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Neuronal differentiation in vitro from precursor cells of regenerating spinal cord of the adult teleost *Apteronotus albifrons*

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Abstract. This study documents neuronal differentiation in vitro from undifferentiated precursor cells of caudalmost regenerating spinal cord of the teleost *Apteronotus albifrons*. At 11 days in vitro, cells from the caudalmost tip of the regenerating cord are flat and polygonal in shape, lack neuronal processes and do not stain with antibody against neuron-specific filaments. At 15 days in vitro, some of the caudalmost cells have developed short, neurite-like processes; at 18 days in vitro, some cells react positively with antibody against neuron-specific filaments. At 26 days in vitro, many of the caudalmost cells have long branching neurites and react positively with anti-neurofilament antibody. Addition of insulin-like growth factor-I to the medium accelerates the process of neuronal differentiation from the caudalmost precursor cells in vitro. The source of these precursor cells is ultimately cells of the ependymal layer of adult spinal cord. Further investigation of the factors that control production and differentiation of these cells will be important in defining the developmental potential possible for vertebrate spinal cord cells and may aid in creating an optimal environment for regeneration of axons within mammalian spinal cord.

Key words: Neurons – Spinal cord – Differentiation – Regeneration – Neurofilaments – *Apteronotus albifrons* (Teleostei)

Introduction

Tissue cultures of embryonic neurons have led to much knowledge about processes such as axon elongation and guidance, substrate and target effects on axons, and synapse formation. However, little is known about the steps of neuronal determination and initial neuronal differentiation because neuronal precursor cells have been very difficult to maintain and grow in vitro. Recently, several approaches have led to successful cultures of mitotic cells from embryonic mammalian sources that can differentiate into neurons in vitro (Cattaneo and McKay 1990; DiCicco-Bloom et al. 1990; Reynolds et al. 1992).

While neuronal precursor cells from postnatal mammalian central nervous system (CNS) have been especially difficult to culture (Reynolds and Weiss 1992), another model system is available. Prolific cultures have been established from spinal cord of the Black Ghost Knife Fish, *Apteronotus albifrons* (Anderson and Waxman 1985b; Anderson 1993), a teleost that can regenerate its spinal cord naturally (Anderson and Waxman 1983 a). Studies with ^{3}H-thymidine have shown that mitosis and neuronal differentiation occur in these cultures (Anderson and Waxman 1985 a, b). These processes appear to mirror events that occur during the natural regeneration of spinal cord in this species (Anderson and Waxman 1983b; Anderson et al. 1984).

The purpose of this study is to determine if differentiation of neurons occurs from undifferentiated precursor cells in cultures of *Apteronotus* spinal cord. A second aim is to enrich cultures for precursor cells by plating tissue from caudalmost regenerating spinal cord, which has a higher proportion of ependymal cells, the presumed source of mitotic precursors for the regenerating cord (Anderson and Waxman 1983 a; Anderson et al. 1983, 1986). Cultures enriched in neuronal precursor cells will facilitate analysis of the factors or events that control initial neuronal differentiation.

Materials and methods

Tissue culture

Spinal cord tissue from 25 Black Ghost Knife Fish (*Apteronotus albifrons*) was used in this study. The adult fish, all between 16–21 cm in length and over one year old, were housed in separate 5-gallon tanks, maintained at 27°C. Spinal cord regeneration was initiated by amputating 1–3 cm from the caudal end of a fish anesthetized with 0.01% MS-222 (tricaine methane sulfonate: Argent Chemical Labs, Redmond, Wash., USA). After 3–7 months, the fish

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was re-anesthetized and the regenerated portion of the tail was reamputated for use in tissue culture. The total length of spinal cord in a typical 20-cm uncut fish is approximately 16.5 cm, with the spinal cord extending to within 5 mm of the end of the tail. In fish with regenerating spinal cords, the regenerated spinal cord likewise extends to within 5 mm of the end of the regenerating tail. For cultures of "caudalmost" spinal cord cells, only the caudalmost 0.5–1 mm of regenerating cord was used.

