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This work examined the response of different mouse genotypes to the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA). Genotype specificity for the particular tumors developed was observed; DMBA increased the incidence of and decreased the average age at which these tumors were observed. It does not appear that the genetic instability associated with the Werner Syndrome in humans enhances the predilection of mice to develop mammary cancer, even in animals dosed with the carcinogen DMBA. It was demonstrated that the dietary intervention of calorie restriction reduced the incidence of mammary tumors following carcinogen administration in genotypes predisposed to development of mammary cancers. An important finding is that in addition to genetic and environmental factors, there is a stochastic component involved in tumor development.

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Introduction

Determining whether the clustering of cancer within families is reflective of common carcinogenic exposure, the presence of susceptibility genes not yet identified, a set of environmental conditions or a combination of these factors is a complex problem, especially with human subjects (1). Dissecting this complexity is more approachable using animal models. Inbred strains provide many replicates for analysis. This is not only very valuable for statistical analysis of outcomes; it also permits assessment of the rarely mentioned role for stochastic events to affect the development of cancer. Specific gene substitution provides an opportunity to examine how a particular locus impacts on cancer incidence. Further, the surroundings of all the animals studied can be carefully controlled, allowing assessment of environmental variables to affect cancer incidence.

The work in DAMD17-97-1-7123 compared and documented the predisposition of various inbred strains of mice to develop mammary cancer following exposure to the mammary tumor-causing carcinogen 7,12-dimethylbenz[a]anthracene (DMBA). The first set of experiments in this project were designed to test the hypothesis that responsiveness to caloric restriction, manifest as a reduction in mammary tumor incidence and/or delay in its manifestation, was under genetic control. This was complicated by the difference in malignant lesions preferentially manifest in the various strains of inbred mice examined (2). This was followed by an examination of the interaction between genetic instability and exposure to DMBA in mammary tumor incidence (3).

While caloric restriction (CR) is well documented to delay the onset and to decrease the incidence of tumors in mice and rats (4, 5), the mechanism by which these effects are elicited is unknown. Relegating these effects to either caloric intake or alterations in body composition is not possible. Although the impact of CR is robust, little has been done with regards to direct comparison with the effects of obesity on mammary cancer incidence.
Body

Approved Statement of Work:

A. Determine the range of mammary tumor susceptibility after exposure to the carcinogen 7,12-dimethylbenz[a]anthracene in a panel of 10 inbred strains of mice.

AND

B. Compare the differences in mammary tumor burden between ad libitum and calorie restricted animals for each genotype.

Completed work

The initial work required for this project was development of a protocol for dosing mice with the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) while minimizing acute mortality following carcinogen administration (6) (Appendix 1). It was observed that the acute effects of dehydration could be avoided with prophylactic subcutaneous administration of saline.

The extent to which genetic variability influences the efficacy of calorie restriction (CR) to ameliorate the impact of DMBA to cause mammary cancer was examined using a panel of eight inbred strains of mice. Administration of a single 65mg/kg dose of DMBA was not found to cause mammary cancer in each strain but rather, was observed to accelerate expression of the neoplastic lesions known to develop spontaneously in each strain (2)(Appendix 2). An interpretation of this study, germane to this project, was that a single 65mg/kg dose of DMBA was insufficient to induce an incidence of mammary cancer sufficient for study in most mouse genotypes examined.

Addressing the issue of insufficient mammary cancer incidence, the impact of genetic instability for the development of mammary cancer in conjunction with administration of DMBA was examined. The human disease Werner's syndrome results from mutation in a gene shown to be a DNA helicase (7). Individuals with Werner's
syndrome have an increased incidence of a variety of age-related diseases including cancer (8). Defect in this gene results in genomic instability leading to increased mutation frequency (9). Similar genetic instability may be presumed to occur in mice failing to express the murine form of this protein, encoded by the WRN gene. Therefore, the impact of DMBA to cause mammary cancer was examined in mice both homozygous and heterozygous for the deletion of the exon-encoding region VI of the catalytic helicase portion of the WRN gene. Administration of a single 65mg/kg dose of DMBA to mice lacking either one or both copies of WRN gene did not result in an increased incidence of mammary cancer (3)(Appendix 3).

**Key Research Accomplishments**

- Survival of mice following oral gavage with the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) is optimized by prophylactic support of adequate hydration (Appendix 1).
- Variability in Response to the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) is observed to have genetically controlled variability (Appendix 2).
- The extent to which calorie restriction affects tumor incidence, age of tumor onset and generalized longevity is seen to demonstrate genetic variability (Appendix 2).
- Generalized genetic instability as conferred by elimination of the Werner helicase gene with or without administration of DMBA is insufficient to cause mammary cancer in mice (Appendix 3).
Reportable Outcomes


Conclusions

The work conducted as a result of DAMD17-97-1-7123 demonstrates species differences between mice and rats in apparent sensitivity to the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA). As compared with rats, mice appear to be more sensitive to acute effects of DMBA. It was observed that mice manifest genotypic specificity in the tumors that develop following exposure to this carcinogen. These results suggest that treatment with DMBA increases the incidence and decreases the age of manifestation to at least a major subset of the neoplastic lesions to which the mice are genetically predisposed. Notwithstanding the genetic homogeneity of the various cohorts of inbred mice together with the tight control of environmental conditions, the manifest tumor incidence in these cohorts of mice was not 100%. This is evidence for a stochastic component involved in tumorigenesis.

This work documents that calorie restriction (CR) has a generalized impact of reducing tumor incidence and delaying their manifestation even in carcinogen treated mice. This is, however, the first study to present data clearly illustrating that there is genotypic variability in the magnitude of the murine response to CR. In addition, the generalized genetic instability associated with the Werner's syndrome in humans was shown not to specifically predispose mice to develop breast cancer, even following dosage of the carcinogen DMBA.
References


Improved Survival Rates in Mice that Received Prophylactic Fluids After Carcinogen Treatment

DONALD E. SMITH, BS, MS, RLAT, JEFFREY B. BLUMBERG, PHD, FACN, AND RUTH D. LIPMAN, PHD

Abstract | During the development of a model for 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary adenocarcinoma in mice, a high mortality rate was attributed to dehydration. Therefore, we compared the acute survival of mice given subcutaneous fluids prophylactically immediately following DMBA gavage to that of animals provided treatment only when clinical signs of dehydration were observed. Mortality in the prophylactically treated mice was 5% compared to 47% in animals treated only after the manifestation of dehydration. Prophylaxis with subcutaneous fluids significantly reduces mortality in DMBA-treated mice.

