Scanning Electron Microscopy

Volume 1985
Number 2 *Part II*

Article 33

2-22-1985

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Kim, Seung U.; Kim, Kookmin M.; Moretto, Giuseppe; and Kim, James H. (1985) "The Growth of Fetal Human Sensory Ganglion Neurons in Culture: A Scanning Electron Microscopic Study," *Scanning Electron Microscopy*: Vol. 1985 : No. 2 , Article 33.
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SCANNING ELECTRON MICROSCOPY/1985/II (Pages 843-848)
SEM Inc., AMF O'Hare (Chicago), IL 60666 USA

0586-5581/85$1.00+.05

THE GROWTH OF FETAL HUMAN SENSORY GANGLION NEURONS IN CULTURE: A SCANNING ELECTRON MICROSCOPIC STUDY

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(Paper received March 6 1984, Completed manuscript received February 22 1985)

Abstract

Sensory neurons of 8-week human fetal dorsal root ganglia were dissociated into single cells by trypsinization and cultured on coverslips for 4 months, in either serum-containing or serum-free chemically defined media. At different times the cultures were fixed and prepared for scanning electron microscopy. Fetal sensory neurons in culture regenerated axons within 24 hours which were characterized by axonal growth cones at their tips and the neuronal perikarya assumed spherical or a bonnet-like morphology.

Key Words: Human, neurons, dorsal root ganglion, culture, scanning electron microscopy

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Introduction

Tissue culture of neurons and glial cells provides an excellent opportunity to study some basic properties of these cells under relatively simple and carefully controlled environments.

There have been several studies dealing with the growth and differentiation of human fetal neurons in culture obtained from both central and peripheral nervous systems (Lapham and Markesbery 1971, Choi and Lapham 1974, Kim 1976, Crain et al. 1980, Baron-Van Evercooren et al. 1982, Zeevalk et al. 1982, Kim et al. 1984). No three-dimensional study, however, of these fetal human neurons has been made. This is undertaken in the present study.

The scanning electron microcsope is a useful tool for obtaining a three dimensional view of the features of fetal human neuron isolated and grown in culture, and we hope that the results generated by the study will facilitate a better understanding of the behaviour of neurons during the early phase of neurogenesis details of which are not available at present.

Materials and Methods

Dorsal root ganglia of a human fetus (18 weeks) were obtained 2 hours after the suction abortion (legal abortions were performed in an authorized clinic and consent forms for experimental use of fetal tissue were obtained) and washed in Hanks' balanced salt solution (BSS). The age of fetuses was established by menstrual history. After the removal of connective tissue, the ganglia were incubated in 0.25% trypsin in calcium- and magnesium- free BSS for 20 min. at 36°C, washed twice in BSS and dissociated into single cells by repeated pipetting with siliconized, fire-polished Pasteur pipette. One hundred microliters each of cell suspension in feeding medium was seeded onto polylysine- and collagen-coated 15 mm glass coverslips placed in 60 mm plastic Petri dishes. Feeding medium was composed of Eagle's minimum essential medium supplemented with 5% fetal calf serum, 5 mg/ml glucose, 50 ng/ml nerve growth factor (NGF) and 20 μg/ml gentamicin.

After 24 hours in serum-containing medium,

some cultures were transferred to serum-free chemically defined medium that is modified from the earlier formula specificially designated for rat astrocytes (Kim et al. 1983).

The serum-free chemically defined medium was composed of F12 medium supplemented with insulin (20 μg/ml), human transferrin (10 μg/ml), fetuin (0.5 mg/ml), triiodothyronine (3×10^{-8}M), hydrocortisone (5×10^{-8}M), sodium selenite (3×10^{-8}M), ascorbic acid (10 μg/ml), retinol (50 ng/ml), glucose (5 mg/ml), NGP (50 ng/ml), gentamicin (20 μg/ml) and HEPES (10^{-2}M). Cultures were grown in a CO_2 incubator in an atmosphere of 5% CO_2 - 95% air at 36°C and fed with fresh medium twice a week.

After the various time periods in culture, starting 24 hours after plating, coverslips carrying cells were fixed in 3% glutaraldehyde in 0.12 M phosphate buffer for 20 minutes at room temperature, followed by fixation in 1% osmium tetroxide in the same buffer for another 40 minutes at room temperature. The coverslips were dehydrated in series of ethyl alcohol of ascending concentration and finally in absolute acetone, and critical point dried using a Polaron critical point drying apparatus. The coverslips were then coated with a 10 nm layer of gold-palladium in a sputter coater. Observations were made on an ETEC Autoscan microscope at an accelerating voltage of 25 kV. Photomicrographs were taken on Polaroid Type 55 black and white film.

