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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)
This work examined the response of different genotypes of mice to the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA). Mice demonstrate a genotypic specificity for the particular tumors that they develop; DMBA increase the incidence and decreases the average age at which these tumors are manifest. It does not appear that the genetic instability of the sort associated with the Werner syndrome in humans appreciably enhances the predilection of mice to develop mammary cancer, even in mice dosed with the carcinogen DMBA. Increased adiposity is a risk factor for postmenopausal breast cancer in humans. Data is presented demonstrating that the dietary intervention of calorie restriction reduces the incidence of mammary tumors following carcinogen administration in genotypes predisposed to its development. Conversely, work demonstrated that increased adiposity resulting from hyperphagia present after oral gavage with DMBA increases mammary tumor incidence in mice. Two aspects of this work illustrate aspects of mammary tumor carcinogenesis deserving of further investigation. The first is that since tumor incidence in genetically identical individuals maintained under identical environmental conditions is not 100%, it indicates that there is a stochastic component involved in tumor development. The second is that adiposity may be an important modifiable breast cancer risk factor.

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<tr>
<td>Cover</td>
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<td>SF 298</td>
<td>2</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>3</td>
</tr>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Body</td>
<td>5</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>8</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>9</td>
</tr>
<tr>
<td>Conclusions</td>
<td>10</td>
</tr>
<tr>
<td>References</td>
<td>11</td>
</tr>
<tr>
<td>Appendices</td>
<td>12</td>
</tr>
</tbody>
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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. Examination of this phenomena was complicated by the difference in malignant lesions preferentially manifest in the various strains of inbred mice examined [2]. Next examined was the interaction between genetic instability and exposure to DMBA in mammary tumor incidence [3]. Finally, the hypothesis that within a given genotype, incidence of mammary tumor following DMBA exposure was modulated caloric intake was explored.

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.

Ongoing and completed work

The preliminary work 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).

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).

The supposition that animal models with genetic predisposition to obesity merit further exploration in relation to breast cancer [10] was examined in light of our previously reported epidemiological association between adiposity and breast cancer [11]. The ability of obesity to potentiate the mammary cancer causing effects of DMBA in mice was examined using Ay/Ay mice that are congenic with C57BL/6 mice. The result of this difference in a single gene is that the agouti protein is ubiquitously expressed resulting in mice with a yellow coat color that are hyperphagic and therefore obese [12]. The only genetic difference between Ay/Ay and C57BL/6 mice is the single gene that results in the obesity of the Ay/Ay mice. The interaction between obesity and DMBA induced mammary cancer was examined using Ay/Ay mice dosed either with DMBA in sesame seed oil or sesame seed oil alone as carrier controls compared with DMBA dosed C57BL/6 mice. Mice were dosed at 3 months of age and were followed for up to 18 months of age. Pearson chi-square (3 x2) analysis demonstrated that the single 65mg/kg dose of DMBA in combination with obesity significantly increased the incidence of mammary cancer and/or mammary hyperplasia (p < 0.00015). This interaction between tumor incidence and adiposity was not a generalized phenomenon. While both C57BL/6 and Ay/Ay mice given a single 65mg/kg dose of DMBA had an increased incidence of lymphoma as compared with controls, obesity did not significantly increase the incidence of lymphoma over that observed in non obese controls. A manuscript detailing these results is currently in preparation.
All of the previously described experimental work utilized a single oral dose of DMBA to elicit mammary cancer and it may be that this is an inefficient method for inducing mammary cancer in most strains of mice. A study modeled after a report characterizing chemically induced mouse mammary tumors using multiple doses of DMBA in conjunction with prior administration of progesterone [13] is currently underway. At 18 months of age, the incidence of mammary cancer in four genotypes of mice (C57BL/6, C3H, 129/J and FVB/J) after progesterone administration followed by multiple doses of DMBA will be assessed. While preliminary results demonstrate that none of the genotypes manifest an earlier age of mammary tumor onset, it remains to be established as to whether the overall incidence of mammary tumors elicited by this dosing regime differs significantly from that observed with a single dose of DMBA in these strains.
Key Research Accomplishments

- Survival of mice following oral gavage with the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) is optimized by prophylactic hydration support (Appendix 1).
- Genetically controlled variability in response to calorie restriction has been demonstrated (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).
- In terms of increasing the incidence of mammary cancer, obesity and DMBA administration are observed to act synergistically.
- Multiple doses of DMBA in C57BL/6, C3H, 129/J or FVB/J in conjunction with prior administration of progesterone do not decrease the age at which mammary cancer is observed.
- A strikingly high percentage of DBA/2 mice given a single dosage of DMBA exhibit cardiac calcinosis. This suggests that in a susceptible genotype, DMBA exposure increases the incidence and decreases the age at which this degenerative process is observed (Appendix 4).
Reportable Outcomes

Publications:


3. Lipman, RD. *Genotypic difference in neoplastic lesion and longevity response of mouse strains following treatment with 7,12-dimethylbenz[a]anthracene*. (J. Gerontol. manuscript submitted.)

4. Lipman, RD and Smith DE. *The response of the DBA/2 heart to aging, carcinogen exposure and calorie restriction*. (manuscript in preparation)

5. Lipman, RD. *Obesity and the incidence of malignant lesions in mice* (manuscript in preparation)
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 are more sensitive to acute effects of DMBA and then manifest genotypic specificity in the tumors that develop following exposure. These results suggest that exposure to this carcinogen 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. Further, the work in this project goes on to demonstrate that the opposite of CR, extra adiposity, appears to increase tumor incidence in carcinogen treated animals and may specific enhance mammary cancer.

As part of this project, 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

Appendix 1

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), which forms depurinating DNA adducts in rat mammary epithelial cells. DMBA is widely used as a carcinogen in rodents, with a variety of doses, number, and routes of administrations reported. 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. This method is reported to result in a 100% incidence of mammary adenocarcinoma in susceptible rat genotypes without early mortality. 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/HeN 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. 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 isoflurane (Pfa cult 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 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 gear 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 x 2 chi-square analysis. Average body weights of the mice were compared by using a two-tailed t-test. Statistical analyses were conducted with STATTOOLS (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 some sign 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 < 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 ≤ 0.005). The average weight of the surviving mice 4 weeks after DMBA administration did not differ (21.5 ± 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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Appendix 2

Genotypic differences in neoplastic lesion and longevity response of mouse strains following treatment with 7,12-dimethylbenz[a]anthracene