Because of species differences in physiology, experimental protocols based on one animal model may require modification when applied to a different animal. In light of previous work in rats, we developed a breast cancer model in mice by using the pro-carcinogen 7,12-dimethylbenz[a]anthracene (DMBA), (1,2) which forms depurinating DNA adducts in rat mammary epithelial cells (3). DMBA is widely used as a carcinogen in rodents, with a variety of doses, number, and routes of administrations reported (1, 2, 4-7). We selected the DMBA method described by Haag et al. (2) and Hsu et al. (1) because it appeared to allow for a smaller sample size and minimal stress to the animals (5). This method is reported to result in a 100% incidence of mammary adenocarcinoma in susceptible rat genotypes without early mortality (1, 2). The protocol involves only a single administration of carcinogen and thus minimizes both stress to the animals and the potential for environmental contamination. The modifications necessary to manage the hydration status of DMBA-treated mice and to minimize acute mortality are described.

Material and Methods

This study was approved by the USDA Human Nutrition Research Center on Aging Animal Care and Use Committee. Two cohorts of 20 6-wk-old female C3H/HeNHsd mice (Harlan Sprague Dawley, Indianapolis, IN) were individually housed in 8" x 8" x 8" suspended, polycarbonate cages and provided ad libitum access to NIH-31 diet (Harlan Teklad, Madison, WI) and purified water sterilized by UV irradiation. The mice were acclimated to appropriate environmental conditions for 3 wk prior to carcinogen exposure (9, 10). At this time, all animals were observed daily for clinical signs of disease and weighed each week.

Working within a fume hood, we dissolved DMBA (Sigma Chemical, St. Louis, MO) in sesame seed oil (Sigma Chemical) to a concentration of 5.2 mg/mL. The first cohort of 20 mice were anesthetized with Aerrane (isoflurane; Fort Dodge Animal Health, Fort Dodge, IA) in a negative-pressure hood and orally gavaged with 0.13 mL DMBA to provide 65 mg DMBA kg body weight. The second cohort were similarly dosed, but prior to recovery from anesthesia, each mouse was injected subcutaneously (SQ) with 1.0 mL 0.9% NaCl (Abbott Laboratories, North Chicago, IL). In addition to the water bottle with sipper tube present in each metabolic cage, this second cohort of mice was also given a jar of drinking water. The difference between the two cohorts was the timing of the treatment for dehydration rather than the treatment itself.

Three days after dosing, 80% of a DMBA dose is reportedly present in the excreta (11), and no biologically active carcinogen remains in vivo 5 d after an oral gavage (12). Accordingly, mice were housed in metabolic cages (Lab Products, Maywood, NJ) for 1 wk to facilitate collection of all feces and urine potentially contaminated with DMBA. A plastic bag was used to enclose the entire urine/feces separation unit to minimize potential carcinogen contamination of the area. All excreta were disposed of as chemical waste. Personnel safety procedures including protective face shield and disposable garb were used as previously described (13). Access to the animal room (maintained at negative pressure) was restricted.

Comparison of mortality incidence between groups was carried out with a $2 \times 2 \chi^2$ analysis. Average body weights of the mice were compared by using a two-tailed t-test. Statistical analyses were conducted with STATOOLS (14).

Results

No difficulties were experienced while gavaging the mice, and all animals were ambulatory and active upon recovery from anesthesia, which occurred within 1-2 min after dosing. All mice appeared to be in a similar condition 24 h after receiving DMBA. Between 48 h and 72 h after DMBA dosing, three animals in the first cohort (no prophylactic fluids) were lethargic, with clinical signs of dehydration, including anorexia, anuria, loss of skin elasticity, and skin turgor. Mice observed with any of these clinical signs were given 1.0 mL saline SQ, and a water jar was placed in their cage. Despite this supportive fluid therapy, the condition of these mice did not improve, and they died within 48 h. Another eight mice in this cohort had similar clinical signs up to 4 wk after DMBA treatment and were provided with supportive hydration; these mice subsequently died within 48 h. The two cohorts did not differ with respect to the hydration measures taken, but rather the time point after dosing at which they were initiated.

The difference in post-dosing mortality was significantly different ($p \leq 0.05$) between groups (Figure 1). The cumulative 4 wk post-procedure mortality for the first cohort of mice was 47%. This value compared with a loss of only 5% (one mouse) during the same 4-wk period for mice receiving the prophylactic injection of saline immediately after DMBA dosing. At 2 weeks after dosing, the average weight of the mice in the first cohort that...
died during weeks 3 and 4 (18.6 ± 1.51 g) was significantly less than that of the prophylactically treated mice (22.3 ± 2.3 g; p ≤ 0.003). The average weight of the surviving mice 4 weeks after DMBA administration did not differ (21.3 ± 1.8 g for control animals vs. 22.3 ± 2.3 g for those prophylactically treated).

**Discussion**

The induction of mammary tumors in the rat with the use of chemical carcinogens is a commonly utilized model for the study of breast cancer (15). Although the rat model is ideal for some experiments, there are valid reasons for examining phenomena in other species, including the facilitation of specific analyses or the comparison of effects between species. We highlight here the great importance of prophylactic hydration to survival of mice treated with DMBA.

