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RESEARCH

Diet Induced Obesity Increases the Risk of Colonic Tumorigenesis in Mice

Angelos K. Sikalidis · Mark D. Fitch · Sharon E. Fleming

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Abstract A large body of epidemiological data indicates that obesity increases the risk of colon cancer in humans. There are limited studies using rodent models where the relationship between obesity and colon cancer has been studied. In this study, wild-type diet-induced obese (DIO) mice and lean wild-type controls were used to investigate the influence of obesity on the risk of colon cancer. We hypothesized that the obese phenotype would exhibit increased colonic tumorigenesis. Colon cancer was chemically induced by injecting the mice with azoxymethane (AOM) at levels that we experimentally determined to result in equivalent AOM concentrations in circulating blood. Risk of colon cancer was assessed via microscopic examination of entire colons for aberrant crypts, aberrant crypt foci and proliferation levels. The DIO mice were found to have significantly more aberrant crypts and aberrant crypt foci as well as increased proliferation of colonocytes per mouse compared to wild-type control mice, supporting the epidemiological data that obesity increases the risk of colonic tumorigenesis.

Keywords Obesity · Colon cancer · C57BL/6J · Diet induced obesity · AOM

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A. K. Sikalidis · M. D. Fitch · S. E. Fleming
Department of Nutritional Sciences and Toxicology, University of California, Berkeley, CA 94720, USA

A. K. Sikalidis (✉)
Harvard Institutes of Medicine, Dana-Farber Cancer Institute,
77 Avenue Louis Pasteur,
Boston, MA 02115, USA
e-mail: angelos_sikalidis@dfci.harvard.edu

Introduction

A plethora of epidemiological observations indicates that obesity increases the risk of colon cancer in humans [1–15]. There are limited studies using rodent models where the relationship between obesity and colon cancer has been studied. An association, however, between obesity and increased colon tumorigenesis has been found in rat models [16–18]. Studies investigating the relationship between colon cancer and obesity in mice are limited and, although obesity-related conditions have been studied in mice [19], the experimental design involves mice with a genetic predisposition for gaining weight due to genetic manipulation. This practice distances these studies from the human condition in which obesity is primarily due to excessive caloric intake compared to the energy expenditure. Consequently, the extrapolation of conclusions to humans is not optimal. In animal studies, one possibility is the use of the leptin deficient mouse, a strain derived by a mutation of the gene which controls the production of leptin, a hormone which acts as a satiety regulator. Disruption of the leptin gene leads to satiety deregulation resulting in the obese phenotype. More specifically, in the absence of leptin, mice constantly are hungry, thus develop hyperphagia as a consequence of which, they become obese. This model allows deriving conclusions in an excessively high obesity regime but the obesity condition is partially relevant to the respective obese human condition since the lack of leptin is not typically the cause for human obesity. With limited studies available regarding diet-induced obesity [20–25] and a lack of data regarding the relation between colon cancer and diet-induced obesity, the investigation of the relationship between diet-induced obesity and the risk of colonic cancer seems rather interesting as the diet-induced model of obesity mimics much more realistically the development of obesity in humans, both in terms of rate and order of magnitude.

Obtaining more information regarding the relationship between diet-induced obesity and the risk of colon cancer could improve the understanding of the underlying mechanism pertaining to colon cancer inducement while ideas regarding beneficial interventions for reducing the risk of colonic cancer could also arise. The advantage of a diet-induced model as opposed to a leptin deficient one is that the former allows for the exploration of the potential involvement of leptin in the relationship between obesity and colon cancer.

In the case of diet-induced obesity, wild type animals (no genetic manipulation or abnormality of any kind) are fed a high-fat diet *ad libitum* and they become obese due to their high caloric intake of calorie-dense feed. Although not all strains develop diet-induced obesity equally efficiently as some exhibit low responsiveness to the treatment, there are strains of mice which are good responders to diet-induced obesity, thus providing a good model for studying in vivo the effect of diet induced obesity on health in general. Such an obesity animal model, seems to be closer to describing the real conditions in terms of human obesity development, compared to the ones which involve genetic manipulation or exploitation of an inherent genetic anomaly.

The principal hypothesis was that obese animals would exhibit a higher risk of colon cancer compared to their lean (control) counterparts. The risk was assessed via measurement of numbers of aberrant crypts, aberrant crypt foci and tumors as well as levels of proliferation of colonocytes. The initial hypothesis was that obese mice would exhibit significantly higher risk of colon cancer compared to the lean group. This set of experiments was able to contribute further in vivo evidence for the effect of obesity on the risk of colon cancer (corroborating epidemiological data [1–15]).

