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Studies were undertaken to evaluate   the potential role of the retinoblastoma tumor suppressor gene/protein during chemical carcinogenesis in the medaka (Oryzias latipes), a small fish. Cell lines from the rainbow trout liver and long-term primary cultures of medaka liver cells were established to aid these studies. To better understand the role of the retinoblastoma protein during the cell cycle it was necessary to purify the protein from trout liver, develop antibodies, and evaluate protein expression following treatment of fish liver cells with known promoters. Finally, appropriate molecular biological diagnostic reagents were developed to evaluate alterations in the retinoblastoma gene in retinoblastoma and hepatocarcinomas following induction with known environmental carcinogens. Studies to date suggest the retinoblastoma gene/protein may play a role in oncogenesis in the medaka.

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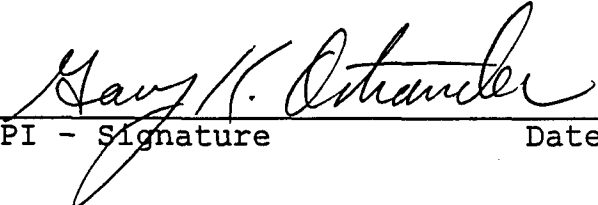
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INTRODUCTION

General Overview

Suppressor genes

The rapidly expanding field of molecular biology has coincidently allowed for rapid advances in our understanding of the mechanisms of malignant transformation. Of primary relevance have been studies that have further elucidated the role of DNA lesions in oncogenesis. One target of carcinogenic agents receiving considerable attention is the anti-oncogene or tumor suppressor gene (For Review see Den Otter et al. 1990). In normal cells these genes appear to suppress, rather than promote cellular proliferation. Consequently, their absence or truncated expression of their gene products will remove the normal constraints on cell growth and replication--giving rise to neoplastic transformation. One of the better studied suppressor gene models is the retinoblastoma tumor suppressor gene model.

The significance of the retinoblastoma tumor model

Retinoblastoma (*Rb*) is an intraocular neoplasm that affects approximately 1 in 15,000 human infants during the first year of life (Weinberg 1988). Development of retinoblastoma, and other types of tumors (Fung et al. 1987; Friend et al. 1986; Lee et al. 1988; Harbour et al. 1988; Lee et al. 1988), has been linked to failure of transformed cells to correctly synthesize and express the *Rb* protein. As discussed below, this has

been tied to mutations in both alleles of the *Rb* gene that inactivate or eliminate the *Rb* encoded protein p105^{Rb}. Finally, the utility of this model extends beyond ocular and other tumors failing to properly express the *Rb* protein. A clear understanding of the etiology of retinoblastoma will provide an opportunity to understand the role of tumor suppressor genes in cancer causation of other, presumably unrelated, cancers.

Limitations of the human retinoblastoma model

The most glaring limitation to studying retinoblastoma is the lack of a vertebrate model capable of expressing retinoblastoma. There is currently no established, available, mammalian model for either heritable or chemically induced retinoblastoma. Currently, we are limited to human surgery and autopsy specimens. Furthermore, owing to the limited window of development of retinoblasts, these tumors are nearly always expressed in the first few years of life (Den Otter 1990). In a preliminary report (Windle et al. 1990), a transgenic mouse that develops intraocular neoplasms similar to human retinoblastoma was created by specific expression of the *Tag* oncogene in the retina. While this will no doubt be a valuable source of retinoblastoma tissue, owing to the way the mouse was engineered, it will not provide a means for understanding the genetics controlling expression of the disease under natural conditions.

Background: Retinoblastoma tumors

In normal individuals retinoblasts are the precursor cells of the retina, the light sensitive layer at the back of the eye. Retinoblasts destine to become cones have been

implicated as being involved in retinoblastoma (Bogenmann et al. 1988). Significantly, once a retinoblast completes differentiation, to a specialized retinal cell (e.g. rod, cone etc), it stops dividing and as such no longer serves as a target for tumorigenesis (Den Otter et al. 1990).

Genetics of retinoblastoma

The genetics of retinoblastoma as originally proposed by Knudson (1971) was verified by Yunis's studies of chromosome 13 and in particular the q arm (Yunis and Ramsey 1978). This arm was often found to have a deletion in part of band 14. Upon further examination it was determined that among children afflicted with familial retinoblastoma, this deletion occurred not only the tumor cells, but also in normal cells throughout the body as well as in the body cells of one parent. However, when the same deletion was found in sporadic cases of retinoblastoma, they were limited to the tumor cells. Thus, available evidence suggests that two events are required to trigger retinoblastoma involving both copies of the *Rb* gene. Each "hit" inactivates one copy resulting in an inactive allele. The outcome being the failure to properly and completely encoded the *Rb* protein, p105^{Rb} (Den Otter et al. 1990; Knudson 1971; Yunis and Ramsey 1978; Shew et al. 1990). Some children are born with one intact and one defective copy of the *Rb* gene. If the intact copy is lost through a somatic mutation in one of their retinal cells, cancer is triggered. In rare instances, individuals fortunate enough to be born with two good copies, may still suffer the loss of both good copies and trigger tumorigenesis (Weinberg 1988).

Molecular biology of retinoblastoma

A number of elegant studies have elucidated much about this neoplasm. The human *Rb* gene was cloned and sequenced and found to contain at least 12 exons distributed in a region of over 100 kilobases (kb) (Lee et al. 1987). The gene encoded a mRNA of 4.6 kb located in close proximity to the esterase D gene. Sequence analysis of complementary DNA clones yielded a single long open reading frame that could encode a protein of 816 amino acids. Furthermore, the authors examined transcription of the gene and found that in two of six tumors the *Rb* mRNA was not detectable, while the other four expressed variable quantities of *Rb* mRNA with a decreased molecular size (4.0 kb) (Lee et al. 1987). In a subsequent study (Fung et al. 1987), 16 of 40 *Rb* tumors were found to have identifiable structural changes in the *Rb* gene including homozygous internal deletions with corresponding truncated transcripts. Furthermore, deletions in the *Rb* gene of an osteosarcoma were also reported (Friend et al. 1986). Eventually, the *Rb* gene was found to encode a nuclear phosphoprotein with DNA binding activity (Lee et al. 1987). Significantly, in this study and subsequent studies by this group and others (Shew et al. 1990; Lee et al. 1987; Bernards et al. 1989; Whyte et al. 1988) resulted in production of an array of polyclonal and monoclonal antibodies to the various epitopes of the *Rb* protein. The utility of these antibodies was amply demonstrated earlier this year when these antibodies were used in conjunction with western blotting to demonstrate that C-terminal truncation of the *Rb* gene product p105^{rb} leads to functional inactivation (Shew et al. 1990). The material for these studies was derived from an osteosarcoma cell line (Saoa-2) which also contained an abnormal

endogenous *Rb* protein of 95 kDa (p95). Though a number of antibodies recognizing several different *Rb* epitopes were reactive; polyclonal antibody anti-C failed to recognize the protein. Subsequent analysis of mRNA and genomic DNA revealed a transcriptional inactive *Rb* allele with a deletion of the C-terminal exons 21-27.

A study with human fibroblasts suggests that failure to phosphorylate the *Rb* protein may be an immediate cause of the failure of these cells to enter S phase (Stein et al. 1990). Likewise, another group has simultaneously demonstrated that a single amino acid substitution, within exon 21, resulted in a retinoblastoma protein defective in phosphorylation and oncoprotein binding (Kaye et al. 1990).

A vertebrate model for retinoblastoma: the medaka

Human surgical and autopsy specimens, established retinoblastoma cell lines (Reid et al. 1974; McFall et al. 1978; Bogenmann and Mark 1983), and a very recent report of heritable ocular tumors in transgenic mice (Windle et al. 1990) represent the only available mammalian material for studying retinoblastoma. Retinoblastoma, however, has been reported in a number of fish species. With one exception all have been single spontaneous cases (Fournie and Overstreet 1985; Harshbarger 1980), however, a published report (Hawkins et al. 1986) and data contained herein conclusively demonstrate the inducibility of retinoblastoma in medaka (*Oryzias latipes*) with the chemical carcinogen methylazoxymethanol acetate (MAMA). The medaka remains is the only model, mammalian or otherwise, in which retinoblastoma can be induced in apparently normal organisms via chemical carcinogenesis. Thus, a unique

opportunity existed to study the etiology of this malignancy in   another vertebrate species. The similar pathology to mammalian models and unique mode of induction insure that information relevant to cancer in general will be gleaned from these studies. In addition, the retina of the medaka continues to develop throughout life, thus allowing for tumor induction at any stage. Finally, the medaka model provided the opportunity to work with a nearly unlimited, inexpensive, supply of fresh tissues with appropriate controls. Consequently, a variety of manipulations were possible.

Introduction to Specific Areas of Investigations

1. Partial Hepatectomy Studies

Rapid hepatocyte proliferation, following partial hepatectomy (PH), was observed by Higgins and Anderson (1931) in the rat model. They documented that compensatory hyperplasia of the remaining lobes of the liver following surgical removal of 65 to 75% of the hepatic parenchyma. Relative to chemical carcinogenesis, the PH procedure with its resulting proliferative response was used to promote tumor development in rats initiated with 2-acetylaminofluorene (Laws, 1959). Subsequently, liver cell proliferation following PH has contributed much to our understanding of the complexities of chemical hepatocarcinogenesis (e.g., Servais et al., 1990; Levy-Favatier et al., 1989; Glauert and Pitot, 1989; Taton et al., 1990; Hasegawa et al., 1989).

Aquatic species, especially fishes, are also proving to be valuable resources for the study of chemically-induced neoplasia in liver and a variety of tissues (reviewed in

Mix, 1986). Studies with the rainbow trout model have documented many similarities with traditional rodent models and have established value of this species for studying certain aspects of hepatocarcinogenesis (e.g., Kirby et al., 1990; Chang et al., 1991; Bailey et al., 1988; Dashwood et al., 1989; Bailey et al., 1987). Also, the trout model has several advantages over traditional rodent models including: 1) large number of animals generated at low cost; 2) thousands of genetically-similar offspring derived from each mating; and 3) external fertilization which provides convenient access to all life-history stages.

The enhancing effect of PH on hepatocarcinogenesis has been previously investigated in a small aquarium fish (medaka) with equivocal results (Kyono-Hamaguchi 1984). To date, however, PH has not been used as a proliferative stimulus in trout liver carcinogenesis. Although, a PH procedure has been described for rainbow trout, details of that surgical procedure were lacking, and the mortality rate for that technique approached 20% (Sanz et al., 1982; Sanz et al., 1986). Moreover, those authors suggested that their surgical manipulation and not liver removal was the principal cause of death (Sanz et al., 1986). Consequently, we undertook our studies to carefully define a reproducible surgical technique which would result in high survival rates (>95%) and to characterize the initial proliferative response of the rainbow trout liver to PH.

2. Establishment of rainbow trout liver cell lines

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; Shigeoka et al., 1987). 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. 1996). 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 et al., 1994; Takagi et al., 1994) and the importance of the medaka as an *in vivo* model for hepatocarcinogenesis has been well established (Harada et al., 1988; Lauren et al., 1990; Ostrander et al., 1992; Aoki et al.,1993; Bunton, 1995).

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 hepatocytes, 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

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novel behavior of both of these cells in the early stages of primary culture. Finally, we report on the successful passage and continuous culture of these cells.

3. Purification of retinoblastoma protein

Having established procedures for examination of regenerating liver and for the isolation, attachment, spreading, and proliferation of trout liver cells in primary culture, it was necessary to isolate the retinoblastoma protein for future studies. Specifically, isolation structural characterization of the protein would tell us much about its potential function. In addition, the isolated protein could be used to make monoclonal antibodies specific for the epitopes expressed in the fish. Availability of these reagents would pave the way for future studies involving immunohistochemistry to study the ontogeny of retinoblastoma formation in the medaka model. The medaka was not used for these studies as it was not possible to secure adequate amounts of tissue from which to purify the protein.

4. Okadaic Acid treatment of rainbow trout cells

Okadaic acid is a know tumor promoter. Tumor promoters such as okadaic acid have been previously shown to alter the phosphorylation state of protein by their selective activity of protein kinases and protein phosphatases. The retinoblastoma protein is a DNA binding protein and as such we proposed that tumor promoter might alter its phosphorylation state in culture. The cells used for these studies were the rainbow trout cell lines developed as these studies were initiated. The antibodies used

were developed from the trout in conjunction with the retinoblastoma protein purification studies described elsewhere in this document.

5. *Establishment of medaka primary liver cell cultures*

Primary cell cultures and cell lines from medaka embryo and fin have been established (Wakamatsu et al, 1994; Saito and Shigeoka, 1994, and have proven valuable in recent a few 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 et al. (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 liver cells in culture.

Blair et al. (1990, 1996) has 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 dishes 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 culture dishes in the presence of serum-free

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media. We also describe several lines of investigation to characterize the origin and novel behavior of these cells in primary culture and our efforts to passage and extend culture period of these cells.

6. Retinoblastoma gene and *protein* expression during chemical carcinogenisis

The importance of environmental pollutants in the etiology of carcinogenesis has been well established. The recent discovery of tumor suppressor genes, which act predominantly to maintain cells in a growth-arrested state, strongly suggests at least one of the steps in the path to malignancy requires alteration in function of a tumor suppressor gene product. Alterations in the *Rb* tumor suppressor gene were first described in retinoblastomas, the most common intraocular tumor of young children. The model for these studies will be a vertebrate species in which a retinoblastoma lesion can be chemically induced; the medaka (*Oryzias latipes*), a small aquarium fish that has been used for many years in chemical carcinogenesis studies. Detailed herein are our initial efforts to characterize *Rb* gene and gene product expression in normal and malignant medaka tissues. We have identified by western blotting a 105 Kd protein that cross reacts with antibodies to the human *Rb* gene product. We also report on the use of reverse transcriptase-polymerase chain reaction to generate and characterize fragments from medaka that hybridize with the human *Rb* cDNA. Preliminary examination of normal and neoplastic eye and liver tissue from medaka suggest there may be difference(s) in *Rb* gene or message expression.

Medaka, exposed briefly (30 min) as fry to methylazoxymethanol acetate (MAMA)

developed a variety of retinal lesions following a latency period as short as 4 weeks (Hawkins et al. 1986). Subsequently, it was determined that at a dose of 50 mg/L, advanced lesions including some resembling retinoblastomas, appeared as early as 8 weeks. However 3 to 4 months was more typical latency period.

The classic two-step model for chemical carcinogenesis consists of initiation and promotion. To understand the potential involvement of tumor suppressor and other genes in tumorigenesis, it is critical to know when promotion occurs. Medaka have been exposed to a wide variety of chemical carcinogens, including aflatoxins, oxides, and aromatic hydrocarbons. 1-1-Dimethylhydrazine and methylazoxymethanol acetate (MAMA) are the only two currently identified compounds that consistently produce retinal lesions among the broad spectrum of lesions seen in this animal. 1-1-Dimethylhydrazine is a component of rocket fuel that is a potential environmental contaminant. *In vivo*, it is metabolized to methylazoxymethanol, a naturally occurring neurotoxin that is found in cycads (Laqueur 1977). Since MAMA produced other tumors in medaka (e.g. liver) and *Rb* expression is altered in many human tumors, the capability of examining the pathology of all medaka tissues simultaneously suggest this fish model will be a powerful experimental tool.

In the Statement of Work for this project we proposed examination of the ability of tumor promoters to impact *Rb* expression in the medaka model. In order to complete these studies it was first necessary to validate the medaka model as being suitable for studying the *Rb* gene. Our studies, detailed herein, were focused on establishing the presence of the *Rb* gene and protein in normal medaka and conducting a preliminary

screening of a few liver and eye tumors for alterations in *Rb* gene/message expression.

BODY

Overview

As described above, six broad areas of investigation were undertaken in the completion of the studies in relation to the Statement of Work outlined in the original proposal. Initially, the primary objectives for each area are briefly described followed by the specific methods, results, and discussion. For convenience each of the six areas is clearly delineated as are the *Materials and Methods*, *Results*, and *Discussion* for each area of investigation.

1. Partial Hepatectomy Studies

As a prelude to studying changes in retinoblastoma protein expression during liver regeneration, it was necessary to develop techniques for performance of partial hepatectomy (PH) in the rainbow trout liver.

Briefly, existing procedures for partial hepatectomy in rainbow trout (*Oncorhynchus mykiss*) liver were modified and a greater than 95% success rate achieved. The initial response of the rainbow trout liver to partial hepatectomy was evaluated. DNA synthesis, determined by tritiated thymidine incorporation into DNA in liver slices, increased significantly in the first 24-48 hours following partial hepatectomy and remained elevated for at least 10-14 days. Protein content based on wet weight of the liver, however, appeared to remain constant in the 2-weeks following partial hepatectomy. The procedure was equally effective on both large (600 grams) and small

(6-80 grams) fish and should be useful for either promotion in complete carcinogenesis regimens or other toxicological studies requiring liver cell proliferation.

Materials and Methods

Materials

Adult rainbow trout, 15-months old, and weighing 600 ± 50 grams were purchased from the Weeping Willow Fish Farm (Miami, OK). Rainbow trout fry, weighing 6-8 grams, were obtained from the Norfork National Fish Hatchery (Mountain Home, AR). [Methyl-^{3}H]thymidine with a specific activity of 85 Ci/mM was purchased from Amersham (Arlington Heights, IL).

Partial Hepatectomy

Partial hepatectomy (PH) method development and the initial experiments detailed below were conducted on the 15-month old trout. However, to validate the use of the methodology on smaller trout, which are more commonly used in chemical hepatocarcinogenesis regimens, replicate experiments were repeated with the 6-8 gram fry.

All trout were maintained in Living Stream (Frigid Units, Inc. Toledo, OH) recirculating raceway aquaria (LSW-700) at 10 ± 0.5° C. Prior to surgery, individual fish were removed from the aquaria and placed into a rectangular 10-liter trough containing 5.0 liters of water from the aquaria, 1.5 liters of crushed ice, and 600 mg of MS-222 (tricaine methanesulfonate, Crescent Research Chemicals, Phoenix, AZ). After 90-120

sec the fish exhibited a total loss of equilibrium and no longer responded to tactile stimulation. A fish specimen was then rapidly transferred to the surgery table and placed, dorsal side down, on a surgery net hung loosely between two upright, parallel plexiglass sheets (Figure 1). A stream of cold water containing anesthetic (MS-222, 50 mg/liter) was continuously pumped into the branchial chamber. A 1.5 cm diameter hose with a flow rate of approximately 2.8 ± 0.2 liters/min maintained anesthesia. Wet paper towels were placed on the exposed ventral surface of the fish to prevent dehydration during the procedure.

The left pectoral fin was grasped with Allis intestinal forceps and pulled gently forward to position the fin out of the surgical field. An incision was made with a sharp surgical scalpel blade (full; convex; size 22) along an imaginary parasagittal line, 1 cm left of the ventral midline, beginning 0.5 cm posterior from the medial edge of the base of the pectoral fin and continuing posteriorly for about 2.5 cm. The incision was made simultaneously through both skin and body wall and care was taken not to cut visceral organs. The incision was held open with a self-retaining spring action retractor (Number C-915, Clay-Adams, Inc., New York, NY), the handle of which was pointed posteriorly. The posterior tip of the liver was easily seen through the incision.

