[CANCER RESEARCH 51, 1851-1854, April 1, 1991]

Influence of Diet and Calorie Restriction on the Initiation and Promotion of Skin Carcinogenesis in the Sencar Mouse Model[1]

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ABSTRACT

Diets were restricted to 60% of the intake of the control mice by feeding less diet (total diet restriction, TDR) or by feeding fewer calories from fat and carbohydrate (calorie restriction, CR) during the initiation or promotion phases of skin tumorigenesis in female SENCAR mice. Skin cancer was initiated by topical treatment with 10 nmol of 7,12-dimethylbenzanthracene in acetone and promoted by twice weekly treatments with 12-*O*-tetradecanoylphorbol-13-acetate in acetone for 20 wk. Dietary restriction preceding and during 7,12-dimethylbenzanthracene treatment did not influence skin papilloma or carcinoma yield. Papilloma incidence and the number of papillomas per effective mouse were reduced in mice restricted by both TDR and CR protocols during and following promotion with 12-*O*-tetradecanoylphorbol-13-acetate. Papilloma size was reduced at experimental wk 16 and 20 in both TDR and CR groups fed these diet regimens during promotion. However, by wk 28 and 32, papilloma sizes were similar in the control and TDR groups, and smaller papillomas were observed only in the CR group. The average carcinoma latency was extended by 26% in the groups restricted during promotion, and incidence was reduced in both groups. The reduction, however, was statistically significant only in the CR group. Body weight gain was reduced during the times when dietary restriction was enforced, and in a short-term study, both restricted diet treatments reduced the percentage of carcass protein.

INTRODUCTION

Restriction of dietary intake has been demonstrated to be the most effective dietary method for inhibition of carcinogenesis and extension of life span. Caloric restriction has inhibited both chemically induced and spontaneous cancer (reviewed in Ref. 1). Previous studies showed striking inhibition of cancer of the skin, breast, lung, liver, colon, pancreas, muscle, lymphatic system, endocrine system, and cheek pouch in laboratory rodents (1).

Most of the investigations into dietary restriction and cancer have fed less total diet. Thus, animals received less protein, vitamins, minerals, and fiber in addition to the reduction in calories from fat and carbohydrate. Early investigations by Tannenbaum (2, 3) studied calorie restriction, but in these studies carbohydrate was added to the control diet and then removed for the restricted animals, thereby feeding fewer calories from carbohydrate. Several recent studies restricted calories from fat or carbohydrate, although the practice of feeding less diet continues (4). Direct comparison of restriction of calories with restriction of total diet has not been reported.

Earlier investigations into the influence of dietary restriction on skin tumorigenesis focused on spontaneous skin cancer and cancer induced by polycyclic hydrocarbons (2, 3). The influence of dietary restriction on the two-stage model of skin carcinogenesis induced in SENCAR mice with DMBA[3] and promoted by TPA has not been reported previously.

This investigation aimed to compare calorie restriction with total diet restriction and to determine if restriction would influence skin tumor initiation by DMBA and promotion by TPA.

MATERIALS AND METHODS

Experimental diets are shown in Table 1. Control diet was formulated according to the recommendations of the American Institute of Nutrition (5, 6), except that we used glucose:dextrin instead of sucrose for the carbohydrate, and we did not add ethoxyquin. TDR groups received 0.6 g of the control diet for each gram consumed by the *ad libitum* control group. Thus, these animals received 40% less of all dietary components as well as 40% fewer calories. CR mouse groups received the diet shown in Table 1. This diet was formulated so that these animals received 40% fewer calories but had the same daily intake of all other dietary ingredients. The calories came from carbohydrate and fat. Intake of the restricted groups was based on the mean intake of mice in the *ad libitum* group fed control diet throughout the experiment. Intakes were adjusted weekly based on the consumption of the control group during the preceding week. All diets were pelleted (California Pellet Mill, Crawfordsville, IN) without heat or steam and stored in a freezer for no longer than 3 wk. Animals fed *ad libitum* were given fresh diet weekly, and diet intakes were recorded on 50% of these animals by computing the diet consumed. Restricted groups were fed daily, with weighed portions representing the 40% restrictions described previously.

