GREAT LAKES FISHERY COMMISSION

2000 Project Completion Report[1]

Large-Scale Production of Petromyzonol Sulfate From Lamprey Liver Cell Cultures and Culture of Sea Lamprey Pituitary Cells

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Final Report

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Abstract

Petromyzonol sulfate (PS) is a bile acid produced by lamprey larvae that is able to stimulate the olfactory system of adult lamprey and function as a migratory pheromone. Utilization of PS in the field may provide an environmentally safe species-specific method for controlling lamprey populations by influencing spawning behavior. Since employment of this strategy will require that a continuous, economical source of PS is available, the goal of this research is to develop a large-scale in vitro production system for PS using lamprey liver cell cultures. In previous work, methods were developed for the in vitro culture of liver cells derived from sea lamprey larvae. Analysis of the cultures demonstrated that the liver cells released PS into the medium, however, production was not stabile and by ten days in culture PS release declined to nearly nondetectable levels. In the current project we have been working to stabilize PS production from the cultures using methods that were successfully utilized to produce bile acids from mammalian liver cell cultures. This approach involves the fusion of PS-producing liver cells to cells of a continuously growing hepatoma cell line. The resulting hybrid cells exhibit the desirable in vitro growth characteristics of the immortalized hepatoma cells along with the capacity for stable bile acid production.

Methods have been established for the fusion of liver cells from lamprey larva with cells from the trout hepatoma cell line RTH-149 and selection of hybrid colonies has been accomplished. The hybrid cells grow rapidly in culture and are easy to maintain in simple medium indicating that once a high PS-producing colony is identified scale-up will be feasible. Three colonies of hybrid cells have been identified that continue to produce low levels of PS (0.1 to 0.5 μg/culture/72 hrs) after several months in culture. Bile acid production is stable in the cultures even after the cells have been stored frozen in liquid nitrogen. However, attempts to increase PS production from the three colonies by the addition of hormones and bile acid precursors to the culture medium were not successful. During the course of this work approximately 30 colonies have been screened for PS production in various culture conditions and a large numbers of colonies have been isolated and stored frozen for future assay.

The second goal of this project was to develop methods for the culture of cells derived from lamprey pituitary gland. The cultures will serve as a valuable tool used for research concerning the action of lamprey gonadotropin releasing hormone. To accomplish this objective, methods were established for the initiation of primary cultures derived from female lamprey pituitaries. The cultures were maintained for several weeks in medium containing FBS, fish serum and insulin on a culture surface pretreated with a combination of fibronectin and collagen.

Introduction

Adult sea lamprey (*Petromyzon marinus*) migrate into tributary streams to lay their eggs before dying. After hatching from the eggs, the larvae burrow into the stream bed and exist for several years feeding on detritus and algae until physiological and environmental factors trigger the larvae to undergo metamorphosis and migrate into open water to begin the parasitic phase of their life cycle. During the larval stages, lamprey produce the bile acids, petromyzonol sulfate (PS) and allocholic acid (ACA) which are released into the water in their urine. Biochemical and electrophysiological data have indicated that these compounds specifically stimulate the olfactory system of migratory adult lamprey with detection thresholds of approximately 10^{-12} M (Li et al., 1995). Results of cross-adaptation experiments have suggested that adult sea lamprey have specific olfactory receptor sites that recognize unique substituents of ACA and PS and are able to stimulate independent signal transduction pathways (Li et al., 1995). These results, along with studies showing that the bile acids are able to influence lamprey migration, strongly suggest that ACA and PS function as migratory pheromones in lamprey. These compounds may be used to control lamprey populations in the Great Lakes by disrupting normal spawning migration behavior or by directing migrating lamprey to traps set in specific streams. Similar pheromone based approaches have been employed to control insect populations (Jacobsen, 1972). In order to exploit this approach for lamprey population control, a continuous large-scale source of the pheromone is needed. Such a source may be obtained by in vitro production of the bile acids from lamprey liver cells grown in culture.

