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SURVIVAL AND GROWTH OF ADULT HUMAN OLIGODENDROCYTES IN CULTURE: SCANNING ELECTRON MICROSCOPY

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Abstract

The oligodendrocytes in culture serve as an important model for the study of demyelination diseases. We have previously originated a method of isolating human oligodendrocytes. In order to establish their three dimensional morphology, scanning electron microscopy of the cultured oligodendrocytes was performed.

The oligodendrocytes bulk isolated from adult human brain were maintained in culture for more than 2 months. At the various periods in culture, the cells were studied by scanning electron microscopy and immunofluorescence staining using marker antibodies for the identification of oligodendrocytes. The three dimensional organization and the surface morphology of the cultured oligodendrocytes were investigated. They displayed an extensive network of the cell processes and characteristic surface morphology.

Key Words: Human, oligodendrocytes, culture, bulk isolation, Cell type-specific markers, Immunofluorescence microscopy, scanning electron microscopy

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Introduction

Oligodendrocytes, as the cells which produce and maintain central nervous system myelin, serve as primary targets for immunopathological reactions in patients with demyelinative neurological diseases that include multiple sclerosis (Lisak 1980, McFarlin and McFarland, 1982). In order to understand the pathogenesis of these diseases, investigation of purified oligodendrocyte population in isolation is desirable.

Several studies have been carried out on the bulk-isolation of oligodendrocytes from non-human mammalian brains and the growth of these cells in culture (Szuchet et al. 1980, Lisak et al.1981, Gebicke-Harter et al. 1981, Norton et al. 1983, Hirayama et al. 1983). Recently, we have developed a method by which a large number of human oligodendrocytes were isolated and then grown in culture for an extended period of time (Kim et al. 1983). These cultured human oligodendrocytes expressed cell type specific markers such as galactocerebroside, myelin basic protein and cyclic nucleotide phosphodiesterase (Kim et al. 1983, Kim et al. 1984). None of these studies, however, have addressed the subject of three-dimensional features of oligodendrocytes growing in culture as seen with the scanning electron microscope. In the present study, we describe the morphological features of human adult oligodendrocytes in culture at different phases of regeneration and development as studied by scanning electron microscopy.

Materials and Methods

A detailed account of the method for the isolation of human oligodendrocytes (HOL) has been given elsewhere (Kim et al. 1983). Briefly white matter of human adult brains obtained at autopsy less than 15 hours postmortem was dissected into small pieces, and incubated in 0.1% trypsin and 0.002% DNase in calcium- and magnesium-free Hanks' balanced salt solution for 1 hour at 37°C in a water bath. The enzyme-treated tissues were washed in Hanks' balanced salt solutiuon (BSS),

dissociated into single cells by repeated pipetting and passed through nylon meshes of 150 and 75 μm. The cells suspended in 20 ml of BSS were mixed with 1 ml of 10x BSS and 9 ml of Percoll (Pharmacia, Piscataway, N.J.) and gradients were formed by centrifuging the mixture for 20 min. at 15,000 rpm at 4°C. After the centrifugation, an upper myelin layer and a lower erythrocyte layer were visible. HOL cells suspended between these two layers (specific gravity of 1.035 - 1.065) were collected, diluted three-fold with BSS and centrifuged 15 minutes at 1,500 rpm. Cells were washed twice with BSS and were suspended in feeding medium and seeded on polylysine- (10 μg/ml in water) or collagen- coated 15 mm glass coverslips at the concentration of 4-10 x 10^3 cells/coverslip. Feeding medium consisted of 5% fetal calf serum, 5 mg/ml glucose and 20 μg/ml gentamicin in Eagle's minimum essential medium with Earle's base. Insulin at the final concentration of 20 μg/ml was also included in the medium in early experiments.

After various time periods in culture, starting 48 hours after plating, coverslips carrying HOL 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, dehydrated in an ascending series of ethyl alcohol 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.