Female SENCAR mice were obtained from the National Cancer Institute, Frederick, MD, at 6 wk of age. They were freely fed control diet from 6 to 10 wk of age. Mouse backs were shaved with surgical clippers at 9 wk of age. Two days later, mice in the initiated groups were treated with 10 nmol of DMBA in 0.2 ml of acetone. TPA treatment began 1 wk later and consisted of twice weekly treatments with 2 μg (3.2 nmol) in 0.2 ml of acetone on the shaved back. TPA was administered for a period of 20 wk. Groups not treated with DMBA and TPA were treated with acetone at the same intervals. The experimental groups are shown in Table 2. Thirty mice were assigned to each group treated with DMBA/TPA, and 15 mice were assigned to each acetone-treated group. The control group was fed control diet *ad libitum* throughout the experiment. Mice were fed the restricted diet protocols during wk 6 to 9 to study the influence of restriction on initiation, or from wk 10 to 55 to study effects on promotion. The control diet was fed *ad libitum* at other times. Groups with DMBA or TPA alone were not included in this experiment because these treatments resulted in few papillomas and no carcinomas in previous experiments in our laboratory. Mice were weighed every other week throughout the experiment and palpated for tumors weekly, beginning at 6 wk of TPA treatment (wk 9 of the experiment). Papillomas were counted and sized until 32 wk of the experiment. The rate of increase in papilloma incidence and number leveled off between wk 24 and 28. Carcinomas began to appear around wk 30 of the experiment, and mice were

Received 9/24/90; accepted 1/23/91.

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[1] This work was supported by NIH Grants R01-CA42986 and K04-CA-01382-RCDA, American Cancer Society Grant SIG-16, and NIH Core Grant CA-36727.

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[3] The abbreviations used are: DMBA, 7,12-dimethylbenzanthracene; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TDR; total diet restricted/restriction; CR, calorie restricted.

Table 1 *Experimental control and restricted diets*

Ingredient[a]	Control diet[b] (%)	Calorie restricted[c] (%)
Corn oil	5.0	3.8
Casein	20.0	31.1
DL-Methionine	0.3	0.5
Glucose	15.0	11.5
Dextrin	50.0	38.1
Fiber[d]	5.0	7.8
AIN mineral mix	3.5	5.4
AIN vitamin mix	1.0	1.5
Choline bitartrate	0.2	0.3
Total	100.0	100.0

[a] Ingredients were obtained from Teklad Test Diets, Madison, WI.
[b] Total diet-restricted mice received 0.6 g of this diet for every g consumed by the *ad libitum* group.
[c] Calorie-restricted mice received 0.64 g of this diet for every g consumed by the *ad libitum* group.
[d] Nonnutritive fiber, purified wood, cellulose (74 to 75% crude fiber).

sacrificed when a carcinoma was identified visually. Squamous cell carcinomas were identified by morphological criteria, including the absence of a papillomatous stalk and invasiveness into underlying muscularis and connective tissue. All remaining mice were sacrificed at wk 50 of the experiment, when papillomas showed no further evidence of developing into carcinomas.

Body composition was determined on 12-wk-old mice, which had been prefed for 4 wk. Mice were fed control diet *ad libitum* and were restricted by the total diet restriction protocol and the calorie restriction protocol. Body water was determined by lyophilizing the chopped carcass. The dried material was ground and extracted in a Soxhlet extraction apparatus, and body fat was determined by weighing the extracted lipid. The defatted, dried material was subjected to Keljdal digestion, and nitrogen was determined and converted to protein using the factor of 6.25 (7).

Differences in *ad libitum* food consumption, body weight, body composition, papilloma size, papilloma number, and carcinoma latency were assessed statistically using analysis of variance (Statistical Analysis Software) (8). Differences in papilloma and carcinoma incidence were compared with χ^2 tests (9).

RESULTS

Intake of control diet averaged 4.9 ± 0.1 g/day/mouse before DMBA treatment in the freely fed groups. In groups fed freely following DMBA treatment, DMBA and TPA treatment influenced calorie intake in the control and TDR groups, but not in the CR group. Intake in the carcinogen-treated animals and in the noncarcinogen-treated CR animals averaged 5.3 ± 0.1 g/day/mouse. Intake in the noncarcinogen-treated control and TDR mice averaged 4.8 ± 0.1 g/day/mouse.

