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LONG-TERM PRIMARY CULTURE OF EPITHELIAL CELLS FROM RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) LIVER

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SUMMARY

Long-term primary cultures of epithelial cells from rainbow trout (*Oncorhynchus mykiss*) liver have been established. Nearly homogenous (>97%) populations of hepatocytes were placed into primary culture and remained viable and proliferative for at least 70 d. In addition to hepatocytes, proliferative biliary cells persisted in the cultures for at least 30 d. Finally, a third type of epithelial cell, which we have termed a "spindle cell," consistently appeared and proliferated to confluence in these cultures. The confluent cultures of spindle cells were successfully subcultured and passaged.

The initial behavior, growth, and optimization of serum and media requirements for these cells is described. All three cell types proliferated as measured by thymidine incorporation, autoradiography, proliferating cellular nuclear antigen analysis, and propidium iodine staining. Further efforts to characterize the cells included western blotting and immunohistochemical staining with antibodies to cytokeratins previously reported in fish liver. From these data, it appears that all three cell populations are epithelial in nature. Furthermore, significant changes in actin organization, often indicative of transformation or pluripotent cells, were observed with increased time in primary culture.

Key words: fish; liver; hepatocyte; biliary epithelial cell; cytokeratin; proliferation.

INTRODUCTION

The development of a reproducible technique to isolate (Berry and Friend, 1969) and maintain hepatocytes in primary culture (Bissell et al., 1973) has led to rapid advances in our understanding of the details of mammalian liver function. Subsequently, the isolation and short-term primary culture of hepatocytes was successfully adapted to teleost systems (e.g., Birnbaum et al., 1976; Hayashi and Ooshiro, 1978; reviewed in Moon et al., 1985). These cultures have been used to study various aspects of teleost liver metabolism including hormonal regulation (e.g., Birnbaum et al., 1976; Mommsen and Saurez, 1984), gluconeogenesis and glycolysis (e.g., Mommsen and Saurez, 1984; Ottolenghi et al., 1984; Cornish and Moon, 1985), protein metabolism (e.g., Plisetskaya et al., 1984; Bhattacharya et al., 1985), and lipid metabolism (e.g., French et al., 1983). In addition, primary hepatocyte cultures have also proven useful in toxicological and pharmacological (Parker et al., 1981; Morrison et al., 1985; reviewed in Baski and Frazier, 1990; Gagnè et al., 1990; Miller et al., 1993a, 1993b), developmental (e.g., Maitre et al., 1986; Vaillant et al., 1988) and chemical carcinogenesis studies (Bailey et al., 1982; Klaunig, 1984; Hightower and Renfro, 1988; Loveland et al., 1988). Nonetheless, a potential limitation to extending these studies has been the failure to maintain hepatocytes in primary culture for extended periods of time.

Cheng et al. (1993) recently described efforts to culture lake trout liver cells in a hormone-supplemented, serum-reduced media. While continuous cultures were established, no data are presented on cell characterization/identification except to state that some cultures have remained epithelioid in cell morphology. Likewise, Lee et al. (1993) has developed a cell line, RTL-W1, by proteolytic dissociation of liver fragments. The cells were described as predominantly polygonal or epitheliallike, but as the cultures reached confluence bipolar or fibroblastlike cells appeared. 7-ethroxyresorufin O-deethylase (EROD) was measured in response to β-naphthaflavone exposure suggesting hepatocyte characteristics were retained. However, with the exception of transmission electron microscopy, no effort was made to identify/characterize the cells. Conversely, Fryer et al. (1981) have described the establishment of a cell line (RTH-149) from an aflatoxin-induced hepatoma. While these cells appear to retain some characteristics of normal hepatocytes, their origin is from a tumor and as such their utility for studies of normal liver function may be limited.

Blair et al. (1990) have described the formation of a hepatocyte monolayer following placement in primary culture. After a few hours in culture, the hepatocytes moved together and formed "mounded aggregates." These mounds were very reminiscent of floating multicellular spheroids previously described in rat hepatocyte cultures

(Landry et al., 1985). Unlike mammalian cells, however, the aggregated mounds observed in rainbow trout cultures maintained contact with the plate through a few individual contact points, simply described as filamentous attachments, appearing to be less than 0.1 μm in diameter. Within 4–6 d, however, most aggregates completely disassociated from the dish but remained viable as judged by trypan blue exclusion and the lack of characteristic ultrastructural features of necrotic cells. However, the investigators did report significant vacuolation was evident among the hepatocytes after 5–6 d in culture. In the absence of extended aggregate attachment, investigation of the long-term viability of the cells comprising the aggregates was not possible.

We now report our success in maintaining rainbow trout hepatocytes in primary culture for extended periods of time on positively charged culture dishes in the presence of serum-containing media in a 5% CO_2 atmosphere. Specifically, we were successful in inducing the mounded aggregates as reported by Blair et al. (1990), followed by their firm re-attachment to the culture dish. Moreover, re-attachment was rapidly followed by the spread of cells from the periphery of the aggregates as they simultaneously flatten and eventually disappeared. In addition to hepatocytelike epithelial cells, we also observed biliary epithelial cells and spindle-shaped epithelial cells growing in these cultures. We describe for the first time, several lines of investigation to characterize the origin and novel behavior of these cells in the early stages of primary culture. Finally, we report on the successful passage and continuous culture of these cells.

Materials and Methods

Materials

Phalloidin-FITC F-actin (F-432) and DNase I-FITC G-actin (D-970) were purchased from Molecular Probes (Eugene, OR). Monoclonal antibodies for western blotting were an anti-pancytokeratin (PCK-26) from Sigma Chemical Co. (St. Louis, MO) and an anti-vimentin (V9) from DAKO Corp. (Carpinteria, CA). Western blots were visualized with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Monoclonal antibodies for immunohistochemical staining of cells included an anti-vimentin antibody (ICN Biomedicals, Lisle, IL), an anti-desmin antibody (MeDiCa, Carlsbad, CA), AE1/AE3 (Boehringer Mannheim Co., Indianapolis, IN) specific to pancytokeratin, Cam 5.2 (Becton Dickinson, Mountainview, CA) specific to low molecular weight cytokeratins, and MAK 6 (Triton Biosciences, Alameda, CA), an anti-pancytokeratin. Mouse control MsIgG-FITC, goat anti-mouse FITC conjugate, and unconjugated mouse isotope control MsIgG1 were purchased from Coulter Source, Marietta, GA. Conjugated mouse isotope control MsIgG1-FITC and propidium iodine were purchased from Sigma. An antibody to proliferating cell nuclear antigen (PCNA) was obtained from DAKO (clone PC10). Human bladder transitional cell carcinoma cell lines were purchased from American Type Culture Collection (ATCC) (Rockville, MD) and primary human peripheral blood lymphocytes were purified from whole blood.

Experimental Animals

Shasta strain rainbow trout (*Oncorhynchus mykiss*) were obtained from the Oregon State University Marine/Freshwater Biomedical Center. Fish were maintained in Living Stream (Frigid Units, Toledo, OH) recirculating raceway aquaria (LSW-900) at 10 ± 0.5° C. The fish, males and females weighing 300–500 g, were fed 2–3 times daily approximately 1 g food/100 g body weight (Purina Trout Chow).

Primary Culture of Rainbow Trout Hepatocytes

Isolation and placement into primary culture. Isolation of hepatocytes followed the method of Blair et al. (1990) with minor modifications. The perfusion medium was continuously gassed (5% CO_2:95% O_2) and maintained at a temperature of 16° C. Collagenase [(50 mg) (Sigma, Product # C-5138)] was added to the perfusion medium and perfusion extended to 35 min to allow for complete digestion of liver connective tissues. All washing steps were carried out at 0 to 4° C and the final cell pellet was resuspended in 2 vol of culture medium (Eagle's minimum essential medium (EMEM) with Earle's salts and L-glutamine without $NaHCO_3$ [MEM (JRH Biosciences, Lenexa, KS) supplemented with $NaHCO_3$ (26 m*M*), L-glutamine (2 m*M*), 1.317 g NaCl, MEM-non-essential amino acids solution (0.1 m*M*), penicillin G (100 000 U/l), streptomycin sulfate (0.1 g/liter), amphotericin B (250 μg/liter, and 10% heat-inactivated bovine calf serum (Sigma, Product # C-6278)]. Cells were resuspended by repetitive manual pipetting (10–20 times) and 0.5 ml aliquots of the suspension pipetted into 14.5 ml of culture media in 100 × 20 *mm Primaria* tissue culture plates (Falcon-Becton Dickinson, Franklin Lakes, NJ, Product # 3803). Plates were maintained in a 16° C, 5% CO_2:95% air atmosphere in a Sheldon Lab-Line Incubator (Portland, OR). To facilitate attachment and growth, cells were not disturbed during the initial 2 wk in culture and media was not changed. Subsequently, 60–80% of the media was changed at 2–3 wk intervals and/or when cells were subcultured.

