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Biochemical and histochemical properties of hepatic tumors of rainbow trout, *Oncorhynchus mykiss*

Lynn M.Parker, Darrel J.Laurén[1], Bruce D.Hammock[2], Bruce Winder[2] and David E.Hinton

Department of Medicine, School of Veterinary Medicine, University of California, Davis, CA 95616, [1]EA Engineering, Science and Technology, 41 Lafayette Circle, Lafayette, CA 94549 and [2]Department of Entomology and Environmental Toxicology, University of California, Davis, CA 95616, USA

Biochemical and histochemical studies were conducted in aflatoxin B_1-induced liver tumors in adult rainbow trout. Specific activities of the phase I enzymes, ethoxyresorufin-*O*-deethylase (EROD), microsomal and cytosolic epoxide hydrolase (mEH and cEH), aldehyde dehydrogenase (ALDH) and DT-diaphorase, and the phase II enzymes, γ-glutamyltransferase (γ-GT), glutathione transferase (GST) and uridine diphosphoglucuronyl transferase (UDPGT) were measured. Cryostat sections of tumor and surrounding liver from the same cohorts were analyzed immunohistochemically for cytochrome P450IA1 and histochemically for ALDH (benzaldehyde and hexanal), DT-diaphorase, γ-GT and uridine diphosphoglucuronyl dehydrogenase (UDPGdH). In tumor tissues, the largest biochemical changes were found with benzaldehyde dehydrogenase, where activity increased from undetectable levels to 7.4 nmol/min/mg protein, and γ-GT, where activity increased 12-fold over controls. Increases in other enzymes ranged from 1.26 to 2.84 times that of control liver, except EROD, which decreased, and cEH and mEH, which were unchanged. Histochemical analyses showed the induction of ALDH, γ-GT, DT-diaphorase and UDPGdH, and the depression of cytochrome P450IA1 in hepatic neoplasms. In addition, marker enzyme histochemistry of neoplasms revealed heterogeneous populations of hepatocytes and absence of necrotic areas.

Introduction

The rainbow trout (*Oncorhynchus mykiss*) and a small aquarium fish, the medaka (*Oryzias latipes*), have proven sensitive to certain known mammalian hepatocarcinogens. These fish have thus been suggested as useful model species for screening chemicals for carcinogenic potential as well as expanding our knowledge of environmental carcinogenesis in wild organisms (1–5). To better understand tissue and cellular biology in these emerging vertebrate models, it is necessary to characterize normal liver and hepatic tumors biochemically and histochemically. Enzymatic properties of neoplastic hepatocytes and their precursor lesions have proven to be important in the characterization of cellular aspects of initiation and promotion (6). Altered protein expression may render cells more capable of withstanding toxicity and thereby better able to divide, leading to growth of specific phenotypes (7). Also, it is important to assess the organism's ability to metabolize xenobiotics during and following carcinogen exposure.

In the rainbow trout hepatocarcinogenesis model, metabolism of chemicals prior to and during initiation and the modulatory effects of these early events on eventual tumor yield has been a commonly employed approach (2,8–10). Far fewer reports have looked at the histochemical (11,12) or immunohistochemical (13,14) properties of cells found within discrete tumors, and only one has explored biochemical properties of these neoplasms (15). Aflatoxin B_1 (AFB_1*)-induced rainbow trout liver neoplasms are principally hepatocellular with some biliary cell contribution and show decreased or absent iron accumulation (12), cytochrome P450 LM_{4b} and LM_2 (13), and immunoreactive glutathione S-transferases (GST; 14).

In the following study, we analyzed the quantitative biochemical differences between control liver and tumor tissue, and compared these results to the qualitative histochemical and immunochemical changes found in tissues from the same experimental cohorts.

