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The Initial Report of the Establishment of Primary Liver Cell Cultures from Medaka (*Oryzias latipes*)

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Abstract We report on the establishment of primary liver cell cultures from medaka, *Oryzias latipes*. We utilized serum-free media, positively-charged Primaria dishes, and collagenase for dissociation of medaka liver cells to establish initial culture conditions. Nearly homogenous (about 96%) primary hepatocyte cultures were initiated and remained viable and proliferative for 30 days. Cultured cells exhibited either a cuboidal/epithelial-like phenotype or spindle-shaped phenotype. Both cell types appeared to have been derived from liver epithelia, as verified by western blotting of cytokeratins and transmission electron microscopy. The predominate cell type was the spindle cell which proliferated to confluence in primary culture.

The initial behavior, proliferation characteristics, and media requirements for attachment and spreading of these cells are described. Primary cultures of medaka liver cells have the potential to serve as a useful model for a variety of investigations including, but not limited to, liver cell development, function, and xenobiotic metabolism in an established fish model.

Introduction

The use of cultured fish cells by investigators interested in aquatic toxicology has received increased attention in recent years. Fish cells have been used for studies of the effects of anthropogenic pollutants impacting aquatic environments (e.g. Aoki and Matsudaira, 1977; Bailey *et al.*, 1984; Babich and Borenfreund, 1987; Sigeoka *et al.*, 1987; Ostrander *et al.*, 1993). Specifically, cells from the fish liver have been used as an *in vitro* cellular analyses model for mutagen and carcinogen metabolism and as such, primary liver cell cultures from a number of fish species have been well established (Bissell *et al.*, 1973; Parker *et al.*, 1981; Bailey *et al.*, 1982; Hightower and Renfro, 1988; Ostrander *et al.*, 1995; Blair *et al.*, 1995). The medaka (*Oryzias latipes*) has been one of the most commonly used fish models for laboratory studies of fish physiology, genetics, and toxicology (Egami, 1954; Yamamoto, 1968; Bunton, 1991; Du *et al.*, 1992; Winkler *et al.*, 1992; Shima and Shimada, 1994; Takagi *et al.*, 1994) and the importance of the medaka as an *in vivo* model for hepatocarcinogenesis has also been established (Harada *et al.*, 1988; Lauren *et al.*, 1990; Hinton *et al.*, 1992; Ostrander *et al.*, 1992; Aoki *et al.*, 1993; Bunton, 1995).

Primary cell cultures and cell lines from medaka embryo and fin have been established (Wakamatsu *et al.*, 1994; Saito and Shigeoka, 1994; Hong and Schartl, 1996) and have proven valuable in recent studies (Arai *et al.*, 1994; Ozato and Wakamatsu, 1995). To date, no studies have been published on efforts to establish primary medaka liver cell cultures. Baldwin and colleagues (1993) studied the ability of seven structurally diverse peroxisome proliferators to induce S-phase synthesis in cultured medaka hepatocytes. Those cells were maintained for only 18–30 hours in serum-free conditions and no data on cell attachment, spreading, or growth were reported. The obvious limitation to extending these and similar studies has been our inability to maintain medaka liver cells in culture.

Blair and co-workers (1990, 1995) reported on their efforts to culture various trout liver cells using serum-free media and various treated culture dishes designed to facilitate attachment and growth of cells. The same investigators (Ostrander *et al.*, 1995) recently achieved long-term primary cultures of trout liver cells by using positively charged culture dished and serum-containing medium. Cells were characterized as to proliferation potential and cytokeratin type. We have now extended these techniques, with modification, to the medaka and report our success in establishing primary cell cultures from medaka liver on positively charged cul-

ture dished in the presence of serum-free media. We also describe several lines of investigation to characterize the origin and novel behavior of these cells in primary culture and to passage and extend the culture period of these cells.

Materials and Methods

Materials

Primaria tissue culture plates, flasks, multi-well plates, and disposable sieves were purchased from Falcon-Becton Dickinson (Franklin Lakes, NJ). trypsin and gentamicin were obtained from Sigma Chemical Co. (St. Louis, MO). Crude collagenase (Type IV, lot 84H6804) was from Sigma Chemical Co. Minimum Eagle's medium (MEM), fetal calf serum, MEM non-essential amino acid (100x), and glutamine (100x) were obtained from Gibco (Grand Island, NJ). Sodium dodecyl sulfate, acrylamine, molecular weight standards and other associated reagents for electrophoresis were purchased from Bio-Rad (Hercules, CA). The monoclonal antibodies for western blotting were an anti-pancytokeratin (PCK-26) from Sigma Chemical Co. and secondary rabbit anti-mouse IgG antibody from PharMingen (San Diego, CA). Immobilon polyvinyidene difluoride membranes were purchased Millipore Corp. (Bedford, MA). Alkaline phosphatase conjugated rabbit antimouse IgG specific polyclonal antibodies were purchased from PharMingen (San Diego, CA). Rainbow trout serum was prepared in our laboratory using standard methods (McLeod *et al.*, 1980).

Experimental animals

Medaka (*Oryzias latipes*) were obtained from our breeding colony at Oklahoma State University. Fish were fed Tetra-Min flake food and live brine shrimp twice daily and held at 21 to 24°C. Adults of both sexes, weighing 0.2–0.34 g, were used in these studies. Rainbow trout (*Oncorhynchus mykiss*) were obtained from the Norfolk Fish Hatchery (Mountain Home, AR).

