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Assessment of Environmental Degradation by Molecular Analysis of a Sentinel Species: Atlantic Tomcod

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NEOPLASIA IN FERAL FISH POPULATIONS

In recent years, reports of epizootics of neoplasia in North American feral fish populations have increased dramatically. These episodes have been observed in marine, estuarine, and freshwater species. In all cases, these outbreaks have afflicted populations in waterways impacted from the anthropogenic discharges from highly urban or industrialized areas. Some of the more publicized and studied cases have involved winter flounder from Boston Harbor,[1] English sole from Puget Sound,[2] brown bullhead from the Great Lakes,[3] and Atlantic tomcod from the Hudson River.[4,5] Hepatocellular carcinomas have been the predominant lesion and, given the liver's proclivity to accumulate and process xenobiotics, this tissue specificity is suggestive of an environmental etiology for the disease. Follow-up studies have correlated the incidence of neoplasia in these fish with elevated sediment, tissue, or metabolite concentrations of organic pollutants, most often polycyclic aromatic hydrocarbons (PAHs).[6] Attempts to correlate neoplasia

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with tissue concentrations of PAHs have, however, been thwarted by the extremely rapid metabolism of this class of xenobiotics by fishes.[7] The apparent sensitivity of selected finfish species to environmental insult and increased concern over environmental degradation have led to the suggestion that sentinel aquatic species may serve as surrogates to monitor the health of our nation's waterways.

POLLUTION AND HUDSON RIVER FISHERIES

The Hudson River serves as a domestic water supply, supports commercial fisheries, provides recreational opportunities, and is in close proximity to a vast urban population. The Hudson has been subject to environmental perturbation from a myriad of xenobiotic sources for decades. These have included organic pollutants such as dibenzofurans and dioxins,[8] PAHs,[9] polychlorobiphenyls (PCBs),[10] and heavy metals.[11] Illustrative of the problem is the designation of two Superfund sites within the confines of the Hudson River estuary. These include the Hudson River PCB Superfund Site which extends from Hudson Falls, NY to the Battery in New York City, a distance of over 200 river miles, and the Foundry Cove Superfund site in Cold Spring, NY (river mile 55) because of remarkably high levels of sediment-borne cadmium and nickel.

Historically, the Hudson River has supported lucrative commercial fisheries for three species of anadromous fishes: striped bass, American shad, and Atlantic sturgeon. Currently, commercial fishermen ply their trade from off upper Manhattan Island to at least Catskill, NY (river mile 125). At the present time, American shad and striped bass populations in the Hudson are at or near record levels of abundance, while sturgeon abundance is probably in moderate decline. Yet, despite the availability and potential lucrative commercial values of these fisheries, commercial fishing is prohibited for resident Hudson River species and striped bass due to their elevated tissue levels of contaminants, primarily PCBs and heavy metals. Recently, PCB concentrations have declined in some Hudson River finfish species; however, overall levels within populations have not reached acceptable standards for human consumption.

USE OF BIOMARKERS IN AQUATIC SYSTEMS

The development and validation of biomarkers in sentinel species has become popular in recent years due to their potential ability to quantify actual exposure history, provide a temporal framework for this exposure, and determine a threshold biological response and possibly detrimental biological consequences. The biomarker approach provides several advantages over the measurement of environmental or tissue concentrations of contaminants. In aquatic systems, these might include integration of bioavailable sediment-borne contaminant levels within a river or estuarine system, possible hypersensitivity of the bioresponse, reduced cost, and most importantly evaluation of an endpoint of a quantifiable biological effect.

Biomarkers which have been evaluated in fish span the range of biological organization from the molecular to the community level. Biomarkers on the molecular level offer the advantage of rapid and often sensitive responses to xenbiotics and a degree of specificity in the response. Molecular biomarkers quantify the level of expression or structural alterations in environmentally responsive genes at the level of their messenger RNA (mRNA). The applicability of this approach to aquatic organisms is currently limited by the number of gene probes available; however, this battery is increasing rapidly. Our studies have focused on an evaluation of molecular biomarkers in Atlantic tomcod as indicators of exposure to Hudson River-borne contaminants and point sources of pollution in other northwest Atlantic estuarine systems.

