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Altered expression of the xenobiotic transporter P-glycoprotein in liver and liver tumours of mummichog (*Fundulus heteroclitus*) from a creosote-contaminated environment

PETER S. COOPER[1], WOLFGANG K. VOGELBEIN and PETER A. VAN VELD*

The College of William and Mary, Virginia Institute of Marine Science, School of Marine Science, Gloucester Point, VA 23062, USA
e-mail: vanveld@vims.edu

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P-glycoproteins (Pgps) are involved in efflux of xenobiotics from drug-resistant cell lines and tumours, and in excretion of toxicants from normal tissues. Recently, investigators have proposed that Pgp activity contributes to resistance or tolerance of certain aquatic species to pollutants. In the present study using immunoblot and immunohistochemical techniques, we found elevation of Pgp in liver and liver tumours of creosote-resistant mummichog from a contaminated site in the Elizabeth River, Virginia. Immunoblots of mummichog liver extracts showed an immunoreactive band at 170 kDa and indicated two- to three-fold elevation of Pgp in livers of resistant fish relative to those from a reference site. Laboratory exposures of reference site fish to a model PAH (3-methylcholanthrene), however, produced no increase in liver Pgp levels as measured by immunoblot. Normal mummichog liver sections showed specific immunohistochemical staining for Pgp on the canalicular surface of hepatocytes. In the majority of hepatic neoplasms we observed a high level of over-expression and altered patterns of Pgp expression. However we did not observe Pgp over-expression in early proliferative lesions. Elevation of Pgp in livers and liver tumours of these resistant mummichog may contribute to their survival in a heavily contaminated environment.

Keywords: P-glycoprotein, immunohistochemical staining, *F. heteroclitus*, hepatocellular carcinoma, pollution resistance.

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Introduction

P-glycoproteins (Pgp) are membrane transport ATPases involved in efflux of diverse cytotoxic agents from many multidrug-resistant mammalian cell lines and chemotherapy-resistant tumours (Arceci 1993, Gottesman and Pastan 1993). A wide range of toxic compounds including natural toxins (Gottesman and Pastan 1993) as well as environmental contaminants such as the polycyclic aromatic hydrocarbon benzo[*a*]pyrene (Yeh *et al.* 1992) were shown to be Pgp substrates in drug-resistant cell lines. Pgps have now been identified in a variety of normal tissue and cell types including the apical surfaces of epithelial cells in the kidney, intestine and liver as well as the endothelium of the blood–brain barrier (Thiebaut *et al.* 1989). These sites of expression and the role of Pgp in conferring drug resistance suggested that Pgps may be involved in defence against toxic compounds. It is now known that Pgps are products of a multigene family which contains two or more members in most organisms studied (Childs and Ling 1994). The individual gene products have differing functions, and not all Pgp gene products are involved directly in xenobiotic transport. For example, the mouse has three Pgp genes termed *mdr1a* , *mdr1b* and *mdr2* (Gros *et al.* 1986, 1988, Hsu *et al.* 1989) while

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humans have two, *MDR1* and *MDR2* (Chen *et al.* 1986, van der Bliek *et al.* 1988). The product of either the *mdr1a* and *mdr1b* genes or human *MDR*1 can confer drug-resistance to sensitive cell-lines (Ueda *et al.* 1987, Devault and Gros 1990). Evidence from mice lacking one or both of the *mdr1* genes suggests that the physiological function of the murine *mdr1* gene products *in vivo* is xenobiotic excretion in organs such as the liver and kidney or in xenobiotic exclusion in the blood–brain barrier and intestine (Schinkel *et al.* 1994, Borst and Schinkel 1996). In humans the product of the single *MDR1* gene apparently provides these functions. In contrast neither the murine *mdr2* gene product nor the product of the human *MDR2*, which are expressed predominantly in the canalicular membrane of hepatocytes (Buschman *et al.* 1992, Smit *et al.* 1994), confer resistance *in vitro* against classical drug-resistance substrates (van der Bliek *et al.* 1988, Gros *et al.* 1988). Evidence from *mdr2* null mice and from studies of the effect of Mdr2/MDR2 proteins on transbilayer movement of phospholids suggests that these proteins function as phospholipid translocases involved in excretion of phosphatidylcholine into bile (Ruetz and Gros 1994, Smit *et al.* 1993, Smith *et al.* 1994). Although the *mdr2/MDR2* gene product may not directly transport toxicants, it may play an important role in excretion of xenobiotics or their metabolites *in vivo* by passive coupling of their transport to Mdr2/MDR2 mediated biliary lipid secretion (Fritjers *et al.* 1997). In addition to the true Pgp genes, a closely related gene termed the sister of P-glycoprotein (*spgp*) was recently identified (Childs *et al.* 1995). The *spgp* gene product is expressed only in liver, where it may also be involved in biliary excretion.