*Abbreviations: AFB_1, aflatoxin B_1; GST, glutathione S-transferase; OTD, Oregon Test Diet; γ-GT, γ-glutamyl transferase; ALDH, aldehyde dehydrogenase; UDPGdH, uridine diphosphoglucuronyl dehydrogenase; UDPGT, uridine diphosphoglucuronyl transferase; cEH, cytosolic epoxide hydrolase; mEH, microsomal epoxide hydrolase; EROD, ethoxyresorufin-*O*-deethylase; GSH, glutathione; CDNB, chlorodinitrobenzidine.

Materials and methods

Treatment of animals

Fingerling rainbow trout, *O.mykiss* (Shasta strain), were continuously fed a diet (semipurified Oregon Test Diet—OTD) (16) containing 8 p.p.b. AFB_1 (Calbiochem, San Diego, CA) for 12 months until necropsy. Control fish were fed with OTD free of AFB_1 addition. All fish were held in circular flow-through tanks supplied with 14°C well water at 3 gal/min and were killed after 12 months. At necropsy, trout were killed by a blow to the head and the liver rapidly dissected free and divided into portions for analysis as described below. Overt hepatic tumors were dissected free from remaining liver and biochemistry was restricted to neoplastic tissue.

Enzyme histochemistry

Hepatic tissue was embedded in OCT medium (Tissue-TEK), snap-frozen in 2-methylbutane, cooled to liquid nitrogen temperature and stored at −80°C. Five serial 6–8 μm thick cryostat sections of the tumor and surrounding non-tumor tissue were made. Each was incubated in appropriate medium for 60 min at room temperature. The methods used were γ-glutamyltranseferase (γ-GT; 17), aldehyde dehydrogenase (ALDH; 18), DT-diaphorase (19) and uridine diphosphoglucuronyl dehydrogenase (UDPGdH; 20). The terminal electron acceptor for the ALDH, DT-diaphorase, and UDPGdH reactions was nitroblue tetrazolium. The γ-GT assay used the azo-dye method in which a reddish-brown precipitate was the positive reaction. The ALDH reactions were run in the dark and employed various substrates including hexanal and benzaldehyde and either NAD^+ or $NADP^+$ as cofactors. All histochemical reactions had previously been optimized for fish liver and were correlated with routine staining by H&E. For details of each enzyme reaction in fish liver, refer to Hinton (21). Histochemical controls included incubation with no or inappropriate substrate and biological controls, companion sections of frozen rat livers incubated under similar conditions (19).

Immunohistochemical staining of P450IA1 in the cryostat sections was done by use of a monoclonal antibody (Mab 1–12–3) and an indirect peroxidase labeling method. Mab 1–12–3, the primary antibody was reactive against scup (*Stenotomus chrysops*) P450IA1 and subsequently for the similar protein in trout (22,23). The secondary antibody was a horse antimouse IgG. Immunohistochemical controls included blocking agents for non-specific peroxidase and the use of inappropriate or no primary antibody and no secondary antibody. An additional control included sections of liver from trout exposed to β-naphthoflavone (50 mg/kg body wt).

Enzyme preparation

Liver tumors were stored at −80°C and then homogenized with a Brinkmann Polytron in 14 vol (w/v) of ice-cold 0.25 sucrose solution (pH 7.6) containing

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1 mM EDTA and 1 mM dithiothreitol (Sigma). Initial sedimentation to remove mitochondria and large particles was performed in a Sorval RC-B2, refrigerated centrifuge at 16 000 *g* for 30 min. The microsomal fraction was then separated from the cytosol by centrifugation at 100 000 *g* for 60 min in a Beckman L5 ultracentrifuge. The resultant microsomal pellet was washed by resuspension and centrifugation repeated as above. The final washed pellet was resuspended in buffer (0.05 M Tris−HCl, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol and 20% glycerol) in a volume equal to that of the initial tissue weight. All centrifugations were carried out at 4°C. After the above, all fractions were stored at −80°C until analysis (24).