CHOICE OF SENTINEL SPECIES: THE ATLANTIC TOMCOD

The Atlantic tomcod is a very common bottom-dwelling fish species in the Hudson River whose overall distribution extends from Labrador to New Jersey.[12] The Hudson River supports their southernmost spawning population and, as a result, at times they may be thermally stressed. Tomcod are not a commercial species; however, recreational fishermen frequently target them within the Hudson River. Atlantic tomcod are anadromous and spend their entire life cycles within the confines of their natal estuaries.[13,14] Within these estuaries, adult tomcod undergo winter spawning migrations to the upper reaches of the estuary, and juveniles and surviving adults then drop back to more saline reaches of the rivers for summer feeding. Tomcod are opportunistic feeders, their diet consisting of many available bottom-dwelling food species. Atlantic tomcod also have unusually high liver lipid concentrations which serve to bioaccumulate many xenobiotics of concern due to their lipophilic nature. Additionally, tomcod collected from the Hudson River have increased hepatic liver lipid levels, perhaps indicative of elevated detoxification of organic xenobiotics.[15] As a result of these factors, their exposure history and physiology potentially integrates the contaminant levels throughout the estuary and its food chain.

The Atlantic tomcod population in the Hudson River exhibits a truncated age class structure in comparison with tomcod from other rivers. More than 90% of the population is comprised of fish up to 1 year of age;[5] 3-year-old tomcod are exceedingly rare in the Hudson (0.1% of the population), although tomcod up to 7 years of age are reported from more northern populations.[16]

Atlantic tomcod collected from the Hudson River have one of the highest incidences of neoplasia in any feral population. For example, >50% of 1-year-old Hudson River collected tomcod exhibit hepatocellular carcinomas and >90% of 2-year-olds display this disease.[5] In contrast, <5% of tomcod from the Pawcatuck River, Rhode Island, or the Saco River, Maine, exhibited liver lesions.[17] To date, liver lesions have not been observed in tomcod from more pristine rivers in the Canadian Maritime Provinces, although other diseases have been observed.[18] The

frequency and severity of liver tumors in Hudson River tomcod increases directly with the age and length of the fish such that within an age class, the frequency of tumors is significantly higher in larger fish.[5] All of these factors suggest an environmental etiology to the elevated level of neoplasia in these fish. Thus, it has been suggested that Atlantic tomcod may serve as an excellent sentinel species of tidal river systems in the northeastern U.S. and in the Canadian Maritime Provinces.[17]

GENETIC ALTERATIONS IN HUDSON RIVER TOMCOD

Initially, we sought to determine if Hudson River tomcod constitute a separate population, perhaps genetically predisposed to neoplasia; or was the population subjected to insult from environmentally borne carcinogenic agents? The NIH3T3 transfection assay was used to determine that tomcod liver tumor DNA could transform these mouse fibroblasts *in vitro*. Soft agar assays confirmed the anchorage independence of these transformed cells and their tumorigenicity was demonstrated in nude mouse tumor assays. The injection of primary transfectant cells from the NIH3T3 assay into athymic mice resulted in the rapid generation of very large tumors from all cell lines tested. Furthermore, in Southern blot analysis, DNA isolated from these nude mouse tumor DNAs exhibited an exogenous tomcod K-*ras* oncogene.[19] This was the first demonstration of an activated oncogene in any feral population of fish and was followed by reports of activated *ras* oncogenes in winter flounder from a highly PAH polluted site in Boston Harbor[20] and in aflatoxin-treated rainbow trout.[21] Polymerase chain reaction (PCR) analysis of panels of tumor DNAs from both the winter flounder and rainbow trout revealed mutations at the 12th codon of the *ras* gene, a sequence consistently mutated in tumor DNA from animal models treated with chemical carcinogens and in human populations. Surprisingly, the same rodent-derived PCR primers used successfully on flounder and trout DNA did not amplify *ras* in tomcod. As a result, we have sequenced the tomcod *ras* gene from a cDNA library.

In addition to this somatic mutation in the *ras* oncogene in tomcod tumor DNA, normal tomcod liver DNA revealed a restriction fragment length polymorphism (RFLP) in the c-*abl* oncogene.[22] Allelic variation was observed at two of the three c-*abl* domains scored. Hardy-Weinberg analysis confirmed the Mendelian inheritance of these c-*abl* hybridizable genotypes. Surprisingly, the frequencies of c-*abl* genotypes differed significantly between the cancer-prone Hudson River and cancer-free Maine tomcod populations. While the relationship between this RFLP in the *abl* oncogene and tumorigenesis in tomcod has yet to be investigated, this was among the first demonstrations of a difference between feral populations in the frequency of variant oncogene genotypes. This difference was even more surprising in view of the results of an RFLP analysis of mitochondrial DNA (mtDNA) between the same tomcod populations.[22] Mitochondrial DNA evolves approximately an order of magnitude more rapidly than single copy nuclear DNA genes[23] (see Chapter 2), and thus it would be expected that mtDNA polymorphisms would be numerous between the Hudson and Maine tomcod populations given the difference in oncogene genotypes. To the contrary, we found no variation in the mitochondrial genome within or between these two tomcod populations, a finding consistent with the short evolutionary time elapsed since their divergence. Considered against this background of mtDNA monomorphism, the divergence in c-*abl* oncogene genotypes between the two populations probably was a very recent event and highlights the hypermutability of the tomcod nuclear genome or a barrage of environmental insult within northeast estuarine systems.

