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Issues Related to the Use of Fish Models in Toxicologic Pathology: Session Introduction

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ABSTRACT

Ready or not, fish models are "here to stay." No longer are fish confined to a few specialized laboratories, nor are they exclusively the purview of zoologists or environmental toxicologists. In fact, the institution that does not house at least 1 fish facility is probably not at the forefront of cutting edge research. In toxicologic pathology, fish models are increasingly being used to provide high animal numbers at relatively low cost in carcinogenicity testing and developmental research, and to provide mechanistic information on fundamental cellular processes. In this session, we attempt to provide some perspective for the pathologist that is faced with planning or performing experiments or testing protocols using fish models, or with reading or interpreting fish studies. First, we cover how to approach fish studies from the contract laboratory standpoint, including sectioning, quality control, and GLP considerations. Then, we discuss specifics on the use of the rainbow trout, zebrafish, and Japanese medaka models. The rainbow trout has a rich history in carcinogenicity and mechanistic cancer research. Similarly, the 2 workhorses in the small fish category, zebrafish and medaka, have found their way into many laboratories doing developmental biology and genomics research as well as carcinogenicity testing. Some fascinating genetically altered fish models have been developed with both of these species. This manuscript provides a session overview of the use of small fish models in toxicologic pathology, along with some historical perspective on how these models have played a role in the current state of the science.

Keywords. Carcinogenicity testing; fish models; Japanese medaka; rainbow trout; zebrafish.

INTRODUCTION

The new century finds us more than ever before in an age of rapid discovery, development, and marketing of pharmaceuticals and other chemicals that affect humans and, ultimately, all other biological systems. Hence, the need for rapid safety assessment of these compounds as well as for mechanistic developments in basic research is greater than ever. Despite calls for reducing the numbers of animals used in these efforts, ever increasing regulatory requirements and awareness of product liability demand more precision in safety testing and necessitate the use of a greater variety of test protocols in *whole* animals. In environmental impact studies, the enhanced resolution afforded by technological advancements in analytical instrumentation in turn demands the assessment of lower and lower (ie, more realistic or "environmentally relevant") dose levels, thus requiring larger and larger numbers of test animals (higher "*n*").

At the same time, societal pressures against animal testing have squeezed toxicologists and risk assessors from the other direction. Short-term, in vitro assays have been useful in mass screening of potentially toxic compounds. Although many of these assays are rapid and economical, their validity has been limited somewhat by false positives and false negatives, and by an inherent inability to determine target organ-specific effects such as carcinogenicity or promoting activity (20). In addition, the total milieu including cell-cell and cell-matrix interactions is not included in such assessments. Because whole animal testing remains the benchmark for safety assessment and even basic research, the use of "alternative" animal models in toxicity and carcinogenicity testing has received considerable attention (40). In 1993, the US Congress instructed the National Institutes of Health to investigate the use of alternative animal models. Specifically, this called for *replacing* animals with in vitro tests, chemical reactions, and computer models; *reducing* the numbers of animals used; and *refining* current methods, emphasizing relief of pain, maximizing information obtained from each animal, and utilizing animals lower on the phylogenetic tree (40). In the year 2000, the National Toxicology Program Interagency Coordinating Committee on the Validation of Alternative Methods was established to assist in these efforts. Transgenic mouse models, such as the Tg.AC (zetaglobin promoted v-Ha-*ras*) transgenic mouse (48), will answer some specific questions that require using whole animals, but these specialized models are costly and often difficult to obtain in large numbers needed for bioassays.

FISH MODELS IN TOXICOLOGIC PATHOLOGY: HISTORICAL PERSPECTIVE

Perhaps because of their fecundity, small size, and economical maintenance and use, fish models are becoming well established in many laboratories. In fact, the facility that does not house at least 1 colony of zebrafish, medaka, or other fish species is probably not at the forefront of biomedical research (46). A number of recent reviews have pointed out the advantages of fish models for laboratory-based testing (1, 6, 21, 26, 38, 55). Small fish models have been shown to be sensitive to a variety of known carcinogens and have a short time to tumorigenesis, yet have an exceedingly low spontaneous tumor rate in potential target organs (16, 19).

Although it is widely recognized that aquatic ecosystems serve as the final sink for many chemicals, and that water serves as the ultimate vehicle for exposure to many toxic agents, relatively few methods exist to precisely and

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practically assess health risks from exposure to pollutants in the aquatic environment (55). In fact, it was historical observations of tumors in wild fishes that prompted the development of carcinogenicity testing utilizing small fish species in the laboratory. Harshbarger and Clark (14) documented 41 geographic regions in North America in which clusters, or epizootics, of cancer in wild fishes have occurred. The occurrence of neoplasms involving epithelial tissues such as the liver, pancreas, gastrointestinal tract, and some epidermal neoplasms appear strongly correlated with environmental contamination, that is, exposure to chemical carcinogens. Recent reviews provide more on these epizootics (5, 8, 9, 14, 15, 34). However, several reports of tumors in wild fish have been pivotal and deserve special mention.