The ability of pollution-tolerant organisms to simultaneously cope with mixtures of diverse toxic compounds is reminiscent of the broad spectrum xenobiotic resistance observed in multidrug-resistant mammalian cells. Numerous investigators have speculated that the activity of Pgps may contribute to pollution tolerance in populations of aquatic species (reviewed in Kurelec 1992). In support of this hypothesis, Pgp-like activities and Pgp-related antigens have been detected in excretory tissues of several pollution or toxin-tolerant aquatic invertebrates (Kurelec 1992, Toomey and Epel 1993, Cornwall *et al.* 1995, Galgani *et al.* 1996). There is also evidence that Pgp expression and activity in aquatic invertebrates may be elevated by exposure to toxicants (Minier *et al.* 1993, Kurelec *et al.* 1995, 1996). Although the majority of work has focused on aquatic invertebrates, Pgp expression has also been detected in fish. Using several anti- mammalian Pgp antibodies, Hemmer *et al.* (1995) demonstrated that tissue and subcellular localization of total Pgp antigens in the guppy was similar to that of mammals.

Our recent studies have focused on histopathological and biochemical changes associated with carcinogenesis in a population of the estuarine killifish known as the mummichog (*Fundulus heteroclitus*), inhabiting a site heavily polluted with PAHs and other organic contaminants associated with creosote. Although adult fish at this site have a high prevalence of hepatocellular carcinoma and other neoplasms (Vogelbein *et al.* 1990, Fournie and Vogelbein 1994), this population is resistant to acute toxicity (mortality) associated with exposure to creosote-contaminated sediments while reference site fish are not (Williams 1994, Vogelbein *et al.* 1996). Mechanisms of resistance are not understood. However biochemical features identified thus far including depressed CYP1A (van Veld *et al.* 1992, van Veld and Westbrook 1995) and elevated glutathione *S*-transferase (Armknecht *et al.* 1998) are similar to those reported in drug-resistant tumours and cancer cell lines

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(Buchman *et al.* 1985, Farber 1990). In the present study we extend our biochemical and immunohistological observations by characterizing the expression of Pgp in livers and liver tumours of the resistant population. We report elevated Pgp expression in non-tumour bearing liver of Atlantic Wood mummichog as well as high level over-expression and altered patterns of expression in a majority of hepatic neoplasms. Our results are similar to those reported for Pgp expression in mammalian liver and liver tumours and may have implications for the tolerance or adaptation of these fish inhabiting a contaminated environment.

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Materials and methods

Collection of samples

Mummichog were collected from two sites described previously (van Veld and Westbrook 1995), a creosote-contaminated site (Atlantic Wood) and an uncontaminated reference site (King Creek). Fish were killed within 48 h of capture by overdose with tricaine methane sulphonate (Sigma, St Louis, MO). Livers were removed, frozen in liquid nitrogen and stored at –70 °C until use.