A pair of blunt 14 cm thumb dressing forceps (tips 3 mm wide, ART # 560; Prestige Medical, Northridge, CA) was modified as follows: 1) nine coarse notches were filed inside each tip to replace the fine serrations found on these forceps, and 2) the tips of the forceps were bent out slightly 1.5 cm from their end so when the forceps were pinched shut, the extreme ends of the tips were still open about 1 mm. These "fish liver"

forceps were used to partially retract the liver by positioning them parallel with the long axis of the organ and advancing the tips anteriorly until about 1.00 to 1.25 cm of liver lay in the grasp of the forceps. The liver was then gently grasped and retracted posteriorly. An additional pair of forceps (fine Halstead Mosquito hemostatic curved forceps 12.7 cm) were used to clamp off the retracted liver transversely just anterior to the tip of the "fish liver" forceps. The clamped off segment (15 - 20% of total liver mass) was then separated from the remainder of the liver by cutting along the caudal edge of the fine forceps with a scalpel. The excised segment of liver was immediately placed in a vial on ice and frozen for later analyses.

The forceps were then removed (usually no hemorrhage was present even after as little as 20 seconds), 2 or 3 drops of Combiotic (penicillin and dihydrostreptomycin in aqueous suspension, Pfizer, New York, NY) were dripped into the incision, and the retractor removed. The incision was closed with interrupted sutures made with 000 silk thread and a 1.5 cm half-circle, cutting tip, suture needle (size 6, Miltex Instrument Co, Lake Success, NY). The sutures went through the skin and body wall, suturing as a single unit. While the next to last suture was being made, the anesthetic solution perfusing the gills was switched to plain cold water. After suturing was complete, 2 or 3 drops of Combiotic were spread on the incision, the incision was dried by wiping it with cotton gauze, and a thick coat of petroleum jelly was spread on the incision. The fish was quickly tagged on its dorsal fin with a numbered clip and returned to an aquarium. The fish was initially pushed through the water, mouth first, to ensure water flow over the gills and accelerate revival. Within 2-5 minutes fish swam on their own.

Modifications necessary for performing this method on the 6-8 gram fry included reducing the diameter of the hose entering the branchial chamber to 4 mm and reducing the water flow through the hose to 0.2 ml/min. Due to the small size of the animal it was not possible to use a retractor, instead the body cavity was held open with two pairs of forceps by an assistant. Finally, a block of styrofoam with a depression roughly the size of the trout fry was attached to the surgery table. Fish were immobilized by placement into the depression in the styrofoam, rather than suspended in the surgical netting, and the procedure completed as described above.

Partial hepatectomies were performed on three or four fish per day, at two-day intervals, for a period of 14 days. Sham-operated controls in which the specimen was opened, liver palpated, and finally closed, were also included on days 0, 1, 2, & 14. After 14 days all fish were sacrificed by MS-222 overdose and the liver tissues were utilized as described below.

DNA Synthesis

To estimate the consequences of PH on DNA synthesis, incorporation of tritiated thymidine into DNA in liver slices was examined at various times post-hepatectomy. From a central area of the liver near the wound site, four paper-thin sections (approximately 1 cm x 1 cm) of liver tissue were taken with a double-edged razor blade. Tissue slices were immediately transferred (2 sections/well) to 6-well petri dishes (#3046, Falcon) in four mls of phosphate buffered saline (PBS) and held in a Shel-Lab (Sheldon Laboratories, Portland, OR) low temperature incubator for about 30 min at $16.5 \pm 0.5°$

C with a gas phase of 95% humidified air/5% CO_2 until all slices were obtained. They were then transferred to modified minimum essential medium eagle (MEM) (NaCl concentration 176 Mm) supplemented with 10% fetal calf serum (FCS) containing 5 μCi ^{3}H-thymidine (specific activity 85 Ci/Mm). Incubation continued at 16.5° C for up to 16 hours. Reactions were stopped by rinsing the individual liver slices 3 times with PBS (NaCl concentration 176 Mm) and slices were then placed in 1 ml of 5% trichloroacetic acid containing 0.05 M pyrophosphate and stored at 4° C.

Individual liver slices were homogenized at 4° C with the addition of 0.5 ml of 10% TCA containing 0.1 M pyrophosphate. Samples were then centrifuged at 325 x g and the supernatant discarded. Samples were washed 2 times with 1 ml of 10% TCA, resuspended in 1 ml of 10% TCA and 500 μl filtered through 0.45 μM HA filters (#HAWP 025 00; Millipore, Bedford, MA). Filters were successively washed twice with 3 ml of 10% TCA containing 0.1 M pyrophosphate and once with 10 ml 0.01 of M HCl. Filter discs were dried and counted. Incorporation of radiolabled thymidine into DNA in tissue slices was found to increase linearly over the 16 h incubation, indicating that the liver slices remained viable during this period. Data at each time point is presented as the mean CPMs $\pm$ SE of either 12 or 16 samples (3 or 4 fish/day; 4 samples/fish).

The DNA assay (Giles and Mayers, 1965) was modified from the original method of Burton (1956). From each liver slice, 15 μl of homogenate was added to 600 μl of 10% perchloric acid (PCA), heated at 70° C for 15 min, and centrifuged at 1,310 x g for 4 min. Then 500 μl of the resulting supernatant were transferred to a clean tube with 0.5 ml of 4% diphenylamine in acetic acid and 25 μl of 0.4% acetaldehyde, mixed well,

sealed and incubated at 30° C for 16 hr. Absorbance was determine at 595 nm with calf thymus DNA used as a standard. Data from individual slices from each fish for each time interval were pooled and CPMs/μg DNA/8 hr was determined for each time point. An analysis of variance was used to test for significant differences across all days and a follow-up Dunnett's T test was used to examine individual differences between days.

Protein Content

Protein content of liver tissue was determined by the method of Lowry et al. (1951) with crystalline bovine serum albumin as the standard.

Results

Partial Hepatectomy

Overall survival 24 hours following partial hepatectomy (PH) was more than 95% (n=51) for large trout and more than 80% (n=47) for trout fry. Of those surviving 24 hours, greater than 95% were alive at one month following PH, regardless of size. The mean length of time for surgery for adult trout and fry was 7.8 and 7.7 min respectively, including initial anesthesia which typically took 1-2 min.

Regardless of the length of surgery or size of fish, rainbow trout on the surgical table responded to tactile stimulation about 4 min after resumption of freshwater flow to the gills. Specimens regained equilibrium within 3 min following return to the aquaria and resumed normal feeding within 48 hr.

Some specimens, which were held longer than three months following surgery,

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were sacrificed and the surgery site examined. Externally the skin had healed to the extent that the sutures were barely visible and only a slight depression was apparent along the suture line. Internally, occasional adhesions were observed between the visceral peritoneum of liver, intestinal caeca, and the parietal peritoneum of the body wall. Postoperative infection was never observed in these studies.

DNA Synthesis

No significant differences in DNA synthesis were observed between liver slices taken proximal to and at the surgery site. Consequently, all samples for each liver and each time point were pooled (n = 4 slices/liver; 3 or 4 livers/time point). Incorporation was found to be linear for at least 24 hr (determined during pilot studies) and is expressed as CPMs/mg DNA/8 hr exposure period. An analysis of variance indicated significant differences existed among the various time periods.

Figure 2 shows the relative rate of DNA synthesis as estimated by incorporation of tritiated thymidine into DNA in liver slices from adult rainbow trout (A) and trout fry (B) at various times following PH. In adult sham-operated controls and untreated controls, incorporation of tritiated thymidine into DNA in tissue slices during an 8-hour incubation was 9,603 ± 1,773 CPMs (mean ± S.E.) representing the basal rate. A statistically significant increase in DNA synthesis was noted by 24 hr after PH when mean CPMs reach 15,727 ± 1,299 CPMs/mg DNA. A further increase to 19,869 ± 1,402 CPMs/mg DNA was noted at 48 hr, representing an approximate doubling over controls. The adult trout liver maintained an elevated rate of DNA synthesis for at least 14 days following

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PH. Among rainbow trout fry sham-operated controls and untreated controls, incorporation tritiated thymidine into DNA in tissue slices during an 8-hour incubation was nearly 30% higher than that of adult trout (13,275 ± 3,128 CPMs). A statistically significant increase in DNA synthesis was again noted by 24 hr following PH when mean CPMs reach 29,766 ± 3,383 CPMs/mg DNA, more than double that of controls. DNA synthesis in the livers of trout fry remained elevated over that of controls for 10 days following PH. Beginning on day 12 however, DNA synthesis rates were no longer significantly different from control values.

Protein Content

On a wet weight basis, no significant differences in the liver protein content were observed in the days following PH. Sham control and day 0 adult fish exhibited a mean value of 123.3 ± 11.5 μg protein/mg liver and partially hepatectomized animals ranged from 106.8 ± 10.8 μg protein/mg liver (day 6) to 137.1 ± 9.4 μg protein/mg liver (day 12). Among sham control and day 0 rainbow trout fry the mean μg protein/mg liver was 114.2 ± 19.6. Following PH values ranged from 99.8 ± 23.2 (day 12) to 121.9 ± 21.1 (day 8) and did not differ significantly from control values.

Discussion

Partial Hepatectomy

The procedure detailed here was rapid and allowed one to complete 5 to 6 trout hepatectomies in an hour. Moreover, initial and long-term survival rates were high.

Three-months after PH, few residual effects were observed. Incisions had healed with only a small depression visible along the external suture line. Internal adhesions were only occasionally detected. No scar tissue was visible on the liver surface and the only evidence of surgery was the failure of the regenerating liver to assume its original (pre-surgery) gross morphology. Furthermore, histological examination revealed that proliferation of cells was not a focal feature at the surgery site, instead a general hyperplasia of the remaining liver tissue was encountered. These observations agree with the study of Sanz et al. (1982) who reported that within 30 days of PH the trout liver had recovered its initial weight. Thus, the morphological response of the trout liver to PH appears similar to but slightly slower than the 14-21 days originally reported for the rat (Higgins and Anderson, 1931).

Our initial method development and experiments were conducted on 600 gram fish. However, most chemical hepatocarcinogenesis regimens utilize much smaller rainbow trout fry which are rapidly growing and extremely sensitive to carcinogen initiation. With a minimal effort we were able to successfully modify this technique for smaller (6-8 gram) rainbow trout fry with similar results. In fact, the mean weight of all fry which continued to grow throughout the experiment, was only 8.8 grams at termination. We attribute the slightly lower survival rate at 24 hr among trout fry (>80%), as compared to adults (>95%), to internal injuries sustained during the surgical procedure. When necropsying these animals we observed that other internal organs had sustained serious trauma during the PH procedure. Because of the limited room in the body cavity with which to work, extra care must be taken when conducting PH on such

small animals. Nonetheless, we have successfully performed this procedure on fish as small as 4 grams. Thus, in a carcinogenesis regimen requiring cell proliferation, PH could be implemented on young specimens at any stage.

DNA Synthesis

DNA synthesis, as estimated by the incorporation of ^{3}H-thymidine into the DNA of liver slices, increased significantly following PH in both adult trout and fry and remained elevated for the ensuing 10-14 days. Bucher et al. (1964) reported 2 waves of DNA synthesis in the first 50 hr following PH in rat. Thus, while elevation in synthesis is apparent in both trout and rat, a cyclic pattern was not readily apparent in the trout. It appears that the magnitude of the response following PH may be higher in trout fry when compared to adults. This may be explained in that the liver is growing more rapidly in the younger animals.

Protein Synthesis

In rats, protein content/mass fresh tissue drops sharply in the first day following PH and gradually returns to normal values over the next 2 weeks (Bauer et al, 1976; Riboni et al. 1990). This may be due to fatty infiltration or simple edema. We did not observe any changes in protein content immediately following PH or during regeneration in the rainbow trout liver. Nor did histological observation reveal evidence of fatty infiltration in the regenerating liver.

2. Establishment of Rainbow Trout Liver Cell Lines

Studies were undertaken to establish and characterize cell lines from rainbow trout liver.

Materials & Methods

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

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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 Inc., Toledo, OH) recirculating raceway aquaria (LSW-900) at $10 \pm 0.5°$ C. The fish, males and females weighing 300-500 g, were fed 2-3x daily approximately 1g food/100g body weight (Purina Trout Chow).

Primary Culture of Rainbow Trout Hepatocytes

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 Chemicals, Product # C-5138) was added to the perfusion medium and perfusion extended to 35 minutes 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 volumes of culture medium (Minimum Essential Medium Eagle with Earle's salts and L-glutamine without $NaHCO_3$ [MEM (JRH Biosciences, Lenexa, KS) supplemented with $NaHCO_3$ (26 mM), L-glutamine (2 mM), 1.317 g NaCl, MEM-non-essential amino acids solution (0.1 mM), penicillin G (100,000 U/l), streptomycin sulfate (0.1 g/l), amphotericin B (250 μg/l, and 10% heat-inactivated bovine calf serum (Sigma Chemicals, 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 X 20 mm Primaria tissue culture plates (Falcon, Product # 3803). Plates were maintained in a 16° C, 5% CO_2:95% air

atmosphere in a Sheldon Lab-Line Incubator (Portland, OR).

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, 10% fetal calf serum + 2% bovine serum albumin, 10% newborn calf serum, or 10% bovine calf serum. All plates were examined daily for 30 days 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 established 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 months, periodically thereafter, and evaluated for attachment, aggregation, spreading, degree of confluence, cell integrity, and overall appearance.

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 days. In addition, replicate 32-day cultures were subcultured 1:3 and sampled on

Day 33 along with the primary cultures. Cells were washed with isotonic PBS and the resulting cell pellet homogenized with 10 vol of EBC buffer (50 mM Tris-pH 8.5, 120 mM NaCl, 0.5% non-ident P-40, 100 mM sodium fluoride, 200 μM sodium ortho-vanadate, 10 μg/ml aprotinin, 10 μg/ml phenylmethylsulfonyl fluoride, 2 mM EGTA, and 2mM 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 bovine serum albumin as a standard. Western blotting with PCK26 and V9 followed manufactures protocols using Immobilon polyvinylidene difluoride membranes (Millipore, 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 hour 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 hours, followed by 70% ethanol for at least 24 hours. Cells were removed from culture plates by gentle scrapping and rafts of cultured cells pipetted into a 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 microns 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

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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.

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, 4 plates were incubated with ^{3}H thymidine (specific activity, 85 Ci/mM) for 16 hr (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 xg, resuspended in 10 vol of a cell lysis buffer (Promega, product number E153A), and disrupted with 5 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 days. Following development of the emulsion plates were stained for 30 min with hematoxylin, rinsed for 5 min with deionized water, and counter-stained 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 1,000x) 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 than resuspended in 0.5 ml of 2.5% Noble agar (Difco Laboratories, Detroit, MI) at 50° C (Allred et al., 1993), centrifuged at 3000 xg 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

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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 3x for 2 min each with two 1 min cooling periods (modified from Foley et al., 1994). Following antigen retrieval the slides were rinsed 2x with ddH_2O and 1x 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 hr, biotinylated secondary antibody (BioGenex, 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 1,000 nuclei from random fields and scoring positive staining cells (i.e. indices are expressed as positive cells per 1,000).

For propidium iodide staining cells were washed 3X with PBS and finally pelleted at 600 xg 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

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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 xg 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 3x with PBS, and finally pelleted at 600 xg for 5 min. The resulting cell pellet was resuspended in 10 ml of PBS, and 5 x 10^5 - 5 x 10^6 cells were aliquoted into separate 12 x 75mm 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 2x 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 tube and 5 μl (5 μg) of Mouse IgG1-FITC conjugate was added to each control tube. All samples were incubated at 4° 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 xg for five minutes. 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 xg 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 hours). FCM analysis was with an Epics Profile II (Coulter Cytometry, Inc., Hileahia, 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, Inc., Seattle, WA.)

Continuous culture of rainbow trout liver cells

Upon reaching confluence (approx. 30 days), cells were subcultured 1:3. Briefly, media was decanted, cells rinsed once for 60 sec with 0.05% trypsin in 0.53 mM 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. Confluent cultures were harvested and centrifuged at 250 g for 5 min to pellet the cells. Cells were resuspended in freezing medium (Eagles MEM with 10% calf serum and 10% DMSO) and approximately 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 hr vials were stored in liquid nitrogen.

As necessary, 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 hours culture media was replaced to remove residual DMSO.

Results

Isolation and placement into primary culture

Depicted in Figure 3 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 minutes cells began to attach to the culture dish and form small aggregates (Figure 3a). Within 24-48 hours the small aggregates fused into larger aggregates of which the majority remained weakly attached to the plate. After 48 hours about 20% of the aggregates were detached and floated freely in the media. These clumps of cells, as originally reported by Blair et al.

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(1990), remained viable. Over about 8-10 days 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 (Figure 3b).

Initial growth or movement of cells from the periphery of the aggregates, over the next 6-10 days (Figure 3c), was relatively slow. However, the rate of growth/movement accelerated markedly beginning about Day 19 (Figure 3d) and continued through days 24, 26, and 30 (Figures. 3e, 3f, and 3g). 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 spindle-like 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 spindle-like (Figure 3h) or hepatocyte/epithelial cell-like (Figure 3i). Moreover, foci of the cells with the hepatocyte/epithelial-like phenotype could also be seen prior to confluence, often within the first 21 days. 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 scattered intact bile ducts. 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 hepatocytes, irregular bile

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ducts with attenuated epithelium, and pleomorphic spindle cells. Finally, by Day 70, the culture was completely composed of pleomorphic spindle 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 1,200 days with bi-weekly 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 culture (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 months. For example, at 3 months, 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 the those cultures with no serum added. Conversely, cultures with 5% serum added were 80% confluent, had a spindle cell morphology (Figure 3h), and were not stellate or vacuolated. Finally, cells at 10% serum were 90% confluent (with some piling or layering), most cells had a

"fibroblast-like" morphology (Figure 3h), 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 (Figure 3g).

Visualization of cytokeratin and vimentin expression via western blotting

As shown in Figure 4a significant alterations in cytokeratin expression were evident beginning about Day 4 (lane 5) when compared to Time O (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 not shown).

Vimentin, as expected, was not observed in these cells at any time in culture (Figure 4b, lanes 4-9). A strong signal was evident from NIH3T3 cells (Figure 4b, 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 bile ducts of intact rainbow trout liver with no staining of hepatocytes, periductular connective tissue, or arterial smooth muscle (Table 1). Among the liver cell cultures, similar results were observed with the AE1/AE3 antibody having a strong affinity for the few remaining intact bile ducts and being negative for hepatocytes. In the Day 28 sample the AE1/AE3 antibody exhibited decreased binding to bile ducts 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.

The results of staining with the anti-desmin antibody in the intact control liver was consistent with our observations of control canine 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 non-specific. No staining of the spindle cells with the anti-vimentin antibody.