English sole from contaminated areas of the Puget Sound, Washington, had high prevalences of liver lesions that ranged from megalocytosis to neoplasms (37). Several detailed studies (eg, 28–32) established statistically significant associations between the presence of polycyclic aromatic hydrocarbons (PAH) in the sediments and the prevalence of liver neoplasia. Malins et al (33) identified a novel DNA adduct, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, in neoplastic livers of English sole from carcinogen-impacted areas of the Puget Sound.

On the East Coast, epizootic hepatic neoplasia in winter flounder from Boston Harbor, Massachusetts, has been reported (35, 36). As in the case of the Puget Sound sole but not as firmly established, the hepatic lesions in the winter flounder were highly correlated with anthropogenic chemical contamination.

Although many incidences of cancer epizootics have occurred in fresh water fishes (5), none have been as well studied as the epizootics in the marine species the English sole and winter flounder. Epizootics of neoplasia in fish populations of brown bullhead catfish (*Ictalurus nebulosus*) and Atlantic tomcod (*Microgadus tomcod*) also should be noted. Sediments rich in PAH have generally been considered the principal causes of skin and liver neoplasia in brown bullheads in the contaminated Black River (Ohio), a tributary of Lake Erie (2–4). In laboratory tests, medaka exposed to extracts and fractions of PAH-contaminated sediments from tributaries of the Great Lakes, including the Black River, developed liver neoplasia (11). Similarly, epizootics of hepatic neoplasia have been reported from Atlantic tomcod from the Hudson River (7, 41). Those liver neoplasms have been associated with elevated tissue levels of polychlorinated biphenyls (PCBs) (22).

White suckers from industrially polluted areas of Lake Ontario exhibited increased prevalences of hepatic and skin neoplasia (17, 42). As in other epizootics, the neoplasms have been associated with PAH contamination. Stalker et al (43) showed that the progression of hepatocellular and bile duct neoplasms in the white sucker is accompanied by a loss of immunoreactive glutathione *S*-transferases that usually catalyze a major detoxification pathway.

Only a small number of reported cancer epizootics have dealt with small fish species. Vogelbein and colleagues reported high prevalences of liver neoplasms in mummichog (*Fundulus heteroclitus*) from a creosote-contaminated site in the Elizabeth River, Virginia (53). Exocrine pancreatic neoplasms also apparently induced by contaminant exposure were later reported in these fish (12, 52).

THE NEED FOR MECHANISM-BASED RESEARCH

Studies involving fish in toxicology currently use these models either as surrogates for human health problems or as indicators of environmental health. Human health and environmental health are, of course, inexorably linked, so the 2 concepts should not be separated. Instead, it is critical that we provide as much mechanistic information as possible in order to further validate these alternative test methods and take every advantage of their utility for many types of studies.

Mechanistic information garnered across phyletic levels may be more accurately applied to help substantiate findings from field work. Additionally, it may unlock untold mysteries of the basic mechanisms of cellular pathology and neoplasia (recall the wealth of information garnered on apoptosis from the lowly nematode, *C. elegans*) (13).

The basic metabolic machinery in small fish species, with regard to Phase I and Phase II metabolism, is similar to that in mammals. The Phase I metabolizing enzyme system, the cytochromes P450 (CYPs), have been perhaps the best characterized in aquatic species (44, 45). At this time, it appears that in fish only members of the CYP 1A subfamily are induced by environmental toxicants, and thus would have a major impact on the activation or detoxification of carcinogens (54). Similar to mammals, compounds such as polycyclic aromatic hydrocarbons, polyhalogenated biphenyls (PCBs), and polychlorinated dioxins have been shown to induce CYP1A in fish. One important difference to note between fish and mammals, however, is in the response to phenobarbital. Whereas phenobarbital classically induces the mammalian CYP2B subfamily, fish CYP2B appears to be refractory to phenobarbital induction (23, 24). Recent studies indicate that phenobarbital can instead induce CYP1A in fish, perhaps via enhancement of Ah receptor activation (10, 39). In a recent review, Williams et al (54) point out that xenoestrogens, an important class of aquatic pollutants, may alter the response to carcinogens in fish through modulation of CYPs.