Immunoblotting of mummichog liver extracts

Livers from male fish without grossly visible tumours were used in immunoblot analyses. Processing of mummichog livers and subsequent immunoblot analysis followed the protocols given previously (Cooper *et al.* 1996) using the monoclonal antibody (mAb) C219 (Kartner *et al.* 1985). Livers were homogenized with a Polytron (Brinkman Instruments, Westbury, NY) in 10 volumes of ice cold lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 % w/v sodium deoxycholate, 0.1 % w/v SDS, 5 mM EDTA, 2 % v/v aprotinin solution (Sigma), 380 μg ml^{-1} *N*-toluenesulphonylamido-L-arginine methyl ester, 100 μg ml^{-1} phenyl methyl sulphonyl fluoride, 10 μg ml^{-1} leupeptin). Protein concentrations were determined using the Lowry assay with bovine serum albumin (BSA) as a standard. Extracts were diluted to 0.5 mg ml^{-1} total protein with standard SDS–PAGE sample buffer, heated (65 °C, 2 min) and loaded (2 μg per lane) onto a 5.6 % (total) acrylamide SDS–PAGE mini-gel. Electrophoresis and electro-transfer to nitrocellulose membranes followed the manufacturer's recommendations (Mini-Transblot, BioRad, Richmond, CA). Membranes were blocked in TTBS (100 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.02 % Tween-20) containing 5 % (w/v) non-fat dry milk and then incubated with mAb C219 (Signet, Dedham, MA) (2.5 μg ml^{-1}, in TTBS, 1 % (w/v) non-fat dry milk, 1 h, room temperature). After two washes in TTBS, immune complexes were detected with goat antimouse IgG-alkaline phosphatase conjugate (BioRad) using BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (*p*-nitro blue tetrazolium chloride) as chromogenic substrates for alkaline phosphatase.

Estimation of relative Pgp content in mummichog liver extracts

Intensity of staining of immunoblots was measured with a Shimadzu CS-930 scanning densitometer (λ = 550 nm). Densitometric peak area for each Pgp band was normalized to a standard curve of peak area vs total protein for varying amounts of pooled extract of reference site (King Creek) mummichog liver loaded on each blot. Pgp content in each sample was expressed in arbitrary units made by assigning a value of 100 to the signal obtained from 1 μg of the pooled King Creek liver extract.

Laboratory exposure of mummichog to 3-methylcholanthrene (3-MC)

Male reference site fish were given intraperitoneal (i.p.) injections of 3-MC (100 ml kg^{-1} body weight) in corn oil (10 mg ml^{-1}) or the equivalent volume of corn oil alone. Fish were maintained in 10-gallon aquaria provided with flow-through seawater for 36 h following treatment. Fish were killed and livers frozen as described above. Liver extracts were prepared and analysed for Pgp expression as described (Cooper *et al.* 1996). Extracts were also analysed by immunoblot for relative levels of CYP1A using mAb 1-12-3 (Park *et al.* 1986a, generous gift of John Stegeman, Wood's Hole Oceanographic Institute). Samples were separated on 10 % acrylamide mini-gels. All other electrophoresis, transfer and incubation conditions were identical to those for immunoblot detection of Pgp with mAb C219.

Student's *t*-test was used to assess significant differences between expression levels in the contaminated and reference site fish and between 3-methylcholanthrene exposed fish and corn oil controls.

Immunohistochemical staining of mummichog liver and liver lesions

Many mummichog liver and tumour specimens used in this study were archival specimens collected from Atlantic Wood (Vogelbein *et al.* 1990). Additional specimens were collected in 1992 from both

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Atlantic Wood and King Creek. Fixation, processing and immunohistochemical staining of tissue sections followed previously presented protocols (Cooper *et al.* 1996). Sections were deparaffinized and rehydrated to phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked with 3 % aqueous H_2O_2. After rinsing in PBS and blocking with normal horse serum (10 % v/v in PBS), sections were incubated with mAb C219 (1.5 μg ml^{-1} in PBS containing 1 % w/v BSA, overnight, 4 °C). To control for non-specific binding of the primary and secondary antibodies, an adjacent section was incubated with matched isotype mouse myeloma protein (IgG 2a, kappa, Sigma, St Louis, MO) under identical conditions. Bound antibody was detected with biotinylated horse antimouse IgG and avidin-biotinylated horseradish peroxidase complex (Elite Avidin-Biotin Complex Immunohistochemical Staining Kit, Signet Laboratories, Dedham, MA). 3,3' Diaminobenzidine and H_2O_2 (Sigmafast tablets, Sigma, St Louis, MO) served as chromogenic substrates for horseradish peroxidase, and Harris' haematoxylin was used as a counter stain. Sections were dehydrated and mounted in synthetic mounting medium. An adjacent section was processed for routine haematoxylin and eosin histology.

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Results

Immunoblot detection of Pgp from mummichog liver

Immunoblots of mummichog liver from both the Atlantic Wood site and the King Creek reference site probed with mAb C219 had a highly immunoreactive band at 170 kDa (figure 1). The intensity of this band was significantly higher (Student's t-test, $p < 0.05$) in the Atlantic Wood mummichog livers at both collection times, indicating consistent elevation of Pgp expression in mummichog from the contaminated site (figure 1, table 1). The average increase in expression of the Pgp was 3.2-fold and 2.6-fold (table 1) in the livers of Atlantic Wood fish in 1996 and 1994 respectively.