DNA content/synthesis

DNA synthesis was initially measured by thymidine incorporation and quantified by fluorometry. Reported in Figure 5 are values (CPMs/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 four days in primary culture a statistically significant increase in thymidine incorporation was observed as CPMs/μg DNA $\pm$ S.D. rose from 10,145 $\pm$ 3,779 after 24 hours in culture to 20,707 $\pm$ 2,832. Thymidine incorporation remained active for about three weeks and then gradually decreased to 11,613 $\pm$ 3,149 by Day 33 as the cells became confluent. In an additional experiment, we examined hepatocytes immediately (1 day) after subculturing confluent cells from 32-day primary cultures. Following one day in subculture, DNA synthesis as measured by thymidine incorporation had not been initiated (9,721 $\pm$ 744 CPM's/μg DNA). Finally, hepatocytes from an aflatoxin induced tumor were examined by this method and incorporation of thymidine was elevated when compared to freshly isolated hepatocytes as we observed a mean level of 16,840 $\pm$ 7,529 CPMs/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. 6. Throughout the first 10 days in culture, 22-24% of the cells were observed to have 3 or more silver grains over the nucleus, suggesting a modest level of DNA replication. On Day 19 the percent of cells with 3 or more silver grains had dropped to 8% and a similar value of 11% was observed on Day 33 (33a). As in the previous experiment, one plate of cells was subcultured on Day 32 and thymidine incorporation examined after one additional day in culture. Data from a single plate (33b) suggest DNA synthesis in increasing as nearly 20% of the cells exhibited 3 or more grains. Finally, examination of cells from the hepatocellular carcinoma demonstrated that greater than 44% of these cells presented 3 or more grains over the nucleus suggesting a high level of DNA synthesis (Figure 6, "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 hepatocytes were visible including those comprising islands and disseminated hepatocytes. 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 hepatocytes 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. Hepatocyte PCNA index had risen to 232 indicating that 23%

of the hepatocyte 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-G_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 days in primary culture are tabulated in Table 6 and graphically presented in Figure 7. As expected, at Time 0 (Figure 7, 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 (Figure 7, panel A). After only 4 days nearly identical levels of G- and F-actin were noted and only a single peak for each was present in the hepatocyte populations (Figure 7, panel B & 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 discernable for F-actin and MCN of 30 and 57 were detected (Figure

7, panels C & E). The Day 40 MCN for G-actin was 120. Finally, after 50 days 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 days 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 hepatocytes. Actin expression were also examined in cells from a hepatocellular carcinoma isolated from a rainbow trout. As depicted in Figure 8, the predominant actin expression was in the form of G-actin (MCN = 225) as compared to F-actin (MCN = 135).

Subculturing and long-term storage

After 30 days 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 year in storage. Characterization of these "cell-lines" is in progress.

Discussion

This is the first report of the establishment of long-term primary cultures of epithelial cells from fish liver. Nearly homogenous (>97%) primary cultures of hepatocytes were initiated and hepatocytes remained viable and proliferative for at least 44 days. In addition, proliferative biliary cells persisted in the cultures for at least 30 days. Finally, a third epithelial cell type, we have termed spindle cells, was persistent in our cultures and could be passaged. We now report on 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 long-term aggregate culture was not possible. 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-weeks after hepatocytes were placed in culture. As a consequence, 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, while the initial culture conditions differed from those of Blair et al. (1990), the specific mechanism may lie in the secretion of specific growth factors, mitogens, or cytokines by the cells and their accumulation in culture in the absence of media changes

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during the first 2 weeks.

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 comes from at least four sources. First, DNA synthesis as measured by thymidine incorporation was elevated significantly over the first 4 days in primary culture (Figure 5). Between days 1 and 4 thymidine incorporation more than doubles. Unfortunately, due to the necessity of a 16-hour 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 (Figure 6) demonstrated high activity throughout the first 10 days 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 (Figure 6; 33b), which were passaged 24 hours previous nearly doubled their rate of thymidine incorporation and with 19% of the cells exhibiting ≥ 3 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 hours the number of cells in S-phase had increased to 8% and 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 corresponds with the appearance of actively

growing cells (Figure 3c). Likewise, PCNA analysis was possible on cells immediately after placement in culture and at multiple time-points during the first 10 days in culture (Table 4). Again, a significant   increase in cell proliferation and cell division was seen as the hepatocyte PCNA index value rose nearly 4-fold 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 establish long-term cell cultures following hepatocyte isolation, establishment of the identity of these cells was undertaken. Examination of cells by phase microscopy (Figure 3) and electron microscopy following fixation and staining (results not shown) suggested that following initial placement into culture the majority of cells were hepatocytes with some contaminating biliary epithelial cells which probably originated from bile ducts. As the length of time in culture increased at least three types of epithelial cells appeared to be growing including hepatocytes, biliary epithelial cells, and a cell type we have termed spindle cells with 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 hepatocytes. The positive cytokeratin staining via western blotting at all time-points in culture coupled with negative staining with the anti-vimentin antibody (Figure 6) 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

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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 bile ducts 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 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. Since the anti-cytokeratin antibodies were also

specific for bile ducts in the intact control liver and 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. Friedman et al. (1984) observed a pronounced decrease in actin cytoskeletal organization as cultured human colonic epithelia progressed from a benign to a malignant phenotype. Verderame et al. (1980) measured F-actin cytoskeletal expression utilizing fluorescein isothiocyanate (FITC) phalloidin conjugates and correlated lower F-actin content with cellular transformation. 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 chemically transformed as well as differentiated cell lines. Transformed phenotypes contained less F-actin than their more differentiated counterparts and therefore bind less FITC-phalloidin reflected by a lower mean channel number in flow cytometric analysis.

Using cultured trout hepatocytes, our studies indicated a time dependent loss of F-actin expression with concomitant increase in G-actin expression (Figure 7, Table 6). Although the appearance of these two conformations of actin seemed inversely related, the kinetics of the two processes appeared independent (Figure 7). 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 pre-malignant or malignant phenotype

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(1,4) and, this hypothesis was further substantiated when a hepatocellular carcinoma from the rainbow trout was examined (Figure 8). The tumor cells exhibited the highest G-actin levels (MCN = 225) relative to any cells in primary culture. Whether these cells have assumed a pathway towards malignancy remains to be investigated; however, these data, coupled with the rapid changes in cytokeratin expression observed in the first few days of culture (Figure 4a), suggest that these cells may be progressing toward a transformed phenotype.

Finally, these cells long-term primary cultures are amenable to continuous culture. We have established 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.

3. Purification of retinoblastoma protein

As a prelude to other studies described below and for future studies it was necessary to purify the retinoblastoma protien from the rainbow trout.

Materials and Methods

Peptide Synthesis

A peptide with a known human retinoblastoma protein (pRb) binding motif was

prepared to use as an affinity ligand to immobilize on a resin. The consensus sequence GGELLCGEGG, was chosen based on the sequences of several known pRb binding proteins. Synthesis was done via solid phase 9-Fluoroenylmethoxycarbonyl (Fmoc) chemistry on a Perkin Elmer ABI 433 A automated peptide synthesizer (need reference here).

K 562 Cell Culture

Human leukemia myeloma tumor cells (K 562 cells from ATCC) were grown in RPMI-1640 media (from Sigma) (10.4 g/l RPMI-1640 media: 50 ml of heat inactivated fetal bovine serum: 2 g/l sodium bicarbonate: pH 7.2). Cells were harvested and washed with sterile isotonic saline, pelleted and stored without solution at -85 °C until lysed. Cells were lysed by treatment with 1.8 ml of EBC modified lysis buffer (10 mM HEPES (pH 7.5): 0.15 M NaCl: 10 mM EGTA: 10 mM EDTA: 1 μg/ml aprotinin in PBS: 1 μg/ml of PMSF in isopropanol) for 30 min. at 4 °C. Cell debris was pelleted by centrifugation at 2000 rpm and 4 °C for 15 min. Cell lysates were then used for resin binding experiments.

Affinity Matrix Preparation

Four cyanogen bromide (CNBr) activated Sepharose 4-B resins (from Sigma) were prepared. One milliliter of resin was prepared with a synthesized peptide ligand containing the LXCXE known pRb binding motif (a consensus sequence from known pRb binding proteins (Iami et al. 1991; Soni et al. 1995). A second batch (1 ml) of resin was

prepared, as a control, with the blocking molecule ethanolamine as the ligand. The anti-human Rb monoclonal mouse antibody (Rb-mAb-1) was ligated to a third batch (0.4 ml) of CNBr-Sepharose (purchased from Oncogene Science). The fourth batch of resin (1 ml) was prepared with goat anti-yeast pyruvate kinase serum (Pk-Ab) in 1985 Blair (unpublished data) and was used as a second control.

Affinity Chromatography

Three different resin binding experiments were conducted to determine whether the rainbow trout pRb 50 kD protein had affinity for the Rb binding motif and its specificity. Two additional experiments were preformed to judge the affinity of the trout pRb 50 kD for Sepharose. The conditions of each resin binding was a little different.

The first resin binding experiment only used the pRb binding motif resin (LXCXE) and the ethanolamine blocked control resin. Four grams of rainbow trout liver were homogenized in 20 ml of cold modified EBC lysis buffer. The homogenate was filtered through cheese cloth, to remove large solid material, and then centrifuged at 10000 g for 30 min. at 4 °C. The supernatant, approximately 20 ml, was evenly divided and added to the two resins. The resins and supernatant were gently mixed for 1 hour at 4°C, to allow proteins to bind to the resin. After the 1 hour incubation the unbound proteins were removed by centrifugation at 2000 rpm for 1 min. Resins were then washed six times with cold HEPES saline buffer (10 mM HEPES (pH 7.5): 0.15 M NaCl). Any proteins not washed away were then eluted overnight in 2 ml of 2% sodium dodecyl sulfate (SDS) at room temperature. The resins were then washed an additional 4 times

with 2 ml of 2% SDS each. A final wash with cold HEPES saline and 2-mercaptoethanol (BME) (10 mM HEPES (pH 7.5): 0.15 M NaCl: 1 mM BME) was done in order to elute any proteins which may have formed disulfide bonds with the cysteine of the LXCXE motif. Fractions were taken at each step and subjected to western blot analysis.

The second resin binding experiment used all four resins mentioned in matrix preparation. For this experiment 1 mM of BME was added to solutions used on the LXCXE and control resins. The BME was to keep the sulfhydryl group of the cysteine of the LXCXE motif reduced. No BME was added to the antibody resins. In order to keep the amount of protein proportional in all fractions of the resin bindings 10 ml of liver homogenate was added to 1 ml of the LXCXE, ethanolamine control, and PK-Ab resins and 4 ml was added to 0.4 ml of the commercial Rb-Ab-1 resin. Fractions were collected subjected to western blot analysis.

A more rigorous washing technique was preformed in third resin binding experiment, in order to determine whether the binding was non-specific. Almost everything was the same as the second resin binding except that after elution with 2 ml of 2% SDS: 1 mM BME the resin was washed four times with 7 ml of 2% SDS: 1 mM BME instead of 2 ml. Fractions were collected at each step and subjected to western blot analysis. In addition duplicate lanes were run and western blotted without primary (1° Ab) antibody in order to determine non-specific binding of the secondary antibody (2° Ab).

To determine the affinity of the rainbow trout pRb 50 kD for Sepharose 4-B-200 (from Sigma) without any affinity ligand had a fourth resin binding experiment was

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conducted. Only the PK-Ab resin and a Sepharose 4-B-200 resin was used in this experiment. No BME was included in any of the solutions since there were no sulfhydryl groups to protect. Binding procedures were as explained for experiment 3. All fractions were collected at each step and analyzed by western blotting.

A fifth resin binding experiment was preformed to assess whether the human pRb 110 showed similar affinity for Sepharose as did the trout pRb 50 kD. Two resins were used in this experiment: Sepharose 4-B-200 and the PK-Ab resin. Cell lysates of K 562 cell (from ATCC) were loaded on the resins in proportional amounts to the trout liver homogenates. The proteins were bound and eluted as described earlier for the trout liver homogenates. Fraction were collected and subjected to western blot analysis.

Western Blot Analysis

Fractions from each resin binding experiment were subjected to western blot analysis. Samples were prepared by a 2:3 dilution in sample buffer (0.125 M Tris (pH 6.8): 4 % SDS: 20% glycerol: 10 % 2-mercaptoethanol (BME): 2 grains of bromophenol blue). Molecular weight standards were prepared by a 1:10 dilution of standards from Sigma in sample buffer. Approximately 40 μl of the original sample was loaded in each well for the first three resin binding experiments. For resin binding experiment 4 only 33 μl of original sample was loaded and for experiment 5 only 26 μl of original sample was loaded.

Samples were loaded on a 9 % SDS-PAGE gel and run for 3 hours at increasing voltage settings. Gels were then electroblotted on to PVDF membrane (Immobilon-P

Millipore) for 1.5 hours at 25 V and 200 mAmps. Lanes with high molecular weight standards were stained with coomassie. Lanes blotted with chromatography fractions were blocked overnight at 4 °C in blocking buffer (25 mM Tris (pH 8.0): 125 mM NaCl: 0.1 % Tween 20: 4 % BSA). Membranes were then treated with the 1° Ab, Rb-mAb-1 (from Oncogene Science), for 1.5 hours at 4 °C, followed by two washes with cold TBS, 10 min. each, [Tris (pH 8.0) NaCl: KCl] and one wash with cold TBS and Tween 20, 10 min. A 2°Ab solution, alkaline phosphatase-conjugated affinity pure rabbit anti-mouse IgG (H+L), was then applied. Protein bands which were reactive with the 1° Ab and 2° Ab were visualized by color formation after treatment with bromochloroindoyl phosphate (BCIP from Sigma) and nitro blue tetrazolium (NBT from Sigma). In order to determine non-specific binding of the 2° antibody for proteins duplicates of some lanes were western blotted without the 1° antibody.

Results

Determination of Affinity of pRb for the LXCXE Motif

Resin binding experiments 1, 2 and 3 were done to determine whether trout pRb has any affinity for a peptide with the LXCXE motif. The ethanolamine control resin was included to show that the CNBr Sepharose 4-B resin itself did not interact with the trout pRb or that if it did then to a much lesser degree than the LXCXE resin. The pRb-mAb-1 resin was included to compare the affinity of the LXCXE motif and the anti-human pRb antibody for the rainbow trouts 50 kD pRb. A PK-Ab resin was included as a second control to show that the CNBr activation of Sepharose and the ligation of another protein

would not cause nonspecific binding. Figure 9 (from experiment 1) displays all of the fractions for both the LXCXE resin and the ethanolamine control resin. The SDS fractions (lane 6 and 7) show almost equal strengths of the trout pRb 50 kD bands. It is important to note that some protein is not binding (see flow through lanes 2 and 3 for the LXCXE and control resins respectively) and that a little is coming out in the wash (lanes 4 and 5). These data indicate that the ethanolamine control resin bound trout pRb about as well as the LXCXE binding motif resin and that the affinity for both resins is much less than the amount of protein added.

In order to show that the binding (affinity) shown in experiment 1 was not caused by non-specific disulfide bonding BME was added to all solutions used on the LXCXE and control resins. Figure 10 shows the SDS fraction from experiment 2. It can be seen that the LXCXE resin, the ethanolamine control, and the pRb-mAb-1 resins all show about equal strengths of bands (lanes 2, 3, and 4 respectively). The PK-Ab resin (lane 5) shows an even stronger 50 kD band. It appears that all of the resins, no matter what ligand is present, have a measurable affinity for the trout pRb 50 kD. In this experiment, as in the last, the flow through fractions of all three resins shows a strong 50 kD band that reacts with the human Rb-mAb-1(data not shown).

To determine whether the results from the second experiment was reproducible and to assess the washing techniques a third experiment was preformed. Figure 11 shows the SDS fractions from this experiment. The LXCXE resin and the ethanolamine control resin (lanes 4 and 5 respectively) show a much weaker 50 kD band than was seen in either Figures 9 or 10. However, both the pRb-mAb-1 resin and the PK-Ab resin

show a very strong 50 kD band (lanes 2 and 3). Based on these data the pRb-mAb-1 resin and the PK-Ab resin seem to show affinity for the trout 50 kD pRb protein and the LXCXE and the ethanolamine control resins seem to have lost or weakened their affinity. It is important to note that while the 50 kD band is absent or weak in all of the LXCXE and control resin fractions, it is very strong in all of the Rb-Ab-1 and PK-Ab fractions (data not shown). In order to test for non-specific binding between the 2° Ab, rabbit anti-mouse IgG, and the trout 50 kD pRb duplicates, of each sample were run and treated with only 2°Ab and no 1° Ab, pRb-mAb-1(lanes 6-9). This indicates that these bands are not due to non-specific binding but are real pRb-mAb-1 reactive proteins.

Affinity of pRb for Sepharose

The results of the earlier experiments raised the question: what in these resins was showing affinity for the trout pRb 50 kD? Since all resins, irrespective of what ligand was attached, showed similar affinities, it appeared the resin itself not the attached ligand was binding the trout protein. In order to test this a resin of Sepharose 4-B, the type used for gel filtration chromatography, was prepared by washing with HEPES saline several times. The same liver homogenate was applied to the Sepharose resin, along with the PK-Ab resin as a control. Figure 12 shows the different fractions from the resin bindings. Both the PK-Ab and the Sepharose resins show strong pRb 50 kD bands in the SDS fractions (lanes 4 and 5 respectively). Note that some came off in the flow through (unbound) and the washes (lanes 8 and 9 and lanes 6 and 7 respective for PK-Ab and Sepharose). However, the SDS seems to remove most of the protein. (see lanes

2 for Sepharose and 3 for PK-Ab which are further SDS wash fractions).

Since Sepharose 4-B seemed to show affinity for the pRb 50 kD protein from trout it was important to determine whether the human pRb 110 kD would show a similar characteristic. (If the human pRb 110 showed similar results this would give more support for the 50 kD trout protein being pRb.) A human leukemia myloma tumor cell line, K 562, was used as a source of wild type human pRb 110. The lysate from these cells was allowed to bind to a Sepharose 4-B resin. As can be seen in Figure 13 the human pRb 110 kD gives a similar profile as the trout pRb 50 kD. A fairly strong band can be seen in the SDS fraction at ≈ 110 kD (lane 6). It is important to note that the before binding fraction (lane 2) and the flow through or unbound fraction (lane 3) seem to show strong bands at ≈110 kD as well. This indicates that while some of the protein binds the Sepharose resin not all of it does. Some of the protein is passing through the resin unhindered. Another point of interest is that little of the protein which binds is being eluted off in the general washes (lanes 4 and 5). Most of the protein comes off specifically in the SDS fraction which consists mainly of the 110 kD band. The other bands of lower molecular weight get lighter from the beginning to the SDS fraction (lanes 2 and 6).

Discussion

The results of the earlier resin binding experiments (experiments 1, 2 and 3) are somewhat confusing. In the first experiment (Figure 9) the LXCXE resin and the control resin both showed about equal binding. The second experiment showed similar results.

Interpretation of these results indicates the control resin binds to p50 kD Rb at least as well as the LXCXE resin. A puzzling observation in the second experiment makes this conclusion less sound. In lane 5 of figure 10 the Pk-Ab (a polyclonal antibody for yeast pyruvate kinase) seems to have higher affinity for p50 kD Rb than did the others. This trend is seen throughout the other experiments. This observation may be explained in a few ways. One possibility is that the antibody is leaking off the resin (after all it is about 11 years old) in large amounts. This large concentration of protein leaking off could be overloading the gel resulting in an increase in nonspecific binding with the pRb-mAb-1. A second possibility is that nonspecific binding between the 2^0 Ab and the proteins being eluted off the column (whether they are Pk-Ab or proteins form the homogenate) would give similar results. This second scenario, however, can be precluded due to the blot of experiment three (lanes 6-9) where the 1^0 Ab was not used and essentially no bands were visible (Figure 11).