Enzyme assays

DT-diaphorase (25) assay was adapted for trout hepatic cytosolic fractions and modified to pH 7.6 and 25°C. Specific activities for DT-diaphorase, ALDH (18) and GST (26) were estimated from the linear portions of the continuous curves obtained over a 5 min period using the CPS kinetic mode of a Shimadzu 160 recording spectrophotometer. Activity of the microsomal enzyme, uridine diphosphoglucuronyl transferase (UDPGT), with *p*-nitrophenol as a substrate, was measured by the method of Castren and Oikari (27) and was modified by addition of digitonin (0.2% final concentration) to solubilize protein (28). UDPGT and the cytosolic enzyme, γ-GT (Sigma; optimized for trout liver), were also assayed spectrophotometrically by calculating the amount of product formed after a 20 min, 25°C waterbath incubation. Cytosolic and microsomal epoxide hydrolase (cEH, mEH) activity were measured radiometrically in cytosolic and microsomal fractions using the procedure of Laurén *et al.* (26) with [^{3}H]*trans*- and *cis*-stilbene oxides as substrates respectively.

Ethoxyresorufin-*O*-deethylase (EROD) activity was monitored continuously for 5 min using a Shimadzu spectrofluorimeter by measuring the formation of resorufin (29). The trout microsomal fraction was analyzed for EROD activity at 25°C with a pH of 7.6, conditions found to be optimal in another fish species (30).

Specific enzyme activity was expressed in nmol product/min/mg of protein. Protein was measured by the micromethod of Bradford (31) using bovine serum albumin as the standard. Substrates and cofactors used are listed in Table I.

Results

Enzyme histochemistry

Histochemical examination on a subset of the tumors of this study showed that the major constituent was neoplasm with limited, non-tumorous parenchyma at the periphery (Figure 1). Tumor hepatocytes were homogeneously basophilic due to apparent increased granular endoplasmic reticulum (32) and glycogen depletion (33). Trout hepatic neoplasms were characterized as mixed hepato- and cholangiocellular carcinomas (Figure 1), with the former predominating. Portions of biliary structures revealing variable cellular anaplasia (not shown) were contained within these lesions of predominantly basophilic hepatocytes. Serial sections reacted for a specific enzyme (Figures 2−5) demonstrated that the neoplastic tissue differed from surrounding liver by variously enhanced enzyme products.

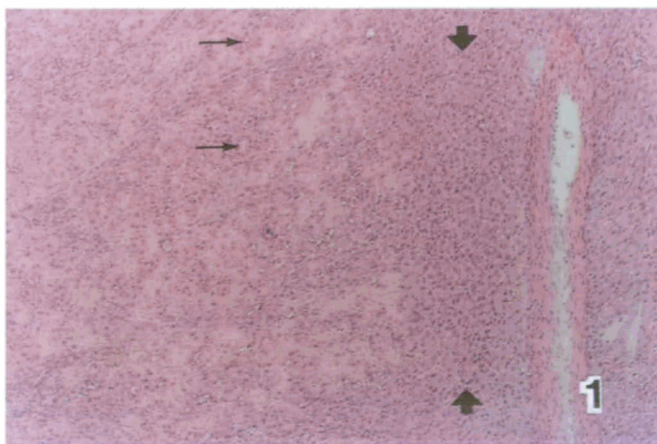

Fig. 1. Cryostat section of fresh frozen trout liver containing portion of mixed hepato-cholangiocellular neoplasm and non-involved hepatic parenchyma (to the right of the line between vertical arrows). Hepatocytes of neoplasm were basophilic to amphophilic. Bile ductal and ductular elements (horizontal arrows) are in neoplasm. H&E, ×35.

DT-diaphorase enhanced (Figure 2a) tumor cells are purple in micrographs due to the reduction of nitroblue tetrazolium salt by DT-diaphorase. Under higher magnification (Figure 2b), DT-diaphorase marked cellular components of the lesion heterogeneously. Hepatocytes were either enhanced or weakly reactive while biliary epithelial cells were moderately stained. Connective tissue surrounding bile ducts of tumor was non-reactive (Figure 2b).

