Advances in Brief

Calorie Restriction Induces a p53-independent Delay of Spontaneous Carcinogenesis in p53-deficient and Wild-Type Mice[1]

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Abstract

We reported previously that calorie restriction (CR) delays spontaneous carcinogenesis in p53-deficient ($p53^{-/-}$) mice, suggesting that CR modulates carcinogenesis by p53-independent mechanisms. To further evaluate the role of p53, we monitored tumor development in $p53^{-/-}$ and wild-type ($p53^{+/+}$) mice fed *ad libitum* (AL) or a CR regimen (60% of AL calorie intake). CR delayed tumor mortality in $p53^{-/-}$ and $p53^{+/+}$ mice (mean time to death, 169 and 648 days, respectively) relative to AL feeding (104 and 470 days). The estimated age-specific cancer death rate AL:CR ratios were 4.3 for $p53^{-/-}$ mice and 4.4 for $p53^{+/+}$ mice. Thus, despite the accelerated onset of carcinogenesis in $p53^{-/-}$ mice, the tumor-delaying effect of CR was similar in the two genotypes.

Introduction

CR[4] is one of the best documented and most effective experimental manipulations for suppressing carcinogenesis (1) and extending life span (2) in rodents. Recent reports of beneficial effects of CR on physiological parameters associated with aging and cancer in nonhuman primates suggest that CR may exert in humans the antiaging and anticancer effects observed in rodents (3). Great interest exists currently in the translation of this phenomenon to prevention strategies for human cancer, not necessarily by the direct application of CR in human populations but through the identification and manipulation of new prevention targets that are expected to emerge from a better understanding of the mechanisms underlying the antitumor effects of CR. $p53^{-/-}$ mice, in which both alleles of the *p53* tumor suppressor gene are inactivated by gene targeting, provide an attractive carcinogenesis model for cancer prevention studies, because tumor development in these mice is rapid and spontaneous (4) and because p53 mutations are the most commonly observed genetic lesions in human cancer (5). We reported previously that CR delays spontaneous carcinogenesis in $p53^{-/-}$ mice (6), suggesting that CR modulates carcinogenesis by p53-independent mechanisms. However, other reports suggest that the antiproliferative effects of CR may be mediated through a p53-dependent pathway (7). In the study reported here, we compared the effect of CR on spontaneous carcinogenesis in $p53^{-/-}$ and $p53^{+/+}$ mice to further evaluate the role of p53 in the antitumor effects of CR. Our findings show that, although p53 status clearly influences the rapidity with which spontaneous tumors develop, the

effect of CR, relative to AL feeding, was similar in both genotypes, consistent with a p53-independent mechanism underlying the tumor-suppressive effects of CR.

Materials and Methods

Weanling (4–7 week-old) male $p53^{-/-}$ and $p53^{+/+}$ mice (GenPharm International, Mountain View, CA) were housed individually in polycarbonate cages on hardwood bedding and maintained on a 12-h light-dark cycle at 24°C. The $p53^{-/-}$ mice and their $p53^{+/+}$ litter mates had the same genetic background (~94% C57BL/6 and ~6% 129/Sv), differing only at the *p53* locus. After a 2-week acclimation period, during which all mice were fed an AIN-76A purified diet (Bioserv, Inc., Frenchtown, NJ) AL, the mice were randomized to one of two dietary treatment groups (28–30/group): (*a*) the AL group, which received AIN-76A diet AL, or (*b*) the CR group. As described previously (6), the CR group received daily aliquots of a specially modified AIN-76A diet formulated such that the reduction in calories was entirely from the carbohydrate ingredients of the diet, with all other factors isonutrient relative to the AL group when consumed at 60% of the average daily intake of the AL group. Food was administered to both groups as 1-g dustless precision pellets (Bioserv) in standard feeders and was provided to AL mice in weekly aliquots and to CR mice in daily aliquots. All mice received distilled water AL. Food intakes and body weights were recorded weekly, and all mice were observed daily for clinical signs of ill health. Moribund mice were killed by CO_2 asphyxiation. All animals that were killed or found dead were subjected to necropsy, and representative tissues were fixed and evaluated as described previously (6). Mean body weights were compared by ANOVA (8). The Mantel-Haenszel statistic was used to compare the proportions of tumor-bearing animals among experimental groups (9). The Cox proportional hazards model was used to compare survival and to estimate the relative age-specific mortality rates between treatment and genotype groups (10).