Results

Since no significant morphological difference was found in fetal neurons grown in serum-containing or serum-free media during the first 7 days in culture, the morphological features of neurons in these two groups are dealt with together (Figs. 1-7). At 18-24 hours of culture, after dissociated cells were plated on polylysine- or collagen- coated coverslips at a concentration of 2 to 5×10^3 cells per coverslip, almost all neurons, Schwann cells and fibroblasts were found attached to the substrate.

The first axonal growth was found at 18-24 hours of culture, and these axons usually were thin and long, the length of the axons could be 5-10 times that of the cell body. Some neurons, however, had unusually thick axons (Fig. 1). Only 10-20% neurons initiated axonal growth during the first 24 hours; by 4-5 days most other neurons would follow the earlier ones and send out axons. The cell bodies of some neurons were "rounded up" (Figs. 1 and 2), and had a limited degree of contact with the substrate so that the neurons were precariously perched on the coverslip surface (Fig. 3); while others were seen flattened on the substrate surface to varying degrees. Some were almost totally flat, others were spherical in the center and flattened at the edges so that they resembled "bonnets" (Fig. 4). Extremely fine microspikes were found extending from the cell bodies and the proximal parts of the neurites. The surface of the neuronal perikarya was usually smooth and with a few small projections (Fig. 2), however, neurons in early culture phase showed furrows, folds and ridges on their surface (Fig. 3). The neuronal surface became very smooth by 7 days *in vitro*.

The axonal growth cones were found at the tips of axons and were broad and flat membranous extensions (Figs. 5 and 6). A large number of microspikes was also found extending from the main body of the growth cones. Ruffles were usually found at the leading edges of the palm-like membrane extentions (Figs. 6 and 7).

After 5-7 days *in vitro*, the axons became long and straight and their branchings occurred at some distance from the neuronal perikarya. Microspikes were also found extending from the sides of axons (Fig. 6).

Isolated single neurons formed small clumps of 10-20 cells; several thick axonal bundles could extend from these neuronal clumps (Fig. 8). The surface of these neurons was smooth and often with a small number of studs.

After 2 weeks and onward, numerous neurons made an extensive network of axons, and these neurons usually located on the top of Schwann cells and fibroblasts (Figs. 9 and 10). The surface of these neurons was quite smooth and often with microvilli (Fig. 9). The longest time that the cultures were maintained in either serum-containing or serum-free media without any ill health on the part of neurons was 4 months.

Discussion

Several previous studies have described the three dimensional features of the cultured neurons revealed by scanning electron microscopy. These studies include cultures of chick spinal cord (Boyde et al. 1968), chick sympathetic ganglion (Hill et al. 1974), rat cerebellum (Silberberg 1975), mouse dorsal root ganglion (Shahar et al. 1977), cerebrum and retina (Meller 1979), normal and neoplastic nervous tissues of several species (Manuelidis and Manuelidis 1979). In most of these studies cultured neurons were described as large spherical cells located on the top of non-neuronal elements and having long and thin neurites. In this paper, we wish to give a more detailed account of the growth pattern of fetal human neurons as these neurons differentiate *in vitro*.

The results obtained by scanning electron microscopy of the fetal human sensory neurons isolated and grown in culture, demonstrate that fetal neurons regenerate their axons, characterized by growth cones at their tips within 24 hours of isolation; and that the attachment patterns of neuronal perikarya may vary: some neurons became spherical or "rounded up" while others flatten or achieve a "bonnet-like" form.

A recent study of rat sympathetic neurons in culture has shown that the surface of rat neurons continuously exposed to NGF had a very smooth surface, a finding compatible with our results (Connolly et al. 1981). When the neurons which were deprived of NGF for 4-5 hours and then exposed to NGF, there were rapid changes of the surface that included the formation of microvilli and ruffles and pit

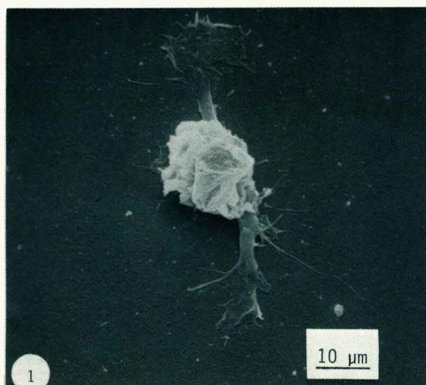

Fig. 1 A bipolar neuron from a human fetal dorsal root ganglion 24 hours after dissociation. Note two thick axons which terminate as axonal growth cones. Fine microspikes extend from the perikarya and the neurite.

