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Chemical Engineering Science 59 (2004) 5931–5936

Chemical Engineering Science

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Analysis of cell aggregation in the culturing of cerebellar granule neurons

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Received 18 October 2002; received in revised form 5 July 2004; accepted 6 July 2004
Available online 26 August 2004

Abstract

We have proposed previously that an increased concentration of growth factor secreted by neurons themselves has a direct effect on survival of the neurons, and thereby cell density serves as a regulator of survival of neurons. In this study, the same idea was used to analyze the aggregation of cerebellar granule neurons in culture experimentally and theoretically. Assuming the transport of growth factor and substrate within an aggregate is by molecular diffusion, the metabolic efficiency of neurons, on the basis of an autocatalytic phenomenon, was increased within an aggregate compared to an identical quantity of dispersed cells. A good agreement in the size of neuronal aggregate between the theoretical prediction and the experimental result was found. This illustrates that growth factors produced by neurons acting in either an autocrine or paracrine manner play an important role during the development of cultured neurons.

Keywords: Neuron aggregation; Growth factors; Diffusion; Metabolism; Mathematical; Modeling

1. Introduction

During the past several decades, tremendous effort has been directed toward the development of methods for treating injuries in central and peripheral nervous systems by means of tissue transplantation or peripheral nerve grafting (Aebischer et al., 1987; Langone et al., 1995; Shoichet et al., 1994; Valentini et al., 1992). Therefore, the neuronal cell behavior on a biomaterial has attracted much attention because of its clinical importance and scientific interest (Carbonetto et al., 1982; Ruegg and Hefti, 1984; Schugens et al., 1995; Yavin and Yavin, 1974). A critical element in the design of strategies for neuronal regeneration is to understand the factors that promote the survival, growth and integration of neuronal cells. The neuronal survival and activities of specific populations of neurons involves a variety of growth factors and hormones (Snider, 1994). Many of these growth factors are produced by the neurons and act in either an autocrine or paracrine manner. In the previous paper (Young et al., 2000), we have described the dependence of survival of cerebellar granule neurons on the cell density. We suggested that specific local autocrine and paracrine growth factors are of importance in the neuronal survival. A mathematical model relating the average concentration of growth factor between cells necessary for cell survival was proposed.

In this work, the aggregation of cerebellar granule neurons, which often occurs in neuronal cultures, was observed experimentally and analyzed theoretically. The use of aggregated cellular transplants has been shown to enhance transplant function in comparison to singly transplanted cells (Hrewson and Saltzman, 1996). Although the self-association phenomenon of neuron cells can be considered as natural behavior of neurons, the explanation for why and how it occurs is not clear. A possible cause for this phenomenon may be that the metabolic efficiency of neurons inside an aggregate is higher than that of cells in the dispersed phase. In our theoretical model, a quantitative analysis describing cell–cell interaction by secreted growth factors acting in an autocrine or paracrine fashion promoting the neuronal metabolic efficiency within an aggregate

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doi:10.1016/j.ces.2004.07.027

was proposed. However, the metabolic efficiency of neurons in the central portion of a large clump may decrease the nutrient uptake compared to disperse cells due to diffusion limitations of nutrient. Therefore, the size of an aggregate should have an optimal magnitude. The main purpose of our investigation is to achieve a proper understanding of what is the optimal size of an aggregate.

2. Experimental

Commercially available poly (ethylene-co-vinyl alcohol) (EVAL) (105A, Kuraray Co. Ltd, Japan), having an average ethylene content of 44 mole%, was used as the cell culture substrate material. The EVAL polymer, used in the form of a membrane with smooth surface, was prepared by the direct immersion precipitation method (Cheng et al., 1998). Briefly, a 25 wt% of EVAL solution dissolved in DMSO was cast uniformly on a glass plate (the casting thickness was ca. 100 μm) using an autocoater (KCC303, RK Print-Coat Instruments, UK) and then immersed directly in water bath to form a membrane.

