Diet, Nutrition, and Cancer: Development of Hypotheses and Their Evaluation in Animal Studies

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Abstract

Research in diet, nutrition, and cancer is multidisciplinary. As a result there are many factors to consider in the conceptualization and design of experiments that may not be readily apparent to someone entering this field. In this article issues are identified that should be considered in the development of hypotheses about the effects of diet and nutrition on the carcinogenic process and the evaluation of these hypotheses using animal models. The questions considered are: what comprises an acceptable rationale for diet, nutrition, and cancer experiments in which animal models are used; what factors should be considered in the design of these experiments; and what constitutes a mechanistic component in such animal studies.

Introduction

During the last decade there has been a progressive shift in the emphasis in cancer control research toward prevention (1–3). From this has emerged an effort to identify possible intervention strategies that will reduce cancer prevalence. Interventions defined thus far are primarily behavioral, chemical, and medical in nature (1–6). Examples of each are, respectively, smoking cessation campaigns, chemoprevention, and participation in early detection programs, e.g., Pap smear and mammography. Potential benefits attributable to these measures are considerable and portend the impact that prevention efforts may have in reducing cancer prevalence and mortality. Successes in this direction necessarily prompt the question of what else can be done.

In 1981 Doll and Peto (7) estimated that dietary modifications could result in a 35% reduction of fatal cancers. Despite the uncertainty that was noted in making this estimate, their analyses and others kindled interest in characterizing critical relationships among diet, nutrition, and cancer (7–9). While considerable progress has been made, the research now necessary to advance this field is increasingly multidisciplinary. As a result investigators with varied backgrounds must deal with issues with which they have little or no familiarity, e.g., diet formulation and tumor induction. The purpose of this article is to identify factors that should be considered in the development of research projects in which animal models are used to investigate diet, nutrition, and cancer hypotheses. Three questions will be addressed: what comprises an acceptable rationale for experiments in which animal models are used; what factors should be considered in the design of these experiments; and what constitutes a mechanistic component in such studies.

What Constitutes an Acceptable Rationale for Experiments in Which Animal Models Are Used to Study the Role of Diet and Nutrition in Cancer?

In order to answer this question, it is important to establish the sequence of events that are typical of laboratory research in diet, nutrition, and cancer. In general, epidemiological data, in concert with clinical observations, give rise to hypotheses about potentially important diet and cancer relationships. However, these observations are correlational in nature and a means is required to determine if a causal relationship exists. While prospective clinical trials could be designed for this purpose, they are expensive and take years to complete. Moreover, there are limitations on the number of subjects available for such studies and it would be considered inappropriate to test certain diet-cancer associations based on only correlational data. In comparison, animal studies provide a direct means by which to determine the casual nature of an apparent relationship in a timely and cost-effective manner. This is achieved through the manipulation of key dietary variables in a defined tumor model system. Animal experimentation provides the investigator with a level of control and ability to manipulate critical variables not possible in clinical trials. If a causal relationship is established in an animal experiment, the finding can be evaluated for its potential clinical merit and/or investigated further to determine the basis of causality at the cellular and molecular level. Frequently these experiments give rise to additional hypotheses about how to alter diet to reduce disease occurrence which again must be validated in animal studies. From such animal experiments a determination is made of whether a particular hypothesis merits clinical evaluation. This brief overview identifies the central role animal studies play in evaluating both hypotheses based on epidemiological and clinical data as well as those resulting from laboratory investigations. Animal studies that are designed for these purposes and intended to establish causality or validate a cause and effect relationship in vivo that is implied by in vitro data are in general considered to be well justified. However, there are other factors that determine whether or not the rationale for an animal experiment is compelling.

