Growth Cones in Developing Cultured Cortical Neurons

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ABSTRACT. Large numbers of growth cones were present in 6-day-old primary cultures of cerebral hemispheres from fetal rats. The average size of the growth cones was 24 by 28 μm . Many of these growth cones had both veil-like lamellipodia and filopodia. A few cones remained in 21-day-old cultures. These also had lamellipodia and filopodia. Ganglioside GM₁ was present in both 6-day-old and 21-day-old cultured growth cones.

Growth cones at the terminals of outgrowing neurites are responsible for the formation of correct neuronal connections during development because they are the part of the neuron that recognizes its specific target cell. The structure of the growth cone and its function *in vivo* (10, 25, 26) and *in vitro* (1, 8, 15, 18, 20) recently has received much study. A major obstacle in doing such studies is the difficulty in obtaining pure nerve cell cultures after seeding. Non-neuronal cells, especially astrocytes, readily proliferate during culture and appear as the major contaminant within a short period. We largely overcame this problem by using an astrocyte-deficient culture in which neurons are allowed to grow but glial cell growth is limited by the culture conditions. This was accomplished by inhibiting glial cell growth by the complement-dependent, antibody-mediated destruction of glial cells (6).

We are interested in determining the mechanism by which an outgrowing growth cone selects the appropriate pathway to its target cell. Cell surface molecules are assumed to behave as an antigen-antibody system by which a neuron-neuron connection is established. One candidate for the active cell membrane constituent that functions in the formation of correct connections is the gangliosides (17). Gangliosides are abundant in neural tissue and may act as receptors for circulating ligands or in intercellular interactions (14). Therefore, the cell surface membrane and gangliosides may indeed be intimately related. We here report on our investigation of whether ganglioside GM₁ is localized in the growth cones of outgrowing neurites.

MATERIALS AND METHODS

The technique used to produce astrocyte-deficient cultures from the embryonic rat cerebral cortex has been described elsewhere (6). Briefly, cerebral hemispheres were dissected from the

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Abbreviations used: MEM, Eagle's minimum essential medium; SFCS, semi-fetal calf serum; SEM, scanning electron microscopy; ABC, avidin-biotin complex; NF, neurofilament; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

brains of 17-day-old fetal rats then dissociated by extrusion through a stainless steel mesh (140 μ m opening size, 100 mesh screen) into a culture dish that held Eagle's minimum essential medium (MEM) containing 15% semi-fetal calf serum (SFCS) (Mitsubishi Chemical Industries Ltd. Tokyo); 0.6% glucose; 0.05% NaHCO_3 ; 292 mg/l of L-glutamine; 100 μ g/ml of kanamycin; anti-GFAP antiserum obtained from DAKO, CA, USA, diluted 1:1000 in MEM; and lyophilized guinea pig serum obtained from Nippon Biosupply Center, Tokyo, diluted 1:700 in MEM, titer 1:71.4 for the complement-dependent, antigenic antibody-mediated cytotoxic cultures.

After a 30 min incubation with shaking, the cells were separated from the medium by centrifugation at $500\times g$ for 7 min. The experimental medium then was replaced with fresh MEM without antibody and complement. Cell density was 10^4 visible cells per 73×15 mm glass Leighton tube (Matsunami glass Inc. Tokyo) topped with a poly-L-lysine-coated microcoverglass (12×32 mm). The medium was replaced after the first day and renewed every fourth day during culture for 21 days in 3% CO_2 and 97% air at 37°C . The biotinylated cholera toxin that was used to identify ganglioside GM_1 was prepared as described elsewhere (4, 5). Briefly, cultured cells were fixed with a 4% solution of paraformaldehyde then treated at 4°C overnight with biotinylated cholera toxin at 5 μ g/ml in PBS. After several washings with PBS containing BSA (2 mg/ml), the cells were treated for 30 min at 37°C with avidin-peroxidase at 5 μ g/ml in PBS.

A fluorescence microscopic study was done to identify neurons (5), with anti-neurofilament antibody as the cell marker (3). Immunocytochemical staining was done to analyze the growth cone. The method of Sternberger (23) was used to measure the growth cone dimensions, the intersection of the long axis of the axon with the leading margin of the growth cone being measured according to the method of Bray and Chapman (8) (see Fig. 2b).

Scanning electron microscopy (SEM) was used after rinsing the cell cultures on the coverslips with PBS and slowly infusing them for 120 min at room temperature with a fixative containing 1% glutaraldehyde in 0.15 M PBS, then rinsing them with PBS and dehydrating the preparation sequentially with 50, 70, 80, 90 and 100% ethanol.

After dehydration, the coverslips were critical point dried (Sorvall Apparatus) then coated with Au-Pd in a Sputter Coater and viewed with an S-450 Hitachi (Japan) scanning electron microscope (2).

RESULTS

Neurons from 3-day-old astrocyte-deficient cultures were identified by immunofluorescent staining with the avidin-biotin complex (ABC). Neurons and a lesser number of oligodendrocytes were the major cell components. The percentage of GFAP-positive cells had been reduced to a range of 2–8% in these cultures. Biotinylated anti-neurofilament antiserum was used to identify neurons (Fig. 1). Table 1 gives the average dimensions of 56 growth cones in 6-day-old cultures of cortical neurons. Types 2 and 3 accounted for 65% of the total growth cones. Equal numbers of types 1 and 4 were present. Immunoperoxidase staining of 6-day-old cultures from rat embryonic cerebral cortex (Fig. 2) for ganglioside GM_1 with biotinylated cholera toxin showed that ganglioside GM_1 may be localized in the growth cone. Cones also were stained positively with anti-ganglioside GM_1 antibody (data not shown) (6). Many neurons bearing various veil-like lamellipodia and prominent filopodia were present. Although there are many variations in form, type 1 growth cones are larger, more articulated and have a variety of lamellipodia (some with long

Fig. 1. Immunofluorescence staining for NF protein in 3-day-old primary cultures. Neurites and growth cones of neurons stained with anti-NF antibody (asterisk). Bar=20 μ m

microtips of filopodia) than do types 2, 3 and 4.

