *Neuron* 11: 321–331.
- Kumar S, Huber J, Pena LA and de Vellis J (1990) Characterization of functional nerve growth factor-receptors in a CNS glial cell line. *J. Neurosci. Res.* 27: 408–417.
- Kumon Y, Sakaki S, Kadota O, Matsuda S, Fujita H and Sakanaka M (1993) Transient increase in endogenous basic fibroblast growth factor in neurons of ischemic rat brains. *Brain Res.* 605: 169–174.
- Merlino JP, Ernfors P, Kokaika Z, Middlemas DS, Bengzon J and Persson H (1993) Increase production of the trkB protein tyrosine kinase receptor after brain insults. *Neuron* 10: 151–164.
- Pterson PH and Hawa H (1993) Neuronal differentiation factors/cytokines and synaptic plasticity. *Cell* 72: 123–137.
- Roush W (1996) New neurons use “lookouts” to navigate nervous system. *Science* 271: 1807–1808.
- Rukenstein A and Greene LA (1983) The quantitative bioassay of nerve growth factor: use of frozen primed PC12 pheochromocytoma cells. *Brain Res.* 38: 177–180.
- Schorderet M (1995) Alzheimer’s disease: fundamental and therapeutic aspects. *Experientia* 51: 99–105.
- Silos-Santiago I and Snider WS (1992) Development of commissural neurons in the embryonic rat spinal cord. *J. Comp. Neurol.* 325: 514–526.
- Wang AZ, Ojakian GK and Nelson WJ (1990) Steps in the morphogenesis of a polarized epithelium. *J. Cell Sci.* 95: 137–151.
- Willis WD and Coggeshall RE (1991) Sensory mechanisms of the spinal cord. *J. Comp. Neurol.* 310: 285–299.

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