Results

p53 status did not influence the effect of CR on body weight; the growth curves of the $p53^{-/-}$ mice on either dietary regimen were virtually identical to the growth curves of the $p53^{+/+}$ mice through the 1st 20 weeks of the study (Fig. 1). CR mice regardless of p53 status were significantly smaller ($P < 0.01$) than AL mice throughout the 1st year of the study. The average body weights of the $p53^{+/+}$ mice on either diet regimen fluctuated considerably during the 2nd year of the study due to the onset of tumor development in many of the mice.

Marked differences were seen in the survival curves for the different genotypes and treatment groups (Fig. 2). On either diet regimen, $p53^{-/-}$ mice spontaneously developed tumors and died much faster than the $p53^{+/+}$ mice. For the AL diet, the mean time to death for tumor-bearing $p53^{-/-}$ mice was 104 days compared to 470 days for $p53^{+/+}$ mice ($P < 0.001$); for the CR diet, the mean time to death for tumor-bearing $p53^{-/-}$ mice was 169 days compared to 648 days for $p53^{+/+}$ mice ($P < 0.001$). As reported previously (6), in $p53^{-/-}$ mice, the CR diet produced a statistically significant delay in tumor-related mortality relative to the AL diet. A Cox proportional hazards
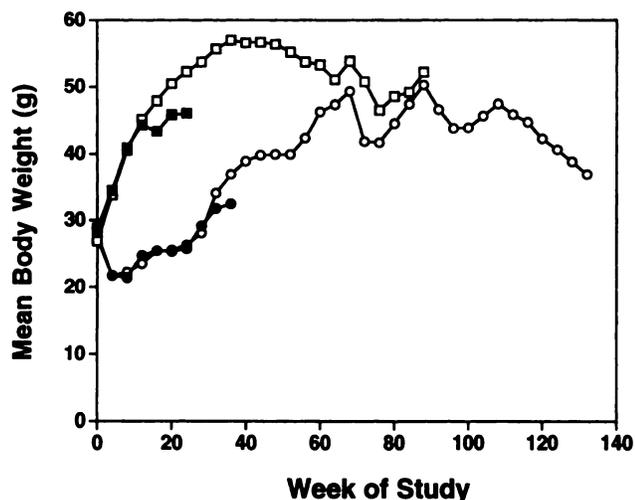
Fig. 1. Mean body weights of male p53$^{-/-}$ (■ and ●) and p53$^{+/+}$ (□ and ○) mice either fed AIN-76A diet AL (■ and □) or calorie-restricted diet (● and ○; n = 28–30/group). The values represent the mean body weights at every 4th week throughout the 132-week study period.

analysis estimated that the AL p53$^{-/-}$ mice had an age-specific cancer death rate that was 4.3-fold that of the CR mice ($P < 0.0001$). The CR regimen also produced a statistically significant delay in tumor-related mortality relative to the AL diet in p53$^{+/+}$ mice. The estimated age-specific cancer death rate AL:CR ratio for the p53$^{+/+}$ mice was 4.4 ($P < 0.0001$), nearly identical to that of the p53$^{-/-}$ mice. These findings indicate that, although p53 status strongly influences the rapidity with which spontaneous tumors develop, the tumor-delaying effect of CR was independent of p53 genotype, with CR-treated mice of either genotype surviving significantly longer than AL-fed mice.

