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Carcinogenesis Studies in Rodents for Evaluating Risks Associated with Chemical Carcinogens in Aquatic Food Animals

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Fish and shellfish caught in polluted waters contain potentially dangerous amounts of toxic and carcinogenic chemicals. Public concern was heightened when a large percentage of winter flounder taken from Boston Harbor was found to have visible cancer of the liver; winter flounder outside the estuary area had no liver lesions. Long-term chemical carcinogenesis studies could be easily and feasibly designed using laboratory rodents offered diets containing fish caught in polluted waters. Induced cancers in rodents would corroborate field observations in fish; positive results from these studies would provide further evidence about potential human health hazards from eating substantial amounts of chemically contaminated fish. Nonetheless, fish and aquatic organisms should be viewed as environmental biological monitors of pollution or of potential human health hazards, and authorities responsible for assuring clean and safe rivers, bodies of water, and biota should give more attention to these valid biological indicators or sentinels of environmental pollution. Consequently, fish and other sea creatures alone should serve as alarms regarding whether water areas constitute public health hazards.

Introduction

The increase in public awareness that fish and shellfish caught in polluted waters contain potentially dangerous amounts of toxic and carcinogenic chemicals (*1–4*) has led to greater attempts to determine the possible health hazards from eating contaminated mollusks, crustaceans, and fish. Concern on the East Coast was heightened when a large percentage of winter flounder (*Pseudopleuronectes americanus*) taken from Boston Harbor was found to have visible lesions in the liver (cholangiocarcinomas and hepatocarcinomas) whereas similar catches of winter flounder outside the estuary area had no liver lesions.

Fish and shellfish examined from other coastal and inland water areas of North America and around the world likewise contained worrisome concentrations of chemicals: coastal regions (*5*), coast of New England (*6*), West Coast (*7*), and various locations within and around the U.S. (*8*); Puget Sound, Washington (*9,10*), Hudson River estuary, New York (*11*), Yaquina Bay, Oregon (*12*), Delaware River (*13*), Great Lakes basin (*14,15*), and freshwaters (*16*) and various watch sites (*17*); and Port Phillip Bay, Australia (*18*), and North Atlantic areas, mainly the Irminger Sea (*19*). The distribution of indigenous fish having tumors is geographically focal, and the highest incidences occur near heavily industrialized areas (*20*).

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Design Strategy and Confounding Factors

What are the most reasonable (and practical) experimental options for determining the potential human health hazards from eating contaminated fish known to have chemically induced cancer of the liver (or other organs)? One option is to do absolutely nothing more. That is, fish and aquatic organisms should be viewed as environmental biological monitors of pollution or of potential human health hazards (*21*), and those responsible for assuring clean and safe rivers, bodies of water, and biota should give more attention to these valid biological indicators or sentinels of environmental pollution (*22*). Pritchard and Miller (*23*) underscore this necessity by emphasizing: "In our effort to identify, understand, and resolve pollutant problems, we cannot afford to overlook the potential of aquatic organisms to provide answers." Consequently, fish and other sea creatures alone should serve as alarms regarding whether water areas are relatively "clean" or are indeed "dirty," and constitute real public health hazards—hazards not only from the contaminated water and environs, but also from catching, cleaning, and eating chemically polluted biota in and around the oceans, seas, and other waters.

A second option would be to identify the chemicals within the fish, shellfish, and biota (sediments and natural diets), and then rely on published reports of their carcinogenicity as determined in long-term studies using rodents (*24–29*). This approach is fraught with uncertainty because many toxic and cancer-causing

chemicals have been detected; Malins et al. (*10*), for instance, identified 25 aromatic hydrocarbons, 26 chlorinated organic chemicals, 37 metals, and other elements in sediment and biota samples from Puget Sound.

So, the question remains, should long-term studies using contaminated fish be done in rodents? Key responses to both sides of this issue follow. No, because *a*) evidence from fish is sufficient to signal human health hazard concerns, *b*) fish should be regarded as environmental sentinels or biologic indicators of potential harm, *c*) feeding fish to rodents would be an insensitive and difficult assay, and *d*) negative results would not be equivalent to no hazard. And the obverse, yes, because *a*) induced cancers in rodents would authenticate field observations in fish, *b*) positive findings would identify and confirm potential human health hazards, and *c*) results in rodents are relevant to humans.