Materials and Methods

Animals and Diets

All mice used were male, purchased at 4 weeks of age from Jackson Lab (Bar Harbor, ME) and were housed five per cage at the Northwest Animal Facility at U.C. Berkeley, under controlled conditions of a 12 h light/dark cycle, 22 °C room temperature. All mice were immediately put on their corresponding experimental diets upon arrival. The animal use protocols were approved by the U.C. Berkeley Animal Care and Use Committee prior to commencing experiments. For the diet-induced obesity study, hereafter referred to as DIO, wild type male C57BL/6J mice were randomly assigned into two groups upon arrival. The first group, comprised of 80 mice, served as the control group, and was fed a low-fat diet (LFD), #D12450B. The second group, comprised of 92 mice, served as the treatment group, and was fed a high-fat diet (HFD), #D12492. Sample size was determined by power calculations. Corresponding diets and water were provided to all animals *ad libitum*. All diets were prepared by Research Diets Inc. (New Brunswick, NJ). A layout of the experimental design is presented in the supplement, for readers' convenience (Table S1).

Carcinogen Administration

For the study using DIO mice, azoxymethane (AOM) a specific chemical inducer for colon cancer was injected into lean and obese mice at 15 mg/Kg body weight and 13.5 mg/Kg body weight, respectively. More specifically, pilot pre-studies performed during the DIO study showed that equal pre-carcinogen exposure of tissues of lean and obese DIO mice was achieved by injecting lean mice subcutaneously (sc) with AOM at 15 mg/Kg body weight and obese DIO mice with 13.5 mg/Kg body weight. After measuring plasma AOM concentrations in mice from both groups at regular intervals up to 60 min following AOM injections, the average area under the curve (AUC) of AOM concentration versus time differed for the lean and obese animals by only 1 %. Thus, with this differential dosing, tissues of the lean and obese animals were equally exposed to AOM.

Feed Intake Assessment

Feed intake was assessed under both AOM and saline administration regimes for both LFD and HFD mice. The aim was to assess feed intake and calculate energy intake in order to justify the different rate of weight gain between the LFD and HFD fed mice. Another aim was to see for a potential effect of AOM on feed intake and consequently energy intake. Feed intake was measured every 3 days for 5 consecutive times (a total period of 15 days), on a per cage basis (number of mice per cage used to calculate feed intake/mouse) and was measured via diet disappearance. A pre-weighed amount of diet was provided to the animals every 3 days and the remainder was weighed at the end of the three-day period. Feed spillage was also accounted for in the calculations. The feed intake data were used to calculate the energy intake of the animals by multiplying the amount of diet consumed by a mouse, by the caloric content of each diet (LFD=3.8 Kcal/g, HFD=5.2 Kcal/g).

Risk of Colon Cancer Assessment via Aberrant Crypts and Aberrant Crypt Foci Number

Aberrant crypt foci and aberrant crypts (ACF—AC), reliable intermediate markers of colonic tumorigenesis [26–28], are thought to be the earliest identifiable pre-neoplastic lesions in the colon carcinogenic model [28–30]. The difference between the two types of lesions is the multiplicity of ACF compared to AC. As ACF are composed of several

AC the former are thought to be more associated with the risk of colon cancer, even though in colon cancer risk assessment studies both indicators are used [26–30]. To assess risk of colon cancer via ACF and AC, the entire colon was opened longitudinally and pathology examined for blood and gross lesions, fixed in Carnoy's fixative (3 parts 100 % ethanol : 1 part glacial acetic acid) overnight and then in 100 % ethanol. The colon was then stained with methylene blue. Gross pathology was observed using a dissecting microscope, and the entire mucosal surface of the unsectioned colon was viewed at 40–60× power to identify and quantify AC and ACF per colon [26].

Assessment of Colonic Epithelial Cell Proliferation

Measurement of cell proliferation, a good indicator for assessing risk of cancer, was assessed via the Bromodeoxyuridine (BrdU) method. BrdU an analogue of thymidine that is incorporated into newly synthesized DNA during the S phase of the cell cycle, is used to label proliferating cells. BrdU was dispersed in sterile water and injected at 10 μL/g of body weight [31] 1 or 2 h prior to animal sacrifice. Colonic segments were prepared using the "swiss roll" method [32] to improve alignment of crypts in the fixed sections and provide a complete profile of crypts of the entire length of colon, embedded in paraffin, cut into histological sections, and processed for BrdU immunohistochemistry [31] using a commercial stain system kit (Zymed, South San Francisco). Anti-BrdU antibody was applied on colon sections according to manufacturer's protocol. Using a light microscope, the right sides of at least 20 crypts per colon were inspected to determine the crypt height (in number of cells), number of labeled cells per crypt column, and positions of highest and lowest labeled cells. These data were used to calculate labeling index; [number of labeled cells / number of cells per crypt column × 100] and proliferation zone; [position of highest labeled cell-position of lowest labeled cell+1], proliferation zone as percentage of crypt height [(position of highest labeled cell-position of lowest labeled cell+1) / number of cells per crypt column × 100], percent highest labeled cell; [(position of highest labeled cell × 100) / crypt height] and percent lowest labeled cell; [(position of lowest labeled cell × 100) / crypt height].