Dehydration in mice leads to diminished food intake, generalized weakness, decreased ability to regulate body temperature, hypovolemia, and electrolyte imbalances with renal and cardiovascular failure. Basic veterinary care, i.e., provision of supportive fluid therapy upon presentation of clinical signs, was insufficient to prevent the high mortality associated with the effective DMBA dose. The timing of fluid administration as a supportive measure is an important factor and may be narrowly defined in rodents (16). Prophylactic interventions to facilitate hydration may be prudent for the adaptation of other rat protocols to mice, as a variety of important physiologic functions including immune responses, renal cortical blood flow, and drug distribution are altered by hydration status in mice (17–19). Subcutaneous fluid administration as a means of rehydration is effective in other species, including humans (20). As also suggested by Dieterich et al. (17), this study reinforces the necessity of performing small-scale pilot studies to adapt published protocols from one animal model to another prior to initiating large experiments.

**Acknowledgments**

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**References**


Effect of Calorie Restriction on Mortality Kinetics in Inbred Strains of Mice Following 7,12-dimethylbenz[a]anthracene Treatment

Ruth D. Lipman

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Calorie restriction (CR) has long been known to increase longevity and to delay the onset and to decrease the incidence of many age-related disease processes. The mechanism(s) by which these outcomes are attained is unidentified. This experiment was designed to examine whether differences exist in the extent to which various inbred strains of mice respond to CR. This work explored whether carcinogen-treated animals could be used to facilitate this aim by decreasing the time needed to observe differences in mortality kinetics between CR mice and ad libitum (AL) fed controls. Female mice from each of eight strains (A/J, BALB/c, C3H, C57BL/6, DBA/2J, FVB/J, NMRI, and 129/J) were given a single oral dose (65 mg/kg) of the carcinogen 7,12-dimethylbenz[a]anthracene and subsequently fed AL or calorically restricted. Following carcinogen treatment, the spectrum of lesions observed demonstrated genotypic variability, thereby complicating comparison among the inbred strains examined. However, in terms of the magnitude of alteration in mortality kinetics observed, a statistical analysis revealed that differences exist among the various strains of mice in their response.

The experimental paradigm of calorie restriction (CR) has been repeatedly demonstrated to increase both mean and median life span in mice and rats. Early implementation of CR maintains youthful physiology such that when compared with age-matched ad libitum (AL) fed controls, CR animals show fewer age-associated changes and live significantly longer (1).

The list of age-related parameters affected by CR continues to expand. However, the mechanism(s) by which CR ameliorates age-related changes, reduces disease burden, and increases longevity in rodents has not been identified. CR has been postulated to have its fundamental effect at the cellular organelle, macromolecular, or metabolic level. The effect of CR has been viewed as fundamentally influencing cell turnover by affecting proliferation (2), apoptosis (3), or both. One suggestion for the genesis of the broad-based changes observed with CR is that they are derived from basic alterations in free radical generation or the various mechanisms for managing the ravages of oxidative stress (4,5). A related hypothesis as to the origin of the beneficial outcomes obtained from CR is that they are derived from its effect on mitochondria (6). Alternatively, it has been proposed that the effects resulting from CR are a consequence of its effect on the fat mass, which in turn modulates a host of endocrine factors, cytokines, and other peptides (7). Discriminating among these and other proposed hypotheses is difficult because of the multitude of biological parameters that are altered by CR.

Few studies have compared the effects of CR among different genotypes. The Biomarkers of Aging Program of the National Institute on Aging was the most multigenotypic study of CR. It included four genotypes of mice (C57BL/6, DBA/2, B6C3F1 hybrid, and B6D2F1 hybrid) and three genotypes of rats (Fischer 344, Brown Norway, and F3BNF1 hybrid) (8). The data presented by Turturo and colleagues showed that the response to CR ranged from an approximate 10% increase in the age at which 50% mortality was reached in the DBA/2 mice to nearly a 35% increase in the B6C3F1 hybrids as compared with the appropriate AL fed male controls (8). These data suggest a genotypic variability in the magnitude of response to CR.

This study was designed to both follow up this observed variability and to determine whether carcinogen exposure could be exploited as a means of decreasing the time required for experimentation with the CR paradigm. This article is a first step in exploring a role of genetics in CR responsiveness using the mortality kinetics of CR and AL cohorts of eight inbred strains of mice after carcinogen treatment.

METHODS

Six-week-old virgin female mice were obtained as follows: A/J, BALB/c, C3H, C57BL/6, and DBA/2 were obtained from Harlan Sprague (Indianapolis, IN); 129/J and FVB/J were obtained from The Jackson Laboratory (Bar Harbor, ME) and NMRI were obtained from B&K Universal (Fremont, CA). The mice were maintained under a cycle of 12 hours of light and 12 hours of dark at 23°C and 45% humidity. Following arrival, all the mice had AL access to NIH-31 diet (Harlan Teklad, Madison, WI) and sterilized water. A total of 37-42 mice of each genotype were utilized in this study. To facilitate the logistics of the experi-
Figure 1. Mouse genotypes are arranged in descending order of the percentage increase in lifespan attained by caloric restriction.
mental protocol, mice were obtained over a period of 14 weeks. The various genotypes were then housed in the facility concurrently. All the mice except for the NMRI were obtained from facilities purported to be specific pathogen free. The NMRI mice were obtained at 1 month of age and were quarantined for 2 months. After that time, two NMRI mice were used to test for exposure to common mouse pathogens and were found to be positive. Sentinel mice housed among the study animals were tested monthly for exposure to or the presence of common pathogens and were never found to be positive.

The mice were acclimated to the laboratory environment for 3 weeks before being dosed with 7,12-dimethylbenz[a]anthracene (DMBA). Each mouse was anesthetized with isoflurane (Fort Dodge Animal Health, Fort Dodge, IA), orally gavaged with 65 mg of DMBA/kg of body weight, and given 1.0 ml subcutaneous injection of 0.9% NaCl to prevent dehydration (9). For 1 week after the mice were dosed, they were maintained in metabolic cages to facilitate safe collection of carcinogen contaminated excreta as previously described (9). The mice were then individually housed in polycarbonate cages with wood chip bedding until they were observed to have a tumor, appeared moribund, or reached 78 weeks (18 months) of age. When the effect of diet on life span was analyzed, only those animals surviving to the age at which the CR diet was initiated for the CR cohort (12 weeks) were included. The number of animals succumbing acutely to carcinogen administration ranged from zero to two mice per genotype with no significant differences detected among the genotypes. Important to understanding the experimental design is that mice were not subjected to CR until well after the time required for metabolism of the carcinogen DMBA.