Isolation and placement of liver cells into primary culture

Isolation of hepatocytes followed non-perfusion procedures for fetal rat hepatocytes with minor modifications (Devirgiliis *et al.*, 1981). Individual medaka were anesthetized by placing in an ice bath for 10 min prior to washing 5 times with sterile water. The fish were then euthanized by cervical dislocation, emerged in 70% of ethanol for 2 min, and washed twice with sterile perfusion medium (0.154M NaCl, 0.154M KCl, 0.154M KH_2PO_4, 0.194M $NaHCO_3$, and 0.154M $MgSO_4$). As livers were excised they were soaked in perfusion medium and then, when all livers had been removed, livers (3–5) were washed twice with perfusion media. To facilitate the digestion, livers were placed into a 100-mm culture dish and minced with a surgical blade. Perfusion medium containing 0.05% collagenase was used to digest the livers, at room temperature with gentle shaking, on an orbital shaker for 40 min. At 10-min intervals cells were gently pipetted to disassociated the cell clumps. Large resilient pieces of the liver were separated from single cells by sieving using a Falcon disposable sieve (100 μm). The resulting cell suspension was centrifuged for 5 min at 300 *xg*, and the pellets washed twice with perfusion medium. The final cell pellets were resuspended in 1 ml of culture media [serum-free MEM with Earles' salts supplemented with 10 ml/L of glutamine (100x), MEM non-essential amino acid solution (100x), and gentamicin (100 μg/ml)]. The cell were resuspended by manual pipetting (5–10 times) and 0.25 ml aliquots of the suspension were pipetted on to the Primaria tissue culture plates (100 mm) containing 4.75 mls of culture media. The resulting primary cell cultures were maintained in a 24°C, 5% CO_2: 95% air atmosphere. Initiation of attachment was measured by checking the movement of cells after gently shaking plates under a light microscope.

Cell proliferation assay

To assess proliferation characteristics of medaka hepatocytes in primary culture, cells were seeded into 6-well Primaria tissue culture plates at the density of 2.5×10^5 cells/well in serum-free MEM culture media in a 24°C, 5% CO_2: 95% air atmosphere. Cells were trypsinized with 0.25% trypsin and cell numbers were determined after 2, 4, 6, 8, 10, and 12 days in culture. Triplicate measurements were made at each time point. A Levens test indicated homogeneity of variances. Therefore, significant differences were assessed by an analysis of variance ($F = 81.2$, $P < 0.01$) with follow-up least significant differences tests ($P < 0.05$). Average variation of single determinations from the mean was less than 12%.

Sensitivity of cells to trypsin during enzymatic disassociation

To compare the effects of enzymes for dissociation of medaka liver, perfusion media containing

0.05% or 0.25% trypsin or collagenase, respectively, were used to isolate the cells. Duration of digestion was 20 min or 40 min and examination for viability was performed by trypan blue exclusion. The final cell pellets were resuspended in serum-free MEM and seeded onto Primaria tissue culture plates. Plates were maintained at 24°C in a 5% CO_2: 95% air atmosphere and observed at 20 min, 1, 4 and 24 h for attachment and spreading. The degree of confluence, cell integrity, and overall appearance were further observed twice daily for 7 days.

Serum requirements for attachment, spreading and proliferation of cells in primary culture

Triplicate experiments were performed to observed if the serum-free media was necessary for attachment, spreading, or proliferation of medaka liver cells in primary culture. Both serum-free and varying concentrations of serum-containing media were used. After isolation, cells were resuspended in serum-free culture media and pipetted onto replicate plates containing serum-free MEM, MEM complemented with 1%, 2%, 5% or 10% fetal calf serum, or rainbow trout serum. All plates were observed daily for 10 days and evaluated for attachment, spreading, degree of confluence, cell integrity, and overall appearance. Cells failing to attach or failing to remain attached were examined for viability by trypan blue exclusion.

Optimal temperature in primary culture

To determine the optimal temperature for primary culture of hepatocytes, cell cultures were maintained at 16°C, 24°C, or 30°C, examined daily or 7 days, and evaluated for attachment, spreading, degree of confluence, cell integrity, and overall appearance.

Ultrastructure of hepatocytes in primary culture

After the conditions of isolation and culture of medaka liver cells were optimized, cells were examined by transmission electron microscopy. Cells were placed into primary culture in Primaria 6-well dishes and reached about 90% confluence after 10 days of culture. Cell processing was initiated by three 30-sec washes with 3 ml of washing buffer (0.1 M cacodylate with 5.5% sucrose, pH 7.4) followed by fixing (1.6% glutaraldehyde in 0.1 M cacodylate, pH 7.4 with 5.5% sucrose) for 2 h at room temperature. The cells were then washed with three 20-min washes with washing buffer and stored at 4°C overnight. Cells were postfixed for 1 h with 1% osmium in 0.1 M cacodylate buffer, 4.2% sucrose, pH 7.4. Cells were then transferred into an ethyl alcohol dehydration series (30% for 30 sec, 50% for 30 sec, 70% for 1 min, 80% for 2 min, 95% for 3 min, 100% 4 times for 3 min each). The final ethanol bath was replaced with absolute ethanol: polybed medium (1:2) (Polysciences, Warrington, PA) for 30 min. This solution was replaced with 100% polybed for 15 min, rinsed and incubated in 100% polybed at 60°C for 2 days. Samples were broken into small pieces, mounted on stubs, thin-sectioned (approximately 70 nm) with an MT 6000 Sorvall ultramicrotome, and post-stained with 2.5% uranyl acetate for 4 min and Reynold's lead stain for 5 min. Sections were then examined with a JEOL 100 CX II scanning transmission electron microscope at 80 kV accelerating voltage.

