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Special Issue

Cell cultures derived from early zebrafish embryos differentiate *in vitro* into neurons and astrocytes

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Abstract

The zebrafish is a polular nonmammalian model for studies of neural development. We have derived cell cultures, initiated from blastula-stage zebrafish embryos, that differentiate *in vitro* into neurons and astrocytes. Cultures were initiated in basal nutrient medium supplemented with bovine insulin, trout serum, trout embryo extract and fetal bovine serum. After two weeks in culture the cells exhibited extensive neurite outgrowth and possessed elevated levels of acetylcholinesterase enzyme activity. Ultrastructural analysis revealed that the neurites possessed microtubules, synaptic vessicles and areas exhibiting growth cone morphology. The cultures expressed proteins recognized by antibodies to the neuronal and astrocyte-specific markers, neurofilament and glial fibrillary acidic protein (GFAP). Poly-D-lysine substrate stimulated neurite outgrowth in the cultures and inhibited the growth of nonneuronal cells. Medium conditioned by the buffalo rat liver line, BRL, promoted the growth and survival of the cells in culture. Mitotically active cells were identified in cultures that had undergone extensive differentiation. The embryo cell cultures provide an *in vitro* system for investigations of biochemical parameters influencing zebrafish neuronal cell growth and differentiation.

Introduction

Zebrafish provide some advantages over mammals for studies of neural development. The optical clarity and rapid development of the zebrafish embryo make it suited for *in vivo* experimental approaches utilizing cell labeling (Eisen *et al*., 1990; Kimmel and Warga, 1986) ablation and transplantation techniques (Eisen, 1989; Eisen *et al*., 1989). These techniques have been employed for studies of cell lineage, synapse formation and gene expression during neurogenesis (Eisen, 1991). Although *in vivo* approaches to the study of zebrafish development have been productive, little emphasis has been directed toward *in vitro* strategies ultilizing zebrafish cell culture systems. In other vertebrates, cell cultures provide an *in vitro* system for the study of extracellular factors affecting survival, growth and differentiation of neuronal cells (Pleasure and Lee, 1993; Reynolds and Weiss, 1992; Sakai *et al*., Stemple and Anderson, 1993; Temple, 1989; Wright *et al*., 1992) and for the identification of lineage specific markers (Levine and Flynn, 1986; Maxwell and Forbes, 1991; Stainer *et al*., 1991). Neuronal cell cultures have also been used for *in vivo* studies through the production of chimeric animals (Cattanneo and McKay, 1991; Huszar *et al*., 1991; Whittemore *et al*., 1991). Similar approaches have not been employed with the zebrafish due to the absence of appropriate cell culture systems.

Previously, we established methods for the culture of cells derived from blastula stage zebrafish embryos (Collodi *et al*., 1992a; Ghosh and Collodi, 1994). The zebrafish blastula is comprised of pluripotent cells (Ho and Kimmel, 1993; Lin *et al*., 1992). In the present study we demonstrate that primary cultures of blastula-derived cells differentiate *in vitro* into neurons and astrocytes, providing a system for the study of neuronal cell growth and differentiation and a source of zebrafish neuronal precursor cells.

Materials and methods

Cell culture

Blastula-stage zebrafish embryos, harvested approximately 4 hr post-fertilization, were rinsed three times in LDF medium (50% Leibovitz's L-15, 35% Dulbecco's modified Eagles's and 15% Ham's F-12 media) (Collodi *et al*., 1992b) containing sodium selenite (10 nM), sodium bicarbonate (0.15 mg/ml), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes; 15 mM, pH 7.4), penicillin (200 U/ml), streptomycin sulfate (200 μg/ml), and ampicillin (25μg/ml). The embryos were treated with bleach solution (0.5%) for 2 min, rinsed in LDF and dechorionated by incubating in pronase E solution (0.5 mg/ml in Hanks buffer) for approximately 15 minutes and rinsed in LDF. Embryo cells were dissociated by treating the dechorionated embryos with trypsin [0.2% (wt/vol.) trypsin (Sigma)/1 mM EDTA in phosphate-buffered saline (PBS)] for 1 min followed by gentle pipetting. The cells were collected by centrifugation and plated in 96-well tissue cultures dishes (approximately 10 embryos/well) precoated with poly-D-lysine (1 mg/ml; Boehringer Manheim). Cultures were maintained in LDF medium conditioned by buffalo-rat liver cells (BRL-CM) supplemented with bovine insulin (10 μg/ml; Sigma), trout embryo extract (40 μg/ml), trout serum (0.5%), and fetal bovine serum (FBS) (1%; GIBCO). Trout serum, embryo extract and BRL-CM were prepared as previously described (Collodi and Barnes, 1990; Collodi *et al*., 1992b). Cultures were fed every 5 to 7 days by replacing half of the medium.