Serum requirements in primary culture. Replicate experiments were conducted in which the type and the concentrations of serum in the culture medium varied. After isolation, cells were resuspended in serum-free culture medium and pipetted onto triplicate plates containing 10% fetal calf serum (FCS), 10% FCS + 2% bovine serum albumin (BSA), 10% newborn calf serum, or 10% bovine calf serum. All plates were examined daily for 30 d and evaluated for attachment, aggregation, spreading, degree of confluence, cell integrity, and overall appearance. Cells failing to remain attached were examined for viability by trypan blue exclusion. We did not detect any obvious differences between the different serum types or different vendors of the same serum type. Considering cost, we elected to optimize culture conditions with bovine calf serum.

In the second round of experiments primary cultures were again conducted in triplicate with either 0%, 1%, 5%, or 10% bovine calf serum added to the culture medium. Again, plates were examined daily for 2–3 mo., periodically thereafter, and evaluated for attachment, aggregation, spreading, degree of confluence, cell integrity, and overall appearance.

Identification of Cells in Primary Culture

Visualization of cytokeratin and vimentin expression via western blotting. Hepatocytes were placed into primary culture in 100-mm dishes for either 1, 4, 10, or 33 d. In addition, replicate 32-d cultures were subcultured 1:3 and sampled on Day 33 along with the primary cultures. Cells were washed with isotonic phosphate-buffered saline (PBS) and the resulting cell pellet homogenized with 10 vol of EBC buffer (50 m*M* Tris-pH 8.5, 120 m*M* NaCl, 0.5% non-ident P-40, 100 m*M* sodium fluoride, 200 μ*M* sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml phenylmethylsulfonyl fluoride, 2 m*M* EGTA, and 2m*M* EDTA) and subjected to sodium dodecyl sulfate polyacrylamide-gel electrophoresis (Laemmli, 1970). Protein concentration of the cell homogenates were determined by the method of Lowry et al. (1951) with BSA as a standard. Western blotting with PCK26 and V9 followed manufacturers protocols using Immobilon polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) and probing with the Vectastain ABC kit.

Immunohistochemical identification of cell types in primary culture. Trout hepatocytes were isolated and seeded onto 100-cm Primaria dishes as described above. At Time 0 (about 1 h after attachment), and Days 2, 10, 28, and 70 of primary culture plates were drained of media, flooded with 10% neutral buffered formalin for 2 h, followed by 70% ethanol for at least 24 h. Cells were removed from culture plates by gentle scrapping and rafts of cultured cells pipetted into 2-ml centrifuge tubes and pelleted. The pellet was wrapped in tissue paper, cassetted, and routinely paraffin processed. Paraffin blocks were cut at 5–7 μm and stained with Harris hematoxylin and eosin. Additional serial sections were deparaffinized, rehydrated, and stained with five commercially available antibodies including anti-vimentin, anti-desmin, AE1/AE3 (specific to cytokeratins 1–8, 10, and 14–16), Cam 5.2 specific to cytokeratins (8 and 18), and MAK 6 (specific to cytokeratins 8, 14–16, 18, and 19) (Bunton, 1993). Control tissues, used in each analysis, included intact rainbow trout liver and a set of canine tissues including liver, lung, skin, skeletal muscle, thyroid, and salivary gland. Staining was subjectively scored on a scale of 0 to 4 with 0 = no staining and 4 = strong staining.

Behavior of Cells in Primary Culture

DNA content/synthesis. Due to the initial clumping and spreading of the cells in primary culture, it was necessary to verify that cells were actually dividing and growing as well as spreading. Four measures of DNA content/synthesis were utilized including fluorometry, autoradiography, proliferating cell nuclear antigen (PCNA) analysis, and propidium iodide staining of intact cells. For the initial two methods of analysis, fluorometry and autoradiography, hepatocytes were isolated and replicate 10-cm plates were maintained in primary culture for the duration of the experiment. Sampling time points were Days 1, 4, 10, 19, and 33. Prior to the designated time point, four plates were incubated with ^{3}H thymidine (specific activity, 85 Ci/m*M*) for 16 h (Ostrander et al., 1993). Three of the plates containing cells were washed 3 times with PBS and cells collected in 1.5 ml Eppendorf tubes for DNA quantification by fluorometry and scintillation counting. The fourth plate was fixed with 10% neutral buffered formalin and stored for subsequent autoradiography. Cells used for DNA fluorometric analysis were pelleted at 1500 ×*g*, resuspended in 10 vol of a cell lysis buffer (Promega, Madison, WI, Product # E153A), and disrupted with five strokes of a homogenizer; the analysis then followed published procedures (e.g., An et al., 1982).

Plates used for autoradiographic analysis were initially coated with NTB-3 autoradiographic emulsion (Eastman Kodak Co., Rochester, NY, Product # 1654441) and stored at −90° C for 21 d. Following development of the emulsion, plates were stained for 30 min with hematoxylin, rinsed for 5 min with deionized water, and counterstained for 2 min with eosin Y. Each plate was then overlaid with an identical template and 10 randomly arranged x/y coordinates were marked. Subsequently, at each of the 10 coordinates, the number of silver grains over the nuclei of 20 cells (approximately one microscopic field at 400 × 1000 were counted for a total of 200 cells scored for each time point. The mean number of silver grains per cell was then determined for each time point. An additional plate containing a primary culture of trout tumor cells from a hepatocellular carcinoma was also examined.

In order to measure DNA synthesis by PCNA analysis and propidium iodide staining, replicate culture dishes of cells were washed with PBS and fixed in 70% ethanol at 4° C overnight with gentle mixing and stored at 4° C until processed. PCNA followed standard procedures for fish tissue sections (Ortego et al., in press) with modifications for cell cultures as described below. Briefly, processing was initiated with the removal of ethanol and washing of cells with PBS. Cells were then resuspended in 0.5 ml of 2.5% Noble agar (Difco Laboratories, Detroit, MI) at 50° C (Allred et al., 1993), centrifuged at 3000 ×*g* for 30 min, agar plugs containing cells cassetted, and routinely processed and embedded in paraffin. Sections were cut at 4.5 μm, mounted on poly-L-lysine-coated slides, and air-dried overnight. Sections were then deparaffinized as previously described (Ortego et al., in press), blocked with 0.1% sodium azide and 3% H_2O_2 in 25% methanol for 15 min, and rinsed with ddH_2O for 5 min. The antigen retrieval procedure used was modified from Ortego et al. (in press) as follows. Slides were immersed in citrate buffer (Leong, 1993) containing 12.75% sodium citrate monohydrate and 11.75% trisodium citric acid dihydrate in ddH_2O, pH 3.8 (modified from Danscher et al., 1993). The slides were heated in a 700 watt microwave oven 3 times for 2 min each with two 1-min cooling periods (modified from Foley et al., 1994). Following antigen retrieval, the slides were rinsed 2 times with ddH_2O and once with automation buffer equivalent for 5 min. Slides were covered with Shandon coverplates and then inserted into the staining tray. All subsequent steps through diaminobenzidine (DAB) incubation were carried out in total darkness. Following blocking, slides were incubated with primary antibody for 1 h, biotinylated secondary antibody (BioGenex Laboratories, San Ramon, CA) for 30 min, buffer rinsed, and incubated with peroxidase-conjugated streptavidin solution for 30 min. Visualization occurred following incubation in freshly prepared 0.05% DAB in 0.18% H_2O_2 and counterstained with Harris hematoxylin (Poly Scientific, Bay Shore, NY). PCNA quantitation was accomplished by counting 1000 nuclei from random fields and scoring positive staining cells (i.e., indices are expressed as positive cells per 1000).