Cultures were processed for the immunofluorescence staining using rabbit anti-galactocerebroside serum and monoclonal anti-glial fibrillary acidic protein (GFAP) for the identification of oligodendrocytes and astrocytes (Raff et al. 1979, Lisak et al. 1981, Kim et al. 1983). Cultures were first incubated in diluted rabbit anti- galactocerebroside serum (1:50) in BSS plus 2% horse serum and 10 mM N-2-Hydroxyethylpiperazine-N' -2-ethanesulfonic acid (HEPES) buffer (BSS+) for 20 minutes, washed in BSS+, then followed by rhodamine-coupled goat anti-rabbit immunoglobulin (1:40) in BSS+ for 20 minutes. After three changes of washing, cultures were fixed in acid-alcohol (5% acetic acid in 95% ethyl alcohol) at -20°C for 10 minutes, washed in BSS+, incubated in monoclonal anti-GFAP antibody followed by fluorescein-coupled goat anti-rat immunoglobulin (1:40), washed in phosphate buffered saline (PBS), and embedded in glycerine-PBS. All the procedures were performed at room temperature. The coverslips were examined on a Zeiss Universal fluorescence microscope equipped with phase contrast, fluorescein and rhodamine optics and epi-illumination. Photographs were taken on Kodak Ektachrome color film (ASA 400) or Kodak Tri-X black and white film (ASA 400).

Results

Most human oligodendrocytes (HOL) did not attach to the plastic Petri dishes and formed floating cell clumps of 3-50 cells after 24 hours in vitro. These floating clumps were dispersed into single cells by repeated pipetting and replated on Petri dishes or on collagen- or polylysine-coated glass coverslips. The replating process was repeated once more at 48 hours in vitro. After these replating procedures, typically 90-95% of the cells were found positive for galactocerebroside and negative for glial fibrillary acidic protein (GFAP) by immunofluorescence microscopy, indicating these cells were oligodendrocytes (Figs. 1 and 2). The remaining 5% of the cells were mostly astrocytes identified by their reaction to GFAP antibody, and flat fibroblasts or endothelial cells.

Following the dispersion and replating procedures, small round HOL cells (8-10 μm diameter) singly or in small clusters were found to attach to the glass surface with fairly limited contact.

After 5-7 days in vitro, many HOL cells became polymorphic and manifested themselves as bipolar, tripolar or multipolar cells. The surface of the HOL cell was either quite smooth (Fig. 3) or with small spherical studs all over the cell bodies (Fig. 6). The HOL cell bodies gave rise to two or three thick and several short fine processes (Figs. 3 and 5). Broad and flat membranous extensions similar to axonal growth cones were usually found in the middle of or on the tips of the processes (Figs. 3 and 5). Ruffles were observed at the leading edges of these palm-like extensions (Figs. 4 and 6).

Later in culture, 2 weeks in culture and onward, many HOL cells were found to form an extensive meshwork of cell processes (Fig. 7). The surface of the cells was smooth with a small number of spherical studs (microvilli) and all processes were oriented radially (Fig. 8). Most of these processes were thick and long and some of these processes were 5-10 times the diameter of the cell body. As in earlier cultures, flat and broad palm-like extensions were found on the tips of the processes (Fig. 9). These growth cone-like extensions are thought to be important for the physiological role of oligodendrocytes as they wrap around axons to form myelin sheaths when axon-oligodendrocyte interaction takes place. These structures should be called oligodendroglial growth cones in order to distinguish them from the axonal growth cones.

Although the cultures we have studied were enriched with oligodendrocytes, a small number of other cell types such as astrocytes and fibroblasts were also present. Astrocytes were

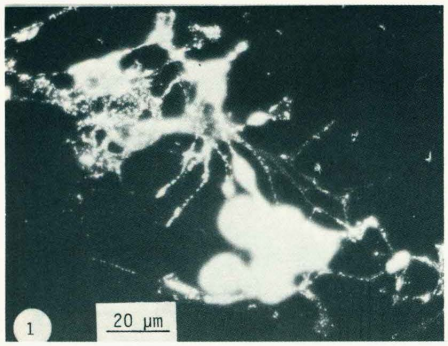


Fig. 1 Immunofluorescence staining of cultured human oligodendrocytes by rabbit anti-galactocerebroside serum. All the cells present are galactocerebroside-positive. The cells were isolated from the brain of a 62 year old male 6 hours postmortem. 42 days in vitro.