Pgp expression following administration of 3-MC

We observed no significant increase in intensity of staining for Pgp in immunoblots of liver extracts of King Creek fish following i.p. injections of 3-MC compared with liver extracts of fish injected with corn oil. The relative Pgp contents in the two treatments were 144 ± 30 units per μg protein and 128 ± 32 units per μg protein for the 3-MC and corn oil treatments respectively. Immunoblots of these liver extracts for CYP1A expression indicated a 10-fold increase in staining intensity of the CYP1A band in the livers fish given the 3-MC compared with the corn oil-treated fish (figure 2).

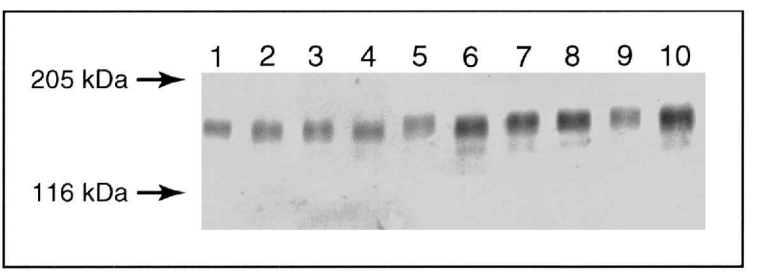

Figure 1. Immunoblot (mAb C219, 1.5 μg ml^{-1}) detection of Pgp in mummichog liver extracts from an uncontaminated reference site (King Creek) and from the creosote-contaminated site (Atlantic Wood). Lanes 1–5 King Creek mummichog. Lanes 6–10 Atlantic Wood mummichog. Positions of molecular weight standards are shown at the left.

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Table 1. Relative content of Pgp antigens in liver extracts of mummichog from the creosote-contaminated site (Atlantic Wood) and an uncontaminated reference site (King Creek) determined by densitometric analysis of immunoblots probed with mAb C219.

	Site		
Date	Atlantic Wood	King Creek	Relative increase
July 1996	374 ± 167[a]	115 ± 11	3.25
October 1994	199 ± 66[a]	75 ± 52	2.65

Values are arbitrary Pgp units per microgram of liver protein, average of five livers from each site ± SD.
[a]Significantly different from reference site (King Creek) (Student's t-test, $p < 0.5$). Relative increase is the ratio of expression in livers of Atlantic Wood fish to expression in King Creek fish.

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Detection of Pgp expression in mummichog liver tumours

Histologic sections of normal liver from either Atlantic Wood or King Creek mummichog stained with mAb C219 showed typical specific immunohistochemical staining of the canalicular surface of hepatocytes (figure 3 (A)). Consistent with previous results (Cooper *et al.* 1996), we observed no staining of mummichog liver or liver tumour sections when a matched isotype mouse myeloma protein was used in place of mAb C219 in the immunohistochemical staining protocol.

Many of the hepatocellular carcinomas and adenomas had obvious alterations in expression of P-glycoprotein antigens (figure 3 (B–D)). The histopathology of the eight adenomas and 16 hepatocellular carcinomas used here were described in previous studies (Vogelbein *et al.* 1990, van Veld *et al.* 1992). The majority of these lesions had elevated immunohistochemical staining, indicating over-expression of Pgp (figure 3 (B–D)). Ten of the 16 hepatocellular carcinomas and six of the eight adenomas had clear over-expression compared with the adjacent parenchyma while the remainder had reduced or approximately equivalent expression to surrounding liver. Many carcinomas had aberrant localization of the Pgp antigen including cytoplasmic staining (figure 3 (C and D)), and varying degrees of loss of polarity ranging from an increase in size of the region stained surrounding the bile canaliculus (figure 3 (B)) to staining of the entire plasma membrane (figure 3 (D)). We saw no evidence of altered expression of Pgp in putative early preneoplastic lesions (altered foci) of Atlantic Wood mummichog (not shown) although many of the livers exhibited regional heterogeneity of staining for Pgp.