In addition to markedly affecting the time course of spontaneous carcinogenesis in p53$^{-/-}$ and p53$^{+/+}$ mice, the CR regimen also altered the distribution of malignant tumor types (Table 1). As we reported previously (6), hematopoietic neoplasms (mostly lymphomas) were the most commonly observed tumors in p53$^{-/-}$ mice: 57% of AL-fed p53$^{-/-}$ mice and 71% of CR-treated p53$^{-/-}$ mice developed at least one malignant hematopoietic neoplasm. Most of the lymphomas in these mice on either diet treatment were of lymphoblastic origin, although pleomorphic lymphomas and lymphomas of immunoblastic or follicular center cell origin were also observed. Nonhematopoietic tumors (mostly hemangiosarcomas or osteosarcomas) were observed in 47% of AL mice and 32% of CR mice. Forty % of AL mice and 29% of CR mice developed multiple tumors. Thus, in p53$^{-/-}$ mice, CR increased the ratio of hematopoietic to nonhematopoietic tumors and decreased the total tumor burden.

The CR-induced shift from nonhematopoietic to hematopoietic tumors was also observed in p53$^{+/+}$ mice (Table 1). Sixty % of AL mice and 23% of CR mice ($P < 0.01$) developed at least one nonhematopoietic tumor (primarily sarcomas and liver tumors), whereas 30% of AL mice and 50% of CR mice ($P < 0.01$) developed at least one hematopoietic tumor (lymphomas or histiocytic sarcomas). Much of the CR-induced shift in tumor types in p53$^{+/+}$ mice appeared to be a result of a CR-induced suppression (relative to AL feeding) in the number of nonhistiocytic sarcomas (from 6 to 2) and liver tumors (from 12 to 2; $P < 0.01$). As in the p53$^{-/-}$ mice, the number of p53$^{+/+}$ mice developing multiple tumors was reduced by calorie restriction (16 AL mice versus 8 CR mice).

The effect of CR (relative to AL feeding) on the distribution of tumor types was found to be consistent for the two genotypes. As

shown in Table 1, analysis of the combined CR and AL data from the two genotypes yielded statistically significant CR effects on the proportion of animals with nonhematopoietic malignant tumors ($P = 0.003$), with malignant liver tumors ($P = 0.004$), and with multiple tumors ($P = 0.009$). CR also suppressed the development of benign tumors in both genotypes. Benign tumors were observed in six AL/p53$^{-/-}$ mice (four thymomas, one cecal adenoma, and one squamous cell papilloma in the stomach) compared with three CR/p53$^{-/-}$ mice (thymomas in each case). Eleven AL/p53$^{+/+}$ mice developed benign tumors (mostly odontomas or benign liver tumors) compared with four CR/p53$^{+/+}$ mice (one with an odontoma, one with a hepatocellular adenoma, one with both an odontoma and a hepatocellular adenoma, and one with an alveolar adenoma). Thus, AL-fed mice of either genotype had a 2–3-fold higher incidence of benign tumors than the CR-treated mice. As shown in Table 1, combining the CR and AL data from the two genotypes yielded a borderline statistically significant CR effect on the proportion of animals with benign tumors ($P = 0.08$).

As shown in Table 2, CR delayed death from all tumor types in both genotypes, suggesting that the main effect of CR was a universal (in terms of tumor types) delay in tumor development and death in these mice. A Cox analysis estimated that AL mice of both genotypes had an age-specific death rate from hematopoietic tumors that was 2.7-fold that of CR mice ($P = 0.001$) and a death rate from nonhematopoietic tumors 9.5-fold that of CR mice ($P < 0.001$). In terms of average time on study, CR lengthened survival of mice that died from hematopoietic neoplasms by 53% in p53$^{-/-}$ mice and 48% in p53$^{+/+}$ mice. This includes 47% longer survival in p53$^{-/-}$ mice and 77% longer survival in p53$^{+/+}$ mice that died from lymphomas as well as 30% longer survival in p53$^{+/+}$ mice that died from histiocytic sarcoma. CR also lengthened survival of mice that died from nonhematopoietic tumors by 86% in p53$^{-/-}$ mice and 33% in p53$^{+/+}$ mice. This includes lengthened survival (by 90% in p53$^{-/-}$ mice and 45% in p53$^{+/+}$ mice) in mice that died from nonhistiocytic sarcomas.