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running page headline: Genotype and Calorie Restriction Efficacy
Abstract
Calorie restriction (CR) has long been known to increase longevity and to delay the onset and to decrease the incidence of tumors. The mechanism(s) by which these outcomes are attained, however, has not been identified. This experiment was designed to test the hypothesis that gene(s) for which polymorphisms exist are involved in the control of the response to CR and that allelic differences among inbred strains could be demonstrated. 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 (DMBA) and were subsequently fed ad libitum or were calorie restricted. Significant variability was found among the various strains of mice in their response to CR in terms of lifespan extension, tumor incidence and age at which tumors were observed. In addition, this experiment demonstrated that the spectrum of tumors and other disease processes induced by exposure of adult female mice to DMBA are genotype specific. The administration of single dose (65 mg/kg) DMBA in adult female mice of these strains appeared to accelerate the expression of the neoplastic lesions to which they have previously been reported to develop spontaneously. Using separate ranking systems for the response to CR in terms of its impact on lesion incidence or age at which specific lesions were observed, the responses to CR for individual strains as well as for particular lesions were calculated. In terms of the overall incidence of neoplastic lesions, the C3H, A/J and FVB/J strains had the most robust responses while the NMRI, BALB/c and C57BL/6 had the smallest response. Similarly, in terms of the cumulative effect on the age at which neoplastic lesions were observed, the calculated response of the C3H and FVB/J were among the most robust while the C57BL/6 and BALB/c were again among the least responsive. Paradoxically, although the response to CR in terms of neoplastic lesion incidence and age at which lesions were observed were among the most robust for the C3H and the FVB/J, these two strains had the smallest response in terms of increased longevity, the classic response to CR. The incidence of neoplastic lesions, the age at which they are
observed or the two combined may be useful phenotypes for mapping genes controlling the CR response.
Introduction

The experimental paradigm of calorie restriction (CR) has been repeatedly demonstrated to increase both mean and median lifespan in mice and rats (1). 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. Historically, the impact of this dietary intervention has been shown to increase longevity (2, 3) and decrease the incidence or delay the onset of most disease processes, especially cancer (4-8). The earliest observation that CR had an effect on cancer (9) has been extended to include the observation that CR decreases tumorigenesis caused by endogenous viruses (10), carcinogen exposure (11), radiation (12) and ablation of the p53 gene (13). Calorie restriction has been demonstrated to result in an overall decrease in total lesion burden as well as reducing the variety of lesions observed (8).

The list of age-related parameters affected by CR continues to expand. However, the mechanism(s) by which CR reduces lesion burden and increases longevity in rodents have not been identified. CR has been postulated to have its fundamental effect at the level of the cell, organelle, macromolecule or metabolism. The effect of CR has been viewed as fundamentally influencing cell turnover by affecting proliferation (14) and/or apoptosis (15). It has been suggested that the broad-based changes observed with CR are derived from basic alterations in free radical generation and/or the various mechanisms for managing the ravages of oxidative stress (16, 17). A related hypothesis is that the basis of the beneficial outcomes obtained from CR are derived from its effect on mitochondria (18). Alternatively, it has been proposed that the effects resulting from CR are a consequence of its affect on the fat mass, which in turn modulates a host of endocrine factors, cytokines
and other peptides (19). Discriminating among these and other proposed hypotheses (1) 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 Institutes of Aging was the most multigenotypic study of
CR that included 4 genotypes of mice (C57BL/6, DBA/2, B6C3F1 hybrid and B6D2F1
hybrid) and 3 genotypes of rats (Fischer 344, Brown Norway and F3BNF1 hybrid) (20).
The data presented by Turturro, et al. showed that in male mice fed NIH-31 diet, the
response to CR ranged from an approximate 10% increase in the age at which 50%
mortality was reached in the DBA/2 to nearly a 35% increase in the B6C3F1 hybrids as
compared with the appropriate AL fed male controls (20). These data suggest that there is
variability in the magnitude of response to CR obtained in different genotypes.

This study was designed to follow up this observed variability in response to CR and to
determine whether the response to CR might be under genetic control. Aging is a complex
phenotype and CR has multiple effects. For this reason, the effect of CR on cancer was
used as a surrogate for its effects on aging, since the impact of CR on neoplastic lesions is
among its most reproducible and profound. As a first step in investigating the genetics of
CR responsiveness, the response to carcinogen treatment when followed by CR or AL
feeding in 8 strains of inbred strains of mice was characterized.
Materials and methods

Six week old virgin female mice were obtained as follows: A/J, BALB/c, C3H, C57BL/6 and DBA/2 (Harlan Sprague, Indianapolis IN), 129/J and FVB/J (The Jackson Laboratory, Bar Harbor ME) and NMRI (B&K Universal, Fremont CA). The mice were maintained under 12 h light/12 h dark at 23°C and 45% humidity. Following arrival, all the mice had ad libitum (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.

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 DMBA / kg body wt and given 1.0 cc subcutaneous injection of 0.9% NaCl to prevent dehydration (21). The mice were maintained in metabolic cages for one week after dosing to facilitate safe collection of carcinogen contaminated excreta as previously described (21). 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 72 weeks (18 months) of age. To facilitate comparison among AL and CR fed cohorts within a genotype, only those animals surviving to the age of 12 weeks, when the CR cohort of each genotype had had their food intake adjusted to its lower level, were included when analyzing the effect of diet on lifespan, lesion incidence or age of lesion observation.

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. 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% that of the AL cohort for that genotype. The ratio of the body weights of the CR vs. AL cohort for each genotype was
used to titrate the food intake of each strain as long as the AL cohort survived. CR cohorts surviving beyond their genotype controls 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 (20), the mice in the CR cohorts were fed vitamin and mineral supplemented NIH-31 diet (Harlan Teklad, Madison WI), while the AL controls consumed NIH-31 diet throughout the study. Mice were weighed biweekly for the duration of the study (Figures 1a-h). Mice with grossly visible tumors or which demonstrated weight loss greater than 20% in 2 weeks, exhibited pain or distress or failed to consume food in a two day period or reached 72 weeks of age were terminated with the use of CO₂ inhalation.

Dead or sacrificed mice were necropsied. Tissues including lung, liver, pancreas, spleen, ovary, mammary gland and uterus were routinely collected from each mouse. Mammary tissue was allowed to adhere to paper prior to fixation to prepare a flat surface for sectioning. In addition, samples of any grossly abnormal appearing tissues were taken. All tissues were fixed in Tellyesniczky’s fixative (20:2:1 of 70% ethanol, 37% formalin and glacial acetic acid). Tissues were dehydrated and then embedded in paraffin. Five μm sections were cut and stained with hematoxylin and eosin.

The data for lesion incidence and lifespan were compiled using the relational database, FoxPro (Microsoft, Redmond WA). A multivariate distribution-free significance test (22) which utilized a Wilcoxon rank test was used to compare the growth curves for the AL control and CR cohorts within each genotype. Significant (P ≤ 0.001) differences in body weight between diet groups were observed for all 8 genotypes, demonstrating that caloric intake was successfully restricted for every strain in this study. The average body weights of the two diet cohorts for each genotype are presented in Table 1. Within each genotype, lifespan data were analyzed by the Lifetest Procedure (SYSTAT version 7.0.1 for
Windows, SPSS Inc., Chicago, IL). Significant differences in longevity ($P \leq 0.02$) between diet groups within genotypes are indicated in Table 1 along with the average lifespan ± standard deviation for each group. The individual mortality curves of each diet cohort for the genotype studied are presented in Figures 1a-h.