Visualization of cytokeratin via western blotting

Medaka hepatocytes were placed into 100-mm dishes and cultured under the optimized conditions for either 0, 4, 8, 12, 16, or 24 days. Cells were harvested by gentle scraping, washed twice with isotonic phosphate-buffered saline (PBS), and lysed with 10 volumes of lysate buffer (50 mM Tris-Cl, 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1% nonidet P-40, 0.5% sodium deoxycholate, pH 8.0) by direct addition to the cell pellet with gentle pipetting. Resulting homogenates were centrifuged at 4,000 xg for 10 min and the supernatants were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970). Protein concentrations of cell homogenates were determined by the method of Lowry *et al.*, (1951) with bovine serum albumin as a standard. Proteins were then transferred to Immobilon polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) and incubated with monoclonal anti-pancytokeratin antibodies. Cytokeratins were visualized with an alkaline phosphatase conjugated rabbit anti-mouse IgG specific polyclonal antibody and a warm (37°C) visualization buffer of 45 ml alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris, pH 9.5), 438 μl 5% NBT in 70% dimethylformamide, and 150 μl 5% bromochloroindolyl phosphate in 100% dimethylformamiden.

Subculture of medaka liver cells

Upon reaching confluence (approx. 8–10 days), cells were rinsed twice with perfusion media, followed by detachment with 1 ml of 0.05% typsin or 0.05% collagenase. Once cells were completely disassociated, 5 ml of serum-free media was added to the culture dish, gently mixed, and cells were sub-divided into three dishes. Alternatively, disassociated cells were washed with perfusion media twice followed by centrifugation at 300 *xg* and split into three dishes. Three additional mls of media were then added sub-cultured cells an they were incubated in a 24°C, 5% CO_2: 95% air atmosphere.

Results

Isolation and placement into primary culture

As shown in Fig. 1, hepatocytes undergo a sequence of events typical for fish liver cells following isolation and placement into primary culture. Initially, a single-cell suspension was distributed uniformly over the tissue culture dishes and clumping/aggregating began immediately (Fig. 1A). Cells began to attach to the dish within 10 min and most completed attachment within 8–10 h. Greater than 99% of the viable cells, as measured by trypan blue exclusion, were attached at 24 h. In a manner reminiscent of what we have previously reported for trout liver cells (Ostrander *et al.*, 1995), some cells formed small aggregates after placement into culture which became firmly attached. The initial observation of individual cells spreading was observed after two days (Fig. 1B) and most cells, except those comprising the aggregates, were spreading by four days (Fig. 1C).

Cell proliferation

Cell proliferation began within 2–4 d and was relatively slow. Cell growth/proliferation accelerated beginning about Day 4 (Fig. 1C) and through Days 7 and 10 (Figs. 1D & 1E) at which time dishes were about 90–95% percent confluent. During the initial period of the rapid proliferation, a shift in the cells to a elongated, spindle-shaped morphology occurred which was especially obvious in the cells growing out from the periphery of the aggregates (Fig. 1D). Primary cultures could be maintained for at least 30 days at which time plates were confluent. Confluent cells in primary culture exhibited one of these two basic morphologies. The majority of cells were spindle-like. However, about 15–20% of plates continued to exhibit foci of cuboidal "hepatocyte-like" cells (Fig. 1F).

As shown in Fig. 2, cell number per well did not change significantly during the initial four days in culture as the cells were actively spreading. Typically, the number of cells per well were about 2.4×10^5. However, on Day 6 in culture, a significant increase ($P < 0.05$) in cell number to 6 3.9×10^5 cells per well were observed. The number of cells per dish continued to increase steadily through Day 10 (confluence) reaching about 9×10^5 cells per well.

