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Partial lipectomy reduces dimethylhydrazine-induced carcinogenic initiation in the colon of rats

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ABSTRACT

This study investigated whether visceral adipose tissue directly modulates the development of preneoplastic lesions in the colon of carcinogen-treated rats. Wistar rats ($n = 64$) were randomly assigned to 8 experimental groups in two experiments. In one experiment, 32 rats were exposed or not to either carcinogen treatment (dimethylhydrazine, DMH; 125 mg/kg) or high-fat diet (standard chow enriched with 14% lard) or both for 56 days. In a second experiment, 32 rats were exposed to a carcinogen or they underwent partial lipectomy or both for 30 days (partial lipectomy groups underwent ablation of mesenteric and parametrial fat pads, whereas sham groups did not; all rats were fed with standard chow). Colon was collected for histopathological analysis. After 56 experimental days a high-fat diet increased carcinogenic mutations in the colonic epithelia. Partial lipectomy reduced weight gain in carcinogen-exposed rats and decreased the *de novo* formation of mesenteric and parametrial fat pads. Partial lipectomy significantly inhibited the mutational process after 30 days: there were fewer colonic preneoplastic lesions and less proliferation, apoptosis, and inflammation. These data suggest that visceral adipose tissue promotes colon carcinogenesis and enhances the establishment and expansion of genetically mutated cells in colonic epithelia.

1. Introduction

In humans abdominal obesity is associated with increased visceral adipose tissue density and risk of colon cancer (Pischon et al., 2008). Adipose tissue is considered an endocrine organ that can secrete a number of adipose tissue-specific or -enriched hormones that promote inflammation (Rajala and Scherer, 2003). In recent years, inflammation has emerged as a unifying link for many features of colon carcinogenesis, including increasing age and greater body fat, and neoplastic risk (Basterfield et al., 2005). In addition, the expression of inflammation-associated genes, including cyclooxigenase-2 (COX-2) is upregulated in both inflamed mucosa and in colon cancer (Itzkowitz and Yio, 2004).

An imbalance between cell proliferation and apoptosis plays a key role in colon carcinogenesis. In rats, dimethylhydrazine (DMH) is a carcinogen which induces proliferation and apoptosis in the colon (Smith et al., 1998; Yamada et al., 1992). DMH also promotes COX-2 expression in the early stages of colon cancer (Barthold and Jonas, 1977). We reported previously that the increase in COX-2 expression mainly occurs in colonic sub-epithelial cells (Kannen et al., 2011a,b).

Partial lipectomy has been shown to reduce skin carcinogenesis and to induce apoptosis in ultraviolet B (UVB) irradiated cells (Lu et al., 2006, 2012). In the preneoplastic colon, we found that adipose tissue, mainly visceral fat pads, promotes the formation of dysplastic lesions (Kannen et al., 2012b). However, it remains unclear whether adipose tissue, which seems to have much more complex activity than one might expect, directly modulates colonic carcinogenesis. Our previous report revealed that inhibiting hepatic fat absorption metabolism enhances early steps in colonic tumor initiation, because colonocytes are exposed to high-levels of fatty acids in stools (Garcia et al., 2006). Further, rats that underwent food deprivation (40% less standard chow) showed enhanced development of preneoplastic lesions, probably due to increased peroxidative stress levels in liver and colon tissues (Kannen et al., 2013).

In the present study, we first investigated whether a high-fat diet inducing high-visceral adipose tissue density also increased mutations in colonic crypts. Second, we investigated the effects of partial lipectomy on the development of colon preneoplastic

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lesions in rats fed a standard chow diet. Our findings demonstrated that the ablation of mesenteric and parametrial fat pads markedly inhibited the manifestation of colonic preneoplastic lesions in non-obese rats.

2. Materials and methods

2.1. Animals and ethics statement

Male and female 30-day-old Wistar rats (150–170 g) were supplied by Ribeirão Preto School of Medicine. Rats were housed in plastic cages in a temperature controlled room at 24 °C (±1 °C) and maintained on a 12:12-h light–dark cycle from their birth to the end of the experiments described herein. All experimental protocols were approved by the Animal Care and Use Committee.

2.2. Experimental design

First, 32 male Wistar rats were randomly assigned to one of four groups. Control rats were not treated with DMH (no-DMH) and were fed a standard diet (SD; SD/no-DMH, $n = 8$) or a high-fat diet (HF; HF/no-DMH, $n = 8$). Alternatively, rats were carcinogen-treated with DMH [single intraperitoneal (i.p.) injection of 125 mg/kg DMH (Wako Pure Chemical Industries, Osaka, Japan) the day before the diet change] and fed either a standard diet (SD/DMH, $n = 8$) or a high-fat diet (HF/DMH, $n = 8$). The SD consisted of standard Purina rat chow, whereas HF diet was standard chow enriched with 14.6% lard (Garcia et al., 2006). Rats were fed either with SD or HF diets for 56 days, and then sacrificed (see below).