The two ways in which CR has been demonstrated to affect neoplastic lesions are by altering the incidence of the tumor ($\alpha$) and/or the age at which the tumor is observed ($\beta$). Both $\alpha$ and $\beta$ as well as their combination were evaluated in this study. The values for $\alpha$ and $\beta$ ± standard deviation for each genotype-diet group are presented in Table 2. The second column of Table 2 shows the results of a $2 \times 8$ analysis of the homogeneity of proportions (23) comparing lesion incidence among the AL controls of each genotype to determine whether the incidence of specific lesions differed significantly among them.

The data in Table 2 were analyzed to detect significant difference in $\alpha$ or $\beta$ between the diet groups within each genotype. The summary of these analyses are shown in Table 3. Determination as to whether differences in $\alpha$ were statistically significant were obtained using $2 \times 2$ analysis of the homogeneity of proportions (23) to compare the $\alpha$ for the various tumors between diet groups within each genotype. The significance of differences between diet groups in $\beta$ for specific tumors within each genotype were analyzed using a two tailed t-test (23). For all of these analyses, differences at the $P \leq 0.05$ were considered to be statistically significant.
Separate rankings for the possible effects on tumor $\alpha$ and $\beta$ for specific tumors are presented in Table 4. The effects on $\alpha$ and $\beta$ were each graded on scales of 1 to 5, with 5 being the most profound impact, e.g. the case in which CR eliminated the occurrence of the specific lesion. This ordinal ranking of effects could be used for analysis of beneficial or harmful response to any intervention.

The rankings detailed in Table 4 were applied to the data in this study and are presented in Table 5. The extent to which CR affected $\alpha$ and $\beta$ were calculated for each neoplastic lesion in each genotype. The total responsiveness to CR of the various genotypes was calculated as the sum of the effects of CR on each lesion observed to occur in that strain divided by the maximum effect possible ($5 \times$ the number of lesions observed in the strain). For example, the calculation of the overall response of the C3H to CR in terms $\beta$ is as follows: 3 lesions were observed to occur in the C3H and the sum of the rank for the affect of CR on $\beta$ for these ($3 + 4 + 4 = 11$), divided by the maximum effect that could have been observed ($5 \times 3 = 15$) is $11/15 = 0.73$. Similarly, the responsiveness of individual tumors to CR was calculated as the sum of the effects observed in all the strains in which the lesion occurred divided by the maximum cumulative effect possible after accounting for the number of strains in which the tumor was observed. For example, calculating the rank of the effect of CR on the $\alpha$ for lymphoma is as follows: lymphoma was observed in 8 strains and the sum of the rank for each of these in terms of effect on incidence ($3 + 3 + 4 + 3 + 3 + 5 + 3 + 3 = 27$) divided by the maximum effect possible multiplied by the number of strains in which it was observed ($5 \times 8 = 40$) is $27/40 = 0.68$. Using these calculations, presented in Table 5, it is possible to compare the responsiveness of each strain and each lesion to CR.
Results

The mice of all genotypes were active within a few minutes after dosing as previously reported (21). There was no genotypic difference in terms of acute effects of DMBA administration resulting in death within 10 days of dosing. These acute deaths occurred prior to separating the mice into diet groups and these individuals were not included when calculating the percentage of mice within each genotype-diet cohort which did or did not have specific lesions.

As seen in Figures 1a-h and summarized in Table 1, the average weight over the course of the experiment of the CR cohort was significantly less than that of the AL fed controls in all 8 genotypes ($P \leq 0.001$), as determined by multivariate distribution-free significance analysis. The average body weight for each genotype-diet cohort is shown in Table 1, where body weights of the CR cohorts ranged from 22 to 35% less than that of the AL controls. The fact that the body weights of the CR cohorts of each genotype was significantly less than that of the controls demonstrates that caloric restriction was successfully attained in each genotype.

Mortality Kinetics

The proportion of mice found dead to those that were sacrificed when moribund or at 18 months of age, did not differ significantly among the 8 genotypes studied. Post-hoc comparison of the average lifespan of the AL controls demonstrated that only longevity of the shortest lived strain (129/J) and the longest lived strain (C57BL/6) differed significantly. This means that with the possible exception of these two strains, differences in tumors seen among the various strains cannot be the result of one strain not living long enough to manifest a particular tumor. Thus, lifespan is not responsible for differences in the incidence of tumors observed among the genotypes studied.
The longevity curves for each genotype are presented in Figures 1a-h. Within each genotype, longevity was analyzed by the Lifetest Procedure, which accounted for the mice which were sacrificed at 72 weeks of age. A significant effect of CR on longevity following a single 65 mg/kg dose of DMBA was observed in 7 out of the 8 inbred strains of mice in this study (Table 1). The notable exception were the FVB/J mice. Although calorie restriction was attained in the FVB/J strain as demonstrated by the significant difference in body weight between the two diet groups, the typical increase in longevity obtained with CR was not observed in this group (Figure 1f).

Lesions

Common lesions were defined as those which were observed in at least 10% of the mice in at least one genotype-diet group. A total of 20 lesions were found to occur commonly among the mice studied within the tissues sampled (data not shown); only the neoplastic lesions are reported here. Table 2 lists the 7 common tumors observed in this study along with the number of cases and the average age at death of affected individuals in each genotype-diet group. Within the AL controls, 4 of the commonly observed tumors (lung adenoma, mammary adenocarcinoma, mammary adenoacanthoma and uterine hemangiosarcoma) demonstrated significantly different α among genotypes examined (Table 2). Thus the tumors observed following DMBA administration differed among the genotypes studied.

The effects of CR on tumors were divided into its impact on α and on β. The various combinations of these CR effects on the tumors observed in this study are presented in Table 3, where strains are listed only if the tumor occurred in ≥ 10% of one or both diet cohorts. From these data, it is clear that the impact of CR on tumors was not uniform. Some strains showed a more robust response to CR than others.
For lymphoma, neither α nor β were affected by CR in most of the strains. The two exceptions were FVB/J and C3H. No lymphoma occurred in any FVB/J mouse in the CR cohort and only one case occurred in the C3H CR cohort at approximately the same age as in the C3H AL controls (Tables 2 and 3).

For lung adenoma, neither α nor β differed between diet groups for the DBA/2 and the 129/J mice. In the remaining 4 genotypes in which lung adenoma was commonly observed, there was no impact of CR on α but β was significantly greater in the CR cohort (Table 3).

