Evaluation and Validation Issues in the Development of Transgenic Mouse Carcinogenicity Bioassays

Raymond W. Tennant

Laboratory of Environmental Carcinogenesis and Mutagenesis, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

Transgenic mouse models have emerged as plausible alternatives to long-term bioassays for carcinogenicity. Three transgenic lines evaluated to date have shown a clear capability to discriminate between carcinogens and noncarcinogens, using long-term bioassay results as the standard. The data also suggest that the transgenic lines will not fully duplicate long-term bioassay results. It is proposed that these models do not respond to chemicals that have induced highly restricted species or strain-specific tumor responses in mice or rats. Rather, the value of the transgenic models is predicated on a preferential response to transspecies carcinogens (i.e., those positive in both rats and mice, often including tumors in the same tissues). Thus, although results in transgenic models may not be completely concordant with long-term bioassays, the data can be used effectively in chemical and drug safety assessments. Further, it is proposed that validation of the models is readily achievable via ongoing studies. Validation of any alternative model is best achieved by sufficient mechanistic understanding of the model to reasonably predict the outcome of bioassays conducted in the models and use all available information on the drug or chemical. This goal can now be met with the transgenic mouse lines. — Environ Health Perspect 106(Suppl 2):473-476 (1998). http://ehpnet1.niehs.nih.gov/docs/1998/Supp1-2/473-476tennant/abstract.html

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Introduction

In the hierarchy of data used in the identification of causes of human cancer, clinical observation and epidemiologic studies provide the most definitive sources. However, because these methods are intrinsically retrospective, the long-term two-species rodent bioassay is the most generally accepted standard for identifying potential carcinogens or, conversely, recognizing chemicals, drugs, or environmental factors that probably do not represent a potential human carcinogen. In this context, the application of in vitro alternative methods refers specifically to alternatives to the conventional rodent bioassay.

We have learned a great deal about issues of validation and evaluation of alternatives through the search for in vitro methods that can replace or complement long-term rodent studies, principally in the field of genetic toxicology. It is estimated that over the past two decades at least 100 different assays have been developed and proposed to serve as alternative systems. The evaluation of these methods has been at times controversial, or at best difficult, but the efforts nonetheless serve as a guide to the types of issues that must be resolved for methods to be generally used in place of 2-year rodent bioassays. Substantial amounts of literature were created on genetic toxicity test methods and various aspects have been reviewed elsewhere (1,2). One of the most thorough and objective evaluations was conducted by the National Institute of Environmental Health Sciences (NIEHS)/U.S. National Toxicology Program (U.S. NTP) and ultimately involved the evaluation of 114 chemicals in four in vitro assays (3,4). Subsequently, additional information has been provided for in vitro assays such as induction of chromosome aberrations or micronuclei (5). The objectivity of the effort included the use of coded samples and, when possible, samples from the same lot of chemical as were used in the 2-year bioassay. Chemicals were chosen for which definitive 2-year bioassay results existed and samples were tested under code. The results of this extended evaluation served to verify the hypothesis that a significant portion of rodent carcinogens probably act via nonmutagenic mechanisms and that existing in vitro assays were inadequate to detect such nongenotoxic carcinogens. However, the results summarized elsewhere also permitted an interesting comparison of the four genotoxicity assays that were used (3,4). An interesting aspect of the consequences of this effort relates to the mouse lymphoma (TK+/-) in vitro mutation assay, which demonstrated a relatively high level of false positive results. That is, the mouse lymphoma assay responded to a high proportion of rodent noncarcinogens that were not detected by the other systems. Despite these results, the mouse lymphoma assay continues to be utilized for drug and chemical safety evaluation (6). One reason for this paradox appears to be the general belief that the mouse lymphoma assay detects not only point mutational effects of chemicals but also responds to agents that have some clastogenic potential (7). However, use of this assay is continued with the knowledge that a proportion of agents that produce a positive response are unlikely to be carcinogenic in 2-year bioassays. This example is given to focus on what may be an important aspect of the validation process. That is, that the validation process cannot be proscriptively objectified and that the use or acceptance of any given alternative system will depend principally on subjective notions about the uses and limitations of the assay system. What then constitutes an appropriate validation of new, more complex in vitro alternative assays? In the remainder of this paper, results of the use of transgenic mouse models as bioassays will be presented and related to how such systems are scientifically validated.