The cut portion of tail for tissue culture was dipped briefly (30 s) in 70% ethanol, then rinsed in a series of 4 dishes of sterile Dulbecco's phosphate-buffered saline (PBS). Spinal cord was dissected out in sterile culture medium (Liebovitz's L-15, supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units penicillin and 100 µg streptomycin per ml, final pH = 7.3). All tissue culture components were from Gibco (Grand Island, N.Y., USA). After dissociation, the cells were plated onto plastic tissue culture dishes (4-well plates, 15 mm diameter wells: Nunclon, InterMed, Thousand Oaks, Calif., USA) coated with 1 mg/ml poly-L-lysine (Sigma, St. Louis, MO, USA) as described in Letourneau (1975).Cells were observed and photographed on a Nikon inverted microscope using phase-contrast optics.

Dissociated cells

The spinal cord was separated into regenerated and caudalmost (regenerated) portions. Meninges were removed from the rostral regenerated cord by dissection. Meninges cannot easily be removed from the caudalmost 1 mm of regenerated cord by dissection. Therefore modified dissociation methods (below) were used for caudalmost tissue. The more rostral regenerated cord was minced into pieces (approximately 0.5 mm square) with an iridectomy knife. Spinal cord pieces were dissociated by incubation with 0.4% Trypsin (Sigma, Type XI) in calcium- and magnesium-free PBS (CMF-PBS) at 4°C for 2 1/2 h, then at 37°C for 25 min. The reaction was stopped with 0.4% Soybean Trypsin Inhibitor (Sigma) in CMF-PBS for 8–10 min, room temperature. Tissue was then transferred to a small volume of culture medium containing serum and triturated gently 10–20 times with a Pasteur pipet. The cell suspension was filtered through 150 µm Nylon mesh (to remove undissociated clumps), then plated as a single droplet in the middle of each polylysine-coated culture dish well (50–80 µl, containing 0.5–1.0×10^5 cells, per well). Cultures were incubated at 27°C (the temperature of the fish) in a humidified air incubator. Cultures were left undisturbed for 12 h, to allow cells to adhere to the substratum; then medium was added to cover the bottom of the well. After 48 h, the cultures were gently washed with fresh medium to remove debris. Cultures were fed weekly by removing 1/2 of the medium and adding 1/2 fresh medium to each well.

Caudalmost cultures

The caudalmost 1 mm of regenerated spinal cord was dissociated and cultured separately. Caudalmost tissue was dissociated by incubation first in 2% crude collagenase (Sigma) in CMF-PBS for 8 min (37°C) to degrade the dural layers. Tissue was then incubated in 0.4% trypsin in CMF-PBS for 30 min at 4°C, then 10 min at 37°C. Tissue was transferred to a small amount of L-15 culture medium (containing serum), triturated gently 10–15 times, and plated by droplet onto polylysine-coated tissue culture plastic.

Explants

Some initial experiments were done with explant cultures. Spinal cord was cut into small pieces (0.3–0.5 µm diameter) and the explants were plated directly in a small amount of L-15 culture medium (supplemented as stated above). The level of medium was kept very low for the initial 2 h, to ensure good adhesion of the explants to the dish (Anderson 1993). After 2 h, medium was added to cover the explants. Cultures were fed weekly by removing half the medium and replacing it with fresh culture medium.

Anti-neurofilament staining

Cultures were stained with SMI-31 (Sternberger et al. 1982), a monoclonal antibody against phosphorylated neurofilament protein (150kD and 200kD subunits) from rat brain. All antibodies were from Sternberger Monoclonals, Jarrettsville, Md., USA. The cultures were first rinsed with temperature-equilibrated PBS, then fixed with ice cold 100% methanol for 5 min, and air-dried. Cultures were always stained on the same day that they were fixed. All staining procedures were carried out at room temperature. All dilutions were made in TRIS buffer containing 0.05 M TRIS, 0.85% NaCl, pH 7.6