The mammary tumors, adenocarcinoma and adenoacanthoma, were observed in a small number of strains, 3 and 1 respectively. The CR effect on mammary adenocarcinoma was robust in BALB/c, where this tumor was only observed in AL fed controls, and in the C3H where CR significantly reduced α and increased β. For DBA/2 mice, there was no effect on either parameter for this lesion. Mammary adenoacanthoma was observed in only a single strain, the A/J. In these animals, CR reduced α, but had no effect on β.

CR had no effect on α for ovarian granulosa cell tumors, ovarian hemangiosarcoma and uterine hemangiosarcoma in any of the strains studied (Table 3). The β for ovarian granulosa cell tumors in CR mice of FVB/J, NMRI and C3H genotypes were all observed to be significantly greater than the AL controls of these genotypes. In addition, β for ovarian granulosa cell tumors in the BALB/c and ovarian hemangiosarcoma in the C57BL/6 mice were artificially reduced given these tumors were only observed in mice
sacrificed at 72 weeks of age because of study design, not because these tumors had any earlier effect on morbidity.

Considering the ranking scheme of Table 4 and the data in Table 5, it can be appreciated that although lymphoma occurred in all the strains studied in this experiment, the magnitude of the CR effect on $\alpha$ for lymphoma was the greatest in the FVB/J as was the magnitude of the CR effect on $\beta$. The magnitude of the CR effect for $\alpha$ on lymphoma among the other strains was similar, ranging from 3 to 4; and was identically ranked as 3 for $\beta$ in all genotypes other than FVB/J. The impact of CR on lung adenoma was comparable in all the strains manifesting this lesion on both $\alpha$ and $\beta$. Therefore, lung adenoma would not be an informative phenotype to monitor for differences in response to CR, at least the particular genotypes used in this study. Mammary adenocarcinoma showed identical ranking for responsiveness to CR in terms of incidence and average age of affected individuals. The BALB/c showed the most robust response (5) for both $\alpha$ and $\beta$ as compared with the ranks of 4 for the C3H and 3 for the DBA/2 for each of these parameters. Mammary adenoacanthoma was only observed in the A/J strain with a rank of 4 and 3, respectively, for the affect of CR on $\alpha$ and $\beta$. As this lesion occurred in only a single genotype, there is no comparison among strains for the effect of CR to be made. Ovarian granulosa cell tumor showed variability among genotypes in response to CR in terms of its impact on $\alpha$ and $\beta$. Of the strains manifesting this tumor, the C3H demonstrated the most robust response in terms of both $\alpha$ and $\beta$, ranking 3 and 4 respectively. Similarly, the response of the BALB/c was the weakest for the two parameters with a ranking of 1 for each. Lastly, the magnitude of the effects of CR within a given genotype on hemangiosarcoma
observed in either the ovary or uterus were consistent for $\alpha$ and $\beta$. For example, with ovarian hemangiosarcoma, the response for $\alpha$ and $\beta$ was greater in the FVB/J than the C57BL/6. Similarly, the response to CR for uterine hemangiosarcoma was greater for $\alpha$ and $\beta$ in the 129/J than the BALB/c.

The analysis shown in Table 5 provides the opportunity to calculate cumulative response to CR for each strain for both overall $\alpha$ and $\beta$. It is possible to calculate the overall responsiveness of individual tumors to CR using the cumulative response of each strains in which the tumor occurred. The FVB/J had the largest response to CR in terms of both overall $\alpha$ and $\beta$. This is an interesting contrast to the failure of this strain to demonstrate any response to CR in terms of longevity. It suggests that although the FVB/J do manifest a response to CR in terms of neoplastic lesions, that some disease process(es) unaffected by CR, killed these mice. Using the phenotype of the CR affect on $\alpha$, the two mammary lesions, with a rank of 0.8, showed the largest response to CR, while the hemangiosarcoma demonstrated the weakest response. Mammary adenocarinoma and ovarian hemangiosarcoma, respectively, were the most and least responsive to CR in terms of its effect on $\beta$. 
Discussion

Caloric restriction is well documented to increase longevity, and while the mechanism(s) by which this effect is attained is heavily speculated upon, it remains unknown. As there appear to be genotypic differences in the extent to which CR increases lifespan (20), one hypothesis is that the response to CR is regulated by a definable number of genes. The purpose of this experiment was to use the effects of CR on tumors as a surrogate for the effects of CR on aging as a means of documenting the variability in response to CR among different genotypes. This is the first step in assessing whether there are gene(s) controlling the biology of the response to this dietary intervention. In this study, three measures were used to assess response to CR: right censored longevity data, neoplastic lesion incidence and the age at which mice were observed with individual neoplastic lesions.

To understand cancer in mice, it is important to recognize that tumors occur commonly in laboratory mice and that their incidence demonstrates an age-related increase (24). It is well documented that there are genotypic differences in the incidence of spontaneous lesions including tumors (24-25). Nearly all of the various neoplastic lesions observed following DMBA administration in this study have previously been reported to occur spontaneously in these strains (24-28) with the sole exception of ovarian granulosa cell tumors in A/J mice. Thus, the difference among the strains studied in terms of the specific tumors observed in this experiment did not arise merely as a result of DMBA administration. The general effect of DMBA as administered in this study was to accelerate the onset of at least a subset of the age-related tumors to which the individual genotypes were genetically predisposed. This may not, however, be a generalizable effect of carcinogen administration as it has been demonstrated that the treatment of F344 rats with alkylating carcinogens does not accelerate development of the spontaneously occurring tumors (29).
Genotypic differences in lesion incidence have previously been reported in mice following DMBA administrations during the neonatal period for lesions including lung adenoma, leukemia and hepatoma, in the genotypes BALB/c, C3H, C57BL/6 among others (30). Mittlestaedt, et al. has reported that the mutational profile following DMBA administration differs between genes using endogenous hprt and a lacI, a transgene, for analysis (31). In addition, the rate and extent to which DNA adducts are induced by aromatic hydrocarbons have been demonstrated to differ among genotypes using C57BL/6 and BALB/c for comparison (32) as well as for different tissues within a given genotype (33). These observations suggest that different alleles of individual genes could manifest different sensitivity to the number and types of mutations that occur following carcinogen administration, leading to the differences in the kinds of tumors that commonly develop in the different strains of mice. For example, genetic differences at the aryl hydrocarbon hydroxylase (AHH) locus have long been known to be responsible for genotypic differences in inflammation and tumorigenesis following exposure to polycyclic hydrocarbons (34-36). However, as all the mice in this study developed tumors and this study included genotypes which were both AHH responsive (C57BL6, C3H, BALB/c) as well as AHH non-responsive (DBA/2, 129/J), this locus cannot be the source of the differences observed. Taken together, it is highly likely that allelic differences among genotypes of mice studied give rise to the specificity of the effects observed in this study following DMBA exposure.