Statistical Analysis

All data were statistically analyzed by ANOVA either with or without repeated measures depending on the parameter tested at each test, using the SPSS data analysis program. Tukey's follow-up tests were performed when making comparisons among 3 or more groups. A probability of type-2 error of 5 % was used to indicate statistical significance (i.e. statistical significance was set at $p<0.05$).

Results

Relative Risk of Colon Cancer for Lean Versus Obese DIO Mice

Feed and Energy Intake

A multiple ANOVA statistical analysis with interactions and repeated measures was conducted for both the feed intake and the energy intake parameters, considering the factors "Diet", "Time", "AOM status" and the combinations of those 3 factors. Regardless of "Time" and "AOM status", the LFD animals consumed significantly more feed (g/mouse/day) than the HFD animals, but not significantly more energy (supplement Table S2). A significant "Time × AOM" interaction was found for both feed intake and energy intake parameters (supplement Table S3). The feed intake and energy intake data were not parallel. That was due to different relative intakes of the two diet groups. While the LFD group consumed significantly larger amounts of diet compared to the HFD group, the HFD group was found to have consumed significantly more calories than the LFD group. These patterns were independent of "AOM status" and/or "Time".

Comparing within the same time-point, neither the feed intake nor the caloric intake of the saline treated mice was significantly different from the AOM treated mice for all time-points (supplement Table S4).

Body Weights and Weight Gain

In the DIO study, all animals were individually weighed on a regular basis. The HFD mice gained weight faster than their LFD counterparts (Fig. 1) which is shown by the progressive increase in the ratio of body weights for the obese versus lean animals (Fig. 2).

The weights of mice at termination were compared using a two-way ANOVA with interactions without repeated measures (as in every time-point there were different mice weighed) statistical analysis considering the factors "Diet", "Latency" and "Diet × Latency" interaction. The term "latency" is the time following AOM/carcinogen exposure, with 0 latency referring to the control prior to AOM exposure. Follow-up of the observed significant Diet × Latency interaction indicated that the HFD mice gained proportionally more weight than the LFD mice between time-points 1 & 2 (Table 1).

The weights of mice (for each of the 3 time-points) were also measured. The times at which weights were measured for time-point 1 and 2 were: 1st AOM injection and 6 weeks post AOM (which is also the termination time for mice at time-points 1 & 2). For time-point 1 there was a significant Diet × Latency interaction. Weights of HFD mice were significantly higher than LFD mice at both times (1st AOM and

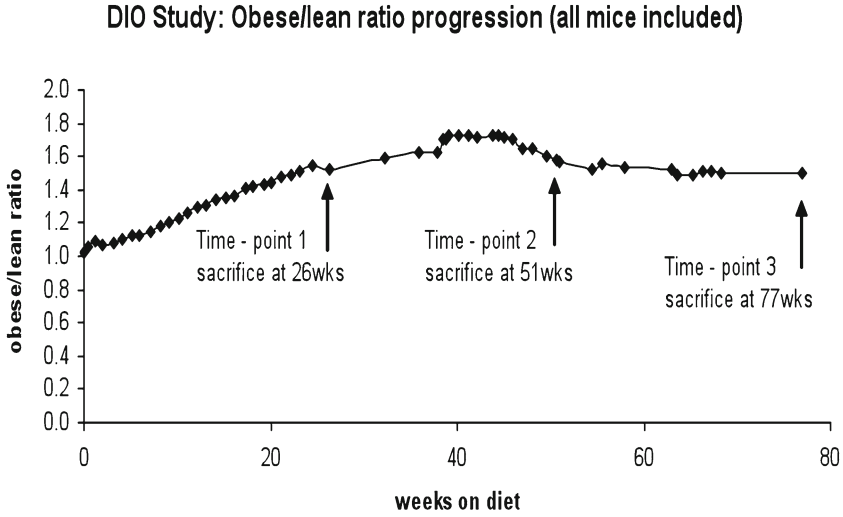

Fig. 1 Obese/lean ratio progression with time. Body weight of mice begins to plateau at approximately 40 weeks on diet

termination), and animals from both groups lost weight following AOM injection, with the 3 g loss constituting a higher proportion of body weight for the LFD group than for the HFD group. The Diet × Latency interaction was not statistically significant for the time-point 2 data, and mice in the HFD group were significantly heavier than mice in the LFD group.