EVAL membranes were cut into circular discs of 16 mm diameter suitably sized for tissue culture plate wells. The membranes were sterilized with 70% alcohol ultraviolet light overnight and then rinsed extensively with phosphate-buffered solution (PBS). Subsequently, membranes were placed in 24 well tissue culture polystyrene plates (Corning, New York, USA) by placing a silicon rubber ring on top of each membrane (DeFife et al., 1995). Before cell culture, membranes were covered with 10 mg/l poly-L-lysine (Sigma) in PBS and incubated 2–3 h. The excess poly-L-lysine solution was then removed by suction and dried for another hour.

Cerebellar granule neurons were prepared from 7-day-old Wistar rats since the granule neurons can relatively easily be prepared from postnatal rat cerebellum and have been extensively used as a model system for neuronal survival and differentiation (Burgoyne and Cambray-Deakin, 1988). Briefly, neurons were dissociated from freshly dissected cerebella by mechanical disruption in the presence of trypsin and Dnase (Levi et al., 1984). Following preparation, cells were seeded onto EVAL membranes at a density of 4×10^5 cells/well in basal Eagle's medium (BME; Gibco) supplemented with 10% fetal calf serum (FCS; Gibco), 25 mM KCl, penicillin G (100 IU/ml) and streptomycin (100 μg/ml). The cell density was determined by a hemocytometer. Cultures were maintained at 37 °C in a humidified atmosphere of 95% air/5% CO_2. One day after plating the medium was changed and Cytosine arabinoside (10 μM) was added to prevent replication of non-neuronal cells. Immunocytochemical analysis of these primary cultures has shown that they contain 95% granule neurons (Thangnipon et al., 1993).

Cell aggregation was observed by using a scanning electron microscope (SEM) after 5 days of incubation since most development occurs during the first 3 days of culture (Mahoney and Saltzman, 1999). The cells adhering to the membrane were washed with PBS and then fixed with 2.5% glutaraldehyde in PBS for 1 h at 4 °C. After thorough washing with PBS, the cells were dehydrated by graded ethanol changes and then critical point dried. The membranes were then gold sputtered in vacuum and examined using a Hitachi S-800 microscope at 20 kV. In addition, cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay. The method of Mosmann (1983) was modified and used in this study. Briefly, after 1, 3 and 5 days of incubation, the 0.35 ml MTT (0.5 mg/ml in PBS) solution was added to each well. Since mitochondrial dehydrogenases of viable cells cleave selectively to the tetrazolium ring, yielding blue/purple formazan crystals, the level of the reduction of MTT into formazan can reflect the level of cell metabolism. After the cultures were incubated with MTT for 5 h, the medium was aspirated and the formazan reaction products were dissolved in 0.4 ml of 10% sodium dodecyl sulphate (SDS, Sigma) in PBS and the plates were then shaken for 15 h. The optical density of the formazan solution was read on an ELISA plate reader (ELx800, BIO-TEK,) at 570 nm. The absorbance was proportional to the cell viability on the membrane surface. Values are expressed as mean ± standard error of mean of four independent cultures and determinations. Statistical analyses were performed using Student's t-test.

3. Results and discussion

SEM of critical point membranes confirmed the presence of the neurons on the EVAL membranes throughout the 5 days of culture (Fig. 1). When neurons are cultured in vitro, neurons will undergo their morphological changes to stabilize the cell–biomaterial interface if an environment similar to the natural one is maintained. Fig. 1 shows that neurons exhibited typically spherical or ellipsoid morphology and extensive neurite development. In addition, after 5 days in culture large aggregates formed on the EVAL membrane that varied in size from a few to several tens of cells. Overall, these cells already showed features of neuronal differentiation. Cellular aggregates contained neurites and were connected to other aggregates. Thus, these neurites formed a web-like interconnecting network.