In previous work, we have derived and characterized a cell line from zebrafish liver and demonstrated that after more than 200 population doublings in culture, the liver cells retained many characteristics of differentiated hepatocytes (Ghosh et al., 1994). Utilizing techniques and information gained from our work with the fish liver cell cultures, we have developed methods for the long-term culture of cells derived from the liver of larval and adult sea lamprey (Ma and Collodi, 1996). Working in collaboration with Dr. Peter Sorensen at the University of Minnesota, we have demonstrated that the lamprey liver cells are able to synthesize and release PS into the culture medium. The lamprey cells were maintained in serum-free medium in order to eliminate undefined components from the medium and facilitate the detection and purification of the bile acids by HPLC. Results from approximately 100 samples of conditioned medium show that the level of PS synthesis is highest during the first week of culture ($7.30 \pm .14$ μg/culture/72hrs) and then decreases after 7 to 10 days ($1.22 \pm .29$ μg/culture/72 hrs). After two weeks in culture detectable amounts of PS continued to be produced by the liver cells ($0.57 \pm .35$ μg/culture/72hrs) and production is maintained for up to 4 weeks ($0.20 \pm .07$ μg/culture/72hrs). After this time PS is not detected in the cultures. The amount of PS (calculated per mg cell protein) synthesized by the lamprey cultures was similar to published levels of bile acid produced by mammalian liver cells in culture (Davis et al., 1983). In addition to PS, some of the lamprey cultures also produced detectable levels of petromyzonol but allocholic acid was not detected in any of the cultures. Addition of bile acid precursors, cholesterol and mevalonic acid did not have any effect on PS production and extracts prepared from lamprey liver and gall bladder in water, acetone and chloroform also had no effect. However, addition of the

glucocorticoid hormone, dexamethasone, did enhance PS production from the lamprey cells.

As we have observed with the lamprey cultures, researchers studying bile acid synthesis from mammalian liver cells in culture have reported that production declined to nondetectable levels by the end of the first week (Davis et al., 1983; Blumrich et al., 1994). This problem was solved by fusing the mammalian liver cells to cells of a hepatoma cell line derived from a liver tumor. Continuous long-term bile acid synthesis has been achieved from the resulting immortalized rat hepatocyte cell line, HPCT (Petzinger et al., 1994; Blumrich et al., 1994). HPCT cells continued to synthesize several bile acids without a decrease in rate even after more than 60 passages in culture. During a 48 hr period the cells secreted the following bile acids (per mg cell protein): cholate (344 $\pm$ 68 μg), glycocholate (145 $\pm$ 33 μg), taurocholate (1537 $\pm$ 501 μg) and glycochenodeoxycholate (179 $\pm$ 51 μg). Bile acid synthesis by HPCT cells was dependent on the presence of glucocorticoid hormones in the culture medium and synthesis was induced by the addition of mevalonic acid (Blumrich et al., 1994).

The goal of the present study is to optimize PS production from the lamprey cultures using techniques that have been successfully employed to stabilize bile acid production from mammalian liver cell cultures. During the course of this research methods were developed to fuse the lamprey cells to cells of a rainbow trout hepatoma cell line and colonies of hybrid cells have been isolated. Three hybrid colonies have been identified that continue to produce low levels of PS after several months in culture.

A second goal of this research is to development methods for the culture of lamprey pituitary cells. To accomplish this objective, primary cultures were initiated from pooled pituitary tissue and the cells were maintained for several weeks in culture.

Results

In order to stabilize pheromonal bile acid production from lamprey liver cell cultures we have developed methods to fuse lamprey PS-producing cells to a continuously growing hepatoma cell line from rainbow trout (RTH-149) (Fryer et al., 1981). The cells are fused in polyethyleneglycol (PEG), and the cell hybrids are selected in LDF medium containing 10% FBS and ouabain. Ouabain is an ATPase inhibitor that is also used in mammalian cell selection systems. The selection method that we developed for the lamprey-trout hybrid cells is outlined in Figure 1. Lamprey liver cells and lamprey liver-RTH hybrid cells are resistant to ouabain whereas nonfused RTH and RTH-RTH hybrids are sensitive to the drug and die within 10 days. Nonfused lamprey liver cells are not able to proliferate in LDF containing only 10% FBS. Using this selection method, we have isolated several colonies of RTH-lamprey liver cell hybrids. The hybrid cells do not exhibit the epithelial morphology that is characteristic of each parent cell and ultrastructural analysis by electron microscopy has revealed that the ouabain selected colonies exhibit characteristics that are typical of hybrid cells including a large irregular shaped nucleus (Fig. 2). The hybrid cells also contained a greater DNA content than either of the parent cell lines when measured by flow cytometry (Fig. 3) and they exhibited a growth rate that is similar to RTH when assayed in LDF medium

supplemented with 10% FBS. Lamprey liver cells are unable to grow in this simplified medium.

