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RESEARCH

Risk of Colonic Cancer is Not Higher in the Obese Lep^{ob} Mouse Model Compared to Lean Littermates

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Abstract Epidemiological data suggest that obesity increases the risk of colorectal cancer in humans. Given that diet-induced obesity mouse models verified the epidemiological data, the present study aimed to determine whether obese C57BL/6J-Lep^{ob} male mice (a different obesity in vivo model) were at greater risk of colonic cancer than their lean male littermates. Risk of colonic tumorigenesis was assessed by numbers of aberrant crypts, aberrant crypt foci and colonic tumors. Proliferation of the colonic epithelia was assessed histochemically following administration of BrdU. Availability of the procarcinogen, azoxymethane (AOM) to target tissues was assessed by quantifying via HPLC plasma AOM concentrations during the 60 min period following AOM injection. When obese and lean mice were injected with azoxymethane (AOM) at doses calculated to provide equivalent AOM levels per kg lean body mass, obese animals had significantly fewer aberrant crypts/colon and fewer aberrant crypt foci/colon than the lean animals. Tumors were identified in the colonic mucosa of lean (4 tumors in 14 mice) but not obese (0 tumors in 15 mice) mice. Colonic cell proliferation was not significantly different for obese and lean mice. Because these results were unexpected, plasma AOM concentrations were measured and were found to be lower in the obese than lean mice. When plasma AOM levels were comparable for the lean and obese mice, the Lep^{ob} mice continued to have significantly fewer aberrant crypt foci/colon than the lean mice, but differences were not statistically different for aberrant crypts/colon. Interestingly, obese Lep^{ob} mice did not exhibit increased risk of colonic cancer as expected. Instead, Lep^{ob} mice exhibited equivalent or lower risk of colon cancer when compared to the lean group. These results taken together with in vivo results from diet-induced obesity studies, imply that leptin may be responsible for the increased risk of colon cancer associated with obesity.

Keywords Leptin · Aberrant crypts · Tumors · Proliferation · Azoxymethane

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Introduction

Colorectal cancer is the second leading cause of cancer-related deaths in the United States for both men and women combined, surpassing both breast and prostate cancer in mortality and second only to lung cancer in the numbers of cancer deaths. Early detection reduces deaths due to this disease and, as the colon is more accessible than many organs to observation and biopsy, it is reasonable to expect further gains in treating and preventing this form of cancer.

There is compelling epidemiological data indicating that risk of colonic cancer is higher in people who are overweight or obese than in people with normal weight for height. In case control studies, increased BMI has been associated with increased colon cancer risk in men [1–5]. In women, the relative risk due to high BMI is often shown to be lower than in men, although increased BMI in women has been associated with increased colon cancer risk in many studies [3, 5–12]. Increased risk of colonic cancer due to high BMI was found for both men and women also in a meta-analysis of the results of 19 studies [13]. While obesity is generally found to increase colon cancer risk, especially in men, the effects have been shown to be greater in the distal (left) than proximal (right) colon [3, 11]. This association is not uniquely an American phenomenon, as increased risk of colon cancer associated with obesity was observed also in Japan,

the U.K., Sweden, Italy and Australia. In epidemiological studies, adjusting for potentially confounding variables such as age, physical activity, energy intake and sucrose intakes has not eliminated the robust effect of obesity. Additionally, in a recent cross-sectional study, colorectal polyps were found in 51 % of obese subjects but in only 29 % of lean subjects [14].

The association between obesity and colonic cancer has been studied to only a limited extent in animal models. In one of these studies, administering a breast carcinogen (N-methyl-N-nitrosourea) produced colon tumors in Zucker *fa/fa* rats but not in wild-type Zucker rats [15]. Others have corroborated these findings emphasizing the obesity phenotype as the cause for increased risk for colon tumorigenesis [16]. Using this same animal model, others observed more colonic tumors in Zucker *fa/fa* rats than in wild-type Zucker rats when the colon-specific carcinogen, azoxymethane (AOM), was used [17]. Another study concluded that C57BL/KsJ-db/db-Apc mice exhibit an increased incidence of intestinal neoplasms and associated it to the obese phenotype [18].