Fig. 2 shows the time course of formazan accumulation for the EVAL membrane and control. Control group is a glass coverslip placed in the tissue culture polystyrene well, which was treated in the same way as the test membrane-containing wells. The tendency of these two curves was similar. However, the EVAL membrane had a significantly higher accumulation of formazan compared to control ($p < 0.01$) at all time points. Since the level of the reduction of MTT into formazan can reflect the level of cell metabolism, the use of an EVAL membrane as a neuronal culture biomaterial appears to promote cell viability, which was consistent

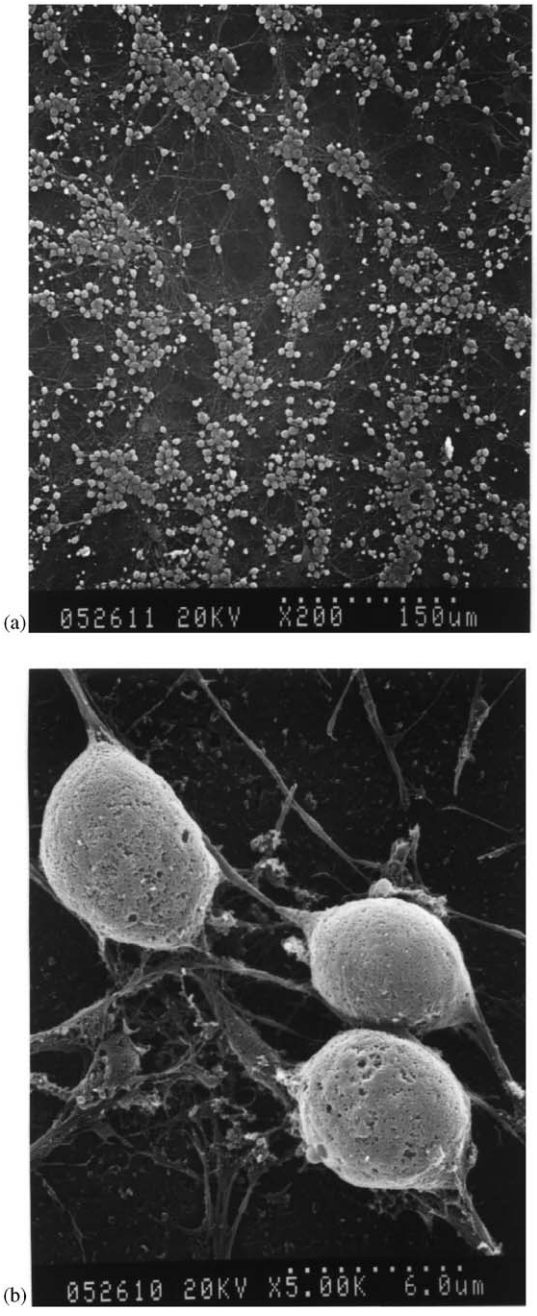

Fig. 1. (a) Scanning electron microphotographs of neurons seeded onto the EVAL membrane after 5 days in culture. (b) A high magnification of (a).

with observations of SEM that neuronal development was good (Fig. 1).

Primary cultures of dissociated neurons are used frequently in neurobiological studies. During cultured neuronal survival and differentiation, neurons are affected by cell–cell interactions, involving many growth factors secreted by neurons. In addition, neuronal behavior is also influenced by specific interactions between cell surface receptors and binding sites on the biomaterial. Thus, the

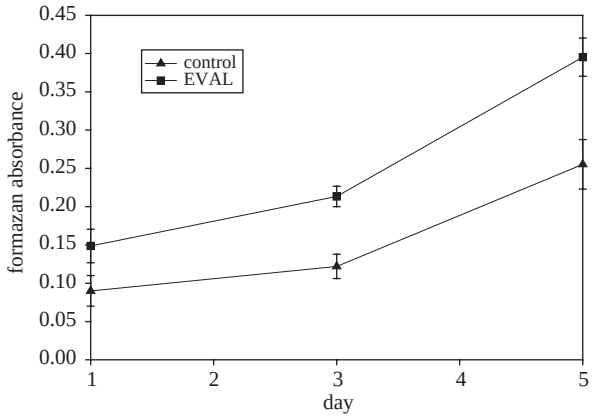

Fig. 2. MTT-tetrazolium assay. Formazan absorbance expressed as a function of time from cerebellar granule neurons seeded onto the EVAL membrane and control ($n = 4$).