Acetylcholinesterase assay

Cells were harvested by gentle pipetting, washed twice with PBS and collected after each wash by centrifugation (500xg). The cell pellet was suspended in PBS containing 1% triton- X-100. Insoluble material was removed by centrifugation and the supernatant assayed for acetylcholinesterase (AChE) activity (Whittaker, 1984). The reaction mixture contained 100 mM phosphate, 0.47 mM acetylcholineodide, 0.32 mM 5,5'dithiobis(2-nitrobenzoic acid) (DTNB), 0.56 mM sodium bicarbonate and the cell supernatant. Nonspecific cholinesterase was inhibited by quinidine sulfate (0.02 mM). Enzyme activity was expressed as nmoles of substrate hydrolyzed per min per mg protein. Protein concentration was determined according to Bradford (1976). For acetylcholinesterase inhibition assays stock solution of technical carbaryl (1-naphthyl methylcarbamate, 99.8%, Rhone-Poulenc) was prepared in LDF medium and technical malathion (diethyl[dimethoxyphosphinothioylthio]succinate, 93%) was prepared in dimethyl sulfoxide (DMSO). Dilutions of each compound were added to the medium of 5 day-old cultures and assays were performed 5 days later. Cell viability, measured by the neutral red cytotoxicity assay (Babich and Borenfreund, 1990), was not affected by either compound at the concentration tested.

Bromodeoxyuridine incorporation

Cells were transferred into Leibovitz's L-15 medium containing 5-bromo-2'-deoxyuridine (BrdU) (10μM) and incubated overnight. After BrdU incorporation the cells were fixed with ice-cold methanol (4 °C; 10 min) and cellular DNA was denatured by the addition of 2N HCl (37 °C; 1hr). The HCl was replaced by 0.1 M borate buffer, pH 8.5, followed by PBS, and the pH was checked for neutrality. The buffer was replaced by 3% bovine serum albumin (BSA) in PBS and incubated overnight at 4 °C. Anti-BrdU monoclonal antibody (50 μg/ml; Sigma) was added and the plate incubated at room temperature for 3 hr. The cells were labeled with tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG (1:75 dilution; Sigma) (1 hr) in the dark at room temperature. Fluorescence was visualized with a rhodamine filter and photographed.

Immunoblotting

Cells were harvested by pipetting in PBS containing 2.5 mM ethylene glycol-bis (b-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA; Sigma) and collected by centrifugation. Brain tissue was dissected from adult zebrafish and rinsed in cold PBS. Cell pellets and brain tissue were extracted in two volumes of extraction buffer (1% triton X-100, 5 mM ethylenediamine tetraacetic acid (EDTA), 4 mM EGTA, 1 mM magnesium chloride, 125 mM Tris, pH 6.8) supplemented with 250 units/ml DNase and protease inhibitors (1 mM leupeptin, 1 mM pepstatin, 0.3 mM aprotinin, 1 mM Nα-p tosyl-L-arginine methyl ester) followed by the addition of 6M urea, 0.5% SDS, and 0.5% 2-mercaptoethanol. Extract was combined with an equal volume of 2X electrophoresis sample buffer containing mercaptoethanol. The proteins were separated by electrophoresis on a 7.5% polyacrylamide slab gel and then electrophoretically transferred to PVDF