A typical UDPGdH reaction is shown (Figure 3). Tumor hepatocytes were either weakly reactive, similar to surrounding non-tumorous tissue, or were moderately enhanced. The slight reactivity of biliary epithelial cells contrasted with the pale regions of connective tissue. However, biliary staining was not strong (Figure 3).

ALDH activity against hexanal was uniformly distributed over control liver sections, with no apparent activity against benzaldehyde (not shown). When compared to H&E preparations, basophilic hepatocytes of the tumor reacted much more strongly when hexanal was the substrate (Figure 4).

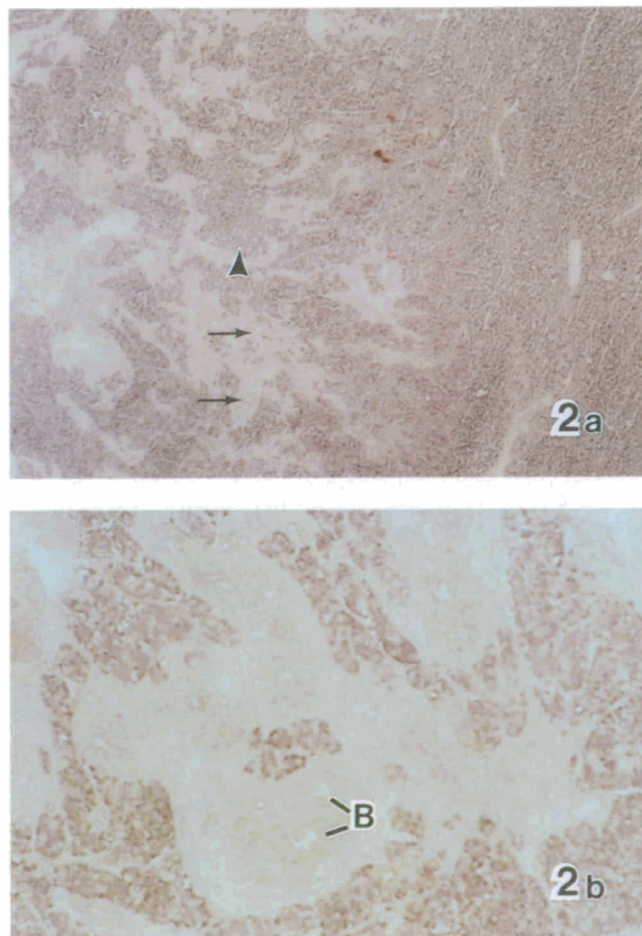

Fig. 2. (**a**) DT-diaphorase preparation in cryostat section at margin of mixed hepato-cholangiocellular carcinoma. Neoplasm (left of field) contains abundant hepatic tubules and broader forms resembling trabeculae (vertical arrow) whose cells were dark purple (reduced nitrotetrazolium salt) and enhanced over surrounding parenchyma (right of field). Horizontal arrows indicate biliary passageways, cells of which were only lightly reactive. ×35. (**b**) Enlargement of area from same neoplasm as in (a). Variable staining of components is shown. Most hepatocytes were enhanced over control tissue. Others reacted only slightly. Biliary epithelial cells of ducts (B) were slightly reactive. ×120.

Benzaldehyde activity was also apparently induced, though to a lesser extent (not shown in figure). Bile ductal and ductular epithelial cells were also positive for ALDH, but to a lesser degree than that of hepatocytes. The connective tissue surrounding biliary passageways within the carcinoma was negative.

As in medaka (33), normal trout liver tissue was negative for γ-GT activity, while tumor proved positive, indicated by a reddish-orange azo-dye precipitate over both biliary epithelial cells and diffusely over hepatocytes (Figure 5).

