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Effects of Caloric Restriction and Dietary Fat on Epithelial Cell Proliferation in Rat Colon[1]

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ABSTRACT

Epidemiological studies indicate that caloric intake and dietary fat content influence colonic carcinogenesis. In rodents, caloric restriction reduces, and some fats increase, carcinogen-induced colon cancer incidence. The present study was designed to investigate the effects of caloric restriction on colonic cell proliferation (CCP) in carcinogen-treated or control rats fed low- or high-fat diets. F344 rats were treated with azoxymethane (15 mg/kg ×2) and then fed an isocaloric AIN 76A diet containing either 5 or 23% corn oil, *ad libitum* or calorie-restricted to 70 or 80% of the kilocalories consumed by *ad libitum* rats. Biopsies of the distal colon were taken at 10 and 20 weeks, and rats were sacrificed at 21 or 34 weeks on the experimental diets. Distal CCP was determined by microautoradiography after [^{3}H]thymidine labeling *in vitro* or presacrifice administration *in vivo*. The labeling index and number of labeled cells per crypt column were significantly reduced by caloric restriction at all time points (10, 20, 21, 34 weeks). Caloric restriction reduced CCP in high fat- and low fat-fed rats and in azoxymethane-treated and control rats. High fat resulted in decreased CCP in the distal colon compared to low fat at 34 weeks but not earlier. The findings indicate that: (*a*) caloric restriction is effective in favorably modulating CCP, an intermediate biomarker of colon cancer risk; (*b*) a high fat *ad libitum* diet, which increased tumor yield, does not increase distal colon proliferation; (*c*) dietary fat intake alters proliferation in a manner differing from that induced by changing dietary caloric intake.

INTRODUCTION

Population and case control studies have shown positive associations between dietary fat and caloric intake and risk of colon cancer (1–6). In those studies, the independent contributions of total caloric intake, dietary fat content, and other components of energy balance (*i.e.*, metabolic rate, exercise, body composition, and body mass index) cannot be evaluated, although information on each factor and risk for colon cancer would be important.

In recent years there has been a revival of interest in the relationship between caloric intake and the risk of development of cancer. Caloric restriction has reduced spontaneous and carcinogen-induced tumors in rodent models. Early studies by Moreshi (7) and Rous (8) noted growth retardation of implanted sarcomas in underfed mice. In the 1940s Tannenbaum (9) observed that caloric restriction, produced by limiting carbohydrate calories, lowered the incidence of several spontaneous and induced tumors in rodents.

Subsequent investigators have expanded these findings (10, 11), showing that caloric restriction reduced the incidence of carcinogen-induced colon cancer in rats (12–15). The question of the relative importance of fat and total calories in colon carcinogenesis has been recently addressed. Klurfeld *et al.* (14) showed that rats fed a CR[3] diet at 60% of AL caloric intake had a lower incidence of carcinogen-induced breast and colon tumors than AL-fed rats consuming one-half the total fat (14). Kumar *et al.* (15) found a dose-dependent reduction of carcinogen-induced colon tumors in CR rats consuming a HF, 23% corn oil, diet at 80 and 70% of AL caloric intake. In the latter study, both fat and caloric intake were positively related to colon tumor risk (15), but body composition and weight had less association with risk. Other determinants of metabolic balance, including exercise, have reduced the incidence of carcinogen-induced colon tumors (16, 17), and may have a role in colon carcinogenesis.

The long-term effects of caloric and fat intake upon intermediate biomarkers of colon cancer risk have not been determined. An intermediate biomarker that has been related to colonic neoplasia is epithelial cell proliferation, measured by [^{3}H]dThd or bromodeoxyuridine incorporation into nuclear DNA of proliferating cells in rodent or human colonic crypts (18). Previous data on the effect of fat intake on colonic cell proliferation were conflicting, since a 23.5% corn oil diet in mice increased labeling indices after 10 or 15 weeks (19), while 30% corn oil or 27% lard diet for 4 weeks had no effects in rats (20). Studies of food restriction have been more consistent. A 3-day fast lowers colonic proliferation by 70% in rodents (21), and food restriction to 25% of AL intake for 7 month reduced [^{3}H]dThd incorporation in several tissues, including the colon (22). In our laboratory, lowering dietary intake to 60% of AL intake reduced colonic cell proliferation in young and aging rats (23).