The other 32 female Wistar rats were randomly divided into four groups ($n = 8$ rats/group). The groups Lipec/C (no carcinogen-treatment) and Lipec/D (carcinogen-treatment) underwent lipectomy. The groups Sham/C (no carcinogen-treatment) Sham/D (carcinogen-treatment) were subjected to surgery in which no fat pads were removed but that was otherwise the same as the lipectomy procedure. The groups Lipec/D and Sham/D were carcinogen-treated (single i.p. injection of DMH, 125 mg/kg body weight) 3 days after the lipectomy or the simulated surgery (Kannen et al., 2011a,b, 2012b). The lipectomy protocol was performed as described by Lu et al. (2012) with minor modifications. Briefly, female rats were injected i.p. with ketamine_HCl (120 mg/kg) and xylazine (10 mg/kg). The abdominal skin of the anesthetized rats was sterilized (70% alcohol), shaved, and an incision was made along the abdominal midline. The parametrial and mesenteric fat pads were pulled out of the abdomen and resected. The, uterus and stomach were returned to the peritoneal cavity, and the abdominal muscle layers and skin were sutured separately. During the sham surgery, organs were pulled out and then placed back into the abdominal cavity; the fat pads were not removed. Rats were monitored carefully over the next three days. These 4 groups were sacrificed after 30 days (see below).

2.3. Sacrifice

All rats were euthanized by CO_2 exposure at the time-points described above. At the autopsy, the large bowel was longitudinally opened as close as possible to the mesenteric border throughout its extension. The colon was fixed in 10% neutral buffered formalin for 24 h.

2.4. Histopathological analysis of colonic epithelia

The number of aberrant crypt foci (ACF) was counted *per* microscopic field under low power magnification by light microscopy according to our previous description (Kannen et al., 2012a). Colonic samples were transversally sectioned from *en face* paraffin-embedded preparations for identification and enumeration of ACFs (Demarzo and Garcia, 2004; Kannen et al., 2012a). ACFs ranging from mild to moderate dysplasia were counted in 20 microscopic fields for each carcinogen-treated animal. Values are expressed as ACF *per* cm^2.

Apoptosis was identified in H&E stained sections as apoptotic bodies with scattered karyorrhectic basophilic globular intracytoplasmic debris (Supplementary Fig. S1). The number of apoptotic cells *per* 50 crypts was counted at 40× magnification as described previously (Shidham et al., 2003). Results were expressed as the proportion of the total number of apoptotic bodies *per* crypt. All analyses were performed with coded slides to avoid intra-observer bias.

2.5. Immunohistochemistry and analysis

In accordance with previous descriptions (Garcia et al., 2006; Kannen et al., 2011a,b), 4-μm paraffin-embedded colonic sections were stained with the following primary antibodies: anti-metallothionein antibody (MT; clone FL-61 at 1:100) from Santa Cruz Biotechnology (USA); and, anti-PCNA (clone PC 10 at 1:100), anti-CPP32 (CASP-3) (clone JHM62 at 1:300), and anti-COX-2 antibodies (clone 4H12 at 1:200) from Novocastra (USA). Primary antibodies were detected in longitudinal sections using the PictureMAX Polymer Kit (Invitrogen, Carlsbad, CA, USA). Using this kit, there is a brown precipitate in the nucleus for positive reactions against MT, PCNA, or CASP-3 (Supplementary Fig. S2), as well as in the cytoplasm for those against COX-2. Values for PCNA were expressed as the ratio between positively stained and unstained nuclei *per* 100 crypts. To estimate the extent of CASP-3 and COX-2 expression, 100 colonic crypts and their surrounding connective tissue were observed and the cells were counted (Oshima et al., 1996). The results were expressed as the number of positive cells per colonic crypt (CASP-3) or its surrounding area (COX-2). Analyses were performed with coded slides to reduce intra-observer bias.

2.6. Statistical analysis

Data were analyzed using GraphPad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA). Two-way ANOVA (Bonferroni *post hoc* test) was used to analyze different categorical independent endpoints on one dependent variable. The Mann–Whitney test was applied for analysis between two datasets. A probability of $P < 0.05$ was considered to be statistically significant. All values represent mean ± standard deviation.

3. Results

3.1. The relationship between visceral adipose tissue and mutations in epithelial cells

We showed previously that feeding rats HF diets enlarges the visceral adipose tissue and promotes the development of preneoplastic lesions in the colon (Garcia et al., 2006; Kannen et al., 2012b). Because MT is a reliable marker related to genetic mutations in colonic epithelial cells (Mori et al., 2012), its expression was evaluated in colonic samples from SD and HF rats that were or were not treated with DMH. This revealed that enhancing adipose tissue density with a HF diet increased significantly MT expression in colonic epithelial cells from DMH-exposed rats (Fig. 1A; CTRL and HF without DMH = 0; CTRL/DMH, 11.13 ± 3.64 *vs* HF/DMH, 21.5 ± 4.07, % MT (+) crypts per rat; $P = 0.0023$). Hence, the enlargement of the adipose tissue promotes genetic changes in colonic epithelial cells.