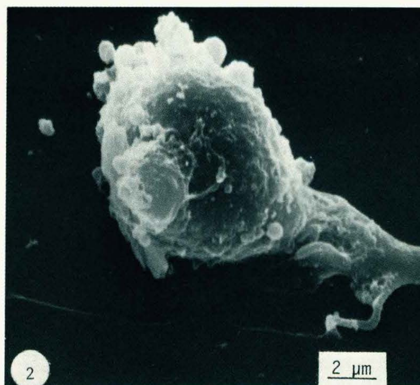

Fig. 2 The neuron grown for 7 days shows a unipolar morphology characteristic of the dorsal root ganglion neurons. The large bump on the lower left side of the neuron might be a closely associated satellite cell. The neuronal surface is smooth except for some small studs.

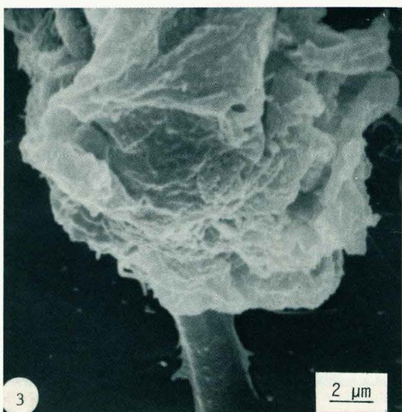

Fig. 3 A higher magnification of the neuronal perikaryon shown in Fig. 1. Note the furrows, folds and ridges forming an uneven surface. These features disappear and the surface becomes smooth later in the culture.

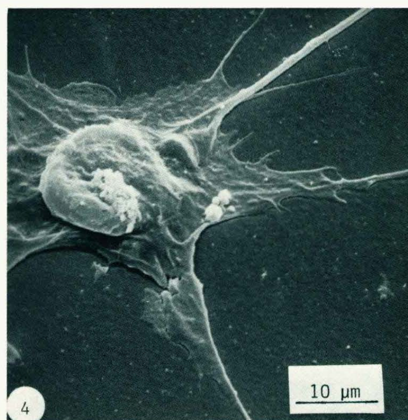

Fig. 4 A neuron of "Bonnet-like" morphology. The neuron is flattened at the parts of the periphery and its surface feature is similar to that of the axonal growth cone. 24 hours in vitro.

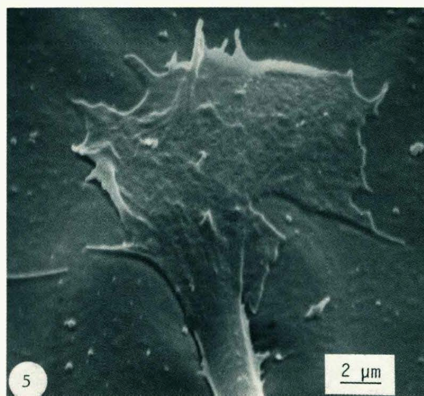

Fig. 5 A higher magnification of the axonal growth cone shown in Fig. 1. The broad and flat membranous extension shows some ruffles at the leading edge of the outgrowth.

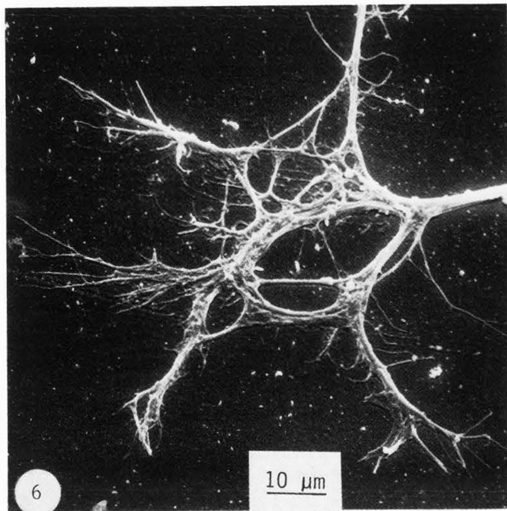

Fig. 6 Terminal portions of several axons growing together. Several profiles of axonal growth cones may be recognized. Large numbers of lateral spikes are noted to extend laterally from the axons. 7 days in vitro.