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Address correspondence to Dr. R.W. Tennant, Laboratory of Environmental Carcinogenesis and Mutagenesis, National Institute of Environmental Health Sciences, P.O. Box 12233, MD B3-09, Research Triangle Park, NC 27709. Telephone: (919) 541-4141. Fax: (919) 541-1460. E-mail: tennant@niehs.nih.gov

Abbreviations used: p53<sup>TM</sup>, heterozygous p53 knockout mouse line; NIEHS, National Institute of Environmental Health Sciences; tg.AC, zetaglobin promoted v-Ha-ras transgenic mouse line; U.S. NTP, U.S. National Toxicology Program.
Implications of Long-Term Bioassay Results

The significant advances of the past two decades in identifying specific genes that play critical oncogene or suppressor gene roles in the process of carcinogenesis have revealed objective targets for the action of chemicals or other potential carcinogenic agents. In conventional long-term rodent bioassays, mice and rats are exposed for a significant portion of their lifespan to concentrations of chemicals and lengths of exposure that ensures systemic exposure of all tissues of the body, as they are administered at minimally toxic doses for 2 years. It has been generally assumed that this method provides the greatest opportunity for identifying potential carcinogens because all of the tissues of the animals are put at risk for possible neoplastic effects. The bioassay identifies any cancers that are induced in comparison to both a concurrent control and historical control data. The concurrent control group plays an intrinsic part in the evaluation process because rodent strains develop specific spontaneous tumors over their lifespan that become confounding factors when evaluating whether a chemical, individual environmental exposure, or drug has induced cancer. Although there are many important genes that are highly conserved between rodents and humans, there are specific genetic differences that determine spontaneous tumors (8,9). Because the pattern and tissue type of spontaneous tumors are characteristic of individual inbred rodent strains, it is obvious that these spontaneous tumors have a specific genetic origin. However, relatively few of the specific genetic determinants of spontaneous cancers in rodents have been defined. One particular locus that plays a role in the development of hepatocarcinogenesis in the B6C3F1 mouse is the hec locus (8). Efforts to link spontaneous and induced hepatocarcinogenesis in B6C3F1 mice to mutations in the H-ras gene have failed to show any clear pattern (10). However, since the hec locus has not been cloned, it is not possible to determine the specific relationship that this locus may have to other identified genes, or whether this is a mutation or a polymorphism of a gene that plays a role in the normal regulatory processes in hepatic cells. A major implication of the existence of genes that determine spontaneous cancers is that long-term exposure to chemicals, rather than indicating an intrinsic property of the chemical to induce carcinogenic effects, may alternatively only show that a chemical has the capability to modulate the expression of genes. If a specific gene is a determinant of spontaneous tumorigenesis in the rodent, then a chemical could also affect genes that alter the expression of the spontaneous tumor gene. In such a case it is likely that the presumed carcinogenic response may only represent a strain- or species-specific effect that results from a specific pattern of inheritance. Thus, in terms of implications for human health assessment such chemicals would be unlikely to pose a hazard.

A second factor that may complicate the interpretation of the rodent bioassays is strain or species susceptibility genes, which can also influence the response of specific strains or species to chemical effects. For example, genes that are derived from the highly polymorphic superfamiliy genes influence interactions with and metabolism of chemicals and drugs (e.g., P450s or various cellular receptors) and may determine strain-specific responses to chemicals. Such strain-specific responses would predictably show as single sex, species, and site of tumor induction (9).

Evaluation of Transgenic Mouse Models as Carcinogen Bioassays

With foreknowledge of the existence of genetic determinants of spontaneous tumors or strain-specific carcinogenicity in the rodents used for the bioassay, it is possible to continue to use the bioassay and provide for discriminatory interpretation of results. However, it is also possible that because there have been important oncogenes or tumor suppressor genes identified that play critical roles in carcinogenic process, these can be made the proximate targets for the action of chemicals, thus bypassing the potential for the influence of strain or species-specific genes. We believe that this goal has been met through the development of transgenic mouse models in which specific genetic determinants have been either inserted or deleted from the mouse genome, rendering the animals specifically sensitive to carcinogenic effects. The principal transgenic models currently available and most extensively utilized as bioassays are presented in Table 1. Although hundreds of transgenic and knockout mouse lines have been developed, most failed to demonstrate phenotypic characteristics that would make them generally useful in the task of chemical and drug safety assessment for carcinogens.