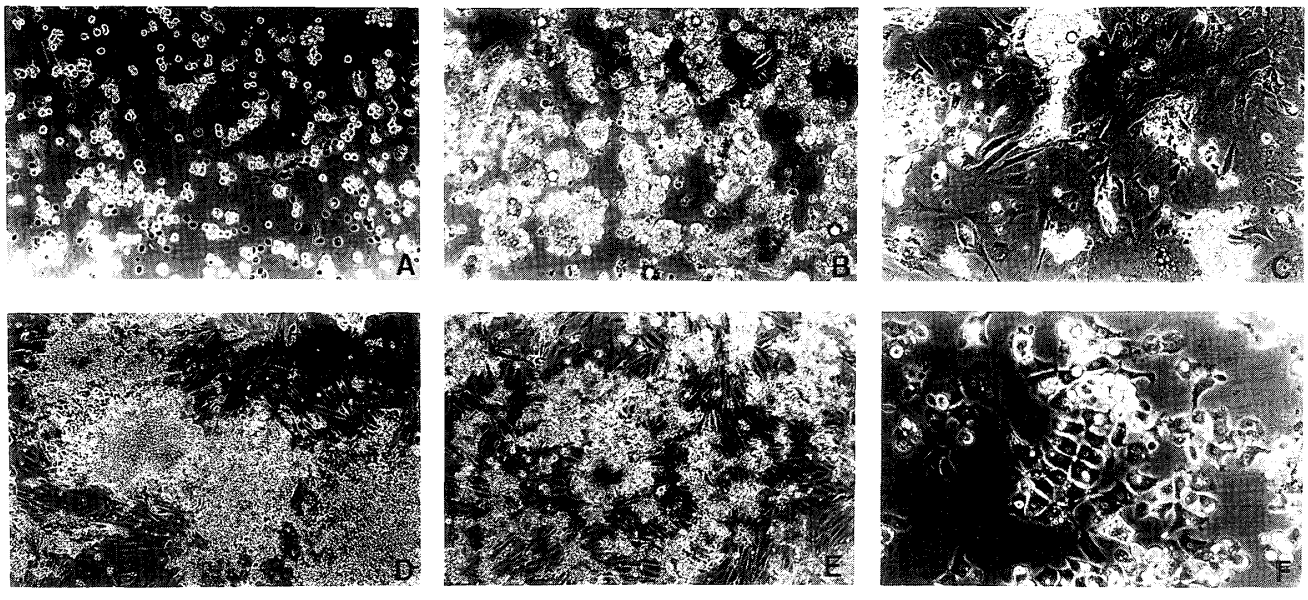

Fig. 1. Primary culture of medaka hepatocytes. *Panel A*, Day 0; *panel B*, Day 2; *panel C*, Day 4; *panel D*, Day 7; *panel E*, Day 10; *panel F*, Day 24. Magnification of panel A is 100x, panels B, D and E are 200x, and panels C and F are 400x.

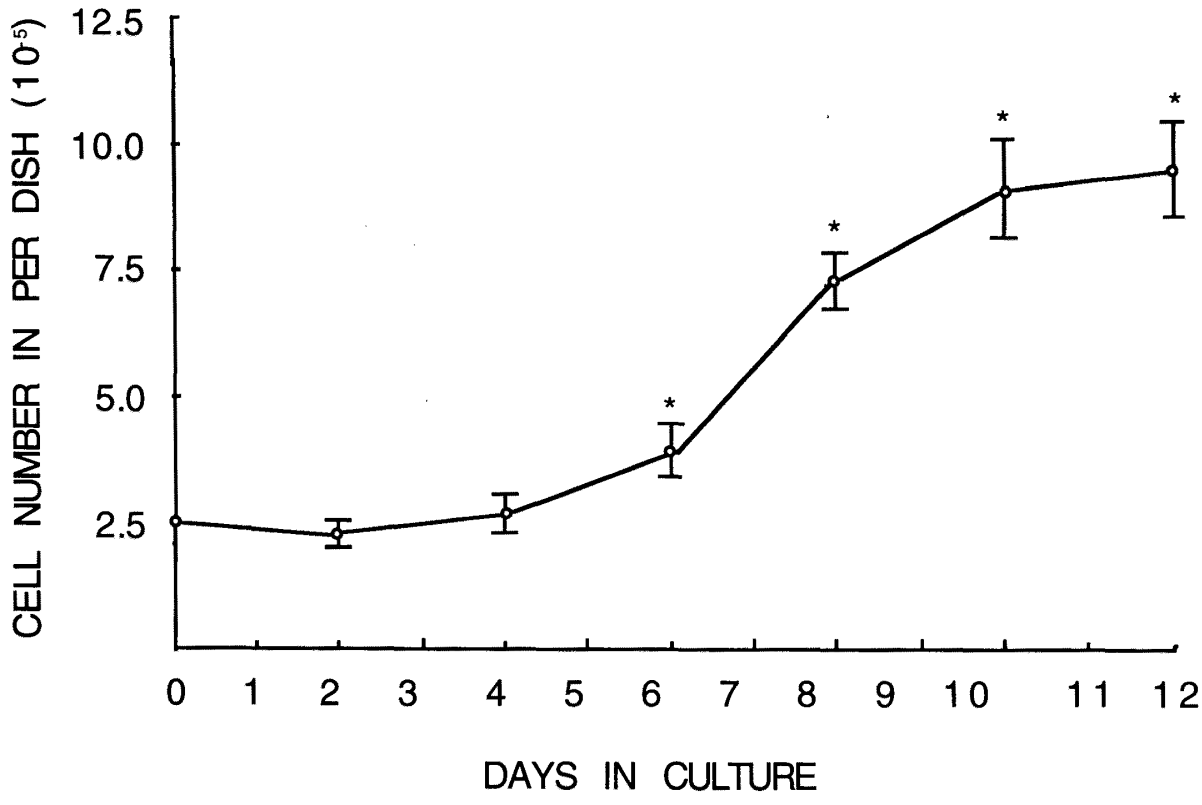

Fig. 2. Proliferation curve of primary culture of medaka hepatocytes in a serum-free MEM medium. As detailed in "Materials and Methods", cells were disassociated with 0.05% collagenase and seeded at 2.5×10^5 per well onto 6-well Primaria tissue dishes. Cell number was determined after 2, 4, 6, 8, 10, and 12 days in culture by counting suspensions of trypsinized cells. Error bars represent one standard deviation about the mean as determined from triplicate experiments. " * " denotes days mean cell number was when statistically significantly higher than mean cell number on Day 0 ($P < 0.05$).