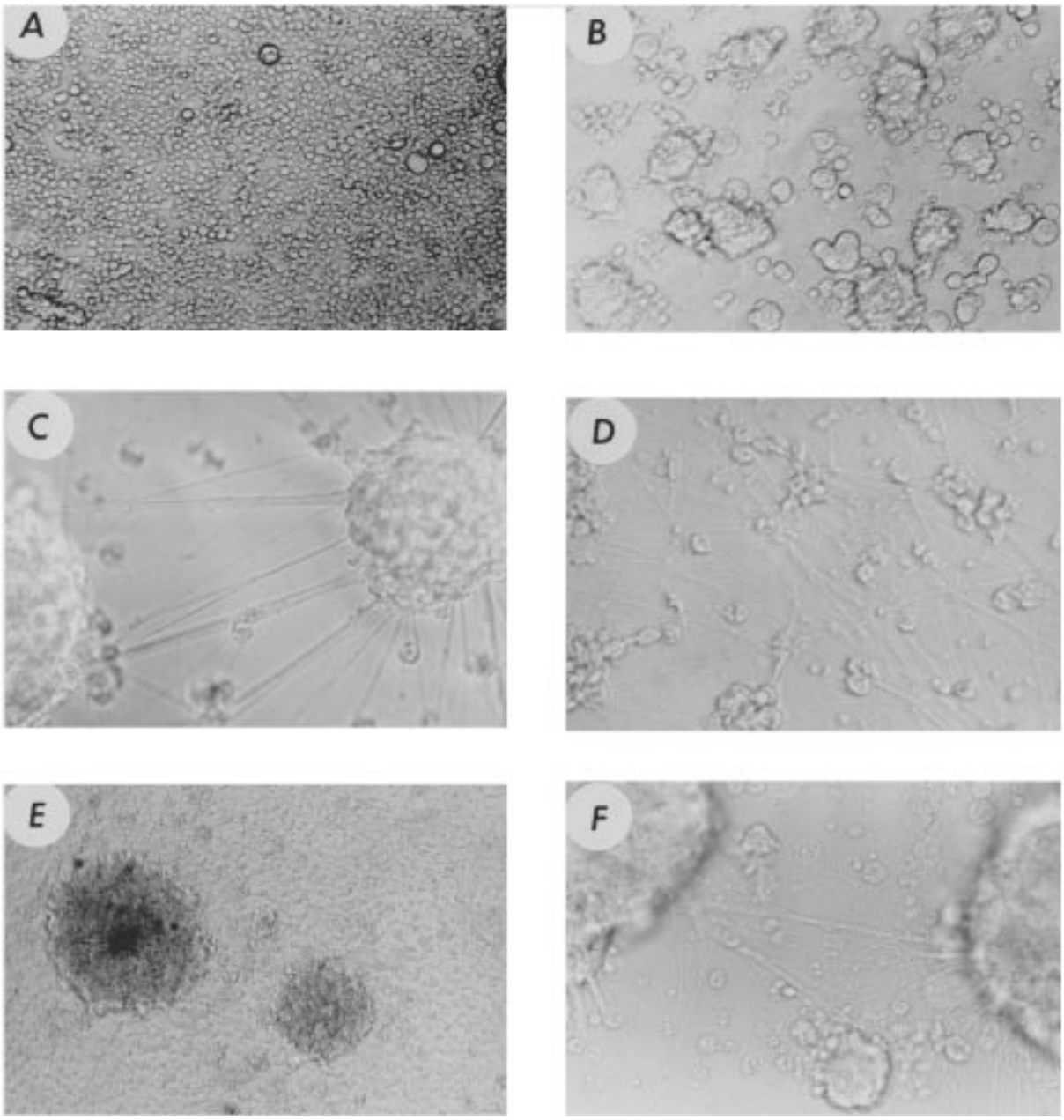

Figure 1. Primary cultures of zebrafish blastula-derived cells. (A) 1 hr after plating, (B) 24 hr-old culture showing cell aggregates, (C) 7-day old culture showing neurites extendng from the aggregates, (D) 14-day old culture showing secondary aggregates and branching network of neurites, (E) 10-day old culture initiated in the absence of poly-D-lysine, (F) culture shown 72 hrs after first passage. Microphotographs were taken with a Nikon inverted microscope equipped with Nomarski optics; magnification, 200X.

filters (BioRad). The filters were blocked with 5% non-fat dry milk in TTBS (500 mM sodium chloride, 0.05% tween-20, 20 mM Tris; pH 7.5), washed, and incubated with a 1:1000 dilution of anti-bovine neurofilament 200 or anti-human GFAP rabbit antiserum (Sigma). After washing, the blots were incubated for 1.5 hrs with peroxidase conjugated anti-rabbit IgG (1:3000 dilution). The blots were visualized by immunoperoxidase staining using 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate. Zebrafish cell lines derived from