Body weights of mice treated with DMBA and TPA are shown in Fig. 1. Diet and calorie restriction before DMBA

Table 2 *Experimental design for addressing specific effects of caloric restriction on two-stage skin carcinogenesis*

Wk of age: 6	9	10					30	56
C[a] ———								→
TDR ———		C ———						→
CR ———		C ———						→
C ———		TDR ———						→
C ———		CR ———						→
C ———	↓	V ———	V	V	V	V	V	→
TDR ———	↓	C/V ———	V	V	V	V	V	→
CR ———	↓	C/V ———	V	V	V	V	V	→
C ———	↓	TDR/V ———	V	V	V	V	V	→
C ———	↓	CR/V ———	V	V	V	V	V	→

[a] C, *ad libitum* control diet; ↓, treatment with DMBA; V, twice weekly treatment with TPA.

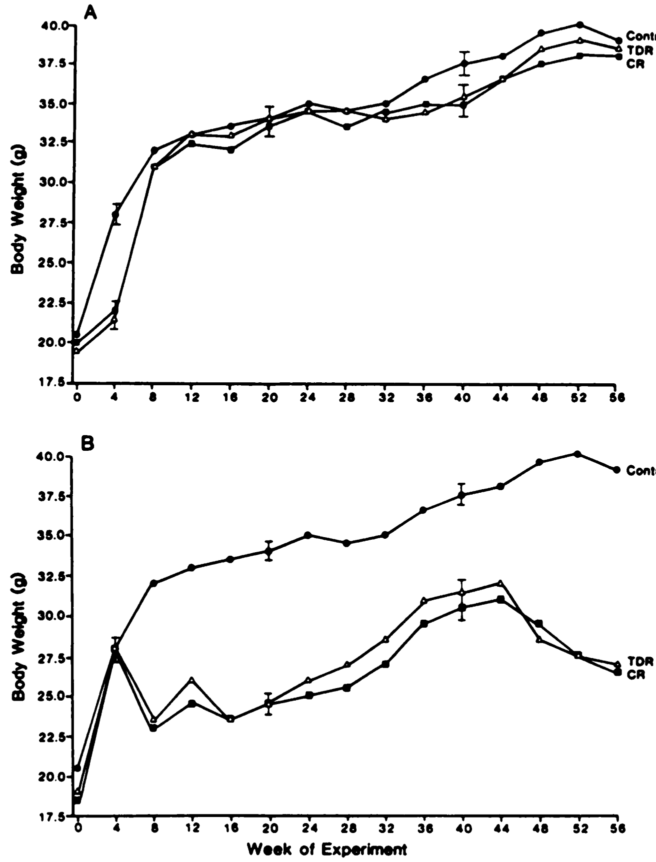

Fig. 1. Body weight of DMBA- and TPA-treated mice. *A,* mice restricted during DMBA treatment; *B,* mice restricted following DMBA treatment. *Points,* mean; *bars,* SEM. Statistical differences are described in the text.

treatment resulted in reduced weight gain at wk 4, recovery by wk 8, and slightly depressed body weight at wk 40. Restriction following DMBA treatment resulted in a loss of body weight by wk 8 and continued reduced weight in comparison with controls throughout the study. There were no significant differences between the two forms of restriction on body weight. Treatment with DMBA and TPA reduced the body weight in all diet groups, but the reduction was greatest in the control diet group (7% reduction in comparison with noncarcinogen-treated control) in comparison with the restricted groups (less than 2% reduction in comparison with noncarcinogen-treated controls) (data for noncarcinogen-treated mice not shown).

Feed efficiency was compared in DMBA/TPA-treated mice from wk 1 to 5 in the groups restricted at the time of DMBA and from wk 6 to 20 in the groups restricted during and following TPA treatment. Values were calculated by dividing the difference in body weight of an individual mouse between the end and the beginning of the period by the calories consumed by that mouse during the period. Feed efficiencies for mice restricted from wk 1 to 5 were the same as for control mice: 0.01 ± 0.001 g/kcal. Feed efficiencies for mice restricted by the CR or TDR protocol from wk 6 to 20 were −0.009 ± 0.002 and −0.011 ± 0.002 g/kcal, respectively, in comparison with mice fed control diet during this period: 0.001 ± 0.002 g/kcal ($P < 0.001$).

