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Obesity promotes colonic stem cell expansion during cancer initiation

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ABSTRACT

There is an urgent need to elucidate the mechanistic links between obesity and colon cancer. Convincing evidence for the role of Lgr5$^+$ stem cells in colon tumorigenesis has been established; however, the influence of obesity on stem cell maintenance is unknown. We assessed the effects of high fat (HF) feeding on colonic stem cell maintenance during cancer initiation (AOM induced) and the responsiveness of stem cells to adipokine signaling pathways. The number of colonic GFP$^+$ stem cells was significantly higher in the AOM-injected HF group compared to the LF group. The Lgr5$^+$ stem cells of the HF fed mice exhibited statistically significant increases in cell proliferation and decreases in apoptosis in response to AOM injection compared to the LF group. Colonic organoid cultures from lean mice treated with an adiponectin receptor agonist exhibited a reduction in Lgr5-GPF$^+$ stem cell number and an increase in apoptosis; however, this response was diminished in the organoid cultures from obese mice. These results suggest that the responsiveness of colonic stem cells to adiponectin in diet-induced obesity is impaired and may contribute to the stem cell accumulation observed in obesity.

Introduction

Colorectal cancer is the 2nd leading cause of cancer mortality in North America [1] and up to 20% of all cancer related deaths may be attributed to obesity [2]. A growing body of data suggest that the relative risk of colon cancer increases in proportion to body mass index tertiles, particularly in men [3–9]. With the rising prevalence of obesity, there is an urgent need to elucidate the mechanistic links between chronic inflammation in adipose tissue and colon cancer risk in obesity. The development of obesity is characterized by excess nutrient delivery to the adipose tissues and an expansion in adipose mass which disrupts the dynamic role of the adipocyte in energy homeostasis, resulting in the alteration of adipose-derived hormones and development of chronic inflammation [10,11]. This is significant because studies in both humans and mouse models have provided a clear link between inflammation and cancer [12–14]. The chronic low-grade inflammation associated with obesity may play a key role linking excess adipose tissue, altered adipokine status and the development of colon cancer by promoting tumor development. Diet-induced obese mice exhibit increased numbers of colonic tumors as well as elevated circulating levels of several cytokines and adipokines [15]; however, the underlying mechanisms responsible for the promotion of colon cancer development in obesity remain to be determined.

The best characterized adipokines relevant to colon cancer are leptin and adiponectin. These two adipokines are primarily produced by adipose tissue, are altered in obesity, and have been shown to play a role in colonic tumorigenesis [16–21]. Unlike most other adipokines, adiponectin is considered anti-inflammatory and is inversely associated with obesity [22,23]. Epidemiological data suggest that low adiponectin levels are correlated with increased risk of colon cancer [16–18] and supporting evidence for the protective role of adiponectin against colon cancer has been reported both in mice and cell culture studies [20,24–26]. However, whether specific adipokines directly influence critical target cell populations such as the colonic stem cells, which are implicated in colon tumorigenesis, remains to be determined.

A long-lived pool of rapidly cycling stem cells in the adult mouse small intestines and colon can be identified by the expression of the leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5). Lgr5$^+$ crypt stem cells have been shown to be the cells of origin of intestinal cancer by breeding of genetically modified Apc$^{flox/flox}$ mice and stem-cell-specific Lgr5-EGFP-IRES-creER^{T2} knockin mice [27]. Both *in vivo* and *in vitro* experiments have demonstrated the role of *LGR5 in cell* proliferation, migration and colony formation [28]. Nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to effectively prevent colon cancer in humans and rodent models [29,30]. The chemopreventative mechanism appears to

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involve the elimination of Lgr5⁺ stem cells that are inappropriately activated by oncogenic events by inducing apoptosis [31]. There is convincing evidence for the role of *Lgr5⁺* cells in colon tumorigenesis; however, the influence of obesity on stem cell maintenance is unknown. Thus, the objectives of this study were to determine: (1) the effects of high fat feeding on colonic stem cell maintenance during cancer initiation; and (2) the responsiveness of stem cells from obese mice to the activation of adiponectin signaling pathways.