Prior to carcinogen administration, the mice in each genotype were assigned to either the AL or CR diet group and the body weights for these diet groups within each genotype were matched at that time. Cage position on the shelves in the animal room intermingling the diet cohorts was also assigned at this time. Three weeks after dosing, when the mice were 12 weeks of age, the food intake of the CR mice for each genotype was gradually reduced so that the body weights of the restricted mice were between 60% and 70% of those of the AL cohort for that genotype (Figure 1). The ratio of the body weights of the CR versus AL cohort for each genotype was used to titrate food intake for each strain as long as the AL cohort survived. CR cohorts surviving beyond the control individuals in their genotype continued to receive the same amount of food they had been fed prior to the loss of the controls. Modeled after the protocol used in the Biomarkers of Aging experiment (8), the mice in the CR cohorts were fed vitamin- and mineral-supplemented NIH-31 diet (Harlan Teklad), while the AL controls consumed the NIH-31 diet through the study. Mice were weighed biweekly for the duration of the study (Figure 1). Mice with grossly visible tumors or that demonstrated a weight loss greater than 20% in 2 weeks, that exhibited pain or distress, or that failed to consume food in a 2-day period or reached 78 weeks of age were terminated by CO₂ inhalation. With the use of these criteria, only six mice were lost to analysis of pathology caused by severity of autolysis.

Table 1. Average Body Weight of AL and CR Mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>AL</th>
<th>CR</th>
</tr>
</thead>
<tbody>
<tr>
<td>129J</td>
<td>18.91 ± 1.7ₐ</td>
<td>14.57 ± 1.6ₐ</td>
</tr>
<tr>
<td>A/J</td>
<td>21.66 ± 1.5ₐ</td>
<td>18.41 ± 1.5ₐ</td>
</tr>
<tr>
<td>BALB/c</td>
<td>23.89 ± 3.0ₐ</td>
<td>18.40 ± 1.4ₐ</td>
</tr>
<tr>
<td>C3H</td>
<td>27.61 ± 2.8ₐ</td>
<td>19.12 ± 1.4ₐ</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>23.00 ± 2.2ₐ</td>
<td>18.40 ± 0.3ₐ</td>
</tr>
<tr>
<td>DBA/2</td>
<td>22.05 ± 1.8ₐ</td>
<td>16.62 ± 1.1ₐ</td>
</tr>
<tr>
<td>FVB/J</td>
<td>24.97 ± 2.3ₐ</td>
<td>18.75 ± 1.0ₐ</td>
</tr>
<tr>
<td>NMRI</td>
<td>27.27 ± 2.5ₐ</td>
<td>19.90 ± 1.3ₐ</td>
</tr>
</tbody>
</table>

Notes: AL = ad libitum (fed); CR = calorie restriction. The average body weight ± standard deviation for the AL fed control and CR cohorts of each genotype is presented with superscript letters identifying statistically significant differences (p < .001) in weight between diet groups in each genotype.

Dead or sacrificed mice were necropsied. All tissues were fixed in Tellysesniczky’s fixative (20:2:1 of 70% ethanol, 37% formalin, and glacial acetic acid). Tissues were dehydrated and then embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin and eosin. Lesions were considered as commonly occurring if they were observed in at least 10% of one genotype.

A multivariate distribution-free significance test (10), which utilized a Wilcoxon rank test, was used to compare the growth curves for the AL control and CR cohorts within each genotype. The average body weight of the two diet cohorts for each genotype are presented in Table 1, and the average age ± standard deviation for each genotype—diet cohort are in Table 2. Mortality kinetics within each genotype were analyzed by the Lifetest Procedure (SYSTAT version 7.0.1 for Windows, SPSS Inc., Chicago, IL). A listing of the commonly observed lesions is presented in Table 3.

RESULTS

The mice of all genotypes were active within a few minutes after dosing as previously reported (9). There were no genotypic differences in terms of acute effects of DMBA admin-

Table 2. Average Age Attained by AL and CR Mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>AL</th>
<th>CR</th>
<th>% Increase in Longevity in Reference Population (8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>129J</td>
<td>40.2 ± 7.5</td>
<td>32.0 ± 11.4</td>
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</tr>
<tr>
<td>A/J</td>
<td>47.7 ± 16.4</td>
<td>32.7 ± 19.7</td>
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<tr>
<td>BALB/c</td>
<td>42.3 ± 18.3</td>
<td>32.6 ± 24.4</td>
<td>33.6ₐ</td>
</tr>
<tr>
<td>C3H</td>
<td>53.4 ± 13.2</td>
<td>46.7 ± 15.5</td>
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</tr>
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<td>C57BL/6</td>
<td>55.2 ± 22.1</td>
<td>47.9 ± 7.0</td>
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<tr>
<td>DBA/2</td>
<td>42.2 ± 15.3</td>
<td>32.9 ± 13.7</td>
<td>63.5ₐ</td>
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<tr>
<td>FVB/J</td>
<td>51.6 ± 21.1</td>
<td>42.7 ± 21.7</td>
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</tr>
<tr>
<td>NMRI</td>
<td>52.7 ± 8.6</td>
<td>44.3 ± 14.5</td>
<td>26.8ₐ</td>
</tr>
</tbody>
</table>

Notes: AL = ad libitum (fed); CR = calorie restriction. The average age in weeks ± standard deviation at a maximum of 18 months of age is provided for each genotype—diet group with asterisks signifying statistically significant differences (p < .02) for calculated mortality kinetics. Data for the referent population (C57BL/6 and DBA/2) is from the National Center for Toxicological Research Project on Calorie Restriction (8).
Table 3. Manifestation of Commonly Observed Lesions by Genotype