As suggested by the discussion above, it is essential that diet, nutrition, and cancer research be hypothesis driven. Since the rationale for conducting animal experiments will be derived from the hypothesis being tested, the acceptability of the rationale directly relates to the merit of the hypothesis. This prompts the question of what constitutes a meritorious hypothesis. A list of the sources of data that generally form the basis of diet, nutrition, and cancer hypotheses is shown in Table 1. It can be argued that the strongest hypothesis will be one for which there is a solid base of support from the sources listed. Of these sources, the greatest importance is generally attributed to hypotheses that are based on epidemiological data and clinical observations because unlike hypotheses derived from other data sources, the likelihood of human applicability is inherent in such hypotheses. In developing a hypothesis, it is often found that epidemiological data is contradictory or that certain aspects of the results of animal experiments or other laboratory data are not consistent with the hypothesis. In such cases, both the strengths and limitations of all data should be critically evaluated and a determination should be made about whether there is sufficient support to warrant evaluation of the hypothesis. It is common for many hypotheses to be discarded as a result of this process. Despite inconsistencies among sources of data, certain high potential hypotheses will emerge. For these it is highly desirable that preliminary data be obtained. Almost without exception, preliminary data demonstrating the feasibility of evaluating a hypothesis in an appropriate animal model is essential in building a strong rationale in support of studies designed to evaluate that hypothesis. A third factor that determines the strength that a hypothesis is accorded is its potential applicability and relevance to human health issues. There is a high expectation for direct human applicability of results in...
the diet, nutrition, and cancer field. Because of this, it is insufficient to simply obtain knowledge about the effect of a dietary factor on the carcinogenic process without evidence that such knowledge has potential applicability. While exceptions to this viewpoint exist, the sense of urgency and the need for perceived human applicability to this area should not be underemphasized.

In summary, it can be argued that a well rationalized experimental plan in which animal models are used is one designed to test a hypothesis for which there is a strong base of support from categories listed in Table 1; for which preliminary data have been obtained which indicate that evaluation of the hypothesis is feasible in an appropriate animal model; and for which there is a clear-cut case for human relevance and in which the dietary factor could have a significant impact on disease outcome. Unfortunately the number of hypotheses for which all these conditions will be met is small. This situation makes it paramount for investigators developing hypotheses about specific dietary factors and cancer to discriminate among the many possible areas of inquiry that could be pursued and select for study those hypotheses with the strongest support and to propose experiments designed to investigate causal relationships.

What Factors Should Be Considered in the Design of Animal Studies for Evaluating Diet, Nutrition, and Cancer Hypotheses?

While there are many facets of experimental design that need to be addressed in developing a research plan to test diet, nutrition, and cancer hypotheses, three elements of any protocol are particularly critical. They are shown in Table 2 and discussed below.

Selection of Diet. The most typical dietary approach that is encountered in the nutrition and cancer literature is that in which a single agent is added to either an unrefined or a purified diet formulation. The rationale for this approach is based on the objective of attributing causality to a specific dietary factor and thus single agent dietary interventions best fit this objective. If this approach is used, an initial decision that needs to be made is whether an unrefined diet or a purified diet is most appropriate for the hypotheses being tested (Table 3). While there may be justifiable reasons for using an unrefined diet, the use of a purified diet formulation is generally recommended because it affords the investigator complete control over both the quality of the diet and the ability to manipulate its composition (10). When a purified diet formulation is judged to be appropriate for the hypothesis under investigation, a strong rationale exists for considering the use of a standard reference diet formulation, especially when an investigator does not have expertise in dietary methodology (11). The reasons that the selection of a standard reference diet should be considered include: many formulations that are commercially available fail to meet nutrient requirements of the species for which it was intended; nutrient requirement data for a particular species are difficult to translate into a nutritionally balanced dietary formulation; and the use of a common dietary formulation permits comparisons of results from different laboratories (10-12). The formulation of the diet most widely recommended as a standard for use in rodent studies is AIN-76A (10). Because of its extensive use, there is a large base of carcinogenesis data in which it has been fed. Nonetheless, the AIN-76A formulation was not developed to be optimal for long-term studies which are common in carcinogenesis research and, under certain conditions, this formulation should be modified or an alternative formulation should be selected (13). It is important to emphasize that the use of a “reference formulation” does not ensure appropriateness of a diet for the evaluation of a specific hypothesis nor does the failure to use a specific formulation necessarily constitute a critical flaw. What is essential is that the process used to select a diet be clearly defined and that the formulation selected be the most appropriate one for the hypothesis under investigation.