Sensitivity of cells to trypsin during enzymatic disassociation

The abilities of two proteolytic enzymes, commonly used in cell culture, to dissociate medaka liver tissue were evaluated. As evidenced in Table 1, collagenase was judged to be slightly better than trypsin at all the concentrations and durations evaluated. In all situations tested we observed greater than 90% cell viability with collagenase. With trypsin, cell viability dropped as low at 74% at the highest concentration and duration tested. Viability never exceeded 89%.

The ability of cells, isolated by trypsin or collagenase disassociation, to attach and spread was evaluated at various time pints over the initial 24 h primary culture. Within the initial 20 min in culture it appeared that higher percentage of cells isolated with collagenase were attached when compared to cells isolated by trypsin. At each time point examined slightly more of the collagenase isolated cells appeared to have attached. By 24 hours nearly 100% of viable cells isolated by collagenase were attached; whereas only ~80% of the cells isolated with trypsin had attached at 24 hours (data not shown). No significant differences in he time to spreading (Day 4) was observed in three independent experiments and no differences were apparent in side-by-side comparisons of the cultures on Day 7.

Table 1. Viability of medaka liver cells after enzymatic disassociation

Enzyme (concentration)	Percent viable after enzyme incubation	
	20 min	40 min
Collagenase (0.05%)	97%	94%
Collagenase (0.25%)	95%	92%
Trypsin (0.05%)	89%	83%
Trypsin (0.25%)	82%	74%

Serum requirements for attachment and spreading of cells in primary culture

In order to determine the serum requirements of medaka liver cells in primary culture, triplicate experiments were performed in which the type and

serum concentrations of the culture media were varied. Very few cells (<5%) resuspended and cultured in MEM complemented with either 10% fetal calf serum or 10% rainbow trout serum attached. Consequently, little spreading and proliferation was observed in primary culture. However, cells placed into primary culture with serum-fee MEM attached, spread, and proliferated. To determine the relationship between the concentrations of serum, and attachment and spreading of cells, culture media containing either 0%, 1%, 2%, 5% or 10% fetal calf serum or rainbow trout serum was tested. As shown in Fig. 3, the ability of cells to attach dramatically decreased as serum concentration, regardless of type, was increased. Media containing either 10% fetal calf serum or rainbow trout serum produced low rates (<5%) of cell attachment 2 days after placement. Decreasing serum concentration, regardless of type, resulted in a parallel increase in the ability of medaka hepatocytes to attach and spread in primary culture. Finally, an additional experiment was performed in which primary cultures established with serum-fee media were supplemented with either 5% rainbow trout or fetal calf serum. Within 24 h reduced cell attachment was apparent. Forty-eight hours after addition of serum greater than 80% of previously attached cells were observed floating in the media. However, examination of floating cells 72 h after media supplementation revealed that most of the floating and weakly attached cells were viable.

Optimal temperature in primary culture

Three different culture temperatures, 16°C, 24°C, or 30°C, were evaluated for optimal cell attachment, spreading, and proliferation. At all temperatures cells reached confluence. However, 24°C was judged to be optimal. Cells cultured at 16°C took longer to reach 90% confluence, not achieving this until about Day 18. Cells cultured at 30°C reached 90% confluence in about 6 days as compared to 10 days for those at 24°C. However, these cultures contained more cellular debris, floating cells, and attached cells exhibited increased vacuolization compared to those at 24°C.