ACF—AC

The risk of colon cancer in mice was assessed via the measurement of the numbers of aberrant crypts (AC) and aberrant crypt foci (ACF). The number of AC and ACF was microscopically assessed in the entire colons of mice. A microscopic examination for tumors in every colon also took place. A two-way ANOVA analysis (with "Diet" and "Time" as main factors) with interactions and without repeated measures was conducted for both AC and ACF.

In the DIO study the statistical test was conducted for time-points 2 and 3. In terms of AC and ACF assessment, time-point 1 was handled separately compared to time-points 2 and 3. This difference was because at time-point 1 the levels of body hydration among the mice were not normalized since the saline intraperitoneal injection for normalization of hydration levels of the mice was introduced at time-point 2. The non normalized body water levels of mice at time-point 1, is considered the reason of the relatively high variability of AC and ACF observed at this time-point. Therefore, the data-points from time-point 1 were not included in the statistical analysis, which included the data from time-points 2 and 3. Instead, time-points 2 and 3 were statistically analyzed together (see below), while time-point 1 was analyzed separately. The statistical analysis for time-points 2 and 3 showed a "Diet × Time" interaction. At both time-points 2 & 3 the HFD mice exhibited significantly higher AC and ACF compared to the LFD mice (Table 2). The interaction effect was due to a general lower degree of difference between the two diets for time-point 3 than for time-point 2. For the LFD group the number of AC was not significantly different comparing time-points 2 & 3. For the HFD group however the number of AC was significantly lower at time-point 3 compared to time-point 2. For both the LFD and HFD groups the numbers of ACF were significantly lower at time-point 3 compared to time-point 2 (Table 2). For time-point 1, even though the variability was relatively high, the trend illustrated by the data was pretty similar to the one seen at time-points 2 & 3 (Tables 2 and 3).

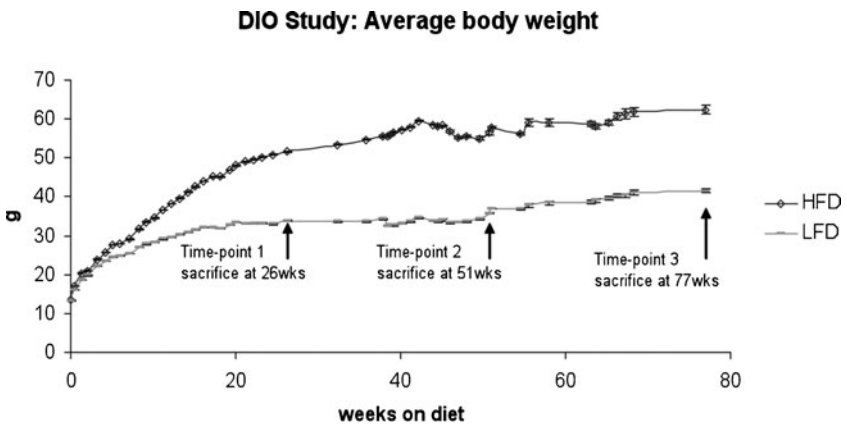

Fig. 2 Body weight progression with time for lean and obese mice. Mean values are graphed ± SEM. A clear divergence of body weights from early on (before the first 10 weeks on diet) is observed

Table 1 DIO study: influence of diet and latency on body weight of mice at termination

	Time-point		
	1	2	3
LFD	29.4^{a}±0.5 (n=20)	35.7^{b}±0.9 (n=10)	41.2^{c}±1.2 (n=14)
HFD	43.0^{c}±0.9 (n=21)	55.6^{d}±1.5 (n=10)	64.6^{c}±3.8 (n=6)

There was a Diet × Latency interaction (p<0.05), values are means ± SEM (p<0.05)

Weights reported in (g)

Colonic Tumors

At time-points 1 & 2, no tumors were identified in any of the animals at termination. At time-point 3, however, a total of three tumors were identified in the 20 surviving mice, one tumor in the HFD group and two tumors in one mouse of the LFD group. The number of tumors divided by number of mice in group as a % was for the LFD group 14 % (2/14) whereas the value for the HFD group was 17 % (1/6). The percentage of mice with tumors was 7 % (1/14) for the LFD and 17 % (1/6) for the HFD group. The small number of tumors did not allow for statistical analysis, although higher risk for colon cancer was implied for the HFD group, which exhibited higher obesity levels.