The CR-induced shift in tumor types from nonhematopoietic to hematopoietic tumors in both genotypes may also have contributed to the longer survival of CR-treated mice. Mice of either genotype with hematopoietic tumors tended to survive longer than mice with nonhematopoietic tumors, regardless of diet treatment. The total mean time to death for both diet treatments was 142 days for the 35 p53$^{-/-}$ mice that died from hematopoietic tumors and 119 days for the 15 p53$^{-/-}$ mice that died from nonhematopoietic tumors ($P = 0.04$).
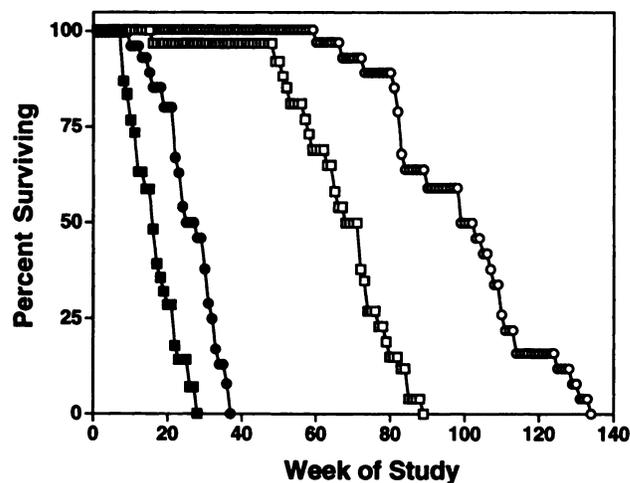

Fig. 2. Kaplan-Meier survival curves by week of study of male p53$^{-/-}$ (■ and ●) and p53$^{+/+}$ (□ and ○) mice either fed AIN-76A diet AL (■ and □) or calorie-restricted diet (● and ○; n = 28–30/group).

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Table 1 *Tumor development in AL-fed or calorie-restricted p53-deficient and wild-type mice*

	p53$^{-/-}$		p53$^{+/+}$		
	AL (n = 30)	CR (n = 28)	AL (n = 30)	CR (n = 30)	P value[a]
Mice with malignant neoplasms	26	24	22	19	0.20
Mice with malignant hematopoietic neoplasms	17	20	9	15	0.21
Lymphomas	17	19	4	6	0.71
Lymphoblastic	7	10	2	1	0.92
Immunoblastic or FCC[b]	4	6	2	5	0.34
Pleomorphic[c]	6	3	0	0	0.41
Histiocytic sarcomas	1	1	5	11	0.19
Mice with malignant nonhematopoietic neoplasms	14	9	18	7	0.003
Nonhistiocytic sarcomas	7	7	6	2	0.41
Liver neoplasms[d]	1	0	12	2	0.004
Other nonhematopoietic neoplasms	6	2	6	5	0.39
Mice with benign neoplasms	6	3	11	4	0.08
Mice with multiple neoplasms[e]	12	8	16	8	0.009

[a] Testing the equality of proportions of tumor-bearing AL and CR mice, adjusted for genotype (Mantel-Haenszel statistic).
[b] FCC, follicular center cell lymphoma.
[c] The pleomorphic lymphomas display morphological features and immunohistochemical staining profiles characteristic of both T- and B-cell lymphomas (15).
[d] Hepatocellular carcinomas, hepatocholangiocellular carcinomas, or hepatoblastomas.
[e] Tumor-bearing mice with two or more neoplasms, malignant or benign.

Table 2 *Mean time to death in AL-fed or calorie-restricted p53-deficient and wild-type mice[a]*

	Mean days on study ± SD (n)			
	p53$^{-/-}$		p53$^{+/+}$	
Tumor types causing death	AL (n = 30)	CR (n = 28)	AL (n = 30)	CR (n = 30)
All malignant neoplasms	104 ± 46 (26)	169 ± 54 (24)	470 ± 135 (22)	648 ± 157 (19)
All hematopoietic neoplasms[b]	110 ± 50 (16)	168 ± 58 (19)	435 ± 187 (8)	643 ± 166 (15)
Lymphomas	110 ± 50 (16)	162 ± 59 (16)	384 ± 187 (4)	679 ± 198 (6)
Histiocytic sarcomas	(0)	143 (1)	486 ± 199 (4)	619 ± 150 (9)
All nonhematopoietic neoplasms	93 ± 39 (10)	173 ± 35 (5)	490 ± 98 (14)	652 ± 146 (4)
Sarcomas (nonhistiocytic)	88 ± 42 (5)	167 ± 37 (4)	452 ± 77 (5)	655 (1)
Liver neoplasms	(0)	(0)	525 ± 101 (7)	(0)
Other neoplasms	98 ± 40 (5)	199 (1)	462 ± 148 (2)	668 ± 163 (3)