A third option, and the basis for this paper, is to use the well-established, long-term chemical carcinogenesis protocol in rodents (*30,31*) for examining the potential carcinogenic hazard of eating fish contaminated with cancer-causing agents. Few others have used this laboratory model for monitoring and identifying public health risks associated with the consumption of fish or fish products. Some investigators have exposed rodents to diets containing contaminated fish for 28 days (*32*), for 13 weeks (*33*), for 4 months (*34*), and for 102 weeks (*35*).

Beginning from a typical carcinogenesis protocol (*30*) and choosing the dietary (or feed) route of exposure, there are several obvious options to consider regarding what would be the most relevant "sample" to which the laboratory rodents should be exposed. Singly or in combination these are potential "samples": water, effluents (industrial and sewage), fish food sources (e.g., polychaete worms), sediments, whole fish, edible tissues/organs, affected organs (liver and kidney for example), stomach contents, or identified organic/inorganic chemicals as a representative core standard mixture; multiples of environmental concentrations; and drinking water as the exposure route. If one adopts the notion that whatever "sample" is selected could be viewed simplistically as a single "chemical," then the task of collecting and using the "chemical" becomes less problematic. Thus, "chemical" (fish) considerations would include *a*) supply (type fish or fish food, source, variability, transport, and frequency of delivery), *b*) storage (stability in various temperature and humidity conditions, in the basal diet mixture, and in the food hoppers), *c*) routes of exposure (intubation or feed), and *d*) form selected for exposure (whole fish, edible parts, or affected organs; raw, freeze dried, processed, cooked, or extracts).

Further, some have suggested that a simulated and analytically defined chemical mixture should be concocted (*10*), following the lead of Yang and colleagues for studying hazardous dump sites (*36–38*). This is not only extremely complicated and costly, it most importantly could not guarantee that the causative chemicals would be included, or that all chemicals would be ever identified or quantitated. Thus, *in situ* environmental samples should be used—whole fish meal, edible portions of fish, food source of fish (e.g., polychaete worms), or fish milieu (e.g., sediment, shellfish, and biota)—recognizing the fact that consistency of content is likely compromised, depending on sampling strategy. Nonetheless, with careful sample collection, the question of human hazard would be at least addressed by exposing laboratory animals to the *Gemisch* that humans are ordinarily exposed to from eating contaminated biota.

Other confounding factors include not knowing the chemical content of the samples, not knowing if the samples selected would be uniform from batch to batch or catch to catch, and not knowing if the samples (e.g., fish) would have "enough" chemicals at the time of catch to induce a biologic/toxicologic response. The latter uncertainty would argue for the use of sediment as "the chemical" (*39–41*), and because flounder are bottom feeders, these fish should be considered reasonably representative of sediment and ocean floor organisms (mainly polychaetes) that flounder eat. Masahito et al. (*21*) strengthen this conjecture: "bottom dwelling/feeding fish species have the highest rates of neoplasia ". . . and provide. . ." evidence that exposure to sediment-bound chemical carcinogens may play an essential role in tumor induction in these fishes." Nonetheless, no feral sample would conform close enough to selecting optimum concentrations that would approach those exposure levels used typically in long-term chemical carcinogenicity studies (*30,42*).

Suggested Experimental Outline

To study the potential adverse effects to humans from eating contaminated fish, a modified protocol is proposed that would likely be as sensitive as any for detecting chemically associated responses. Typically used in long-term chemical carcinogenesis studies are both sexes of two rodent species (Fischer 344 inbred rats and $B6C3F_1$ hybrid mice); 50 animals of each species, strain, sex per control and 2 to 3 exposure groups; and a duration of 24 months (*30*). Major differences from the core design include: one sex of each species [has been shown to identify at least 96% of the positive and negative carcinogenicity responses observed in 266 studies of both sexes of both species (*25*)], and in this design male rats and female mice provide for potential gender-specific influences (*43*); exposed groups contain 100 rats and 100 mice, using double the typical number per group to increase sensitivity; a single "fish-feed" concentration; and a prolonged duration of 30 months, 6 months longer than usual. Even so, this design would still be invariably insensitive (low power) for detecting carcinogenic effects, given that the chemical content of even using whole fish (including fat) would predict that only a mixture made up of particularly potent carcinogens would induce neoplasia in this experiment.

Estimates of the amount of fish needed to complete the long-term study indicated that sufficient fish should not be difficult to obtain. For this study a total of 3 to 5 tons of prepared diet would be needed; using a 25% proportion for both control and contaminated composition, about one or two tons of fish would be adequate: 660 to 1320 pounds of control fish and 1340 to 2680 pounds of contaminated fish. Scheduling, logistic, and quality assurance factors would have to be overcome to ensure a steady and consistent supply of enough (similarly aged/sized) control and contaminated fish caught within the same geographic boundaries. Transport to the study laboratory, storage, and diet mix preparations must be dealt with as well.