The present study was designed to investigate the effects of caloric restriction and HF and LF intake on AOM-induced colon tumors in rats; whether parallel changes occurred in cell proliferation, and whether modified cell proliferation was associated with differences in the development of colonic tumors. Effects of caloric restriction and HF and LF on cell proliferation in rats not treated with AOM also were studied.

MATERIALS AND METHODS

In 2 separate experiments (A and B), groups of rats were treated similarly. One part of this study, the effects of caloric restriction on colon tumor formation (experiment B) was described previously (15).

Inbred male F344 rats obtained at weaning (Charles River Breeding Laboratories, Wilmington, MA) were randomly divided at 5 weeks old into an AL-fed LF 5% corn oil diet or a HF 23% corn oil diet group. The composition and intake of the experimental semi-purified diet is shown in Table 1. The rats were treated with AOM (15 mg/kg body weight s.c.) or saline s.c. (controls) once weekly for 2 weeks. Four days later, animals were further separated into AL-fed and CR groups resulting in 4 dietary groups in each study: HF AL, HF CR, LF AL, and LF CR rats. HF CR rats were CR to 80% of HF AL rat intake in experiment A, and to 70% of HF AL rat intake in experiment B. The LF CR rats were restricted to 80% of HF AL food intake in both experiments. CR rats were pair fed to their respective AL groups. The composition of all diets was adjusted so that all animals consumed similar amounts of minerals, vitamins, and nonnutritive fiber (Table 1).

In experiment A, 8 AOM-treated and 6 control rats were studied in each of the 4 dietary groups. After 10 and 20 weeks on the experimental diets, the

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[3] The abbreviations used are: CR, calorie-restricted; [^{3}H]dThd, [^{3}H]thymidine; AL, *ad libitum*; HF, high fat; LF, low fat; AOM, azoxymethane; LI, labeling index.

Table 1 *Mean daily intake of experimental diets*

	Low-fat diet: 5% corn oil		High-fat diet: 23% corn oil	
	AL	20% CR	AL	30% CR
Casein, vitamin-free	3.43	2.72	2.99	2.09
DL-Methionine	0.05	0.04	0.04	0.03
Corn starch	8.84	6.54	4.39	2.83
Dextrose	2.21	1.63	1.11	0.71
Corn oil	0.85	0.68	2.99	2.09
Alphacel	0.85	0.82	0.77	0.70
Mineral mix, AIN	0.60	0.57	0.53	0.49
Vitamin mix, AIN revised	0.17	0.16	0.15	0.14
Choline bitartrate	0.03	0.03	0.03	0.03
Total food intake	17.0	13.6	13.0	9.1
Calories (kcal/day)	68.0	54.5	62.5	43.7

Mean data shown in g/day in each experimental group in experiment B.

animals were subjected to colonoscopy to the splenic flexure under Nembutal sedation, using an Olympus pediatric bronchoscope (BF482),[4] and 3 to 10 biopsies (average, 8) were taken from the descending colon (Olympus biopsy forceps BF-22C). Four of 56 rats died after this procedure. Both AOM and control rats were sacrificed after 21 weeks on the experimental diets. Biopsies and autopsy sections of distal colon were processed for cell proliferation studies. In experiment B, 27 AOM-treated rats in each of the four dietary groups were sacrificed after 34 weeks on the experimental diet for analysis of the number and type of colonic tumors. Six rats from each dietary group were randomly selected for colonic cell proliferation studies of the proximal and distal colon.

Colonic Cell Proliferation Analysis. One h prior to sacrifice, rats were given injections of [^{3}H]dThd, 1 μCi/g of body weight i.p. Sections of normal appearing distal colon from the mid-descending colon (experiments A and B), and from the ascending colon (experiment B) were fixed in 10% phosphate-buffered formalin and processed for autoradiography and histology as previously described (24). Twenty-five longitudinally oriented crypts were scored to determine crypt height defined as total cell number per crypt column, and the number and position of labeled cells within each crypt column. The LI was calculated as

$$\frac{\text{Labeled cells}}{\text{Total cells}} \times 100$$

Calculations were made for each of 5 crypt compartments of equal size, compartment 1 being at the base of the crypt and compartment 5 near the luminal surface. The distribution of the proliferative zone was measured as percentage of total labeled cells present per crypt compartment. Colonic biopsies were incubated with [^{3}H]dThd (1 μCi/ml) *in vitro*, and processed as above, except that less than 25 crypts were obtained in some specimens.