To determine if the hybrid colonies are able to stably synthesize PS in culture, medium was collected from 30 colonies and examined by HPLC for bile acid content. Results of these experiments revealed the presence of PS in medium from three of the colonies (Fig. 4). Each colony consisted of approximately 5 x 10^5 cells that were derived from a single hybrid cell and levels of PS production ranged from 0.1 to 0.5 µg/culture/72hrs. The positive colonies were expanded into tissue culture flasks and frozen in liquid nitrogen. PS remained stable in cells that were thawed and reinitiated in culture. Addition of the glucocorticoid, dexamethasone at several different concentrations had no effect on PS production in any of the three colonies. Also, mevalonic acid and cholesterol incorporated into lipid vessicles did not increase PS production in the cultures (Kalen et al, 1990).

Conclusions

Results from this project indicate that fusion of lamprey liver cells to cells of a rainbow trout hepatoma line (RTH-149) will provide a method to successfully stabilize PS production. Three colonies producing low amounts of PS after many months in culture were identified from the approximately 30 colonies screened in this study. Since the lamprey-trout hybrid cells exhibit a high growth rate in simplified medium the cultures will be convenient to maintain and easy to grow on a large scale. To use this approach for large-scale production of PS a high producing colony must be identified. Successful identification of a high-PS producing colony and optimization of culture conditions for bile acid production will be greatly facilitated by the availability of a rapid and sensitive assay method that will make it possible to screen and provide immediate feedback on large batches of colonies such as those grown on a 96-well microtiter plate. Such an assay system will make it possible to immediately identify PS-producing colonies that can be scaled up and stored frozen for further study while the negative colonies are discarded.