For propidium iodide staining cells were washed 3 times with PBS and finally pelleted at 600 ×*g* for 5 min, resuspended in 4 ml of PBS, and filtered through 44 μm nylon mesh to remove clumps. Cells were pelleted and the supernatant removed; 850 μl of RNAse A (Sigma) were added to each tube, tubes mixed, and incubated for 45 min at room temperature. Finally, 185 μl of propidium iodide solution (55.6 mg/500 ml H_2O) was added to all tubes prior to a 20-min incubation at 4° C in the dark. After the final incubation, the cells were pelleted at 500 ×*g* and all but 300 μl of supernatant removed. Samples were analyzed on a Coulter Epics Profile II cytometer with 25 mW laser system at a sample flow rate of 17 μl/min, sheath pressure at 7.5 psi, in a sample volume of 150 μl.

G-actin and F-actin expression. Freshly isolated rainbow trout hepatocytes, cells from various time intervals in primary culture, and cells from a rainbow trout hepatocellular carcinoma were examined for G-actin and F-actin expression. Cells were washed with PBS, fixed in 70% ethanol at 4° C overnight with gentle mixing, washed 3 times with PBS, and finally pelleted at 600 ×*g* for 5 min. The resulting cell pellet was resuspended in 10 ml of PBS and 5 × 10^5–5 × 10^6 cells were aliquoted into separate 12 × 75 mm polyurethane tubes by filtering through a 44 μm nytex filter to eliminate clumping. 100 μl of 0.1% Triton-X-100 were added to each tube, tubes vortexed, and incubated at 4° C for 10 min. Cells were then resuspended, washed 2 times in 4 ml of PBS, and finally resuspended in 4 ml PBS with gentle mixing. 10 μl of FITC-conjugated phalloidin or FITC-conjugated DNase I conjugate was added to each sample

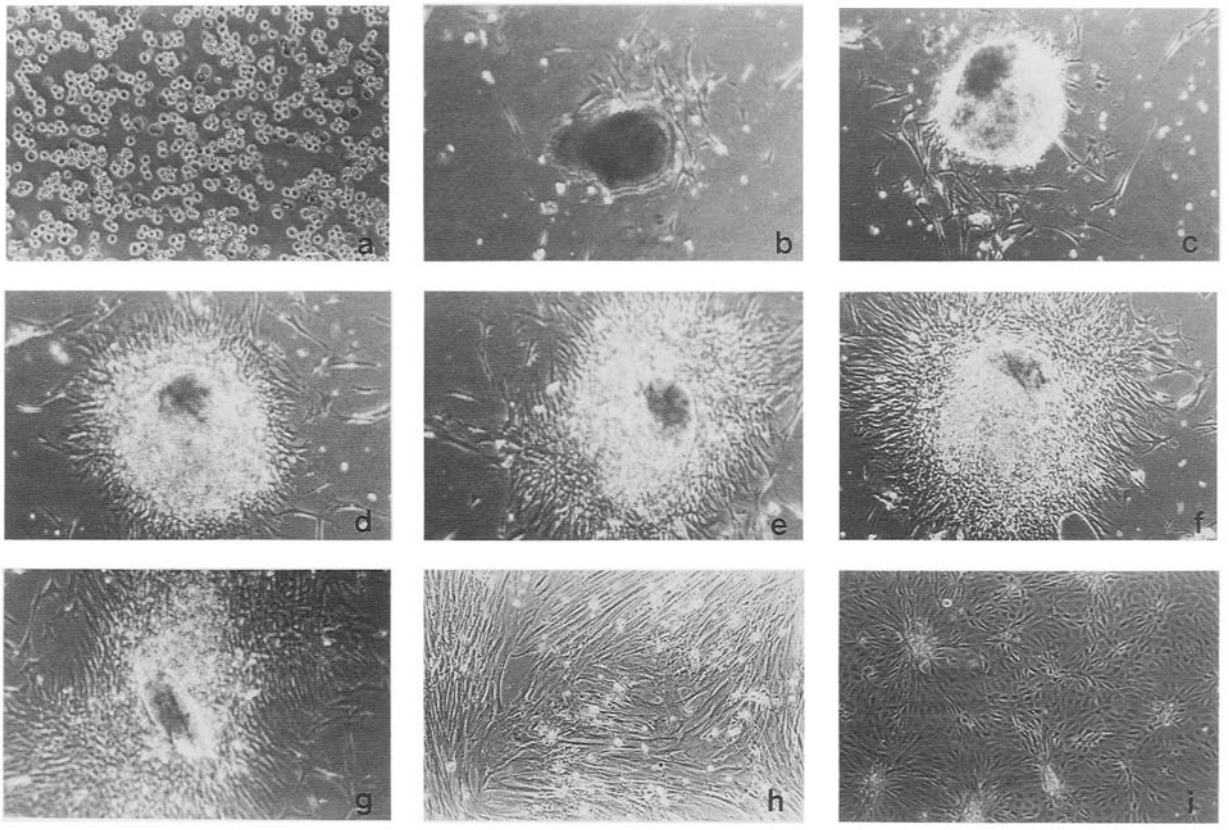

FIG. 1. Primary culture of rainbow trout hepatocytes. *Panel a*, Day 0; *panel b*, Day 7; *panel c*, Day 14; *panel d*, Day 19; *panel e*, Day 24; *panel f*, Day 26; *panel g*, Day 30; *panel h*, Day 30; *panel i*, Day 30. Panels *a–g* are ×100 and *h* and *i* are ×400.

tube and 5 µl (5 µg) of Mouse IgG1-FITC conjugate was added to each control tube. All samples were incubated at 4° C in darkness for 45 min. Following incubation 4 ml PBS were added to each sample and control tube, cells briefly vortexed, and cells pelleted at 500 ×*g* for 5 min. The supernatant was removed, cell pellet briefly vortexed, 1.850 µl RNase (67 units/ml) added to each sample tube, vortexed, and incubated an additional 45 min in darkness. Following incubation, 0.815 µl of propidium iodide (pH 7.2) was added to each tube, gently mixed, and centrifuged at 500 ×*g* for 5 min. Supernatant was aspirated (leaving about 300 µl of cell suspension), which was briefly vortexed. Samples were stored on ice in the dark until flow cytometric (FCM) analysis (maximum duration of storage: 2 h). FCM analysis was with an Epics Profile II (Coulter Cytometry, Hialeah, FL) 25mW 488 nm laser and analyzer system. Data were collected and transferred to an IBM 386 PC for processing and statistical analysis using ReproMan (TrueFacts Software, Seattle, WA.)

Continuous Culture of Rainbow Trout Liver Cells

Subculturing. Upon reaching confluence (approx. 30 days), cells were subcultured 1:3. Briefly, media was decanted, cells rinsed once for 60 s with 0.05% trypsin in 0.53 m*M* EDTA. Immediately after removal of trypsin-EDTA solution, 2 ml were again added to the flask or plate and the cells were incubated at room temperature until they were released into suspension. Once cells were completely disassociated, 8 ml of media was added to the original flask, gently mixed, and 3 ml aliquots transferred to two additional flasks. Finally, an appropriate volume of media was added and cells were incubated 16° C, 5% CO_2:95% air atmosphere. Cells were routinely subcultured at a ratio of 3:1 as they reached confluence.

Long-term storage. Periodically, either primary or continuous cultures were frozen for long-term storage. Cultures were harvested and centrifuged at 250 g for 5 min to pellet the cells. Cells were resuspended in freezing medium (EMEM with 10% calf serum and 10% DMSO) and 1 ml aliquots were dispensed into Corning cryogenic vials (Corning, NY, Product # 25724–2). Vials were placed in a polystyrene box and placed at −90° C to achieve the desired freezing rate of 1° C/min. After 24 h, vials were stored in liquid nitrogen. Stored cells could be recovered and returned to culture. Vials containing cells were removed from liquid nitrogen and thawed rapidly in a 24° C water bath with gentle agitation. Vial contents were transferred to flask with culture media and returned to the incubator. After 24 h, culture media was replaced to remove residual DMSO.

Statistical Analysis

Proliferation data on thymidine incorporation and autoradiography were analyzed with SAS (1985). The Levenes test indicated equal variances ($P = 0.6004$ and $P = 0.72$, respectively) so, an analysis of variance with follow-up least significant difference (LSD) tests were performed.