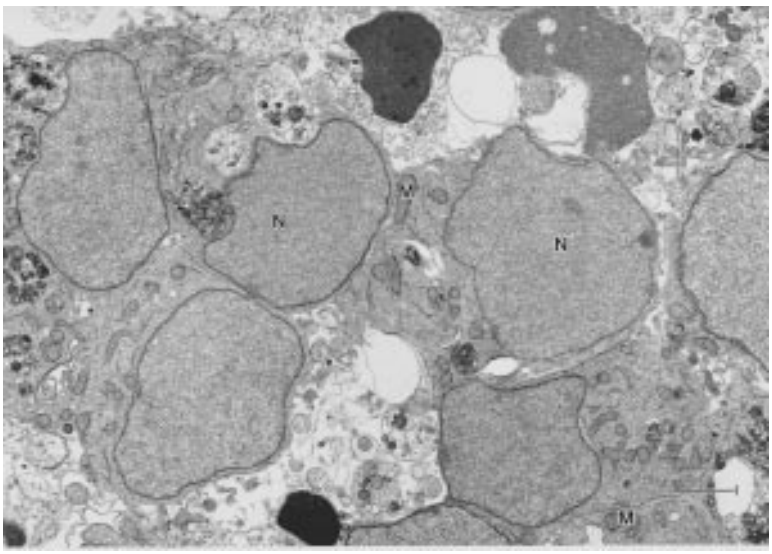

Figure 2A.

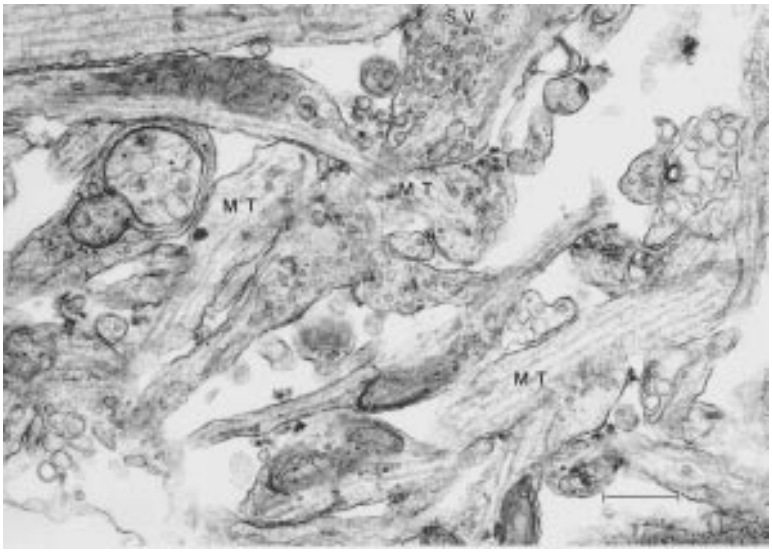

Figure 2B.

embryos (ZEM-2) (Ghosh and Collodi, 1994) and liver (ZF-L) (Ghosh *et al*., 1994) were used as controls.

Transmission electron microscopy

Cells to be examined by electron microscopy were grown in 35 mm tissue culture dishes precoated with poly-D-lysine (Falcon). The cells were fixed by gently removing one half of the culture medium from the dish and replacing it with the same volume of a 3% glutaraldehyde solution in Millonig's buffer. After 15 minutes, the dish was aspirated and fresh glutaraldehyde solution was added. After rinsing three times with Millonig's buffer the cells were incubated in postfixative solution (1% OSO_4, 1.5% KFeCN) for 1.5 hrs (4 °C) followed by three additional rinses in Millonig's buffer. The cells were then dehydrated in an ethanol series and embedded in a 1.3 mm block of Epon (Polybed 812, Polysciences). The block of Epon was separated from the dish and areas of interest were marked and cut from the Epon disc and mounted onto a larger Epon block for sectioning. Thin sections were

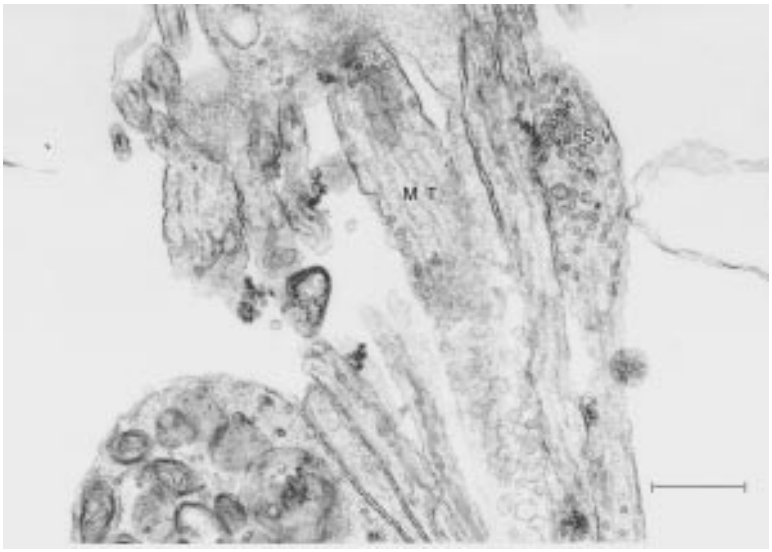

Figure 2C.