Culture wells were rehydrated with TRIS buffer for 20 min, then incubated with blocking serum (normal rabbit serum, 10% in TRIS buffer) for 1 h. A low concentration of blocking serum (1%) was included in all further incubation solutions to reduce potential background staining. After blocking serum was removed, the cultures were incubated with SMI-31 (1:1000 in TRIS buffer) for 16 h on a rotary shaker. Control wells were incubated with TRIS buffer containing 1% blocking serum, instead of the primary antibody (SMI-31). Following the primary antibody, the cultures were washed 3 × with TRIS buffer (5 min each), and incubated 1 h with the secondary ("bridge") antibody: rabbit anti-mouse IgG (1:200). Cultures were then washed 3 × with TRIS buffer, and incubated with peroxidase-antiperoxidase complex (mouse monoclonal), 30 min, 1:100. Culture wells were washed 3 × with TRIS buffer before addition of diaminobenzidine (DAB) and hydrogen peroxide (10 mg DAB in 10 ml TRIS buffer, plus 6 µl H_2O_2). Color reaction developed within 2–7 min. Cultures were washed 4 × with distilled water before observation and photography.

Growth factors and substrates

Dissociated cells from caudalmost regenerated, more rostral regenerated, and normal (unregenerated) spinal cord were cultured in the presence of the following potential growth-controlling factors (Collaborative Research, Bedford, Mass., USA): Nerve growth factor (1–100 B.U./ml), fibroblast growth factor (FGF; 100 ng/ml) and basic fibroblast growth factor (bFGF; 2 ng/ml-5 µg/ml). Recombinant insulin-like growth factor-I (IGF-I, Bachem, Torrance, Calif., USA; 50 ng/ml) was also used. In each case, dissociated cells were plated in culture medium containing the growth factor, and all subsequent medium changes were done with medium containing fresh growth factor. Alternative substrata tested were: Cell-Tak (3.5 µg/cm^2, Biopolymers, Farmington, Conn., USA), laminin (10–25 µg/ml; Gibco), Type IV collagen (Sigma), and fibronectin (40 µg/ml; Collaborative Research).

Cross-sections of spinal cord

In order to determine the cellular composition of the cord used in the "caudalmost" cultures, some regenerated spinal cords were fixed in 10% buffered formalin for 24 h and embedded in glycol-methacrylate. Cross-sections (5–10 µm) were stained with toluidine blue.

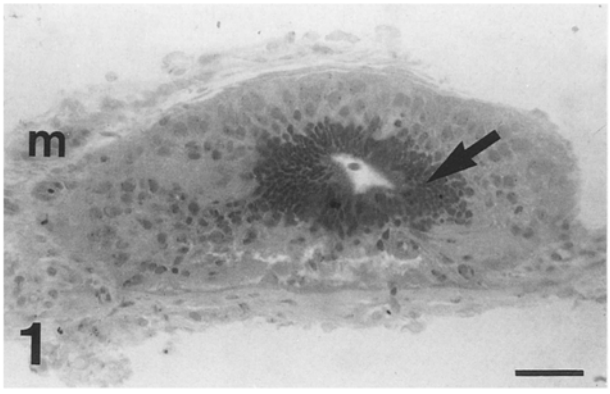
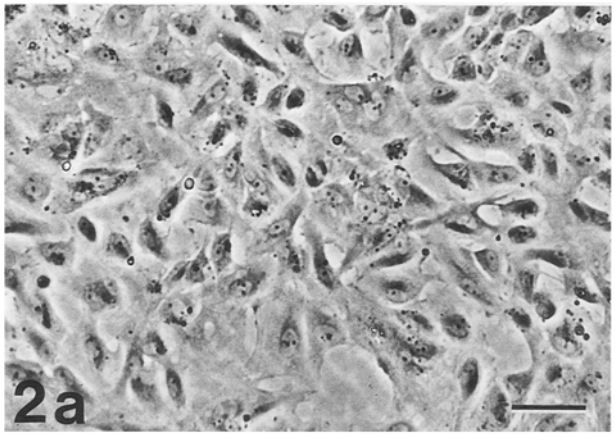
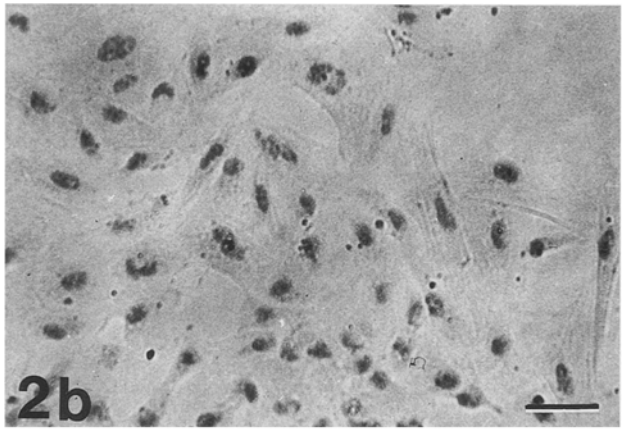
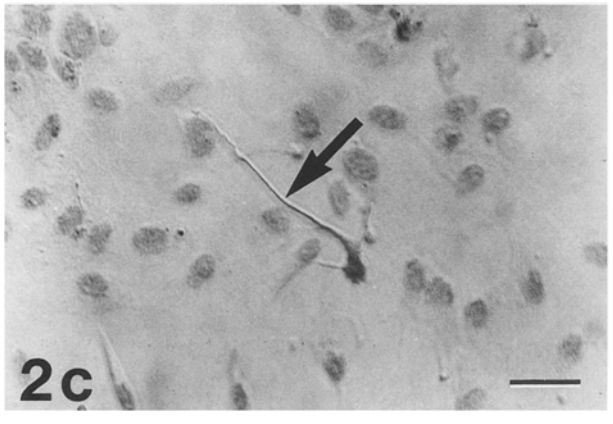