In experiment B, 4 to 6 animals from each group were randomly selected at 34 weeks for body composition analysis, performed by Nutrition International, Inc. (New Brunswick, NJ) (15). Proximate analysis for protein, fat, and ash was conducted by the standard methods of the American Association of Official Analytical Chemists (25). Briefly, this method includes the measurement of water content of the carcass by lyophilization. The carcass is then ground and an aliquot (about 10 g) is used for determination of total fat and protein by chemical assay. Total ash is determined gravimetrically following the ashing of the sample at >500°C in a furnace. The difference between the protein, fat, ash, and dry weight of sample represents carbohydrate (not measured directly).

Statistical Analysis. Differences in [^{3}H]dThd labeling parameters between groups were analyzed by unpaired *t* tests.

RESULTS

General Observations. Mean caloric intake was similar in the high and LF AL-fed groups (Table 1, experiment B), but the HF-fed rats consumed over 3-fold more fat than was consumed by the LF group. The body weight of CR rats was lower than that of the AL-fed animals, but all groups gained weight throughout the experiment

Table 2 *Body weight, body composition, and tumor incidence in rats fed low and high fat ad libitum and calorie-restricted diet*

Tumor yield and body composition are derived from experiment B.

	Body wt (g)			
	LF AL	LF CR	HF AL	HF CR
Experiment A				
AOM-treated				
11th wk	284 ± 16[a]	255 ± 18	339 ± 15	250 ± 13
21st wk	329 ± 20	282 ± 19	395 ± 18	278 ± 21
Saline-treated				
11th wk	324 ± 16	250 ± 21	351 ± 12	252 ± 24
21st wk	386 ± 31	284 ± 23	422 ± 13	276 ± 16
Experiment B				
AOM-treated				
16th wk	360 ± 17	302 ± 13	365 ± 18	280 ± 17
34th wk	436 ± 29	336 ± 18	462 ± 25	288 ± 20
Body composition at necropsy (% of dry wt)				
Fat	42.4 ± 2.0	31.9 ± 10.6	49.8 ± 12.4	28.9 ± 4.9
Protein	36.0 ± 0.9	36.5 ± 0.7	37.8 ± 0.7	36.0 ± 1.0
Ash	5.5 ± 0.1	7.5 ± 2.1	6.6 ± 0.9	6.3 ± 0.9
Tumor incidence (% of rats with tumors)	56	41	85[b]	52
Tumor multiplicity (tumors/rat)	0.78 ± 0.2	0.6 ± 0.2	1.5 ± 0.2[b]	0.7 ± 0.17

[a] Means ± SE.
[b] $P < 0.05$ *versus* other groups.

(Table 2). Body composition after 34 weeks on the four dietary regimens showed no difference in percentage of body weight that represented protein; however, the HF AL animals showed more body fat than the LF AL animals (49.8 ± 12% *versus* 42.4 ± 2%), $P < 0.05$. The CR rats also showed less fat than either HF or LF AL animals (Table 2).

Tumor Data. The CR rats at 34 weeks showed decreased tumor incidence and multiplicity, significant ($P < 0.05$), in the HF group (Table 2) (15). No gross tumors were found in the colon at the time of autopsy 21 weeks after administration of AOM in any animal group.

Epithelial Cell Proliferation. Caloric restriction reduced epithelial cell proliferation as expressed by the number of labeled cells per crypt column and the LI in the HF and LF groups of rats at all time points (10, 21, 34 weeks). Generally, this was seen in both treated and control rats (Table 3; Figs. 1 and 2). Crypt height was not consistently altered by caloric restriction.

The HF diet was associated with decreased proliferation (labeled cell number and LI) in the distal colon and increased proliferation (labeled cell number) and hyperplasia in the proximal colon as compared to the LF diet at 34 weeks (Table 3). At the earlier time points distal colonic proliferation measurements were similar in the LF and HF diets. The HF diet widened the crypt proliferative zone in the proximal colon but not in the distal colon at 34 weeks (Fig. 2). In the proximal colon the majority of labeled cells were found in the mid-crypt (compartment 3) in the LF group but the proliferative zone shifted towards the second and first compartments in the HF group.