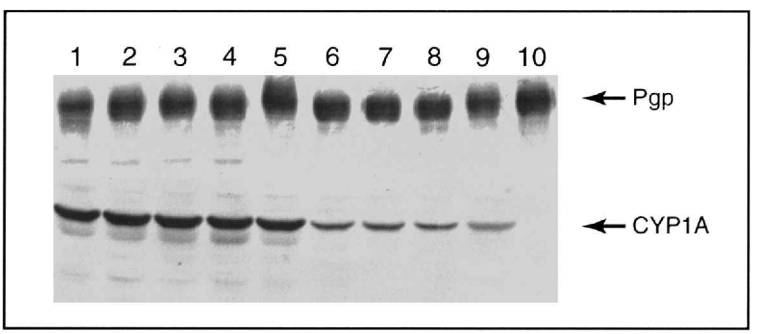

Figure 2. Immunoblot of liver extracts of mummichog (4 µg per lane) 36 h following i.p. injections of 3-methylcholanthrene in corn oil (lanes 1–5) or corn oil alone (lanes 6–10). Upper blot was probed with anti Pgp antibody (mAb C219). The lower blot was probed with anti-scup CYP1A (mAb 1-12-13).

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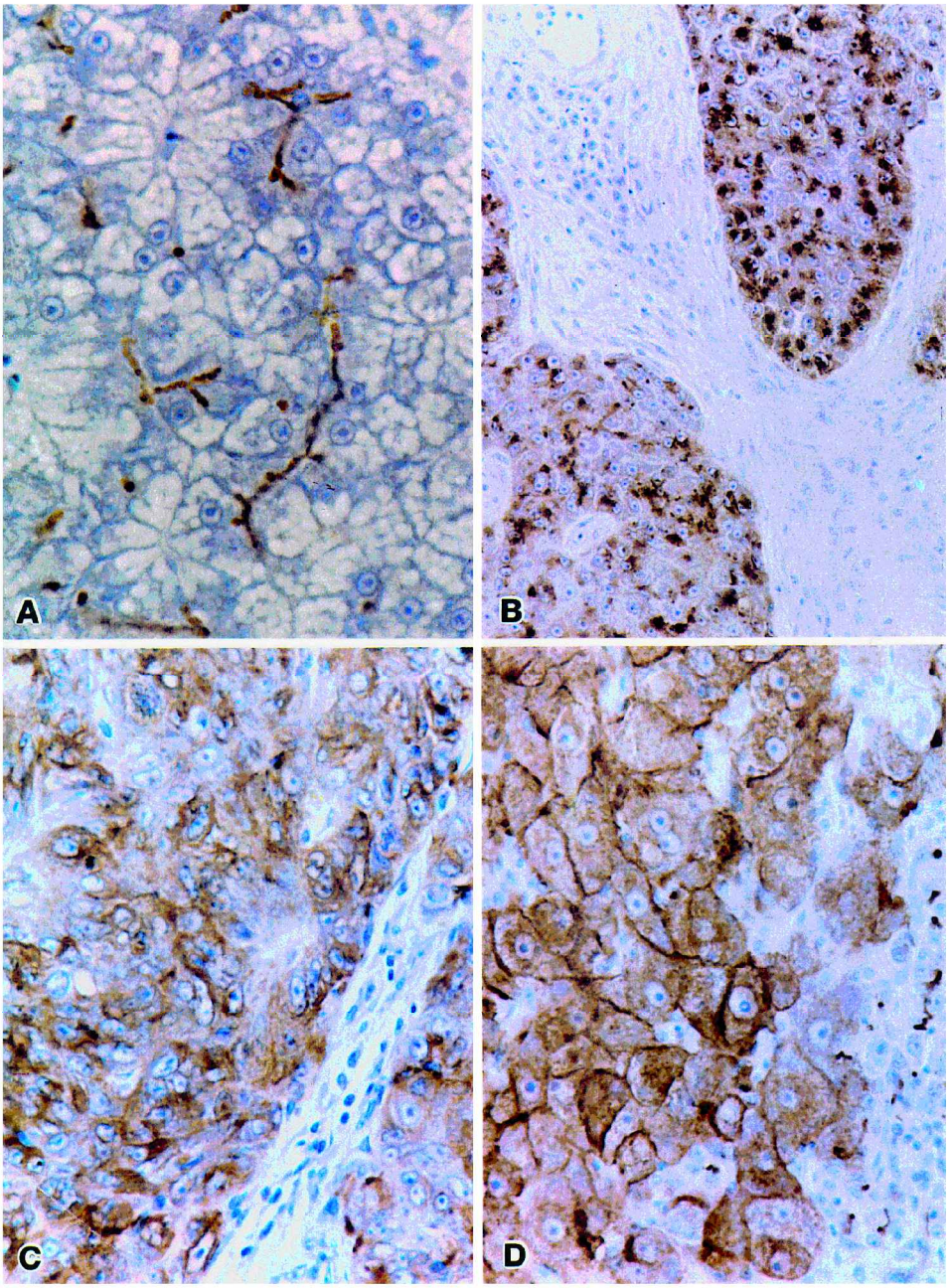