Only occasional hepatocytes were labeled in tumor and normal liver reacted for immunostaining with P450IA1 MAB. These findings are not shown due to a lack of difference observed between tumor and normal liver. In our experience, without the use of the P450 inducer, β-naphthoflavone, activity against the P450IA1 antibody is seen in a few randomly scattered hepatocytes (34). Companion sections of liver from β-naphthoflavone-induced trout showed dark peroxidase staining over cytoplasm of hepatocytes. These results are described in detail in Lester *et al.* (35).

Specific enzyme activities in control and tumor-bearing liver

When compared to control trout liver, the specific activities for DT-diaphorase, UDPGT, ALDH, γ-GT and GST from the mixed hepatocholangiocarcinomas were significantly enhanced and EROD significantly depressed (Table I). No significant difference was found for either cEH or mEH.

DT-diaphorase activity of control trout liver ranged from 22 to 36 while tumor activity ranged from 58 to 80 nmol/min/mg cytosolic protein for a mean increase of 2.38-fold. UDPGT (*p*-nitrophenol as substrate) was increased >200% in the microsomal fraction of tumor versus control liver.

As with the histochemical assays, hexanal, rather than benzaldehyde, was a more favorable substrate for the measurement of control ALDH. In fact, as shown previously (18), benzaldehyde dehydrogenase activity was non-detectable in control tissue. However, neoplastic benzaldehyde dehydrogenase activity increased dramatically and was not significantly different from the induced hexanal dehydrogenase activity. Activity in tumor-bearing tissue ranged from 7 to 10 nmol/min/mg protein when either hexanal or benzaldehyde was used.

γ-GT activity increased significantly (12-fold) from control to tumor-bearing tissue. Since activity was virtually non-dectable in control liver tissue, this enzyme makes a potentially good biomarker. On the other hand, GST, which is much more abundant in the liver (36), showed only slight (1.3- to 1.4-fold) increases in neoplastic tissue, regardless of the substrate, i.e. chlorodinitrobenzidine (CDNB) or ethacrynic acid).

EROD activity was used to indicate changes in cytochrome P450IA1. EROD activity of the neoplastic liver was depressed to 26% of the control value. The more sensitive fluorometric assay, rather than the spectrophotometric procedure (30) was required to detect these low levels of EROD.

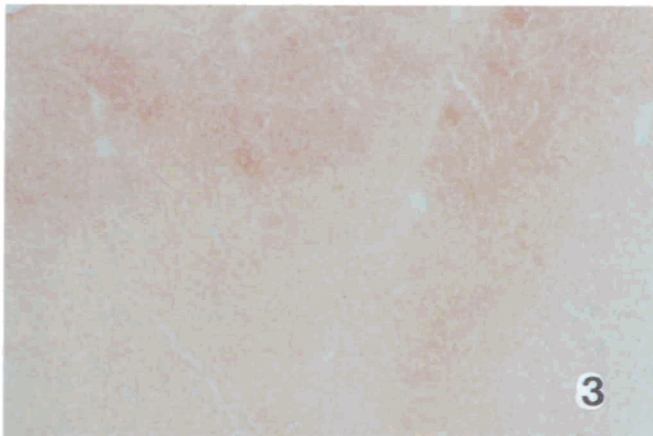

Fig. 3. Neoplasm, top of field, shows collection of hepatocytes that were enhanced (purple, reduced nitrotetrozolium salt) for UDP-glucuronyl dehydrogenasae. Normal liver (bottom of field) reacted weakly. ×35.