Further variables and potential difficulties include the amount of protein mixed in the diet, the palatability and stability of the diet mixture, the likely low concentration of the metabolically activated proximate or carcinogenic chemicals present in the fish

diet *Gimisch*, and the scientific veracity of negative results. High dietary protein levels (approximately 25%) over long periods lead to various kidney perturbations, often resulting in diffuse toxic nephropathy that may compromise study results (*44,45*). Villeneuve et al. (*32*) and Chu et al. (*33*) used concentrations of freeze-dried fish up to 5.8% of the rodent diet for 28 days or 13 weeks; Cleland et al. (*34*) used diets containing 33% minced adult coho salmon for 4 months; and Takahashi et al. (*35*) offered diets to hamsters with up to 40% fish meal pyrolsate. Thus, our selection of 25% (or 250,000 ppm) should be well tolerated.

Regarding the chemical residues in the fish to be fed to rats and mice, one needs to recognize that these bottom-dwelling winter flounder are exposed continually to a large number of both structurally similar and dissimilar chemicals and classes of chemicals known to be carcinogenic in rodents. Most chemicals need metabolic activation to exert their carcinogenicity. The major problem is that the fish when caught will actually contain only a relatively small amount of the overall exposure burden to the various chemical carcinogens. Accordingly, the tissue content will underrepresent the actual cumulative exposures to fish, and thus the studies will have "reduced" sensitivity. Because of this insensitivity and the less-than-optimum levels of chemical exposure, negative findings would be considered inadequate for judging absolute safety for humans ingesting diets made up primarily of contaminated fish and shellfish. Nonetheless, many wonder why one would even do a study of this type, given that the "real experiments" have already been done.

Carcinogenesis Studies Using Fish

A complementary area of expanding and exciting research centers on using fish or shellfish either in the laboratory or as environmental sentinels for the identification of chemically or pollution-induced cancers (*8,46–58*). Hendricks (*51*) compiled a particularly useful review of chemical carcinogenesis in fish. A later review of the importance of fish tumors (*21*) together with various monographs and symposia proceedings on fish toxicology indicate promise for developing and accepting these models in chemical carcinogenesis (*59–63*). Positive outcomes of these models would certainly fit the definition of a carcinogen introduced by Zwickey and Davis (*64*): "Carcinogens are those substances which produce a significant increase in tumor incidence when administered at any dosage level by any route of administration in any species of animal as compared to controls."

The use of mammals or fish and aquatic animals in carcinogenesis studies was debated successfully in a humorous but serious and reflective exchange between Dawe and Couch — "The Devil's Advocate" versus "The Fishy Side" (*65*). These authors agreed on a common ground of trying to achieve an understanding of the strengths and weaknesses of the two systems (i.e., rodents and fish) to allow use of the best aspects of each to greatest advantage (*65*). Further, the U.S. Environmental Protection Agency is studying the applicability of using medaka (*Oryzias latipes*) (*66,67*) in a validation effort on the reliability of detecting known mammalian chemical carcinogens and noncarcinogens. The project is ongoing with 26 chemicals in various stages ranging from still exposing medaka to data being interpreted; eventually 60 chemicals (48 carcinogens and 12 noncarcinogens) will be evaluated in this small (3.0–3.5 cm long and weigh 300–500 mg) Japanese fish (R. Johnson, personal communication, 1990).

Fish and Rodent Liver

As one example of morphologic comparability between fish (i.e., winter flounder) and rodents, the progressive sequence of lesions leading to liver neoplasms is similar in English sole to that in laboratory rodents exposed to various heptocarcinogens (*9*). Even though the structure of some fish tissues may differ from those of mammals (lobular structure of mammalian liver, for example, and the sheetlike arrangements of parenchymal cells with interlacing sinusoids and a few bile ducts of fish liver tissue), histologically tumors in fish do not generally differ markedly from the same site-specific tumors in mammals such as the liver (*21,68*).