Notwithstanding genotypic differences in the specific tumors observed, the impact of CR on the age of the mice in which various tumors were observed, β, and to a lesser extent on their incidence, α, in this experiment strongly suggests that CR acts to slow the progression of the various neoplastic lesions, regardless of genotype. CR was begun only
after DMBA administration and subsequent clearance and therefore could have had an affect
only on tumor promotion, not on initiation.

A study by Fischer and Lutz reported that CR did not influence the formation of mouse
skin papilloma formation by chronic dermal application of DMBA. The authors concluded
that CR was not protective under conditions of chronic exposure to a genotoxic carcinogen
(37). This observation is in conflict with other studies in which CR was found to be
protective against chronic exposure to genotoxic agents (38). Another interpretation of the
Fischer and Lutz data is that the observed lack of CR response was specific to the strain of
mice, the NMRI, in which they conducted their experiment. Indeed, in the study reported
here, while the NMRI did demonstrate an increase in longevity when calorie restricted
followed administration of DMBA, they showed no decrease in tumor incidence. Although
this may be partially explained by the increased age of the CR cohort as compared with the
AL fed controls, it is still an unusual observation within the literature on CR and may
suggest that the response to CR of this mouse strain is only partial. Examination of the
robustness of their CR response in terms of lesion incidence (Table 5) demonstrates that the
NMRI are among the strains with the poorest response. Further study of NMRI mice may
provide some useful insight into the mechanism(s) by which CR affects aging.

The results of this experiment show sufficient variability between mouse strains in their
responsiveness to suggest CR a genetic component regulating the response to CR and that
the genes controlling the response might be identified as quantitative trait loci (QTLs).
Table 5 could be used to choose the strains to be used in such a study. A case can be for
focusing on lymphoma since it occurred in all genotypes following DMBA treatment.
FVB/J mice showed the more robust response to CR for both α and β, with a rank of 5 for
lymphoma, while the other strains were almost exclusively ranked at 3. Out of these other
strains, the C57BL/6 is a prudent choice for linkage analysis in comparison with the FVB/J
because they had the lowest overall ranking for $\alpha$ and $\beta$. The experiment would be conducted as a search for QTLs. FVB/J and C57BL/6 mice would be mated together to produce first an F1 generation from which an F2 generation would be produced. In this F2 population, CR responsive loci would segregate along with short sequence length polymorphic markers. F2 mice derived in this way would all be dosed with DMBA and then subsequently subjected to CR. The mice would be followed until the development of lymphoma, and the trait to be mapped would be $\beta$, the age at which mice developed lymphoma. This experiment would require at least 475 mice using the data in this study to estimate the variability in the response to CR and the assumption that at least 50 F2 mice manifesting the lymphoma phenotype would be needed for identification of QTLs.

It is generally recognized that a complex relationship exists between genetic susceptibility, environmental factors and their interaction(s) (39). In the case of CR, individuals with favorable alleles of CR response genes would live longer and have decreased age-adjusted disease incidence than would individuals with unfavorable alleles. While a recent paper has confirmed and summarized a subset of the changes in gene expression that occur with CR (40), 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 response is likely to be confounded by sharply variable responses among genotypes to the parameters longevity, incidence of lesions and their age of onset.
Acknowledgement

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References


26. Mouse Tumor Biology Database; URL: http://www.informatics.jax.org/


34. Schmid A, Elmer I, Tarnowski GS. Genetic determination of differential inflammatory reactivity and subcutaneous tumor susceptibility of AKR/J and


Figure Legends

Figures 1a-h: Genotype Specific Mortality Kinetics and Body Weights Following the administration of 65 mg/kg DMBA for Mice Subsequently Fed Ad Libitum (AL) or Calorie Restricted (CR)
The longevity curves and the average body weights for the AL controls and CR cohorts of each genotype are presented. The body weights are presented with dashed curves using ● as the symbol for the AL fed controls and ○ as the symbol for the CR cohort. The longevity curves are represented with solid lines using ■ as the symbol for the AL fed controls and □ for the CR cohort of each genotype.

Table 1: Average Body Weight and Longevity for Ad Libitum (AL) and Calorie Restricted Mice (CR)
The average body weight ± standard deviation for the AL fed control and CR cohorts of each genotype are presented with superscript letters to identify statistically significant differences (P ≤ 0.001) in weight between diet groups for each genotype. The average lifespan in weeks ± standard deviation is provided for each genotype-diet group with differences in superscript letters signifying statistically significant differences (P ≤ 0.02) in calculated longevity kinetics.

Table 2: Incidence of Tumors (α) and the Average Age at which they were Observed (β)
The number of affected animals, their average age ± standard deviation are provided for each genotype-diet cohort.

Table 3: Impact of Calorie Restriction (CR) on Tumor Incidence (α) and Age of Affected Individuals (β) by Genotype
Mouse strains are listed for given lesions only if the incidence observed in the strain was ≥ 10% in at least one diet cohort for that genotype. The statistical significance of strains presented as having a difference in incidence between diet groups and/or a difference in average age of affected individuals between diet groups was accepted at the P ≤ 0.05.

Table 4
Ranking the Effects of Calorie Restriction on Lesion Incidence (α) and Age at which Lesions are Observed (β)

Table 5: Calculated Rank for the Effects of Calorie Restriction on Neoplastic Lesions and in Various Genotypes
The effects of each genotype for every lesion which occurred in at least 10% of one diet cohort for that genotype using the rankings listed in Table 4. These were summed for each genotype and divided by the maximum rank that could have been attained to calculate the cumulative rank for the strain. Similarly, these were summed for each lesions and divided by the maximum rank that could have been attained to calculate the cumulative rank for the lesion.
References


26. Mouse Tumor Biology Database; URL:http://www.informatics.jax.org/


34. Schmid A, Elmer I, Tarnowski GS. Genetic determination of differential inflammatory reactivity and subcutaneous tumor susceptibility of AKR/J and


Mortality Kinetics and Average Body Weight for A/J mice
Mortality Kinetics and Average Body Weight for BALB/c mice
Mortality Kinetics and Average Body Weight for C57BL/6 mice
Figure 1e

Mortality Kinetics and Average Body Weight for DBA/2 mice
Mortality Kinetics and Average Body Weight for FVB/J mice
Figure 1h

Mortality Kinetics and Average Body Weight for 129/J mice
Table 1

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| uterine hemangiosarcoma        | 0.0001                      | 1            | 1           | 2              | 543 ± 106      | 1           | 430         | 0              | 1              | 541         | 0              | 1              | 541 ± 100      | 541 ± 100      | 240 ± 57       | 368 ± 67       | 0              | 0              | 0              | 0              | 0              | 0              | 0              | 0
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<td>CR significantly older than controls (AL &gt; CR)</td>
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### Table 5