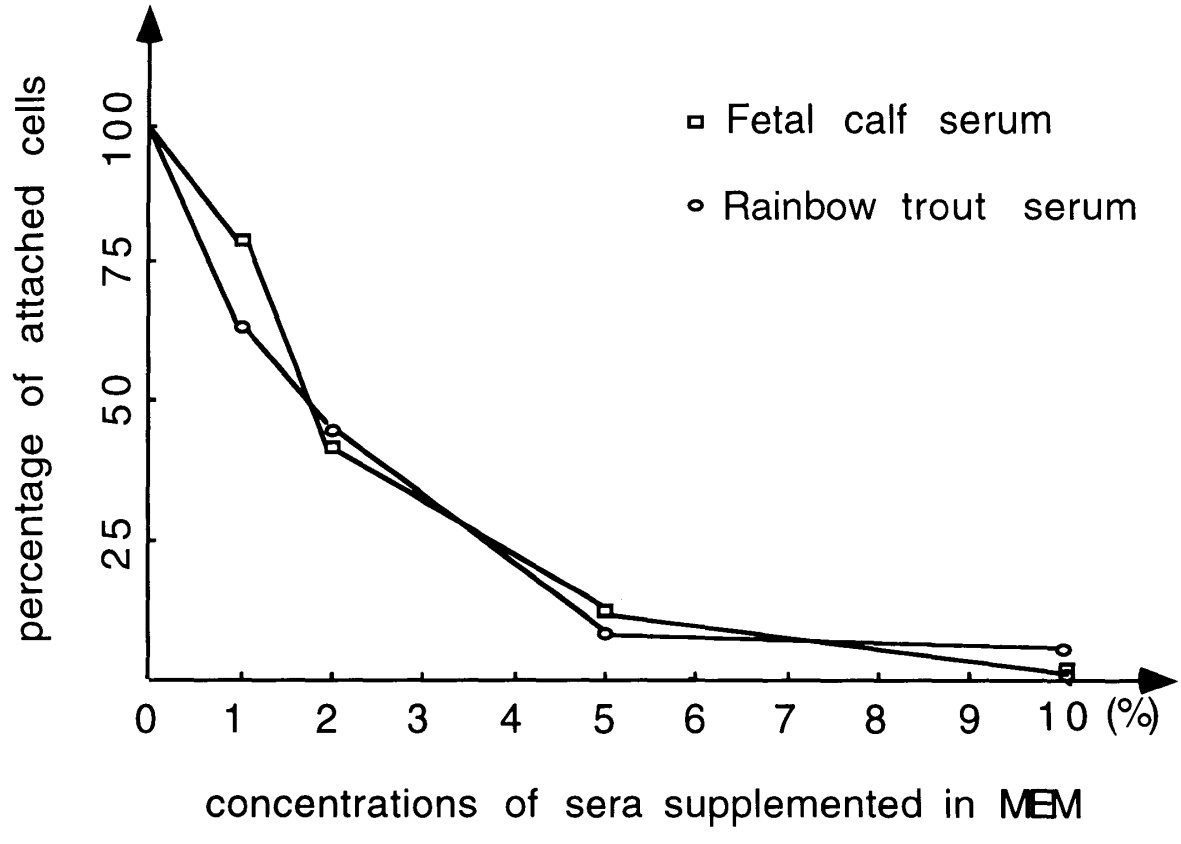

Fig. 3. Serum inhibition of medaka hepatocyte attachment in primary culture. As detailed in the "Materials and Methods", after isolation cells were resuspended in serum-free MEM and pipetted onto replicate plates containing serum-free MEM, MEM complemented with either 1%, 2%, 5%, or 10% fetal calf serum, or rainbow trout serum. Cell attachment was determined 2 days after placement into primary culture. The percentage of the attached cells in serum-free MEM was nearly 100% and all experiments were conducted in triplicate.

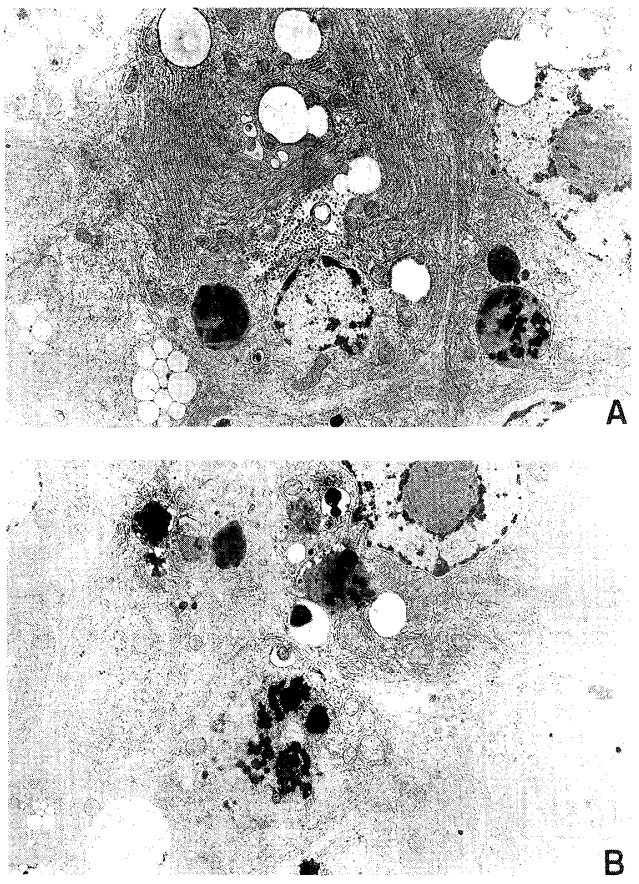

Fig. 4. Transmission electron microscopy of medaka liver cells after 10 days in primary culture. *Panel A*, the region of cytoplasm adjacent to nucleus contained a considerable amount of granular endoplasmic reticulum, mitochondria, and variable amounts of glycogen storage granules; *Panel B*, the junctions of the multiple cells are evident including occasional desmosomes. In addition, lipid storage vacuoles and peroxisomes are evident.

Ultrastructure of cells in primary culture

As shown in Fig. 4A, after 10 days in primary culture, medaka hepatocytes typically displayed a single spherical euchromatic nucleus within a distinct cytoplasmic compartment. The region of cytoplasm adjacent to nucleus contained a considerable amount of granular endoplasmic reticulum and variable amounts of mitochondria and peroxisomes. Storage products such as glycogen were seen adjacent to the nucleus (Fig. 4A) and fat droplets were also seen distributed throughout the cytoplasm (Fig. 4, panels A & B). Gap junctions jointed the hepatocytes laterally and an occasional desmosome was seen (Fig. 4B). The ultrastructure of hepatocytes at Day 10 of primary culture was consistent with normal healthy hepatocyte ultrastucture.