Liver neoplasia is highly prevalent in winter flounder taken from Boston Harbor and involves mutant K-*ras* oncogenes (*69*), specifically point mutations in K-*ras* oncogenes in the 12th codon (*70,71*). Activated K-*ras* was observed in 7 of 13 liver tumors from winter flounder (*71*), whereas K-*ras* was found in 2 of 13 furan-induced and 1 of 13 furfural-induced liver tumor transfectant DNAs from $B6C3F_1$ mice (*72*); H-*ras* was the most common activated oncogene observed in hepatocellular cancer in the $B6C3F_1$ mice (*72*). The relatively high incidence of malignant tumors of the liver, with K-*ras* oncogenes (*70,71*) in winter flounder taken from chemically polluted regions (*8,73*) compared with the near absence of liver lesions in the same genus and species of fish taken from adjacent less chemically contaminated locales (*74*) "could signal DNA damage resulting from environmental chemical exposure" (*71*).

Because the liver is a relatively common site for chemically induced cancer in laboratory rodents (*75–77*), and progressive liver lesions including hepatocellular carcinomas and cholangiocarcinomas are observed frequently in chemically or pollution-exposed fish (*8,9,21*), further and extensive correlation comparisons and experiments (like the 60-chemical project by the U.S. EPA) should be undertaken to strengthen the concept that fish models are relevant to identification of potential health problems. Dawe (*50*) has proposed a 10-step procedure for identifying "carcinogen-indicator fishes" in feral habitats different from simply doing random fish surveys from "clean" or chemically contaminated aquatic areas for locating fish with neoplasia. Alternatively, "Advances in understanding carcinogen metabolism and the pharmacokinetics of carcinogens in fishes suggest an alternative approach . . . that could strengthen the rationale for using neoplasms in feral fishes as indicators of environmental carcinogens in aquatic environments" (*50*). Several useful comparative studies and reviews have been done on mixed-function oxygenase enzymes and drug metabolism in fish (*78–81*).

Chemical Carcinogenesis

Clearly, the accumulated experience in the field of carcinogenesis supports the concept that cancer development is a multistep process and that multiple genetic changes are required before a normal cell becomes fully neoplastic (*82,83*). Likewise, studies of human tumors suggest that the multistep paradigm together with similar genetic events are involved in the development of cancer in humans. And that the carcinogenic process is

clearly similiar among mammals, for instance, laboratory rodents and humans. As more and more advancements are made in molecular carcinogenesis, the mechanisms of cancer induction within the mammalian domain (and likely within the teleosts as well) will allow us to shed more light on the major objectives of using animals (and fish) as predictive surrogates for humans. In basic cellular functions, *ras* genes are likely to play a fundamental role based on their high degree of conservation throughout eukaryotic evolution; using the H-*ras* gene as a particular example, the human and rat protein sequence is identical (*84*). This certainly would argue that the *ras* oncogenes observed in fish liver tumors would be relevant to rodents and thus to humans.

Proto-oncogenes are cellular genes that are expressed during normal growth and development processes. These proto-oncogenes can be activated to cancer-causing oncogenes by point mutations or by gross DNA rearrangement (chromosomal translocation or gene amplification) (*85*). These lesions are especially revealing for chemicals that are apparently nonmutagenic and yet cause point mutations in chemically (furan and furfural) exposed B6C3F$_1$ mice (*86*). Distinct oncogene activation in spontaneous versus chemically induced neoplasms (*87,88*) and in benign versus malignant neoplasms (*87,89,90*) greatly enhances the use of molecular events in the risk assessment process. Moreover, loss of specific regulatory functions (i.e., tumor suppressor genes) represents an important feature in neoplastic transformation (*91–93*). This further permits us to come closer to the public health objective of preventing (or reducing) chemically induced and chemically associated cancers in humans (*94–99*).

Conclusions

Long-term chemical carcinogenesis studies could be easily and feasibly designed using laboratory rodents that might allow an interpretative conclusion about human health hazards from eating substantial amounts of chemically contaminated fish. Still, two additional and important questions must be approached in depth and debated at length before any studies of this magnitude and cost are undertaken: *a*) will results from such studies be considered and accepted as valid and relevant to the human situation? And *b*) what would these newly generated data add to our knowledge that something (chemicals?) in these habitats causes cancer in fish and shellfish? For the second question, positive results (e.g., induced cancer in laboratory rodents) would confirm environmental observations in native fish, thus further convincing public health officials to the realness of the potential hazards.

For the first question, a virtual plethora of papers, articles, books, and symposium proceedings have been written on the issues of extrapolations—from individual to individual, from sex to sex, from strain to strain, from species to species, from race to race—and no clear consensus of interpretation or harmonization of thought has been reached. Nonetheless, there does seem to be an expanding belief among the scientific community that experimental data and interpretative information obtained from whole animals (and perhaps fish as well) are relevant and applicable to the expected or observed responses in humans; this is especially true for chemically associated cancers in humans and in rodents (*100,101*).

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