Our research has indicated an increased risk of colonic tumorigenesis in diet-induced obese mice compared to their lean counterparts [19]. The aim of the current study was to determine the relationship between obesity and risk of colon cancer using an obese in vivo model namely C57BL/6J-Lep^{ob} mice and add to in vivo studies in diet-induced obese mice that showed positive correlation between obesity and colon cancer risk [19]. We were particularly interested in seeing whether the premise of increased risk in the obese state would be irrespective of the obese phenotype. In other words we wanted to investigate if obesity as a status is a sufficient factor to increase the risk of colonic tumorigenesis. Guiding the current studies was the hypothesis that obese mice would exhibit a higher risk of colon cancer than their lean littermates. The results of our studies show that obese C57BL/6J-Lep^{ob} mice are not at greater risk of colonic cancer, contrary to what we observed in other obesity models [19]. As Lep^{ob} mice are deficient in leptin, it appears that leptin may mediate the association between obesity and colorectal cancer in humans.

Materials and Methods

Animals, Diets and Carcinogen

Male C57BL/6J-*ob/ob* mice (hereafter referred to as "obese" mice) and lean male littermates (hereafter referred to as "lean" mice) were purchased at 6 wk of age from Jackson Labs (Bar Harbor, ME). Mice were housed five per cage at 22 °C room temperature, and with a 12 h light/dark cycle. Animal use protocols were approved by the U.C. Berkeley Animal Care and Use Committee. All animals were provided a standard rodent chow diet (#7912, Harlan Teklad, Madison WI) and water ad libitum. Azoxymethane (AOM), a colon-specific carcinogen was either purchased from Sigma Chem Co (St Louis MO) or from the NCI Chemical Carcinogen Reference Standard Repository (Midwest Research Institute, Kansas City CO).

Azoxymethane Analysis in Plasma

The source AOM was determined to be pure by observing a single peak by HPLC and the identity was confirmed by mass spectroscopy. Concentrations of source AOM were similarly determined prior to use by UV spectrometry using an extinction coefficient (215 nm) of 4545, and these values were used to prepare injection solutions of the desired concentration. AOM plasma concentrations were measured via an HPLC technique adapted from a previously described protocol [20]. Briefly, HPLC was performed using a 25-cm×4.6 mm C-18 column (Absorbospheretts, Alltech Inc., Deerfield IL) and a 1 cm C-18 guard column. AOM was eluted isocratically with 6 % acetonitrile at a flow rate of 1.0 ml/min and a column temperature of 40 °C. AOM was detected in HPLC effluent by UV absorbance at λ=215 nm.

Animal Experiments to Determine AOM Dose

All lean and obese mice used in this experiment were 8–10 wk old, and they were injected with AOM only on the day of experimentation. Mice used in this experiment were randomly assigned to groups as defined by the time lag between AOM injection and sacrifice (5, 10, 15, 20, 30 & 60 min; 3 lean and 3 obese mice per timepoint). Mice were anesthetized with isofluorane and blood was rapidly taken by heart puncture and processed for analysis of plasma AOM concentrations. The area under the curve of plasma AOM concentration versus time (0 to 60 min) was integrated using the trapezoid method and used to determine what dose of AOM was needed to produce comparable plasma AOM levels (as defined by area under the curve) for lean and obese mice.

Animal Experiments to Assess Colonic Cancer Risk

In Experiment 1, lean and obese mice were randomly assigned (15 mice per group) to receive either saline or AOM injection. At 7 weeks of age, lean mice were injected subcutaneously (sc) and weekly for 4 wk with AOM at 10 mg/kg body weight, and obese mice were injected with the average amount of AOM delivered to their lean littermates. Consequently, lean and obese mice received comparable amounts of AOM per animal, and per kg fat-free mass (Table 1). Colonic tissue was harvested 6, 12 and 32 wk following initial exposure to AOM, and these tissues were used to assess risk of colonic tumorigenesis.