<table>
<thead>
<tr>
<th>Lesion</th>
<th>129/J</th>
<th>A/J</th>
<th>BALB/c</th>
<th>C3H</th>
<th>C57BL/6</th>
<th>DBA/2</th>
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Notwithstanding genotypic differences in the specific lesions observed, the impact of CR on mortality kinetics was clearly observable in seven out of the eight genotypes examined. The impact of CR is a most robust phenomenon that has been reproduced in numerous laboratories assessing a wide range of parameters. The failure to observe a statistically significant change in the mortality kinetics is strongly suggestive that the FVB/J genotype is less responsive to CR than the other strains studied. This is consistent with the generally recognized complexity of the relationships existing among genetic susceptibility, environmental factors, and their interaction(s) (13). Although recent reports summarize subsets of the changes in gene expression that occur with CR (11,12), there has been little study of the interaction between genotype and CR. The data reported here suggest that CR response gene(s) do exist but that the genetic analysis of CR responsiveness is likely to be confounded by differences in commonly occurring genotype-specific age-related changes.

DISCUSSION

Caloric restriction is well documented to increase longevity, and although the mechanism(s) by which this effect is attained is heavily speculated upon, it remains unknown. Nonetheless, CR clearly alters the expression of many genes (11,12). This work fails to demonstrate that the use of carcinogen-treated mice provides any utility in terms of reducing the time needed for experimentation or providing greater homogeneity in commonly occurring lesions (Table 3). The data presented are consistent with the previous report (8) supportive of hypothesized differences in the magnitude to which various genotypes respond to CR. Carcinogen treatment may amplify such differences.

Acknowledgments

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This work could not have been done without the meticulous attention to the animals’ health and care provided by Maureen Kelly. I am grateful to Dr. R. T. Bronson for the many valuable discussions we have had regarding this project, to Dr. G. Dallal for assistance with the statistical evaluation of these data; and to Dr. J. A. Taylor for indulging my interest in this area of research.

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GENOTYPE AND CALORIE RESTRICTION EFFICACY


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Mutations in the WRN Gene in Mice Accelerate Mortality in a p53-Null Background

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Werner’s syndrome (WS) is a human disease with manifestations resembling premature aging. The gene defective in WS, WRN, encodes a DNA helicase. Here, we describe the generation of mice bearing a mutation that eliminates expression of the C terminus of the helicase domain of the WRN protein. Mutant mice are born at the expected Mendelian frequency and do not show any overt histological signs of accelerated senescence. These mice are capable of living beyond 2 years of age. Cells from these animals do not show elevated susceptibility to the genotoxins camptothecin or 4-NQO. However, mutant fibroblasts senesce approximately one passage earlier than controls. Importantly, WRN+/−; p53−/− mice show an increased mortality rate relative to WRN−/−; p53−/− animals. We consider possible models for the synergy between p53 and WRN mutations for the determination of life span.

Werner’s Syndrome (WS) is a recessive genetic disease which shows premature onset of many pathologies normally associated with old age (18). Patients with WS appear normal during the first decade of life. The first manifestation of this disease is typically growth failure during adolescence. Subsequently, these patients suffer prematurely from a variety of age-related disorders: skin changes, osteoporosis, diabetes, accelerated atherosclerosis, and cancer, particularly sarcomas. Fibroblasts derived from individuals with WS divide many fewer times prior to senescence than do fibroblasts from age-matched control individuals (13). Genomic instability has been observed in WS cells, as chromosomal rearrangements (5, 19, 21) and as mutations within the hypoxanthine phosphoribosyltransferase gene (HPRT); in vivo, an increased frequency of HPRT mutant cells has been observed in patients with WS (2, 3, 14). The gene defective in WS, WRN, encodes a protein of 1,432 amino acids with similarity to the RecQ subfamily of DNA helicases (26). Although mutations throughout the WRN gene have been observed in the homozygous state, homozygosity for a mutation very near the 3′ end of the WRN open reading frame is sufficient to lead to the disease (15).

A mouse knockout (KO) of the WRN gene has been described (10). Lebel and Leder deleted exons III and IV in the catalytic helicase domain of the WRN locus, a mutation predicted to eliminate catalytic function. Cells containing this mutation express an internally deleted, nearly full-length WRN protein. Homozygous mutant mice are viable, indicating that this particular mutation is not lethal. However Lebel and Leder showed a decreased embryonic survival of their mutant: on a C57BL/6-129/SvEv outbred background and on a 129/SvEv inbred background, the ratios of +/+; +/+; ++/−; −/− mice born are 1:2:0:0.8 and 1:1:9:0.6, respectively. Mutant embryonic stem (ES) cells have an approximately sixfold increased mutation rate at the HPRT locus. They are also 10-fold more sensitive to camptothecin, a topoisomerase I inhibitor, and are two- to threefold more sensitive to etoposide, a topoisomerase II inhibitor. Late-passage mutant embryonic fibroblasts also show decreased saturation density in culture, although this was not evident in early-passage cells. The mice themselves, however, are healthy and fertile, showing no signs of premature organ/cytic aging or increased rates of tumor formation. Thus, this KO does not recapitulate many of the phenotypes of human WS.

Here, the generation and characterization of a WRN-null mouse mutant is described. Most phenotypes in the mutant are remarkably similar to the wild type. Cells from these animals are not hypersensitive to camptothecin, unlike those of Lebel and Leder. Most interestingly, the WRN−/− homozygous animal displays a shorter life span in the p53−/− background. We discuss this shortening with respect to a possible aging phenotype.

MATERIALS AND METHODS

Cloning of WRN. A size-selected murine cdNA plasmid library was screened by standard methods (20) by using an 820-bp probe derived from the 3′ end of the human WRN-coding sequence. This probe was generated by PCR from human cdNA with the following oligonucleotides: 5′ AGG TCC AGA TTG GAT CAT TGC 3′ and 5′ GCC CAA CAT GGC AGC TTT GCC 3′. Hybridizations were performed at 55°C. Twenty-two clones were isolated, and preliminary restriction mapping and 3′ sequencing suggested that they were all products of the same gene. The largest clone was sequenced on both strands.