competition between cell–cell and cell–biomaterial interactions can be reasonably considered to determine cell migration, leading to cell aggregation. That is to say both growth factors and components of the extracellular matrix are believed to guide nerve cells to their targets in culture. However, if a controlled environment such as the cell seeding density, the presence of serum or chemically defined medium is maintained, neuronal cells can tend to form aggregates even cultured on a variety of biomaterials (Ruegg and Hefti, 1984) (Mahoney and Saltzman, 1999). Consequently, although the biomaterial supporting nerve cells has an influence on cell migration, it seems that the interaction between biomaterial and neuron does not have a vital effect on the cell aggregation. In fact, the use of aggregated cellular transplants has been shown to enhance transplant function in comparison to singly transplanted cells (Hrewson and Saltzman, 1996). This allowed us to propose the cell–cell interaction other than cell–biomaterial interaction leads to cell aggregation. Since most neuronal cells require the presence of specific growth factors for cell survival and differentiation (Lindholm et al., 1993), the aggregation phenomenon in neuronal culture may be due to specific interactions between cells controlled by growth factors secreted by neurons. Growth factors may be certain neurotrophic compounds like brain-derived neurotrophic factors or multiple neurotrophic factors overlapping in the neuronal activities (Lindholm et al., 1993). In fact, growth factors have many physiologically important roles during and after the development of the nervous system.

3.1. Mathematical model

To elaborate quantitatively the dependence of neuronal aggregation on growth factors, a mathematical model was proposed. Although growth factors are certainly involved in the complex neuronal behavior, we assumed that growth factors are also necessary for cell aggregation. Suppose that a

neuron is capable of secreting some growth factors which, upon diffusing to receptors on its surface, serves to accelerate its metabolism. For simplicity, only the behavior of a representative growth factor denoted by X is considered in the subsequent.

Suppose that rate of substrate uptake, μ, is of Monod type, that is,

$$\mu = \mu_{\max} \frac{S}{K_m + S}, \tag{1}$$

where $\mu_{\max}$, S, and K_m denote the maximum value of μ, the concentration of substrate, and a constant, respectively. If K_m is relatively larger than S, Eq. (1) reduces to

$$\mu = K_s S, \tag{2}$$

where $K_s = \mu_{\max}/K_m$. We assume that μ is a function of the concentration of X (denoted by C), and for simplicity, they are assumed to be linearly correlated, that is, $K_s = k_s C$, where k_s is a constant. This implies that the rate of the substrate uptake of a neuron is controlled by growth factor produced from it or from other adjacent neurons. A pseudo steady-state material balance on the substrate in an aggregate gives

$$D_s \nabla^2 S = k_s C S, \tag{3}$$

where D_s denotes the effective diffusivity of substrate in a neuron aggregate, and ∇^2 represents the Laplacian operator.

A pseudo steady-state material balance for X in a neuron aggregate yields

$$D_c \nabla^2 C = k_d C - R_x, \tag{4}$$

where D_c is the effective diffusivity of X in a neuron aggregate, and k_d is a constant. The first term on the right-hand side of this equation represents the rate of dissociation of X, and the second term denotes the rate of production of X. We assume that the rate of production of X (R_x), takes the form

$$R_x = k_p C, \tag{5}$$

where k_p is a constant. This implies that the production of X is autocatalytic in nature. Substituting this expression into Eq. (4) yields

$$D_c \nabla^2 C = -k_c C, \tag{6}$$

where $k_c = k_p - k_d$.

