行政院國家科學委員會專題研究計畫成果報告

神經細胞在高分子薄膜上行為之研究
Studies on the behavior of cultured cerebellar granule neurons on the polymer membranes

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一、　中文摘要

本研究計畫為「神經細胞在高分子薄膜上行為之研究」，以 MTT 細胞計數實驗，及掃描式電子顯微鏡來觀察細胞的貼附情形和生長情形，並以理論探討神經細胞培養在人造環境所引發的相關問題，藉不同狀況的臨界接種密度觀察神經細胞之存活率外，同時將建立數學模式分析臨界接種密度與細胞本身分泌生長因子分佈情形的關係，藉此評估神經細胞在薄膜表面的行為表現，開發適合神經細胞培養的高分子薄膜，期盼有實用之價值，能應用於臨床實際之需求。

關鍵詞：神經細胞、高分子薄膜、數學模式

Abstract

In this projct, the in vitro interaction of cerebellar granule neurons and poly (ethylene-co vinyl alcohol) (EVAL) membranes will be investigated. Cell viability is studied using MTT assay. The membrane structure and its relationship with cells are examined by scanning electron microscopy. In addition to experimental observation, the dependence of survival of cerebellar granule neurons on the cell density will be examined theoretically. In general, cell survival index is inappreciable if cell density is below a critical level. If cell density exceeded this critical value, cell survival index increases with the increase in cell density. We suggest that not only cell density promotes survival of neurons but also an increased concentration of growth factors produced by neurons has a direct effect on survival of the neurons. Therefore, in this project, a quantitative model describing the distribution of the growth factor at different cell densities will be proposed to investigate the role of cell density in the survival of the neurons.

Keywords: neuron , polymer membranes, mathematical model

INTRODUCTION

The behavior of neuronal cell on a biomaterial has attracted increased attention in recent years because of its scientific interest and clinical importance. Besides extracellular matrix substrates, other mechanisms such as growth factors and hormones both *in vitro* and *in vivo* can affect neuronal survival and activities of specific populations of neurons[1].

In the present study we analyzed the effect of cell density on the survival of neurons in culture. Regardless of the effect of substrate, a minimum seeding density of 2.2×10^5 cells/cm^2 was required. In addition, neuronal survival was significantly increased by using conditioned medium from dense cultures. Therefore, not only cell density promotes survival of neurons but an increased concentration of the growth factor produced by neurons has a direct effect on survival of the neurons. A quantitative model, which relates the survival behavior and cell density describing the distribution of the growth factor, is proposed.

MATERIALS AND METHODS

Poly (ethylene-co-vinyl alcohol) (EVAL)

was used as substrate material. The substrate used in the form of a membrane with dense structure was prepared by solvent evaporation in a vacuum oven.

Cerebellar granule neurons were prepared from 7-day-old Wistar rats[2]. Following preparation, cells were seeded onto EVAL membranes with cell densities ranging from 0.55×10^5 cells/cm^2 to 35.2×10^5 cells/cm^2. One day after plating the medium was changed and Cytosine arabinoside was added to prevent replication of non-neuronal cells.

After 5 days of incubation, cell survival was determined by the MTT colorimetric assay. The optical density of the formazan solution was read on an ELISA plate reader at 570 nm. The absorbance was proportional to the number of living cells present. Statistical analyses were performed using Student's t-test.

MATHEMATICAL MODEL

To elaborate quantitatively the dependence of neuronal survival on cell density, a mass transfer model was proposed. Suppose that the growth of neuronal cells is controlled by one or several growth factors, that is, the growth of cells occurs only if the concentration of these growth factors exceeds a critical level. We assume that the growth factor at cell surface diffuses toward the bulk liquid phase driven by the concentration gradient established near the surface. Apparently, the concentration of the growth factor near a neuron is dependent upon cell density. The phenomenon under consideration can be simulated by Fig.1. Here, we consider two adjacent cells each has radius R. Let L be the center-to-center distance between two cells and C be the concentration of the growth factor. At steady state the spatial variation in the concentration of the growth factor is governed by

$$\nabla^2 C = 0 \qquad (1)$$

Let C_b be the concentration of growth factor far away from substrate surface and C_s be the average concentration of growth factor in a cell. Then the boundary conditions associated with Eq.(1) are

$$C = C_b \quad \text{as} \quad \sqrt{x^2 + y^2} \to \infty \qquad (2)$$

$$\partial C / \partial y = 0 \quad \text{at y=0} \qquad (3)$$

$$-D\frac{\partial C}{\partial r} = k(C_s - C_b) \quad \text{at cell surface} \qquad (4)$$