Generation of antibodies against WRN. A polyclonal antiserum was raised in chickens (Covance) against a His6-tagged protein fragment corresponding to amino acid residues 1191 to 1390 of the WRN protein. Immunoglobulin Y was isolated from eggs by using a commercially available kit (ECCO; Stratagene) and was further purified over a diaminopropylamine column (Pierce) containing 5 mg of bound immunizing antigen.

Tissue Western blotting. Fragments of various mouse tissues were placed in Laemmli buffer, macerated with a polytron, and boiled. Equal amounts of proteins were loaded into each lane and assayed by Coomassie blue staining of a duplicate gel (20). Horseradish peroxidase-conjugated antichicken antibodies were used to detect bound anti-WRN. ECL reagent (Amer sham) was used to develop the bound secondary antibody.

Targeting the WRN locus. Several genomic clones in lambda phage encoding portions of the WRN locus were recovered by screening a genomic library in EMBL 3A with a full-length WRN-coding region probe by standard methods (20). Two clones encoding portions of the catalytic helicase domain were subcloned into pBR322 and were extensively mapped with restriction enzymes. To
construct the 5′ homology arm of the targeting vector, a 3.0-kb SalI/HindIII fragment from the larger pBR322 clone was subcloned into the SalI/HindIII sites of pSL190 (PharMacia). For the 3′ homology arm, a 4.9-kb BamHI/SalI fragment was ligated into the BamHISalI sites of the pSL190 vector. A KpnI/NcoI fragment containing the β-globin cassette (β-galactosidase/neomycin' fusion gene) was then inserted into the KpnI/NcoI sites of the pSL1190 construct. An internal SalI site in the β-globin cassette was obliterated by partial digestion followed by blunting and religation, and then the completed cassette was excised from the vector with SalI digestion. All cell culture and mouse embryo manipulations were as previously described except that no negative selection step was employed (11). Chimeric founders were crossed with BALB/c animals, and progeny from these matings were intercrossed to obtain homozygotes.

The genotypes of neomycin-resistant ES cell clones were checked with two external flanking probes. The 3′ probe consisted of a 1.2-kb ScaI/Nhel restriction fragment (see Fig. 2a) which yielded a 9-kb wild-type band and a 6-kb mutant band on genomic DNA digested with Nhel. The 5′ probe consisted of an Ndel/KpnI fragment which was isolated and further digested with EcoRI and the upper- and lower-500-bp portion was used as the probe. On EcoRI-restricted genomic DNA, this probe detected a large (>13 kb) band representing the wild-type allele and an approximately 10-kb band representing the mutant allele. A PCR genotyping assay was also developed based on the results of genomic sequence. The oligonucleotides used were as follows: pSL3093, 5′ GCC TGC ACG TGG CCG CAT C3′; COMMON2, 5′ CAA TAA CCA ATG GAA TTC TAA GC3′; and WT1, 5′ TAC ATT TGC CAT TTT AAG GTG GC3′. The PCR conditions were 95°C for 3 min, followed by 30 cycles of 94°C, 30 s; 55°C, 30 s, and 72°C, 30 s, followed by a final 5-min incubation at 72°C. This combination of oligonucleotides produces an approximately 250-bp band in the presence of the mutant allele and an approximately 150-bp band in the presence of the wild-type allele.

Splenocyte culture. Spleens were isolated from mice of the indicated genotypes, erythrocytes were lysed, and the splenocytes were resuspended at a concentration of 2 × 10^6/ml in plating media (10% fetal bovine serum–Gin-HEPES–6.0 × 10^{-3} M β-mercaptoethanol in RPMI medium [Gibco]). To determine response to mitogenic stimulation, 2 × 10^5 lymphocytes/well were plated in triplicates in 96-well plates. Anti-CD3 was added with the indicated dilution, and the cultures were left for 72 h. During the last 24 h, the cultures were pulsed with 1 μCi of [H]thymidine per well. The wells were then harvested, and proliferation was quantified on a scintillation counter. The results shown are representative of two separate experiments.

Embryonic fibroblasts. Murine embryonic fibroblasts were generated from day-13.5 embryos as previously described (6). Fibroblasts were cultured in media consisting of 10% fetal bovine serum in Dulbecco modified Eagle medium (Gibco). To determine response to mitogenic stimulation, 2 × 10^5 lymphocytes/well were plated in triplicates in 96-well plates. Anti-CD3 was added with the indicated dilution, and the cultures were left for 72 h. During the last 24 h, the cultures were pulsed with 1 μCi of [H]thymidine per well. The wells were then harvested, and proliferation was quantified on a scintillation counter. The results shown are representative of two separate experiments.

RESULTS

Cloning and protein expression studies of the mouse WRN homolog. A size-selected murine cDNA library derived from activated lymph node and spleen was screened at reduced stringency with a hybridization probe derived from the 3′ end of the human WRN-coding sequence. The largest clone was sequenced in its entirety on both strands. This 6,476-nucleotide cDNA encodes a putative protein of 1,401 amino acids which is 72% identical to the human WRN protein at the amino acid level. Others have independently cloned the mouse WRN homolog (7). The inferred protein sequence reported by Imamura et al. is identical to that reported here at all but three residues: the Imamura et al. sequence contains a Q rather than a K at position 800, an A rather than a T at position 1145, and a V rather than an L at position 1181. These differences may represent polymorphisms between the two strains of mice used to generate the libraries from which these cDNAs were derived or errors in reverse transcription. Outside the coding region, the Imamura et al. sequence shows several nucleotide differences from that described here. The WRN nucleotide sequence reported here also contains two exons in the 5′ and 3′ untranslated regions absent in the Imamura et al. sequence as well as 1,347 nucleotides of 3′-UTR sequence not reported by Imamura et al.