Results

Primary Culture of Rainbow Trout Hepatocytes

Isolation and placement into primary culture. Depicted in Fig. 1 is the typical sequence of events following isolation of hepatocytes and placement into primary culture. Initially, a single cell suspension was distributed uniformly over the tissue culture dish. Within 30 min cells began to attach to the culture dish and form small aggregates (Fig. 1 *a*). Within 24–48 h, the small aggregates fused into larger aggregates of which the majority remained weakly attached to the plate. After 48 h, about 20% of the aggregates were detached and floated freely in the media. These clumps of cells, as originally reported by Blair et al. (1990), remained viable. Over about 8–10 d, the weakly attached large aggregates became firmly attached and the first evidence of cells spreading or growing-out from the periphery of the larger aggregates was evident (Fig. 1 *b*).

Initial growth or movement of cells from the periphery of the aggregates, over the next 6–10 d (Fig. 1 *c*), was relatively slow. However, the rate of growth/movement accelerated markedly beginning about Day 19 (Fig. 1 *d*) and continued through Days 24, 26, and 30 (Figs. 1 *e*, 1 *f*, and 1 *g*). During this period of rapid growth, the size of the original large aggregates decreased by dorso-ventral compression toward the surface of the dish. From the periphery of the aggregates, large numbers of spindlelike cells were observed to migrate in all directions. By Day 30, cells from the aggregates had migrated to the point of making contact with cells from neighboring aggregates and, depending on initial seeding density and temperature, plates were confluent. Confluent cells in primary culture exhibited one of two basic morphologies; either spindlelike (Fig. 1 *h*) or hepatocyte/epithelial-cell-like (Fig. 1 *i*). Moreover, foci of the cells with the hepatocyte/epithelial-like phenotype could also be seen prior to confluence, often within the first 21 d. The appearance of these foci occurred in about 20% of the plates. The number of foci per plate varied from 1–4 with 2 being typical.

Examination of H & E stained sections of the primary cell cultures revealed that at Time 0 and Day 2, the liver cell cultures were composed of >90% mature, well-differentiated hepatocytes together with aggregated biliary ductular epithelia, which appeared tubular in nature. By Day 10, some necrosis of hepatocytes and small foci of proliferating spindle cells was observed. By Day 28, the cultures were composed of a mixture of small epithelial cells with a hepatocytelike appearance, irregular biliary epithelial cells with attenuated epithelium, and pleomorphic spindle cells. Finally, by Day 70, the culture was composed of mostly pleomorphic spindle cells with a few foci of hepatocyte/epithelial-like cells.

Once cells reached confluence, they could be subcultured or frozen (discussed below). Alternatively, if left in culture and supplied with regular changes of media, the cells remained viable for an extended period of time in an apparent state of quiescence. For example, we have maintained one primary culture of confluent cells for over 1200 d with biweekly media changes. The cells are no longer dividing but remain viable.

Serum requirements in primary culture. Among the various sera tested no obvious differences were observed in attachment, degree of aggregation, rate of cell growth/spreading, time to confluence, cell integrity, and overall appearance of the cultures (data not shown). Considering cost and availability, bovine calf serum was selected for all further studies.

Primary cultures with different serum concentrations in the culture media exhibited significant differences within a few weeks, which typically remained constant through termination of the experiment at 10 mo. For example, at 3 mo., cells plated with no serum were only 10% confluent, most exhibited a stellate morphology, and some vacuolation was present. Cells growing in 1% serum were only 20% confluent and exhibited a stellate morphology and similar vacuolation to those cultures with no serum added. Conversely, cultures with 5% serum added were 80% confluent, had a spindle cell morphology (Fig. 1 *h*), and were not stellate or vacuolated. Finally, cells at 10% serum were 90% confluent (with some piling or layering), most cells had a fibroblastlike morphology (Fig. 1 *h*), and no stellate or vacuolated cells were observed. In all cultures, except 0%, we have observed foci with a distinct hepatocyte/epithelial-cell-like morphology. Frequency of foci appearance did not appear to be linked to serum concentration. On a number of occasions, we have subcultured these foci and the resulting cultures are primarily spindle cells with a few areas exhibiting the hepatocyte/epithelial cell-like morphology (Fig. 1 *g*).

Identification of Cells in Primary Culture

Visualization of cytokeratin and vimentin expression via western blotting. As shown in Fig. 2 *a*, significant alterations in cytokeratin expression were evident beginning about Day 4 (*lane 5*) when compared to Time 0 (*lane 4*) cultures. As the cells continued in culture, other changes in cytokeratin expression occurred as evidenced by the appearance of faster migrating bands appearing about Day 19 (*lane 7*). Cytokeratin expression in tumor cells (*lane 10*) looked remarkably similar to cells that had been held in long-term primary culture (*lane 9*). As expected, NIH3T3 cells were negative for cytokeratin expression (data now shown).

Vimentin, as expected, was not observed in these cells at any time in culture (Fig. 2 *b*, *lanes 4–9*). A strong signal was evident at about 40 Kd from NIH3T3 cells (Fig. 2 *b*, *lane 2*), which served as a positive control. The weak signal in *lane 3* is from a homogenate of total trout liver. This was expected since the homogenate included blood vessels and connective tissue, which express vimentin.

Immunohistochemical identification of cell types in primary culture. Antibody staining of intermediate filaments of intact rainbow trout liver and liver cell cultures was very informative. The anti-cytokeratin AE1/AE3 antibody was specific for biliary ductular epithelia of intact rainbow trout liver with no staining of hepatocytes, periductular connective tissue, or arterial smooth muscle (Table 1). Among the initial liver cell cultures, similar results were observed with the AE1/AE3 antibody having a strong affinity for the few biliary epithelial cells and being negative for hepatocytes. In the Day 28 sample, the AE1/AE3 antibody exhibited decreased binding to biliary epithelia with attenuated epithelium and strong staining of the spindle cells. The spindle cells maintained strong staining in the Day 70 cultures as well. Staining with the MAK 6 antibody (Table 2) resulted in similar findings with MAK 6 being specific for biliary epithelium. However, when the older cultures (Day 28 and Day 70) were examined, there was a decreased intensity of staining of the spindle cells. Staining with CAM 5.2, an antibody against low molecular weight cytokeratins, revealed no specificity with any liver cell types in the rainbow trout (results not shown).

The results of staining with the anti-desmin antibody in the intact control liver was consistent with our observations of control canine

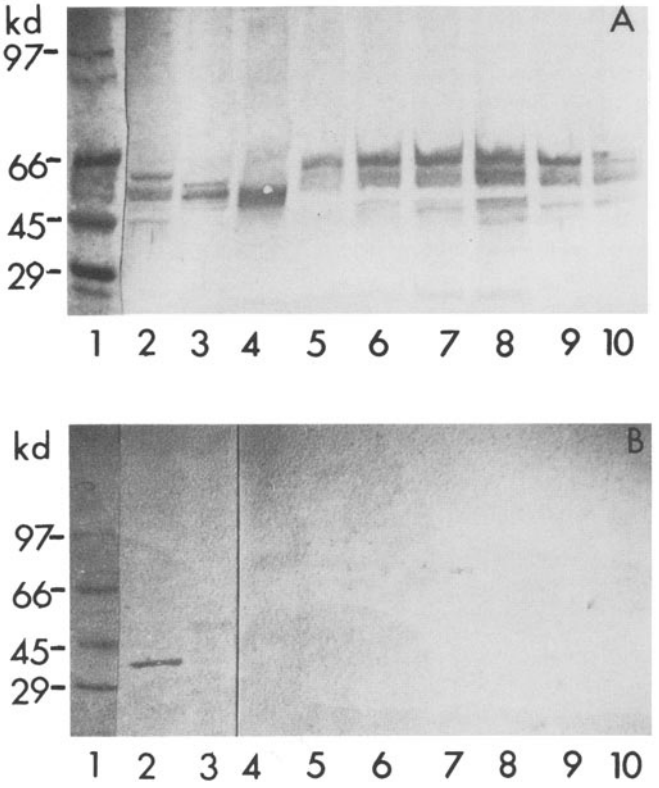

FIG. 2. Western blot analysis of cytokeratin (*A*) and vimentin (*B*) in expression in rainbow trout epithelial cells at various time intervals in primary culture. *A. Lane 1*, Molecular weight standards; *lane 2*, homogenate of rat liver; *lane 3*, homogenate of trout liver; *lane 4*, Day 0; *lane 5*, Day 4; *lane 6*, Day 10; *lane 7*, Day 19; *lane 8*, Day 33a; *lane 9*, Day 33 (these cells had been passaged after 32 d in primary culture); *lane 10*, tumor cells from a primary culture of an aflatoxin-induced hepatocellular carcinoma. *B. Lane 1*, Molecular weight standards; *lane 2*, NIH3T3 cells; *lane 3*, homogenate of rainbow trout liver; *lane 4*, Day 0; *lane 5*, Day 4; *lane 6*, Day 10; *lane 7*, Day 19; *lane 8*, Day 33; *lane 9*, Day 33 (these cells had been passaged after 32 d in primary culture); *lane 10*, tumor cells from a primary culture of an aflatoxin-induced hepatocellular carcinoma.