Immunoperoxidase staining for ganglioside GM₁ in 21-day-old cultured cells (Fig. 3) showed that ganglioside GM₁ is localized in the growth cones. Neuritic processes or cortical neurons clearly were present in these long term cultures (Fig. 3a, arrow). The number of growth cones in 21-day-old neurons were few, but they still had a very few veil-like structures. Various short filopodia-like structures could still be seen in these cultures. The surprising finding was that a form of the type 2 growth cone and its phenomena (veil-like lamellipodia and filopodia-like structures) survived for 3 weeks (Fig. 3b, large arrow).

TABLE 1. CHARACTERISTICS OF GROWTH CONES IN PRIMARY CULTURES OF EMBRYONIC RAT CEREBRAL CORTEX

Measurements	Type of Growth Cone (μ m)			
	Type 1	Type 2	Type 3	Type 4
Overall Width (Wf)	35.5 $\pm$ 4.1	23.7 $\pm$ 2.8	13.9 $\pm$ 2.6	6.2 $\pm$ 1.2
Base Width (Wi)	12.6 $\pm$ 1.2	9.6 $\pm$ 2.4	6.2 $\pm$ 1.4	2.3 $\pm$ 0.8
Overall Length (Lf)	36.1 $\pm$ 5.5	28.1 $\pm$ 7.5	22.2 $\pm$ 5.5	14.4 $\pm$ 3.9
Base Length (Li)	23.6 $\pm$ 6.2	18.6 $\pm$ 8.3	13.3 $\pm$ 2.4	8.0 $\pm$ 3.3
Proportion of Growth Cone (%)	17.9 $\pm$ 4.8	42.9 $\pm$ 5.9	21.4 $\pm$ 3.4	17.8 $\pm$ 2.3

The culture was 6 days old at the time of measurement. Data given are the Means $\pm$ SD for the 56 growth cones measured.

Fig. 2. Immunoperoxidase staining for ganglioside GM₁ with the avidin-biotin complex system in 6-day-old primary culture growth cones of embryonic rat cerebral cortex. Bar=7.6 μ m (2a), Bar=15 μ m (2b). Various veil-like lamellipodia bearing numerous filopodia were stained with ganglioside GM₁ (2a, arrows). One of the microtips of the filopodia of the cone extends to the target neurite (Fig. 2a, b, arrowhead). The dimensions given are for overall length (Lf), base length (Li), overall width (Wf) and base width (Wi) as described by Bray and Chapman (1985).

Fig. 3. Immunocytochemical staining for ganglioside GM₁ in 21-day-old primary culture growth cones and neurites of rat cortical neurons. Note that growth cones are still present in the culture (arrowhead) and various short filopodia-like structures also are present (3b, large arrow). Many growth cone-like structures remain (arrowhead).
Bar=7.6 μ m (3a), Bar=15 μ m (3b)

Fig. 4. Scanning electron micrograph of cortical neurons in culture. Growth cones are visible (arrow). Bar=14.5 μ m

The scanning electron microscopic techniques used provided us a convenient means with which to survey the surface of the neurons in order to characterize their morphological features. A growth cone with filopodia (microspike-like) structure is clearly visible in Fig. 4.

DISCUSSION

Results of numerous morphological studies of growth cones *in vivo* and *in vitro* have been reported (7, 9, 19, 21, 24, 27), but no statistical analysis of the dimensions of the growth cone structure in cultured cerebral cortical neurons has yet been published. We here have identified growth cones that have both filopodia and lamellipodia.

Four types of growth cones also have been characterized by size. About 65% of the total cones had the dimensions of types 2 and 3 (Table 1). One of the microtips of the filopodia extends to the target neuron (Fig. 2a, b, arrowhead). Our observations suggest that filopodia may interact specifically with particular neurons in their pathway.

Recently, Algiro et al. (1), who used the superior cervical ganglia of the rat, reported that the postnatal nerve growth cones lacked definitive lamellipodia and the filopodia were few. These findings are comparable to our findings with 21-day-old nerve growth cones in an astrocyte-deficient culture. These cones appeared considerably more flattened and had short lamellipodia with branched filopodia (Fig. 3b, large arrow). The veil-like lamellipodia in the long term developing cultures appear to differ from those early in culture. A surprising finding was that one of the tips of the filopodia of the cone that extended to the target neurite still was present in the 21-day-old culture (Fig. 3b, small arrow). Our findings indicate that neurons retain the ability to seek out and extend their processes to target cells for at least 21 days in culture. This ability was retained in the absence of astroglial cells, evidence that these cells have no role in this process.

Little is known about the chemistry of the growth cone membrane (12, 16, 22) and of how its components are inserted. We have shown that ganglioside GM₁ is one such constituent in both 6-day-old and 21-day-old primary cultured cortical neurons. Movement of the growth cone is mediated by various environmental factors present in cultures (11, 13), and ganglioside GM₁ may function in the establishment of intercellular connections. The culture system we have described appears to be a useful model system with which to study the biochemical, physiological and morphological aspects of the growth cone function.

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