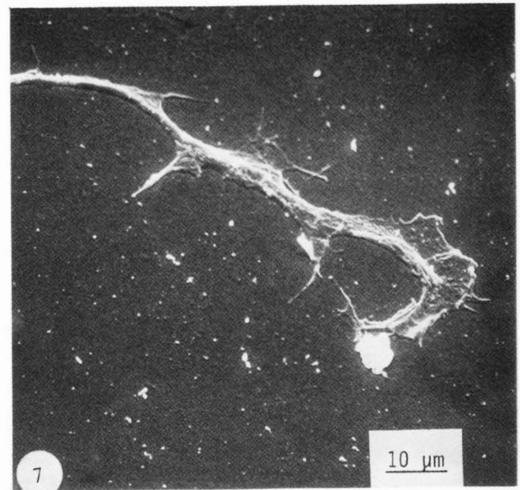

Fig. 7 Another example of an axonal growth cone. Several microspikes are noted. 7 days in vitro.

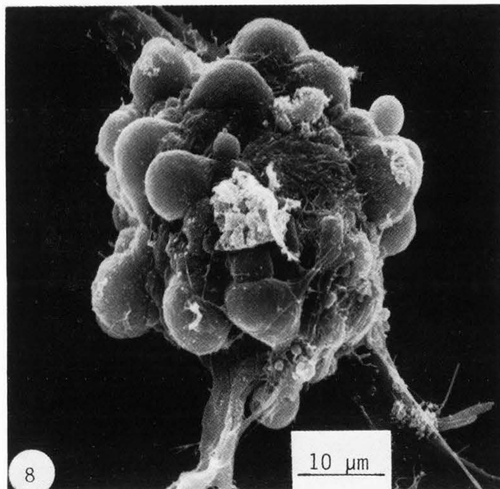

Fig. 8 A clump of neurons from which three thick axonal bundles are emerging. The surface of these neurons is very smooth except for some microvilli present. 14 days in vitro.

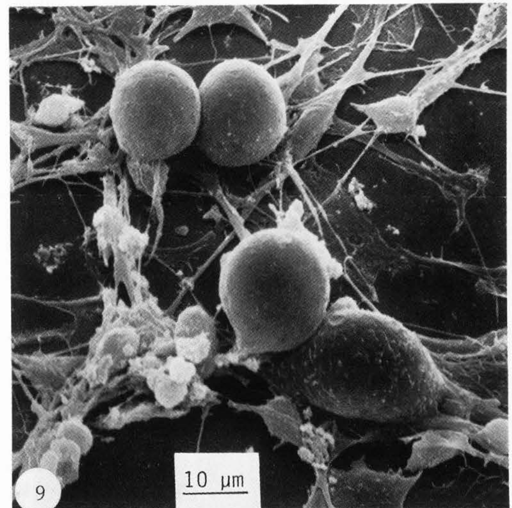

Fig. 9 Four spherical neurons are shown here sitting on the top of flat fibroblasts or spindle-shaped Schwann cells. The surface of neurons is quite smooth but one of them has numerous microvilli. 32 days in vitro.

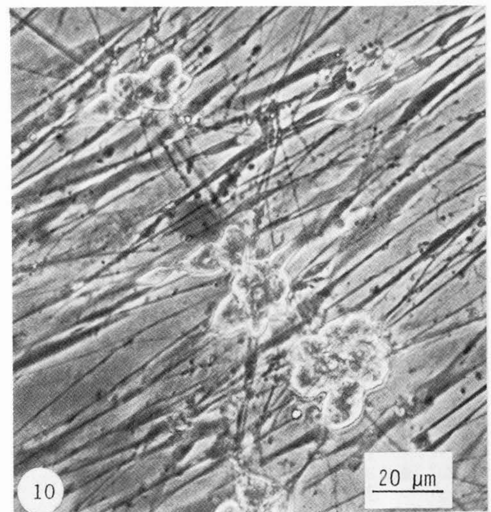

Fig. 10 Several clusters of fetal dorsal root ganglion neurons can be recognized here. These neurons form an extensive network of interconnecting axons. 45 days in vitro. Phase contrast microscopy.

formation. These changes were short-lived and lasted less than 7 minutes (Connolly et al. 1981). In our study, we did not detect any such transient and sequential changes on the neuronal surface.

The scanning electron microscopy of cultured neurons and glial cells would present an ideal starting point for the study of growth patterns of neurons and visualization of interactions between different cell types. The cultured neurons may be exposed to growth factors, hormones or toxic agents under controlled conditions, and then evaluated carefully for their surface morphology and general organization.