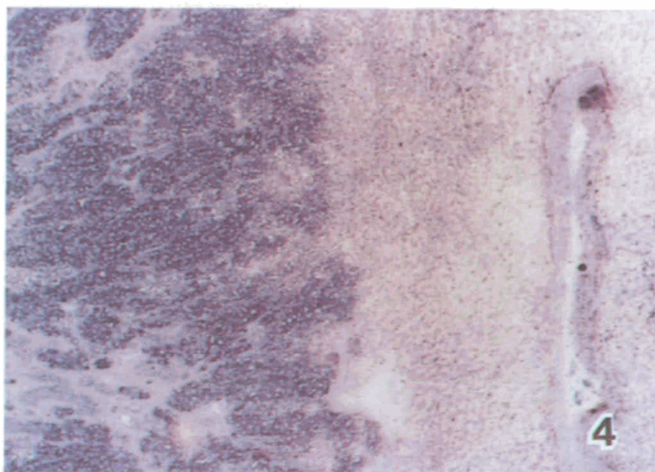

Fig. 4. Hepatocytes of neoplasm (left of field) show enhanced reaction for ALDH using hexanal as substrate and NAD$^+$ as a cofactor. ×35.

Discussion

Hepatic neoplasms of trout have been reported after exposure to a wide variety of carcinogens (1,2,37,38). Cellular components of resultant neoplasms vary. Some are highly differentiated hepatocellular, while others are mixed hepatocellular and biliary. Rarely, pure biliary neoplasms occur. From our review of the above literature and the specimens analyzed herein, trout tumors have shown neither extensive necrosis nor diffuse connective tissue accumulation. Therefore, estimation of enzyme activity in the subcellular fractions of these neoplasms likely reflects prior changes within component cells.

Quantitative biochemical and qualitative histochemical analyses of enzymes in trout liver neoplasms were correlated. Where enhanced activity was suggested by histochemistry (Figures

Fig. 5. γ-GT was diffusely positive over both hepatocytes and biliary epithelial cells of neoplasm. Portion of normal tissue at right edge (see dotted line) of micrograph did not react. ×35.

Table I. Specific enzyme activities (nmol/min/mg liver protein) in normal and tumor bearing liver

Enzyme	Substrate	Control liver	Tumor-bearing liver
DT-diaphorase	2,6-dichlorophenol indophenol	29.09 ± 7.14	69.23 ± 11.05[a]
UDPGT	*p*-nitrophenol	1.66 ± 0.46	3.71 ± 1.09[a]
ALDH			
Cofactor NAD^+	hexanal	3.45 ± 1.10	9.80 ± 2.60[a]
Cofactor $NADP^+$		3.85 ± 1.45	8.73 ± 1.25[a]
ALDH			
Cofactor NAD^+	benzaldehyde	ND[b]	7.43 ± 2.61[a]
Cofactor $NADP^+$		ND	6.8 ± 3.29[a]
γ-GT	γ-glutamyl-*p*-nitroanilide	0.07 ± 0.01	0.85 ± 0.25[a]
GST	CDNB	277.7 ± 40.2	384.2 ± 28.0[a]
GST	ethacrynic acid	54.9 ± 18.5	71.0 ± 13.2
EROD	ethoxyresorufin	0.035 ± 0.002	0.009 ± 0.004[a]
cEH	*trans*-stilbene oxide	4.34 ± 1.51	6.89 ± 1.36
mEH	*cis*-stilbene oxide	1.74 ± 0.76	2.2 ± 0.30

Values are the means ± standard deviations for three different animals.
[a]Determined by Student's *t*-test to be significantly different from controls ($P < 0.05$).
[b]Not detectable.

