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High-fat diet feeding promotes stemness and precancerous changes in murine gastric mucosa mediated by leptin receptor signaling pathway

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Obesity increases the risk for gastric cancers. However, the occurrence and mechanisms of precancerous atrophic gastritis induced by high-fat diet (HFD) remain unclear. Here, we show that HFD-associated lipotoxicity induces precancerous lesions that are accompanied by the disruption of organelle homeostasis, tissue integrity, and deregulated expression of stemness genes in the gastric epithelium mediated by leptin receptor (ObR) signaling. Following HFD feeding, ectopic fat accumulated and expression of LAMP2A in lysosome and COX IV in mitochondria increased in the gastric mucosa. HFD feeding also led to enhanced expression of activated-Notch1 and stem cell markers Lgr5, CD44, and EpCAM. In addition, HFD-fed mice showed intracellular β-catenin accumulation in the gastric mucosa with increased expression of its target genes, Nanog, Oct4, and c-Myc. These observations were abrogated in the leptin-deficient *ob/ob* mice and ObR-mutated *db/db* mice, indicating that these HFD-induced changes were responsible for effects downstream of the ObR. Consistent with this, the expression of the Class IA and III PI3Ks was increased following ObR activation in the gastric mucosa of HFD-fed mice. Together, these results suggest that HFD-induced lipotoxicity and deregulated organelle biosynthesis confer cancer stem cell-like properties to the gastric mucosa via signaling pathway mediated by leptin, PI3K and β-catenin.
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1. Introduction

Gastric carcinoma is the second leading cause of cancer death worldwide. Since about 20% of all cancers are associated with excess of body weight [1], obesity is considered to be a critical risk factor for a variety of cancers, including gastric cancer and, in particular, gastric cardia adenocarcinoma [2,3]. The progression to gastric cancer from normal tissue typically arises on a background of atrophic gastritis, intestinal metaplasia, and dysplasia of the gastric mucosa, which may occur following deregulation of the balance between self-renewal and differentiation of stem cells [4]. However, the mechanisms by which high-fat diet (HFD) damages the gastric mucosa and affects the self-renewal of stem cells during the precancerous stage remain unknown.

The stomach is responsible for the initial digestion of dietary fat and the integration of dietary signals. The gastric epithelium is sustained by stem cells located in the isthmus of the gastric glands in the corpus and the pyloric antrum. β-catenin is a multifunctional protein involved in both maintaining physiological homeostasis and sustaining the integrity of the epithelial barrier by acting as an adherens junction molecules along with E-cadherin. β-catenin also acts as a transcriptional co-regulator to control the Wnt/β-catenin signaling that regulates the pluripotency of stem cells and cell fate decisions during development [5–7]. Target molecules of β-catenin include leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5), CD44, the epithelial cellular adhesion molecule (EpCAM), which are known as markers for cancer stem cell as well as normal stem cells [8]. Aberrant expression and intracellular localization of β-catenin leads to various diseases, including cancer. Nuclear localization and intracellular accumulation of β-catenin have been

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reported in both intestinal- and diffuse-type gastric cancer, where they are positively associated with increased metastatic potential [9]. It is therefore critically important to determine whether HFD-induced development of precancerous lesions and malignancies involves in aberrant β-catenin signaling in order to reduce disease progression in high-risk patients.

Leptin, a product of the *obese* (*ob*) gene, is primarily produced by adipocytes and acts on its receptor (ObR) in the hypothalamus to suppress food intake and increase energy expenditure [10]. Leptin signaling is mediated through Janus kinase 2 and signal transducer and activator of transcription 3 (JAK2-STAT3), phosphoinositide 3-kinase (PI3K), and extracellular signal-regulated kinase 1/2 (ERK1/2) [11]. The stomach also spontaneously produces leptin and expresses ObR [12,13]. Although high levels of serum leptin have been associated with colonic cancer [14,15], there is little direct evidence for its involvement in the onset of gastric cancer. Gastric leptin, in contrast, is thought to be involved in the development of gastric malignancies, including gastric cancer [16]. We have previously demonstrated in a gene-targeting murine model that excess leptin and leptin signaling activation in the stomach cause gastric tumors by suppressor of cytokine signaling 3 (Socs3)-deletion in the gastrointestinal epithelium [17]. More recently, we reported that the gastric mucosa in HFD-fed mice exhibits increased leptin production and the activation of leptin signaling. These mice exhibit increased expression in the gastric mucosa of ectopic molecules associated with intestinal epithelium, such as Muc2, an intestinal-type mucus protein; PLA2, a Paneth cell marker; Cdx2, a transcription factor involved in the development of intestinal epithelial cells. In contrast, HFD-fed mice exhibited decreased expression of H^+K^+ ATPase, a parietal cell marker, and of Sox2, a transcription factor in gastric epithelial cells, but these alterations have not been observed in leptin-deficient *ob/ob* or ObR mutated *db/db* mice [18]. Based on these findings, in this study, we investigated the mechanism by which dietary fat causes fat toxicity for the epithelium and organelle by assessing the expression of markers including stem cell markers, and its regulation by the leptin receptor signaling pathway.