As illustrated in Fig. 3, we assume that a neuron aggregate can be simulated by a disk of radius a and height h. Let S_b and C_b be the concentrations of substrate and X in the bulk phase. The cylindrical coordinates (r, θ, z) are adopted. The symmetric nature of the problem under consideration suggests that both S and C are independent of θ. Therefore, Eqs. (3) and (6) reduce to, respectively,

$$\frac{1}{r}\frac{\partial}{\partial r}\left(r\frac{\partial S}{\partial r}\right) + \frac{\partial^2 S}{\partial z^2} = (k_s C)\frac{S}{D_s} \tag{7}$$

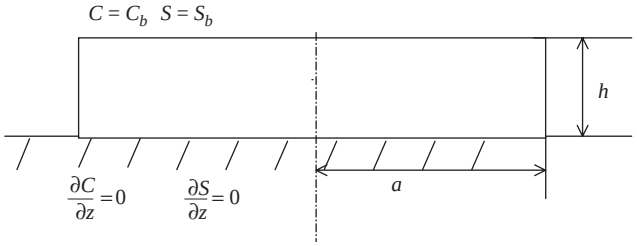

Fig. 3. A schematic representation of the mathematical model under consideration. A neuron aggregate is simulated by a disk of radius a and height h.

and

$$\frac{1}{r}\frac{\partial}{\partial r}\left(r\frac{\partial C}{\partial r}\right) + \frac{\partial^2 C}{\partial z^2} = -k_c C\frac{1}{D_c}. \tag{8}$$

The associated boundary conditions are assumed as

$$C = C_b \text{ and } S = S_b \text{ at } r = a, \quad z = h, \tag{9}$$

$$\frac{\partial C}{\partial z} = 0 \text{ and } \frac{\partial S}{\partial z} = 0 \text{ at } z = 0. \tag{10}$$

The last expression implies that the bottom of an aggregate is impermeable to both substrate and X. For a simpler mathematical manipulation, Eqs. (7)–(10) are rewritten in scaled forms as

$$\frac{4}{x}\frac{\partial}{\partial x}\left(x\frac{\partial S'}{\partial x}\right) + \beta^2\frac{\partial^2 S'}{\partial y^2} = C'\beta^2\alpha^2 S', \tag{11}$$

$$\frac{4}{x}\frac{\partial}{\partial x}\left(x\frac{\partial C'}{\partial x}\right) + \beta^2\frac{\partial^2 C'}{\partial y^2} = -C'\beta^2\delta^2 \tag{12}$$

$$C' = 1 \text{ and } S' = 1 \text{ at } x = 1, \quad y = 1, \tag{13}$$

$$\frac{\partial C'}{\partial y} = 0 \text{ and } \frac{\partial S'}{\partial y} = 0 \text{ at } y = 0. \tag{14}$$

In these expressions $x = r/a$, $y = z/h$, $S' = S/S_b$, $C' = C/C_b$, $\alpha = h[k_s(C_b/D_s)]^{1/2}$, $\delta = h(k_c/D_c)^{1/2}$, $\beta = 2a/h = (4N/\pi)^{1/2}$, N being the number of neurons in an aggregate.

Following the approach of Logan and Hunt for the case of plant cells (Logan and Hunt, 1988), we define the metabolic efficiency for neurons ε as $\varepsilon =$ (metabolic rate of aggregated neurons/metabolic rate of dispersed neurons). Suppose that the metabolic rate of neurons is proportional to the rate of substrate uptake. Then,

$$\varepsilon = \frac{\int_0^h \int_0^a k_s C S(2\pi r)\,\mathrm{d}r\,\mathrm{d}z}{(\pi a^2 h/\pi(h/2)^2 h)\left[\int_0^h \int_0^{h/2} k_s C S(2\pi r)\,\mathrm{d}r\,\mathrm{d}z\right]}. \tag{15}$$

The metabolic efficiency greater than unity identifies conditions whereby neuron association within an aggregate is more favorable to growth than dispersed neurons. Fig. 4 shows the variation of metabolic efficiency ε as a function of the size of a neuron aggregate N at various values of α.