TABLE 1

RESULTS OF ANTI-CYTOKERATIN ANTIBODY STAINING OF INTACT RAINBOW TROUT LIVER AND LIVER CELL CULTURES

	Immunohistochemical Staining with Anti-Cytokeratin Antibody AE1/AE3[a]				
Day	Bile Ducts	Hepatocytes	Connective Tissue	Smooth Muscle	Spindle Cells
Intact Liver	4	0	0	0	NP
0	4	0	NP	NP	NP
2	4	0	NP	NP	NP
10	4	0	NP	NP	NP
28	2	0	NP	NP	4
70	NP	NP	NP	NP	3

[a] Anti-filament staining was subjectively scored on a scale of 0 to 4 with 0 = no staining and 4 = strong staining. NP = indicated cell type not present in the culture.

TABLE 2

RESULTS OF ANTI-CYTOKERATIN ANTIBODY STAINING OF INTACT RAINBOW TROUT LIVER AND LIVER CELL CULTURES

	Immunohistochemical Staining with Anti-Cytokeratin Antibody MAK6[a]				
Day	Bile Ducts	Hepatocytes	Connective Tissue	Smooth Muscle	Spindle Cells
Intact Liver	4	0	0	0	NP
0	4	0	NP	NP	NP
2	4	0	NP	NP	NP
10	4	0	NP	NP	NP
28	0	0	NP	NP	2
70	NP	NP	NP	NP	1

[a] Anti-filament staining was subjectively scored on a scale of 0 to 4 with 0 = no staining and 4 = strong staining. NP = indicated cell type not present in the culture.

TABLE 3

RESULTS OF ANTI-VIMENTIN ANTIBODY STAINING OF INTACT RAINBOW TROUT LIVER AND LIVER CELL CULTURES

	Immunohistochemical Staining with Anti-Vimentin Antibody[a]				
Day	Bile Ducts	Hepatocytes	Connective Tissue	Smooth Muscle	Spindle Cells
Intact Liver	0	0	2	2	NP
0	0	0	NP	NP	NP
2	0	0	NP	NP	NP
10	1*	1*	NP	NP	NP
28	1*	1*	NP	NP	1*
70	NP	NP	NP	NP	0

[a] Anti-filament staining was subjectively scored on a scale of 0 to 4 with 0 = no staining and 4 = strong staining. NP = indicated cell type not present in the culture; 1* = diffuse non specific staining.

tissues where the antibody is strongly positive for skeletal muscle, but only variable and inconsistently positive in arterial smooth muscle. No significant staining of cultured liver cells with the anti-desmin antibody was observed (results not shown).

Staining with the anti-vimentin antibody in intact control trout liver occurred as expected with the antibody exhibiting specificity for the connective tissue surround medium and large bile ducts and the smooth muscle walls of large arteries. Mesenchymal tissues for which vimentin is specific includes both fibroblasts and muscle cells. Staining in cultured liver cells (Table 3) was either completely negative or only slightly positive. However, the mild positive staining observed was diffuse and we consider it nonspecific. There was no staining of the spindle cells with the anti-vimentin antibody.

Behavior of Cells in Primary Culture

DNA content/synthesis. DNA synthesis was initially measured by thymidine incorporation and quantified by fluorometry. Reported in Fig. 3 are values (CPM/μg DNA) at various time points in primary culture following hepatocyte isolation. Incorporation of thymidine into rainbow trout hepatocytes was found to be linear for least 24 h (results not shown). During the initial 4 d in primary culture, a statistically significant ($P < 0.05$) increase in thymidine incorporation was observed as CPM/μg DNA ± SD rose from 10 145 ± 3779 after 24 h in culture to 20 707 ± 2832. Thymidine incorporation remained active for about 3 wk and then gradually decreased to 11 613

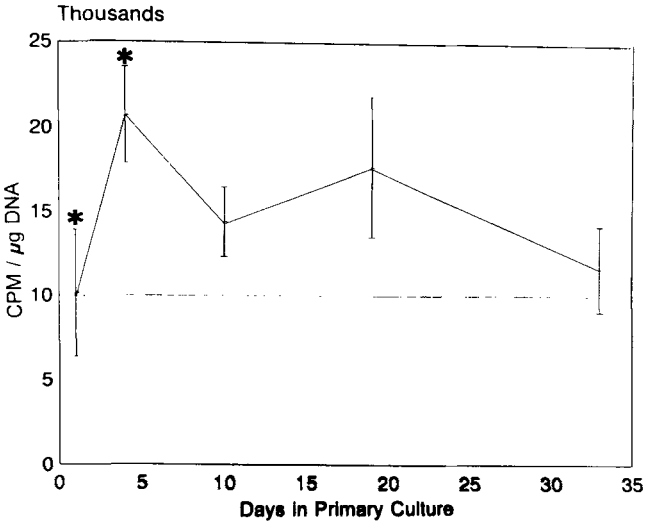

FIG. 3. DNA synthesis as measured by thymidine incorporation into rainbow trout epithelial cells in primary culture. Rainbow trout hepatocytes were maintained in culture for 33 d and DNA synthesis, as determined by thymidine incorporation, was determined at various time points. *Error bars* include one standard deviation about the mean of triplicate samples. ★ indicates a significant difference from all other time points.

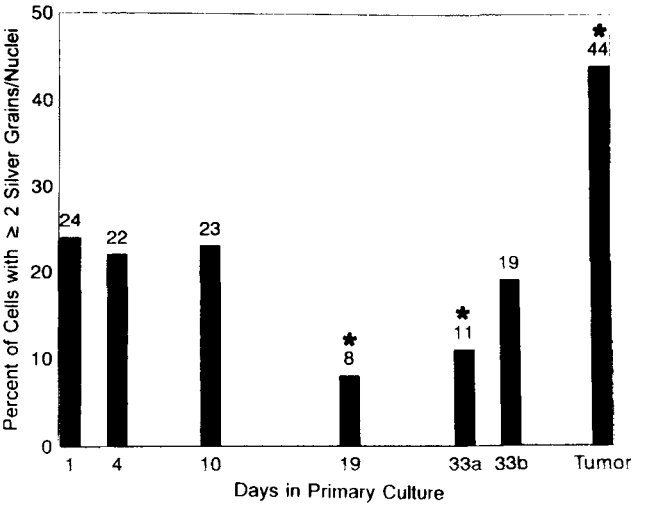

FIG. 4. Autoradiographic analysis of rainbow trout epithelial cells and a hepatocellular carcinoma in primary culture. As detailed in "Materials and Methods," prior to the designated time point, each plate of cells was incubated with ^{3}-H thymidine for 16 h, coated with emulsion, incubated at −90° C for 21 d, and stained. The mean number of silver grains per cell was then determined for each time point for 200 cells. Day 33a cells were nearly confluent after 33 d in primary culture and did not differ from Day 19 cells. Day 33b cells had been passaged on Day 32 and were beginning to undergo DNA synthesis. An additional plate containing a primary culture of rainbow trout tumor cells from an aflatoxin-induced hepatocellular carcinoma was also examined. ★ indicates a significant difference from all other time points.