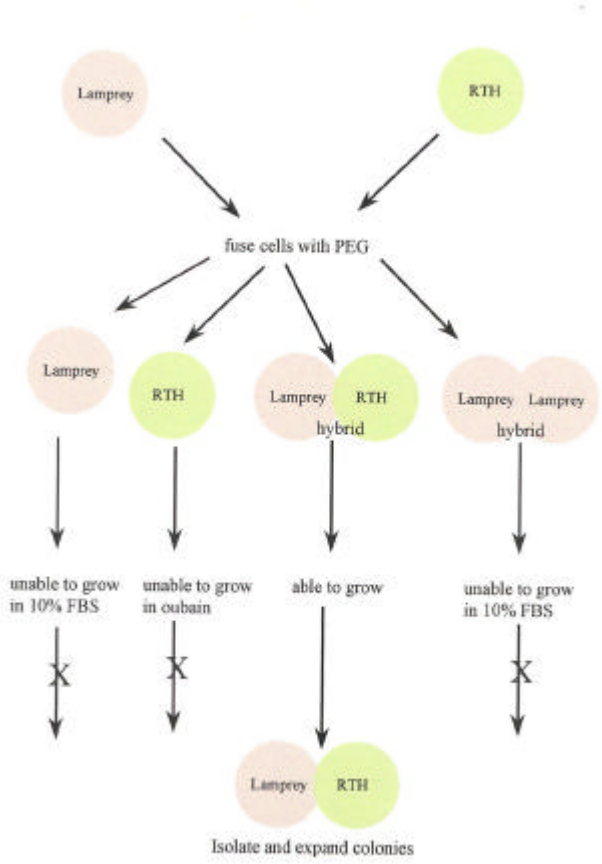

Figure 1. Selection of lamprey liver-rainbow trout hepatoma cell hybrids

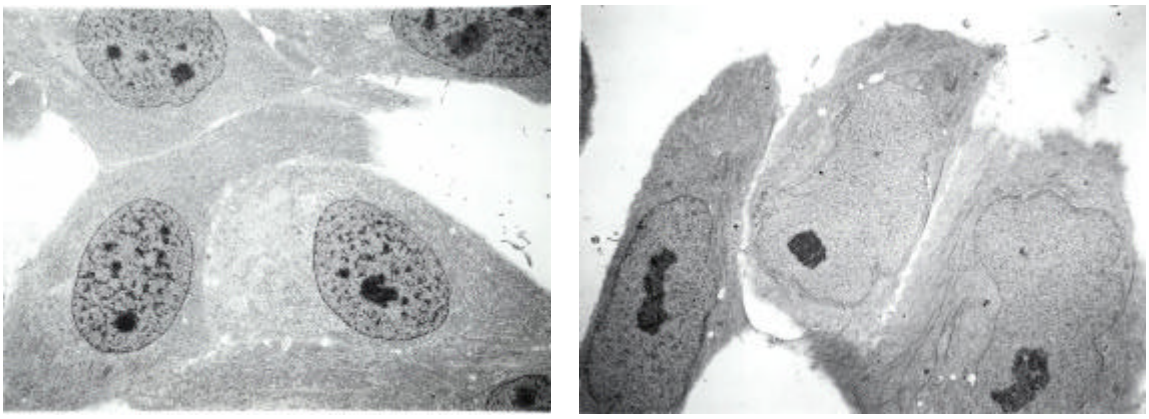

Figure 2. Ultrastructural analysis of RTH cells (left) and lamprey-RTH hybrid cells (right). Hybrid cells possess a larger irregular shaped nucleus than the RTH cells.

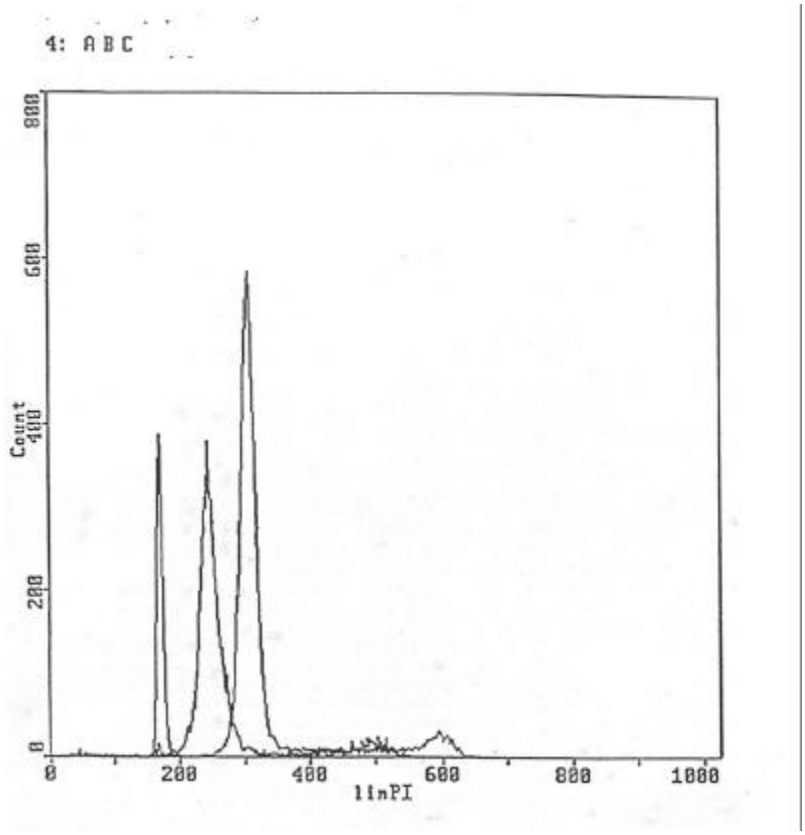

Figure 3. Flow cytometric analysis of lamprey (left peak), RTH (middle peak) and hybrid cells (right peak). Hybrid cells selected by the method outlined in Figure 1 possess a different chromosomal number than either of the parent cells.

