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Research Paper

Reciprocal Inflammatory Signaling Between Intestinal Epithelial Cells and Adipocytes in the Absence of Immune Cells

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Visceral fat accumulation as observed in Crohn's disease and obesity is linked to chronic gut inflammation, suggesting that accumulation of gut adipocytes can trigger local inflammatory signaling. However, direct interactions between intestinal epithelial cells (IECs) and adipocytes have not been investigated, in part because IEC physiology is difficult to replicate in culture. In this study, we originally prepared intact, polarized, and cytokine responsive IEC monolayers from primary or induced pluripotent stem cell-derived intestinal organoids by simple and repeatable methods. When these physiological IECs were co-cultured with differentiated adipocytes in Transwell, pro-inflammatory genes were induced in both cell types, suggesting reciprocal inflammatory activation in the absence of immunocompetent cells. These inflammatory responses were blocked by nuclear factor-κB or signal transducer and activator of transcription 3 inhibition and by anti-tumor necrosis factor- or anti-interleukin-6-neutralizing antibodies. Our results highlight the utility of these monolayers for investigating IEC biology. Furthermore, this system recapitulates the intestinal epithelium–mesenteric fat signals that potentially trigger or worsen inflammatory disorders such as Crohn's disease and obesity-related enterocolitis.
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1. Introduction

Crohn's disease (CD) is a recurrent inflammatory bowel disease (IBD) characterized by abdominal pain, diarrhea, weight loss, and fever. Around 40% of patients suffer from chronic inflammation in both small and large intestines, while 30% exhibit symptoms limited to the small intestine (Annese et al., 2012). Development of IBD is usually accompanied by dysregulation of intestinal epithelial cells (IECs), which function in nutrient absorption and as physical and immunological barriers to pathogen invasion (Peterson and Artis, 2014). It has been recognized for decades that the onset of CD is associated with accumulation of mesenteric white adipose tissue (WAT) (Desreumaux et al., 1999; Drouet et al., 2012; Goncalves et al., 2015; Peyrin-Biroulet et al., 2007), with CD patients exhibiting four times more adipocytes per unit area than healthy controls (Fink et al., 2012). The net effects of mesenteric fat abnormalities on CD patients, whether beneficial or harmful, have been debated. Some suggest that the balance of cell types in mesenteric fat abnormalities could exert an immunoprotective effect including M2 macrophage increase, while others stress the pathological effects based on clinical observations (Coffey et al., 2016). Emerging evidence suggests that mesentery including, the mesenteric fat tissues, could be a therapeutic target for CD by both operative and

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; CD, Crohn's disease; ChgA, Chromogranin A; CM, conditioned medium; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DSS, dextran sodium sulfate; ECM, extracellular matrix; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FGF, fibroblast growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IBD, inflammatory bowel disease; IBMX, 3-isobutyl-1-methylxanthine; IEC, intestinal epithelial cell; IFN, interferon; IL, interleukin; iPSC, induced-pluripotent stem cell; MMP, matrix metalloproteinase; Muc2, mucin 2; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; pIgR, polymeric immunoglobulin receptor; PPAR, peroxisome proliferator-activated receptor; RT-PCR, reverse transcription polymerase chain reaction; RPMI, Roswell Park Memorial Institute; R-spo1, R-spondin1; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; STAT3, signal transducer and activator of transcription 3; TER, transepithelial electrical resistance; TNF, tumor necrosis factor; UC, ulcerative colitis; WAT, white adipose tissue.

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pharmacological means. In many cases, patchy or linear mucosal ulcerations are observed just beneath WAT, which implies local pathogenic signaling between mesenteric fat and intestinal epithelium (Peyrin-Biroulet et al., 2007). In addition, several pro-inflammatory cytokines, including tumor necrosis factor (TNF) and Interleukin (IL)-6, are produced in excess by WAT in CD patients (Goncalves et al., 2015). Similarly, obesity is linked to IBD pathogenesis (Boutros and Maron, 2011; Ding et al., 2010; Ding and Lund, 2011; Goncalves et al., 2015; Zhang et al., 2012), partly due to mesenteric WAT hypertrophy. It was reported that around 38% of IBD patients are overweight (Steed et al., 2009). High-fat diet and/or associated obesity can also cause mild bowel inflammation in experimental animal models (Ding et al., 2010; Gulhane et al., 2016). Furthermore, chemically or genetically induced enterocolitis is exacerbated by diet-induced obesity (Paik et al., 2013; Teixeira et al., 2011).

Adipocyte development and fat storage may be regulated by local tissue signaling. Transcriptional cascades for adipocyte differentiation, initiated by peroxisome proliferator-activated receptor (PPAR)γ, have been investigated *in vitro* using 3T3-L1 cells or mouse embryonic fibroblasts (MEFs) (Rosen and MacDougald, 2006). Differentiated adipocytes can release free fatty acids (FFAs) in response to lipolytic stimuli such as fasting that are utilized by peripheral tissues (Fruhbeck et al., 2014). However, hypertrophied adipocytes tend to release more FFAs in the steady state, which act as lipotoxicity and can lead to insulin resistance and inflammation in many other tissues (de Luca and Olefsky, 2008). Adipocytes also secrete various cytokines, such as leptin, adiponectin, and IL-6 (Peyrin-Biroulet et al., 2007; Rosen and Spiegelman, 2006). For instance, the secretion of some pro-inflammatory adipokines including TNF and resistin is augmented in obesity and is directly brought about by β-cell dysfunction or apoptosis, resulting in the progression of type II diabetes (Dunmore and Brown, 2013). Based on these findings, it would be possible that changes in the local number and activity of adipocytes induce the inflammation of IECs in CD and obese patients as IECs are prone to external stimuli and stress (Hosomi et al., 2015; Zeissig et al., 2004), but no direct evidence for this currently exists.