Other issues related to diet formulation also must be considered. Included among these is the effect of the dietary modification under investigation on nutrient density of the diet (14). If a dietary modification, e.g., addition or deletion of a dietary component, results in diets that will be used in the same experiment and that will differ in energy content, adjustments are required in the way the diets are formulated. Several approaches can be taken to resolve this problem, the most common of which is to maintain nutrient density/kcal as a

<table>
<thead>
<tr>
<th>Source of data</th>
<th>Strengths</th>
<th>Limitations</th>
<th>Comment</th>
</tr>
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<tbody>
<tr>
<td>Human studies</td>
<td>Population-based source of hypotheses</td>
<td>Associations identified but causality not determined</td>
<td>More weight typically attributed to this source</td>
</tr>
<tr>
<td>Epidemiological data/clinical observations</td>
<td>Human applicability</td>
<td>Data often imprecise and subject to biases</td>
<td></td>
</tr>
<tr>
<td>Animal studies</td>
<td>Variables controlled; direct test of causality</td>
<td>Extent to which the data can be extrapolated to humans</td>
<td>If this source of data is used, the issue of human applicability should be addressed</td>
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<tr>
<td>Carcinogen-induced and spontaneously occurring tumorgenesis data</td>
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<tr>
<td>In vitro studies</td>
<td>Simple, well defined models; direct tests of causality at cellular and molecular level</td>
<td>Applicability of observation to more complex levels of organization, e.g., tissue, organ, organism</td>
<td>While powerful in establishing causality, appropriate caution required in extrapolating to higher systems and disease process</td>
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Table 1 Development of hypotheses about relationships among diet, nutrition, and cancer

Table 2 Elements of design to be considered in planning an animal experiment

<table>
<thead>
<tr>
<th>Selection of diet</th>
<th>Formulation</th>
<th>Feeding</th>
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<tbody>
<tr>
<td>Selection of animal model</td>
<td>Species/strain</td>
<td>Carcinogen</td>
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<tr>
<td>Biological and molecular characteristics</td>
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<tr>
<td>Experimental design</td>
<td>Treatment group configuration</td>
<td>Animal Assignment</td>
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<td>Endpoints</td>
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Table 3 Questions that should be considered in selection of a diet

Is the use of a purified diet appropriate to the working hypothesis? if not, why not and what is an appropriate alternative?

If a purified diet is appropriate, is the reference formulation designated AIN-76A suitable? If not, why not and what is the suitable alternative?

Does the dietary manipulation(s) being used potentially alter the nutrient density of the diet? If yes, how can the diets be formulated to avoid introduction of uncontrolled variables?

Does the dietary treatment to be studied have the potential to alter the amount of food consumed? If yes, what controlled feeding technique is most appropriate?
constant among treatment groups except for the dietary factor(s) under investigation (15). A related question is what feeding methodology should be used when it is anticipated that a dietary modification will alter the food intake of animals. Under such circumstances, a controlled feeding procedure is required and the most widely described method is referred to as pair-feeding. However, there are other methods, e.g., restricted feeding which frequently may be more desirable for controlling food intake in long-term studies. It is important to emphasize that more than one approach to both nutrient density and controlled feeding is available. The key is that the approaches chosen are the most appropriate to the hypothesis being evaluated.

As noted above, single agent experiments are by far the most common in nutrition and cancer research. However, there is a growing need for other dietary approaches. Until recently the investigation of dietary/nutrient interactions that may affect carcinogenesis has been neglected (16). However, the nutrition literature contains numerous examples documenting the importance of such interactions in affecting the health and well-being of an organism and work in this area should be encouraged. In selecting suitable dietary formulations, the same general principles referenced above underlie the formulation of a nutritionally adequate diet in which several factors are varied. When possible, existing paradigms for formulation should be considered for adaptation to cancer-specific questions. A major point to consider is whether evidence exists to indicate that the dietary formulation that is proposed is suitable for the maintenance of animals in long-term feeding studies.

A concern of some investigators working in diet and cancer is that the purified diets generally used in this field are not representative of the way people eat and that a more realistic approach to dietary formulation is required in order to identify relationships that are directly applicable to human populations. While this subject can be the topic of heated debate, it should be recognized that food-based dietary/nutrient interactions that may affect carcinogenesis has been neglected (16). However, the nutrition literature contains numerous examples documenting the importance of such interactions in affecting the health and well-being of an organism and work in this area should be encouraged. In selecting suitable dietary formulations, the same general principles referenced above underlie the formulation of a nutritionally adequate diet in which several factors are varied. When possible, existing paradigms for formulation should be considered for adaptation to cancer-specific questions. A major point to consider is whether evidence exists to indicate that the dietary formulation that is proposed is suitable for the maintenance of animals in long-term feeding studies.