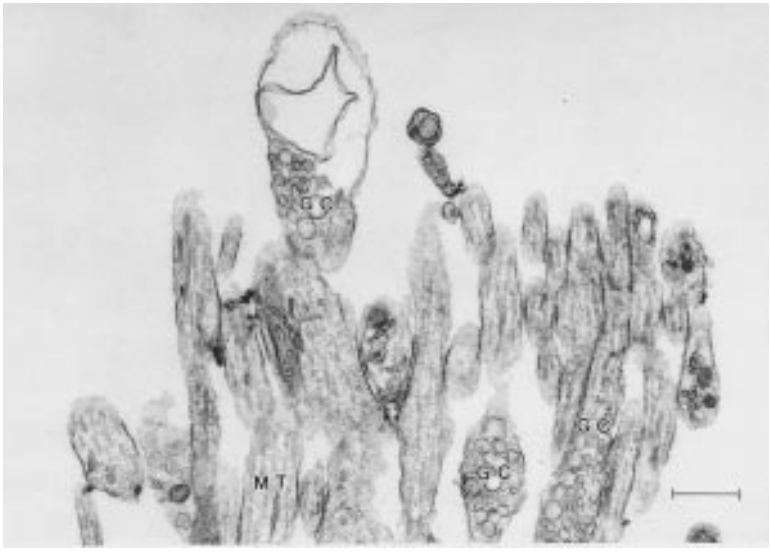

Figure 2A–D. Ultrastructure of zebrafish embryo cells in culture. Cells cultured for 10 days on poly-D-lysine were fixed, sectioned and observed by transmission electron microscopy as described in Materials and Methods. (A) section through a cell aggregate showing the cell bodies containing nucleus (N) and mitochondria (M), (B) section through neurites showing presence of microtubules (MT) and synaptic vessicles (SV), (C) synapse-like contact, containing synaptic vessicles, formed between neighboring neurites, (D) regions exhibiting growth cone-like morphology (GC) present in the neurites. (A) Bar = 2 μM; (B-D) Bar = 0.5 μM.

cut parallel to the culture surface of the dish using a Reichert Ultracut E microtome. The sections were stained in 2% uranyl acetate/lead citrate solution and viewed on a JEOL 100 XL electron microscope.

Results

Morphology of zebrafish embryo cell cultures

Primary cell cultures were derived from zebrafish blastulas in LDF medium containing trout serum, trout embryo extract, insulin, and FBS. Embryo extract and a low concentration of trout serum have been shown to