2. Materials and methods

2.1. Animals and diets

Male C57BL/6J (wild-type: WT), *ob/ob*, and *db/db* mice (Japan SLC, Inc., Hamamatsu, Japan) were studied at 7 weeks of age. The mice were housed individually in plastic cages at 24 °C ± 1 °C with lights on from 0700 to 1900 h. The mice were provided with either control-diet (CD, 10% of calories from fat, D12450J) or a high-fat diet (HFD, 60% of calories from fat, D12492; Research Diets Inc., New Brunswick, NJ) and water *ad libitum*. Animal care and experiments were conducted in accordance with the guidelines of the Prefectural University of Hiroshima Animal Care and Use Committee.

2.2. Immunohistochemical analysis

Paraffin-embedded sections of 10% formalin-fixed tissues were stained with either hematoxylin and eosin (H&E) or immunohistochemical regents as described in our previous reports [17]. Primary antibodies (Abs) used in this study are shown in Supplemental Table 1. For immunofluorescence staining, the slides were incubated with the primary Abs and then reacted with Alexa 488 or Alexa 594-conjugated rabbit, mouse or rat IgG Ab as appropriate. Slides were mounted using ProLong Gold Antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies, Carlsbad, CA). For alkaline phosphatase staining, after reacting with the primary Abs, slides are reacted with alkaline phosphatase-conjugated secondary antibody (Histofine Simple Stain AP, NICHIREI BIOSCIENCE INC., Tokyo, Japan), developed using a substrate for alkaline phosphatase (ImmPact™ Vector Red, VECTOR Laboratories, Burlingame, CA), and for detection using a ZEISS Axio Imager 2 (Carl-Zeiss, Oberkochen, Germany). For each stomach specimen, all epithelial cells in randomly selected four high-power fields were evaluated for positivity of each stem cell marker. Three sections of the stomach from three CD- and HFD-fed mice were evaluated.

2.3. Detection of lipid accumulation in the stomach

For Oil Red O staining to evaluate lipid accumulation in the mouse stomach, the tissues fixed with 4% paraformaldehyde were embedded in a compound including fish gelatin, snap-frozen in liquid nitrogen, and sectioned on a Leica CM3050S cryostat at 5-μm thickness at −20 °C. The sections were incubated in 60% isopropanol for 1 min and then the in the Oil Red O solution (SIGMA-ALDRICH, St. Louis, MO) for 20 min, followed by counterstaining with hematoxylin.

2.4. Western blot analysis

Lysates were prepared from gastric tissues and analyzed by western blotting, according to a previously published method [17]. The Abs used in western blotting are summarized in Supplemental Table 1.

2.5. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA from murine gastric mucosa samples were extracted using RNeasy Mini Kits (QIAGEN, Valencia, CA), according to the manufacturer's protocols. cDNA was synthesized from approximately 100–200 ng RNA from 1 to 2 μg RNA from gastric mucosal cells using the ReverTra Ace® qPCR RT Kit (TOYOBO, Co., Ltd., Osaka, Japan) according to the manufacturer's protocol. qRT-PCR was carried out using the Power SYBR Green PCR Master Mix (ThermoFisher Scientific, Watham, MA) with specific primer sets (400 nM at the final concentration; Supplemental Table 2) according to the manufacturer' s protocol. Relative changes in gene expression were calculated using the ΔΔCt method, and the 18S rRNA gene was used for normalization.

2.6. Statistical analysis

The Mann-Whitney *U* test and the Kruskal-Wallis test were used to determine significant differences. A p-value of less than 0.05 was considered significant. Statistical analyses were performed using Prism software version 6 (GraphPad, San Diego, CA).

3. Results

3.1. HFD-fed mice exhibit gastric lipotoxicity and the disruption of organelle homeostasis

We recently reported that HFD feeding induces atrophic gastritis with intestinal metaplasia [18]. As reported in our previous study, HFD-fed mice exhibited dysplasia with increased chromatin condensation, nuclear enlargement, pseudostratification, and mucus-producing like cells with cellular atypia in the gastric mucosa at 20 weeks after feeding (Supplement Fig. 1A). Even at an early stage in the development of hyperplasia at 3 weeks after HFD feeding, abundant leptin was expressed in the gastric mucosa in accordance with robust phosphorylated ObR (Supplement Fig. 1B).

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