The three transgenic models presented in Table 1 each possess a phenotype that permits selective responses to carcinogens (15,16). Though the total number of chemicals tested to date in each line is limited, the data are sufficient to clearly indicate ways in which the models could be used as carcinogen bioassays. The specific chemicals tested to date in each model are provided in the references cited in Table 1 and will not be retabulated here. Each model has shown the capability to respond in a specific manner to exposure to carcinogens. Tumors have been induced within 6-month exposure periods at doses that are the same or comparable to those utilized in 2-year bioassays. With the heterozygous p53 knockout mouse line (p53del), the responses were in some cases at the same site and of the same histogen type as those induced in animals in the 2-year bioassay. In the p53del model only ionizing radiation (17) and those chemicals of known mutagenic potential (16) induced tumors. The animals failed to respond to comparable exposures to two nonmutagenic carcinogens. These observations support the inference that the p53del line can be used to derive mechanistic insights about the carcinogenic potential of chemicals.

The zetaglobin promoted v-Ha-ras transgenic mouse line (Tg.AC) possesses a unique phenotype that is the product of two primary properties that include the activation of the mutated ras gene via a fetal globin promoter and the site of integration (13). The induction of skin papillomas by topical exposure to tumor promoters and carcinogens appears directly related to functional properties of the zetaglobin promoter and the location of the site of integration in a domain that permits the transgene to be expressed in the epidermis. The model is consistent with the role of the mutated endogenous Ha-ras gene in skin tumor induction in the conventional two-stage initiation-promotion process in mice (18). That is, mutation of the endogenous
Ha-ras gene is an initiating event in the initiation–promotion protocol (19). However, in the Tg.AC model the induction of papillomas appears to proceed via transcriptional activation of the transgene. Transgene expression has not been detected in normal adult skin in the absence of exposure to carcinogens or development of papillomas (20,21). Thus the mechanism of the model is related to the capability of both mutagenic and nonmutagenic carcinogens and tumor promoters to activate expression of the transgene. The expression of the transgene appears to alter the terminal differentiation pattern of basal keratinocytes, resulting in a growth imbalance that leads to the development of the papillomas. A high proportion (approximately 40%) of animals that develop a papilloma also develop squamous cell carcinomas that likewise highly express the transgene. A useful way of viewing the model is to consider the induction of papillomas as a report phenotypic for carcinogenic potential. Of the chemicals tested to date for which 2-year bioassay data are available, the model has shown a highly specific response pattern and has yielded no false positive results (16). In addition to skin exposure, preliminary results with dimethyl vinyl chloride administered via gavage indicates that the fore stomach epithelium is also sensitive; the chemical induced fore stomach papillomas that expressed the transgene. It is possible therefore that the oral route of exposure may be useful, but more data are needed.

The Tg-ras-H2 transgenic mouse line was developed utilizing an endogenous human c-Ha-ras gene with its endogenous promoter sequence. Preliminary results suggested that the model demonstrated an increased sensitivity to chemically induced carcinogenesis (15). Subsequent studies increased the number of chemicals to which the animal is sensitive; the study of a large group of chemicals is currently in progress at the Japanese National Institute of Environmental Health Sciences (Tokyo) and the Japanese Central Institute for Experimental Animals (Kawasaki). This model differs from both the p53md and the Tg.AC lines in that the transgene is endogenous protooncogenes. Studies are currently underway to understand the mechanism by which various carcinogens induce tumors in this model.

Papers published to date providing information on the chemicals that have been tested in these transgenic models, and details of their genotype and phenotypic characteristics are listed in the bibliography (11,13,14,16). It is also possible to obtain the latest information available on the studies in transgenic models conducted by the NIEHS/U.S. NTP via the Internet (22). This Internet site will be used to solicit nominations for further studies in transgenics, list chemicals currently being studied, and provide summary results as they become available.

Validation of Transgenic Bioassays

The basic proposition of this paper is that although the data are limited for the number of chemicals that have been evaluated in the three transgenic models, the results are sufficient to allow incorporation of results from these models into the safety assessment of chemicals and drugs. The safety assessment, hazard identification, and risk evaluation processes are intrinsically subjective and by their very nature must be done by weight of evidence. That is, all the available data on the properties and biologic effects of the chemical or drug must be used in evaluating human risk (1,2). There is no proscriptive formula to effectively safeguard human health and permit the valid utilization of chemicals and drugs. Although the rodent bioassay is viewed by some as the gold standard for safety assessment, it is actually one tool with which to judge the biologic effects of chemicals. It is obvious that rodents are imperfect surrogates for the human population. Results in the rodent can be validly compared to those in humans for a variety of substances, and though many genetic determinants are highly conserved between the species, there are many more genetic differences that significantly impact on judgments of hazard evaluation. As stated above, the principal manifestations of these differences in regard to the bioassay are the high background rates of spontaneous tumors and evidence of strain-specific responses to chemicals. These observations do not invalidate the bioassay, as known human carcinogens are also carcinogenic in rodent models, but conversely, it does not mean that every effect observed in the rodent is necessarily predictive of human health hazard. We have advocated that the transgenic models can complement and eventually supplant the use of the long-term rodent bioassays by minimizing strain-specific responses without significantly diminishing the capability to recognize the transspecies carcinogens that are likely the most proximate human health hazard identified in the long-term bioassay. Those who hold to a literal interpretation of bioassay results, i.e., any effect in the rodent is probably indicative of a human health risk, will not endorse the use of such models because they are predicated on the principle that they will not respond to the majority of chemicals that have shown highly strain- or site-specific effects in the 2-year bioassay.