Proliferation of Colonic Epithelial Cells

Proliferation of colonic epithelial cells was assessed as one indicator of colon cancer risk. Since differences between diet groups were assessed at three time-points, data for all proliferation parameters were statistically compared using a two-way ANOVA of the main factors (diet and time) and their interaction (Diet × Time). As different animals were used to acquire data for each time/diet treatment, data were independent therefore repeated measures was not appropriate. The significance of differences as output following statistical analyses is shown in Table 4. Because there were significant differences due to both time and diet, and since the Diet × Time interactions were not statistically significant for any proliferation parameter, the effects of diet and time were assessed using cumulative means.

The differences between the HFD and LFD groups were statistically significant for most, but not all, proliferation parameters assessed (Table 4). Although colonic crypt heights for the HFD and LFD groups were not significantly different, the HFD group had significantly more labeled cells/crypt and a significantly higher labeling index than the LFD group. Also, the position of the highest labeled cell was significantly higher up the crypt column for the HFD group than the LFD group, and this difference remained when calculated as relative position along the length of the crypt column (i.e. percentage of crypt height). Although differences by diet were not statistically significant for position of the lowest labeled cell, the zone of proliferation was significantly larger for the HFD group than the LFD group (Table 5).

Treatment time had a significant effect on most measures of cellular proliferation (Table 6). Specifically, values for time-point 3 were significantly lower than for time-points 1 & 2 for proliferation parameters including number of labeled cells/crypt, labeling index, positions along the crypt column of both the lowest and highest labeled cells, and relative position (i.e. % of crypt height) of the highest labeled cell. The size of the proliferation zone (proportion of the crypt column that contained proliferating cells) also was significantly lower for time-point 3 than for time-points 1 & 2.

Table 2 DIO study: influence of diet × time on AC and ACF for time-points 2 & 3

	Time-point 2	Time-point 3
Condition	45 weeks on diet at 1st AOM	45 weeks on diet at 1st AOM
	51 weeks on diet at sacrifice	77 weeks on diet at sacrifice
	6 weeks post AOM	36 weeks post AOM
Aberrant Crypts		
LFD	25.9^{a}±1.4 (n=10)	20.1^{a}±2.0 (n=14)
HFD	53.8^{c}±2.3 (n=10)	35.5^{b}±2.2 (n=6)
Aberrant Crypt Foci		
LFD	20.2^{b}±1.0 (n=10)	12.4^{a}±0.8 (n=14)
HFD	44.0^{c}±1.5 (n=10)	23.2^{b}±1.4 (n=6)

Statistics apply per on a row—parameter basis. Values are means ± SEM

Superscript letters indicate a significant Diet × Time effect for the parameter indicated (p<0.05)

Time-point 1 was excluded because of not normalized hydration levels of the mice, which affects the AOM concentration (note that plasma AOM concentrations were assessed following this pretreatment hydration protocol to ensure equivalent delivery of carcinogen to mice in the two diet groups)

Table 3 DIO study: influence of diet on AC and ACF for time-point 1

Condition	20 weeks on diet at 1st AOM 26 weeks on diet at sacrifice 6 weeks post AOM	
	Aberrant Crypts	Aberrant Crypt Foci
LFD (n=20)	40.1±3.4	29.0±2.4
HFD (n=21)	49.1±3.7	31.2±2.6
	p-value=0.120	p-value=0.543

Values are means ± SEM. Not statistically significant differences were attributed to high variability within group. Serendipitous observations suggest this may be due to variability in the hydration level of the mice. Introducing a pretreatment with saline 2 h prior to AOM injections was found to reduce variability within group. See Table 2

Discussion

Based on epidemiological evidence [1–15] and some in vivo obtained data [16, 26] we hypothesized that obese mice would exhibit higher risk of colon cancer compared to lean counterparts. We tested our hypothesis by putting wild type male C57BL/6J mice on either a Low Fat Diet (LFD) or a High Fat Diet (HFD) in order to produce a lean and an obese physiologically meaningful/relevant phenotype respectively, and measure colon cancer risk. The expectation was that the HFD group would exhibit significantly higher risk of colon cancer compared to the LFD one, because of higher body fatness and correspondingly higher levels of circulating leptin in the blood. The results obtained on the outcome variables tested verified our hypothesis.

Body Weights

Body weights of mice were monitored as an indirect indicator of levels of obesity. The obese/lean body weight ratio at the beginning of the DIO study was 1.02, which shows that the randomization of the mice into two groups was successful and the two groups started from the same point in terms of their weights (weight-matched). As the two groups of mice were put on different diets (HFD Vs. LFD) upon arrival, they immediately started consuming diets of different caloric density. Although body weights of mice on both diets increased over time, a gradual increase was observed in the obese/lean body weight ratio, indicative of the fact that the HFD group was gaining weight faster than the LFD group. Such an effect, has been reported by others [33]. Initially, the increase of body weight, regardless of the group, was more rapid as the mice were in their rapid growth phase. The body weight for the HFD group increased at a higher rate compared to the LFD group due to the higher caloric intake of the former, which resulted in higher fat deposition (see supplement; Table S5). These findings are consistent with the findings of others [16–25].