Methods and experimental design

Animals and diet

All experimental procedures adhered to the guidelines approved by the Public Health Service and the Institutional Animal Care and Use Committee at Texas A&M University. A total of 20 male Lgr5-EGFP-IRES-creERT2 transgenic mice (3 months of age) were randomized to 2 different experimental groups. Mice were fed either a high fat (HF; 60% kcal from fat; n = 10) or low fat (LF; 10% kcal from fat; n = 10) diet (Research Diets, New Brunswick, NJ) for 12 weeks. Body weight and food intake were monitored weekly. At the end of 12 weeks on diet, 3 mice per group were injected with saline as a control and 7 mice per group received subcutaneous injections of Azoxymethane (AOM; Sigma-Aldrich) at 15 mg/kg body weight and 13.5 mg/kg body weight in lean and obese groups, respectively. In order to provide similar amounts of AOM to target tissues, differential doses of AOM for lean and obese mice were used in order to generate equivalent circulating AOM concentrations as previously described [32,33]. All mice were sacrificed 12 hours after injection.

Serum cytokine and adipokine profiles

At the time of sacrifice, cardiac blood was collected and allowed to clot at room temperature for 30 min, then centrifuged at 1500 *g* for 15 min and the serum was stored at –80 °C. Serum levels of IL-1β, IL-6, IL-17a, leptin, resistin and adiponectin were measured by customized Bio-Plex immunoassays on the Bio-Plex 200 System using Bio-Plex Manager 6.0 software (BioRad). All plasma measurements were analyzed in duplicate.

Immunohistochemistry

Two hours prior to termination, mice were injected with 30 μg/g body weight of 5-ethynyl-2′-deoxyuridine (EdU) for analysis of cell proliferation. At the time of sacrifice, the colon was removed and perfused with PBS to remove the contents. A 1 cm section of the distal colon was fixed in 4% PFA in PBS for 4 hours at 4 °C, embedded in paraffin, sectioned and used for immunohistochemical studies. The remaining colon was used for isolating colonic crypts that were used for apoptosis assays, cell sorting, and organoid culture experiments as described below.

Fluorescence immunohistochemistry was used to assess markers of cancer initiation, e.g. apoptosis (TUNEL), cell cytokinetics (EdU), and Lgr5 stem cell lineage (GFP) as previously described [34]. GFP was assessed using an anti-GFP primary antibody (Abcam ab6673) followed by an Alexa-488 anti-goat secondary antibody (Jackson Immuno Research Cat. #705-545-147). Cell proliferation was measured using the Click-IT EdU kit (Invitrogen A-21222) and apoptosis was measured using a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) kit (Trevigen #4810-30). For quantification of immunohistochemical staining, all GFP expressing crypts were counted and the total number of apoptotic and proliferative cells, both GFP⁺ and GFP⁻, were recorded.

Organoid cultures

Colonic crypts were isolated as we have previously described [35,36]. Briefly, crypts were isolated by incubation with 20 mM EDTA, mechanical disruption and centrifugation. Isolated crypts were resuspended in Matrigel at a density of 15 crypts/μl (BD Biosciences, San Jose, CA), plated onto 24-well plates and maintained in complete medium containing advanced Dulbecco's modified Eagle's medium/F12 (ADF; Life Technologies, Grand Island, NY), epidermal growth factor (50 ng/ml; Life Technologies), Noggin (100 ng/ml; Peprotech, Rocky Hill, NJ), R-Spondin (500 ng/ml; Sino Biological, Beijing, China), N2 supplement (1; Invitrogen), B27 supplement (1×; Life Technologies), *N*-acetylcysteine (1 μM; Sigma, St Louis, MO) and Wnt-conditioned medium as described previously by Barker et al. [37]. Colonic crypts begin to bud around 2–3 days and on day 3 the organoid cultures from lean and obese Lgr5-GFP mice were treated directly with the recombinant leptin (5–100 ng/ml, BioVision Cat. #4367), adiponectin (5–50 μg/ml, BioVision Cat. #4902), the adiponectin receptor agonist AdipoRon (5–50 μM BioVision Cat. #42565) or dimethyl sulfoxide (DMSO; control vehicle) for 48 hours.