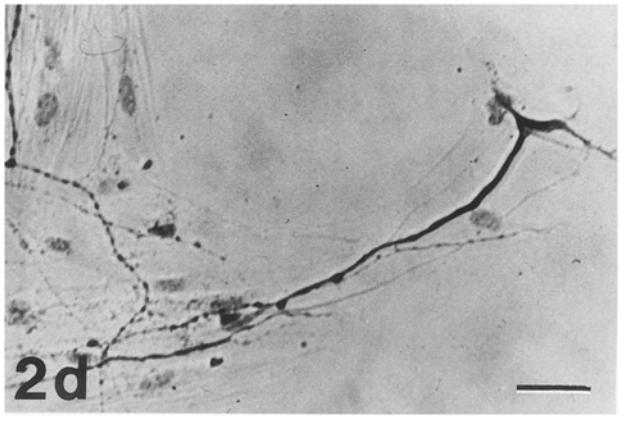
Fig. 1. Cross-section of caudalmost regenerating spinal cord, showing cellular composition at rostral end of tissue used in caudalmost cultures. Ependymal layer (*arrow*) with many more cells than in non-regenerating cord. Meningeal tissue (*m*) attached to part of cord. × 110. *Bar*: 80 μm

Fig. 2a–d. Cell cultures at 11 days in vitro. **a** Caudalmost cells at 11 days in vitro, flat and undifferentiated viewed with phase optics. **b** Caudalmost cells at 11 days in vitro, stained with SMI-31 (anti-neurofilament antibody). Reaction negative (nuclear staining non-specific). **c** Cells from section of spinal cord just rostral to caudalmost 1 mm, stained with SMI-31. One cell with positive staining of soma and neurite-like process (*arrow*). **d** Cells from spinal cord 3 mm rostral to that in **a**., stained with SMI-31. Note many antibody-positive neurites. **a, b** × 200. *Bar*: 50 μm. **c, d** × 300. *Bar*: 30 μm









Results

Cross-section of caudalmost cord

In order to prepare cultures enriched for mitotic precursor cells, the caudalmost 0.5–1 mm of regenerating cord was dissociated and cultured separately. Fig. 1 shows a cross-section of spinal cord at the rostral end of the tissue used in "caudalmost" cultures. In this cross-section, approximately one third of the cord is composed of ependymal cells. The other two thirds of the cord consist of morphologically undifferentiated cells. Due to the gradient of differentiation that exists along the length of regenerating spinal cord, with the caudalmost tip of the cord being the site of continuous cell generation (Anderson et al. 1984), the proportion of ependymal cells will be greater in sections of cord caudal to the one shown in Fig. 1. Thus, Fig. 1 shows the maximum number of non-ependymal cells that would be included in any "caudalmost" culture.