In Experiment 2, lean mice were injected sc and weekly for 4 wk with AOM at 10 mg/kg body weight, and obese

Table 1 AOM doses administered to mice

	AOM mg/kg body weight	AOM mg/mouse	AOM mg/kg fat-free mass[1]
Experiment 1			
Lean (n=45)	10.00±0.03	0.23±0.003	10.9±0.07
Obese (n=45)	6.35±0.06	0.23±0.001	11.1±0.11
Experiment 2			
Lean (n=15)	10.0±0.010	0.26±0.003	11.00±0.010
Obese (n=15)	7.5±0.004	0.33±0.003	12.99±0.007

Values are means of the 4 weekly AOM injections administered to each animal

[1] Fat-free mass was previously reported to account for 90.9 % of the mass of lean mice and 57.7 % of the mass of obese mice, when lean and obese mice weighed 26.1±0.9 and 38.6±0.4 g, respectively [47]. These values were used to calculate fat-free mice in the current studies since lean and obese mice averaged 22.9±0.23 and 36.3±0.32, respectively in Experiment 1; and 26.0±0.35 and 44.2±0.46, respectively, in Experiment 2, when exposed to AOM

mice were injected with 7.5 mg/kg body weight (Table 1). Using this protocol, we showed experimentally that the lean and obese mice had comparable plasma AOM concentrations, when area under the curve of plasma AOM concentration versus time was integrated over the 60 min post-injection time-period. Colonic tissues were harvested 6 wk following initial exposure to AOM and used to assess risk of colonic tumorigenesis.

Aberrant Crypts and Aberrant Crypt Foci

The entire colon was opened longitudinally and examined for blood and gross lesions, fixed in neutral buffered formalin, then stained with methylene blue. Gross pathology was observed using a dissecting microscope, and the mucosal surface of the unsectioned colon was viewed at 40–60× magnifying power using a light microscope to identify and quantify aberrant crypts and aberrant crypt foci per colon [21].

Identifying and Classifying Tumors

When lesions were evident on the mucosa, histological sections were prepared, microscopically analyzed and classified using a grading scheme into “adenomas”, “adenocarcinomas” or “carcinomas in situ”. Tumor incidence (number of animals with tumors) and multiplicity (number of tumors per animal) were calculated.

Cellular Proliferation Assays

Immunohistochemistry was performed to determine degree of cell proliferation. Bromodeoxyuridine (BrdU), which is an analogue of thymidine and is incorporated into newly synthesized DNA in S phase cells, was used to label proliferating cells. BrdU was dispersed in sterile water and injected at 0.2 mg/g body weight [22] 2 h prior to animal sacrifice. Colonic segments were prepared using the ‘swiss roll’ method [23] to improve alignment of crypts in the fixed sections, embedded in paraffin, cut into histological sections and processed for BrdU immunohistochemistry [22] using commercial stain system kit (Zymed, South San Francisco). AntiBrdU antibody was applied on colon sections according to manufacturer’s protocol. Using a light microscope, the right side of at least 20 crypts per colon were inspected to determine the crypt height, 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]/number of cells per crypt column×100).

Statistical Analysis

Data were compared by ANOVA, and results were considered statistically significant if the p-values were<0.05.