DIO Mice Obese Mice Exhibited Higher Risk of Colonic Cancer as Assessed by Number of Aberrant Crypts and Aberrant Crypt Foci Compared to Their Lean Counterparts

Colonic aberrant crypt foci (ACF) [16, 26–29] are regarded as premalignant lesions [34]. The principal hypothesis was that the higher obesity status group (obese HFD group) would exhibit a higher risk of colon cancer because of increased body fatness by exhibiting higher numbers of AC & ACF compared to the lean wild type / LFD group.

The colon cancer risk in the DIO study, as assessed via AC and ACF measurements, was significantly higher for the HFD mice than for the LFD mice for time-points 2 and 3. Although the same trend was clearly observed for time-point 1 data as well, high variability was observed within diet groups for the first time-point. Because of a serendipitous observation, we hypothesized that this variability may be

Table 4 DIO study: proliferation of colonic epithelial cells: significance of differences (p-values) for the main variables, "Diet" and "Time", and for their interaction (Diet × Time)

	p-values for main variables		
	Diet	Time	Diet × Time
Crypt height (cells/crypt side)	0.600	0.000	0.556
Total number of labeled cells (cells/crypt side)	0.000	0.000	0.303
Labeling Index (%)	0.000	0.000	0.452
Position of Highest labeled cell (cell position)	0.000	0.000	0.839
Position of Lowest labeled cell (cell position)	0.547	0.390	0.959
Relative position of Highest labeled cell (% of crypt below highest labeled cell)	0.000	0.001	0.904
Relative position of Lowest labeled cell (% of crypt below lowest labeled cell)	0.789	0.087	0.808
Proliferation zone (% of crypt column with labeled cells)	0.000	0.000	0.770

Table 5 DIO study: influence of "Diet" on proliferation of colonic epithelial cells

	Diet*	
	LFD (n=44)	HFD (n=37)
Crypt height (cells/crypt side)	21.67[a]±0.09	21.74[a]±0.05
Total number of labeled cells (cells/crypt side)	1.47[a]±0.01	2.02[b]±0.02
Labeling Index (%)	6.80[a]±0.07	9.25[b]±0.10
Position of Highest labeled cell (cell position)	5.07[a]±0.05	6.18[b]±0.07
Position of Lowest labeled cell (cell position)	2.91[a]±0.03	3.02[a]±0.04
Relative position of Highest labeled cell (% of crypt below highest labeled cell)	23.45[a]±0.21	28.22[b]±0.31
Relative position of Lowest labeled cell (% of crypt below lowest labeled cell)	13.50[a]±0.12	13.89[a]±0.17
Proliferation zone (% of crypt column with labeled cells)	17.23[a]±0.17	21.23[b]±0.21

*Since the "Time × Diet" interaction was not statistically significant for any of the proliferation parameters, differences were determined on pooled means for each diet. Each mean value represents the mean of values for the 3 time-points evaluated in this experiment. Values are means ± SEM

Superscript letters indicate a significant diet effect for the parameter indicated (p<0.05)

due to differences in hydration status of the animals. To offset this as a possible source of variability, mice used for time-points 2 and 3 data were comparably hydrated 2 h prior to AOM injection. Interestingly, consistent with this working hypothesis, variability within group was dramatically decreased in these subsequent experiments. As a result, differences between diet treatments were statistically significant for time-points 2 and 3 and, at both treatment times, the HFD mice had more AC and ACF than the LFD mice did, suggesting that the HFD group is at a higher risk for colon cancer compared to the LFD group. This observation is consistent with the literature [16, 26, 35–39].

Between time-points 2 and 3 the numbers of AC for the LFD were not significantly different whereas for the HFD group there was a significant decrease from time-point 2 to time-point 3. However in the case of ACF both HFD and LFD groups exhibited significantly fewer ACF at time-point 3 compared to time-point 2, an observation consistent with what other workers have reported [40]. A possible explanation for such a tendency for reduction of both AC and ACF could constitute the fact that the time interval between last AOM injection and termination was 2 weeks for time-point 2 as opposed to 28 weeks for time-point 3. Possibly, the long time interval between 1st AOM injection and termination time for time-point 3, allowed the ongoing healing process to reduce numbers of aberrant crypts as some of the abnormal cells which constitute the aberrant crypts might be sloughed off, due to epithelial cell turnover.