AOM-treated animals had more labeled cells per crypt column and a higher LI than control rats at 21 but not at 10 weeks (Table 3). In rats bearing a colonic carcinoma or adenoma, no differences were found in proliferative indices compared to similar non-tumor-bearing rats.

Caloric restriction significantly reduced proliferative indices in biopsy specimens incubated with [^{3}H]dThd *in vitro*, as well as in autopsy specimens after [^{3}H]dThd *in vivo*. Less variation between individual rats was found in the autopsy specimens, probably because histological definition was superior. In biopsies taken at 20 weeks, specimens from only 3–4 rats/group were processed, confirming changes seen with *in vivo* labeling, but statistical significance was not reached in all groups because of small sample numbers.

[4] We thank Olympus Corp. of Woodbury, NY, for the gift of a pediatric bronchoscope to perform this work.

Table 3 *Effect of caloric restriction, fat intake, and carcinogen treatment upon colonic crypt cells number, labeling index, and number of labeled cells per crypt column, from biopsies at 10 weeks and autopsy at 21 and 34 weeks*

	LF AL	LF CR	*P*	HF AL	HF CR	*P*
			34-wk autopsy			
AOM-treated distal colon						
LI (%)	9.1 ± 0.9[a]	3.9 ± 0.2	<0.001	6.0 ± 0.1[b]	3.0 ± 0.2[b]	<0.005
Cells (no.)	37.4 ± 1.2	33.4 ± 0.7	<0.05	36.2 ± 1.0	35.9 ± 0.6[b]	0.24
Labeled cells (no.)	3.4 ± 0.4	1.3 ± 0.1	<0.001	2.1 ± 0.2[b]	1.1 ± 0.1	<0.001
AOM-treated proximal colon						
LI (%)	6.0 ± 0.6	4.0 ± 0.6	<0.05	6.9 ± 0.4	4.0 ± 0.5	<0.05
Cells (no.)	23.4 ± 0.59	22.5 ± 0.8	0.85	29.4 ± 2.4[b]	22.6 ± 0.9	<0.05
Labeled cells (no.)	1.4 ± 0.12	0.9 ± 0.2	<0.05	2.1 ± 0.2[b]	0.9 ± 0.1	<0.05
			21-wk autopsy			
Saline-treated						
LI (%)	3.3 ± 0.4	1.6 ± 0.4	<0.05	3.0 ± 0.6	1.8 ± 0.2	0.14
Cells (no.)	32.2 ± 0.4	33.8 ± 0.4	0.05	33.1 ± 1.4	34.6 ± 0.3	0.37
Labeled cells (no.)	1.1 ± 0.1	0.5 ± 0.1	0.05	1.0 ± 0.2	0.6 ± 0.1	0.14
AOM-treated						
LI (%)	7.5 ± 0.9[c]	3.2 ± 0.2[c]	<0.05	7.9 ± 1.0[c]	3.4 ± .3[c]	<0.06
Cells (no.)	32.2 ± 0.9	37.4 ± 2.0	<0.05	32.9 ± 1.4	36.0 ± 0.4[c]	0.11
Labeled cells (no.)	2.4 ± 0.2[c]	1.2 ± 0.0[b]	<0.05	2.6 ± 0.3[c]	1.2 ± 0.1[c]	<0.05
			10-wk biopsies			
Saline-treated						
LI (%)	7.1 ± 0.2	5.4 ± 0.9	0.08	7.9 ± 0.7	4.8 ± 0.4	<0.05
Cells (no.)	30.7 ± 0.7	30.4 ± 1.2	0.77	29.9 ± 1.2	29.5 ± 1.0	0.80
Labeled cells (no.)	2.2 ± 0.1	1.7 ± 0.3	0.12	2.4 ± 0.3	1.4 ± 0.1	<0.05
AOM-treated						
LI (%)	8.3 ± 0.7	5.5 ± 0.8	<0.05	8.9 ± 0.7	5.5 ± 0.6	<0.05
Cells (no.)	33.8 ± 1.1[c]	32.3 ± 1.6	0.45	30.4 ± 0.1[b]	30.0 ± 1.0	0.77
Labeled cells (no.)	2.8 ± 0.2[c]	1.2 ± 0.3	<0.05	2.7 ± 0.3	1.65 ± 0.2	<0.05

[a] Mean ± SE.
[b] Significantly different from its respective LF group, $P < 0.05$.
[c] Significantly different from its respective saline control group, $P < 0.05$.