USE OF CYP1A INDUCTION IN ENVIRONMENTAL MONITORING

For the past 15 years, studies conducted on levels of cytochrome P450 gene products in fish from contaminated environments have generally shown a positive correlation between overall levels of contamination and expression of the gene.[24,25] Thus, it has been proposed that induction of the cytochrome P450 system could serve as an effective biomarker of exposure and effect to selected organic pollutants. The cytochrome P450 system encodes for protein products responsible for the metabolism of both endogenous and exogenous substrates. Phase I enzymes encoded for by this system oxidize xenobiotic agents to an inactive form in which they may be excreted from the body by the action of Phase II conjugative enzymes. Alternatively, classes of environmental procarcinogens are activated to their penulimate carcinogenic state, diolepoxides, by CYP1A enzymes.[7] Activated carcinogens then form DNA adducts at critical genetic loci, protooncogenes or tumor suppressor genes, and initiate the neoplastic process. Thus, it may be argued that induction of CYP1A gene expression is not only indicative of xenobiotic exposure, but also detrimental biological effect.

Levels of CYP1A transcription in fishes may be induced by several classes of organic pollutants. These include polycyclic aromatic hydrocarbons, coplanar PCB congeners, and dibenzofurans and dioxins.[26] Therefore, it has been suggested that levels of CYP1A gene products can serve as effective biological markers of exposure to these environmental toxicants.[27] Levels of CYP1A induction may be quantified by measurement of its enzyme activities (EROD or AHH), quantification of CYP1A protein concentration by western blotting, or analysis of CYP1A mRNA by northern or slot blot analyses. Given the incidence of liver cancer in Hudson River tomcod, the presumed role of CYP1A enzymes in the neoplastic process, and their responsiveness to organic pollutants of concern, we proceeded to evaluate CYP1A gene expression as a potentially sensitive marker of exposure and early biological effect.

To date, most studies using CYP1A induction to monitor environmental levels of these xenobiotics have focused on the protein level. Potential use of CYP1A mRNA levels as a biomarker of exposure requires an examination of several aspects of the response. These include quantification of the sensitivity of the CYP1A mRNA response in comparison to levels of induction detected of the CYP1A proteins. What are the kinetics of induction and clearance of CYP1A

mRNA in fish treated with known inducers? Is induction of CYP1A mRNA sufficiently persistent to serve as an effective biomarker? Can differences among inducers in temporal aspects of induction and clearance of CYP1A mRNA be used to identify environmental inducing agents? How much genetic variability is there in the inducibility of CYP1A mRNA? This can be measured between species, populations, and among individuals within a species. Background levels of intraspecific variability in CYP1A mRNA inducibility in feral populations need to be quantified.

Levels of CYP1A mRNA in Tomcod Collected from Natural Systems

Initially, levels of hepatic CYP1A mRNA were quantified in tomcod collected from the Hudson River and other more pristine estuarine systems to determine if levels of gene expression were sufficiently sensitive, but also followed gradients in known levels of contamination among systems and therefore validate its use as a suitable biomarker of exposure. Detectable levels of CYP1A mRNA were observed in almost all individuals, even those collected from pristine rivers. Not unexpectedly, levels of hepatic CYP1A mRNA were significantly higher in tomcod collected from the Hudson River than seen in fish from all other rivers. The extent of variability in the response among Hudson River fish was substantial, whereby some individuals exhibited a highly induced phenotype and other fish showed very low levels of CYP1A mRNA.[28] This may have reflected genetic variability among individuals in terms of inducibility of the CYP1A gene or difference among individuals in their recent exposure history. The Margaree River drainage, Nova Scotia, has no urbanization or industrialization, and Margaree-collected tomcod showed extremely low levels of CYP1A mRNA. No variability was seen in the response among the Margaree-collected tomcod; all fish showed low levels of gene expression. Tomcod collected from the St. Lawrence River, Quebec, the Saco River, Maine, and the Miramichi River, New Brunswick, showed intermediary levels of CYP1A mRNA consistent with their moderate levels of anthropogenic influences.[29] These levels were significantly higher than levels of gene expression detected in Margaree tomcod, yet lower than levels detected in Hudson River-collected fish. Once again, interindividual variability in the response was considerable.