[a] All values across rows are statistically different from each other at P < 0.05, except histiocytic sarcoma in p53$^{+/+}$ mice and 0 values.
[b] Two p53$^{-/-}$ mice receiving the CR regimen died from leukemia.

Similarly, the mean time to death was 571 days for the 23 p53$^{+/+}$ mice that died from hematopoietic tumors and 526 days for the 18 p53$^{+/+}$ mice that died from nonhematopoietic tumors (P = 0.08).

Discussion

The rapid development of tumors and the sharp increase in mortality in the AL/p53$^{-/-}$ mice, beginning around the 10th week of the study, demonstrated the extraordinary susceptibility of p53$^{-/-}$ mice to spontaneous carcinogenesis. In contrast, AL/p53$^{+/+}$ mice showed virtually no tumor development through the 1st year of the study; however, these mice did begin to develop spontaneous tumors by the 60th week of the study, and all AL/p53$^{+/+}$ mice were dead by 90 weeks of the study. These findings are consistent with previous reports on spontaneous carcinogenesis in p53$^{-/-}$ and p53$^{+/+}$ mice (4, 6, 11).

Numerous studies have shown that CR increases longevity and inhibits a variety of spontaneous and experimentally induced tumors in rodents, including spontaneous lymphomas in C57BL/6 mice (1), the main background strain of the mice we used. The hypothesis that p53 could mediate the antitumor effects of CR is certainly plausible (7). Two recent studies of rat liver show that CR is associated with enhanced rates of apoptosis, decreased DNA synthesis, and a marked reduction in the number and volume of preneoplastic foci (12, 13). CR has been shown repeatedly to suppress mitogenesis (1, 14), enhance DNA repair (13), and decrease oxidative damage to cellular macromolecules (11); each of these processes is potentially regulated by p53. Thus, it is feasible that CR exerts its antitumor effects through p53-regulated mitogenic/apoptotic control pathways. However, our previous report that CR lengthened survival in p53$^{-/-}$ mice and

slowed the cell cycle in lymphocytes isolated from p53$^{-/-}$ and p53$^{+/+}$ mice suggested that p53-independent pathways regulating mitogenesis and carcinogenesis were involved (6).

To further explore the role of p53 in the tumor-delaying effects of CR, we continued to monitor tumor development throughout the entire life span of the AL/p53$^{+/+}$ and CR/p53$^{+/+}$ mice used as controls in the original report. Not surprisingly, CR delayed spontaneous carcinogenesis in p53$^{+/+}$ mice, consistent with the numerous reports in a variety of spontaneous and chemically induced tumor models in mice with normal p53 tumor suppressor activity (1). In addition, our present finding that the relative effects of CR on survival curves and tumor development were similar in p53$^{-/-}$ and p53$^{+/+}$ mice provides strong evidence that the wild-type p53 gene product is not necessary for CR to exert its suppressing effects against spontaneous carcinogenesis.

In conclusion, although p53 status influenced the latency of spontaneous carcinogenesis, the effects of CR (relative to AL feeding) were virtually identical in the two genotypes, suggesting the mechanism underlying the tumor-delaying effects of CR is independent of p53. In addition, the observation that a tumor-modulating intervention such as CR can exert similar effects on tumor development in p53$^{-/-}$ and p53$^{+/+}$ mice suggests that p53$^{-/-}$ mice may provide a rapid, highly relevant, and responsive model of spontaneous carcinogenesis that will be useful for cancer prevention studies.

Acknowledgments

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