A concern of some investigators working in diet and cancer is that the purified diets generally used in this field are not representative of the way people eat and that a more realistic approach to dietary formulation is required in order to identify relationships that are directly applicable to human populations. While this subject can be the topic of heated debate, it should be recognized that food-based dietary formulations that reflect particular eating patterns have been used in other nutrition research areas (17). However, investigators interested in such an approach must: (a) carefully define the hypothesis that will be evaluated; (b) identify adequate dietary controls that ensure the validity of the experimental approach; and (c) develop methodology by which the diets that will be used can be reproducibly formulated. Evidence that these diets will support the health and maintenance of animals in long-term feeding studies is also required. Because of the inability to specifically identify how various food-based diets differ chemically, there are constraints on the extent to which specific cause-and-effect relationships can be investigated. Given that a major advantage of using animal model systems is the ability to study causal relationships directly, a compelling rationale must be presented for the use of animal models in food-based research.

### Selection of Animal Models for Cancer

Animal models for cancer provide a critical link in the continuum of research necessary to evaluating diet, nutrition, and cancer hypotheses because they permit the investigation of specific cause-and-effect relationships during defined stages of the disease both prior to and after the occurrence of clinically detectable lesions. This is an important advantage of animal studies since epidemiological observations on which many diet, nutrition, and cancer hypotheses are based provide only evidence of associations, and it is generally not possible to establish when during the disease process a particular association is manifest. For this reason and others, the best animal tumor model is one that closely parallels the biology of the human disease and in which the characteristics of the disease process are compatible with the hypothesis being tested. Some of the questions that need to be addressed in choosing an animal model are outlined in Table 4.

While numerous model systems exist for the study of cancer(s) at certain sites, options are limited for other sites. All tumor models have inherent advantages and shortcomings and, needless to say, perfect models of the human disease do not exist. While obviously it is important to emphasize that models need not be perfect to be useful, the question of how to overcome deficiencies of a particular model is critical. Until recently, the principle response to this question would have been to suggest that more than one model system for the cancer of interest be used to study a hypothesis and to choose models that are complementary. However, the recent development of new approaches to cancer induction, prominent among which are transgenic models and in vitro or in situ transfection/infection models using oncogene/tumor suppressor genes, has opened new avenues for investigators (18–19). The use of these systems should be encouraged when they are appropriate to the study hypothesis since they afford the opportunity to address directly how dietary/nutritional factors affect the development of cancer induced by alterations in specific genes. However, these newer approaches should not be considered as substitutes for other models in which chemical, viral, or physical agents are used to initiate tumor development. While it is recognized that the new technologies now available offer the opportunity to gain insights not previously possible when these models are used in appropriate contexts, the perception that such approaches are better than other well characterized tumor models must be questioned. Experience with these new technologies is restricted and the inherent advantages and limitations of these new approaches are only beginning to be defined. In this regard, reference is made to the work of Weinberg (19) in

### Table 4 Questions that should be considered in selection of an animal model

<table>
<thead>
<tr>
<th>Question</th>
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<tbody>
<tr>
<td>Why is the use of an animal model required?</td>
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<tr>
<td>What are the strengths and limitations of the available animal tumor models relative to the human disease process?</td>
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<tr>
<td>What are the advantages and disadvantages of a particular animal tumor model as it relates to the study hypothesis?</td>
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<tr>
<td>Are there alternative animal model systems that offer advantages that are lacking in the animal model that is being considered?</td>
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### Table 5 Questions that should be considered in the design of an experiment

<table>
<thead>
<tr>
<th>Question</th>
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</thead>
<tbody>
<tr>
<td>What are the endpoints to be measured?</td>
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<tr>
<td>What is the variability inherent in the measurement of the endpoint(s)?</td>
<td></td>
</tr>
<tr>
<td>What difference in the endpoint(s) is expected to be affected by the experimental treatment relative to the control?</td>
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<tr>
<td>With what statistical power is it desirable to detect changes in the endpoint(s) being measured?</td>
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### Table 6 What constitutes a mechanistic component in animal studies