2–5), subsequent biochemical detection of DT-diaphorase, γ-GT, ALDH and UDPGdH supported these findings. Pioneering studies by Scarpelli *et al.* (15), following outbreaks of AFB_1-induced carcinogenesis in trout, established activities of specific hepatic enzymes such as markers of cell degeneration (glutamic oxalacetic and glutamic pyruvic transaminases), four Kreb's cycle mitochondrial enzymes, and three found later to be excellent biomarkers of foci in rodent tumor studies (alkaline phosphatase, glucose-6-phosphate dehydrogenase and glucose-6-phosphatase) in normal and neoplastic trout liver. Years later, after the eventual development of marker enzymes in mammalian species, Hendricks *et al.* (11) confirmed the earlier glucose-6-phosphatase results histochemically. They showed nodular masses in trout liver to be deficient in this enzyme. Our findings are, however, the only biochemical and histochemial couplings in trout liver neoplasms. In general, enhanced activity in trout liver neoplasms agrees with previous findings in Morris hepatoma cell lines (25,39) and in rodent nodules (39–41). We are aware that the contribution of biliary versus hepatocytic components in a neoplasm may vary, and this would likely cause variation in enzyme activity. Despite the fact that biliary epithelial cells are shown to be numerous in normal (42) and neoplastic (37) trout liver, trout hepatic biliary cell chemical data are unfortunately not available. Blair *et al.* (43) recently isolated and maintained in cell culture biliary epithelial cells from trout, but chemical characterization has not followed. One procedure, *in vivo* exposure to α-napthyl isothiocyanate, in rats causes proliferation of biliary epithelial cells (44) without neoplasia. We are attempting such enrichment procedures in the trout liver to obtain sufficient cells for biochemical measurements.

Our observation of both DT-diaphorase positive and negative hepatocytes within an individual neoplasm reinforces the necessity of using more than one enzyme as a biomarker and also reflects possible phenotypic heterogeneity within a trout tumor. This heterogeneity of trout tumors, plus the fact that during different time sequences specific phenotypes may dominate (45–47), delineates the need for additional studies and markers.

Morphological and biochemical results illustrate that neoplasms of rodent liver take on fetal characteristics and the substrates, *p*-nitrophenol with UDPGT (48) and CDNB or ethacrynic acid with GST (49,50), used in this study, have been shown to be indicative of the fetal form of the enzyme. Verification followed demonstration of increased activity towards the substrate during embryogenesis. In trying to make comparisons between neoplasms of fish liver and those of their mammalian counterparts, it is important to understand that we are not certain what the fetal equivalent period of trout development is. However, studies should focus on time periods prior to rupture of the chorion (hatching) and early, sac-fry, larvae prior to swim-up that may be the fetal equivalent.

UDPGT activity of tumor microsomal fractions of this study showed that enzyme activity was doubled over that of control liver. Interestingly, Astrom *et al.* (40) reported rat liver UDPGT increased 200% in microsomal fractions from neoplastic nodules. GST activities were measured in well-described hepatic lesions (51) of mummichog (*Fundulus heteroclitus*) taken from heavily creosote-contaminated sites and adjacent normal liver tissue from the same fish (52). While no significant differences were reported in GST activity between non-tumorous and tumor-bearing liver, masking may have obviated detection of tumor increments. Table I of that study showed site (environmental)-specific induction of GST in whole liver (52). Although the observation of increased GST activities in trout tumors of this study does not correlate with previously reported immunohistochemical findings of decreased levels of an isozyme of GST in trout tumors (14) and those in mummichog (52), our findings are in agreement with rodent hepatocarcinogenesis assays in which increased GST was measured biochemically (40,50) and immunohistochemically (53). Therefore increases in enzyme activity with these substrates in fish neoplasms may reinforce the hypothesis that neoplastic cells take on putative fetal characteristics.

Although γ-GT, DT-diaphorase and EH have been studied extensively in rodents, only one study in fish has been performed (54). γ-GT, an enzyme also found to be highly active in fetal rat and mouse tissue (55,56), identifies more foci than other markers in rat liver tumor models (46). Yet, because adult control rat (55) and trout hepatic tissue has low constitutive activities, biochemical measurements have not frequently been made in these species. Values from control trout correlate with rodent findings of undetectable levels of γ-GT, biochemically and

histochemically. As in rat neoplasms (39) trout tumors have elevated levels of γ-GT.