Results

Experiment 1

In Experiment 1, lean mice were injected with AOM at 10 mg/kg body weight, providing 0.232±0.12 mg AOM per mouse (Table 1). Obese mice also received 0.232 mg per mouse. Using previously published body composition data of lean and obese *ob/ob* mice of comparable body weights to those used in our study [24], we calculated that the lean and obese animals would be provided with comparable amounts of AOM when calculated per unit of fat-free mass (Table 1). This protocol was selected for several reasons since we wanted to avoid protocols that might provide physiologically higher AOM doses to the obese than lean animals. We rejected the approach used by others [17] of injecting lean and obese animals with equivalent amounts of AOM per kg body weight since the higher body weight of *ob/ob* animals has been reported to be largely due to excess body fat. Since, in a previous study, providing equal amounts of AOM per animal resulted in equivalent levels of DNA methylation in colonic epithelial cells of lean and obese rats [15], and since our calculations indicated that this approach would provide equivalent AOM per unit of fat-free mass, Experiment 1 was performed using a protocol in which lean and obese animals were provided with equivalent amounts of AOM per animal, and equivalent amounts of AOM per unit of fat-free mass. As we fully expected that the risk of colonic

cancer would be higher for obese than lean animals we reasoned that this would avoid concerns that the results could be attributed to higher AOM levels in the obese than lean animals.

When assessed 6, 12 or 32 wk following first exposure to AOM, obese mice had significantly fewer aberrant crypts (AC)/mouse and significantly fewer aberrant crypt foci (ACF)/mouse than lean mice (Fig. 1). At 32 wk, 4 tumors (1 tumor in each of 4 mice) were observed in the 14 lean mice (1 of the 15 mice assigned to this group died for no identifiable reason at 4 wk following initial exposure to AOM) whereas none were observed in the 15 obese mice. No tumors were observed in lean or obese mice killed 6 or 12 wk following AOM, and saline-treated mice (ie. no AOM treatment) exhibited neither aberrant crypts nor tumors (data not shown). Proliferation of epithelial cells within the colonic crypts was not significantly different for lean versus obese mice when assessed 6, 12 or 32 wk following initial exposure to AOM (data not shown). Proliferation parameters that were evaluated included number of cells/crypt column, number of labeled cells/crypt, labeling index, position of highest labeled cell, proliferation zone, and proliferation zone as percentage of crypt depth.

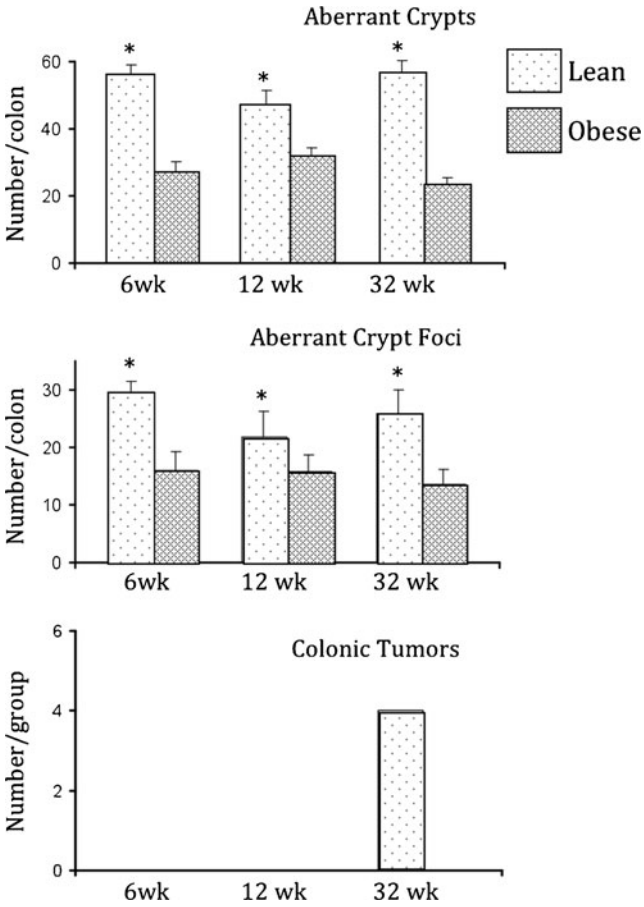

Fig. 1 Aberrant Crypts, Aberrant Crypt Foci, and Colonic Tumors in Lean versus Obese Mice: Experiment 1. Values are means + SEM, n=14−15. *Asterisks* indicate that values for lean and obese animal groups are significantly different at $p<0.05$