<table>
<thead>
<tr>
<th>Preconceptions</th>
<th>Questions</th>
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<tbody>
<tr>
<td>To be of high merit, an experimental plan must have a mechanistic component, and to be cutting edge, research must involve molecular biology and especially oncogenes/tumor suppressor genes.</td>
<td>What is meant by mechanistic research?</td>
</tr>
<tr>
<td>To be “molecular” is of “higher merit” than to be “organismic.”</td>
<td>At what stage of hypothesis testing in animal studies is a cellular/molecular research component appropriate?</td>
</tr>
</tbody>
</table>
which some of the limitations in the use of transgenic animals are defined. While many issues can be debated about the usefulness of animal models in general and what models are most appropriate, two principles invariably emerge from such discussions, that animal models play a central role in diet, nutrition, and cancer research; and that the nature of the hypothesis being tested should be the key determinant in selection of the most appropriate animal model(s).

Experimental Protocol

While the selection of the dietary formulation and tumor model for diet and cancer studies is critical, equal importance should be accorded to the other aspects of designing an experiment (Table 5). For example, the arrangement of treatment groups within an experiment should take into consideration the appropriateness of using more than one dose of carcinogen and/or multiple concentrations of the dietary agent being investigated. Another important question is whether the use of time series comparisons among treatment groups is warranted. Once an experimental design is defined, powers calculations should be used to determine the number of animals to assign per treatment group. This necessarily requires consideration of the endpoints that will be measured and their sensitivity, the level of variation observed in the endpoint(s) among animals, and the magnitude of change in the endpoint(s) that it is desirable to detect. From these considerations emerge not only the number of animals that should be assigned to each treatment group but also the plan for data tabulation and statistical evaluation. It is important to recognize that failure to address such issues at the outset of planning an experiment raises numerous questions, prominent among which is will the experimental design allow a definitive test of the hypothesis under investigation given the nature of the data that will be obtained.

What Comprises a Mechanistic Component in Animal Studies?

Perhaps more than in other areas of investigation, there is a considerable danger in diet, nutrition, and cancer research to engage in the conduct of what is referred to as "descriptive" rather than "mechanistic" research. This is an important issue because scientific merit is frequently equated with the extent to which a research plan has a mechanistic component. Common perceptions about descriptive versus mechanistic research are summarized in Table 6.

There is a natural tendency to characterize research as descriptive if it involves earlier stages of hypothesis testing in which animal experiments are proposed to establish that a relationship suggested by epidemiological, clinical, or laboratory data can be validated in an appropriate tumor model system. Later stages of testing the same hypothesis that are targeted at the cellular or molecular level are generally defined as mechanistic. However, such arbitrary definitions of descriptive versus mechanistic research obscure the critical question. At issue is whether the use of the word "mechanistic" properly refers to whether cellular or molecular approaches are used in a research plan or to the extent to which experiments are designed to establish causality. It is argued that experiments designed to establish causality are mechanistic and the level at which causality is addressed needs to be consistent with what is understood about the hypothesis being evaluated. The point is that animal carcinogenesis studies designed to establish a cause-and-effect relationship between a dietary factor and cancer outcome are mechanistic as are studies targeted at the cellular or molecular level that are designed to establish the basis of causality of an effect(s) observed in a carcinogenesis experiment. Conversely, studies at the animal, cellular, or molecular level that are not designed to investigate causality are descriptive irrespective of the types of techniques that are used. Thus, a goal for investigators working in this area is to develop experiments that investigate causality rather than describe phenomena and to advance to the cellular and molecular level of causality as is feasible.

Conclusion

Experiments in which animal models are used play a key role in the evaluation of diet, nutrition, and cancer hypotheses. The development of well rationalized experiments requires that they be hypothesis driven and have a good likelihood of applicability to the human disease. This is considered essential to the continued progress of diet, nutrition, and cancer research. Because of the nature of the variables studied and the complexity of dietary and nutrient interactions, it is critical that investigators working in this field be reminded of the importance of investigating causal relationships in the experiments they design and that they be encouraged to use, to the extent that is possible, cellular and molecular approaches that enhance the opportunity to determine causality directly and with the greatest precision possible.

References

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