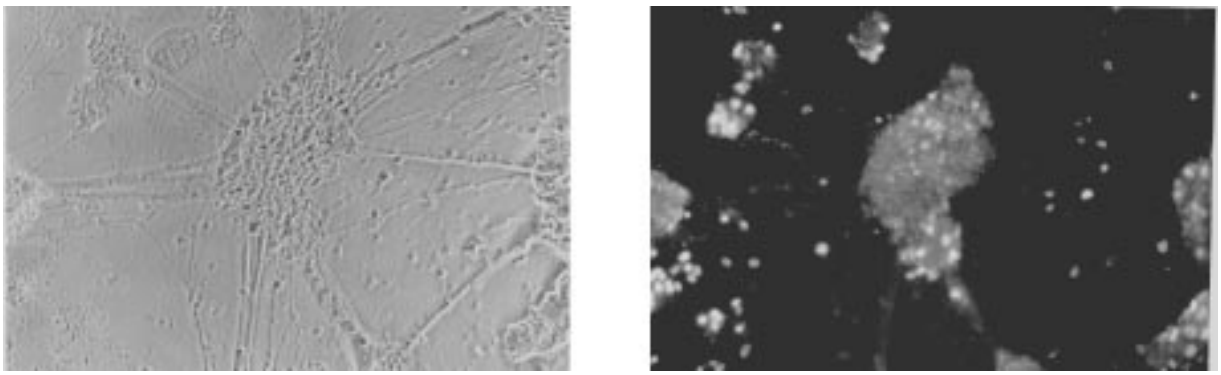

Figure 3. DNA synthesis in zebrafish blastula-derived cell cultures. Ten-day old cultures exhibiting extensive neurite outgrowth were incubated with BrdU and incorporation detected with rhodamine-conjugated monoclonal anti-BrdU. (A) Phase contrast photograph of fixed, labeled cells; (B) same field as in (A) visualized by fluorescence microscopy using a rhodamine filter showing clusters of fluorescent cells located within the cell aggregates. Controls in which BrdU was omitted did not stain.

stimulate *in vitro* growth of zebrafish blastula-derived cells (Bradford *et al*., 1994; Collodi and Barnes, 1990). When cultures were initiated on poly-D-lysine substrate in BRL-CM the cells proliferated rapidly and by 24 hr formed dense aggregates composed of approximately 50 to 100 tightly adherent cells (Figure 1A, B). The cells continued to proliferate and the aggregates increased in size. After approximately 4 days in culture, each cell aggregate produced several neurite-like fibers which grew and made contact with neighboring aggregates (Figure 1C). After two weeks, the fibers produced secondary branches that grew to form a network of interconnected neurites extending throughout the culture (Figure 1D). During this time new cell aggregates began to appear in the culture and increase in size while producing additional neurites that contributed to the network (Figure 1D). Examination of the cultures by transmission electron microscopy revealed that the neurites possessed microtubules and cluster of synaptic vessicles located in areas where contacts were formed with neighboring neurites (Figure 2B, C). Regions of the neurites were also identified that possessed a growth cone-like morphology characterized by a large number of membrane vessicles (Figure 2B, D).

Addition of BRL-CM to the culture medium stimulated cell growth and increased the length of time that healthy primary cultures could be maintained. Poly-D-lysine substrate enhanced neurite formation in the cultures and inhibited the growth of nonneuronal cell types. In the absence of poly-D-lysine the embryo cells proliferated and formed aggregates but neurite outgrowth was greatly reduced (Figure 1E). In many of these cultures nonneuronal cell types such as fibroblasts proliferated to form a confluent monolayer of cells that eventually overgrew the aggregates. In the presence of BRL-CM and poly-D-lysine primary cell cultures exhibiting extensive neurite outgrowth could be maintained for more than 8 weeks.

A large number of cells synthesizing DNA were present in cultures undergoing neuronal differentiation. Cells that incorporated BrdU were identified in approximately 90% of the aggregates contained in 10-day old cultures exhibiting extensive neurite outgrowth (Figure 3). The cultures were passaged by partially dissociating the cell aggregates in trypsin and replating the cells onto new poly-D-lysine treated plates. Cells from a single well of a 96-well dish were transferred into 3 wells. The newly passaged cells immediately adhered to the culture surface and within 48 hrs began to produce neurites (Figure 1F). Addition of BRL-CM to the culture medium promoted the recovery of the cells after passage. Healthy cultures were maintained for a minimum of 14 days after passage.

Embryo cell cultures express neuronal markers

The neuron-like morphology exhibited by the embryo cells led us to examine the cultures biochemically and immunologically for expression of neural differentiation markers. Acetylcholinesterase enzyme activity was detected soon after cell aggregates formed (2 days) and continued to increase, reaching the highest level as neurites appeared in the cultures (7 days) (Figure 4A). The acetylcholinesterase activity was inhibited by the addition of carbaryl and malathion to the culture medium (Figure 4B). Inhibition by carbaryl was greater than

