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Western diet enhances intestinal tumorigenesis in *Min*/+ mice, associating with mucosal metabolic and inflammatory stress and loss of *Apc* heterozygosity[☆,☆☆]

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Abstract

Western-type diet (WD) is a risk factor for colorectal cancer, but the underlying mechanisms are poorly understood. We investigated the interaction of WD and heterozygous mutation in the *Apc* gene on adenoma formation and metabolic and immunological changes in the histologically normal intestinal mucosa of *Apc*$^{Min/+}$ (*Min*/+) mice. The diet used was high in saturated fat and low in calcium, vitamin D, fiber and folate. The number of adenomas was twofold higher in the WD mice compared to controls, but adenoma size, proliferation or apoptosis did not differ. The ratio of the *Min* to wild-type allele was higher in the WD mice, indicating accelerated loss of *Apc* heterozygosity (LOH). Densities of intraepithelial CD3ε$^+$ T lymphocytes and of mucosal FoxP3$^+$ regulatory T cells were higher in the WD mice, implying inflammatory changes. Western blot analyses from the mucosa of the WD mice showed suppressed activation of the ERK and AKT pathways and a tendency for reduced activation of the mTOR pathway as measured in phosphoS6/S6 levels. The expression of pyruvate dehydrogenase kinase 4 was up-regulated in both mRNA and protein levels. Gene expression analyses showed changes in oxidation/reduction, fatty acid and monosaccharide metabolic pathways, tissue organization, cell fate and regulation of apoptosis. Together, our results suggest that the high-risk Western diet primes the intestine to tumorigenesis through synergistic effects in energy metabolism, inflammation and oxidative stress, which culminate in the acceleration of LOH of the *Apc* gene.
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1. Introduction

Colorectal cancer (CRC) is one of the most common cancer types in industrialized countries. An inactive lifestyle, obesity and a high-fat diet with low micronutrient density are well-known risk factors for CRC, and studies in various animal models support the role of a high-risk Western-type diet (WD), that is, a diet high in fat and low in fiber, calcium, vitamin D and folate, in colon tumorigenesis [1–4]. Mechanisms for how the unbalanced, high-energy yielding WD predisposes or promotes the intestinal epithelium to carcinogenesis are poorly understood.

Components of the Wnt signaling pathway are almost ubiquitously mutated in CRC. In the majority of sporadic CRC cases, mutations of APC, a key regulator of Wnt signaling, are found [5]. APC regulates β-catenin pools in cells, and increased nuclear accumulation of β-catenin is one of the driving forces for colon tumorigenesis [6].

Recent research connects the activation of Wnt signaling with dysregulation of energy metabolism so that its activation drives increased use of glucose, that is, Warburg-type cancer metabolism [7]. Increased glycolytic rate in transformed cells elevates the production of glycolytic intermediates, which are used in the biosynthetic pathways to satisfy the anabolic needs of dividing cells [8]. This provides a selective growth advantage for transformed cells. Furthermore, mitochondrial metabolism is shifted to uncoupled oxidation of glutamine and fatty acids to maintain glycolysis. These alterations in cellular metabolism predispose cells to an increased load of reactive oxygen species that activate apoptosis or, if the cell does not undergo apoptosis, may induce tumorigenic proliferation and anchorage-independent growth [9]. Earlier it was believed that the Warburg effect is secondary to transformation, but the metabolic changes seem to occur already during the transformation process and are governed by the same signal transduction pathways as proliferation [10]. Interestingly, an earlier study [4] demonstrated that a WD reprograms

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the intestinal epithelium toward a gene expression pattern resembling that driven by *Apc* mutations. Thus, dietary and genetic factors converge in driving intestinal tumorigenesis.

In the glycolytic switch, pyruvate dehydrogenase kinases (PDKs) act as key regulators. These inactivate the pyruvate dehydrogenase complex, which catalyzes the rate-limiting step for channeling pyruvate into the tricarboxylic acid cycle. Increased pyruvate dehydrogenase kinase 4 (PDK4) expression has been reported in the preneoplastic intestinal epithelium of cancer-susceptible mice and CRC patients. In addition, it is known that PDK inhibition can suppress tumor growth [11,12]. On the other hand, many tumors show lower PDK4 expression in comparison to the normal tissue of origin, indicating that the metabolic demands in the various stages of carcinogenesis are different [13]. PDK4 expression is negatively regulated by the PI3 kinase/AKT and ERK1/2 pathways, which control nutrient uptake and convey growth factor signals to the mTOR kinase, promoting cellular growth and differentiation [13,14]. These pathways are commonly overactivated in CRC and other cancers [15,16].