#### Affect of CR on Incidence (α)

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#### Affect of CR on Average Age (β)

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Appendix 3

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

DAVID B. LOMBARD,1 CAROLINE BEARD,2 BRAD JOHNSON,1 ROBERT A. MARCINIAK,1 JESSIE DAUSMAN,2 RODERICK BRONSON2 JANET E. BUHLMANN,4 RUTH LIPMAN,3 RUTH CURRY,2 ARLENE SHARPE,4 RUDOLF JAENISCH,3 AND LEONARD GUARENTE1∗

Department of Biology, Massachusetts Institute of Technology,1 and Whitehead Institute,2 Cambridge, and School of Veterinary Medicine, Tufts University, and Department of Pathology, Brigham and Women’s Hospital, Harvard Medical School, Boston,4 Massachusetts

Received 7 December 1999/Accepted 31 January 2000

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, WRNΔC;p53−/− mice show an increased mortality rate relative to WRNΔC;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, homozigosity 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 1:19:0:6, 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 organismic 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 WRNΔC−/− 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 marine cDNA plasmid library was screened by standard methods (20) by using an 820-bp probe derived from the 5′ 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′ GGCCAA CAT GGG ATG TCC 3′ Hybridizations were performed at 35°C. Twenty-two clones were isolated, and preliminary restriction mapping and 5′ 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 (EggGistart; Promega) and was further purified over a diaminopropylamide 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 protein were loaded in 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 (Amersham) 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 cloned into pBR322 and were extensively mapped with restriction enzymes. To
construct the 5' homology arm of the targeting vector, a 3.0-kb Sall/HindIII fragment from the larger pBR322 clone was subcloned into the Sall/HindIII sites of pSL1190 (Pharmacia). For the 3' homology arm, a 4.9-kb BamHI/Scal fragment was ligated into the BamHI/Scal sites of the pSL1190 vector. A Kpnl/Nol fragment containing the beta-geo cassette (beta-galactosidase/neomycin' fusion gene) was then inserted into the Kpnl/Nol sites of the pSL1190 construct. An internal Sall site in the beta-geo cassette was obliterated by partial digestion followed by blunting and religation, and then the complete cassette was excised from the vector via Sall 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 mice, 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 Scal/Nhel restriction fragment (see Fig. 2a) which yielded a 9-kb wild-type band and a 6.3-bst band on genomic DNA digested with Nhel. The 5' probe consisted of an Ndel/Kpnl fragment which was isolated and further digested with EcoRl and BamHI to yield 800-bp and 350-bp bands as the probe. On EcoRl-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 Southern blotting. The oligonucleotides used were: pSL3095, 5' GCC TGC AGG TGC CGC CAT C3'; COMMON 2, 5' CAA TAA CCA ATG GAA TTC TAA GC 3'; and WT 1, 5' TAC ATT TGC CAT TGT AAG GTG GC 3'. The PCR conditions were 95°C for 3 min, followed by 30 cycles of 94°C, 30 s, 57°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.

Splenicocyte culture. Splenocytes were isolated from mice of the indicated genotype, washed, and lysed, and the splenocytes were resuspended at a concentration of 2 × 10^6/ml in plating media (10% fetal bovine serum—Gib-HEPES-6.0 × 10^{-5} M beta-mercaptoethanol in RPMI medium [Gibco]). To determine response to mitogen stimulation, 0.5 × 10^6 lymphocytes/well were plated in triplicate in 96-well plates. Anti-CD3 was added at the indicated dilution, and cultures were incubated for 24 h. The last 4 h of the cultures were pulsed with 1 μCi of [3H]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 containing 10% fetal bovine serum—Dulbecco modified Eagle medium (Gibco) and 10% fetal bovine serum. The cultures were grown on 60-mm Falcon dishes with 10-cm2 coverslips and were passaged weekly. The cells were then cultured for 4 days, and cell proliferation was subsequently quantified by using the BrdU/fluorescence cell labeling kit (Becton-Dickinson), following the manufacturer's instructions. In each case, two independent cell lines of each genotype were treated in two wells each, and the results were averaged.

Nucleotide sequence accession number. The 6,476-nucleotide cDNA sequence encoding the murine WRN protein has been submitted to GenBank under accession no. AF234163.

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 acidic 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 an R at position 80, an A rather than an S at position 1145, and a V rather than an L at position 1181. These differences may represent polymorphisms between the strains of mice used to generate the libraries from which these cDNAAs 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/SvJ genomic library (library courtesy of the Housman 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 beta-geo 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 antiserum 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 +/- to 173 +/- to 105 +/- /-. Crosses between heterozygous mice and mutant mice have yielded mice in the ratio of 98 +/- to 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<sup>−/−</sup> or WRN<sup>+/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup>;BLM<sup>−/−</sup> and WRN<sup>−/−</sup>;BLM<sup>+/−</sup> fibroblasts were serially passaged in culture; 10<sup>6</sup> 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<sup>−/−</sup> 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 mutants (8). Animals with the genotypes WRN<sup>−/−</sup>;p53<sup>−/−</sup> or WRN<sup>−/−</sup>;p53<sup>−/−</sup> were monitored over time (Fig. 6). Whereas WRN<sup>−/−</sup>;p53<sup>−/−</sup> animals had an average life span of 149 days, WRN<sup>−/−</sup>;p53<sup>−/−</sup> 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<sup>−/−</sup> 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 p53−/− 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 p53−/− 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 p53−/− cancer-prone strain. This model predicts that the WRN−/− animals will also display a slightly shortened life span in the p53 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-NOQ in WRN-/-:BLM-/- embryonic fibroblasts. (a) Camptothecin treatment. (b) 4-NOQ 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.

ACKNOWLEDGMENTS

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REFERENCES


Appendix 4

The response of the DBA/2 heart to aging, carcinogen exposure and calorie restriction

USDA Human Nutrition Research Center on Aging at Tufts University

keywords: DBA/2 mice, cardiac, DMBA, pathology, calorie restriction

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Introduction

Cardiovascular disease is a common cause of death in humans; notwithstanding its association with mortality, there are relatively few age-related structural changes in the heart. While it is critical not to confuse occult disease with normal aging in the development of a model for human aging, it is pertinent to account for disease related changes and their overall contribution to mortality. The DBA/2 mouse is a strain in which the incidence of heart disease is greater than other strains [Bronson, 1990 #1] and this incidence increases with age [Bronson, 1991 #2]. Although not all humans develop cardiopathology with age, the DBA/2 mouse is an excellent system in which to study cardiac aging compromised by disease.