At time-point 3, colonic tumors were observed in both the HFD and LFD group. More specifically, two tumors were identified in one mouse of the LFD group and one tumor was identified in one mouse from the HFD group. Thus, the proportion of mice with tumors, was higher (although not statistically significantly due to the small n) in the HFD group (1/6) than in the LFD group (1/14).

The AC and ACF measurements showed that the HFD group was at a higher risk of colon cancer compared to the LFD one. This result is consistent with the epidemiology [1–15] but also with other animal studies [37–39]. The tumor results do not conflict with the AC-ACF results. It is concluded that the HFD mice, which exhibit a much higher obesity status compared to the LFD ones (HFD/LFD weight ratio at AOM administration at time-points 1 and 2 was 1.47 and 1.71 respectively), are at a significantly higher risk of colon cancer.

Proliferation Parameters

As cells undergo division, their DNA may well be exposed to mutagens and carcinogenic agents, increasing risk for colon cancer development. Thus, treatments that increase the number or proportion of cells undergoing cell division are considered to increase risk of colon cancer [41]. Also, an upward shift in the position of dividing cells is also considered to increase colon cancer risk [41]. Using these criteria, the HFD group was at higher risk of colon cancer than the LFD group since the former exhibited significantly higher numbers of labeled cells, proportionally more labeled cells (labeling index), a clear upward shift in the position of the highest labeled cell, and a much wider zone of proliferating cells. Conversely, the zone of differentiated cells was notably narrower in the HFD group than in the LFD group, reducing the "safety" zone for the HFD animals. Quite a few studies in rats report that when animals are fed a HFD they exhibit significantly higher numbers of AC and ACF [37–39] as well as higher levels of proliferation of colonocytes [34] compared to the animals fed a LFD. In terms of mere obesity, our results are in accordance with the literature which again supports that the obese animals show significantly higher levels of proliferation of colon epithelial cells

Table 6 DIO study: influence of "Time" on proliferation of colonic epithelial cells

	Time—point		
	1	2	3
Parameter	(n=41)	(n=20)	(n=20)
Crypt height (cells/crypt side)	21.20^{a}±0.02	22.30^{b}±0.16	22.18^{b}±0.24
Total number of labeled cells (cells/crypt side)	1.87^{b}±0.02	1.86^{b}±0.05	1.32^{a}±0.04
Labeling Index (%)	8.69^{b}±0.07	8.23^{b}±0.23	6.04^{a}±0.15
Position of Highest labeled cell (cell position)	5.75^{b}±0.05	6.17^{b}±0.13	4.67^{a}±0.10
Position of Lowest labeled cell (cell position)	3.06±0.03	2.96±0.05	2.81±0.07
Relative position of Highest labeled cell (% of crypt below highest labeled cell)	26.91^{b}±0.24	27.40^{b}±0.60	21.22^{a}±0.40
Relative position of Lowest labeled cell (% of crypt below lowest labeled cell)	14.37±0.15	13.24±0.24	12.71±0.25
Proliferation zone (% of crypt column with labeled cells)	19.40^{b}±0.14	21.79^{b}±0.53	15.63^{a}±0.33

Description of Time–points:

Age of mice at sacrifice: 1=30 weeks, 2=55 weeks, 3=81 weeks

Time between first AOM injection and termination: 1=6 weeks, 2=6 weeks, 3=32 weeks

Body weight ratio at time of first AOM injection (HFD/LFD): 1=1.47, 2=1.71, 3=1.71

Body weight ratio at termination (HFD/LFD): 1=1.46, 2=1.56, 3=1.57

Since the "Time × Diet" interaction was not statistically significant for any of the proliferation parameters, differences were determined on pooled means for each time point. Each time point mean value represents the mean of values for the two diets evaluated in this experiment

Values are means ± SEM

Superscript letters indicate a significant time effect for the parameter indicated (p<0.05)

[26] and numbers of ACF [26]. Additionally, in human experiments it has been reported that caloric restriction reduces levels of proliferation of colonocytes [42].

The crypt height was found to be significantly higher at time-points 2 and 3 compared to time-point 1, but means differed by only 1 cell. This small difference may be due to differences in animal age, since the mice were 30 weeks old at time-point 1 and 55 and 81 weeks old at time-points 2 and 3, respectively. Since crypt heights differed by 1 cell only (less than 5 % of the crypt height) this difference is unlike to be of physiological significance. Again the order of the value of crypt height is comparable to results from other studies 25±0.1 for lean wild type (C57BL/6J) mice (unpublished data).