A third possibility is that the Pk-Ab resin is purifying either pRb or a protein with sequence similarity to both yeast PK and human pRb. Rb-related proteins have been reported in the literature (reviewed Riley et al. 1994); however none have been reported with PK sequence similarities as well. A BLAST search was done to find proteins with pRb similarity. The main hits were pRb, from human and other organisms, and Rb-related proteins. None of the proteins showed any similarity to yeast PK.

Other observations were made which are not very easily interpreted. As mentioned in the results section, strong p50 kD Rb bands can be seen in the SDS fractions of the LXCXE resin, the control, and the Rb-mAb-1 resins for experiments 1

and 2 (Figures 9 and 10); however, only very weak corresponding bands may be seen in Figure 11 SDS fractions (for the LXCXE and control resins). This result was not pursued but indicates a possible loss of binding or a decrease in the capacity of binding in these resins. These resins will need to be tested again to determine whether the apparent loss of binding ability is observed again.

The most interesting results, and the ones most relevant to the purpose of this paper, deal with the binding of trout p50kD Rb and human p110-114 Rb to Sepharose 4-B. Based on the results from the first three resin binding experiments trout Rb has no greater affinity for the LXCXE peptide than for the ethanolamine blocked resin. The results of experiment 4 (Figure 12) indicate also that the trout pRb has about the same affinity for nonactivated Sepharose as it does for the LXCXE Sepharose or pRb-mAb-1 Sepharose. This characteristic was the same for human p110-114 Rb as shown in Figure 13.

These experiments only tell that an interaction is occurring. Further characterization of the interaction needs to be done. The apparent affinity of trout and human pRb for Sepharose could be ionic or hydrophobic in nature. In our experiments SDS was used to elute the protein, which can perturb both ionic and hydrophobic bonds. In support of an ionic bonding nature the most likely result of improper attachment of the LXCXE affinity ligand or ethanolamine to CNBr-Sepharose would have been an exposed ammonium ion ($-NH_3^+$). This would essentially make the resin and anion exchange resin. However, since the CNBr Sepharose columns showed similar affinity as the nonactivated Sepharose this was probably not the case.

Another possibility is that the free hydroxyl (OH) group of Sepharose acted as weak cation exchanger (very weak). However, according to Crustreacasas and Anfinsen (1971) nonspecific adsorption of proteins should be minimal in buffers of ionic strength 0.05M or greater. For all experiments the ionic strength of the buffers was kept at 0.15 M during binding. One final aspect of ionic-interactions was not explored. The pH was kept constant throughout binding and washing. Elution was done in a solution of 2% SDS (unbuffered). If the change in pH (going from buffered to unbuffered solution) changed the ionic state of the resin or the protein then pRb could be eluted. In order to test for a pH effect an elution profile using high or low pH buffers should be tried (such as glycine pH 2-3).

If some kind of hydrophobic interaction is occurring this would agree with the fact that it is not affected by salts in the binding buffer. The pRb only elutes when the salt is removed and SDS is used. This type of interaction could be further characterized by attempting to elute pRb using non-ionic detergents such as triton-X.

As seen in the results section above, a sizable amount of protein appears to not bind Sepharose (see text comments for Figures 9-13). A question arises from these observations: why so much of the pRb (human and trout) does not bind. There are two possible reasons. First it is possible that the columns are being greatly overloaded. In most cases the ratio of homogenate volume to resin was kept constant at 10:1. This ratio is lower or equal to that of other reports using similar purification methods for (Iami 1991; Edwards et al. 1992). In order to test the capacity of the resin further the volume of resin should be increased or the volume of the homogenate loaded should be

decreased.

Second, it is possible that only a fraction of the pRb in the cell is in the proper form to bind. This may be related back to the multiple phosphorylation states of human p110-114 (and maybe of p50kD Rb of trout but we are still unsure whether Rb gets phosphorylated in trout). Some data which supports this is HPV-16 E7 (from which our LXCXE peptide was derived) preferentially binds to underphosphorylated p110 Rb (lami 1991). When further analysis was done on Figure 13 the Sepharose 4-B resin appears to have preferentially purified a hyperphosphorylated form of pRb from K562 cells. This is an opposite affect from what one would expect to see using an LXCXE Sepharose column. To explore this tentative observation trout and human cell lines could be grown under treatment with okadaic acid or TPA and their lysates could be loaded on the Sepharose columns.

While these observations about the interaction of trout and human pRb with Sepharose are interesting and may prove useful, more work needs to be done on characterization. Once these interactions have characterized the use of Sepharose as a purification step of pRb from human and trout cell lines or tissues may be used. The attraction of using Sepharose instead of other purification methods is quite apparent. Other methods are usually expensive (such as commercially produced antibody resins) or are labor intensive to make. These early results indicate that Sepharose purification may be a cheap and easy alternative to what is currently being used in pRb research.

4. Okadaic Acid Treatment of Rainbow Trout Liver Cells

As described in the Statement of Work, we proposed experiment to investigate potential changes in the phosphorylation state of the retinoblastoma protein in response to know tumor promoters such as okadaic acid.

Materials and Methods

The cells used for these studies were the cell lines developed for rainbow trout liver above. SOB 3 cells were split from 3 large single plates into 6, 6 well plates at a density that had previously been determined to give ~ 80% confluence in one day. (1/2 ml of cells into each well with 3 mls of 10% RBT media) Passage numbers were SOB3 p29, 30, and 31. Each well was treated with 10nM Okadaic Acid (OA), 20nM OA, 60 μl ethanol (the solvent for OA), and no treatment.

Five of the 6 well plates were given a designated time of treatment. At the designated times (either 30 min., 1 hour, 6 hrs., 12 hrs., or 24 hrs.) the media was removed and approximately 1ml of cold PBS was added and plates were scraped. After the cells and PBS was removed off the plates were washed with a little additional PBS to detach any remaining cells. Plates were checked under a phase contrast microscope to determine if cells were being removed effectively. At each time point cell morphology was observed under a phase contrast microscope at both 10x and 40x magnifications. (No changes in morphology were observed over the 24 hour time course.) Once cells were removed they were pelleted at ~2000 rpm and frozen at -80 C until lysis and protein assays were conducted.

Lysis and Bradford Assays

Thirty samples were taken over the 24 hour period listed above. Each sample was lysed by the following procedure. 100 μl of modified EBC buffer was added to each pellet. Cell pellets were resuspended by gently flicking (and some mixing by pipette tip). Cells suspensions were incubated at 40 C for 30 min. (mixing of cell suspension was done periodically). Cell lysates were cleared by centrifugation, ~2000 rpm for 15 min at 40C.

Cleared lysates were assayed for total protein concentration by Bradford microassay procedure (Bio-Rad from technical service). 10μl of each lysate was assayed and total concentration was determined by comparison to BSA standard curve (0-10μg BSA). The total protein concentrations for each sample was used to determine amount of lysate needed to load ~8μg of protein on to an SDS-PAGE gel for western blot analysis.

SDS-PAGE and Western Blot Analysis

An attempt was made to load equal amounts of protein into each well of SDS-PAGE gels (9% resolving gels and 4% stacking gels). However, due to low protein concentrations in some samples this was not possible. Total protein concentration for each lane is reported in the legend for each gel's figure. One gel was run for each time point and one well was designated for each treatment, with the end lanes reserved for molecular weight standards.

Western electroblotting was done on PVDF membrane using a Bio-Rad wet

electroblotting system. (Blotting was done for 1.5-2 hours at ~25 ohms, with volts set at 100 and mamps set at 200 but never reaching these values.) After blotting was completed molecular weight standard lanes were cut and stained with coomassie blue stain (R-250). Lanes to be recognized by antibodies were blocked overnight in blocking buffer (4% BSA) at 40C. Primary antibody used was Rb-Ab-1 from oncogene science and the secondary antibody used was alkaline phosphatase conjugated anti-mouse IgG. Visualization was done by alkaline phosphatase color formation using BCIP (bormochloroindolyl phosphate) and NBT (Nitro Blue Tetrazolium).

Results & Discussion

As can be seen from the Figures 14-18 only the 6 hour treatment (Figure 16) membrane and the 24 hour treatment (Figure 17) membrane have $8\mu g$ of protein loaded in to each lane. Based on this these membranes are easiest to compare. Their comparison shows no visible change in the 50 kDa band based on OA concentration or an 18 hour time difference. If specific lanes are compared from gel to gel then an overall idea of how OA treatment affects maybe seen. When lanes 2 from each membrane and lanes 7 from each membrane are compared (each representing 10nM OA and 20nM OA treatments respectively) no general trend is observable. Unfortunately no 0 time point was taken (due to one plate not growing well to begin with) so no comparison between 30 min and 0 time of treatment with OA maybe made. However, I expect the no treatment samples to be similar to what would be seen for the 0 time point.

5. Medaka liver cell lines

Once we had developed necessary reagents with the rainbow trout (which provides large amounts of tissue) we turned our attention to focus on the medaka, a small fish which will be the primary thrust of our future studies.

Materials and Methods

Primaria tissue culture plates, flasks, multi-well plates, and disposable sieves were purchased from Falcon-Becton Dickinson (Franklin Lakes, NJ). Trypsin and gentamicin was obtained from Sigma Chemical Co. (St. Louis, MO). Crude collagenase (Type IV, lot 84H6804) were 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, NY). Sodium dodecyl sulfate, acrylamide, 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).

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 with 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 NaHCO3, 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 disassociate the cell clumps. Large resilient pieces of the liver were separated from single cells by sieving using a Falcon disposable sieve. 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 10ml/L of glutamine (100x), MEM non-essential amino acid solution (100x), and gentamicin]. The

cells were resuspended by manual pipetting (5-10 times) and 0.25 ml aliquots of the suspension was pipetted on to the Primaria tissue culture plates containing 4.75 mls of culture media. The resulting primary cell cultures and 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 with a Coulter particle counter. 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 was 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 was further

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observed twice daily for 7 days.

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

Triplicate experiments were performed to observe 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 for 7 days, and evaluated for attachment, spreading, degree of confluence, cell integrity, and overall appearance.

Ultrastructure of hepatocytes in primary culture

After the conditions for 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 times for 20 min each 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) 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, 150mM NaCl, 0.02% sodium azide,

0.1% sodium dodecyl sulfate, 100ug/ml phenylmethylsulfonyl fluoride, 1 ug/ml aprotinin, 1% nonidet P-40, 0.5% sodium deoxycholate, pH 8.0). by direct addition to the cell pellet with gentle pippetting. Resulting homogenates were centrifuged at 4,000 xg for 10 min and the supernants were subjected to 10% sodium dodecyl sulfate polyacrylamide-gel electrophoresis (Laemmli, 1970). Protein concentrations of cell homogentates 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 a 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, 5mM $MgCl_2$, 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% trypsin or 0.05% collagenase. Once cells were completely disassociated, 5 mls of serum-free media was added to the culture dish, gently mixed, and cells were sub-divided into three dishes. Alternatively, disassociated cells were sometimes washed with perfusion media twice followed by centrifugation at 300 xg and split into three dishes. Three additional ml of media were then added to sub-cultured cells and they were incubated in a 24° C, 5%

CO_2:95% air atmosphere.

Results

Isolation and placement into primary culture

As shown in Figure 19, 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 (Figure 19A). 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 (Figure 19B) and most cells, except those comprising the aggregates, were spreading by four days (Figure 19C).

Cell proliferation

Cell proliferation began within 2-4 d and was relatively slow. Cell growth/proliferation accelerated beginning about Day 4 (Figure 19C) and through days 7 and 10 (Figures 19D & 19E) 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 (Figure 19D). 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 (Figure 19F).

As shown in Figure 20, 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 was 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.

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 7, 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 points over the initial 24 h in primary culture.

Within the initial 20 min in culture it appeared that a 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 the 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.

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-free 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 Figure 21, 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.

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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-free 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.

Ultrastructure of cells in primary culture

As shown in Figure 22A, 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 (Figure 22A) and fat droplets were also seen distributed throughout the cytoplasm (Figure 22, panels A & B). Gap junctions jointed the hepatocytes laterally and an occasional desmosome was seen (Figure 22B). The ultrastructure of hepatocytes at Day 10 of primary culture was consistent with normal healthy hepatocyte ultrastructure.

Visualization of cytokeratins via western blotting

As shown in Figure 23, 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 decrease 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

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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 described 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., 1990). 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, 1977; Smets et al., 1979; and Pleskach et al., 1994). In another experimental system, the use of

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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, 1980). 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 and 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 media 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; Sodho 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 cells 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 levels 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 at 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.

6. Medaka Retinoblastoma Tumor Suppressor Gene Studies

Our final objective was to apply the reagents and techniques we had developed during the initial years of funding to test the potential involvement of the retinoblastoma tumor suppressor gene in the medaka model.

Materials and Methods

Medaka are sexually dimorphic fish that spawn daily. Females produce clusters of eggs within two hours of the initiation of the light cycle. Eggs are fertilized by the males during the next 2 hours. After fertilization, laboratory personnel removed the clumps of fertilized eggs from the females to raise them in petri dishes or allowed the female to scrape fertilized eggs onto sponge filters. Individual eggs were reared from newly fertilized eggs to hatched individuals in a 96-well plate. All exposures were conducted as previously reported (Hawkins et al. 1986).

Western Blotting

Medaka eyes were isolated, lenses removed, washed with isotonic PBS, and the resulting cell pellet homogenized with 10 vols of EBC buffer (50 mM Tris-pH 8.5, 120 mM NaCl, 0.5% non-ident P-40, 100 mM sodium fluoride, 200 μM sodium ortho-vanadate, 10 μg/ml aprotinin, 10 μg/ml phenylmethylsulfonyl fluoride, 2 mM EGTA, and

2mM EDTA). Protein concentrations of the homogenates were determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. One volume of the homogenate containing 20 μg of protein was combined with 2 vols of gel-loading buffer and boiled for 2 min and clarified by centrifugation at 16,000 x g for 2 min. Fifteen μl of each sample was then run on 7.5% SDS-PAGE (Mini-Protean II, Bio-Rad) initially at 48V for 0.5 hr and then increased to 200 V for 0.65 hr. Resulting gels were electroblotted to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked with blocking buffer (25 mM Tris-pH 8.0, 125 mM NaCl, 0.1% Tween-20, and 4% dry milk) for at least 1 hr at room temperature. Antibodies against the *Rb* gene product were obtained from Pharmingen (product # Mh-Rb-02P1) and Triton Diagnostics (product # 100601) and were diluted from manufactures stock to a final concentration of 2.5 μg/ml blocking buffer prior to use. Blots were incubated with gentle shaking for 16 hr at room temperature. The membrane was washed 3 times for 5 min with TTBS (100 mM Tris-pH 7.5, 0.9% NaCl, 0.1% Tween-20) and the blot was then probed with the Vectastain ABC kit.

Rb Gene Expression in Medaka Normal and Neoplastic Tissues

RNA was isolated by modifications of the method of Chomczynski and Sacchi (1987). Our modifications included 3 washes in 70% ethanol at Step 4 followed by breaking up of the resulting pellets with a fine pipet. An additional wash was added to Step 5. Typical yield was 0.6 - 1 μg RNA/mg tissue. The quality of the RNA was examined by spectrophotometry and determined to be undegraded by the clear definition

of the 28s and 18s rRNA bands and the presence of very little low molecular weight RNA. For isolation of tumor RNA, tRNA was added as a carrier (approx. 10 μg tRNA/sample).

Synthesis of cDNA was performed with the SuperScript Preamplification System (Gibco BRL); according to the manufacturer protocol at 43°C with MMLV (Moloney murine leukemia) virus reverse transcriptase. Random primers were used to prime cDNA synthesis. Appropriate negative controls included reaction mixtures without reverse transcriptase and reactions mixtures in which the RNA had been treated with RNAase (to indicate if genomic DNA was present).

The RT-PCR primers used were based on the human *Rb* cDNA sequence and they have been previously published (Murakami et al. 1991; Shew et al. 1990). We initially generated 7 fragments which corresponded to the first 15 exons of the human *Rb* gene. Amplification reactions were prepared in 15 μl volumes containing 10mM Tris·HCl (pH 8.3); 50mM KCl; gelatin (0.001% wt/vol); 1mM $MgCl_2$; 1.7 μM of each primer; 3.7 μl cDNA and 0.75U of AmpliTaq polymerase (Perkin-Elmer Cetus). Amplification was performed using a Perkin-Elmer Cetus GeneAmp PCR System 9600 as follows. Two cycles of 2 min at 94° C, 30 seconds at 55° C, and 2 min at 72° C. This was followed by 40 cycles of 1 min at 94° C; 30 seconds at 55° C, and 2 min at 72° C. A 30 second time ramp was inserted between 55° C and 72° C, and 72° C and 94° C, and a 1-min ramp was inserted between 94° C and 55° C. The samples were then subjected to a 10-min final extension at 72° C at the end of 40 cycles.

Following amplification 5-10 μl of each reaction mixture was analyzed on 1%

agarose gels containing 0.5 μg of ethidium bromide/ml TBE buffer (89 mM Tris·HCl; 89

mM boric acid; 2 mM EDTA; pH 8.0). Intact fragments were isolated and subjected to

hybridization with p0.9R (containing human *Rb* cDNA exons 1-9) and p3.8R (containing

human *Rb* cDNA exons 9-27).

Seven fragments were obtained as described above. Four of these were cloned

into pCR™ II vector using TA cloning kit from Invitrogen. The TA cloning system takes

advantage of the fact that AmpliTaq polymerase adds single deoxyadenosines to the 3'-

end of all duplex amplified fragments. The A-overhangs are used to insert the PCR

product into a specifically designed vector having T-overhangs. Verification that the

plasmids contained the correct right sized fragments was done by PCR and PCR

fragments were sequenced with the Sequenase kit (United State Biochemical).

Plasmids (1 μg) containing the appropriate fragment of human cDNA were

digested with *Eco*RI and loaded onto 1% agarose gels, electrophoresed in TBE buffer,

and transferred overnight to Zeta-Probe membranes with 0.4 M NaOH. Hybridization to

human cDNA (p0.9R and p3.8R) was performed at 42° C for 12 hr in 6X SCC (1x SCC

= 0.15 M NaCl/0.015 M sodium citrate); 10 mM EDTA; 5X Denhardt's solution; 0.5%

SDS; 100 μg/ml denatured salmon sperm DNA; 10% dextran sulphate; 50% formamide

under high stringency. The membrane was washed twice with 0.2X SCC/0.1% SDS at

42° C for 15 minutes, twice with 0.1 X SCC/0.1% SDS at 65° for 15 minutes, and

exposed to Kodak XAR-5 film for 1-2 days at -80° C. The fragments used as probes

were labeled with PCR using 50 μCi ^{32}P-dCTP in a normal PCR reaction. The

unincorporated nucleotides were separated by repeated ethanol precipitation with 1/10

volume of 3M sodium acetate and 2 volumes of cold 95% ethanol.

Results

Western Blotting

Both antibodies cross-reacted with at least one protein band from medaka eye and liver tissues with an approximate molecular weight of 105 kD. These bands migrated in the same region as a similar band from the positive control of mouse tissue (data not shown).