DISCUSSION

Caloric restriction repeatedly has been shown to inhibit tumor formation in experimental animal models. In the present study, caloric restriction to 70 and 80% of AL intake resulted in significant reduction in indices of colonic epithelial cell proliferation as measured by [^{3}H]-dThd incorporation into DNA in normal-appearing mucosa. The effect was seen as early as 10 and 20 weeks of caloric restriction and persisted at 34 weeks. The LI was lowered by caloric restriction both in the carcinogen-treated and in the control rats. Tumor incidence measured at 34 weeks was also reduced (15). Thus, caloric restriction

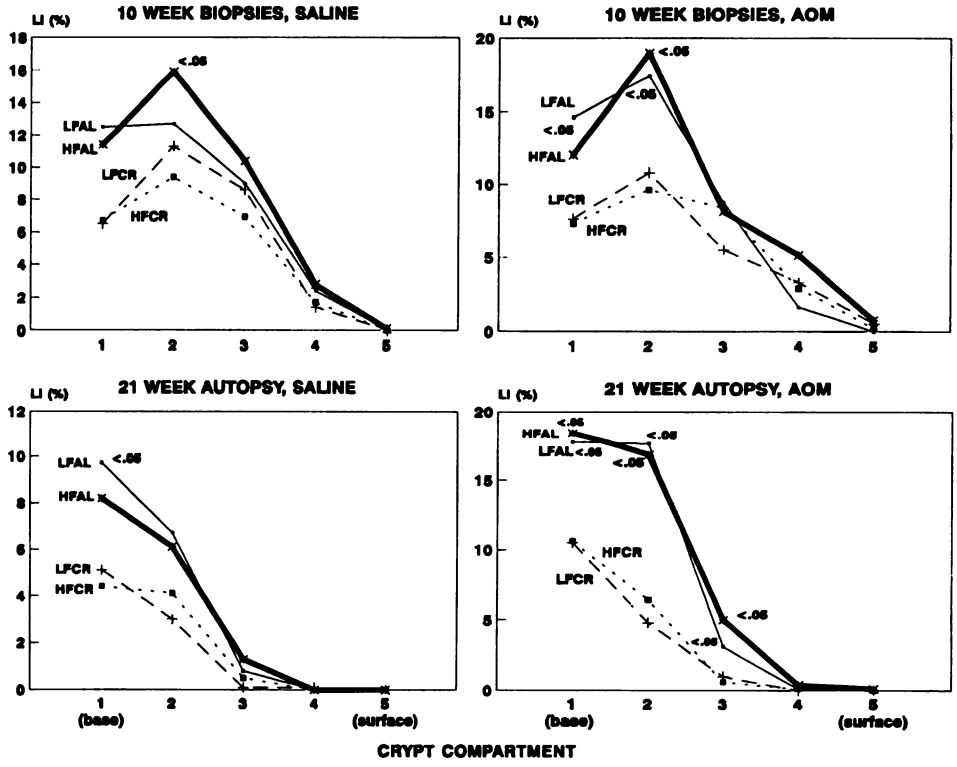

Fig. 1. Pattern of [^{3}H]dThd labeling in colonic crypt compartments following 10 and 21 weeks of CR or AL feeding in LF- or HF-fed rats, and AOM or saline-treated animals. Abscissae show colonic crypt compartments 1 (base of crypt) to 5 (top of colonic crypt). Significant differences between CR and AL-fed rats shown by *P* values.