Additionally, levels of CYP1A mRNA expression were found to be very low in two other species of Hudson River-collected fish, hogchokers and striped bass.[30] Hogchokers are a bottom-dwelling flatfish and share a similar ecological niche and exposures as tomcod and would therefore be expected to exhibit comparable levels of CYP1A expression. Striped bass are a mid-water feeding species and therefore their low levels of CYP1A mRNA expression were not unexpected.

To validate the responsiveness of enhanced CYP1A mRNA expression to levels of environmental contaminants, two other biological markers were measured in a subset of these samples, including levels of fluorescent aromatic compounds in their bile and levels of hepatic DNA adducts. Levels of fluorescent aromatic compounds (FAC) determined by HPLC with fluorometric detection at a wavelength pair appropriate for higher molecular weight PAC such as benzo[a]pyrene and normalized for bile protein concentrations were significantly higher in tomcod from the Hudson River than in St. Lawrence or Miramichi River fish. For example, levels of FACs were approximately eightfold higher in the Hudson River than in Miramichi River tomcod. Levels of hepatic DNA adducts as detected by [^{32}P] postlabeling analysis followed the same general pattern. For example, levels of DNA adducts (nmol adducts/mol DNA) were between 10- and 40-fold higher in Hudson River tomcod than seen in Margaree or Miramichi River fish. Interestingly, levels of hepatic DNA adducts did not decrease significantly in Hudson River-collected tomcod that were depurated for more than 20 days in clean water. Additionally, hogchokers collected from the Hudson River, which did not exhibit induced CYP1A mRNA expression or liver cancer, had levels of hepatic DNA adducts that were comparable to those in Hudson River tomcod.[29] Not only did these results with alternative biomarkers confirm the exposure history of these fish, they also helped in identifying the CYP1A mRNA inducing agents in these systems. Both the FAC and DNA adduct analyses are only sensitive to PAH exposures and thus suggested that PAH exposure contributed to the elevated levels of CYP1A mRNA in Hudson River tomcod.

We also examined the rate of clearance of induced CYP1A mRNA in tomcod collected from a Hudson River site and transferred to clean laboratory water. Levels of expression in fish sacrificed immediately after collection were high. Surprisingly, levels of CYP1A mRNA decreased very rapidly upon transfer: by 4 hr a slight decline in expression (15%) was observed, and by 8 hr a 75% reduction; by 1 to 5 days, levels of gene expression approached basal levels for all individuals.[28] This rapid rate of CYP1A clearance in Hudson River-exposed fish indicates their likely exposure to rapidly metabolized compounds, such as PAHs. Levels of CYP1A mRNA remained at uniformly low basal levels for tomcod maintained in laboratory water for up to 120 days after collection. All depurated fish showed low levels of CYP1A mRNA expression.

Genetic Polymorphism in the CYP1A Gene in Atlantic Tomcod

During the course of quantifying levels of Hudson River tomcod CYP1A mRNA in northern blot analysis, it became evident that there was variability in the number of CYP1A hybridizable mRNA bands among individual fish.[31] All tomcod displayed a 3.0 kb CYP1A mRNA band, while variant individuals exhibited an additional 2.2 kb CYP1A mRNA band. Southern blot analysis was conducted on genomic DNA from these same individuals using a battery of six different restriction enzymes to determine if tomcod with the variant CYP1A mRNA also displayed a polymorphism in the structure of the CYP1A gene. DNA from all tomcod with the normal single 3.0 kb CYP1A mRNA band exhibited a single CYP1A hybridizable DNA band with all six restriction enzymes. All tomcod with

he second variant mRNA band exhibited an extra DNA fragment in relation to omcod with the common CYP1A genotype with five of the six restriction enzymes tested. The molecular size of the second variant DNA fragment was approximately 500 to 600 bp smaller than the normal DNA fragment. Thus, the size difference among DNA fragments was consistent with that observed among mRNA bands. The fact that five of six restriction enzymes tested revealed this smaller DNA fragment in variant individuals suggests that this polymorphism did not represent a single base substitution. These results suggested that this polymorphic DNA fragment represented a second CYP1A allele that contains a 500 bp deletion in comparison to the common CYP1A allele.