Tissue cultures of caudalmost cord

Over 70 cultures of caudalmost cord have been performed. In all of these, the cells are flat and polygonal, or fibroblast-shaped when observed at 7–11 days in vitro (Fig. 2 a). Caudalmost cells at 7–11 days in vitro do not have thin, neurite-like processes and do not stain with anti-neurofilament antibody (Fig. 2 b; antibody staining performed on 12 cultures). Cultures of regenerated spinal cord just rostral to the caudalmost 1 mm show a few

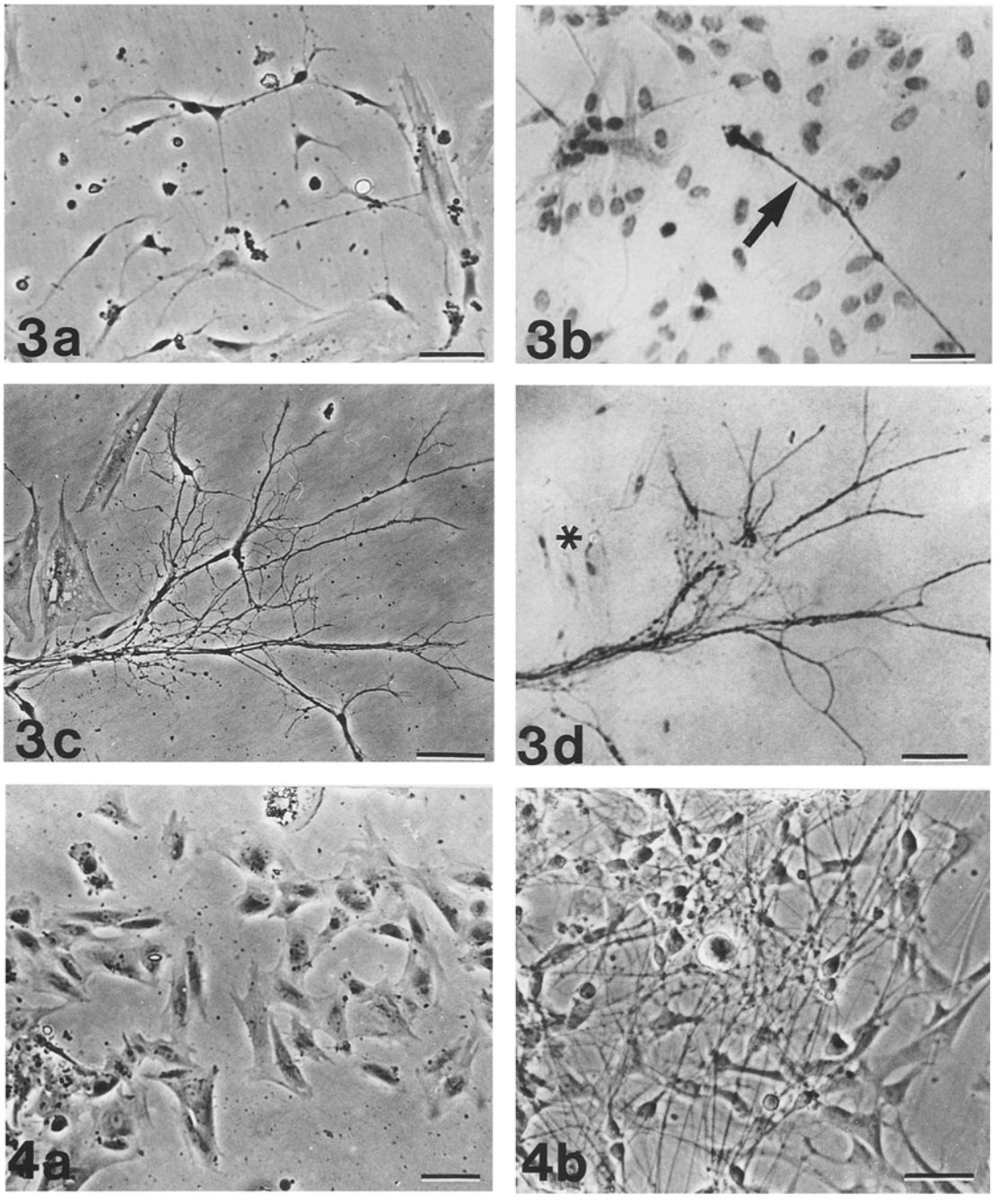


Fig. 3a–d. Differentiation of caudalmost cells over time in vitro. **a** At 15 days in vitro, some cells more rounded (than at 11 days) and with short neurite-like processes. ×160. *Bar*: 60 μm. **b** Caudalmost cells at 18 days in vitro, stained with SMI-31. One neurofilament-positive cell (*arrow*) present in this field, and others that did not react with the antibody. ×230. *Bar*: 40 μm. **c** At 26 days in vitro, many cells with typical neurites and growth cones. **d** The culture in **c**, after fixation and staining with SMI-31. All neuritic processes seen at this stage stained positively for neuron-specific intermediate filaments. Non-neuronal cells (*asterisk*) negative. **c, d** ×145. *Bar*: 70 μm

Fig. 4a, b. Effect of IGF-I on caudalmost cells in vitro. **a** Without IGF-I, caudalmost cells undifferentiated at 4 days in vitro. ×190. *Bar*: 50 μm. **b** Caudalmost cells cultured with IGF-I (50 ng/ml) with marked neuronal differentiation at 4 days in vitro. Note many neurites and rounded neuronal cell bodies. ×200. *Bar*: 50 μm

differentiating neurons (Fig. 2 c). These cells have thin neurite-like processes, and stain positively with anti-neurofilament antibody (SMI-31; antibody staining performed on 6 out of 31 cultures). Cultures from even more rostral spinal cord (Fig. 2 d) show many more differentiated neurons. All of the morphologically-identified neurites in these cultures stain positively with SMI-31 (antibody staining performed in 20 out of 116 cultures).