Rb Gene Expression in Medaka Normal and Neoplastic Tissues

Utilizing RT-PCR we generated 7 fragments using primers from the human *Rb* cDNA. These fragments (MRb-1, MRb-2,...MRb-7) were screened for their ability to hybridize to the human *Rb* cDNA. Thus far, all fragments examined hybridized to human *Rb* cDNA (Figure 24). MRb-2, MRb-3, and MRb-4 were sequenced and found to have approximately 45% to 55% sequence homology to the human *RB* cDNA at the nucleic acid level (Figure 24). Finally, the approximate size of each of the fragments was determined and is reported in Figure 24.

The results of our preliminary probing of normal and neoplastic liver and eye tissues from medaka are presented in Figure 25. Using RT-PCR it was possible to generate fragments MRb-1, MRb-3, and MRb-6 in both normal and neoplastic tissues. Repeated attempts to produce the MRb-2 fragment in using cDNA generated from neoplastic tissues from 3 eye tumors and 4 hepatocellular carcinomas were not

successful. This suggests there may be significant deletions at the DNA or mRNA level in these tissues.

Discussion

To validate medaka as a model for studying retinoblastoma and the *Rb* gene, it was necessary to initially determine whether the *Rb* protein was expressed in normal medaka eye tissue. As a first step, medaka eye and mouse liver tissues were analyzed by western blotting. Two commercial antibodies were used which recognized two different domains of the human *Rb* protein. Both cross-reacted with medaka eye tissues as determined by positive staining of a protein(s) from medaka that co-migrated with the *Rb* protein from an appropriate mouse control tissue.

While these experiments were in progress, Bernards et al. (1989) reported on their analyses of a variety of vertebrate tissues including humans, mice, swordfish, and shark for the *Rb* gene by Southern blotting. Two probes from the human *Rb* gene (p0.9R and p3.8R) were used to demonstrate the presence of the *Rb* gene in lower vertebrates. It was subsequently shown by our group that the Rb protein was present in a variety of primitive and advanced fishes including the ceolacanth, rainbow trout, zebra danio, and English sole) (Ostrander et al. unpublished). Thus, it appears that the Rb protein is expressed in most all vertebrate species.

Positive staining with monoclonal antibodies that appear to cross react with proteins from fish tissues, while encouraging, does not provide conclusive evidence as to the existence of the *Rb* gene/protein in fish tissues. Further studies were initiated.

First, the cloning and sequencing of the gene was initiated and remains in progress. Second, the reverse transcriptase–polymerase chain reaction (RT-PCR) was used to generate fragments using primers from the human *Rb* cDNA (Figure 24). In some cases, the medaka probes are considerably smaller than the corresponding human probe; in others, the medaka probes are considerably larger. Overall, since the medaka cDNA is 8.5 kb, it is not surprising that there is variability.

The human *Rb* cDNA is 4.7 kb long and contains 27 exons of varying length and size. Thirteen medaka probes have been prepared by RT-PCR that extend the entire length of the corresponding human cDNA (data not shown). In these preliminary studies it was noted that three of the four probes tested (MRB-1, MRb-3, MRb-6) were also produced in both normal and neoplastic tissue from the medaka. MRb-2, however, was generated only in normal tissues suggesting some modification had occurred in DNA or mRNA of the neoplastic tissues. MRb-2 corresponds to human *Rb* exons 5-10 and has been sequenced (Figure 26). The uniformity of these results was somewhat surprising. While "hot-spots" for mutation (including deletions) in the human *Rb* mRNA have been suggested (Goodrich and Lee, 1993) no reports indicating a uniform mutation in a particular model system has been reported. Our data suggest that MAMA caused compound-specific alterations in the mRNA. If this observation proves consistent under further investigation, elucidation of the interaction between methylazoxymethanol and DNA or RNA that produces these mutations, and ultimately, the tumor may be possible.

CONCLUSIONS

General conclusions for each of the six areas of study that encompassed what was proposed in our Statement of Work are provided below. Taken in total, we have made significant advances in verification of the continued use of fish models to address fundamental questions of chemical carcinogenesis in response to environmental contamination.

1. Partial Hepatectomy Studies

We have examined the response of the rainbow trout liver to PH. Although similarities exist in regeneration response relative to rodent liver, both qualitative and quantitative aspects of the response are reduced.

The cellular events and cell specific roles in trout liver regeneration are under investigation. Morphometric studies have shown a large contribution of bile preductular and ductular epithelial cells to the liver parenchyma (Hampton et al., 1989). In contrast, these cells are found in small amounts and only at the lobular periphery in rodent liver (Grisham, 1980). Moreover, Sell (1990) cites studies indicating a stem cell function for certain biliary epithelial (oval) cells in mammalian liver. Differences in trout and rodents in the response to PH may reflect relative amounts and roles of specific cell types and their proliferation and subsequent differentiation. The success of the partial hepatectomy technique described herein, suitable for both small and large trout, is encouraging in that these possibilities can now be experimentally addressed.

2. Rainbow Trout Liver Cell Lines

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 30+ 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 an, as yet, incompletely characterized population 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, considering the nearly homogenous starting cultures of hepatocytes, is that the non-hepatocyte 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 necessary. In addition, the ability to establish long-term propagable cultures suggest 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.

3. Protein Purification Studies

The retinoblastoma protein is a nuclear DNA binding protein that exists in small

quantities in all living eukaryotic cells examined to date. We have successfully developed a technique using an antibody bound to a Sepharose resin to purify adequate quantities of the protein for further analysis. The purified protein has proven useful in developing polyclonal antibodies specific to the retinoblastoma protein from the rainbow trout. Moreover, adequate quantities have now been obtained so that it should now be possible to complete sequence analysis.

4. Okadaic Acid Studies

It is not apparent from the western analysis that the trout 50kDa Rb-Ab-1 reactive protein is sensitive to either 10nM OA or 20 nM OA treatments. The expression of the trout 50 kDa Rb-Ab-1 reactive protein also seems to be unaffected by OA treatment over a time course of 30 min - 24 hours. I expect that this may be extrapolated to 0 time. To be more definite a larger number of cells should be used to ensure higher overall protein concentrations. This should be done so that equal protein concentrations may be loaded and an easier comparison of samples be made.

5. Medaka Cell lines

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 disaggregation 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 is 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.

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 at 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 subculture these cells are underway.

6. Medaka Tumor Cell Studies

Retinal lesions resembling retinoblastoma are chemically inducible in medaka, a small fish, and induction appears to be compound specific. If this observation is supported by further investigation, it may be possible to understand the mutagenic interaction of methylazoxymethanol and DNA or RNA. The *Rb* gene is expressed in all

normal medaka tissue examined, including the eye and the RT-PCR technique appears effective in generating medaka DNA probes that cross-react with the human gene and can be used to screen malignant and normal medaka tissue.

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APPENDIX 1

FIGURE   LEGENDS AND FIGURES

Figure 1. Fish Surgery Apparatus.

The anesthetized fish is suspended in a surgical netting, ventral surface upward, on the surgery table. Water, containing anesthetic, leaves the pump (P) via Tygon tubing and is pumped into the buccal cavity (2.8 $\pm$ 0.2 l/min), traverses the gills, and exits via the opercular opening. The water then passes through the perforated surgery table and is deflected into either compartment A or B by the deflector board (C) before returning to the pump. Routinely, one compartment contains anesthetic and the other fresh water.
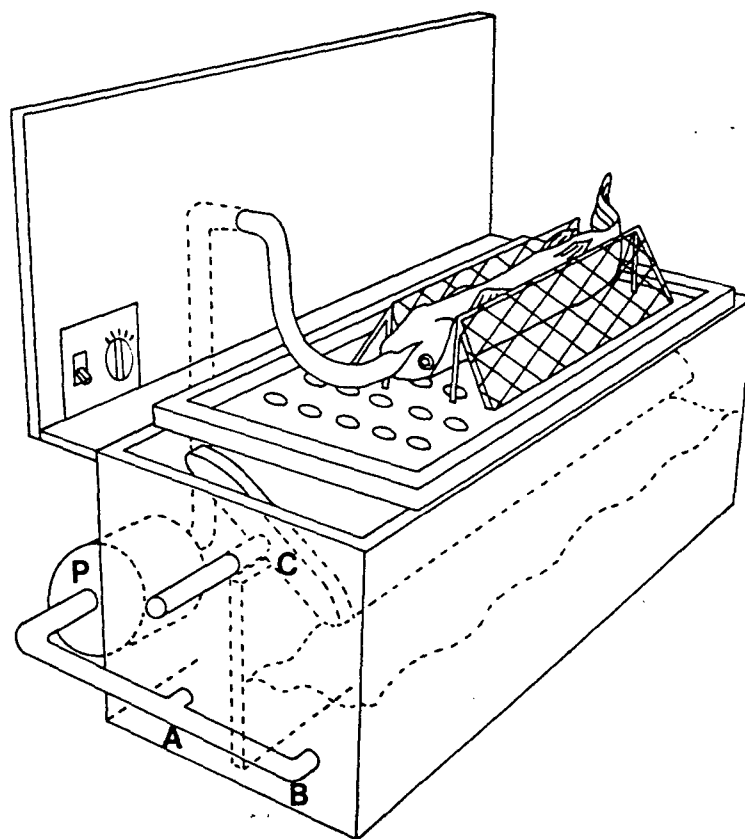
Figure 2. Mitotic response of the liver of rainbow trout following partial hepatectomy.

The Kruskal-Wallis test indicated significant differences existed across all days (P = 0.0109). Subsequently, Mann-Whitney U Tests were used to compare groups at the 0.05 α level. Days 2-12 were significantly different than Days 0 and 14. Values shown are mean # of mitotic figures/11,200 hepatocytes. Liver samples were prepared from fish at indicated time periods following PH and the mitotic index was histologically determined as described in the Materials and Methods.
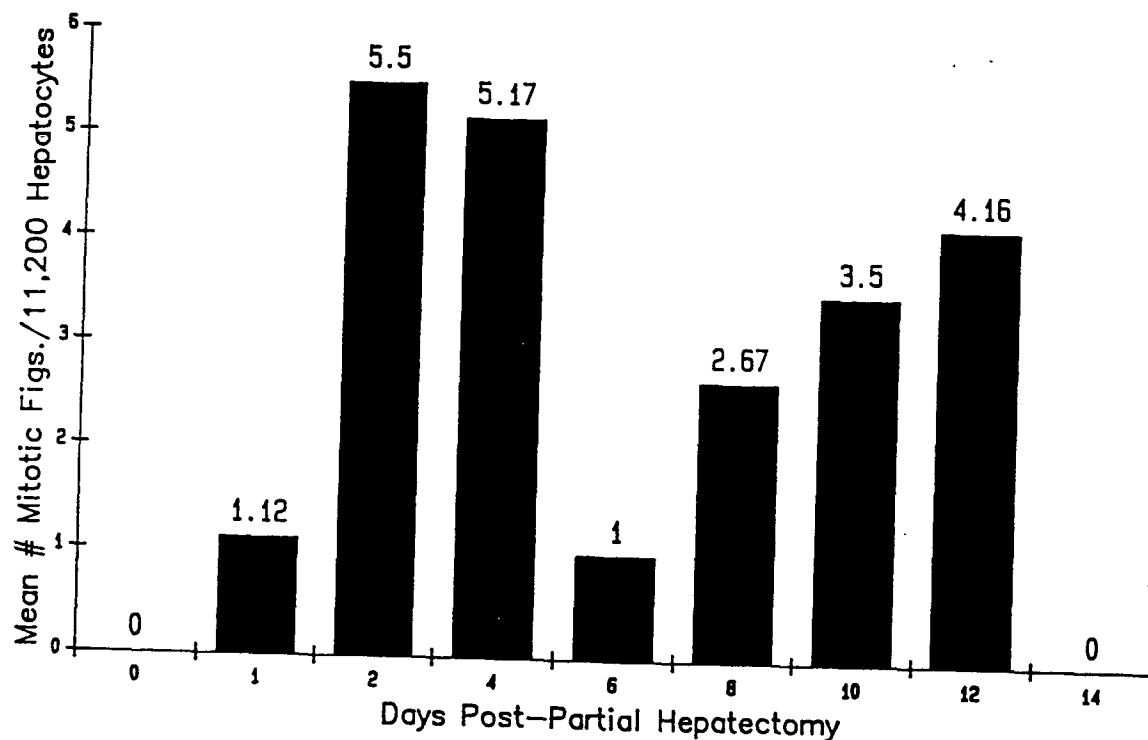
Figure 3. Primary culture of rainbow trout hepatocytes.

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 100x and h & I are 400x.
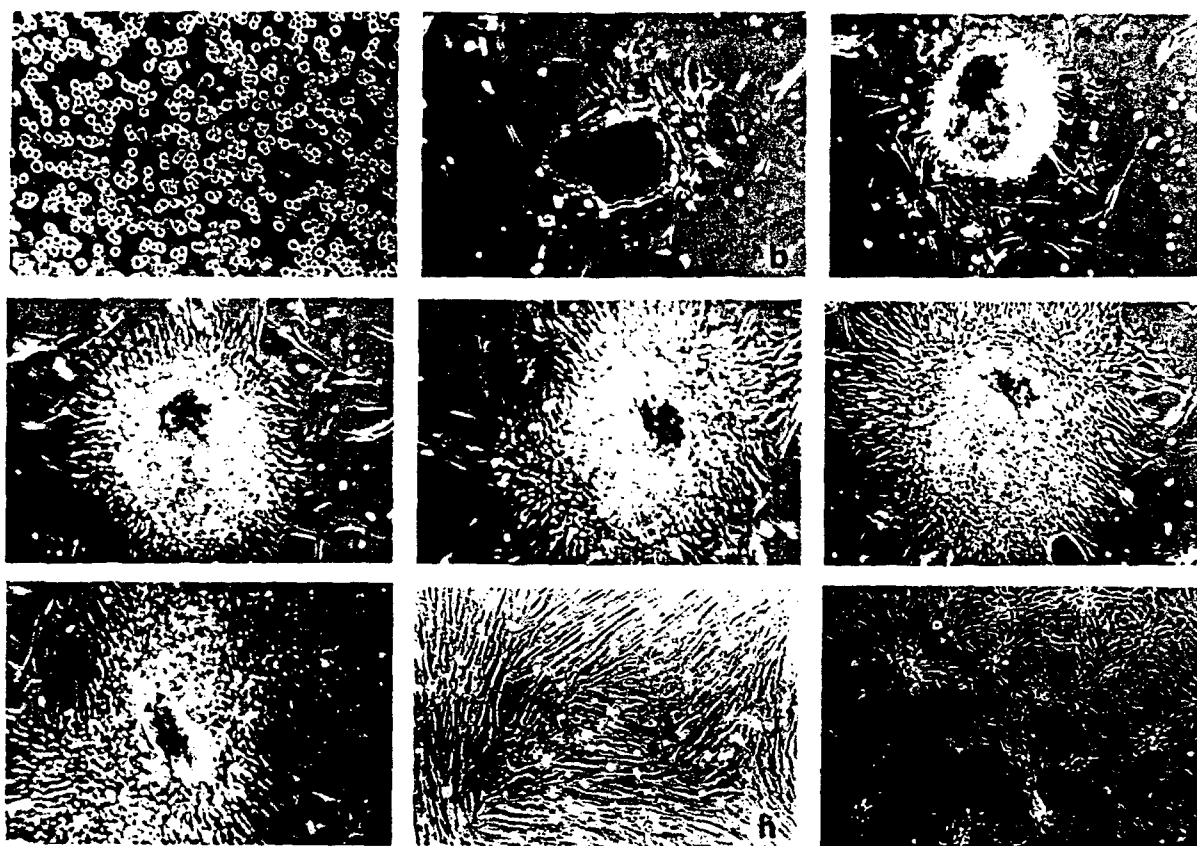
Figure 4. Western blot analysis of cytokeratin (Panel A) and vimentin (Panel B) in

expression in rainbow trout hepatocytes at following various time intervals in

primary culture.

Panel 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 33b (these cells had been passaged after 32 days in

primary culture; *lane 10*, tumor cells from a primary culture of an aflatoxin induced

hepatocellular carcinoma.

Panel 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, however, these cells had been passaged

after 32 days in primary culture; *lane 10*, tumor cells from a primary culture of an

aflatoxin induced hepatocellular carcinoma.
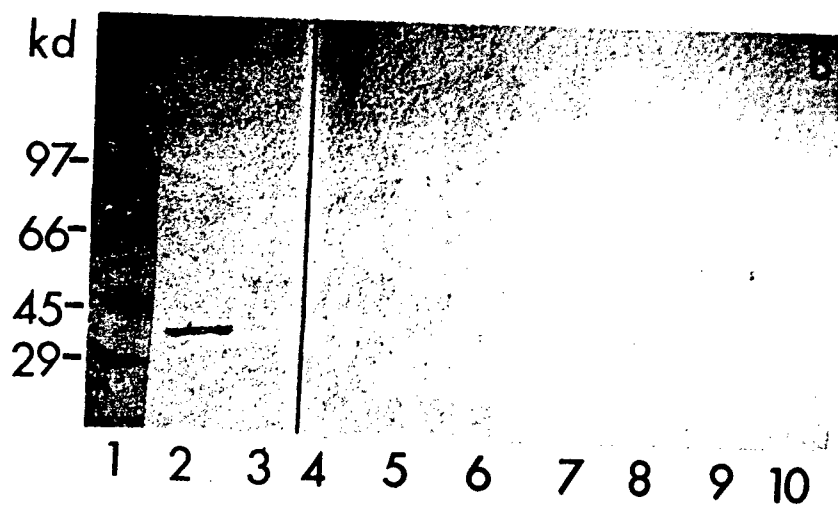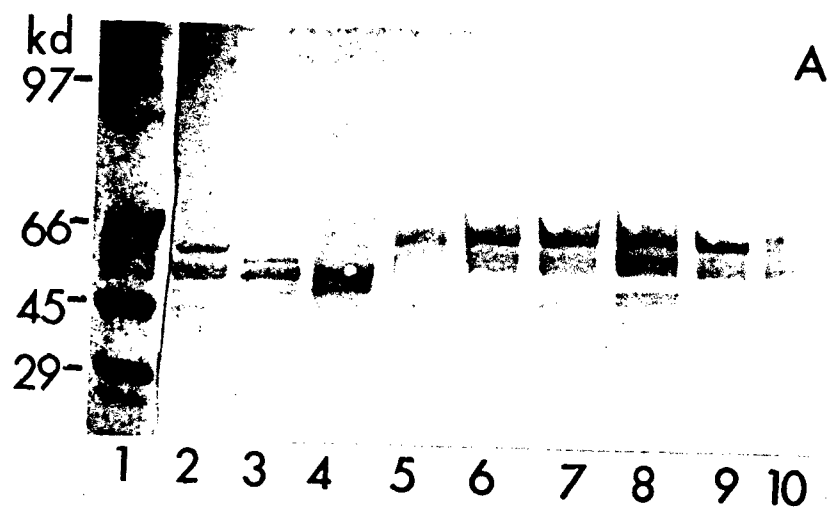
Figure 5. DNA synthesis as measured by thymidine incorporation into rainbow trout hepatocytes in primary culture.