We have earlier shown that high fat content in an otherwise balanced diet does not promote intestinal tumorigenesis in the *Apc*-mutated *Min*/+ mouse [17], a widely used murine model of human intestinal carcinogenesis. Here we have further studied tumor formation in the *Min*/+ mouse by combining the high fat content with marginal concentrations of fiber, calcium, vitamin D and folate to better mimic a typical WD. Several of these components have been shown to interact with β-catenin metabolism [18–20].

We found that the high-risk WD accelerated adenoma formation, but not growth, in the *Min*/+ mouse. Increases in adenoma numbers were accompanied by loss of heterozygosity (LOH) in the *Apc* gene, down-regulation of ERK1/2, AKT and mTORC1 signaling pathways, as well as changes in the density of immune cells in the normal-appearing mucosa of the *Min*/+ mouse. Gene expression analyses showed several changes in the key cellular pathways regulating energy metabolism, tissue organization and cell fate, all of which could predispose the intestinal epithelium to enhanced tumorigenesis.

2. Materials and methods

2.1. Animals and diets

Male and female C57BL/6 J *Min*/+ mice were bred at the Laboratory Animal Centre in Viikki, Helsinki, from inbred mice originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA). After weaning, the mice were polymerase chain reaction (PCR) screened for the *Min*/+ genotype. The 5-week-old *Min*/+ mice were stratified by weight and litter background and assigned into experimental diets for 10 weeks. The mice were housed in plastic cages in humidity- and temperature-controlled facilities with a 12-h light–dark cycle. They were fed *ad libitum* and had free access to tap water. The mice were weighed and monitored weekly and a record was kept on their growth throughout the experiment. One mouse from the WD group had to be sacrificed before the end of the experiment due to considerable weight loss for 1 week and was consequently excluded from the subsequent analyses. The total number of mice included in the data was 12 in the WD diet group and 14 in the control diet group. The control diet was the semisynthetic unmodified AIN-93G diet. The WD was modified from AIN-93G, with high fat and low fiber, vitamin D, calcium and folate (Table 1; Harlan Teklad, Madison, WI, USA). We used our own modification of the WD, which highly resembles the new Western diet (NWD) used by Newmark *et al.* [2] shown to induce benign and malignant neoplasms in the colon of normal B6 mice after an 18-month feeding experiment The main difference between NWD and our WD is the source of fat, which in our diet resembled the more typical consumption of fat in Western populations, and was largely composed of milk fat (66.4% of total fat from milk, and 33.6% from rapeseed and sunflower oil), while in the NWD, the main fat source was corn oil. The Laboratory Animal Ethics Committee, University of Helsinki approved the study protocol.

Table 1
Composition of diets

Component	Control diet (AIN-93G)	WD
Protein, %kcal	18.8	18.5
CHO, %kcal	63.9	42.3
fat, %kcal	17.2	39.2
Milk fat, %fat/%diet	–	66.4/13.2
Oil, %fat/%diet	100/7.0	33.6/6.7
kcal/g	3.8	4.6
Ca, g/kg	5.0	0.5
Folic acid, mg/kg	2.0	0.2
Vitamin D, IU/kg	1000	100

2.2. Evaluation of adenomas and collection of samples

After the 10-week feeding period, the mice were sacrificed by CO_2 asphyxiation. Blood was collected from the abdominal aorta, and after centrifugation, the plasma was stored at −70°C. The small intestine, cecum and colon were removed and opened along the longitudinal axis and rinsed with ice-cold saline. The small intestine was divided into five sections of equal length. The cecum and colon were separated from the small intestine and kept together for analysis. The intestinal sections were spread flat on microscope slides. Each section of the intestine and colon and cecum was analyzed under a stereomicroscope attached to a monitor. The number and diameter of each adenoma were recorded for all intestinal sections separately. The observers keeping record were blinded to the treatment. The intestinal adenomas were excised from the tissue and the normal-appearing mucosa that was left behind was scraped off from lamina propria. The tissue samples were snap frozen in liquid nitrogen and stored at −70°C. For immunostaining analyses, samples were collected from the distal small intestine, fixated in buffered 4% paraformaldehyde and processed for paraffin sections. For microarray analysis, the mucosa was scraped from another 0.5-cm section of the same intestinal area and stored in RNA later solution (Qiagen) at −20°C.