DT-diaphorase activity in tumor tissue of this study was increased at least 2-fold over control liver. This corresponds to the level of change seen in studies of Morris hepatoma cell line (25). Interestingly, in the only other report on DT-diaphorase in fish, our values for this enzyme fell in the range of activities reported by Hasspieler and DiGulio (54) in channel catfish (*Ictalurus punctatus*).

mEH has been shown in numerous rodent hepatocarcinogenesis studies to be elevated in nodules using both biochemical (57,58) and immunohistochemical methods (59,60). Although tumor-bearing liver in this study showed an increase over previusly cited control values for rainbow trout using the same substrates [2.70−3.38 cEH and 0.78−0.98 nmol/min/mg protein mEH (26)], we saw no significant increase over control activity in this study. cEH, induced by peroxisome proliferators in rodent liver neoplasms (61), has, in fish, appeared refractory to induction by typical peroxisome proliferators such as clofibrate (unpublished) and ciprofibrate (62). Calabrese *et al.* (63) have reviewed peroxisome proliferators as potential epigenetic carcinogens. Although they comment that 'extensive data' exist for this class of compounds in fish, they cited few published and few unpublished data on trout and medaka. Furthermore, the magnitude of change was not great and other studies in fish with peroxisome proliferators report difficulties in observing statistically significant changes in marker enzymes due to a wide range of variability in their activities (64). These findings, along with those in the present study, indicate that EH may not be a good biomarker for use in fish hepatocarcinogenesis.

EROD is frequently used as a substrate for cytochrome P450 and has been shown to be indicative of changes in P450IA1 activity (65). Thus, our findings of decreased microsomal EROD and P450IA1 activity in hepatic neoplasms of trout reinforce the immunohistochemical findings of Lorenzano and co-workers (13). Those studies examined P450s in aflatoxin-induced tumors and found decreased levels of LM_{4b} in nodules.

Hepatic neoplastic tissue generally exhibits deviations from the phenotype of normal liver (6,66,67), characterized by a decrease in metabolizing systems responsible for bioactivation of compounds and by an increase in enzyme activities considered as part of the detoxification of substrates. Parker *et al.* (18), studying ALDH of trout liver, showed that this enzyme detoxifies endogenous products of lipid peroxidation. Also, significant changes in cytosolic oxidative ALDH activity have been observed histochemically (68) and biochemically (69) during rat hepatocarcinogenesis. Further evidence that xenobiotic enzymes protect tissue from oxidant damage is seen with DT-diaphorase that catalyzes the formation of hydroquinones from quinones, thereby protecting the cell from damage by reducing semiquinone production and subsequent superoxide radical formation (70). In addition to the kidney function of cleavage of glutamic acid moieties from glutathione conjugates, γ-GT catalyzes the uptake of plasma glutathione (GSH) (71). Therefore, γ-GT-positive hepatocytes may accumulate more GSH, an antioxidant. Also, EH is associated with the metabolism of endogenous fatty acids. In addition to those cellular events produced by the ultimate carcinogens in their targeted cells, other host tissue reponses are occurring. For example, macrophages, active in resolution of cellular lytic phenomena from cytotoxicity, become sequestered in liver and generate superoxide anions (72). Companion studies in this laboratory with medaka showed extensive macrophage infiltration after diethylnitrosamine exposure (33). Also, recent studies have shown that spontaneous DNA damage caused by endogenous oxidants is remarkably frequent (73). Thus, fish lipid membrane, containing greater quantities of the more easily oxidized polyunsaturated fatty acids than mammalian tissue, may have increased susceptibility to endogenous oxidant damage (74,75). In fact, in fish liver taken from polluted and relatively pristine environments, there was no significant difference in the amount of DNA damage measured by the sensitive technique of ^{32}P-postlabeling (76). Thus, it is hypothesized that these biochemical differences in the liver may alter bioactivation and/or protection mechanisms.

Although the present study has found general agreement with previous rodent investigations, these types of analyses need to be extended to other fish species and to other enzymes and substrates.

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