Plasma AOM Concentrations

As the results of Experiment 1 suggested that obese mice are at lower risk of colonic tumorigenesis than their lean littermates, a conclusion that is opposite this study's initial hypothesis and opposite the epidemiological data, explanations for those results were therefore sought. Our first concern was that carcinogen administration was such that may not have led to equivalent/comparable doses for the two animal groups (lean and obese mice). Rather than using an intermediate marker to assess AOM equivalency, plasma AOM concentrations were measured as this directly assesses availability of the pro-carcinogen to the target tissue. Following subcutaneous injection of AOM, plasma AOM concentrations increased rapidly, reaching a peak in approximately 10 min, and returned to near baseline levels by 60 min following AOM injection. The area under the concentration by time curve was integrated and these values were used to assess availability of AOM to colonic tissues.

Lean and obese mice were injected with the same amount of AOM per animal as was done in Experiment 1, and plasma AOM concentrations assessed by taking the area under the 0–60 min post-injection curve. Using this protocol, plasma AOM_{AUC} for obese mice averaged 65 % of the values for lean mice (Fig. 2). This lower value opened the possibility that the lower risk of tumorigenesis observed in the obese mice in Experiment 1 was at least partially due to lower AOM availability to colonic tissue.

Detailed experiments were conducted to determine the AOM dosing regime needed to provide equivalent plasma

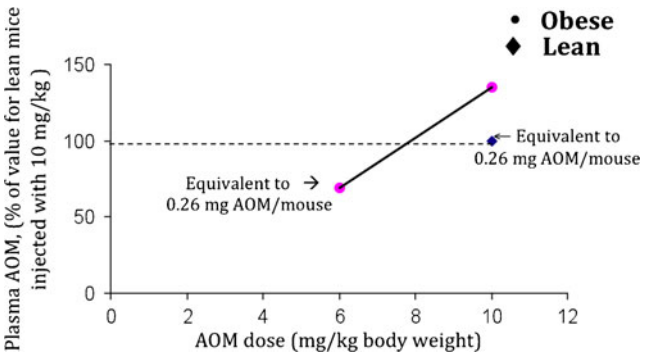

Fig. 2 AOM dose versus plasma AOM concentration in lean and obese mice. Lean mice averaged 26 g; obese mice averaged 42 g. Plasma AOM concentrations were calculated as the area under the curve of plasma AOM concentrations versus time (0–60 min following sc injection of AOM). Values represent the means for 3 mice at each time point, for a total of 18 mice per group

AOM concentrations (assessed as area under the 0–60 min curve) for lean and obese mice. First, obese and lean animals were provided with the same dose of AOM per kg body weight (i.e. 10 mg AOM/kg body weight for obese and lean mice) in accordance with the protocol used by other workers to assess differences in cancer risk between lean and obese rats [17]. When lean and obese mice were all injected with 10 mg AOM/kg body weight, plasma AOM concentrations were significantly *higher* for obese than for lean mice (Fig. 2). Thus, neither approach would be expected to result in equivalent delivery of AOM to tissues of lean and obese mice. The straight line describing the function of AOM dose versus plasma AOM concentration for obese mice shown in Fig. 2 (regression line) was used to predict the AOM dose needed to achieve equivalent plasma AOM concentrations for lean and obese animals. Experimentally, we found that injecting obese mice of ~46 g body weight with 7.5 mg AOM/kg body weight and injecting lean mice of ~24 g body weight with 10 mg AOM/kg body weight produced equivalent plasma AOM concentrations in the lean and obese mice.

Experiment 2

In Experiment 2, obese mice were injected with AOM at doses that were determined to provide plasma AOM concentrations equivalent to those in lean mice. Lean mice in Experiment 2 were injected subcutaneously with 10 mg AOM/kg body weight weekly for 4 weeks, and obese *ob/ob* mice were injected with 7.5 mg/kg body weight. These relative doses were shown in the earlier tests to provide equivalent plasma AOM concentrations when assessed by area under the 0–60 min post-injection curve.