Flow cytometry on organoid cultures

After 5 days in culture, organoids were harvested by washing with cold PBS and then released from the Matrigel by pipetting with cold ADF medium. The dissociated organoids were transferred to a conical tube and residual Matrigel was removed by subsequent PBS washes and centrifugation. Apoptosis was measured using the Dead Cell Apoptosis Kit (Life Tech #V13241) with Annexin V Alexa Fluor 647 conjugate (Life Tech #A23204) following the manufacturer's instructions. The organoids were incubated with trypsin/DNase to produce a single cell suspension for flow cytometry of apoptotic and GFP positive cells using an Accuri C6 flow cytometer (BD Biosciences).

Statistics

Investigators were not blinded to the group allocation during the experiment or when assessing the outcome. GraphPad Prism 6.0 and SPSS 14.0 software were used to perform statistical analyses. Body weight, feed intake and serum data were analyzed by the Student's t-test. Lgr5-GFP⁺ stem cell number, apoptosis and proliferation data were analyzed using a two-way analysis of variance (ANOVA) with the main effects of diet and depot, and followed, if justified, by testing between-mean differences using Bonferroni's multiple comparisons test. The D'Agostino & Pearson omnibus normality test was used to test for normality and the Brown–Forsythe test was used for homogeneity of variances. Data sets not normally distributed were log transformed. Differences were considered significant at $P < 0.05$ and all results are reported as mean ± SEM.

Results

High fat diet increased body weight and altered serum adipokines

High fat diets are regularly used to induce an obese phenotype, including an increase in body weight, fat mass, adipocyte size and plasma glucose and insulin levels, as well as altered adipokines status [38–40]. We employed a similar high fat dietary approach to induce obesity in the Lgr5-EGFP-IRES-creERT2 transgenic mice. After 12 weeks on diet, the HF group had a significantly higher body weight ($p < 0.01$) compared to the LF group (50 ± 0.8 *vs* 37 ± 1.1 g, respectively). Total food intake was essentially identical between the two groups (240 ± 33 g for HF and 240 ± 15 g for LF). Serum levels of the cytokines IL-1b, IL6 and IL17a did not differ significantly between groups; however, serum leptin levels were increased ($p = 0.04$) and serum adiponectin levels were decreased ($p = 0.03$) in the HF group (Table 1).

Effects of high fat feeding on Lgr5-GFP⁺ stem cell proliferation and apoptosis

The total number of cells per crypt did not differ between mice fed HF and LF diets or between AOM-treated and control groups (Fig. 1A). We next investigated diet- and treatment-induced differences in distinct Lgr5-GFP⁺ and non-GFP⁺ cell populations. The number of non-GFP⁺ cells per crypt displayed a pattern similar to total cells per crypt, with neither diet nor AOM treatment exerting a statistically significant effect (Fig. 1B). Likewise, the number of Lgr5-GFP⁺ stem cells per crypt did not differ between HF- and LF-fed mice, which had not been injected with AOM ($p > 0.99$), and no statistically significant difference in the number of Lgr5-GFP⁺ stem cells per crypt was observed between AOM-injected LF and non-AOM-injected HF ($p = 0.09$) (Fig. 1C). In contrast, AOM-injected HF fed mice had a

Table 1
Circulating adipokines and cytokines in response to high fat feeding.

	LF	HF	P-values
IL-1beta (pg/ml)	1379 ± 866	1128 ± 303	0.32
IL-17a (pg/ml)	100 ± 12	205 ± 75	0.07
IL-6 (ng/ml)	4.2 ± 1.4	1.5 ± 0.8	0.13
Resistin (ng/ml)	90 ± 24	83 ± 39	0.32
Leptin (ng/ml)	40 ± 12	72 ± 18	***0.04***
Adiponectin (μg/ml)	8.5 ± 1.6	5.3 ± 0.5	***0.03***

Data are presented as mean ± SEM for all AOM injected mice (n = 7/group). Statistical difference was determined by the Student's t-test.

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