In these expressions D and k are respectively the diffusivity of growth factor and its mass transfer coefficient, and r is the radial distance from the center of a cell. For a simpler mathematical treatment, Eqs.(1)-(4) are rewritten in a scaled form by defining $C'=(C-C_b)/(C_s-C_b)$, $r'=r/R$, $x'=x/R$, and $y'=y/R$. We have

$$\nabla^2 C' = 0 \qquad (5)$$

$$C' \cong 0 \quad \text{as} \quad \sqrt{x'^2 + y'^2} \to \infty \qquad (6)$$

$$\partial C' / \partial y' = 0 \quad \text{at y'=0} \qquad (7)$$

$$-\frac{\partial C'}{\partial r'} = \frac{Rk}{D} \quad \text{at cell surface} \qquad (8)$$

RESULTS AND DISCUSSION

As shown in Figure 2, cell survive index was higher at higher cell densities when seeded cell density less than 8.8×10^5 cells/cm^2. At very lower cell density (less than 1.1×10^5 cells/cm^2), almost no neurons survived.These data suggest cell density promotes neuronal survival. Moreover, optical microscope observation confirmed that neuron culture at higher cell density enriches for differentiated neuronal cells as shown in Figure 3.

Cell survive index increased with increasing cell density when the cell density is above a critical level. Based on the above results, an approximately minimum density of 2.2×10^5 cells/cm^2 was required for neurons to survive and to initiate cellular differentiation.

The density-dependent increase in neuronal survival may be due to specific interactions between neuronal cell surface receptors controlled by growth factors secreted by neurons.[3] To justify this concept, medium from dense cultures

($8.8x10^5$ cells/cm^2) after 5 days of incubation was added to a fresh medium for low density cultures ($2.2x10^5$ cells/cm^2).The composition of conditioned medium from dense cultures and fresh medium is a 1:3 ratio. Therefore, the conditioned medium contained higher concentrations of growth factors than a fresh medium. Figure 4 shows that conditioned medium significantly promoted the survival index of neurons at low density (2.2×10^5 cells/cm^2) after 5 days of incubation. The cell survival index increased from 0.99 ± 0.02 using fresh medium to 2.0 ± 0.03 using conditioned medium, only slightly less than that in the 4.4×10^5 cells/cm^2 of cultures using fresh medium. This clearly indicates that not only cell density promotes neuronal survival but also medium composition has a direct effect on the granule neurons. Thus, the density-dependent increase in neuronal survival could be indirectly due to an increased concentration of growth factors produced by neurons.Furthermore, if cell density exceeds a certain level ($\geq 8.8x10^5$ cells/cm^2), cell survival index starts to decrease, as seen in Figure 2.

Figure 5 shows the simulated contours for the spatial variation in the scaled concentration of growth factor. Due to the symmetric nature of the system under consideration only the right-half contours are presented. The value of L' (=L/R) is based on the cell density of $4.4x10^5$ cells/cm^2 and R=2.5 μ m. Figure 5 reveals that the concentration of growth factor decreases with the distance from cell surface, as expected. In addition, growth factors residing in the culture medium between the cell and the substrate play an important role. As shown in Figure 5, growth factors between the cell and the substrate richly distributed, which suggests a condition favorable for cell survival. Hence, the density-dependent increase in neuron survival may be related to the variation of the concentration of growth factors between cells, but less related to the substrate used.

Suppose that a necessary condition for cells to survive is that the concentration of the growth factor needs to exceed a critical level. For simplicity, the level of growth factor at the midpoint of the line segment joining the centers of two adjacent cells, C_c, is chosen as a measure for the averaged concentration of the growth factor between two cells. The variation in cell survival index as a function of the scaled C_c, $C_c^{'}$, is illustrated in Figure 6. According to this figure, the critical level for $C_c^{'}$ is about 7, that is, to achieve an acceptable degree of survival, $C_c^{'}$ must exceed 7. The dependence of cell survival index on $C_c^{'}$ can also be justified by varying the level of growth factor in culture medium. As described in the last section, a conditioned medium comprised 25% of medium from dense cultures ($8.8x10^5$ cells/cm^2) and 75% of fresh medium was used to culture neurons at cell density of $2.2x10^5$ cells/cm^2. After 5 days of incubation, the $C_c^{'}$ value of the conditioned medium is about 7.86 based on the mass balance principle, and cell survival index is about 2 according to Figure 6 which is very close to the experimental result (Figure 3).

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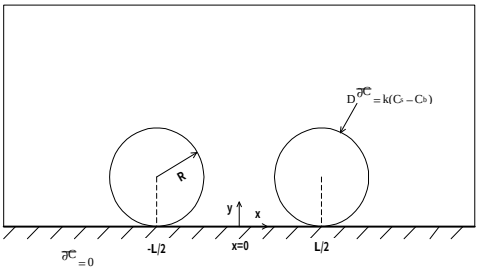


Fig.1. Schematic representation of the model considered. **R** is the radius of a cell, L is the center-to-center distance between two cells, n is the

normal of cell surface, C is the concentration of growth factor, C_s and C_b are respectively the value of C at cell surface and in the bulk phase, **D** and k are the diffusivity and the mass transfer coefficient of growth factor.