Six weeks following first exposure to AOM, all animals were terminated. The number of AC/colon was significantly lower for obese *ob/ob* mice than for lean mice (Fig. 3). Although the same trend was observed, differences for ACF/colon were not statistically significant. As expected, no tumors were observed. Proliferation of epithelial cells within the colonic crypts was not significantly different for lean versus obese mice for any of the several proliferation parameters assessed.

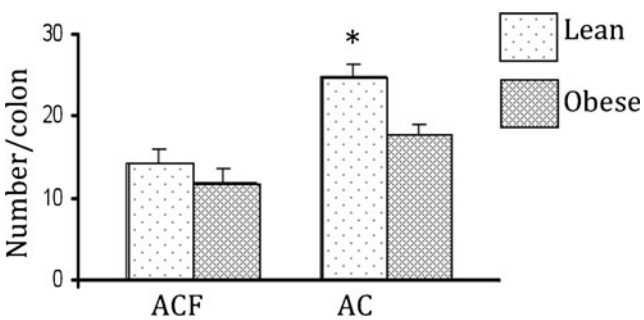

Fig. 3 Aberrant crypt foci and aberrant crypts per mouse colon in lean versus obese ob/ob mice nnn following administration of AOM at doses that provided equivalent plasma AOM concentrations (Experiment 2). Values are means + SEM, $n=15$. *Asterisk* indicates that values for lean and obese animal groups are significantly different at $p<0.05$

Discussion

Based on epidemiological evidence and the results of two rat studies performed by other workers, we hypothesized that obese mice would be at greater risk of colonic tumorigenesis than their lean littermates. Thus, protocols for which increased tumorigenesis in obese animals might be attributed to physiologically higher AOM doses for the obese than lean animals were carefully avoided. For this reason, the approach used by others [17] of injecting lean and obese animals with equivalent amounts of AOM per kg body weight was rejected. Instead, the assumption was made that lean and obese would have equivalent lean body mass since the higher body weight of the *ob/ob* animals has been attributed largely to excess body fat; and, in a previous study with rats, providing equal amounts of AOM per animal resulted in equivalent levels of DNA methylation in colonic epithelial cells of lean and obese rats [15]. Contrary to our original hypothesis, obese *ob/ob* mice had fewer aberrant crypts, aberrant crypt foci and tumors than their lean littermates when injected with equivalent amounts of AOM per animal and, via calculation, per kg lean body mass. The decreased risk of colonic tumorigenesis appeared not to be mediated via decreased cellular proliferation. Our observation that obese mice were at lower, not higher risk of colonic tumorigenesis as expected, prompted us to experimentally determine whether the AOM delivery protocol used in Experiment 1 provided equivalent plasma AOM concentrations. Plasma AOM concentration provides a direct measure of pro-carcinogen delivery to tissues. The results of those experiments suggested that the obese mice received comparatively lower doses of AOM, requiring further experimentation to determine whether the obese *ob/ob* mice would have lower colon cancer risk than their lean littermates when provided with comparable AOM doses.

In Experiment 2 equivalent plasma AOM concentrations were achieved by providing lean mice with 10 mg AOM/kg body weight, and obese mice with 7.5 mg AOM/kg body weight. At 6 weeks following first exposure to AOM, the number of aberrant crypts/mouse was significantly lower for the obese than lean mice. A longer-term study will be needed before it can be known whether the obese *ob/ob* mice are at reduced risk, but these limited data support a conclusion that obese *ob/ob* mice are not at increased risk of colonic tumorigenesis. This conclusion is not consistent with the results of several animal studies and numerous epidemiological studies that have reported obesity to increase risk of colonic cancer.