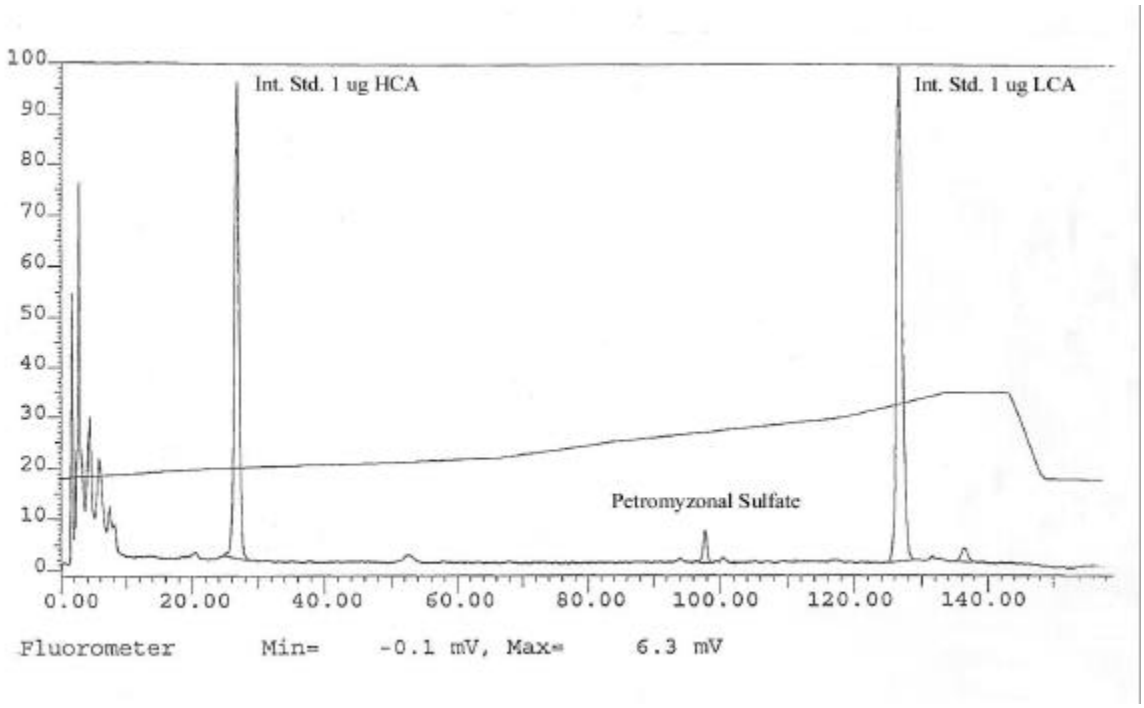

Figure 4. HPLC chromatograph showing the presence of PS in medium collected from a colony of hybrid cells.

Experimental Methods

Lamprey. Lamprey larvae are maintained in 20 gallon aquaria containing a sand substrate and fed a suspension of live yeast once a week. Before use each larva is transferred to clean water and starved for a period of 4 to 5 days. Larvae are sacrificed in 3-amino benzoic acid ethylester and the surface of the animal is sterilized in 95% ethanol. Dissections are done aseptically in a laminar flow hood. Following dissection, each liver is placed in a petri dish containing culture medium supplemented with antibiotics (described below). The tissue is rinsed 4 or 5 times with fresh medium and then enzymatically dissociated by incubating 30 minutes in trypsin/EDTA solution (0.2% trypsin, 1mM EDTA in PBS). Periodically, during trypsinization, the tissue is mechanically disrupted by gently pipetting repeatedly through a 10 ml pipette. When the tissue is disrupted enough to pass through the pipet, the action of the trypsin is stopped by adding FBS (10% final volume) to the tube. The dissociated cells are collected by centrifugation (500xg, 5min), resuspended in culture medium and transferred to a 35-mm tissue culture dish that had been treated with a commercial basement membrane preparation (matrigel, Collaborative Research). To initiate larger cultures, livers from 3 or 4 larvae can be processed together and plated into a 25 cm^2 flask pretreated with matrigel.

Initiation of Cell Cultures. Liver cells are grown in LDF basal nutrient medium. LDF consists of a mixture of Leibovitz's L-15, Dulbecco's modified Eagles and Ham's F-12 media (50:30:20) containing sodium bicarbonate (0.15 mg/ml), HEPES buffer (15mM; pH 7.0), selenium (10^{-8} M), penicillin (200 IU/ml), streptomycin sulfate (200 μg/ml) and ampicillin (25 μg/ml). The LDF medium is supplemented with insulin (10 μg/ml), epidermal growth factor (20ng/ml), trout serum (2%) and FBS (10%). Cultures are incubated at 18^o C and the medium is changed approximately once a week.