By 15 days in vitro, cells in the caudalmost cultures (35 cultures examined) show a change in morphology (Fig. 3 a). Many cells have more rounded cell bodies. Short, thin cellular processes, 5–100 μm long, are observed. Small lamellar areas at the tips of these processes may represent early growth cones. At 18 days in vitro, a few of the caudalmost cells (Fig. 3 b) react positively with anti-neurofilament antibody (antibody staining performed on 7

cultures). However, most of the neurite-like processes at 18 days still do not stain positively for neurofilaments. At 26 days in vitro (Fig. 3 c), many cells in the caudalmost cultures have neuritic processes, tipped with active growth cones. In every case (36 cultures observed; antibody staining performed on 13), all of the morphologically neuronal processes stain positively with anti-neurofilament antibody at 26 days (Fig. 3 d).

When caudalmost precursor cells are plated and grown in the presence of 50 ng/ml insulin-like growth factor-1 (IGF-I), the process of neuronal differentiation is accelerated. At 4 days in vitro, cultures without IGF-I (Fig. 4 a) routinely have cells that are flattened and morphologically undifferentiated. In cultures with IGF-I at 4 days in vitro (Fig. 4 b), rounded cell bodies and profuse neurites are present. Neurites in the cultures with IGF-I continue elongating and reach lengths of up to 370 μm (mean neurite length = 280 μm, $n = 40$) at 18 days in vitro. At the same time-point, precursor cells grown in standard medium (without added IGF-I) have neurite lengths of up to 100 μm (mean length = 64 μm, $n = 62$).

Addition of nerve growth factor (1–100 B.U./ml), fibroblast growth factor (100 ng/ml) or basic fibroblast growth factor (2 ng/ml-5 μg/ml) to the culture medium did not accelerate or otherwise affect the differentiation of caudalmost precursor cells. Alternate substrates of fibronectin (40 μg/ml), laminin (10–25 μg/ml), Type IV collagen, and Cell-Tak (Waite and Tanzer 1981) did not accelerate the timing of neuronal differentiation of the caudalmost cells in vitro, nor the rate of neurite outgrowth.