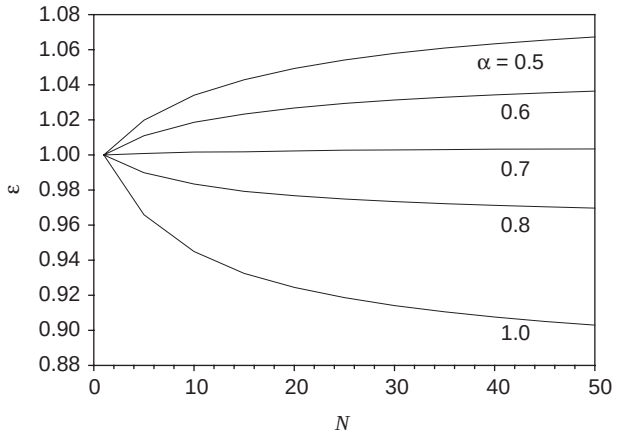

Fig. 4. Variation of metabolic efficiency ε as a function of the size of a neuron aggregate N for various values of α for the case $\delta = 1$.

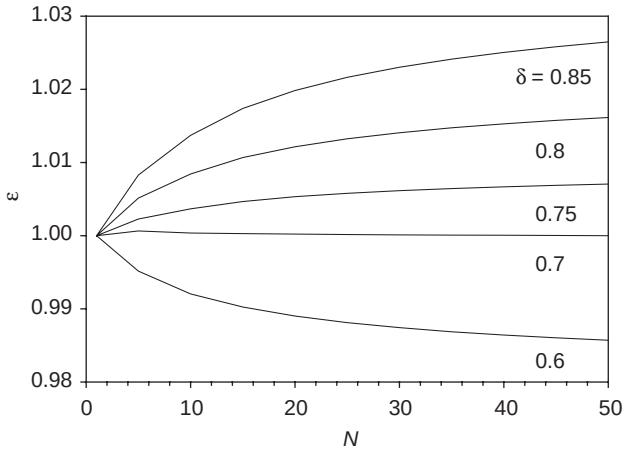

Fig. 5. Variation of metabolic efficiency ε as a function of the size of a neuron aggregate N for various values of δ for the case $\alpha = 0.5$.

As can be seen from this figure, the ε can exceed unity as $\alpha = 0.7$ and the smaller the α the larger the ε for a fixed N. The definition of α suggests that it can be viewed as the ratio (rate of substrate uptake/rate of substrate diffusion). If α is small, the rate of substrate diffusion within an aggregate is faster than the rate of substrate uptake by aggregated cells, and, therefore, the concentration of substrate within the aggregate is close to a uniform distribution. This suggests that substrate supply to aggregated neurons by molecular diffusion is not less than substrate supply to dispersed cells. In this case, the aggregation of neurons is not impeded by substrate supply and even has the effect of increasing the concentration of X (see the next section) and, therefore, the rate of metabolic of neurons. On the other hand, if α is large, the rate of substrate diffusion of within an aggregate is slower than the rate of substrate uptake for the neurons inside the aggregate, that is, the formation of a neuron aggregate becomes disadvantageous. Therefore, the value of ε is always less one at α-values above 0.7 because the aggregate provides a larger resistance for the diffusion of substrate. Also, the larger the N the smaller the ε. This is justified in Fig. 4.

For the case α is small, Fig. 4 reveals that the larger the N the larger the ε, however, the increase rate of ε declines as N increases. This implies that the metabolism efficiency could be higher in larger cell aggregates, but the driving force for cell aggregation is not so strong. Thus, the size of a neuron aggregate tends to have an upper limit, since forming a bigger aggregate has no obvious advantage as far as the metabolic rate is concerned. In fact, during the neuron culture, a very big cell aggregate will lead to rapid depletion of substrate from the medium, limiting the size of aggregate. In practice, h is on the order of 5×10^{-6} m, D is in the range 10^{-10}–10^{-9} m^2/s, $K_s(=k_s C_1)$ is in the range 1–10^7 1/s, k_s is in the range 10^2–10^{10} (m^3/s mol), K_p is in the range 1–10^7 1/s, and C_b is in the range 10^{-6}–10^{-5} M (or 10^{-3}–10^{-2} mol/m^3). Based on these, the value of α is in the range 0.05–5000, and δ is in the range 0.158–1581. According to Fig. 4, the upper limit of a neuron aggregate is about 35 because ε appears to approach a constant value when N exceeds 35 for the case $\alpha = 0.6$. The upper limit is consistent with experimental observation.