Densitometry revealed approximately equal concentrations of both CYP1A hybridizable mRNA bands in polymorphic individuals. Additionally, the optical densities of both CYP1A hybridizable DNA fragments in variant individuals were equivalent following high stringency wash conditions, suggesting equal homology between these two DNA sequences and the 3-MC induced rainbow trout CYP1A cDNA probe.[31] Thus, it is likely that both mRNA transcripts are equally expressed and are both products of the CYP1A gene.

Restriction fragment length polymorphisms in the structure of the CYP1A gene have also been detected in humans, and the frequency of variant genotypes differs among racial groups.[32] It has been hypothesized that the presence of the variant CYP1A genotypes may affect the susceptibility of individuals to cancer. Studies to date have provided mixed results suggesting that these CYP1A polymorphisms may impact significantly on genetic susceptibility to some cancers and not others.

We sought to determine if the CYP1A polymorphism in tomcod was ubiquitous throughout the species' distribution or restricted to the cancer-prone Hudson River population. Southern blot analyses of tomcod genomic DNA demonstrated that more than 10% of over 200 Hudson River tomcod analyzed displayed the CYP1A polymorphism. In all cases, these individuals also displayed the variant CYP1A mRNA. Further analysis determined that this polymorphism was absent in greater than 40 tomcod, each from four other populations: the St. Lawrence River, Quebec; Saco River, Maine; Margaree River, NS; and Miramichi River, NB.[33] Thus, this CYP1A polymorphism was restricted to the cancer-prone Hudson River population. Although it is tempting to speculate that this polymorphism plays a role in the neoplastic process, the functional significance of this variation has yet to be demonstrated. The fact that the variant allele is transcribed and harbors a large deletion suggests a possible selective disadvantage for this genotype. Furthermore, preliminary western blot studies have indicated that the variant CYP1A mRNA is translated to a second CYP1A protein recognizable by a CYP1A monoclonal antibody. Additional studies underway to assess the significance of this polymorphism include a comparison of EROD activity and CYP1A mRNA inducibility in beta-naphthoflavone (β-NF) treated fish and a comparison of the DNA sequence of the variant and normal tomcod CYP1A alleles.

The Effects of Prior Exposure or Genetic Variability in CYP1A mRNA Inducibility

To gain information concerning the identity of environmental inducing agents, Hudson River-collected tomcod were depurated for 20 to 30 days and subsequently treated with pure chemicals to determine which were able to induce CYP1A gene expression. After this period of depuration and prior to treatment, a subset of these fish were sacrificed and expression of their CYP1A mRNA was determined to be at basal levels. A single i.p. injection of β-NF induced a rapid and strong response; levels of gene expression approximated that seen in other species of fish treated with the same compound.[34,35] Unexpectedly, similar treatment of Hudson River-collected tomcod with two halogenated aromatic hydrocarbons, 2,3,7,8-TCDD (dioxin) and 3,3′,4,4′-TCB (coplanar PCB congener 77) did not result in induction of CYP1A mRNA.[36] In contrast, studies have demonstrated the inducibility of the CYP1A gene in other species of fish by i.p. injection of equivalent doses of these two compounds.[37,38] To determine if more extensive depuration, and thus additional opportunity for metabolism of resident hepatic xenobiotics, would permit induction of the CYP1A gene, a second set of Hudson River-collected tomcod was depurated for 120 days and again treated with 3,3′,4,4′-TCB. Despite extensive depuration, these fish did not exhibit induced CYP1A mRNA.

To determine if the noninducibility of CYP1A mRNA in Hudson River tomcod is a function of prior exposure history or a genetic difference between tomcod and other fish species, CYP1A gene inducibility was evaluated in tomcod from a second population. In this case, tomcod were collected from the Miramichi River, New Brunswick, depurated for 20 to 30 days, and treated with doses of 2,3,7,8-TCDD and 3,3′,4,4′-TCB equivalent to those used on Hudson River fish. Levels of CYP1A mRNA were highly induced in these Miramichi River tomcod collected subsequent to treatment with these two halogenated aromatic hydrocarbons, demonstrating that the CYP1A gene could be induced in tomcod by these compounds. The question still remains as to whether the failure of the Hudson River tomcod to respond to these treatments resulted from genetic differences among tomcod populations or their prior exposure history. Monosson and Stegeman[39] observed a similar phenomenon in winter flounder collected from two sites, pristine offshore Georges Bank and a moderately polluted river in Narragansett Bay. Flounder collected from Georges Bank had very low P4501A protein levels, but were inducible by subsequent i.p. injection of 3,3′,4,4′-TCB. In contrast, flounder collected from Narragansett Bay had 80-fold higher levels of P4501A protein, but TCB treatment resulted in no significant change in their P4501A protein content. They concluded that this lack of response was due to the maximal or near-maximal induction in these Narragansett Bay fish as a result of environmental exposure to inducers.