Discussion

The results show that undifferentiated cells in the cultures from caudalmost regenerating *Apteronotus* spinal cord do undergo neuronal differentiation over time in vitro. Changes in morphology of the cells occur first, at approximately 15 days in vitro. Several days later neuron-specific intermediate filaments are immunologically detectable. There could be several explanations for this result. The short processes which appear first in cultures of the caudalmost cells may lack neurofilaments. Neurofilaments are lacking in some primitive axons (crayfish ventral cord: Samson 1971) and were not demonstrable in newborn mouse optic nerve (Peters and Vaughn 1967). Alternatively, the neurofilament protein produced first in the caudalmost *Apteronotus* cells may be non-phosphorylated, and therefore would not react with the antibody used in these experiments. Developmental studies with the antineurofilament antibody used here have shown little staining of phosphorylated neurofilaments in early rat brain development (Sternberger 1986). In these studies, non-phosphorylated epitopes of neurofilament protein appear before the phosphorylated ones.

The time course for neuronal differentiation of the caudalmost *Apteronotus* cells in vitro, as judged by the appearance of neurite-like processes and neuron-specific intermediate filaments, is slower than that observed for mammalian neurons in vitro (Reynolds and Weiss 1992). The difference in rate of differentiation is most probably due to the lower temperature of the fish cultures. *Apteronotus* cells are cultured at 27°C (the temperature of the fish) and do not survive at 37°C, the temperature of mammalian cultures.

Insulin-like growth factor-I (IGF-I) had a remarkable effect on neuronal differentiation in the *Apteronotus* cultures, accelerating the timing of neurite initiation and the lengths of neurites achieved by the differentiating caudalmost cells. It was the only growth factor tested that increased neuronal differentiation in the cultures. Insulin and insulin-like growth factors have been shown to affect neuronal survival, regeneration and possibly mitosis in a number of vertebrate systems (Ishii 1992). IGF-I and IGF-II induce nerve sprouting to innervated skeletal muscle and neurite outgrowth from embryonic cells in vitro (Caroni and Grandes 1990). Insulin and IGF-II enhance neurite outgrowth from human neuroblastoma cells in vitro (Recio-Pinto and Ishii 1984). IGF-II also enhances regeneration of peripheral nerve axons in vivo (Near et al. 1992). Endogenous IGF-I aids in eliciting neuroblast mitosis from embryonic mouse neuroepithelial cells in vitro (Drago et al. 1991). The preliminary results reported here show a dramatic effect of IGF-I on initiation of neurite outgrowth from the caudalmost precursor cells of *Apteronotus* spinal cord. Future work will determine if insulin and IGFs also affect the mitosis of *Apteronotus* spinal precursor cells in vitro and the proportion of cells that become neurons.

One importance of the model system presented here is that the precursor cells cultured are derived from normal adult vertebrate spinal cord, since the ultimate source of new cells in regenerating spinal cord is the ependymal layer of non-growing adult cord adjacent to the site of injury (Anderson and Waxman 1981, 1983 a). A similar role for ependyma is seen in other lower vertebrates that can regenerate spinal cord to some degree (Koppanyi 1955; Simpson 1964; Egar et al. 1970). In this sense, the ependymal layer of regenerating cord serves as a generative neuroepithelium, much like that in embryonic development (Anderson et al. 1986; Jacobson 1991).

Most previous tissue culture studies of neuronal differentiation have employed embryonic (DiCicco-Bloom et al. 1990; Drago et al. 1991), transformed (Hammang et al. 1990; Mellon et al. 1990; Ronnett et al. 1990), or retrovirus-immortalized cells (Bartlett et al. 1988; Frederiksen et al. 1988). In the case of embryonic cell cultures, the cells used may have already had axons (which were cut before tissue culture); thus it is axon regrowth that is being studied, not the initial axon generation. The steps of initial neuronal differentiation from undifferentiated precursor cells may well differ from those involved in the regrowth of axons from already-differentiated cells.Likewise, mitotic precursor cells derived from normal spinal cord may have different growth characteristics and controls than those for transformed cells. These considerations point out the value of studying neuronal differentiation from normal tissue, as well as from embryonic or transformed cells.

Cultures of precursor cells from *Apteronotus* spinal cord provide an important vehicle for testing the biochemical factors that control neurogenesis and axon regeneration from adult vertebrate CNS (Anderson 1993; Anderson and Waxman 1985 a). One long-range goal of

studies with spinal cord cells from *Apteronotus* is to determine the cellular and biochemical conditions that permit natural regeneration in this species, with the hope of applying this knowledge to create an environment that will enhance or permit regeneration in higher vertebrate CNS.

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