In order to examine the tissue distribution of the WRN protein, polyclonal antiserum to a C-terminal fragment of the WRN protein corresponding to amino residues 1191 to 1390 was raised in chickens. This region was chosen because it lies outside the catalytic helicase domain and was therefore unlikely to contain epitopes cross-reactive with other helicases. Affinity-purified antiserum was used to probe a Western blot containing lysates of various murine tissues (Fig. 1). The band corresponding to the murine WRN protein migrates at roughly 170 kDa. Murine WRN protein is expressed in lung, kidney, pancreas, liver, testes, and ovary but is present only at very low levels in cortex, cerebellum, heart, and skeletal muscle.

Targeting the WRN locus. Mice bearing a targeted mutation in the murine WRN gene were generated. The full-length murine WRN cDNA was used as a hybridization probe to recover several clones from a 129/Sv genomic library (library courtesy of the Houseman laboratory). In turn, these clones were used to generate a targeting construct in which the 3′-most exon encoding a portion of the catalytic helicase domain is replaced by a β-globin cassette (Fig. 2a). If there should be splicing around this cassette, this mutation is also predicted to introduce a frameshift mutation. Homozygous mutations in the helicase domain or near the 3′ end of the WRN open reading frame are sufficient to confer the WS phenotype in humans (15). This construct was electroporated into ES cells; of 88 neomycin-resistant clones selected, 11 were heterozygous for the WRN mutation, yielding a targeting frequency of 12.5%. Two correctly targeted clones were used to generate chimeric founders. These mice, representing two independent ES cell clones, were used to generate heterozygotes, and these heterozygotes were subsequently intercrossed to obtain homozygotes. Mice of different genotypes are distinguished by Southern blotting (Fig. 2b) or by PCR assay. Western blot analysis of whole-cell extracts of ear fibroblasts from mice of different genotypes using an antibody directed against the C terminus of the murine WRN protein demonstrates that there is no detectable WRN expression in KO cells (Fig. 2c). Probing of these extracts with antisera directed against the N terminus of the WRN protein did not reveal a truncated WRN protein in mutant animals (data not shown).

WRN KO animals are viable. Heterozygous crosses have produced offspring in the ratio of 108 +/- / 173 +/- to 105 +/- / . Crosses between heterozygous mice and mutant mice have yielded mice in the ratio of 98 +/- / 93 +/- / . Since these are close to the ratios predicted by simple Mendelian segregation, it seems unlikely that the WRN mutation described in this
work confers any prenatal lethality, in contrast to that described elsewhere (10). Mutant animals are grossly normal, and all KO animals tested are fertile. The oldest homozygote obtained is over 2 years old and is still healthy. Histological examination of several aged KO animals ranging in age between 3 and 17 months failed to uncover any unusual lesions, with the exception of bone marrow hyperplasia in one.

**Mutant splenocytes proliferate normally.** The proliferation of cells derived from mutant animals was examined in culture. Splenocytes were derived from two mutant animals, two wild-type animals, and one heterozygote and were treated with various dilutions of anti-CD3, a mitogenic stimulus; the response was measured by [3H]thymidine uptake 3 days later (Fig. 3). No significant differences were noted between mutant and control animals.

**No heightened susceptibility to camptothecin or 4-NQO in mutant embryonic fibroblasts.** WS patient cells show sensitivity to the DNA-damaging agent 4-NQO (4, 16), and WRN KO ES cells described by Lebel and Leder show sensitivity to the topoisomerase I poison camptothecin (10). In order to determine whether WRN mutation would confer sensitivity to these agents, WRN+/− or WRN−/− embryonic fibroblasts were cultured in the presence of these agents, and the number of viable cells was quantitated by using an assay to detect viable cells via their mitochondrial respiration (Cell Proliferation Kit II; Boehringer-Mannheim) 3 days later. These cell lines were also heterozygous for a mutation in the BLM gene (G. Luo and A. Bradley, unpublished data). Neither camptothecin (Fig. 4a) nor 4-NQO (Fig. 4b) affected WRN mutants differentially.

**Modestly accelerated senescence in WRN−/− embryonic fibroblasts.** In humans, a cardinal feature of WS is accelerated senescence in patient skin fibroblasts. Experiments were undertaken in order to determine whether this phenotype might be recapitulated in the WRN KO mouse. WRN+/−;BLM+/− and WRN−/−;BLM+/− fibroblasts were serially passaged in culture; 106 cells were plated at each passage, and the number of cells present at confluence was determined several days later (Fig. 5). The number of cells at confluence has been used as a measure of replicative potential in previous studies (25). WRN KO cultures cease growing approximately one passage earlier than controls.

**Homozygous WRN mutations accelerate mortality in p53−/− animals.** In humans, WS is associated with a heightened susceptibility to tumors. In order to accentuate any predisposition to tumors in WRN KO mice, WRN mutants were bred to p53−/− mice (8). Animals with the genotypes WRN+/−;p53−/− or WRN−/−;p53−/− were monitored over time (Fig. 6). Whereas WRN−/−;p53−/− animals had an average life span of 149 days, WRN−/−;p53−/− animals lived for an average 122 days. The survival curves are statistically different from one another by the Wilcoxon ranked sum test (P = 0.0163). The possible implications of this result are discussed below.

**DISCUSSION**

Here, the cloning of a highly conserved murine homolog of the WRN protein is described. Despite the high degree of sequence identity between these two proteins, the human and mouse WRN homologs do not show similar immunolocalization patterns (12). Mice bearing a targeted mutation in the catalytic helicase domain of WRN are viable and fertile, they do not show any histological signs of premature aging, and they are capable of surviving until at least 2 years of age. Splenocytes from these animals proliferate normally in response to a mitogenic stimulus; however, cells from these animals senesce prematurely in cell culture.