Scanning electron micrographs represented in Figures 3-9 were all taken from the sister cultures of the same series.

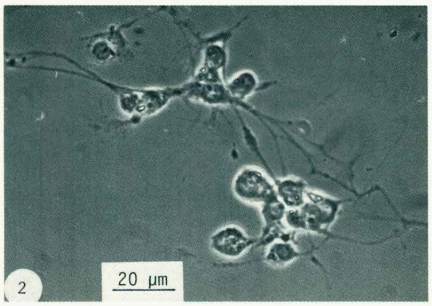


Fig. 2 Same field as Fig. 1. Phase contrast microscopy.

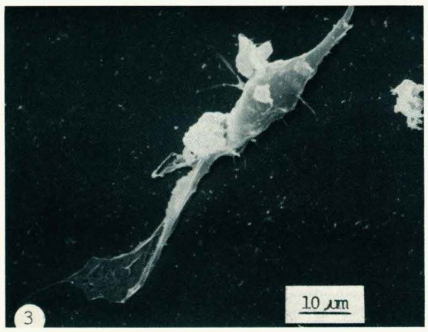


Fig. 3 An oligodendrocyte with bipolar morphology the surface of which is quite smooth. Note several short and thin processes emerging from the cell body. The cells were isolated from the brain of a 62 year old male 6 hours postmortem. 7 days in vitro.

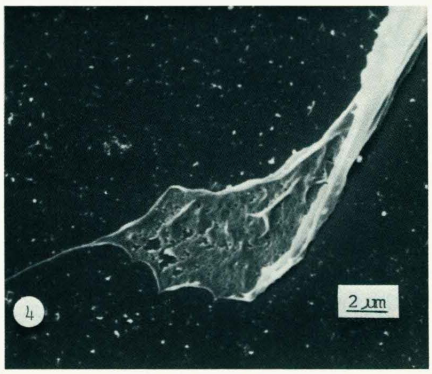


Fig. 4 High magnification of the tip of the oligodendrocyte process shown in Fig. 3. Note the ruffles on the leading edges of the oligodendroglial growth cone. 7 days in vitro.

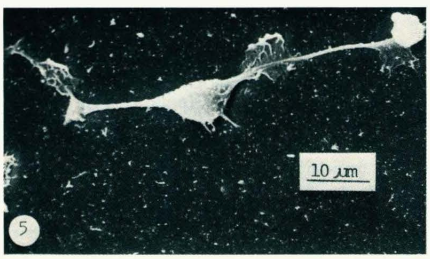


Fig. 5 Another example of a bipolar oligodendrocyte in an early phase of culture. Note the flat and broad membranous expansions in the middle and end of the processes which can be identified as oligodendroglial growth cones. 7 days in vitro.

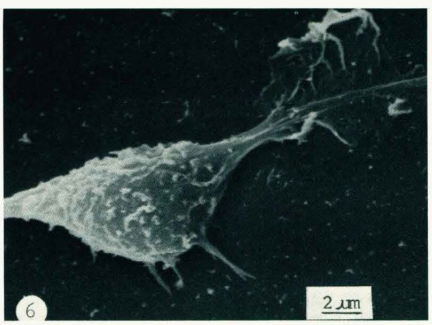
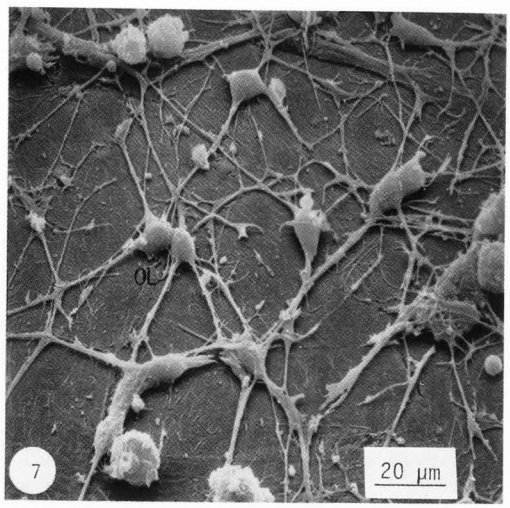
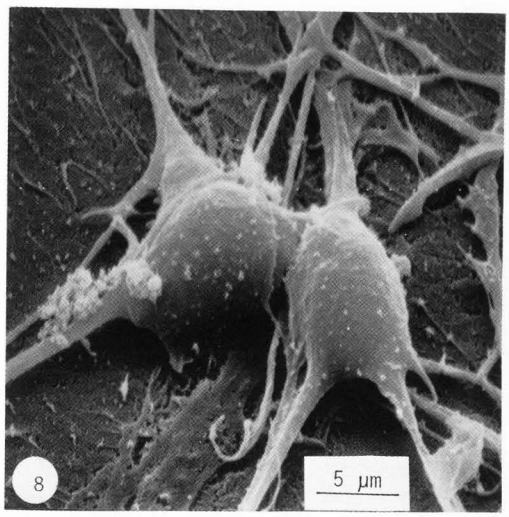