In winter flounder, prior exposure to some environmentally borne compounds resulting in strong CYP1A protein induction may have acted to inhibit further

induction of gene expression. However, in tomcod, levels of CYP1A mRNA were not induced, and in fact were at very low levels prior to TCB treatment. This result at the transcriptional level would argue against prior CYP1A mRNA induction serving to inhibit additional increases in gene expression. Most intriguing is the observation that CYP1A mRNA could be induced in depurated Hudson River tomcod by treatment with β-NF, but not with the two halogenated aromatic hydrocarbons. CYP1A mRNA was inducible in Hudson River tomcod by β-NF despite the fact that hepatic levels of total PCBs and coplanar PCB congeners were very high, at levels comparable to those observed in Narragansett Bay flounder. For example, hepatic levels of TCB in Hudson River-collected tomcod ranged from 11 to 16 ng/g wet weight,[36,40] whereas the level of TCB in Narragansett Bay flounder livers was 8.0 ng/g wet weight.[39] Total coplanar PCB concentrations in Hudson River-collected tomcod ranged from 1.4 to 1.9 μg/g wet weight. These observations lead to the suggestion that perhaps separate molecular pathways lead to CYP1A mRNA induction in halogenated vs non-halogenated hydrocarbon treated tomcod.

Caging of Tomcod in Natural Environments

We tested the efficacy of CYP1A mRNA expression as a monitor of the effects of point sources of pollution or the general levels of contaminants in estuarine systems by reintroducing depurated caged tomcod into natural systems. For example, levels of CYP1A mRNA were measured in tomcod caged on two separate occasions in bleached mill kraft effluents on the Miramichi River, New Brunswick and compared to levels in fish caged at two sites downstream. Control tomcod were caged at a pristine upriver site not impacted by the mill's effluents. A second group of control fish consisted of unexposed tomcod maintained in the laboratory. Levels of CYP1A mRNA were low and did not differ between the laboratory maintained tomcod and those caged at the upriver site. During a winter exposure, with low flow levels in the river, tomcod caged in the mill's effluents exhibited a significant fourfold induction in levels of CYP1A mRNA over controls. A gradient in levels of induction was observed at the two downstream sites, three- and twofold induction, respectively, at stations 5 and 10 km downriver from the mill site. The observed gradient in levels of CYP1A mRNA at two downstream sites is consistent with a single point source of inducers in the system and subsequent dilution of the effluents with ambient river water. Significant 11-fold induction of CYP1A mRNA was also detected in tomcod caged at the mill site in the spring months; levels of gene expression were higher than those observed during the earlier winter exposure.[41] This seasonal difference in levels of CYP1A gene expression in fish caged at the same site could have resulted from several factors, both biotic and abiotic. Several studies have demonstrated that sex and maturational levels significantly impact on inducibility of CYP1A proteins in fishes[42] and, considering that tomcod spawn in midwinter, it is possible that seasonal differences in caging time may have impacted on levels of CYP1A mRNA. It is likely that spawning would have reduced levels of CYP1A mRNA in females during the winter exposure, thus lowering mean levels of gene expression in that group. Experiments are currently underway to quantify the effects of sex, maturation level, and season on levels of CYP1A mRNA inducibility in chemically treated tomcod. Additionally, the exposure and bioavailability of inducers in the effluents may vary seasonally due to differences in river flow which impacts on dilution factors and resuspension of sediment-borne xenobiotics. These results illustrate that seasonal variability must be considered when interpreting the results of caging experiments of feral fishes in natural environments.