Cell lines are widely used as models of the intestinal epithelial monolayer, including Caco-2 and HT-29 cells (Rousset, 1986). However, these lines are derived from cancer cells and so exhibit chromosome aneuploidy and multiple mutations (Ghadimi et al., 2000). For more physiological assays, some recent studies have attempted to establish IEC cultures (Moon et al., 2013; VanDussen et al., 2015; Wang et al., 2015), but several technical issues remain, including recapitulation of *in vivo* physiology, operational simplicity, culture stability over time, and assay throughput.

Gut epithelial organoid culture is an emerging technique for investigating the molecular biology of IECs (Sato et al., 2009, 2011; Yui et al., 2012). Organoids derived from mouse small intestine contain enterocytes, Paneth cells, goblet cells, and enteroendocrine cells, and so may better reflect enteric characteristics *in vivo*. However, it is difficult to evaluate the specific functions of IECs, such as their response to bacterial infection and interactions with other types of cells, because organoid culture is conducted in a solidified three-dimensional (3D) environment and the villus-like luminal domains exist at the organoid core (Sato et al., 2009). Therefore, to improve our understanding of intestinal epithelial biology, it is critical to establish culture methods for more accessible IEC monolayers. On the other hand, it has recently been reported that intestinal organoids can be generated from human induced-pluripotent stem cells (iPSCs) (McCracken et al., 2011). These cells have several major advantages for organoid development; there are fewer ethical issues associated with iPSC technologies compared to embryonic stem (ES) cell studies, and iPSCs can be isolated from patients as disease models for basic research as well as for regenerative medicine.

In this study, we present evidence that intestinal inflammation induced by chronic dextran sodium sulfate (DSS) administration can cause mesenteric fat accumulation and inflammation in mice. To examine potential interactions between IECs and adipocytes, we developed a simple, original method for preparing physiological IECs from physically-fractured organoids. Co-culture of these IECs with mature adipocytes revealed direct inflammatory crosstalk between these cell types in the absence of immune cells, which strongly suggests that adipose tissues themselves can be a promising therapeutic target for chronic inflammatory diseases.

2. Materials and Methods

2.1. Materials

The recombinant proteins and neutralizing antibodies for mouse TNF and IL-6 were obtained from R&D Systems. Defined fetal bovine serum (FBS) was purchased from HyClone, vitronectin was from Thermo Fisher Scientific, and insulin, dexamethazone and a protease inhibitor cocktail were from Sigma. 3-Isobutyl-1-methylxanthine (IBMX) and pioglitazone were obtained from Wako. CAPE and Galiellalactone were from Tocris and Cayman Chemical, respectively. Antibodies for villin1 (ab3304), mucin 2 (Muc2) (ab11197 for human, ab76774 for mouse), and chromogranin A (ChgA) (ab15160) were purchased from Abcam. Anti-E-cadherin antibody (3195) was from Cell Signaling, anti-lysozyme antibody (A0099) was from Dako, and anti-perilipin1 antibody (GP29) was from Progen. Secondary antibodies for immunostaining were from Jackson ImmunoResearch.

2.2. Mice and Tissues

C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). Mesenteric fat tissues from whole intestinal mesentery were isolated from male mice fed three cycles of 2.25% DSS in the drinking water for 5 days, followed by 2-week consumption of water. Mesenteric fat as well as other white adipose tissues were confirmed to float in phosphate-buffered saline (PBS) and were carefully separated from other associated tissues, including mesenteric lymph nodes and blood vessels. The tissues were rapidly immersed in RNA later (QIAGEN), and stored at 4 °C until use. Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacture's instruction. All mice were housed in specific pathogen-free conditions in the experimental animal facility of the Institute of Medical Science, The University of Tokyo. The Institutional Animal Care and Research Advisory Committee at The University of Tokyo approved all animal procedures.

2.3. Cell Culture

Primary MEFs were obtained from embryos at day 13 post-coitum, and adipocyte differentiation experiments were carried out at passage two, as described previously (Kim et al., 2007). HEK293T cells, L cells, Caco-2 cells, and MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. For differentiation, Caco-2 cells were monolayer-cultured in Transwells (Corning 3413) for over 10 days after they reached confluence. Human primary visceral pre-adipocytes were purchased from Lonza. Cell growth and adipocyte differentiation followed the supplier's protocols. The human iPSC line TkDN4-M (Takayama et al., 2010) was supplied by The University of Tokyo and was maintained as colonies in feeder-free conditions on plates coated with vitronectin (Gibco) with Essential 8 medium (Gibco). The cells were passaged every 3–4 days before reaching confluence. All cultures were incubated at 37 °C in 95% humidity with 5% CO_2.

2.4. Preparation of Conditioned Medium

L cells stably expressing human R-spondin1 and human Noggin together with (WRN CM) or without mouse Wnt3a (RN CM) were established by lentiviral infection. The plasmids for lentiviral production

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