Papilloma incidence leveled off after wk 24 in all groups (data at wk 24 are shown in Table 3). Incidence reached 80 to 90% in groups fed restricted diets during initiation, and no differences were seen between these groups. Incidence reached 55 to 56% in the groups restricted during promotion compared

Table 3 *Influence of caloric restriction on skin papilloma incidence and carcinoma incidence and latency in SENCAR mice*

Dietary treatment (during initiation/ during promotion)	Papilloma incidence (wk 24)		Carcinomas (through wk 50)		
	No. of effective mice[a]	% of incidence	No. of effective mice[b]	% of incidence[c]	Av. latency of carcinomas[d] (mean ± SEM)
Control/control	30	90	24	71 (b)	37 ± 2 (a)
Total diet restriction/control	30	80	24	58	36 ± 2 (a)
Calorie restriction/control	28	89	16	69	36 ± 2 (a)
Control/total diet restriction	20	55	17	41	45 ± 3 (b)
Control/calorie restriction	23	56	18	28 (a)	46 ± 3 (b)

[a] Number of effective mice for papilloma data was the number surviving until wk 24 or those killed with at least one papilloma.
[b] Number of effective mice for carcinoma data was the number surviving until the end of the experiment plus those killed because of a developing carcinoma.
[c] Incidence data were analyzed by χ^2 tests: $a < b$ ($P < 0.05$).
[d] Latency data were analyzed by analysis of variance: $a < b$ ($P < 0.05$).

with a 90% incidence in the control *ad libitum* group. Papilloma numbers at wk 12, 16, 20, 24, and 28 are shown in Fig. 2. Restriction during initiation did not influence the number of papillomas/effective animal. Both CR and TDR during promotion reduced the number of papillomas/effective animal ($P < 0.001$), but no significant differences were observed between these two groups. Fig. 3 shows papilloma size at wk 16, 20, 24, 28, and 32 on mice restricted during promotion. At wk 16 and 20, most of the papillomas on restricted mice were <0.1 cm in diameter, while more than half of the papillomas on the control mice were larger than 0.1 cm. By wk 24, CR mice had a larger proportion of papillomas <0.1 cm in diameter than did mice in the control group, and CR mice had fewer papillomas in the ≥0.4- to <0.8-cm range than did the control or TDR mice. A similar pattern was observed at wk 28. By wk 32, the only statistically significant difference was fewer papillomas in the ≥0.4- to <0.8-cm range in the CR mice than in the control group. Also, papillomas larger than 0.8 cm did not develop in mice on the CR protocol, but both control and TDR mice had papillomas in this range at wk 24, 28, and 32.

Table 3 shows cumulative carcinoma incidence and average latency. Restriction during initiation did not influence carcinoma incidence or latency. Restriction during promotion extended latency in both CR and TDR mice and significantly inhibited the incidence in CR mice. Mice were killed when one carcinoma was verified, so carcinoma number and size data are not meaningful and therefore not reported.