**Homozygous WRN mutations accelerate mortality in p53−/− animals.** There are two general possible explanations for this synthetic interaction between the WRN and p53 genes. First, homozygous mutations in WRN may exacerbate the cancer-
prone phenotype of $p^{33^{-/-}}$ animals. The genome instability reported in cases of WS could be the molecular basis of this interaction. WRN and p53 have recently been shown to interact physically, further suggesting that these proteins may cooperate to maintain genome stability (1, 23). A second possibility is that the homozygous WRN mutant animals do have a slightly accelerated aging phenotype. This phenotype might be first evident in the $p^{33^{-/-}}$ background because of its short life span. In this view, the cancer phenotype itself would be under the control of the aging program of mice. Thus, speeding up this program would advance all of the regulated phenotypes, including cancer in a wild-type or $p^{33^{-/-}}$ cancer-prone strain. This model predicts that the $WRN^{-/-}$ animals will also display a slightly shortened life span in the $p^{33}$ wild-type background. Although some $WRN^{-/-}$ animals are now over 2 years old, it is still too early to know whether their life span will be shortened compared to that of the wild type.

Lebel and Leder have described a WRN KO bearing a helicase domain mutation which shows several phenotypes (10). Mutant ES cells are highly sensitive to camptothecin and show an elevated mutation rate, and late-passage embryonic fibroblasts possess a shortened in vitro life span compared with that of wild-type cells. In addition, mutant animals are born at less than the expected frequency, suggesting that this mutation confers some prenatal lethality. By contrast, mutant embryonic fibroblasts described herein are not hypersensitive to camptothecin. The former difference may stem from biological differences between embryonic fibroblasts and ES cells. $WRN^{-/-}$ embryonic fibroblasts generated in this work do possess a modestly shortened in vitro proliferative capacity, in accord with the results of Lebel and Leder; however, we find that WRN mutant mice are born at the expected frequency. Several possible explanations exist for these discrepancies. Modifying loci in ES cells and/or mouse strains may alter the phenotypic consequences of WRN mutations. The nature of the WRN alleles generated represents another potential reason for these discrepant results. The allele described herein deletes an exon in the catalytic helicase domain and introduces a frameshift mutation, resulting in no detectable protein expression, as assayed by immunofluorescence (12) and Western blotting using an anti-C-terminal antibody. As the nuclear localization signal of the human WRN protein lies at the distal C terminus of the protein, it seems likely that this mutation should represent a functional null. By contrast, the mutation described by Lebel and Leder results in the expression of an internally deleted fragment that still has the potential to localize to the nucleus.
WRN protein is spread diffusely throughout the nucleoplasm (12). This suggests that some divergence in WRN function may have occurred between mice and humans. It is also possible that murine WRN is functionally redundant with another helicase, either a RecQ family member or perhaps a member of a different helicase family altogether. In addition, mice may show milder effects of a WRN mutation simply as a result of their smaller size and shorter life span, perhaps not allowing enough time for the full spectrum of effects of WS to manifest themselves.

Another potential reason for the discrepancy between the behavior of WRN mutants in mice and humans is that the nature of the WRN target may differ. One such target of the WRN protein may be the telomeres. In primary human WS cells, telomeres shorten more rapidly than in wild-type cells, though WS cells ultimately senesce with longer telomeres than do wild-type cells (22). One explanation for the latter observation is that telomeres may be more recombinogenic and unstable in WS cells than in normal cells; hence, there may be more variation in telomere length in WS cells than in wild-type cells. This may occasionally produce a single very short telomere in WS cells which overall retain long telomeres; this could lead to senescence in cells which, for the most part, still possess long telomeres. Data consistent with telomeric instability in WS have been obtained in studies of lymphoblastoid cells (24). Recent studies in our laboratory suggest that introduction of telomerase into primary WS cells can rescue their premature senescence (B. Johnson, personal communication). Mice, unlike humans, express telomerase constitutively in multiple somatic tissues and possess very long telomeres (9, 17); thus, if telomeres are an important target of WRN, many of the effects of WS might not be evident in the mouse. One critical test of this model will be to cross WRN mutant mice with mice

where it might exert unpredictable effects. Thus, the effects noted by Lebel and Leder might not represent those of a true null allele in the WRN gene.

Several possible explanations exist as to why murine WRN mutants do not recapitulate the full spectrum of effects seen in human WS patients. Mice may possess more than one WRN homolog; disruption of the putative second WRN gene or both genes in the same animal might be required to recapitulate the human phenotype. Several observations argue against this hypothesis. In this study, 22 clones, all derived from the same gene, were isolated via reduced-stringency hybridization of a splenic library. This same gene has been isolated by using degenerate reverse transcriptase PCR (7, 10). Hence, if there is a second WRN gene in mice, it must be expressed at much lower levels and/or be significantly diverged in sequence from the one that has been described. The WRN gene lies in a chromosomal region in the mouse which is syntenic to human chromosome 8p, the location of the human WRN gene (10, 26). Screening of Northern blots at reduced stringency does not reveal any transcripts which might correspond to a second WRN gene (D. B. Lombard, unpublished data). Finally, antibodies derived against the WRN protein and antibodies against the human WRN protein only recognize the known WRN protein in the mouse (D. B. Lombard and R. Marciniak, unpublished results). Thus, it is unlikely, though still formally possible, that more than one WRN gene exists in the mouse.

Another possible explanation for the failure to produce a strong WS-like phenotype in the mouse is simply divergence between mice and humans in WRN function and/or, more generally, in DNA repair functions. In humans, the WRN protein is concentrated in the nucleolus, whereas the murine

FIG. 4. No elevated sensitivity to camptothecin or 4-NQO in WRN<sup>−/−</sup>, BLM<sup>−/−</sup> embryonic fibroblasts. (a) Camptothecin treatment. (b) 4-NQO treatment. y axis represents the number of cells in the treated well divided by the number of cells in the control well.
lacking the telomerase RNA component to determine whether these double-mutant animals show any synthetic phenotypes. Such experiments are underway.

In summary, we have generated and characterized a murine mutant in the WRN locus. Further studies in both mice and in human cells are necessary to elucidate the role of WRN in normal cellular physiology and its possible role in aging.

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FIG. 6. Mortality of WRN<sup>−/−</sup>, p53<sup>−/−</sup> and WRN<sup>−/−</sup>, p53<sup>−/−</sup> mice. The health status of the mice was monitored several times per week. Mice were sacrificed when obviously moribund or, in some cases, died on their own. The difference between the curves was judged significant (P = 0.0163) by the Wilcoxon ranked sum test.