It is of interest that both normal aging and various progeroid syndromes include cardiac manifestations and increased cancer incidence [Lee, 1997 #10; Yancik, 1997 #8; Pagano, 1998 #7]. Although the response to various insults elicited in mitotically active tissue is likely to include a replicative component, the response of quiescent cells will be something other than replication. For example, as heart muscle cells do not have the capacity to divide, their response to carcinogen exposure must, by definition, be something other than neoplastic in nature.

In this report, the various age-related lesions affecting the heart in the DBA/2 mouse are presented. The effects of calorie restriction (CR), a nutritional intervention long documented to increase longevity [Weindruch, 1989 #9] on the overall incidence and the average age of affected individuals with heart lesions are compared. Finally, the capability of CR to modulate the effects on the heart of carcinogen administration are examined.

Materials and Methods

The cross-sectional experimental design, in which approximately 30 mice of each sex-diet cohort were examined at 6 months intervals utilized DBA/2NNia mice bred and maintained at the National Center for Toxicologic Research as detailed [Turturro, 1999 #3]. The actual number of mice examined and their average ages are shown in Table 1. Briefly, the mice were individually housed and were fed either NIH-31 ad libitum (AL) or NIH-31 supplemented with 1.67X the vitamin mix such that their calorie intake was equal to 60% of the AL intake (CR). The mice were shipped to the USDA Human Nutrition Research Center on Aging by air cargo and were processed for histological examination within 48
hours of their receipt. Prior to sacrifice, each mouse was deeply anesthetized with Avertin, their vasculature was flushed with physiologic saline and then their tissues fixed by intracardiac perfusion with Bouin’s solution, as previously described [Bronson, 1991 #2]. The animals were fixed for several weeks prior to tissue dissection which included the longitudinally bisected hearts and samples of thigh muscle. Tissues were dehydrated in alcohol, embedded in paraffin and 5 μm sections were cut and stained with hematoxylin and eosin. Tissues were graded on the presence or absence of lesions.

The cardiac effects of carcinogen exposure were examined using 6 week old, virgin female DBA/2 mice obtained from Harlan Sprague (Indianapolis, IN) and were maintained in our facility, fed NIH-31 ad libitum as described [Lipman, 2000 #5]. At 9 weeks of age, each mouse was anesthetized with isoflurane and given 65 mg/kg 7,12-dimethylbenz[a]anthracene (DMBA) in sesame oil via oral gavage followed by 1.0 ml of physiologic saline administered subcutaneously to prevent dehydration [Smith, 1999 #6]. Three weeks after DMBA administration, the mice were divided into two groups matched for weight. One cohort was fed ad libitum (AL) and the intake of the other was gradually restricted (CR) until the average body weight of the mice approximately 30% less than the AL cohort. The body weights and mortality kinetics for the AL and CR cohorts are shown in Figures 1 and 2. The mice were sacrificed using CO₂ inhalation at 18 months of age or when they demonstrated 20% loss in body weight, anorexia or appeared moribund. Tissue from animals which were sacrificed or found dead were fixed in Tellyesniczky’s fixative and were processed as above.

The data on age, diet and presence of specific lesions for each mouse was compiled using the relational database FoxPro (Microsoft, Redmond WA). Comparison of the mean ages for mice in the sex-diet cohorts of the cross-sectional experiment were analyzed using a two tailed t-test [Dallal, 1986 #4] and are shown in Table 1. The incidence of myocardial degeneration, cardiac calcinosis and skeletal muscle mineral deposition in these mice are shown in Tables 2-4 and the incidence of each lesion was compared among the different sex-diet cohort using χ² analysis [Dallal, 1986 #4]. Comparison of lifespan between the AL and CR mice which had been DMBA treated used SAS for Windows version 6.12 (SAS Institute, Cary, NC) and involved the log-rank estimate to correct for right censoring of the data. The incidence of cardiac calcinosis were compared between the diet groups for these DMBA treated mice also used χ² analysis [Dallal, 1986 #4].
Results

Determining whether the ages of the mice in each cohort are comparable is a necessary consideration (Table 1) prior to comparing the incidence of various lesions in the various cohorts of mice. A two-tailed student t-test showed that for both the males and females, the CR animals were significantly older than the AL, \( P \leq 0.00001 \) and \( = 0.02 \) and males and females respectively. In addition, the AL males were significantly older than the AL females (\( P = 0.04 \)) and the CR males were older than the CR females (\( P = 0.00000 \)).

The two main effects of CR on myocardial degeneration examined were the age at which individual mice were found to manifest this lesion and its incidence within the cross-sectional populations. The data demonstrates that the incidence of myocardial degeneration was significantly greater among male and female mice that were ad libitum fed as compared with CR cohorts, \( P \leq 0.0001 \) and \( P = 0.003 \), respectively (Table 2). Comparison of the average age of affected individuals with myocardial degeneration demonstrated that the age at which CR mice were observed to be affected was significantly greater than the AL males (\( P \leq 0.0001 \)). The difference in average age of affected individuals between the AL and CR cohorts did not reach significance for the females. Additionally, it was observed that in both dietary groups, the incidence of myocardial degeneration was significantly greater among the males than the females (\( P = 0.00006 \)).

The impact of diet on the average age of affected individuals and the incidence of cardiac calcinosis was more complicated (Table 3). The average age of the animals with heart calcinosis did not differ significantly among diet or sex groups. The incidence of heart calcinosis was significantly greater among the AL males than the CR males (\( P = 0.00000 \)) and in addition, amongst the AL cohorts, the incidence was significantly greater in the males than the females (\( P = 0.00001 \)).

With the observation of cardiac calcinosis, we sought to determine whether mineral deposition was a generalized age-related response of this strain. Examination of skeletal muscle (Table 4) and the tongue (Table 5) demonstrated that this was the case. The average age of individuals with mineral deposition in either skeletal muscle or tongue did not show significant differences among the diet groups. However, for both these lesions, the incidence was significantly greater among the CR females and males than among their AL counterparts (\( P = 0.02 \)). In addition, for both skeletal muscle and tongue mineralization,
among the CR mice, the incidences were significantly greater in the males than in the females ( = 0.01) and the average age of affected individuals was significantly greater for the males than the females (P = 0.02). This pattern of increased incidence as well as increased age of affected individuals in the males is similar to that observed for the cardiac calcinosis in these mice.

Among the female DBA/2 mice dosed with DMBA, the average body of the CR mice was maintained at about 75% that of the AL fed cohort (Figure 1). The average body weight of the AL mice dosed with DMBA was comparable that reported for other DBA/2 mice of the same age which were not given DMBA [Turturro, 1999 #3]. As seen in Fig 2, the longevity of the CR mice was significantly greater than that of AL fed animals (P = 0.00001). While the incidence of cardiac calcinosis did not differ between these two diet cohorts of DMBA dosed individuals (Table 5), the average age of CR mice with this lesion was significantly greater than AL cohort (P = 0.04). The cardiac calcinosis was observed at a significantly younger age in mice that were treated with DMBA than those animals which were part of the cross-sectional study (P = 0.004). In addition, the incidence of cardiac calcinosis was significantly greater in this carcinogen exposed group for both AL and CR cohorts (P ≤ 0.00001).