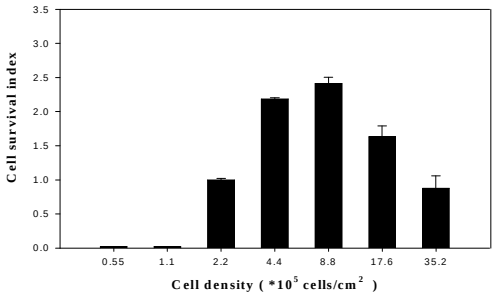
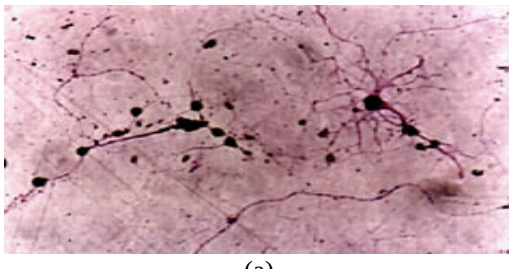
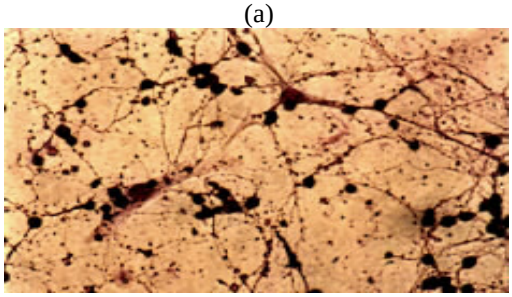


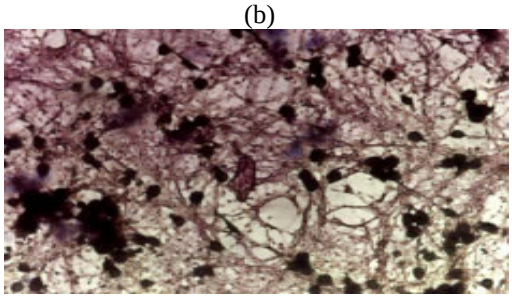
Fig.2. Survival of cerebellar granule neurons plated for five days at different cell densities. Cell survival index was expressed as (MTT colorimetric assay x 10^7 /originally plated cells).



(a)



(b)



(c)

Fig3. Photomicrographs of cerebellar granule neurons seeded onto the EVAL membrane at different cell densities after 5 days in culture (oruginal magnification x400). (a) 2.2 x 10^5 cells/cm^2 (b) 4.4 x 10^5 cells/cm^2 (c) 2.2 x 10^5 cells/cm^2

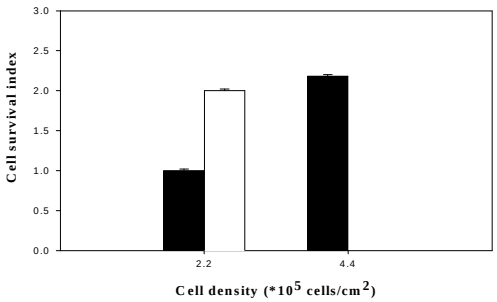


Fig.4. Survival index of cerebellar granule neurons plated for five days using different medium at different densities (¥: fresh medium and ¡: conditioned medium).

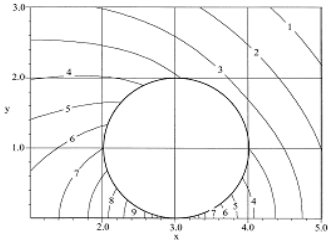


Fig.5. Contours for the spatial variation in the scaled concentration of growth factor.Parameters used are R=2.5μm, L'=L/R=6.03, and Rk/D=1 (Bird et al., 1960).Curves 1, C'=6.0; 2, C'=6.5; 3, C'=7.0; 4, C'=8.0; 5, C'=8.5; 6, C'=9.0;7, C'=9.5; 8, C'=10.5; 9, C'=11.

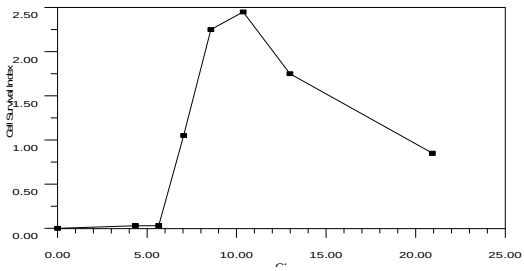


Fig.6. Variation of cell survival index as a function of the scaled concentration of growth factor at the midpoint of the line segment joining the centers of two cells, $C_c^{'}$ for the case R=2.5μm and Rk/D=1 (Bird et al., 1960).