Visualization of cytokeratins via western blotting

As shown in Fig. 5, cytokeratin expression in primary cultured medaka liver cells was maintained at a consistent level throughout the time in primary culture (lane 3–8) when compared to the homogenate of intact medaka liver (lane 2). There were no significant quantitative differences in the patterns of cytokeratin expression observed at different time points during the primary culture of medaka liver cells. However, a slight qualitative difference, manifested as a decreased in higher molecular weight bands on the western blot after Day 4 (lanes 5–8), was observed.

Subculture of medaka liver cells

Primary medaka liver cell cultures typically experienced a growth crisis beginning about 30 days after placement into primary culture. A variety of attempts were made to subculture these cells, usually between days 8 and 10, when proliferation rate appeared maximal. Cells placed into subculture failed to thrive with less than 5% attaching and only limited spreading and cell division. Thus, successful subculturing has not yet been possible.

Discussion

This is the first report of the establishment and characterization of primary cultures of epithelial cells from medaka liver. Nearly homogenous (about 96%) primary cultures of hepatocytes were initiated and remained both viable and proliferative for approximately 30 days. We have descried the isolation, placement into culture, initial behavior serum-free media requirements for attachment and spreading, and our preliminary characterization of the cell cultures.

The small size of the medaka liver precluded cell isolation by the perfusion techniques we have successfully employed for rainbow trout hepatocytes (Ostrander *et al.*, 1995) and rainbow trout biliary cells (Blair *et al.*, 1995). Nonetheless, enzymatic disassociation using two different proteases, trypsin and collagenase, generated differential yield, viability, and efficiency of attachment. The small amount of cells obtained prevented us from utilizing the exhaustive purification steps we had developed for rainbow trout liver. Regardless, cultures of approximately 96% purity of medaka hepatocytes was accomplished. Peakman *et al.*, (1994) reported that trypsin provided the highest yield of monodispersed islet cells and highest viability, whereas lower yields and viability were obtained using collagenase. Yet, it has been demonstrated that trypsinization under routine conditions can be highly cytotoxic (McKeehan,

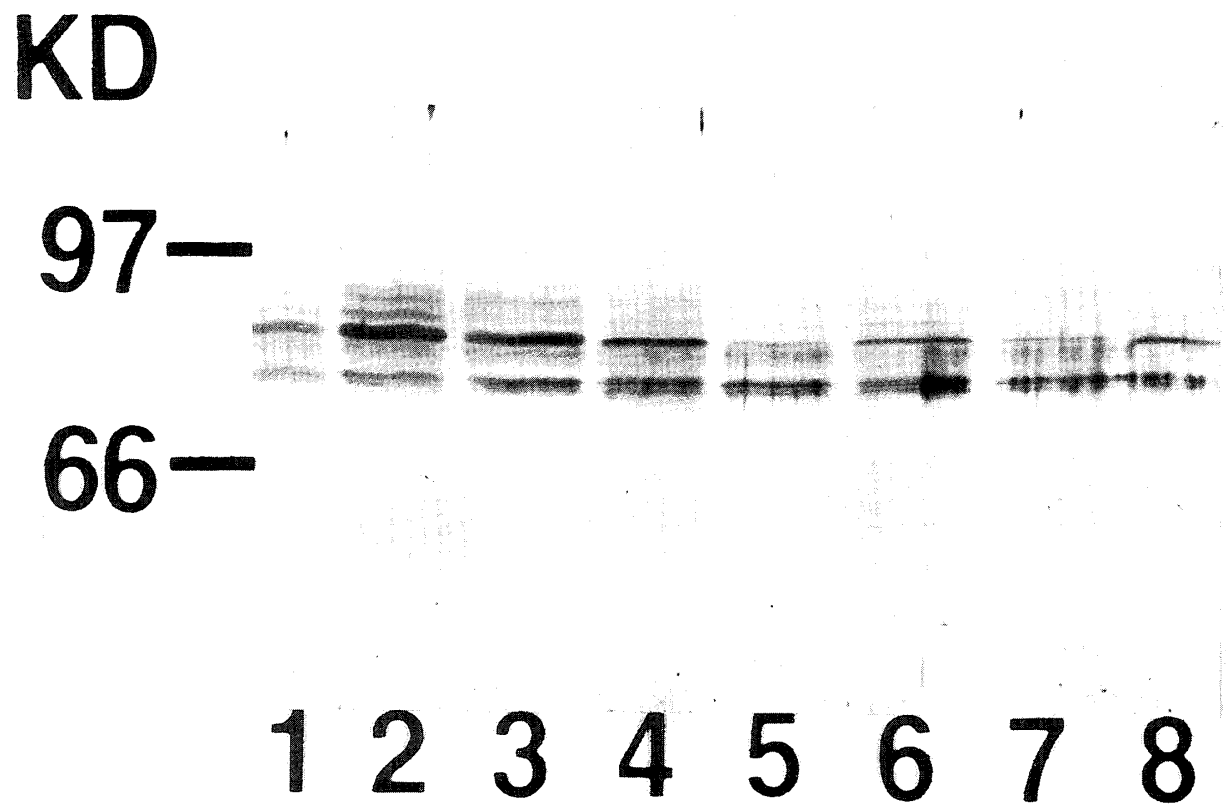

Fig. 5. Western blot analysis of cytokeratin expression in primary liver cell cultures of medaka at various time intervals in primary culture. *Lane 1*, homogenate of rainbow trout liver; *lane 2*, homogenate of medaka liver; *lane 3*, Day 0; *lane 4*, Day 4; *lane 5*, Day 8; *lane 6*, Day 12; *lane 7*, Day 16; *lane 8*, Day 24. Molecular weight standards are shown to the left of lane 1.