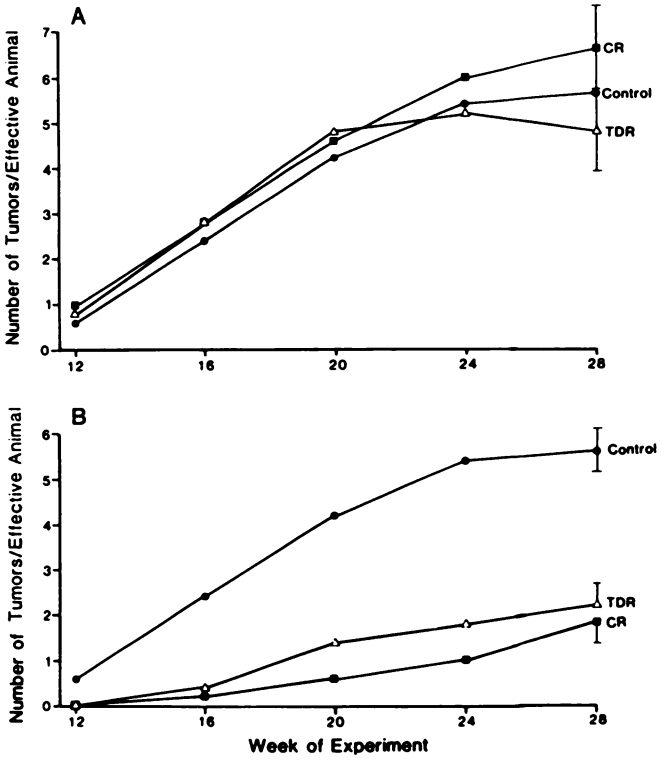

Fig. 2. Number of papillomas per effective animal. *A*, mice restricted during DMBA treatment; *B*, mice restricted following DMBA treatment. *Points*, mean; *bars*, SEM. Statistical differences are described in the text.

Table 4 shows body composition of mice fed the restricted diets for 4 wk. The percentage of water and fat did not differ among the three diet groups, but the percentage of protein and g of nitrogen/100 g of carcass were reduced in the mice on both restricted protocols.

DISCUSSION

Dietary restriction during promotion inhibited skin tumorigenesis in the mouse model initiated by DMBA and promoted

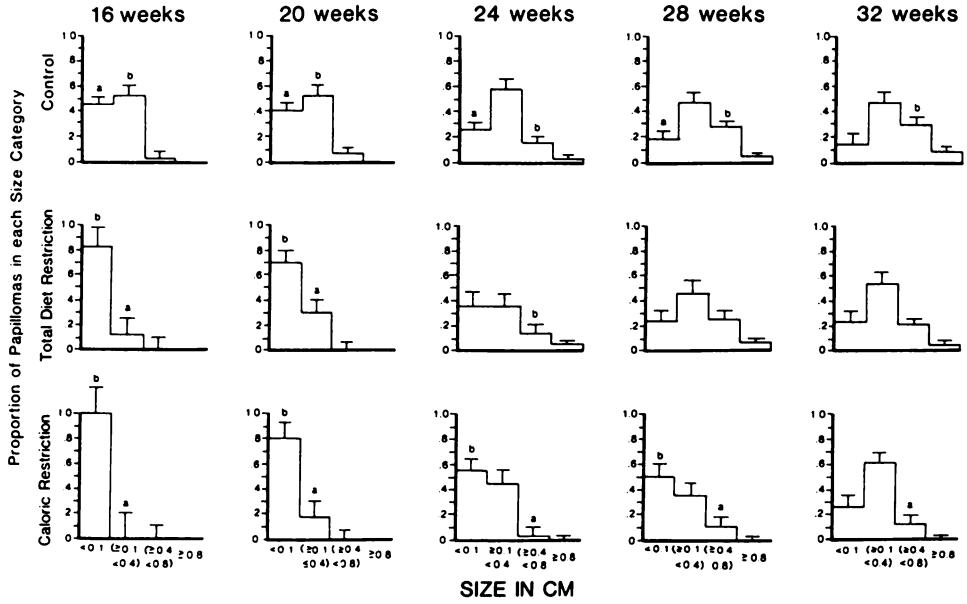

Fig. 3. Papilloma size on mice fed restricted diets following DMBA treatment. Data are presented as the proportion of papillomas in each size category. *Columns*, mean; *bars*, SEM. Statistically different values in comparing diet groups within a time interval are indicated by *superscripts* above the *error bars*, $a < b$ ($P < 0.05$).

Table 4 *Body composition of mice restricted in diet (TDR) or calories (CR) for 4 wk, beginning at 8 wk of age*

Data were analyzed by analysis of variance. A significant difference was observed in body weight ($P < 0.03$) and protein ($P < 0.001$): $a < b$.