Figure 3. Normal liver from King Creek mummichog and neoplasms from Atlantic mummichog showing various types of mAb C219 immunohistochemical staining for Pgp. Positive immunoperoxidase stain is reddish brown. (A) Normal liver from King Creek mummichog showing canalicular staining for Pgp. (B), (C) and (D) Hepatocellular carcinomas showing over-expression and degeneration of polar expression for Pgp including cytoplasmic staining and expression on the entire plasma membrane. Original magnifications; (A) 630x, (B) 250x (C) and (D) 400x.

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Discussion

We found increased levels of Pgp in livers and liver tumours of mummichog inhabiting an environment severely contaminated with creosote. Although there are reports of elevated levels of Pgp antigens in gill and mantle tissue of marine molluscs from contaminated sites (Minier *et al.* 1993, Kurelec *et al.* 1995, 1996), we believe this is the first report of elevated Pgp in a teleost from a polluted environment and the first report of Pgp over-expression in the tumours of a lower vertebrate. The molecular weight (170 kDa) and characteristic broad appearance of the labelled band in immunoblots, indicating a high degree of glycosylation, strongly suggest that the C219 monoclonal antibody specifically detects Pgp homologues in mummichog liver and that the increased staining seen in immunoblots of Atlantic Wood mummichog livers represents elevated levels of one or more Pgp isoforms. Immunohistochemical results reported here localize Pgp to the canalicular surface of hepatocytes in normal teleost liver. Similar staining patterns were reported for histologic sections of guppy liver using C219 antibody as well (Hemmer *et al.* 1995). Immunohistochemical staining patterns of teleost livers indicate that expression of Pgp in fish liver is homologous with the expression in mammalian liver and that Pgps are likely to be involved in biliary excretion in teleosts.

Thorgiersson and colleagues have shown that Pgp gene expression may be induced in mammalian liver by xenobiotics or their metabolites having biliary excretion pathways (Thorgeirsson *et al.* 1991, Gant *et al.* 1995). In fish as well as in mammals biliary excretion is an important route of elimination of PAH metabolites (Varanasi *et al.* 1989, Schintz *et al.* 1993). Thus elevated levels and activity of Pgp in liver may aid in elimination of PAH metabolites and allow these fish to turn over toxic PAHs more efficiently.

In order to test whether the elevated levels of Pgp in Atlantic Wood mummichog could be a result of environmental induction by carcinogenic PAHs present in creosote, we exposed reference site fish to the model PAH 3-MC. However we found no detectable increase in Pgp immunostaining in reference mummichog following exposure to 3-MC at a dose and duration that produced large increases in immunodetectable CYP1A. Previous studies reported elevated Pgp and CYP1A1 expression in mammalian livers following exposure to carcinogenic xenobiotics including several known aryl-hydrocarbon receptor agonists (Burt and Thorgeirsson 1988, Thorgeirsson *et al.* 1991). The PAH 3-MC has been shown to induce Pgp expression *in vitro* in primary rat hepatocytes cultures; however it apparently does not induce Pgp elevation *in vivo* at least in rats perhaps due to differences in metabolites *in vivo* and *in vitro* (Gant *et al.* 1991). Creosote itself is a complex mixture consisting predominantly of PAHs. However phenols and N-, S- and O-containing heterocyclics are also significant components (Mueller *et al.* 1989). Possibly exposure to these other compounds associated with creosote may cause detectable Pgp induction. Alternatively, elevated Pgp in normal liver of the Atlantic Wood fish may be an intrinsic component of their previously reported resistance to creosote (Williams 1994, Vogelbein *et al.* 1996).