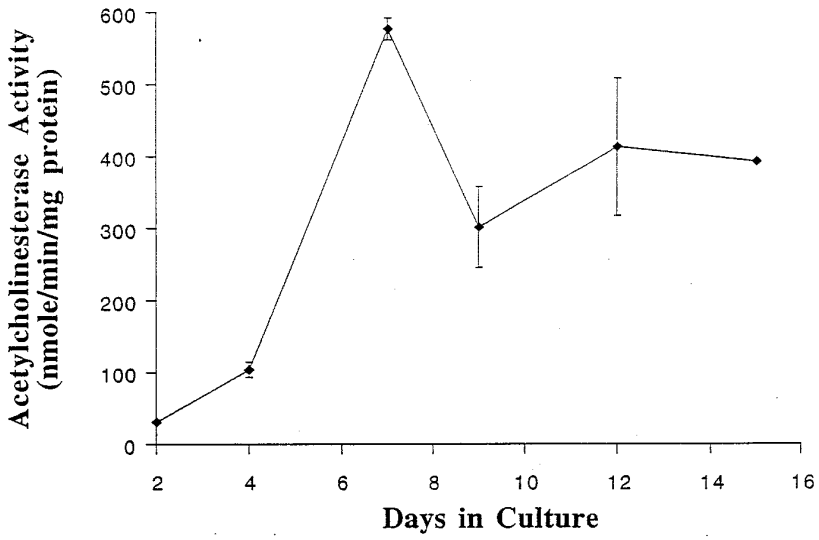

Figure 4A.

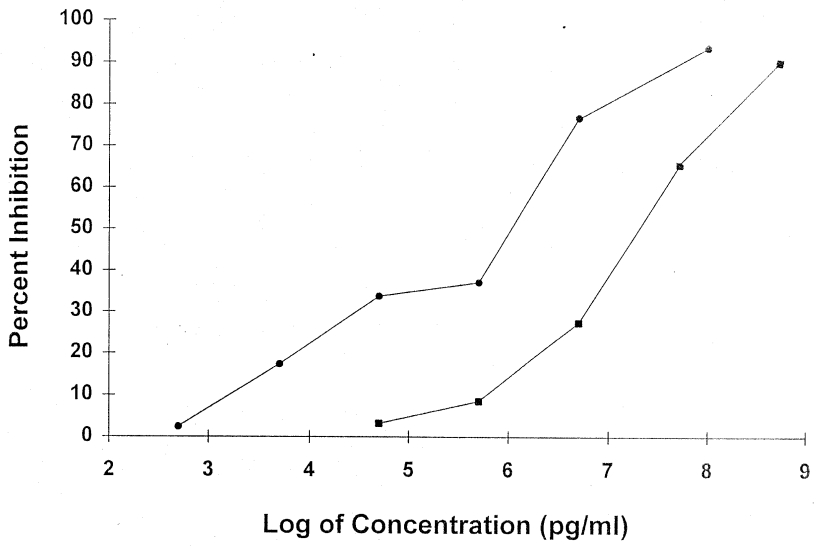

Figure 4B. Acetylcholinesterase enzyme activity in zebrafish embryo cell cultures. (A) Cultures were initiated as described in Materials and Methods and AChE activity was measured on the days indicated. Bars denote standard deviation. (B) Inhibition of acetylcholinerase activity by malathion (■) and carbaryl (●). Cells were cultured as described in Materials and Methods and each compound was added to the culture medium on day 5 and assays were performed on day 10.

malathion. Neither compound reduced cell viability at the concentrations tested (data not shown).

The cultures were examined by immunoblot analysis for expression of neural-specific proteins. Extracts prepared from 20-day old cultures contained a protein (MW_r approximately 160-kDa) that was recognized by antibodies to the neuron-specific marker, neurofilament (Figure 5). The immunoreactive protein was absent from 48 hr-old cultures and present in extracts of zebrafish brain (data not shown).

The embryo cells also expressed a protein (Mw_r approximately 50-kDa) that was recognized by antibodies to the astrocyte-specific marker, GFAP. The immunoreactive protein was present in 20-day old cultures but absent in extracts of 5-day old cultures (Figure 6). A small amount of the protein was present in

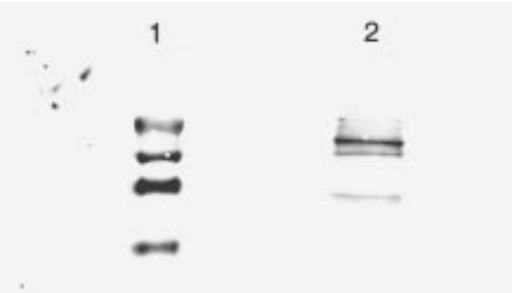

Figure 5. Immunoblot of extract (30 μg/lane) prepared from 10-day old zebrafish cell culture and probed with anti-neurofilament IgG. Extracts were prepared as described in Materials and Methods. Lane 1, molecular weight markers; lane 2, embryo cells. Molecular weight markers include myosin (205-kDa), β-galactosidase (116.5-kDa), bovine serum albumin (80-kDa) and ovalbumin (49.5-kDa).