± 3149 by Day 33 as the cells became confluent. An analysis of variance ($P = 0.05$) followed by the LSD test indicated that Day 1 and Day 4 were significantly different from all other days ($P < 0.05$). In an additional experiment, we examined the epithelial cell cultures immediately (1 d) after subculturing confluent cells from 32-d primary cultures. Following 1 d in subculture, DNA synthesis as measured by thymidine incorporation had not been initiated (9721 ± 744 CPMs/μg DNA). Finally, hepatocytes from an aflatoxin-induced tumor were examined by this method and incorporation of thymidine was significantly elevated when compared to freshly isolated hepatocytes as we observed a mean level of 16 840 ± 7529 CPM/μg DNA (data not shown).

A complementary measurement of intact cells was taken by quantifying silver grains over nuclei following autoradiography. These results are presented in Fig. 4. Throughout the first 10 d in culture, 22–24% of the cells were observed to have three or more silver grains over the nucleus, suggesting a modest level of DNA replication. On Day 19, the percent of cells with three or more silver grains had dropped to 8% and a similar value of 11% was observed on Day 33 (*33a*). Both of these values were significantly different from all other days as determined by an analysis of variance ($P = 0.034$) and LSD test ($P < 0.05$). As in the previous experiment, one plate of cells was subcultured on Day 32 and thymidine incorporation examined after 1 additional d in culture. Data from a single plate (33b) suggest DNA synthesis is increasing as nearly 20% of the cells exhibited three or more grains. Finally, examination of cells from the hepatocellular carcinoma demonstrated significantly ($P < 0.05$) higher levels of thymidine incorporation as greater than 44% of these cells presented three or more grains over the nucleus (Fig. 4, *tumor* lane).

PCNA analysis proved highly effective in demonstrating temporal changes in DNA synthesis of the various cell types in primary culture. Furthermore, this analysis allowed for the simultaneous scoring and identification of each cell type present in the cultures (Table 4). As expected, during the initial 10 days in culture, the predominate cell type was hepatocytes and the PCNA index rose steadily from 10 at Time 0 to 37 on Day 4. On Day 10, two distinct populations of hepatocytelike epithelial cells were visible including those comprising islands and disseminated cells. The PCNA index for these two populations was 64 and 41, respectively. In addition, on Day 10, a few proliferative biliary epithelial cells were beginning to organize. On Day 19, the PCNA index for hepatocytelike epithelia rose to 71. However, the highest activity on Day 19 was seen in spindle cells with an index value of 240. The last time point examined, Day 44, occurred when the culture was confluent. The PCNA index had risen to 232 indicating that 23% of the population was proliferative. Finally, by this time very few biliary cells were present and neither they nor the spindle cells were proliferative in the confluent culture.

Cell cycle analysis was determined by propidium iodide staining of intact cells (Table 5). The majority (96%) of freshly isolated cells (Time 0) were found to be in G_0-$_1$, while 2% were in S-phase and G_2-M. By Day 1, a significant decrease in the percent of cells in G_0-G_1 was noted (86.5%) as was a concurrent increase in cells in S-phase (8.0%) and G_2-M (3.5%). This trend was maintained through Day 10 when 85% of the cells were observed to be in G_0-G_1 and 13% were in S-phase.

G-actin and F-actin expression. Changes in F-actin and G-actin content in rainbow trout epithelial cells during the initial 50 d in primary culture are tabulated in Table 6 and graphically presented in Fig. 5. As expected, at Time 0 (Fig. 5, *panel D*) hepatocytes contained significant levels of F-actin distributed over two peaks with mean channel numbers (MCN) of 165 and 144. Conversely, comparatively little G-actin was present with MCN of 84 and 43 for the primary and secondary peaks (Fig. 5, *panel A*). After only 4 d, nearly

TABLE 4

PCNA STAINING OF PRIMARY HEPATOCYTE CULTURES

Days in Primary Culture	PCNA Index		Description
	HEP	SPN	
Day 0	10	N/C	Predominantly hepatocytes with some areas of ductular organization (i.e., biliary epithelial cells)
Day 4	37	N/C	Predominately hepatocyte aggregates
Day 10	64[a] & 41[b]	N/C	Hepatocyte aggregates[a] and disseminated hepatocytes[b] were scored separately. A few BEC were present, proliferative, and beginning to organize. Too few to index.
Day 19	71	240	Hepatocyte aggregates and ductular areas present. Majority of cells were spindle cells.
Day 44	232	N/C	Hepatic structure intact. Confluent culture comprised of mostly spindle cells and a few BEC, neither were proliferative.

Key: PCNA = proliferating cell nuclear antigen; index value is number of positive cells per 1000 scored. HEP = hepatocytes. BEC = biliary epithelial cells. SPN = spindle cells. N/C = none counted; too few present or proliferative to index.

TABLE 5

CELL CYCLE ANALYSIS OF PRIMARY HEPATOCYTE CULTURES

Days in Culture	Phase of Cell Cycle		
	G0–G1 (%)	S-Phase (%)	G2-M (%)
Day 0	96.0	2.0	2.0
Day 1	86.5	8.0	3.5
Day 4	84.0	11.5	4.5
Day 10	85.0	13.0	2.0

TABLE 6

MEAN CHANNEL NUMBER VALUES OF PRIMARY AND SECONDARY PEAKS OF G-ACTIN AND F-ACTIN IN PRIMARY RAINBOW TROUT HEPATOCYTE CULTURES

Time in Culture	G-ACTIN		F-ACTIN	
	1° PEAK	2° PEAK	1° PEAK	2° PEAK
Day 0	84	43	165	144
Day 4	137	NPD	140	NPD
Day 10	143	NPD	132	NPD
Day 40	120	NPD	30	57
Day 50	164	97	26	70

NPD: No peak detected.

identical levels of G- and F-actin were noted and only a single peak for each was present in the hepatocyte populations (Fig. 5, *panels B* and *E*). Specifically, F-actin levels began to drop, MCN = 140, and a corresponding increase in G-actin was noted, MCN = 137. This trend continued through Day 40 when both a primary and secondary peak were again discernible for F-actin and MCN of 30 and 57 were detected (Fig. 5, *panels C* and *E*). The Day 40 MCN for G-actin was 120. Finally, after 50 d in culture, the F-actin MCN for the primary and secondary peaks had dropped to 26 and 70 and for G-actin had risen to 164 and 97, respectively. Thus, over the initial 50 d in primary culture, a significant decrease in F-actin occurred, possibly through depolymerization, while a corresponding and significant increase in G-actin expression was observed. The contribution of each cell type to the analysis at the later time points is not possible. However, visual and immunohistochemical analysis of replicate cultures suggest that by Day 40 the majority of cells remaining viable are spindle cells and hepatocytelike epithelial cells. Actin expression was also examined in cells from a hepatocellular carcinoma isolated from a rainbow trout. As depicted in Fig. 6, the predominant actin expression was in the form of G-actin (MCN = 225), as compared to F-actin (MCN = 135).

Continuous Culture of Rainbow Trout Epithelial Cells

Subculturing and long-term storage. After 30 d, confluence was achieved in most cultures and passage of the cells was possible. To date, we have successfully subcultured cells from over a dozen different preparations. The oldest active culture has been subcultured 24 times. We have been successful in placing these cells in long-term storage (liquid nitrogen) and reintroducing them into culture after as long as 1 yr in storage. Characterization of these cell lines is in progress.

Discussion

This is the first report of the establishment and characterization of long-term primary cultures of epithelial cells from fish liver. While previous attempts to culture epithelial cells from trout liver have been successful (e.g., Lee et al., 1993; Cheng et al., 1993), those cells and cell lines have not been adequately identified/characterized as to the cell type(s). Nearly homogenous (>97%) primary cultures of hepatocytes were initiated and remained viable and proliferative for at least 70 d. Proliferative biliary cells persisted in the cultures for at least 30 d and a third epithelial cell type, we have termed spindle cells, was persistent in our cultures and could be passaged. We describe the initial behavior of these cells in primary culture and our preliminary steps to characterize them.