1977; Smets *et al.*, 1979; and Pleskach *et al.*, 1994). In another experimental system, the use of collagenase for disaggregation of cultured human kidney proximal tubule cells was recommended (Jung *et al.*, 1995). It is probable that medaka liver cells have different sensitivity to trypsin and collagenase and this results in the differences in viability, ability to attach, and spreading. On the basis of our studies, it was obvious that collagenase was superior as judged by cell viability and attachment.

Cell attachment is essential to achieve successful liver cell primary cultures. Serum at the level of 5% to 10% in the media has been shown to improve attachment of various types of cells in culture, including hepatocytes (Bissell and Guzelian, 1981). Conversely, freshly isolated adult rat hepatocytes in a minimal medium containing insulin but, without serum or other protein supplement, attached and spread on standard tissue culture plastic (Bissell *et al.*, 1973; Blaauboer and Paine, 1979). Rainbow trout hepatocytes were reported to attach to culture dishes either in a serum-free media (Blair *et al.*, 1990) or in a serum-containing media (Ostrander *et al.*, 1995). Previous work with medaka liver cultures suggested that serum-free conditions could facilitate the attachment of isolated medaka liver cells to the substrate (Baldwin *et al.*, 1993). Fish skin extract-coated substrates have also been shown with rainbow trout liver cells (Blair *et al.*, 1990) and in preliminary experiments with medaka hepatocytes (Ostrander *et al.*, unpublished data) to facilitate attachment. Unfortunately, under these conditions we could not maintain the medaka liver cells in long-term primary culture and proliferation was never observed. In our present studies, we have successfully employed a serum-free medium and positively-charged Primaria dishes to promote attachment. These modifications not only allowed for the initial attachment previously reported, but also promoted cell proliferation in a manner that was originally reported for rainbow trout hepatocytes (Ostrander *et al.*, 1995). Moreover, it appears that both fetal calf serum and rainbow trout serum inhibit attachment and subsequent spreading and growth of medaka liver cells in a parallel manner, suggesting a common mechanism.

The specificity of medaka hepatocyte attachment and the mechanism(s) of the inhibitory

effects of serum on cell attachment remains unclear. Specific membrane proteins and/or glycoproteins, which are normal substrates for proteolytic digestion, reside in the cell membrane (Baumann and Doyle, 1979). Disassociation of medaka liver cells with collagenase may release or "unmask" specific cell adhesion molecules, peptide fragments, or other functional groups on the surface of the cells necessary for attachment of the cells to the dishes. Serum contains an abundance of, as yet, incompletely defined negatively charged proteins that may saturate cell surface binding sites on the positively-charged culture dishes, thus, inhibiting the attachment of medaka hepatocytes. Finally, surface composition of culture dishes is important to cellular response. Tissue culture surface chemistry effects the binding of proteins and the attachment, spreading, shape, and behavior of cells (Springer *et al.*, 1976; Klein-Soyer *et al.*, 1989; Sidho *et al.*, 1994). The Primaria dishes used in these studies mimic the extra-cellular matrix and in particular the structure of attachment proteins by intrinsically incorporating amide- and amino-functional groups. These properties appear to benefit medaka hepatocytes as they attached, spread, and eventually proliferated in primary culture.

Our initial effort to characterize these medaka liver cells in primary culture included western blotting for cytokeratins and transmission electron microscopy, both of which revealed characteristics of cell architecture. Cytokeratins are the major structural proteins of intermediate filaments characteristic of epithelial cells (Franke 1987). The demonstration of cytoplasmic keratin leaves little doubt that the proliferating spindle-shaped cells were of epithelial origin as opposed to a fibroblastic origin. Moreover, no significant differences were seen between the cytokeratin expression in the cultured cells when compared to intact liver. A single qualitative difference was observed in cells after 4 days in culture. An apparent slight decrease in slower migrating bands was seen which correlates with both the initial and continued proliferation of these cells in culture. These potential differences are worthy of future investigation. Although no data is available on the half-life of cytokeratin in medaka hepatocytes, the relatively stable level of cytokeratin from Day 0 through Day 24 suggested a constant synthesis of cytokeratin in medaka liver cells cultured in serum-free MEM since the half-life of cytokeratin in normal human cells is 40–80 h (Sundstrom *et al.*, 1990). The conclusion of an epithelial origin to these cells, as opposed to fibroblastic, is further strengthened when the transmission electron micrographs are reviewed. As discussed above, the cells exhibited ultrastructural features characteristic of hepatocytes.

To date we have not been successful in subculturing medaka liver cells from our primary cultures. This is somewhat surprising considering the success we have had with the establishment of rainbow trout cell lines (Ostrander *et al.*, 1995, and unpublished data). Nonetheless, there may be cytotoxic or inhibitory factors released during the process of freeing attached cells from the primary cultures. Further efforts to characterize and subculture these cells are underway.

Acknowledgements

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