We proposed that the rate of clearance of induced CYP1A mRNA in tomcod exposed at the mill site and subsequently depurated in clean water might permit identification of the inducing agents in the mill's effluents. To test this hypothesis, tomcod caged at the mill site for 14 days were transferred to clean water and subsets of these fish were sacrificed 1, 3, and 10 days later. CYP1A mRNA expression in these exposed and depurated tomcod was compared to levels in tomcod caged at the mill and upriver sites and sacrificed immediately after exposure. Levels of CYP1A mRNA in tomcod transferred from the mill site to clean water remained significantly induced for at least 3 days post transfer and even increased over time despite their depuration in clean water. Levels of CYP1A mRNA remained above control values (fourfold) for the entire 10-day duration of the experiment. How does this rate of clearance of CYP1A mRNA in these environmentally exposed tomcod compare to that observed in fish treated with pure chemicals? The rates of induction and clearance of CYP1A mRNA in killifish[34] and rainbow trout[35] treated with single i.p injections of b-NF were very rapid. Maximum gene induction was reached within 40 and 18 hr, respectively, with no secondary peak in gene expression evident. Basal levels of CYP1A mRNA expression in trout were reached by 48 hr post treatment. Clearly, the kinetics of clearance of CYP1A mRNA in tomcod caged at the mill site were very different from that seen in fish treated with a single dose of a rapidly metabolized PAH. In contrast, single treatments of rodents with the halogenated hydrocarbons 2,3,7,8-TCDD and the PCB Aroclor 1254, revealed persistently elevated levels for up to 30 weeks and secondary peaks in hepatic and lung CYP1A gene expression.[42,43] In combination, these results suggest that the temporal characterization of the kinetics of induction and clearance of CYP1A gene expression in exposed tomcod should provide insight into the identity of environmental inducers.

Characterization of Rates of Induction and Clearance of CYP1A mRNA in Tomcod Treated with Three Model Chemicals

Tomcod were collected from natural populations, depurated for 20 to 60 days in the laboratory, and i.p. injected with single doses of three model chemicals — 2,3,7,8,-TCDD (dioxin), 3,3′,4,4′-TCB (PCB congener 77), and β-NF — and then allowed to depurate in clean laboratory water.[44] Doses of each chemical were selected based on their ability to significantly induce CYP1A mRNA in earlier dose-response studies. Subsets of these chemically treated tomcod were sacrificed at

various times following treatment and levels of their CYP1A mRNA were determined. Not surprisingly, single treatment with the two halogenated hydrocarbons yielded a very different profile of CYP1A mRNA induction and clearance than seen in tomcod treated with β-NF. For example, initial significant induction (4- to 19-fold) of CYP1A gene expression in 2,3,7,8-TCDD-treated fish was not observed until 5 days post treatment. Induction levels remained constant from day 5 through day 14, then declined at day 18, followed by a secondary increase to maximum 7.4-fold induction at day 25. In contrast, in β-NF-treated fish, twofold induction of gene expression was detected by 8 hr, maximum induction was observed at 72 hr and expression returned to near basal levels by day 5. In several respects, treatment with the PCB congener 3,3′,4,4′-TCB resulted in a response similar to that observed with dioxin. For example, induction was persistent for the duration of the experiment with both halogenated hydrocarbons, with no decrease in levels of gene expression observed for the durations of the experiments (10 and 25 days). Additionally, treatments with both halogenated hydrocarbons resulted in an initial induction in gene expression, then decline followed by a second and more pronounced peak. Thus, the response with both halogenated hydrocarbons was characterized by persistent induction and a secondary increase in gene expression. We believe that these very different kinetics of mRNA induction and clearance in halogenated vs nonhalogenated hydrocarbon exposures provide signature profiles that could be used to identify environmental inducing agents.

Dose Response of CYP1A mRNA to Three Model Chemicals

In order to calibrate the response of CYP1A mRNA in environmentally exposed tomcod, fish were treated in the laboratory with environmentally relevant concentrations of three model chemicals; β-NF; 3,3′,4,4′-teterachlorobiphenyl (TCB, a coplaner PCB congener); and 2,3,7,8-TCDD (dioxin). In all cases, fish were i.p. injected with these agents and sacrificed at times determined to provide at least 50% of maximum induction. Maximum induction ranged from approximately 50-fold in the dioxin-treated fish to 160-fold in the PCB-injected tomcod. Maximum induction in the β-NF-injected tomcod was 100-fold. In both β-NF- and dioxin-injected tomcod, saturation in levels of CYP1A mRNA was observed at concentrations of 50 ppm and 5 ppb, respectively. Levels of CYP1A was still increasing in TCB-injected tomcod at a concentration of 10 ppm. Significant induction with these compounds was initially observed at the following concentrations: β-NF, 1 ppm; TCB, 100 ppb; and TCDD, 100 ppt. In all three cases, no threshold concentration of inducing agent was required before CYP1A mRNA induction was observed. These studies illustrated that significant induction of CYP1A mRNA was possible in tomcod exposed to environmentally relevant concentrations of these xenobiotic agents. Additionally, it was demonstrated that via this route of exposure and for all three model chemicals, no threshold concentration of inducer was required to elicit an induced response at the transcriptional level.