Bile acid production from the liver cell cultures is determined by HPLC analysis of cell-conditioned medium in Dr. Peter Sorensen's laboratory at the University of Minnesota (Li et al., 1995). Based on our previous work, the concentration of bile acids produced in vitro by the lamprey cells (1 to 5 μg/ml) is well within the HPLC detection limits for PS (approximately 0.1 to 0.5 μg/ml) without concentrating the medium. To obtain serum-free conditioned medium, liver cell cultures initiated as described above are transferred into serum free LDF containing EGF, insulin and dexamethasone (0.5 μg/ml) and medium is collected every 72 hrs and stored frozen (-20^o C). Cultures can be maintained for approximately 4 weeks in serum-free medium before they must be transferred back into serum-containing medium. After one week in serum, the cultures can be transferred back to serum-free conditions and medium can be collected again. This cycle can be repeated several times and serum-free medium can be collected from the same culture for more than 6 months.

Measurement of PS. Medium collected from individual flasks is extracted with activated C_{18} solid phase extraction (SPE)cartridges (J.T. Baker Co.) and bile acids are eluted with methanol (Li et al., 1995). Bile acid analysis is conducted using a reverse-phase Nova-Pak C_{18} 4-μm column (4 mm x 10 cm) eluted with a step-wise gradient of ammonium dihydrogen phosphate (25mM, pH 7.8) and acetonitrile. Eluted bile acids are passed

through a second column (5 cm x 0.5 cm) containing 3α-hydroxysteroid dehydrogenase and bound to glutaraldehyde-treated aminopropyl glass beads. Buffer containing NAD (0.1 M Tris-HCl, pH 8.5, 2.7 mM EDTA, 1.63 mM dithiothreitol and 0.01 mM NAD) is introduced using a tee between the first and second columns at a constant rate of 1 ml/min. This enzyme oxidizes 3α-hydroxyl bile acids into 3α-keto bile acids and reduces NAD to NADH which is subsequently detected by a fluorescence detector with a narrow band excitation filter of 340 nm and a wide band emission filter with a range of 420-650 nm. Peak areas are calculated using a chromatography software program (712 System Controller). Bile acids are identified by comparing their retention times with those of standards. To enhance our ability to detect and quantitate the bile acids produced in the cultures, the bile acids are metabolically labeled by adding ^{14}C-cholesterol (5μCi/ml) to the serum-free medium. In addition to the detection system described above, the labeled bile acids eluted from the C_{18} column are identified and quantitated using a Radiomatic detector.

Cell Fusions. Cell fusions are performed using previously published methods with modification (Yip and Bols, 1982; Blumrich et al., 1994; Petzinger et al., 1994) (Figure 1). Lamprey liver cells and RTH are suspended together (3:1 ratio) in LDF medium containing 10% FBS and added to a 25 cm^2 flask. The lamprey cells can be obtained from previously initiated cultures or directly from whole livers. To isolate the cells from whole liver, the tissue is dissected and prepared for cell culture as described above. The RTH and lamprey liver cells are incubated in the flask for 2 or 3 days and then the medium is removed and 4 mls PEG (15% in PBS, pH 7.0) is added and left on the cells for 10 to 12 minutes. We have found that higher concentrations of PEG that are normally used for cell fusion dissociate the lamprey cells from the culture surface. The PEG is diluted with 4 mls LDF and then the contents of the flask is immediately aspirated and the flask gently rinsed three times with fresh medium. After fusing, the cells are incubated 48 hrs in LDF containing 10% FBS and then ouabain (0.15 μM) is added to select for lamprey-RTH hybrids. Based on repeated control experiments, we have demonstrated that in the presence of ouabain, RTH and RTH-RTH fused cells die after 5 to 7 days and no surviving cells can be visualized or recovered when the ouabain is removed from the medium. Lamprey liver cells and lamprey-lamprey fused cells survive ouabain treatment for more than 14 days. Viable cells can be easily visualized in the cultures but the cells proliferate very slowly in 10% FBS after the ouabain is removed. After fusing mixed cultures of RTH and lamprey cells, we have isolated several colonies that are resistant to ouabain for more than 14 days and proliferate rapidly when the ouabain is removed from the medium.

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