Rainbow trout hepatocytes were maintained in culture for 33 days 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.
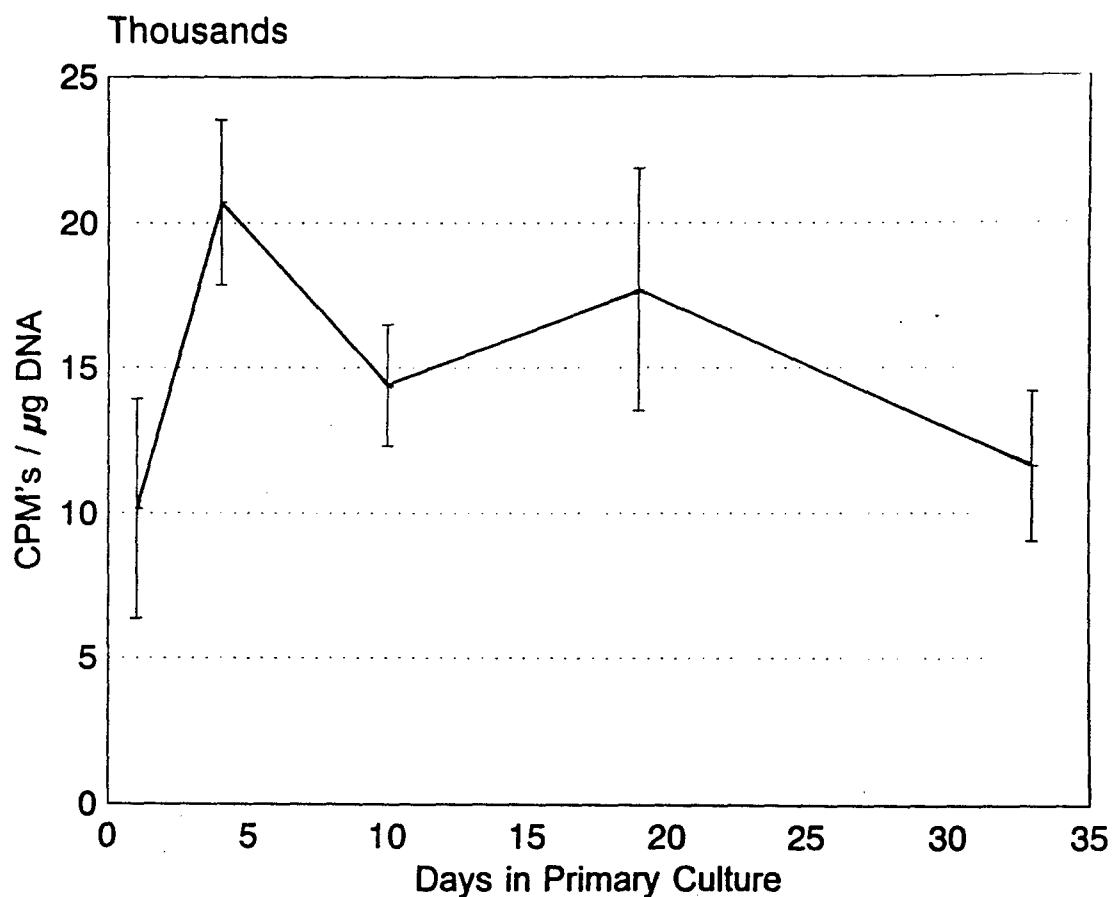
Figure 6. Autoradiographic analysis of rainbow trout hepatocytes and a hepatocellular

carcinoma in primary culture.

As detailed in the Materials and Methods, prior to the designated time-point, each plate

of cells was incubated with ^{3}H thymidine for 16 hr, coated with emulsion, incubated at

-90° C for 21 days, 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 days 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.
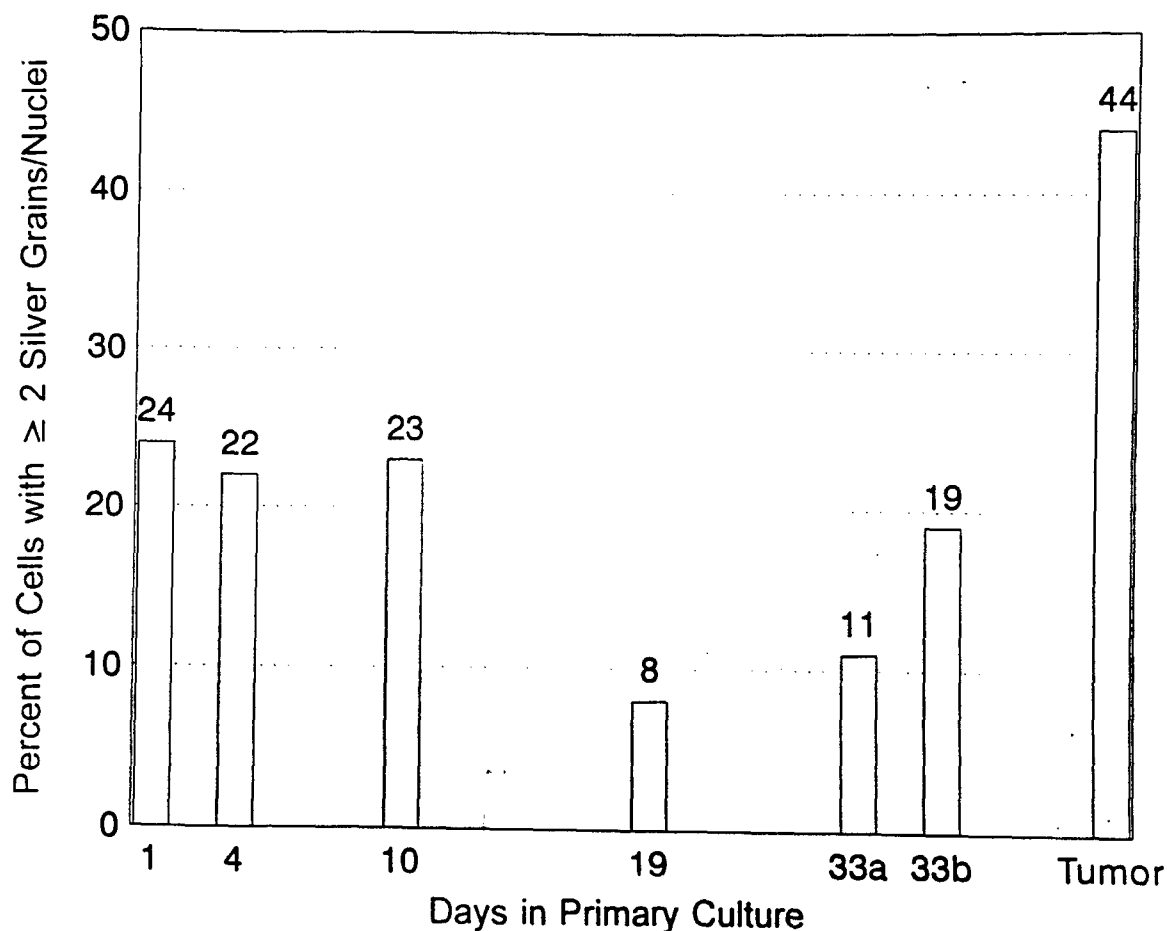
Figure 7. Results of flow cytometric analysis of F-actin and G-actin expression in rainbow trout hepatocytes.

Histograms are of fluorescein (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.

DAY 0 TROUT CELLS G-ACTIN

A

DAY 40 TROUT CELLS G-ACTIN

B

DAY 50 TROUT CELLS G-ACTIN

C

DAY 0 TROUT CELLS F-ACTIN

D

DAY 40 TROUT CELLS F-ACTIN

E

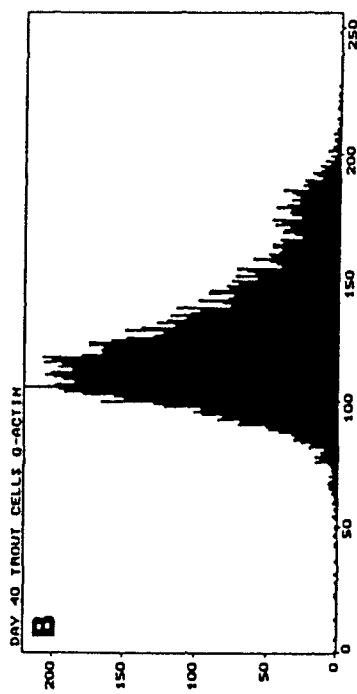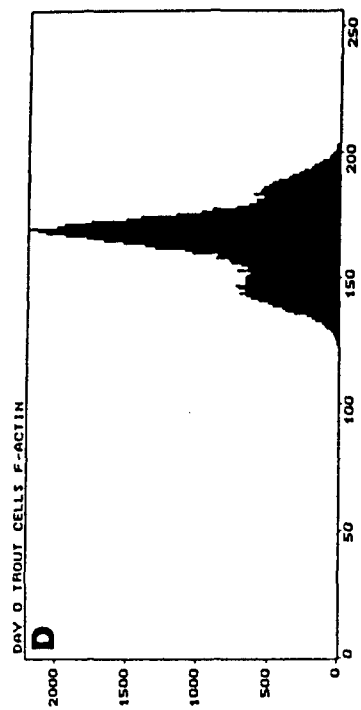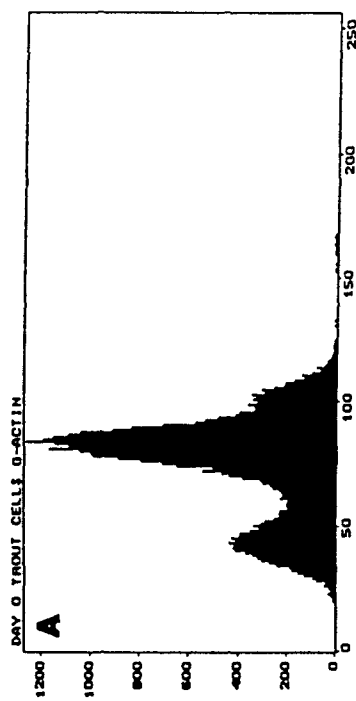DAY 50 TROUT CELLS F-ACTIN

F
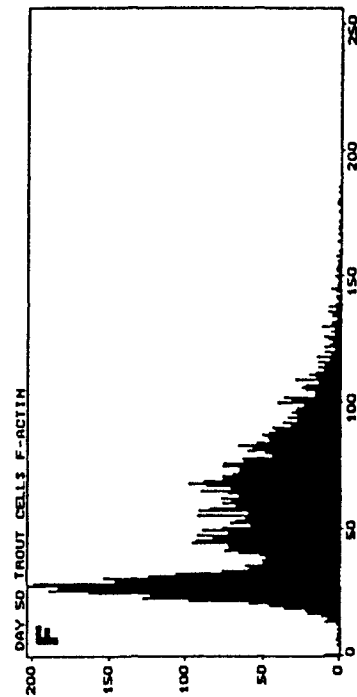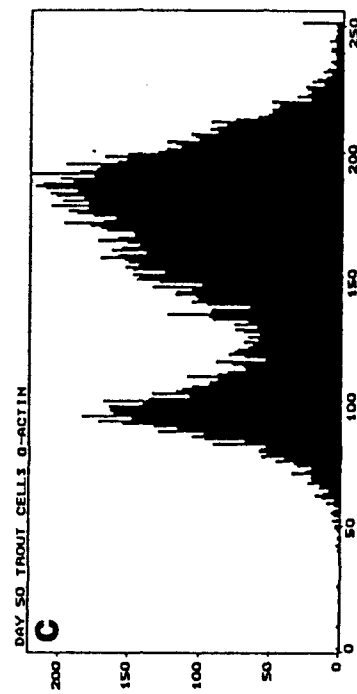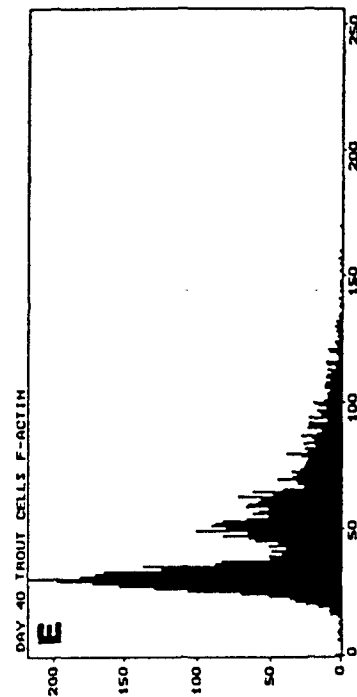
Figure 8. G-actin and F-actin expression in cells isolated from a

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

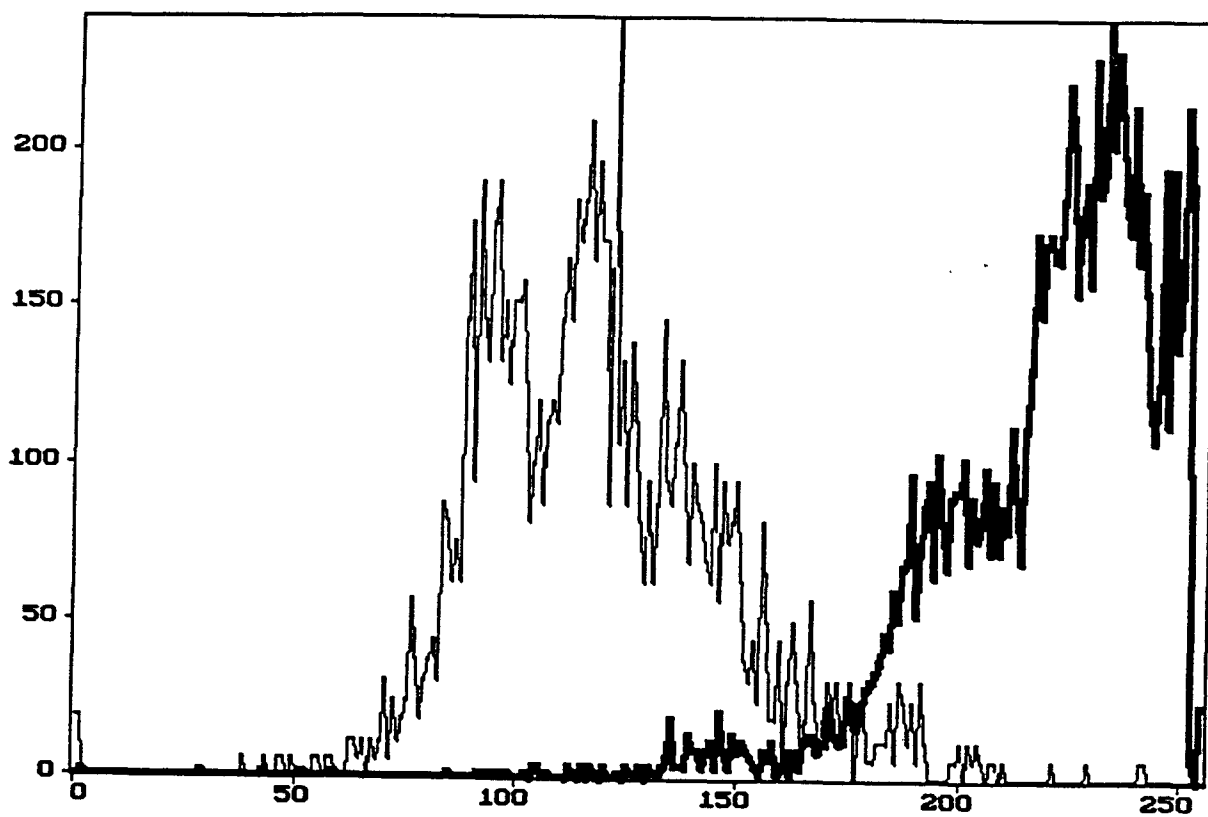the numbers of assayed cells.

119

Figure 9. Western blot using RB-m-AB-1 for an epitope on the human Rb between amino acids 300-380.

This blot resulted from the first resin binding experiment using a CNBr activated Sepharose 4-B with either an LXCXE motif or a control resin with just ethanolamine. The lanes are as follows: *Lane 1*, molecular weight standards; *lane 2*, flow-through fraction from LXCXE motif resin; *lane 3*, flow-through fraction from the control resin; *lane 4*, HEPES/saline wash from the LXCXE resin; *lane 5*, HEPES/saline was from the control resin; *lane 6*, SDS elution fraction from the LXCXE resin; *lane 7*, SDS elution fraction from the control resin; *lane 8*, SDS wash from the LXCXE resin; *lane 9*, SDS wash form the control resin; *lane 10*, a pRB sample previously purified using a commercial antibody resin.
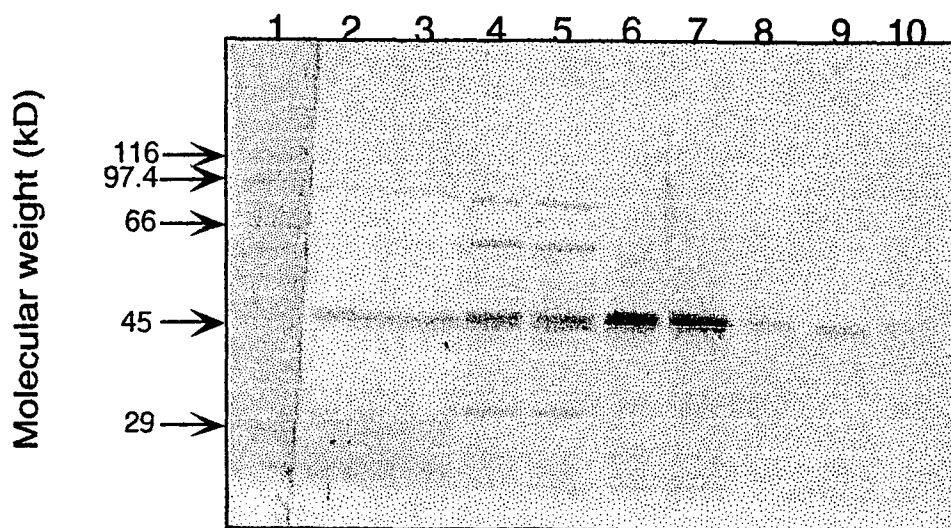
Figure 10. Western blot analysis of SDS fraction for the second resin binding experiment.

Lane 1 is a mixture of high molecular weight standards with the molecular positions marked (molecular weight standards were comassie stained and lanes 2-5 were treated with the same antibody as in Figure 9). CNBr activated Sepharose 4-B resin conjugated to: lane 2, the LXCXE motif; lane 3, to ethanolamine (a blocking group); lane 4, to Rb-mAb-a (a commercial resin); or lane 5, a null antibody (against yeast pyruvate kinase).
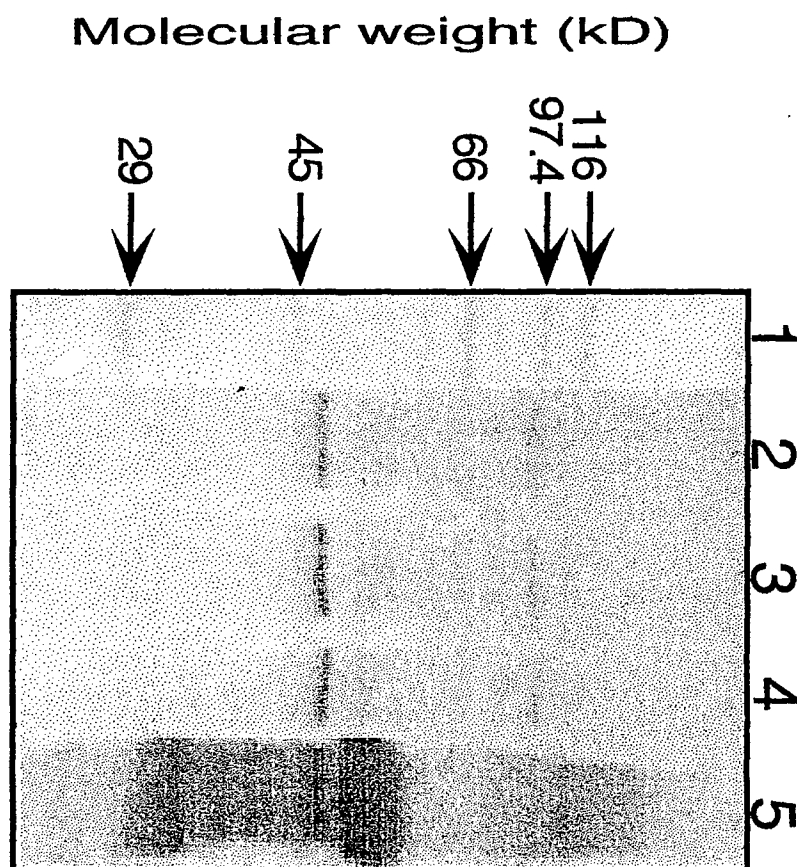

Molecular weight (kD)

Figure 11. Western blot analysis of the SDS fractions for the third resin binding experiment.