	Control ($n = 10$)	TDR ($n = 9$)	CR ($n = 8$)
Body wt at end of experiment (g)	29 ± 1[a] (b)	24 ± 1 (a)	25 ± 1 (a)
Water (%)	58.9 ± 8.3	63.4 ± 3.1	55.8 ± 8.6
Fat (%)	10.7 ± 4.5	13.0 ± 4.3	9.1 ± 4.0
Protein (%)	14.3 ± 2.9 (b)	9.8 ± 1.9 (a)	9.8 ± 1.4 (a)
Water (g)	15.9 ± 2.7	14.6 ± 0.8	12.4 ± 1.4
Fat (g)	2.9 ± 1.4	3.1 ± 1.2	2.1 ± 1.0
g of nitrogen/100 g of carcass	2.3 ± 0.5 (b)	1.6 ± 0.3 (a)	1.6 ± 0.2 (a)

[a] Mean ± SEM.

by TPA. Restriction during initiation, however, did not influence tumorigenesis. Previous studies on the influence of restriction on tumorigenesis demonstrated inhibition of many forms of cancer including breast, colon, and pancreas (1). Two-stage skin carcinogenesis studies described by Boutwell (10) with DMBA-initiated and croton oil-promoted tumors demonstrated an inhibition of papilloma and carcinoma development in calorie-restricted mice. Calorie restriction at the time of promotion inhibited skin tumorigenesis, but restriction imposed during initiation had no influence on tumorigenesis (10). The method of restricting calories was not described. Restriction effects on cancer promotion have been more generalized than effects on initiation, possibly because the mechanisms of cancer promotion are more similar than are the mechanisms of cancer initiation. But, most studies have not investigated separate effects on initiation and promotion.

Observations on body composition of mice fed the restricted protocols do not suggest that the inhibition of tumor promotion was because of a change in body fat content. Restricted mice did not show that their percentage of body fat was altered, and the body protein composition in animals from both restricted groups was reduced. It is important to recognize that the animals in the body composition experiment were restricted for only 4 wk, and long-term restriction may have produced different results.

Restriction of calories alone during promotion in the CR group influenced the reduction in tumor rates slightly more than feeding less of all dietary components during promotion in the TDR groups. This difference was most apparent in the size of papillomas but was also observed in the carcinoma incidence, which was significantly reduced in the CR group compared with the controls but not significantly reduced in the TDR group. Interestingly, papilloma sizes in the CR and TDR groups were very similar when compared at wk 16, but at wk 24, 28, and 32, only the control and TDR groups had papillomas that were 0.8 cm or larger. Fewer large papillomas developed in the CR group, possibly because these animals were deprived of calories only. Mice on the TDR protocol were deprived of all vitamins, minerals, and calories. Deprivation of vitamins such as ascorbic acid and vitamins A and E may have allowed the tumors to grow larger. This hypothesis is consistent with the progression observed; *e.g.*, the difference between the CR and the TDR animals became greater with time and, thus, may have been due to greater depletion of these nutrients.

Our studies used only one level of dietary restriction, *i.e.*, 40% reduction in calorie or diet intake. Studies of mammary tumorigenesis by Klurfeld *et al.* (11) assessed mammary carcinogenesis induced by DMBA in rats fed diets restricted in 10, 20, 30, or 40% of the calories following treatment with DMBA. They fed fewer calories from carbohydrate (sucrose) to their restricted animals. Their results indicated a graded relationship, inversely correlated between tumor burden and the amount of diet restriction. Our future plans include studies of an intermediate level of dietary restriction on skin tumor promotion.

The mechanism of cancer inhibition in diet-restricted animals has not been identified. Lok *et al.* (12) demonstrated a decrease in thymidine-labeling indices in tissues from mice fed calorie-restricted diets. In these studies some tissues, such as the mammary gland, responded to calorie restriction with a much greater decrease in labeling index than in others, such as the colon and esophagus, but a decrease was observed at each site studied (12). Skin was not studied in this investigation. Klurfeld *et al.* (11) reported that fasting serum insulin was reduced in rats restricted by 30 or 40% of the calories, but the same laboratory did not observe a reduction in insulin at lower levels of restriction (13). Other effects of dietary restriction on hormonal, immunological, and growth-regulatory parameters have been suggested as potential mechanisms whereby these treatments reduce cancer rate (14, 15). Recent studies in our laboratory indicated an inhibition of protein kinase C activity in epidermal cells from animals fed restricted diets.[4] This observation may be important because of the suggested role of protein kinase C in cellular proliferation and skin tumor promotion.

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