Figure 6. Immunoblot of extracts (30 μg/lane) prepared from zebrafish cell cultures and brain probed with anti-human GFAP IgG. Extracts were prepared as described in Materials and Methods. Lane 1 molecular weight markers; lane 2, embryo cells cultured for 5 days; lane 3, embryo cells cultured for 20 days; lane 4, brain; lane 5, ZF-L liver-derived cell line. Molecular weight markers are the same as described in the legend to Figure 5.

cells from 10-day old cultures (data not shown). The protein was also detected in extracts prepared from zebrafish brain but absent from a zebrafish liver cell line (Figure 6).

Discussion

We have developed culture conditions that induce zebrafish embryo cells to differentiate *in vitro* into neurons and astrocytes. The cultures formed aggregates of tightly packed cells that produced axon- and dendrite-like structures containing microtubules and synaptic vessicles. The morphology of the cell cultures was similar to that of mammalian neuronal cultures (Ved and Pieringer, 1993). Poly-D-lysine enhanced neurite outgrowth and inhibited the proliferation of nonneuronal cells in the zebrafish cell cultures. Polylysine substrate has been shown to be important for the survival of neuronal cultures from rat (Yavin and Yavin, 1974) and chick embryo (Sensenbrenner *et al*., 1978). The substrate is also critical for neurite elongation and arborization of axons in primary neuronal cultures from rat (Raju *et al*., 1981). The effect of poly-D-lysine on the embryo cell cultures may be partially due to an increase in plating efficiency in the presence of the substrate. Ahmed *et al*. (1983) have shown that neuron survival and neurite outgrowth in culture are influenced by cell density. We have found that poly-D-lysine improved the plating efficiency and thereby increased initial cell density in the cultures. The substrate also prevented detachment of the cell aggregates during routine medium changes.

Addition of BRL-CM to the culture medium stimulated the growth of newly plated embryo cells, promoted the survival of primary cultures exhibiting a neuronal morphology, and contributed to the successful passage of the cultures. The effects of BRL-CM may be due to the neurotrophic factor, leukemia inhibitory factor (LIF), present in the conditioned medium (Smith and Hooper, 1987). LIF stimulates the generation and promotes the survival of mouse sensory neurons derived from the neural crest (Murphy *et al*., 1991). In addition to its neurotropic effects, LIF is able to maintain the pluripotent phenotype of mouse embryonal stem cell cultures and it has been used to derive cultures initiated from zebrafish embryos (Pease *et al*., 1990; Bradford *et al*., 1994) BRL-CM also contains other factors that may influence growth and differentiation of the embryo cells including mitogens such as insulin-like growth factor II (Greenstein *et al*., 1987).

Acetylcholinesterase activity in the cultures increased as the cells differentiated and the activity was inhibited by the pesticides carbaryl and malathion. Carbaryl was a more potent inhibitor of acetylcholinesterase activity in the zebrafish cells reflecting the relative *in vivo* toxicity of the two compounds in fish (Beyers and Sikoski, 1994). Inhibition of acetylcholinesterase activity by malathion indicates that the cultured cells were able to produce active metabolites of the organophosphate compounds. Previously, we demonstrated that zebrafish embryo cell cultures possess inducible cytochrome P450 enzyme activity (Collodi *et al*., 1992b). This activity may be responsible for the activation of malathion in the embryo cell cultures.

The embryo cells synthesized proteins that were recognized by antibodies to the markers neurofilament and GFAP indicating that the cultures contained a mixture of neurons and astrocytes (DeArmond *et al*., 1983; Morrison *et al*., 1985; Sivron *et al*., 1993). Mitotically active cells were present in cultures that had undergone extensive differentiation. $BrdU^+$ cells were identified in 10-day old cultures and new cell aggregates continued to appear in the cultures and increase in size for several weeks. The new aggregates produced neurites that formed connections with existing fibers. In mammals, neuroepithelial stem cells derived from the central nervous system have the capability to differentiate *in vitro* into neurons and astrocytes (Lendahl *et al*., 1990; Pleasure and Lee, 1993; Reynolds and Weiss, 1992). It is possible that neuroepithelial stem cells are present in the population of mitotically active cells that persist in the zebrafish embryo cell cultures undergoing neuronal differentiation. Since the cultures were derived from pluripotent embryo cells, they provide an *in vitro* system for investigations of the early stages of cell commitment to neuronal differentiation.

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