Lane 1 is a mixture of high molecular weight standards with the molecular positions marked (molecular weight standards were comassie stained and lanes 2-5 were treated with the same antibody as in Figure 9). Duplicates of each sample were run (lanes 2-5 and lanes 6-9) and immunodetected using either RB-mAb-1 (lanes 2-5) or PK-Ab (lane 6-9). CNBr activated Sepharose 4-B resin conjugated to: *lanes 5 & 9,* the LXCXE motif; *lanes 4 & 8,* to ethanolamine (a blocking group); *lanes 3 & 7,* to Rb-mAb-a (a commercial resin); or *lanes 2 & 6,* a null antibody (against yeast pyruvate kinase).
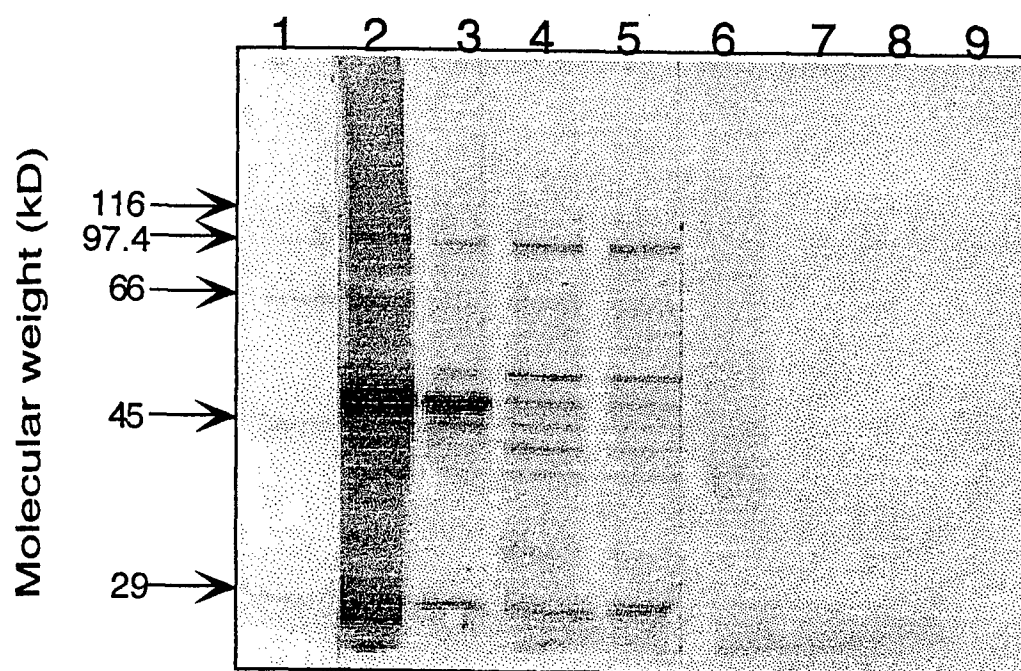
Figure 12. Western blot analysis of all fractions of the fourth resin binding experiment suing a CNBr activated Sepharose resin with PK-Ab conjugated and a Sepharose 4-b 200.

The lanes are as follows: *Lane 1*, high molecular weight standards; *lane 2*, SDS wash of the Sepharose 4-b resin after elution; *lane 3*, SDS wash of the PK-Ab conjugated Sepharose after elution; *lane 4*, SDS fraction from PK-Ab column prior to elution with SDS; *lane 5*, SDS fraction from the Sepharose 4-B column; *lane 6*, washes of the Pk-Ab column prior to elution with SDS; *lane 7*, washes of the Sepharose 4-B column prior to elution with SDS; *lane 8*, flow-through faction (unbound) from the PK-Ab column; *lane 9*, flow-through fraction from the Sepharose 4B column.

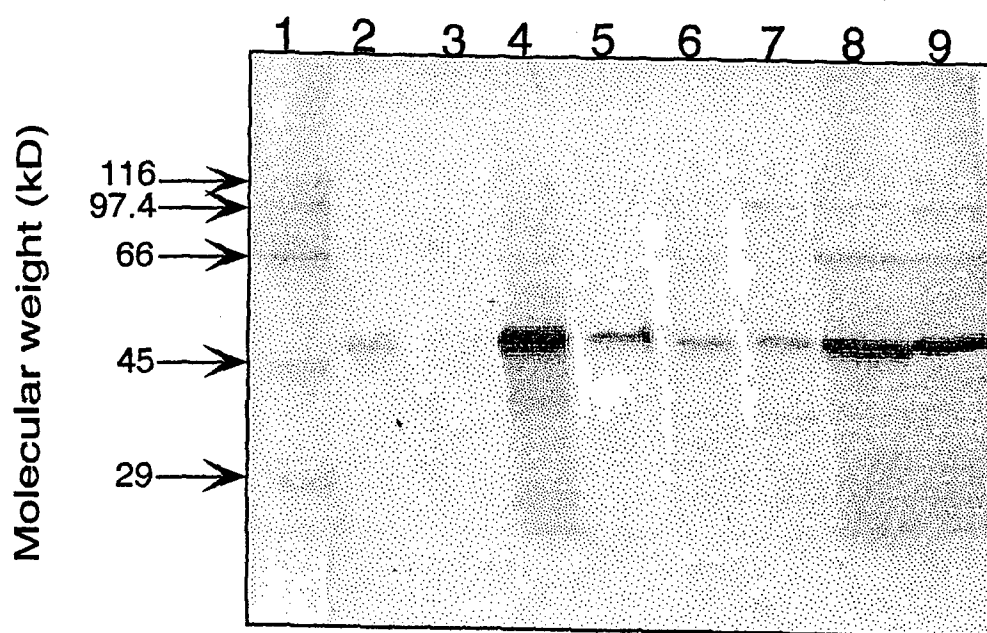Figure 13. Western blot analysis of all fraction for the fifth resin binding experiment using a Sepharose 4-B 200 resin.

Lanes 1 & 9, high molecular weight standards; lane 2, lysate from K562 cells which was applies to the column; lane 3, flow-through fraction (unbound); lanes 4 & 5, washes before elution with SDS; lane 6, SDS elution fraction from Sepharose; lanes 7 & 8, SDS washes after elution.
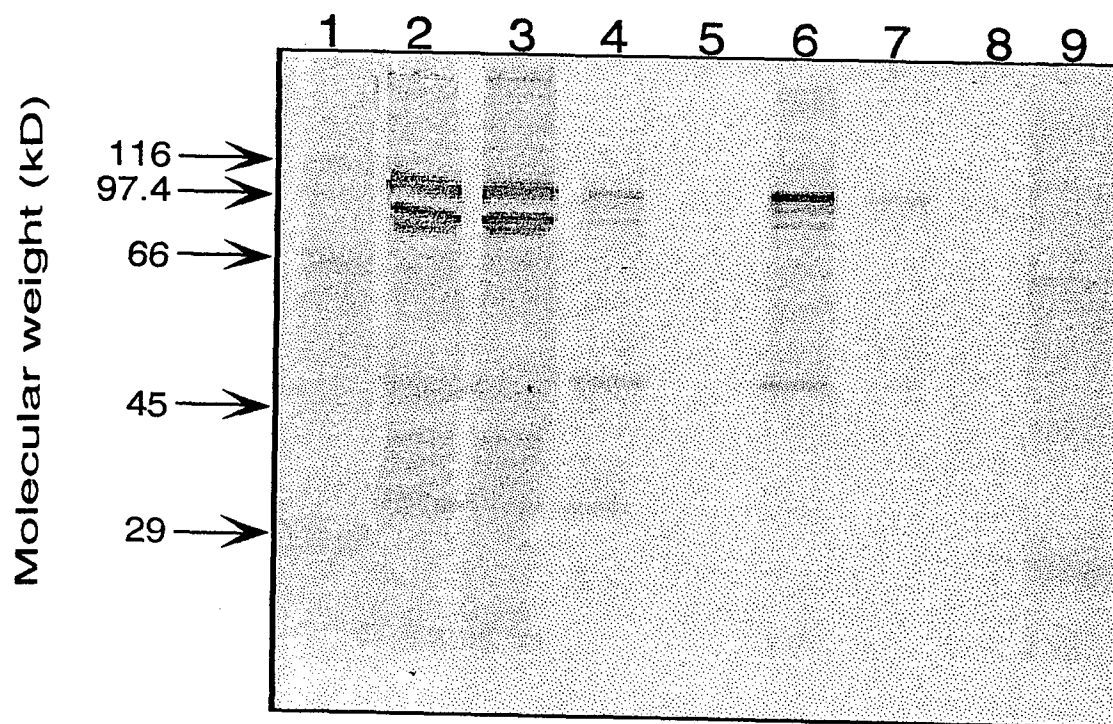
Figure 14. Western blot analysis of lysates after 30 min of treatment with okadaic acid.

Lanes 1 and 8, high molecular weight standards; lane 2, 10nm okadaic acid treatment (6 μg total protein); lane 3, 10nm okadaic acid treatment (0.35 μg total protein); lane 4, control with 60 μl ethanol (8 μg total protein); lane 5, control with nothing added (8 μg total protein); lane 6, 20nm okadaic acid treatment (8 μg total protein); lane 7, 20nm okadaic acid treatment (8 μg total protein).

30 min. of treatment with OA.

1   2   3 , 4   5   6   7 8

Molecular wieght kDa

205 →
116 →
97.4 →
66 →
45 →
29 →

125

Figure 15. Western blot analysis of lysates after 1 h of treatment with okadaic acid.

Lanes 1 and 8, high molecular weight standards; lane 2, 10nm okadaic acid treatment (7 μg total protein); lane 3, 10nm okadaic acid treatment (3.2 μg total protein); lane 4, control with 60 μl ethanol (8 μg total protein); lane 5, control with nothing added (8 μg total protein); lane 6, 20nm okadaic acid treatment (5 μg total protein); lane 7, 20nm okadaic acid treatment (8 μg total protein).

1 hour of OA treatment.

Figure 16. Western blot analysis of lysates after 6 hr of treatment with okadaic acid.

Lanes 1 and 8, high molecular weight standards; lane 2, 10nm okadaic acid treatment (8 μg total protein); lane 3, 10nm okadaic acid treatment (8 μg total protein); lane 4, control with 60 μl ethanol (8 μg total protein); lane 5, control with nothing added (8 μg total protein); lane 6, 20nm okadaic acid treatment (8 μg total protein); lane 7, 20nm okadaic acid treatment (8 μg total protein).
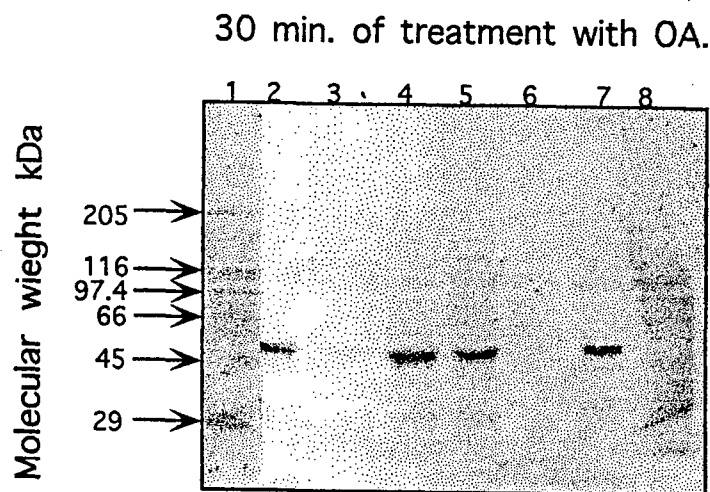

6 hours of OA treatment
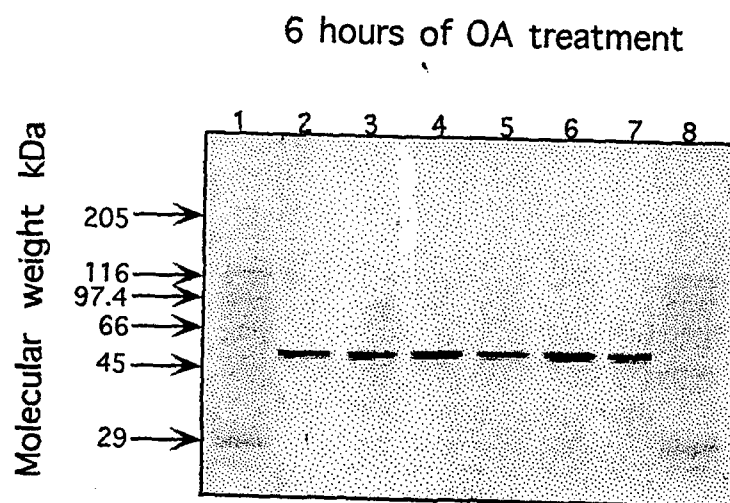
Figure 17. Western blot analysis of lysates after 12 hr of treatment with okadaic acid.

Lanes 1 and 8, high molecular weight standards; lane 2, 10nm okadaic acid treatment (8 μg total protein); lane 3, 10nm okadaic acid treatment (8 μg total protein); lane 4, control with 60 μl ethanol (8 μg total protein); lane 5, control with nothing added (8 μg total protein); lane 6, 20nm okadaic acid treatment (4.7 μg total protein); lane 7, 20nm okadaic acid treatment (3.6 μg total protein).
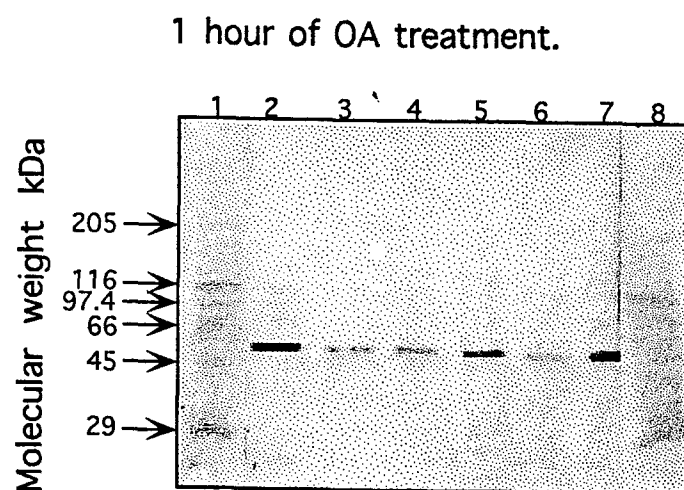

12 hours treatment with OA

128

Figure 18. Western blot analysis of lysates after 24 hr of treatment with okadaic acid.

Lanes 1 and 8, high molecular weight standards; lane 2, 10nm okadaic acid treatment (8 μg total protein); lane 3, 10nm okadaic acid treatment (8 μg total protein); lane 4, control with 60 μl ethanol (8 μg total protein); lane 5, control with nothing added (8 μg total protein); lane 6, 20nm okadaic acid treatment (8 μg total protein); lane 7, 20nm okadaic acid treatment (8 μg total protein).

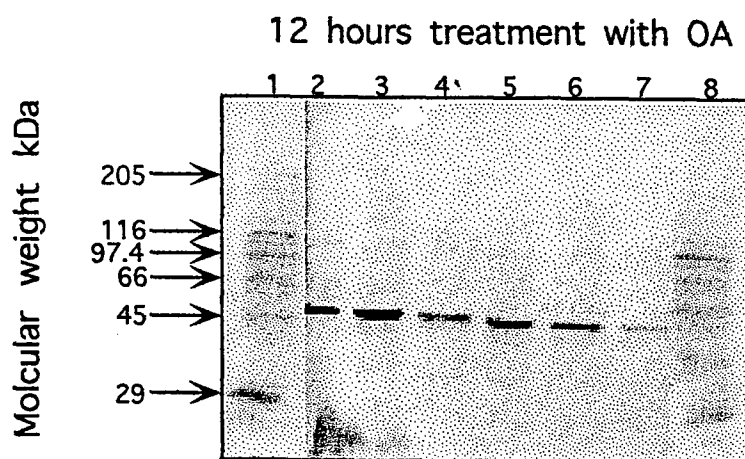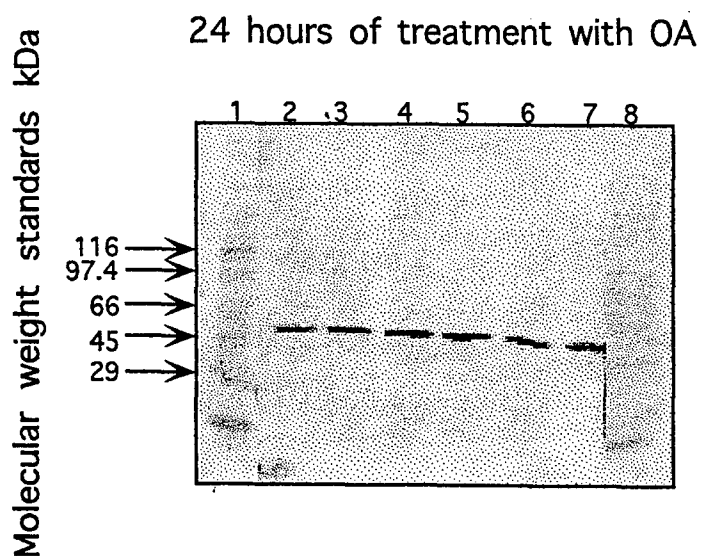Figure 19. Primary culture of medaka hepatocytes.