We suggested that alterations in levels and activities of liver enzymes involved in biotransformation and detoxification may contribute to toxicity resistance in Atlantic Wood mummichog (van Veld and Westbrook 1995, Armknecht *et al.* 1998). These alterations include suppression of enzymes involved in both xenobiotic activation and production of reactive oxygen species (e.g. CYP1A) and

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elevation of beneficial phase II detoxifying enzymes such as GSTs (van Veld *et al.* 1991, Armknecht *et al.* 1998). Overall, the pattern of expression of mummichog proteins and enzymes studied thus far (CYP1A, GST, Pgp) is similar to that believed to contribute to xenobiotic resistance in the early stages of carcinogenesis (Buchman *et al.* 1985, Roomi *et al.* 1985, Fairchild *et al.* 1987, Farber 1991).

The over-expression and altered patterns of Pgp expression observed here in the majority of mummichog hepatic neoplasms are strikingly similar to those reported for mammalian liver tumours (Goldstein *et al.* 1989, Teeter *et al.* 1990, Bradley *et al.* 1992). Pgp over-expression thus is another biochemical property of mummichog tumours in addition to reduced CYP1A (van Veld *et al.* 1992) which is similar to biochemical alterations reported in mammalian liver tumours and associated with xenobiotic resistance (Fairchild *et al.* 1987, Farber and Rubin 1991, van Veld and Westbrook 1995). The cause of Pgp over-expression in mammalian hepatocarcinogenesis models is not well understood. While Pgp over-expression has been suggested to result from selective promotion of Pgp-expressing lesions (Thorgeirsson *et al.* 1987) similar to selection proposed for GGT and GST expression in such lesions (Farber and Rubin 1991), Pgp over-expression in liver tumours was also produced without promotion by xenobiotics (Teeter *et al.* 1990, Bradley *et al.* 1992), and there is good evidence that Pgp over-expression in liver tumours is related to oncogene activation and tumour progression (Burt *et al.* 1988, Teeter *et al.* 1990, Bradley *et al.* 1992). Within the 90 livers we examined, only the most progressed lesions (hepatocellular adenomas and hepatocellular carcinomas) over-expressed Pgp while we saw no elevation of Pgp in several categories of altered foci (Vogelbein *et al.* 1990) which are presumed to be preneoplastic lesions. Therefore our results suggest that Pgp over-expression in these environmentally-induced fish tumours, as in mammalian liver tumours, is related to tumour progression rather than selection of expression early in carcinogenesis. On the other hand, the elevation or induction seen in normal liver of these fish and the intense over-expression seen advanced neoplasms probably result from different causes.

Understanding of the pathways involved in and relevance of increased Pgp expression to the toxicity resistance will require prolonged exposure studies and characterization of the Pgps and other gene products expressed during carcinogenesis in these fish. In particular the question of which mummichog Pgp isoforms are elevated and expressed in mummichog liver and liver tumours needs to be addressed. It is important to note that the monoclonal antibody C219 used in the present study recognizes a highly conserved linear epitope present in all known P-glycoproteins (Kartner *et al.* 1985). In mammals this includes the Mdr1, Mdr2 and Spgp proteins, all of which are expressed in liver (Thiebaut *et al.* 1989, Cordon-Cardo *et al.* 1990, Smit *et al.* 1994, Childs *et al.* 1995). The P-glycoprotein genes of lower vertebrates are poorly known. There is evidence for two Pgp-related genes in teleosts (Chan *et al.* 1992, Ling *et al.* 1992). Partial genomic clones of an *spgp* homolog (*fpgpA*) and what may be the single teleost *mdr* gene (*fpgpB*) were reported from winter flounder (Chan *et al.* 1992, Childs *et al.* 1995). Since both of these teleost sequences also contain the C219 epitope in their deduced amino acid sequences, it seems likely that in normal mummichog liver we are detecting both Spgp and Mdr expression. Elevation of either one or both of these genes may have adaptive value as they both may increase biliary excretion. Possibly different gene products are elevated in tumour and normal liver. Identification of which gene

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products are elevated will require the development of gene specific probes. Work is under way to clone and characterize the specific Pgps expressed in mummichog liver.

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