There were significant differences by "time" in numbers and placements of labeled cells. As diet × time interactions were not statistically significant, differences by time did not alter the relative differences between diets. We expect the difference due to "time" is largely due to a methodological difference. Specifically, animals were terminated 2 h post BrdU administration at time-points 1 and 2 but, for time-point 3, termination occurred only 1 h post BrdU administration. This change in protocol was purposeful. There is debate among researchers as to what the ideal time between label administration (BrdU) and sacrifice should be (1 h Vs. 2 h incubation time) [31]. To offset experts' concerns that our results might be biased by choice of time interval, 2 h was selected for time-point 2, and 1 h was selected for time-point 3. Since animals in groups 2 and 3 were injected with AOM at the same time (i.e. when 49 weeks old and after 45 weeks on diet) the effect of diet on proliferation should be consistent at time-points 2 and 3. Thus, this allowed the dietary effect on proliferation to be assessed with two protocols in order to achieve a solid conclusion regarding the influence of diet. When animals were terminated 2 h post BrdU administration (time-point 2), the total number of labeled cells and the labeling index were both found to be significantly higher compared to when animals were sacrificed 1 h post BrdU administration (time-point 3). This is not surprising, since one would expect more cells to take-up label when exposed to the label for a longer period of time. It is important to note, however, that regardless of the BrdU incubation times, the HFD group always consistently exhibited a significantly higher number of total labeled cells and labeling index compared to the LFD group, which suggests a higher risk of colon cancer for the HFD group consistent with what other groups have reported [43]. The values for both labeled cells and labeling index in the DIO study were comparable to the ones observed in other studies where total labeled cells in lean animals were 1.6 and 2.0±0.1 for wild type animals (C57BL/6J) (unpublished data), while when the PCNA method for proliferation assessment was used (which tends to overestimate the proliferation levels) the value was at 5.0 labeled cells per crypt [43].

An outcome variable measured to assess risk of colon cancer was the level of cellular proliferation. The level of proliferation is used as a means to assess the risk of colon

cancer and is positively associated with HFD and obesity [34, 43]. The proliferation data from all time-points are consistent with the ACF and AC data in the sense that both proliferation and ACF-AC measurements data attribute higher risk of colon cancer to the HFD group compared to the LFD group. More specifically, for proliferation parameters, significantly higher values for the HFD group versus the LFD group were found for total labeled cells and labeling index (the main values used for proliferation levels assessment) as well as for highest labeled cell, highest labeled cell as a % of crypt height and proliferation zone as % of crypt height. Again it is crucial to point out that, regardless of the BrdU incubation times, the position of the highest labeled cell was closer to the lumen surface for the HFD group than for the LFD group, suggesting higher risk of colon cancer for the HFD group. Since there were no differences in the position of the lowest labeled cell, the proliferation zone for the HFD group was significantly higher than for the LFD group, supporting the conclusion that the risk of colon cancer was higher for the HFD group than for the LFD group.

Summarizing the proliferation data, risk of colon cancer as assessed via the proliferation parameters was higher for the HFD group compared to the LFD group. Since the animals consuming the HFD had more body fat than animals consuming the LFD, differences between these two groups may be due to differences in body fatness/obesity. Such a connection, has also been reported by other groups [34, 37, 38]. More specifically, the obese to lean ratio (average weight of mice on HFD diet over average weight of mice on LFD diet) changed from 1.47 for time-point 1 to 1.71 for time-points 2 and 3 (ratios at 1st AOM administration) an overall increase of the ratio by approximately 16. 3 %. Based on growth body weight, the HFD mice were 47 % and 71 % heavier than the LFD body weights measured at 1st AOM administration at time-points 1 and 2 respectively (time-points 2 and 3 are considered together since the 1st AOM administration was common time-wise for both treatments). In terms of body fatness as it was assessed via fad pad collection, the HFD group at time-points 1, 2 and 3 was 129 %, 65.4 % and 73.4 % significantly more fat than the LFD group respectively (at termination). These results suggest the HFD mice were at all times significantly more obese than the LFD mice. Thus, the higher colonic cell proliferation observed in the HFD mice may be due to greater obesity/adipocity in that group. These findings agree with the AC and ACF results of other animal studies [17, 26, 29] and corroborate both the epidemiological data [1–15] and animal data [17, 26, 29], all suggesting that obesity increases the risk of colon cancer.

Taken together, the data generated from this study support a conclusion that wild type diet induced obese mice are at increased risk of colonic tumorigenesis compared to their lean littermates. This conclusion is consistent with the results of several animal studies that used different models of obesity and numerous epidemiological studies that have reported obesity to increase risk of colonic cancer. Further experimental studies are needed to specifically and directly address and elucidate the role of leptin/insulin as a mediator of obesity's stimulatory effect on colon cancer and the precise mechanism of action.

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