Acknowledgements

This work was supported by grants from the Medical Research Council of Canada (MA-7700) and B.C. Health Care Research Foundation (72-83-1). We thank Myong Kim and Blair Ruff for technical assistance and Mary Forkin for typing the manuscript.

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Discussion with Reviewers

A. Peters: Do these neurons always have only two processes each, as do ganglion cells, or are there multiple processes? What is the meaning of the image in Figure 6? Are these groups of axons?

Authors: During the early phase of culture (approx. 4 days), many neurons possess multiple processes as shown in Figure 4, and later lose most of their processes by retraction. After 1-2 weeks in culture, most of the neurons assume bipolar or unipolar morphology. Figure 6 shows the terminal portions of two thick axon bundles shown in the upper right portion of the picture. The axon bundles dissociated into single fibers, fanned out, and formed growth cones at their tips.

A. Peters: With reference to Figures 8 and 9, the authors make no comment about why some of the cells shown are round and have smooth surfaces. Since there seem to be Schwann cells in the culture, could it be that these round cells are Schwann cells ensheathing ganglion cells?

Authors: The cells in question are neurons as they are always labelled with immunofluorescence staining of anti-neurofilament antibodies (specific for 68K or 150K triplet proteins) or by monoclonal A_2B_5 antibody (specific for G_{Q1C} ganglioside).

H. Sobkowicz: At what time after explantation do the supporting cells adjoin the neuronal soma and axons?

Authors: Association of neurons and supporting cells usually occur within a week in culture, however, such neuron-glia interactions may never occur in low-density cultures as shown in Figure 9 in which some neurons are noted without any satellite cells. This is the reason why myelin formation does occur rarely in dissociated cell cultures.

H. Sobkowicz: Can difference in a medium or substrate modify the neuron-glial relations?

Authors: Both the composition of medium and the nature of substrate should modify the neuron-glia interactions. Fetal mouse spinal cord cells were dissociated into single cells, seeded on polylysine- or collagen-coated coverslips, and were grown in serum-containing or serum-free media. Only the spinal cord cells seeded on collagen-coated coverslips and fed with serum-containing medium (plus embryo extract) later formed myelin sheaths (unpublished data). This is one of the most dramatic examples of the importance of the environmental and nutritional factors on the neuron-glia interactions.

H. Sobkowicz: Does the presence of satellite cells influence the shape and attachment of the nerve cells?

Authors: The shape and attachment of the neurons appear not to be affected by the presence or absence of satellite cells.

H. Sobkowicz: What, if any, relations exist between the growth cones and the Schwann cells?

Authors: We do not have any results to indicate the existence of any cause-effect relationship between growth cones and Schwann cells.

J. Walsh: Numerous previous authors (for example, Sensenbrenner and Mandel , Exp. Cell Res. 87:159-167, 1974) have noted that dissociated fetal neurons survive best when maintained on an underlying layer of astrocytes and that their survival is more limited and their differentiation more restricted when they are maintained on collagen- or polylysine-coated coverslips. Have the authors had any experience with neurons maintained on an astrocytic or leptomeningeal carpet and, if so, do the surface features as described with SEM differ from those presented here?

Authors: Fetal human neurons did poorly when cultures were overgrown by fibroblasts. The most important single factor in our fetal human dorsal root ganglion neuron culture was the inclusion of NGF in the medium. There was no obvious surface difference between the neurons on or off the fibroblasts.

J. Walsh: Much difficulty has been expressed in the literature regarding the differentiation of immature neurons and glial cells in culture on the basis of their morphology, especially in regard to their ultrastructural cytoplasmic and surface features. What surface characteristics did the authors observe with SEM that help distinguish these two primitive types of cells? Were other studies such as immunofluorescence staining for cell membrane-bound tetanus toxin used to confirm their identity as neurons?

Authors: We did not encounter any difficulty in identifying neurons and Schwann cells in our dorsal root ganglion cultures. Schwann cells are characterized by their bipolar spindle-shaped appearance and narrow elongated nucleus, and the neurons are identified by their large size and spherical shape.

Immunofluorescence staining of tetanus toxin binding is helpful in identifying neurons but tetanus toxin also binds to non-neuronal cells as well. The best cell type-specific marker for neurons so far is the immunocytochemical staining by neurofilament triplet protein antibodies. We have utilized anti-neurofilament antibodies (specific for 68K and 150K triplet protein) and monoclonal A_2B_5 antibody (specific for G_{Q1C} ganglioside) in our study of fetal human neuron cultures (unpublished data).