2.3. Immunostaining

Paraffin tissue sections were deparaffinized and subjected to heat-induced antigen retrieval (microwave heating for 15 min at 700 W power in 250 ml of buffer, followed by a 20-min cooling period). Automated immunostaining was performed using the LabVision Autostainer 480 and the Ultravision detection system (LabVision, Thermo Fisher Scientific). Proliferating cells were identified with an antibody against Ki67 antigen (clone SP6, Labvision), mitotic cells with anti-phospho-histone H3 (#06-570, Millipore), apoptotic cells with an antibody against cleaved caspase 3 (#CP229, Biocare), T lymphocytes with anti-CD3ε (clone SP7, Neomarkers), regulatory T cells with anti-FoxP3 (clone FJK16s, eBioscience) and B lymphocytes with anti-CD45R (clone RA3-6B2, Invitrogen). Antigen retrieval was performed using Na-citrate buffer, pH 6, for all markers except for cleaved caspase 3, where Tris–HCl–EDTA (pH 8) was used. The immunostained sections were photographed using a Leica DM4000 microscope and an Olympus DP70 camera. The digital photomicrographs were analyzed using ImageJ.

2.4. Western blotting

Samples were prepared as described previously [21]. Briefly, proteins were isolated from morphologically normal mucosa of the distal small intestine, representing approximately 40% of the total small intestine. For proteinase and phosphatase inhibition, 0.4 mM leupeptin, 3.0 μM pepstatin and 1.0 mM PMSF (in DMSO), 0.5 M NaF and Na_3VO_4 were added to the homogenization buffer (20 mM Tris–HCl, pH 7.4; 2 mM EDTA; 10 mM EGTA; 0.25 M saccharose). For the whole mucosa lysate, 10% Triton-X was added to the homogenate, after which the sample was mixed for 20 min with 5-min intervals, followed by centrifugation (15,000×*g* 10 min at 4°C). All protein samples were stored at −70°C. Protein samples were loaded in equal concentrations to SDS-PAGE gels (6.5% gels for beta-catenin and AKT/phospho AKT, 10% gels for others). All samples were analyzed at least as duplicates. After electrophoresis, the proteins were transferred to either nitrocellulose (Hybond ECL membrane, Amersham Pharmacia Biotech) or PVDF (Hybond P membrane, Amersham Pharmacia Biotech) membrane. Membranes were incubated overnight in blocking solution containing 3.5% nonfat soya (β-catenin and lamin B analysis) or 5% nonfat milk (other analyses) in Tris-buffered saline with 0.1% Tween. Membranes were incubated with primary antibodies for 2 h or overnight with phospho-specific antibodies. Primary antibodies were anti-β-catenin (sc-7199, Santa Cruz Biotechnology), anti-phospho-β-catenin (Ser552 #9566 and Ser675 #4176, Cell Signaling Technology), anti-ERK1 (sc-94, Santa Cruz Biotechnology), anti-phospho-p44/42 (#9101, Cell Signaling Technology), anti-AKT (#9272, Cell Signaling Technology), anti-phospho-AKT (#9271, Cell Signaling Technology), anti-S6 (#2217, Cell Signaling Technology), anti-phospho-S6 (#4858, Cell Signaling Technology), anti-PDK4 (NBP1-07,047, Novus Biologicals), anti-lamin B (sc-6216, Santa Cruz Biotechnology) and anti-β-actin (A541, Sigma). For protein detection, secondary antibodies (sc-2030 and sc-2031, Santa Cruz Biotechnology) and enhanced chemiluminescence reagents ECL or ECL+ (GE Healthcare) were used. The levels of phosphorylated and total protein were analyzed using the same membrane. After incubating the membrane with a phospho-specific antibody, the membrane was stripped in stripping buffer [78 mM Tris–HCl, 2% SDS, 0.68% (v/v) mercaptoethanol] for 20 min at 65°C, followed by incubation in TBS–Tween for 20 min at 65°C and incubation in TBS–Tween for 20 min at room temperature. Blots were transferred to X-ray film (Amersham) and scanned and analyzed by GSA-800 Calibrated Imaging Densitometer and Quantity One Program (BioRad Laboratories). β-Actin and lamin B were used to control equal loading of protein samples. Phosphorylated forms of β-catenin and PDK4 were detected using Odyssey infrared imager as described by Marttinen *et al.* [21].

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