Discussion

A significant difference in the age of the mice studied in the cross-sectional study which must be taken into consideration when examining lesion incidence among the four sex-diet cohorts. Thus, the increased incidence of cardiac calcinosis and the mineral deposition in skeletal muscle in the CR males as compared with the AL males may in part, simply reflect the increased average age of the CR cohort. It cannot be concluded from these data that CR increases the risk of manifesting these lesions as age is an associated factor implicated in lesion development. Additionally, the cardiac calcinosis and mineral deposition in skeletal muscle, although observed in all sex-diet groups were not commonly found in either diet cohort of females, nor among the AL males. The significantly increased incidence observed in the CR males coincident with the significantly greater age of this cohort suggests an association may exist between the two.

The myocardial degeneration, however, was observed to have a significantly increased incidence in the AL cohorts, even though they contained a smaller proportion of older individuals. The decreased incidence of this lesions in concert with the increased age of the
population strongly suggests that CR is associated with a decreased risk of developing myocardial degeneration. As the average age of the CR males manifesting this lesion was greater than the AL males, it suggests that the development of the disease is delay as well.

The incidence of the mineral deposition lesions examined appear to be more common among the CR cohorts than the AL mice examined. This may, in part, reflect the older average age of the individuals in these cohorts and perhaps a dependency on age for lesion development.

The high proportion of DMBA treated mice exhibiting cardiac calcinosis is striking, especially in light of the relatively young average age of the individuals examined. Although one response to the stress of carcinogen exposure is cancer, this would be limited to mitotically active tissue. Another response of tissue to injury, however, is mineral deposition at sites of inflammation (ref). Consistent with the observation of this type of response in the DBA/2 mice, it has previous been established that a locus on chromosome 7 determines susceptibility to dystrophic cardiac calcinosis in mice [Ivandic, 1996 #11]. Although this disease process is not well understood, it has been hypothesized to be the generalized manifestation of cellular injury from any number of possibilities including age, infectious agents and chemical agents. The data presented are consistent with the view that the dramatic cardiac calcinosis in relatively young DBA/2 mice is merely reflective of the permissive genetics that potentiates this response. It is arguable that exposure to carcinogen potentiates the development of disease to which these mice were predisposed.
Table 1: Age of DBA/2 mice in the cross-sectional study

<table>
<thead>
<tr>
<th>Sex</th>
<th>Diet</th>
<th>average age ± S.D.</th>
<th>number</th>
</tr>
</thead>
<tbody>
<tr>
<td>female</td>
<td>AL</td>
<td>17.09 ± 6.55</td>
<td>114</td>
</tr>
<tr>
<td>female</td>
<td>CR</td>
<td>19.00 ± 6.81</td>
<td>149</td>
</tr>
<tr>
<td>male</td>
<td>AL</td>
<td>18.57 ± 5.12</td>
<td>98</td>
</tr>
<tr>
<td>male</td>
<td>CR</td>
<td>22.37 ± 6.83</td>
<td>147</td>
</tr>
</tbody>
</table>

Table 2: Incidence of myocardial degeneration in the cross-sectional cohorts of DBA/2 mice

<table>
<thead>
<tr>
<th>Sex</th>
<th>Diet</th>
<th>average age ± S.D.</th>
<th>incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>female</td>
<td>AL</td>
<td>21.87 ± 3.69</td>
<td>40%</td>
</tr>
<tr>
<td>female</td>
<td>CR</td>
<td>23.20 ± 2.58</td>
<td>23%</td>
</tr>
<tr>
<td>male</td>
<td>AL</td>
<td>21.39 ± 3.67</td>
<td>78%</td>
</tr>
<tr>
<td>male</td>
<td>CR</td>
<td>26.60 ± 3.94</td>
<td>46%</td>
</tr>
</tbody>
</table>

Table 3: Incidence of cardiac calcinosis in the cross-sectional cohorts of DBA/2 mice

<table>
<thead>
<tr>
<th>Sex</th>
<th>Diet</th>
<th>average age ± S.D.</th>
<th>incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>female</td>
<td>AL</td>
<td>24.00 ± 0</td>
<td>2%</td>
</tr>
<tr>
<td>female</td>
<td>CR</td>
<td>16.00 ± 7.48</td>
<td>5%</td>
</tr>
<tr>
<td>male</td>
<td>AL</td>
<td>24</td>
<td>1%</td>
</tr>
<tr>
<td>male</td>
<td>CR</td>
<td>27.71 ± 3.31</td>
<td>23%</td>
</tr>
</tbody>
</table>

Table 4: Incidence of mineral deposition in skeletal muscle in the cross-sectional cohorts of DBA/2 mice

<table>
<thead>
<tr>
<th>Sex</th>
<th>Diet</th>
<th>average age ± S.D.</th>
<th>incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>female</td>
<td>AL</td>
<td>20.00 ± 3.64</td>
<td>3%</td>
</tr>
<tr>
<td>female</td>
<td>CR</td>
<td>21.47 ± 5.73</td>
<td>10%</td>
</tr>
<tr>
<td>male</td>
<td>AL</td>
<td>24 ± 0</td>
<td>3%</td>
</tr>
<tr>
<td>male</td>
<td>CR</td>
<td>26.0 ± 3.64</td>
<td>20%</td>
</tr>
</tbody>
</table>
Table 5: Incidence of tongue mineralization in the cross-section component of this study

<table>
<thead>
<tr>
<th>Sex</th>
<th>Diet</th>
<th>average age ± S.D.</th>
<th>incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>female</td>
<td>AL</td>
<td>15.00 ± 9.86</td>
<td>5%</td>
</tr>
<tr>
<td>female</td>
<td>CR</td>
<td>16.29 ± 7.14</td>
<td>14%</td>
</tr>
<tr>
<td>male</td>
<td>AL</td>
<td>no affected individuals</td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>CR</td>
<td>23.14 ± 2.27</td>
<td>5%</td>
</tr>
</tbody>
</table>

Table 6: Incidence of cardiac calcinosis in DMBA treated DBA/2 mice

<table>
<thead>
<tr>
<th>Sex</th>
<th>Diet</th>
<th>average age ± S.D.</th>
<th>incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>female</td>
<td>AL</td>
<td>12.3 ± 3.1</td>
<td>26%</td>
</tr>
<tr>
<td>female</td>
<td>CR</td>
<td>16.4 ± 3.0</td>
<td>40%</td>
</tr>
</tbody>
</table>