Blair et al. (1990) reported that hepatocyte aggregates loosely adhered to culture dishes by long filamentous attachments measuring less than 0.1 μm in diameter. Within 5–6 d of primary culture, the majority of their aggregates had dislodged from the culture dish. Considering the delicate nature of the attachments and the fact that the culture medium was changed daily, it is not surprising that they did not achieve long-term aggregate culture. In our studies, we employed a different medium, 5% CO_2 atmosphere, and positively-charged Primaria dishes. As such, the concerted effect of these modifications precluded a media change for at least 2 wk after initiating hepatocyte in culture. We believe this action not only allowed for the initial attachment and aggregate formation previously reported, but permitted the cells to eventually re-attach to the dish and begin to spread/grow. The exact trigger for this response is presently unknown, the specific mechanism may lie in the secretion of specific

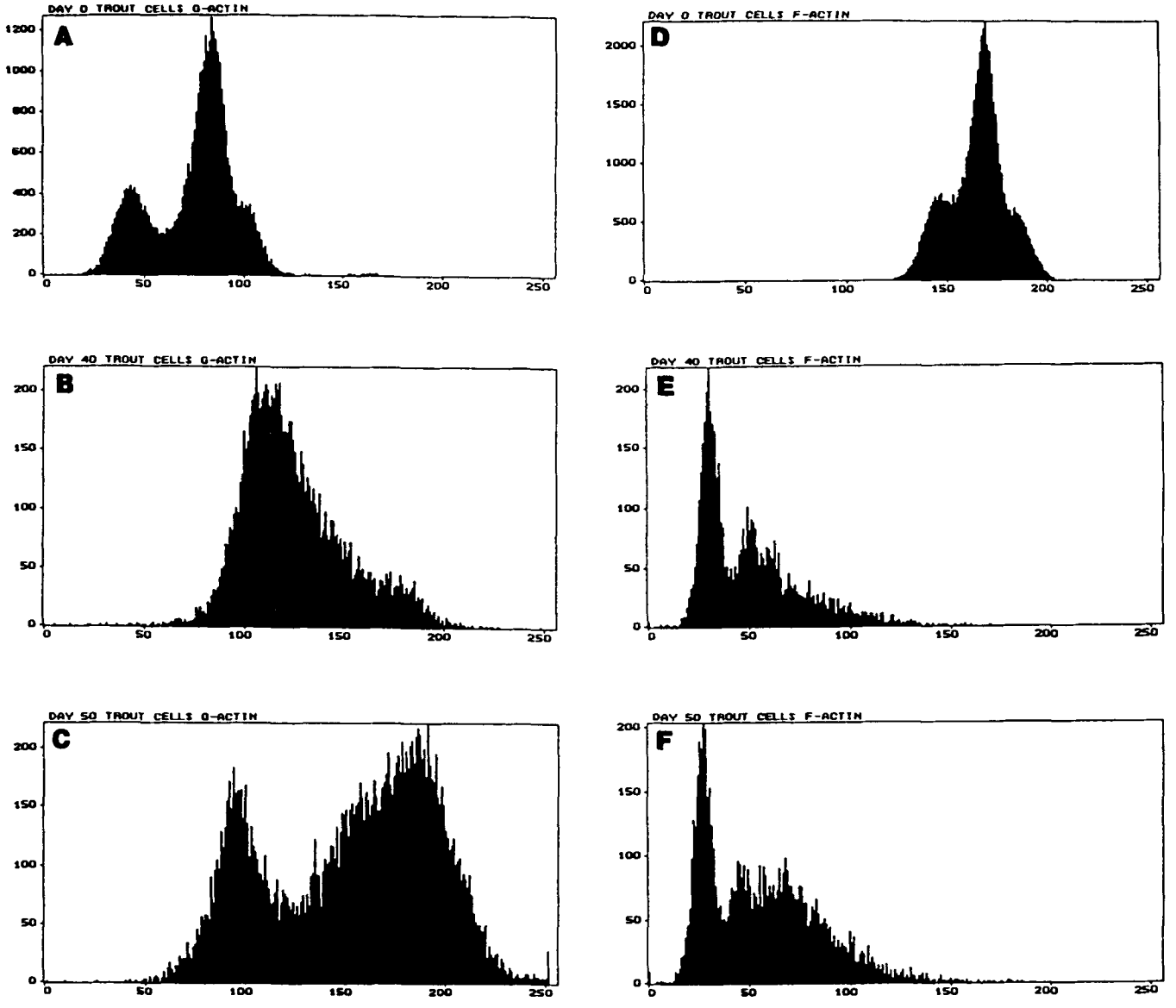

FIG. 5. Results of flow cytometric analysis of F-actin and G-actin expression in rainbow trout epithelial cells. Histograms are of fluorescein isothiocyanate (FITC) binding to rainbow trout hepatocytes at various time points in primary culture. FITC conjugated DNAse I (G-actin) binding at: *A*, zero time; *B*, 40 days in primary culture; and *C*, 50 days in primary culture. F-actin specific FITC phalloidin binding at *D*, zero time; *E*, 40 days in primary culture; and *F*, 50 days in primary culture. X-axis represents fluorescence intensity and y-axis represents the numbers of assayed cells.

growth factors, mitogens, or cytokines by the cells and their accumulation in the absence of media changes during the first 2 wk.

The re-attachment of the aggregates and subsequent spreading of cells from the periphery of the disintegrating aggregates raised questions relative to the growth, as opposed to merely spreading, and the identity of the cells. Data supporting our contention that these cells were actively growing within a few days of culture come from at least four sources. First, DNA synthesis as measured by thymidine incorporation was elevated significantly over the first 4 d in primary culture (Fig. 3). Between Days 1 and 4, thymidine incorporation more than doubles. Unfortunately, due to the necessity of a 16-h thymidine incubation, a true "Time 0" in this experiment was not possible; thus, the magnitude of the response is most likely underestimated. In a similar manner, quantification of silver grains over cell nuclei (Fig. 4) demonstrated high activity throughout the first 10 d in culture. Again, due to the necessity of thymidine incubation, determination of a true Time 0 was not feasible. It was also noted that obviously growing/dividing cells (Fig. 4, *33b*), which were passaged 24 h previous nearly doubled their rate of thymidine incorporation and with 19% of the cells exhibiting $\geq$ three silver grains over the nuclei, these values were approaching that of the early stages of primary culture. A true Time 0 was obtained when cell cycle analysis was performed (Table 5). Ninety-six percent of the freshly isolated hepatocytes were found to be in the resting (G_0-G_1) phase of the cell cycle and only 2% were in S-phase undergoing DNA synthesis. In only 24 h, the number of cells in S-phase had increased to 8% and

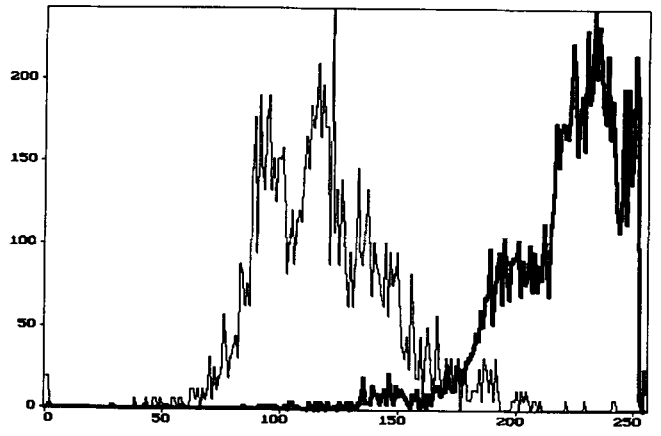

FIG. 6. G-actin and F-actin expression in cells isolated from a hepatocellular carcinoma from rainbow trout. Composite histograms of F-actin specific FITC phalloidin binding (*light line*) and FITC-conjugated DNAse I (G-actin) binding (*heavy line*) against cells isolated from a hepatocellular carcinoma. X-axis represents fluorescence intensity and y-axis represents the numbers of assayed cells.

continued to increase through Days 4 and 10. Our final measures on Day 10 indicated that 13% of the cells were in S-phase and correspond with the appearance of actively growing cells (Fig. 1 *c*). Likewise, PCNA analysis was possible on cells immediately after placement in culture and at multiple time points during the first 10 d in culture (Table 4). Again, a significant increase in cell proliferation and cell division was seen as the hepatocyte PCNA index value rose nearly fourfold from 10 at Time 0 to 37 on Day 4 and continued to rise as the cells remained in primary culture. These final two measurements of cell-cycle activity are probably the most meaningful as cells were fixed within 60 min of isolation. Thus, all four measurements of DNA synthesis and ultimately cell growth occurring soon after placement into primary culture are corroborated. The continual growth of these cells to confluence, after multiple passages, is a final bit of evidence for cell growth as well.