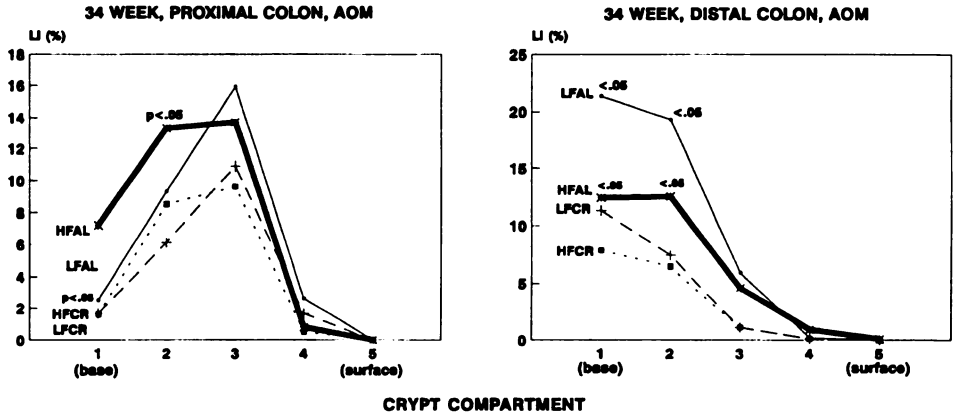

Fig. 2. Pattern of [^{3}H]dThd in colonic crypt compartments following 34 weeks of CR or AL feeding in distal and proximal colon of LF- or HF-fed AOM-treated rats. Significant differences between CR and AL-fed rats shown by *P* values.

in rats is effective in favorably modulating an intermediate biomarker of colon cancer risk in a direction previously associated with lower risk of neoplasia (18).

It is generally held that excessive proliferation is positively associated with tumor risk and that reduction in cell proliferation favorably reduces this risk. The present study supports this theory, since at 34 weeks after carcinogen administration, colonic cell proliferation indices were reduced in parallel with lower tumor incidence. Furthermore, the effect of caloric restriction on proliferation occurred independently of carcinogen treatment and of the presence of tumors, and the effect predated the appearance of tumors in carcinogen-treated rats. These data imply that reduction in cell proliferation by caloric restriction independently and favorably alters tumor risk in this experimental model.

In contrast, the effect of fat on tumor yield was dissociated from effects on cell kinetics in the distal colon. At 34 weeks tumor incidence was greater in HF AL-fed animals as compared to LF AL-fed animals, but colonic crypt LI and labeled cell number were lower in HF AL animals. At the earlier time points of 10 and 21 weeks, however, the HF diet had no effect on indices of colonic cell proliferation in the distal colon. Thus, distal colonic proliferation did not appear to be a reliable indicator of tumor risk induced by a HF diet. This is in contrast to findings in the proximal colon where some hyperplasia and an increase in labeled cell number in response to HF was noted at 34 weeks. There have been conflicting previous findings regarding the effect of HF diet on colonic cell proliferation (19, 20) in rodents, and this needs to be further investigated. Similarly, the chronic effects of high and LF diets in humans, and the possible different effects of such experimental interventions on the proximal and distal colon also remain to be studied.

The relative contributions of fat and of caloric intake to colon cancer risk is an important and unresolved health issue. In this model of colonic carcinogenesis in the rat, fat and caloric intake appear to act independently of each other. The role of fat in tumor promotion independent of its caloric load is supported by observations showing that rats fed a HF diet develop more tumors than rats fed an isocaloric LF diet (15, 26). Caloric intake also appears to be an independent positive modulator of tumor incidence and proliferation, since when fat intake is low and is not a promoting factor, reduction in caloric intake still reduces tumor yield and cell proliferation. The present study demonstrates that caloric and fat intake have differing effects upon colonic proliferation, suggesting that they affect colon carcinogenesis by different mechanisms.

Several epidemiological studies have suggested a positive correlation of colon cancer risk with body mass index or body weight (5, 27, 28). This correlation is difficult to analyze in caloric restriction experiments since caloric restriction decreases tumor yield and body weight simultaneously. In the present study, both dietary fat and total caloric intake influenced tumor incidence and/or cell proliferation indices but body weight and composition, the end points of metabolic balance, did not consistently correlate with tumor yield or proliferation measurements. It remains unclear which aspects of energy balance, other than caloric intake, influence carcinogenesis. Studies which focus on measurements of energy expenditure and storage are needed.

The divergent effects of calories and fat on colonic cell proliferation point out that when colonic cell kinetics are measured these must be interpreted as part of a complex of interrelated factors affecting carcinogenesis. Not only the proliferation rate or LI but also the distribution of label and the location in the colon where changes take place need to be considered. In addition, it is important to study the proliferative response to particular interventions in animal models in relation to other end points in carcinogenesis before interpreting corresponding human data. Based on the present study, proliferation data appear to be valuable end points in human studies on the effect of caloric balance. However, the separate effects of fat and other dietary determinants could confound the results and need to be considered.

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