The metabolic efficiency ε of a neuron aggregate depends also on the value of δ. The variation of metabolic efficiency ε as a function of the size of a neuron aggregate N at various values of δ is presented in Fig. 5. Similar to the value of α, at $\delta = 0.7$, the metabolic rates of aggregated neurons and dispersed neurons are nearly equivalent, i.e. $\varepsilon = 1$, however, the larger the δ the larger the ε for a fixed N. The definition of δ suggests that it can be viewed as the ratio (net production rate of X/diffusion rate of X. If δ is large, the rate of net production of X by aggregated cells is faster than the rate of diffusion of X in a neuron aggregate. In this case the formation of a neuron aggregate has the effect of increasing the concentration of X within the aggregate, and therefore the metabolic rate of neurons.

Also, for the cases $\delta = 0.8$ and 0.85, the larger the size of an aggregate the larger the ε, as can be seen from Fig. 5. However, the metabolic efficiency ε is not significantly increased for $N > 35$ but is sharply increased in small N from 2 to 10. This suggests that the ε can be greatly affected when only several cells are enclosed in a small aggregate. On the other hand, cells will not aggregate to a very large size because the increasing rate of the metabolic efficiency of a neuron aggregate becomes less sensitive to the aggregate size for $N > 35$ even though the metabolic efficiency ε of a neuron aggregate is continuously increased. This is consistent with experimental observation that neuron aggregates varied in size from a few to several tens of cells.

Conversely, if δ is small, the rate of diffusion of X in a neuron aggregate is faster than the net production rate of X in the aggregate, then the growth factor inside the aggregate will be directly depleted, leading to a low concentration of X distribution. In this case, the formation of an aggregate becomes disadvantageous to the metabolic of neurons within

the aggregate. Furthermore, the larger the N, the smaller the ε. This is justified by Fig. 4. Therefore, there is no advantage for cell aggregation as the rate for X diffusing out from a cell exceeds the production rate of X. Even a dispersed cell will be dead in this circumstance since it does not have enough X for metabolism. Thus, the curve at $\delta = 0.6$ is impractical because cells cannot survive. The benefit for an aggregate is that the growth factor secreted by neurons can accumulate to a higher concentration. Every neuron in this environment can make use of the growth factor secreted by itself or other adjacent neurons. This suggests that cell metabolism is accelerated by secreted growth factors acting in an autocrine or paracrine fashion to promote cell aggregation. In contrast, if cells are singly distributed, the concentration of X around a neuron is very low since most of X will be depleted rapidly. This indirectly explains that neuronal survival was inappreciable if inoculum cell density was below a critical level (Young et al., 2000).

4. Conclusion

The model as presented here required several assumptions and the use of empirically experimental data to understand the role of the parameters α and β in cell aggregation during the culturing cerebellar granule neurons. Although considerable uncertainty exists in this model such as diffusivities of substrate and growth factor, there are contributions leading to the understanding of neuronal aggregation. Calculations indicate that the presence of the smaller α and the larger δ-values within an aggregate can offer an advantage to accelerate the metabolic efficiency of aggregated neurons because the concentrations of substrate and growth factor must be sufficiently high enough to increase substrate uptake in response to different conditions. Therefore, substrate and growth factors with high and low diffusivities, respectively, are more favorable to the metabolic efficiency of aggregated neurons than freely dispersed cells. This offers a logical explanation for a commonly observed phenomenon of cell aggregation.

Acknowledgements

The authors thank the National Science Council of the Republic of China for their financial support of this research.

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