While these relationships are best characterized in the rainbow trout model, comparatively little information is available for small fish models. CYP1A was found to be deficient in preneoplastic and neoplastic lesions in mummichog (*Fundulus heteroclitus*), an estuarine small fish model that shows great promise for environmental toxicology research (51). This same group recently demonstrated tissue-specific expression of CYP1A in mummichog exposed to benzo[a]pyrene in both aqueous and dietary exposures, and developed a grading system for CYP1A staining intensity (50). Studying the metabolism of trichloroethylene, a common groundwater contaminant, Lipscomb et al (27) found that CYP1A was readily detectable in medaka liver by immunohistochemistry, whereas CYP2E1 was present at very low levels.

Other enzyme systems have shown somewhat more variable results in fish studies. Immunostaining for gamma-glutamyl transpeptidase (GGT), an important enzyme marker in rodents, detected foci of cellular alteration in medaka exposed to DEN (18). However, GGT staining showed conflicting results in rainbow trout studies (6). Glutathione-*S*-transferase, an important Phase II biotransformation

enzyme, has also shown variable results in preneoplastic and neoplastic lesions in rainbow trout (6).

These reports support the utility of tissue-specific induction patterns for biotransformation enzymes in fish carcinogenesis research. However, it is also clear that there is a need for more information on these enzyme systems, particularly in small fish models. Future bioassays using these models should include a battery of immunohistochemical stains, such as those used by Van Veld et al (1997) or Lipscomb (1998). Immunostains could provide valuable mechanistic information on carcinogen metabolism and help delineate enzyme altered foci that might otherwise be missed using routine staining methods. Furthermore, basic research is needed on induction of enzymes by various classes of carcinogens in small fish models such as the CYPs, GST, DNA repair enzymes, and the caspases involved in apoptosis. This information will be forthcoming as long as adequate funding is maintained and balanced between basic, applied, and clinical cancer research.

A virtual explosion of information is occurring in the area of molecular biology with small fish models, much of which is beyond the scope of this paper. See recent reviews for further discussion of these developments (47, 49). These molecular findings can only serve to bolster the validation of these models. Recent discoveries have supported the use of small fish models for human health risks from the environment. For example, the *ras* oncogene of fish has a high homology to human K-*ras*; in goldfish, this homology is approximately 96% (49). Point mutations in Ki-*ras* occurred in a high proportion of rainbow trout liver tumors induced by aflatoxin B1, dimethylbenzanthracene (DMBA), methyl-*N* = -nitro-*N*-nitrosoguanidine (MNNG), and DEN. The *p53* tumor suppressor gene is another important example of molecular markers. The *p53* gene has recently been cloned and sequenced in medaka, but specific mutations have yet to be demonstrated (25). However, we have seen overexpression of the *p53* protein product in liver neoplasms in several fish bioassays (unpublished work). Stabilization of a nonfunctional form of *p53* protein is thought to account for the increased expression of *p53* in tumors, suggesting a failure of this important gatekeeper of the cell cycle.

Session Overview

In this session, we attempt to provide some perspective for the pathologist that is faced with planning or performing experiments or testing protocols using fish models, or with reading or interpreting fish studies. First, Jeff Wolf of Experimental Pathology Laboratories, Herndon, VA, discusses how to approach fish studies from the contract laboratory standpoint, including special sectioning considerations, quality control, and Good Laboratory Practices (GLP) considerations. Although universal GLP standards have yet to be officially established between laboratories that conduct fish studies, recent contract work conducted "in the spirit of GLP," including quality assurance and peer review, has gained rapid acceptance and helped in moving toward interlaboratory standardization.

Next, David Williams of Oregon State University, Corvallis, Oregon, discusses specifics on the use of the rainbow trout model. Williams reviews some of the fascinating work with rainbow trout carcinogenicity testing done predominantly at Oregon State University, including testing of dietary carcinogens and those with anticarcinogenic properties. Historically, this fish model provided some critical breakthroughs in the links between dietary aflatoxin exposure and liver cancer. Jan Spitzbergen, also of Oregon State in Corvallis, next provides an overview of the multitude of scientific accomplishments afforded by the zebrafish model, particularly in the molecular mechanisms of development. Spitzbergen also reviews the current state of toxicity and carcinogenicity testing with this model, and rightly articulates the need for training of veterinary pathologists that can help make sense of the explosion of transgenic animal models, including transgenic fish models. Last, William Hawkins, Institute of Marine Sciences, Ocean Springs, Mississippi, reviews some recent studies using the Japanese medaka. The medaka has emerged as the preeminent model for large scale carcinogenicity testing, and is helping to define the dose-response curve for genotoxic carcinogens at relatively low and perhaps more environmentally realistic exposure concentrations.

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