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.
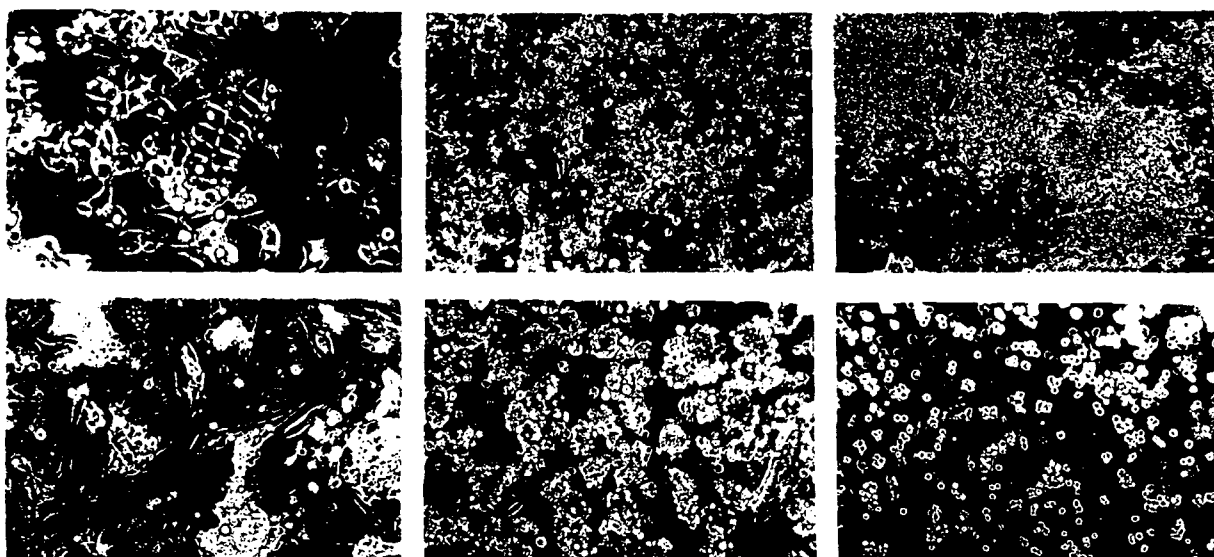
Figure 20. Proliferation curve of primary culture of medaka hepatocytes in a serum-

free MEM media.

As detailed in "Materials and Methods", cells were disassociated with 0.05%

collagenase and seeded at 2.5 x 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 bar represent one standard deviation about

the mean as determined from triplicate experiments. "*" denote days that mean cell

number was statistically significantly higher than mean cell number on Day 0 ($P <$

0.05).
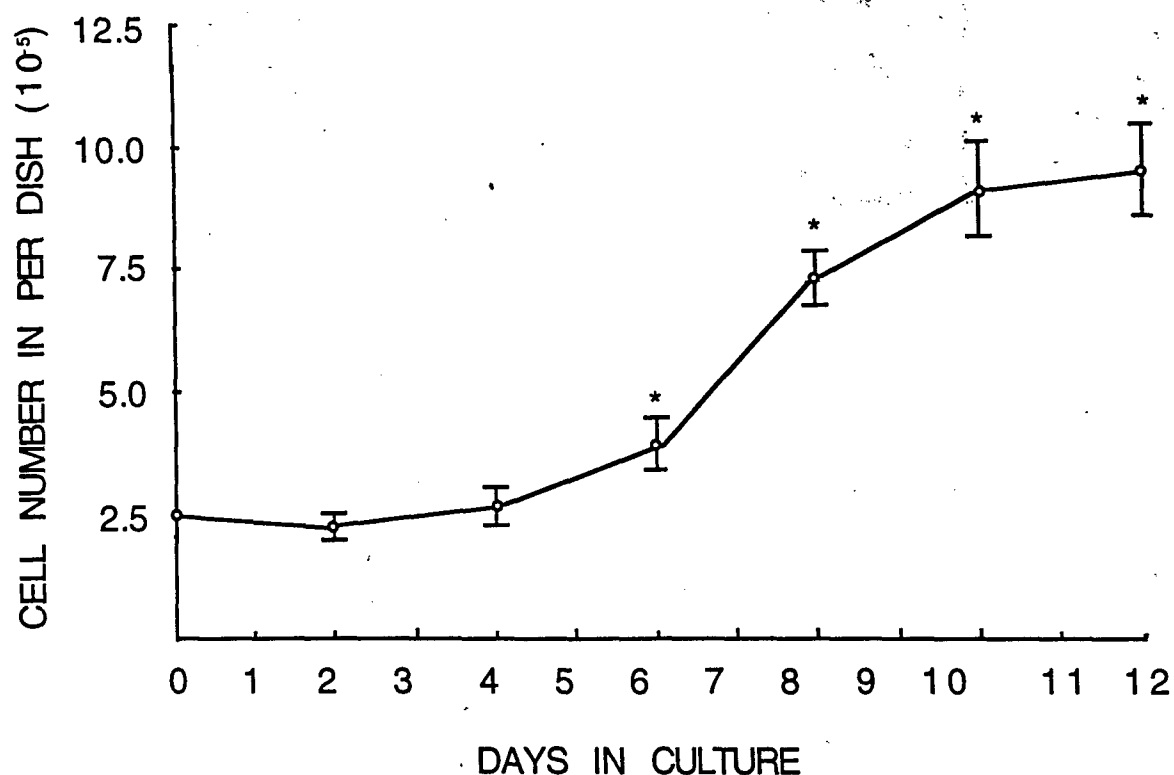
Figure 21.   Serum inhibition of medaka hepatocyte attachment in primary culture.

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.
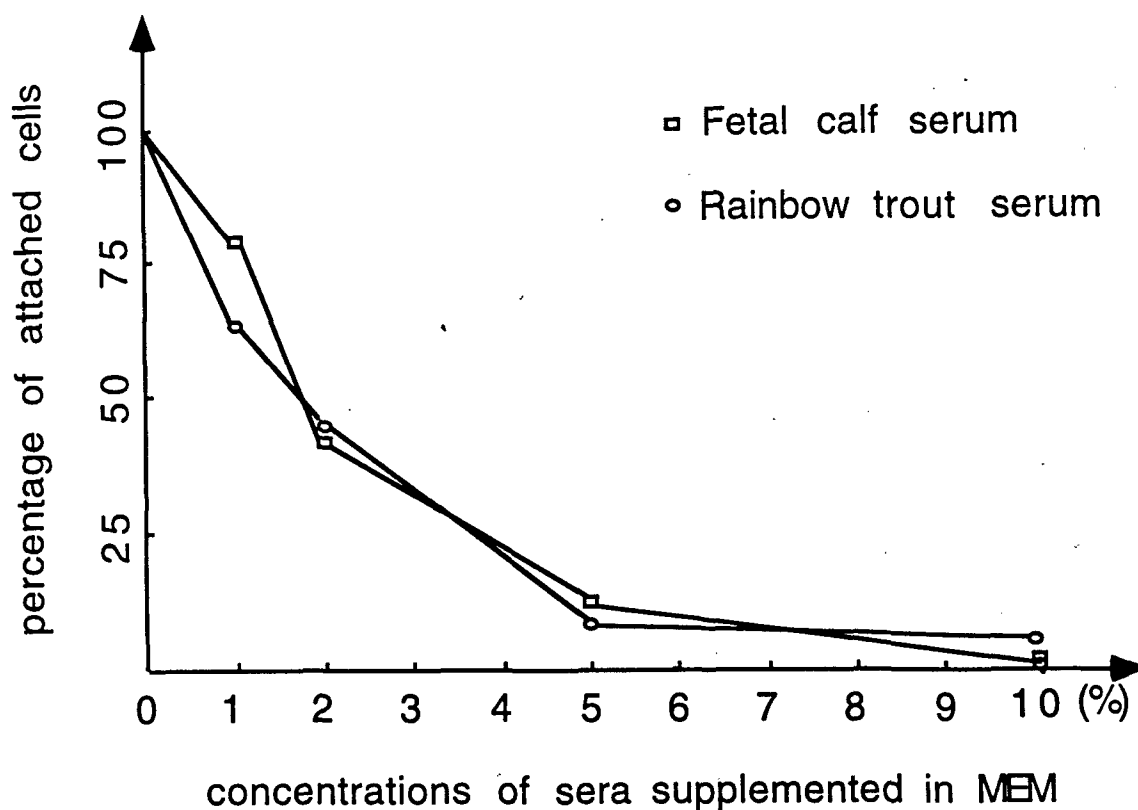
Figure 22. Transmission electron microscopy of medaka liver cells after 10 days in primary culture.

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 demosomes. In addition, lipid storage vacuoles and peroxisomes are evident.
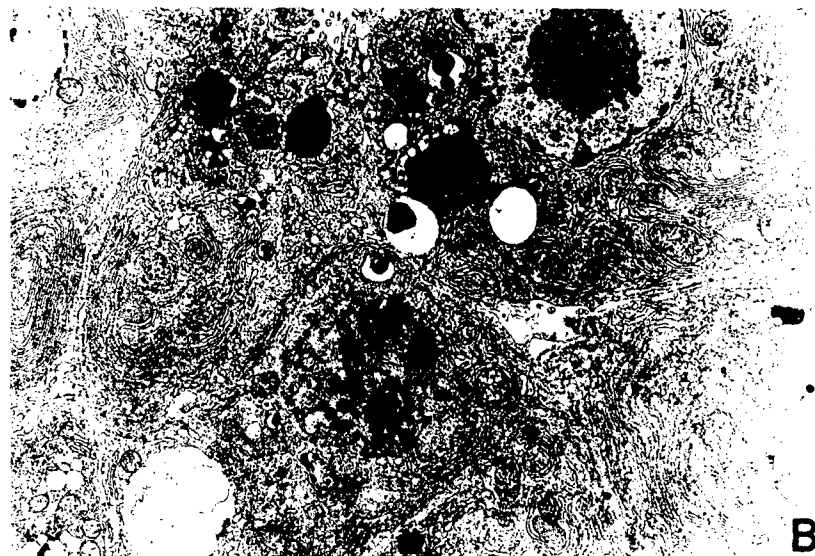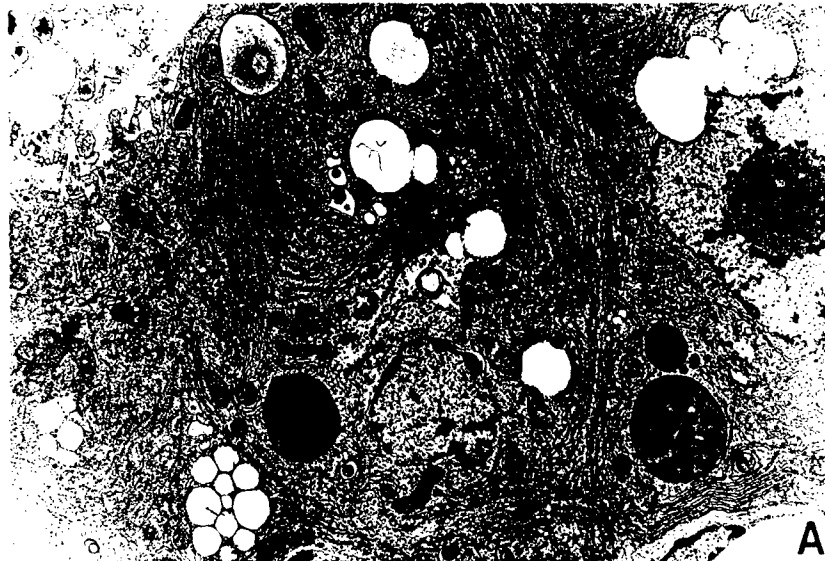
Figure 23. Western blot analysis of cytokeratin expression in primary liver cell cultures of medaka at various time intervals in primary culture.

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.
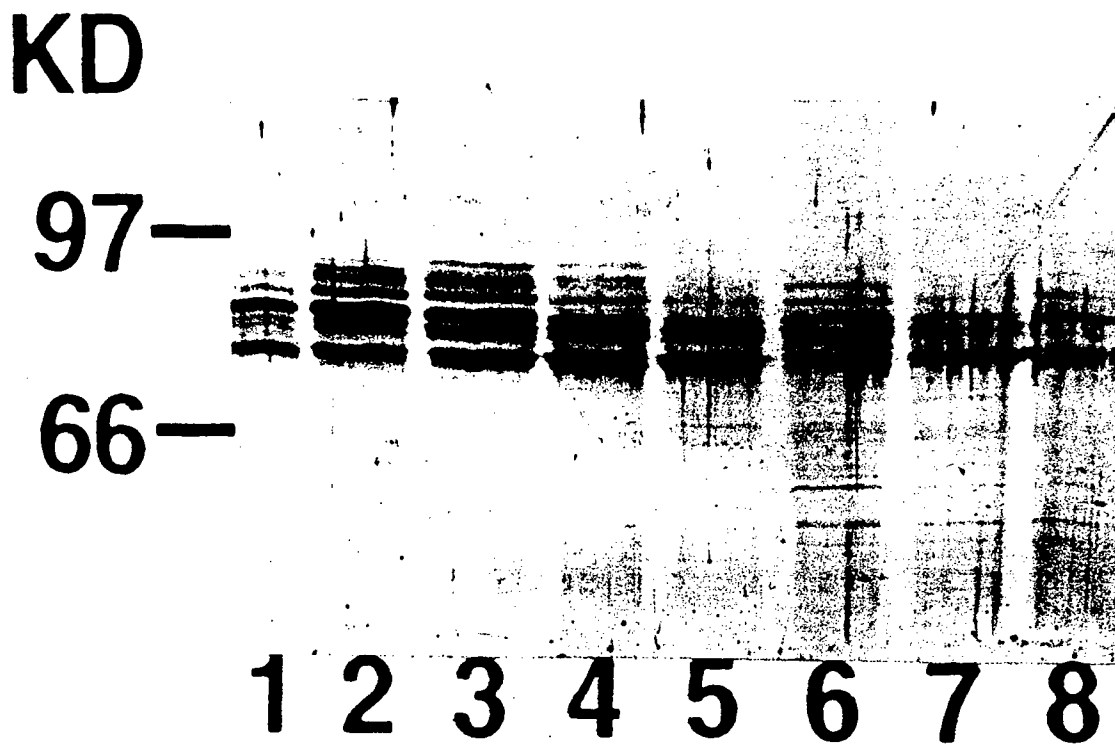
PCR Probes to Rb Generated in Medaka

Human Rb cDNA = 4.7kb;   Medaka Rb cDNA = ~8.5kb

Fragment	Size in Human (bp)	Size in Medaka (bp)	Hybridized to Human Probes*	Cloned/Human Sequence Homology
MRb-1	230	~390	p0.9R	yes**
MRb-2	514	493	p3.8R & p0.9R	yes/54%
MRb-3	124	330	p3.8R	yes/45%
MRb-4	600	535	p3.8R	yes/53%
MRb-5	242	~618	**	**
MRb-6	1276	~640 & ~860	p3.8R (both)	**
MRb-7	989	~690	**	**

* p0.9R (exons 1-9) p3.8R (exons 9-27);   ** Studies in progress

Figure 24. PCR probes to the retinoblastoma (Rb) fragment generated from Medaka.

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Screening of Normal and Malignant Medaka Tissues

PCR Probe	Normal Eye	Normal Liver	Eye Tumors (3)	Hepatocarcinomas(4)
MRb-1	+	+	+	+
MRb-2	+	+	-	-
MRb-3	+	+	+	+
MRb-4	+	+	**	**
MRb-5	+	+	**	**
MRb-6	+	+	+	+

** Studies in progress

Figure 25. Screening of normal and neoplastic eye and liver tissues from medaka by RT-PCR.

SEQUENCING OF MRb-2

```
              10        20        30        40        50        60
MRb-2   CAGCAGTTCG ATATCTACTG TAAGAAATTG TTTATTTTAT GGCCCATCAT ATATGCAGGC
        .......... .......... *..*.....* .**. ..**.  .. *. .* .**.*.**.
HUMAN   CAGCAGTTCG ATATCTACTG AAATAAATTC TGCA-TTGGT -GC-TA--AA AGTTTCTTG-
        670        680        690       700        710        720


              70        80        90       100       110       120
MRb-2   GGCCATAATA TCACCAGAAG ACAAGAGGCA TACAGCCAAT AAAAGGTCTC CTGTTTATAA
        .**..*.*.* .*.**.. ** . ...*..*. ..***.*... **...*.*** ...*.*..**
HUMAN   GATCACATTT TTATTAG-CT A-AAGGGGAA TATTACAAAT GGAAGATGAT CTGGTGATTT
        730        740       750        760        770        780


             130       140       150       160       170       180
MRb-2   TAGATCTGCA CAGCAAGGAA AACAGTTTGT AGTCTCATCA TCCAAAAATA TTAAGCAGCC
        *.**..*.*. **..*.*.** **..*...*. .*.... ... ..****..* ..***.***
HUMAN   CATTTCAGTA ATGCTATGTA TCCACTTTTT ATTCTC-TCA -CCTCCCATG TTGCTCAAAG
                  800        810                   830        840


             190       200       210       220       230       240
MRb-2   AGAGCATCAT AATCATATCA CATAATGAAA CAATTCTTTT CTTGAAAAAT CCATGCTGTA
        . .*...*** ..*..**.** *.. .***.* *...**..** .*.**..*.* ...*... .
HUMAN   A-ACCATATA AAACAGCTAT TAT-ACCCAT TAATGGTTCA CCTCGAACAC CCAGGC-G-A
        850        860        870        880        890        900


             250       260       270       280       290       300
MRb-2   AGGTTTATGT GAAATGTTTT AATATTAAGT AATATACATA AGTGAGAGTA TTGATATAGT
        *.****.*** .*.*..**** *...**..** *.**..*. . .*....*. *. *..**..*.
HUMAN   GGTCAGAACA GGAGTGCACG GATAGCAAAA CAACTAGA-A AATGATA-CA -AGAATTATT
        910        920        930        940        950        960


             310       320       330       340       350       360
MRb-2   GATGAGTAGG TCATAGATTG CAGGCTGTGA ATTGTGAGAA AAGATGGAAG CATGGAACAG
        .. ...*** ***..*.*** ..*. **..* .*.*. ...* *..*. ...* *...***..*
HUMAN   GA--AGTTCT CTGTAAAGAA CATG-AATGT AATAT-AGAT GAGGT-GAAA AATGTTTCAA
                  970        980        990       1000


             370       380       390       400       410       420
MRb-2   TAATTTTGTT ATTAAACTTT TTTTAGTACA TGTGCACTT TGGTGGCGAC ACCTGTTACA
        *......*.. *...****.. *.*.*.*..* ...*... .* .**....*.* .**.***.*.
HUMAN   AAATTTTATT TTTATGAATT CTCTTGGACT TGTAACA-TC TAATGGCCAG AGGTTGAAAA
        1020                 1040       1050       1060                1080


             430       440       450       460       470       480
MRb-2   TTTTTCGACG AACATGAGCT GTCAGGATCA CAGGAAGGTA AAAATTACAG AACCTCTTCA
        .*....*.   ...**..**.  *.  ..*..  *.***.**.*  ....*..*..  .*.****..
HUMAN   TCTTTCTA-- AAC-GA--T -AC--GAAGA AATTTATCTT AAAAATAAAG ATCTAGATC-
        1090                 1100       1110       1120       1130


             490
MRb-2   GACTGATTCT ATA
        .. *....*. *.
HUMAN   GA-AGATTAT TT-
```

Figure 26. Sequence analysis of the MRb-2 probe from medaka.

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APPENDIX 2

TABLES

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[1]					
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

[1]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.

	Imunohistochemical staining with anti-cytokeratin antibody MAK6[1]				
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

[1]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

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Table 3. Results of anti-vimentin antibody staining of intact rainbow trout liver and liver cell cultures.

	Immunohistochemical staining with anti-vimentin antibody[1]				
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

[1]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

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Table 4. PCNA Staining of Primary Hepatocyte Cultures.

Days in Primary Culture	PCNA Index HEP	SPN	Description
Day 0	10	N/C	Predominately 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

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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

Table 7. 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%

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