The issue of what constitutes an appropriate validation can be best addressed by viewing experience with the long-term bioassay. The bioassay as it is conducted today has evolved for over two decades in concept, methodology, and interpretation, and some have asserted that the model has never been appropriately validated. I believe that validation has been achieved via experience. We have come to recognize the potential uses and limitations of the bioassay and scenarios have been developed to address the various circumstances in which the outcome of bioassays may be at variance with anticipated results based on chemical structure, toxicity, or other properties. Mechanistic inference rarely can be used to assess the results. Some notable exceptions are the case of the 62-microglobulin-induced tumors in male rat kidneys. The most problematic interpretations involve increases only in incidence of tumors that have a high spontaneous rate.

Despite these interpretive uncertainties it has been possible to demonstrate that a high proportion of the outcomes of bioassays can be predicted based on utilizing information on chemical structure, genotoxicity, subchronic toxicity, etc. The least predictive certainty is associated with chemicals that produced highly selective carcinogenic effects (i.e., single-site or single-sex species tumor induction) and for those that induce equivocal responses. The highest degree of predictive confidence is associated with the chemicals that produced transspecies carcinogenic effects and that were genotoxic and/or relatively highly toxic in subchronic studies. Thus, although a formal validation has never been conducted for the long-term rodent studies, the capability to predict bioassay results to a reasonable degree confirms the fact that there is understanding and confidence in the use of this method for chemical and drug safety assessment.

A syllogism to justify the use of transgenic bioassays is that a) if the transgenic bioassays can reproduce the predictable responses of the long-term bioassay, and do so with significant reduction in time and cost, and b) if the transgenic bioassay result likewise can be predicted based on similar information relating to chemical structure, genotoxicity, and systemic toxicity, then c) the transgenic bioassays should provide a
plausible and acceptable alternative to the long-term studies. At the current stage of development and understanding, the highest level of predictive confidence is associated with the p53def transgenic model. The circumstantial evidence that associates mutagenic potential with carcinogenic potential is high (though not certain, as approximately 30% of mutagens are not tumorigenic in the long-term rodent bioassays). Because a high proportion of human tumors carry mutations in the p53 gene it is plausible to predict that mutagenic chemicals may produce a positive response in the p53def animal via mutational inactivation or loss of the functional p53 allele. This hypothesis must be tested experimentally, but the results obtained thus far provide some confidence that the mechanistic basis of the response of the p53def line is consistent with the other data. Under these circumstances it is plausible at present to adapt the use of the p53def line to verify the carcinogenic potential of mutagenic chemicals; a positive response in this model should form the basis for effective chemical safety assessment. In addition, because group sizes can be much smaller than in long-term bioassays, it is plausible to conduct extensive dose-range evaluation to determine whether possible no effect levels exist for the action of the chemical. Sample sizes required to achieve high levels of confidence for very low frequency events are still large, but the absence of induced effects in sequential doses of chemicals in the transgenic model can provide useful information about the relative carcinogenic potential of a chemical. The absence of any induced tumors in a transgenic bioassay with the p53def model could indicate that despite its mutagenic properties, the chemical is not intrinsically carcinogenic, or it could mean that the p53 model is unresponsive in tissue sites at which the chemical might demonstrate a carcinogenic effect. At this stage of our understanding, these two alternatives cannot be discriminated effectively, but as additional experience with a greater variety of chemicals or drugs is obtained, distinction between these possibilities should be resolvable.

The Tg.AC transgenic model has shown clear-cut responses to both mutagenic and nonmutagenic chemicals administered topically. Apparently, induction of papillomas is a direct consequence of the activation of transgene expression in a specific population of cells that appear to reside predominately within the upper follicular epithelium.

In summary, the available though limited data for chemical effects in transgenic mouse models indicate that selected models can play a role in drug and chemical safety evaluation. The validation of the models is an evolving process, but sufficient understanding of the mechanisms of tumor induction in transgenic lines exists to make plausible predictions for the outcome of transgenic bioassays.

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