It is possible that our use of leptin-deficient *ob/ob* mice may account for our observation that obese *ob/ob* mice are not at increased risk of colonic tumorigenesis. Leptin is a hormone and cytokine secreted primarily from adipose tissue, and its concentration in plasma is highly correlated with body fatness [25, 26], except in humans or animals that harbor a mutation in the leptin gene that prevents formation of a functional leptin protein. While absence of leptin in animal models and low leptin levels in humans have been shown to increase infectivity by pathogens [27–29], leptin-deficient mice have been shown to be protected from some T-cell mediated and autoimmune diseases [30–32, 34] and from intestinal inflammation [33, 34].

Inflammation is generally recognized as an important determinant of cancer risk [35]. Risk of colonic cancer is significantly increased in the presence of inflammatory bowel disease [35, 36], and anti-inflammatory agents have been shown to reduce risk. Recent molecular data have unequivocally linked inflammation to colonic tumorigenesis [37] and liver cancer [38]. Thus, it is possible that the absence of leptin in obese *ob/ob* mice is responsible for our observation that obese *ob/ob* mice are not at higher risk of colonic tumorigenesis than their lean littermates. In support of this suggestion, risk of breast cancer was shown to be lower in leptin deficient obese mice than in lean mice [39] even though the epidemiological data show that obesity increases risk of breast cancer. Furthermore, increased levels of serum leptin were found to be a risk factor for the recurrence of stage I/II hepatocellular carcinoma after curative treatment [40].

Leptin has been found to function as a growth factor in colonic epithelial cells [41, 42]. Mechanisms via which leptin might be exerting a pro-carcinogenic effect include the STAT pathway, the MAPK pathway and NF-κB, the JAK2 tyrosine kinase pathway, phosphoinositide PI3 kinase pathway, the mTOR kinase and protein kinase C (PKC) pathway [41–43].

Leptin might influence colon cancer risk in vivo via mechanisms other than via it's inflammatory effect since leptin has been shown to stimulate growth of colonic [42], esophageal [39], prostate [44], and breast [45, 46] epithelial cells. Other data suggest that leptin may increase cancer risk by stimulating the invasive capacity of cells [47], or by promoting angiogenesis [48].

Further experimentation is needed to determine whether leptin mediates the stimulatory effect of obesity on colonic tumorigenesis. In support of this suggestion, the results of our studies using leptin-deficient obese mice suggest that these mice are not at increased risk of colonic cancer whereas the results of two studies with Zucker *fa/fa* rats (obese due to leptin receptor deficiency), suggested that obesity does stimulate tumorigenesis. Also, C57BL/KsJ-*db/db* mice (leptin receptor deficient) were reported to be at greater risk of colonic cancer than their lean wild-type controls, even though body weights were normalized to that of lean control mice by feed restriction [49]. These *db/db* mice were reported to be both hyperinsulinemic and hyperleptinemic in comparison to their lean littermates, and the investigators pointed to both factors possibly mediating the increased risk of tumorigenesis. As the obese *ob/ob* mice used in our studies are known to be hyperinsulinemic but leptin deficient, we suggest that leptin rather than insulin is more likely to serve as the key hormone mediating the increased risk of colonic tumorigenesis associated with obesity in humans. Contrary to our suggestion, however, infusing leptin into AOM treated lean F344 rats reduced formation of aberrant crypts [50]. That observation is consistent with results of an earlier study showing that injection of leptin into starved and lean mice did not stimulate mucosal proliferation in vivo [24]. Whether the effects of leptin differ for lean than obese animals is unknown at this time. Observations with lean *db/db* mice [49] as well as other works [51, 52] suggest that several interacting factors may be involved.

In summary, our results indicate that obese *ob/ob* mice are not at higher risk of colonic tumorigenesis than their lean littermates. Studies that directly test the role of leptin as a key mediating factor are warranted since it is plausibly possible that it is the absence of leptin that accounts for findings that are inconsistent with studies conducted with obese but hyperleptinemic rats, lean hyperleptinemic mice, and with the large body of human epidemiological data.

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