Fig. 6 High magnification of the cell shown in Fig. 5. Note the glial growth cone and numerous spherical studs on the surface of the cell body. 7 days in vitro.





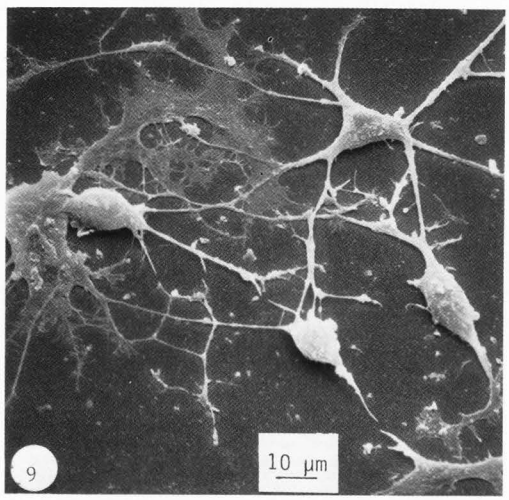


Fig. 7 Several oligodendrocytes (OL) are shown here to form an extensive network of processes. Most of the cells acquired multipolar morphology. 35 days in vitro.

Fig. 8 High magnification of the cells indicated by OL in Fig. 7. Moderate numbers of microvilli (studs) are seen on the surface of these oligodendrocytes. The surface is rather smooth as compared to the cell shown in Fig. 6. These cells were cultured on collagen substrate. 35 days in vitro.

Fig. 9 Several oligodendrocytes are shown here to form close contacts among themselves. The flat cell shown on the left is an astrocyte with an elaborate branching pattern. Note the palm-like oligodendroglial growth cone at the lower right. 66 days in vitro.

flat cells with several broad processes which covered a large area of the substrate and were in close contact with polymorphic oligodendrocytes (Fig. 9).

The cultures were routinely maintained for 6 months and longer but the oldest culture studied in the present study was maintained for 66 days in vitro.

Discussion

There have been several previous studies describing the morphological features of different cell types found in neural tissue culture detected by scanning electron microscopy. These studies include cultures of spinal cord (Boyde et al. 1968), sympathetic ganglion (Hill et al. 1974), dorsal root ganglion (Shahar et al. 1977), cerebellum (Silberberg 1975), cerebrum and retina (Meller 1979), and normal and neoplastic nervous tissues (Manuelidis and Manuelidis 1979). Although these studies have reported the three dimensional organization and cell surface morphology of various cell types, none of them has addressed specifically the oligodendrocytes.

We have, therefore, attempted in the present paper to present the basic information on the three dimensional morphology of cultured oligodendrocytes.

The results presented here show that oligodendrocytes isolated from adult human brain by the Percoll gradient method can be maintained for months in culture and that the typical morphology of the cultured oligodendrocyte revealed by scanning electron microscopy is a small round cell with several thick processes by which these cells form an extensive network. It is also demonstrated that the growing ends of processes usually terminate in flat and broad membranous extensions similar to axonal growth cones and that the leading edges of the extensions become ruffles.