Having determined that we could initiate long-term cell cultures following hepatocyte isolation, determination of the identity of these cells was undertaken. Examination of cells by phase microscopy (Fig. 1) and electron microscopy following fixation and staining (results not shown, but *see* Blair et al., 1990) suggested that following initial placement into culture the majority of cells were hepatocytes with some contaminating biliary epithelial cells that probably originated from the bile ducts. As the length of time in culture increased, at least three types of epithelial cells appeared to be growing. These cells included hepatocyte like epithelial cells, biliary epithelial cells, and a cell type we have termed spindle cell, which has a fibroblast-like appearance. Our initial effort to characterize these cells included western blotting and immunohistochemical staining with a panel of antibodies to various cytokeratins. Cytokeratins are characteristic of epithelial cells and are involved in functions related to the differentiation state of cells (Franke, 1987). Moreover, transformed epithelial cells, including tumor cells, will continue to express the cytokeratins of their cell of origin (Van Eyken and Desmet, 1992). The anti-pan cytokeratin antibody PCK-26 cross-reacts with cytokeratins of all major phyla including mammals, birds, reptiles, and fish. Specifically, the 58 kD cytokeratin 5, the 56 kD cytokeratin 6, and the 52 kD cytokeratin 8 are most often visualized by western blotting. These cytokeratins are typically expressed in various epithelial tissues and as such this antibody would be expected to recognize intermediate filaments expressed in rainbow trout hepatocytes. Cytokeratin 8 has been previously reported to be present in both biliary cells and hepatocytes of a variety of mammalian (Van Eyken and Desmet, 1992) and fish tissues (Bunton, 1993), including rainbow trout (Markl and Franke, 1988; Markl et al., 1989). The anti-vimentin antibody (V9) reacts with vimentin, which is a intermediate filament protein found in cells of mesenchymal origin. Consequently, this antibody would not be expected to recognize intermediate filament proteins from rainbow trout liver epithelia. The positive cytokeratin staining via western blotting at all time points in culture, coupled with negative staining with the anti-vimentin antibody (Fig. 2), supports our assertion that these cells are epithelial in nature and even the spindle cells have an epithelial origin and are not contaminating fibroblasts.

Immunohistochemical staining with a variety of anti-cytokeratin antibodies further supports the assertion of an epithelial origin of all cells in the cultures. Though expected, the lack of cross-reactivity of these specific antibodies with trout hepatocytes was not surprising as recent studies by Bunton (1993) demonstrated that these antibodies would cross-react with biliary cells, but not hepatocytes, of striped bass and medaka. Perhaps, the cytokeratins of the fish (in particular, cytokeratin 8) do not express the same epitopes as their mammalian counterparts. Alternatively, trout hepatocytes, when examined via electron microscopy, appear to contain fewer intermediate filaments than biliary cells (Okihiro, unpublished observation). In the Day 28 sample, the antibodies exhibited decreased binding to biliary ductular epithelia with attenuated epithelium but strong staining of the spindle cells. Thus, the spindle cells appear to be of epithelial origin and to have originated from the bile ducts. Staining with the MAK 6 antibody (Table 1) resulted in similar findings with MAK 6 being specific for biliary epithelium. However, when the older cultures (Day 28 and Day 70) were examined, a decreased intensity of staining of the spindle cells occurred. This observation may indicate that some intermediate filaments recognized by MAK 6 (cytokeratins 8, 14, 15, 16, 18, and 19) are not maintained as well as those structures recognized by AE1/AE3 (cytokeratins 1–8, 10, 14, 15, and 16) in the older cultures (e.g., 1–7). The spindle cells were not stained with the anti-vimentin antibody, again supporting the hypothesis that these cells are not mesenchymal in origin.

Although the pleomorphic spindle cells, which appeared in large numbers in the Day 28 and Day 70 samples, had morphological characteristics similar to mesenchymal (connective tissues) cells, staining with antibodies specific for intermediate filaments is more consistent with cells of epithelial origin. Specifically, the persistent spindle cells were negative for the intermediate filaments desmin and vimentin, but were strongly positive for various cytokeratin antibodies known to cross-react with liver epithelia in various mammalian and fish models. Because the anti-cytokeratin antibodies were also specific for bile ducts in the intact control liver and biliary ductular epithelia in the cell cultures, the persistent spindle cells probably originated from biliary epithelium.

Another cytoskeletal filament, actin, exists in the cytoplasm in a reversibly polymerized filamentous conformation (F-actin) or a monomeric globular conformation (G-actin), the cytosolic precursor of F-actin. Rao et al. (1990) demonstrated that F-actin depolymerization is a marker of cellular transformation relative to cellular differentiation by correlating FITC-labeled phalloidin binding to both chemi-

cally transformed and differentiated cell lines. Transformed phenotypes contained less F-actin than their more differentiated counterparts and correspondingly more G-actin. Alternatively, a pluripotent stem cell, such as our spindle cells, may be expected to exhibit the precursor G-actin prior to differentiation or during dedifferentiation, if it occurs. Liver epithelial cells are thought to be multipotent in nature and may originate from stem cell compartments located in bile ductular structures. Thus, they may have the capacity to differentiate into either biliary epithelial cells or hepatocyte lineages (Bisgarrd and Thorgeirsson, 1991). Data presented herein support the hypotheses that the spindle cells cultures may represent either transformed liver epithelial cells or stem cells.

Our studies indicated a time-dependent loss of F-actin expression with concomitant increase in G-actin expression (Fig. 5; Table 6) as the time in culture increased. Although the appearance of these two conformations of actin seemed inversely related, the kinetics of the two processes appeared independent (Fig. 5). The proliferation index, or percentage of cells in S + G_2M, was significantly higher after Day 0 (Table 5). These observations are consistent with the hypothesis that cytoskeletal disorganization is an empirical phenomenon associated with the transformed phenotype (Rao et al., 1990). This hypothesis was further substantiated when a hepatocellular carcinoma from rainbow trout was examined (Fig. 6). The tumor cells exhibited the highest G-actin levels (MCN = 225) relative to any cells in primary culture. However, evidence for the assertion that the spindle cells may be liver stem cells can also be gleaned from the cytokeratin data as antibodies specific for both bile duct epithelial cells and hepatocytes were positive. Whether these cells represent a stem cell population or have assumed a pathway towards malignancy remains to be investigated. Ongoing studies in our laboratory with the rainbow trout hepatoma cell line RTH-149 (Fryer et al., 1981), and embryonic liver cells should prove beneficial.

Finally, these cells long-term primary cultures are amenable to continuous culture. We have initiated over a dozen cultures (cell lines?) that have been passaged multiple times and continue to exhibit both the spindle cell and hepatocyte/epithelial cell morphology. With the exception of minor differences in growth characteristics, the initial behavior of all these cultures was as described herein. Studies aimed at characterization of these cultures are underway.

Conclusion

We have successfully extended the temporal maintenance of rainbow trout hepatocytes in primary culture from the 5–6 d reported by Blair et al. (1990) to 70+ d. In doing so, we have observed that even nearly homogenous initial cultures of trout hepatocytes will give rise to populations of biliary epithelial cells and, as yet, incompletely characterized populations of spindle cells. All cell types identified in the cultures are actively growing and appear to be of epithelial origin. The most parsimonious explanation of cell behavior in the primary cultures is that the nonhepatocyte cell populations are arising from the clonal expansion of contaminating cells to include, possibly, facultative stem cells as have been described for mammalian liver (Grisham, 1980; Sell, 1990; and reviewed in Thorgeirsson and Evarts, 1992). Alternatively, the speed at which these changes occur suggests the possibility of a de-differentiation or foetalization of the hepatocytes as had been suggested for mammalian cells (reviewed in Berry et al., 1991). Further study of the initial events occurring in the primary culture of these cells are in progress. In addition, the ability to initiate long-term propagable cultures suggests that these may have utility in studies of the fish liver in a variety of areas to include development, toxicology, chemical carcinogenesis, etc. As such, efforts to further characterize these cell cultures are underway.

Acknowledgments

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