CONCLUSIONS AND RECOMMENDATIONS

Clearly, CYP1A mRNA induction in Atlantic tomcod is a sensitive marker of exposure and early biological effect. Monitoring can be conducted via three alternative strategies: surveying levels of CYP1A mRNA in fish collected from feral populations, *in situ* caging exposures at suspected contaminated sites, or laboratory exposures to field collected sediments. Field studies showed that levels of CYP1A mRNA expression correlated well with environmental levels of aromatic hydrocarbon contamination, and concordance was seen between relative levels of CYP1A gene expression and a second biological marker of exposure (bile metabolites detected by FACs) and a biological marker of advanced effect (levels of hepatic DNA adducts). Furthermore, elevated levels of DNA adducts, oncogene activation, and genomic DNA mutability in tomcod highlight the probable detrimental consequences of this xenobiotic exposure and processing.

Laboratory studies demonstrated that expression of CYP1A mRNA is sensitive to extremely low levels of exposure to xenobiotics and a dose response was observed between extremely low and saturating levels of inducers. Temporal characteristics of induction and clearance of CYP1A mRNA varied widely among classes of inducers and suggested that this relationship could be used to identify environmental inducers. In most cases, the persistence of the mRNA response was sufficient to validate its use as a marker of exposure in field studies.

However, our results highlight several precautions which should be considered when interpreting results from environmental monitoring programs. First, genetic differences in the inducibility and activity of CYP1A mRNAs should be quantified and evaluated before instituting environmental monitoring programs. This variability exists at varying levels of taxonomic comparisons, including among individuals within a population, among populations, and among species. We observed significant variation among individuals from a single genetically defined population and between species in fish exposed under laboratory controlled conditions to pure model inducers. Furthermore, levels of gene expression varied widely among induced individuals from feral populations exposed to environmentally borne inducing agents. Because of the large interspecific variability in CYP1A inducibility, selection of the appropriate sentinel species is critical. Additionally, variation in the structure of the CYP1A mRNA and protein suggests that the biological consequences of induction may vary widely among individuals. However, a definitive answer to this question awaits characterization of the variant proteins, quantification of their enzymatic activities, and evaluation of more advanced biological effects such as levels of DNA adducts or oncogene activation.

Second, prior exposure history may be a significant factor impacting on levels of CYP1A gene expression in exposed populations of feral fishes. In two fish species, prior exposure to environmental inducers apparently inhibited subsequent increases in levels of CYP1A gene expression. In the case of Hudson River tomcod, this inhibition of further gene induction at the mRNA level was observed

despite low levels of CYP1A mRNA. In the case of winter flounder, inhibition of additional CYP1A gene expression and even reduction of CYP1A enzymatic activity was detected in fish which already exhibited induced levels of CYP1A proteins. Thus, it is possible that different mechanisms of inhibition of CYP1A induction are operative in different species. Alternatively, these interspecific differences in levels of gene expression in fish from contaminated sites prior to subsequent treatment with inducing agents may have resulted from the gene products examined. *De novo* CYP1A mRNA and protein synthesis may have shut down, and induced protein levels may have resulted from their decreased lability. In either case, it is clear that further characterization and understanding of this inhibitory effect is needed to interpret the results obtained from environmental monitoring programs.

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Molecular Epidemiology of Common Diseases

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INTRODUCTION

Etiology-oriented epidemiologic research has been successful in many areas; for example, many risk factors of cardiovascular diseases and carcinogenic agents have been established, which has helped to design preventive measures.[1,2] However, even modern epidemiological methods have limitations when multiple etiologic factors and small risk ratios are involved. Furthermore, epidemiologic findings rarely establish the mechanism of the disease. The lack of mechanistic handles has been called "the black box" (Figure 1). It has been suggested that the opening of the black box by the exploitation of molecular biology will be a key to future success in etiology-oriented epidemiological research.[3,4]

Over the years, epidemiology and biochemical research have conceptually interacted with each other. Epidemiologic research, the identification of causes and risk factors, has provided clues for biochemists to study, and vice versa. Examples of studies where biochemical parameters have been extensively used to refine epidemiological studies include those where blood lipids, vitamins, and microelements have been correlated to risks of cardiovascular disease and cancer.

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