Although we are reasonably sure of the identification of cells we have labelled as oligodendrocytes by immunofluorescence microscopy using cell type-specific markers such as galactocerebroside (Raff et al. 1979, Lisak et al. 1981, Kim et al. 1983), it would be more desirable if we can identify cultured

oligodendrocytes by immunocytochemical scanning electron microscopy using immunogold particles as described by previous authors (Horisberger 1981). Our preliminary results with the method have been encouraging and a large number of immunogold particles were found on the surface of the cells we identified in the present study as oligodendrocytes.

Acknowledgements

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Discussion with Reviewers

K. Meller: What is the rate of division of the oligodendroglia and the astrocytes during the 6 months cultivation?

Authors: Following the exposure to H^3-thymidine for 24-48 hours, cultured cells were labelled by either galactocerebroside or GFAP antibody, and then processed for the autoradiography. It was noted that none of the galactocerebroside+ or GFAP+ cells incorporated H^3-thymidine except for very few flat fibroblasts.

K. Meller: Are the oligodendroglia cells also described MBP positive?

Authors: Yes, the example of myelin basic protein (MBP)-staining was shown in a previous article (Kim et al., 1983, text reference).

K. Meller: What is the opinion of the authors as to whether the astrocytes and the oligodendrocytes originate from a common or from different precursor cells?

Authors: We believe, as many other scientific colleagues do, that oligodendrocytes and astrocytes are derived from same precursor cells. We do not have any evidence to suggest

the contrary. Recently, however, we have demonstrated that adult human oligodendrocytes in culture could acquire astrocytic phenotype by revealing GFAP+ reaction. This transformation was reversed by a short term treatment with cyclic AMP and its derivatives (Unpublished data).

J. Walsh: The authors state that 5% of the cells in the preparations were probably astrocytes and that the cells were grown on polylysine- or collagen-coated slips. Were any oligodendrocytes seen that adhered on top of the astrocytes and was the morphology of these cells different from those adhering directly to the coverslip?

Authors: Adult human oligodendrocytes grown in culture rarely adhere to astrocytes or fibroblasts. They attach directly to substrates which usually were collagen or polylysine-coated surfaces. In double labelling experiments in which oligodendrocytes were labelled by rabbit galactocerebroside-rhodamine and astrocytes were stained by rat monoclonal GFAP- fluorescein, oligodendrocytes and astrocytes were associated very closely but never grew on top of others.

J. Walsh: Did the authors observe any oligodendrocyte-oligodendrocyte or oligodendrocyte-astrocyte junctions and if so, what were the morphological features of these contacts? Could any variation in morphology corresponding to light, medium, or dark oligodendrocytes be detected and were any mitotic forms seen?

Authors: Transmission electron microscopic examination of cultured human oligodendrocytes revealed that there are two types of junction complexes between oligodendrocytes. They are puncta adhaerentia and gap junction. Most of the oligodendrocytes found in culture falls in the class of medium shade cells of Mori and Leblond (Mori S and Leblond CP (1970) electron microscopic identification of three classes of oligodendrocytes and a preliminary study of their proliferative activity in the corpus callosum of young rats. J. Comp. Neurol. 139:1-28). Our autoradiographic study has demonstrated that none of galactocerebroside+ human oligodendrocytes incorporates H^3-thymidine.

Reviewer 4: In the present study you have not attempted to immunologically determine the presence of myelin basic protein. This has been carried out in an earlier study. Can you simply describe where in the cell this substance was identified in the earlier studies?

Authors: Myelin basic protein is present in cultured human oligodendrocytes intracytoplasmic and not on the surface of the cells. Double labelling experiments using mouse anti-galactocerebroside serum and rabbit anti-MBP antibody performed in a previous publication clearly demonstrated the unequivocal identification of our cultured cells as oligodendrocytes (Kim et al., 1983, text reference).

Reviewer 4: Have you tried to carry out any correlative scanning electron microscopical and immunological studies where the same cell is examined with both methods?

Authors: Yes we did. By using rabbit anti-galactocerebroside serum and 40 nm gold particles conjugated with goat anti-rabbit immunoglobulin (Jansen Pharmaceutica), we found a large number of gold particles attached to the surface of the cells we identified as oligodendrocytes under the scanning electron microscope. We will publish these results elsewhere shortly.