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Defective Intestinal Mucin-Type *O*-Glycosylation Causes Spontaneous Colitis-Associated Cancer in Mice

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BACKGROUND & AIMS: Core 1– and core 3–derived mucin-type *O*-linked oligosaccharides (*O*-glycans) are major components of the colonic mucus layer. Defective forms of colonic *O*-glycans, such as the Thomsen-nouveau (Tn) antigen, frequently are observed in patients with ulcerative colitis and colorectal cancer, but it is not clear if they contribute to their pathogenesis. We investigated whether and how impaired *O*-glycosylation contributes to the development of colitis-associated colorectal cancer using mice lacking intestinal core 1– and core 3–derived *O*-glycans. **METHODS:** We generated mice that lack core 1– and core 3–derived intestinal *O*-glycans (DKO mice) and analyzed them, along with mice that singly lack intestinal epithelial core 1 *O*-glycans (IEC *C1galt1*$^{-/-}$ mice) or core 3 *O*-glycans (C3*Gnt*$^{-/-}$ mice). Intestinal tissues were collected at different time points and analyzed for levels of mucin and Tn antigen, development of colitis, and tumor formation using imaging, quantitative polymerase chain reaction, immunoblot, and enzyme-linked immunosorbent assay techniques. We also used cellular and genetic approaches, as well as intestinal microbiota depletion, to identify inflammatory mediators and pathways that contribute to disease in DKO and wild-type littermates (controls). **RESULTS:** Intestinal tissues from DKO mice contained higher levels of Tn antigen and had more severe spontaneous chronic colitis than tissues from IEC *C1galt1*$^{-/-}$ mice, whereas spontaneous colitis was absent in *C3GnT*$^{-/-}$ and control mice. IEC *C1galt1*$^{-/-}$ mice and DKO mice developed spontaneous colorectal tumors, although the onset of tumors in the DKO mice occurred earlier (age, 8–9 months) than that in IEC *C1galt1*$^{-/-}$ mice (15 months old). Antibiotic depletion of the microbiota did not cause loss of Tn antigen but did reduce the development of colitis and cancer formation in DKO mice. Colon tissues from DKO mice, but not control mice, contained active forms of caspase 1 and increased caspase 11, which were reduced after antibiotic administration. Supernatants from colon tissues of DKO mice contained increased levels of interleukin-1β and interleukin-18, compared with those from control mice. Disruption of the caspase 1 and caspase 11 genes in DKO mice (DKO/*Casp*1/11$^{-/-}$ mice) decreased the development of colitis and cancer, characterized by reduced colonic thickening, hyperplasia, inflammatory infiltrate, and tumors compared with DKO mice. **CONCLUSIONS:** Impaired expression of *O*-glycans causes colonic mucus barrier breach and subsequent microbiota-mediated activation of caspase 1–dependent inflammasomes in colonic epithelial cells of mice. These processes could contribute to colitis-associated colon cancer in humans.

Keywords: Mucin-Type *O*-Glycans; Mucus Barrier; Ulcerative Colitis; Mouse Model.

BASIC AND TRANSLATIONAL AT

Colorectal cancer may be initiated by mutations in key proto-oncogenes or tumor suppressors that occur somatically or are inherited,[1] or induced by unresolved chronic intestinal inflammation such as inflammatory bowel disease (IBD).[2] The pathogenesis of inflammation-associated cancer is less well understood relative to sporadic colorectal cancer, although components of the innate immune system and microbiota are regarded as pivotal players.[3,4]

The colonic mucus layer constitutes an innate defense barrier necessary for homeostasis of the host with the microbiota.[5,6] Mucin 2 forms the major structural basis of the mucus layer in human beings and mice.[6] Mucin 2 is composed primarily of *O*-glycans, which have 2 main subtypes known as core 1– and core 3–derived *O*-glycans.[7,8] The primary *O*-glycan structure linked to Ser or Thr is referred to

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Abbreviations used in this paper: **ASC, apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain; BM, bone marrow; C3GnT, core 3 β1,3-*N*-acetylglucosaminyltransferase; DKO, double knock-out; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IL, interleukin; iNOS, inducible nitric oxide synthase; NLR, Nod-like receptor; qPCR, quantitative polymerase chain reaction; TLR, Toll-like receptor; Tn, Thomsen-nouveau; UC, ulcerative colitis; WT, wild-type.**

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as the Thomsen-nouveau (Tn) antigen (GalNAcα-*O*-Ser/Thr).[9] The Tn antigen normally is extended to form core 1–derived glycans by the core 1 β1,3-galactosyltransferase (C1GalT1), which is expressed in most tissues,[9] or core 3–derived *O*-glycans by core 3 β1,3-*N*-acetylglucosaminyl transferase (C3GnT), which is expressed mainly in the intestine.[7]

Both ulcerative colitis (UC) and colorectal cancer tissues show defective mucin *O*-glycosylation, including exposure of Tn antigen.[10,11] Tn antigen is considered a tumor-associated carbohydrate antigen,[12] although its role in tumor development in vivo remains elusive. We previously showed that mice lacking core 3–derived *O*-glycans are susceptible to chemically induced colitis and colon cancer,[7] and that mice lacking intestinal core 1–derived *O*-glycans (intestinal epithelial cell [IEC] *C1galt1*$^{-/-}$) develop spontaneous colitis.[8] The primary defect in both strains, especially IEC *C1galt1*$^{-/-}$ mice, was impaired mucus barrier function, enabling rapid breach of the mucus layer by bacteria, which initiates colitis in a myeloid cell–dependent manner.[8] However, Toll-like receptors (TLRs), which are important for microbial recognition, are not essential for bacterial-driven disease in IEC *C1galt1*$^{-/-}$ mice because loss of TLR4, or the TLR family adaptor Myd88, does not protect these mice from developing colitis.[8]

Recently, much attention has focused on deciphering the role of mucosal inflammasomes in IBD and cancer.[3,13–15] Caspases 1 and 11 are key components of canonical and noncanonical inflammasomes, respectively, which are multiprotein complexes whose formation is dependent on diverse stimuli of microbial, environmental, or endogenous origin, and usually require additional components including members of the Nod-like receptor (NLR) family and adaptor proteins.[16] Both types of inflammasomes function to activate caspase 1 or 11, leading to proteolytic processing of pro-interleukin (IL)1β and pro-IL18, which then are secreted and promote inflammation, and/or pyroptotic cell death.[16,17] Caspase 1– and 11–dependent inflammasomes modulate inflammation and/or carcinogenesis in chemical models of colitis,[18,19] but their role in spontaneous colitis and cancer is unclear.

Here, we show that mice lacking both core 3 and intestinal core 1 *O*-glycans spontaneously develop colitis and colorectal cancer. Our results show that defective *O*-glycosylation causes impaired mucin expression and a defective mucus barrier, which leads to microbiota-driven chronic colitis and colitis-associated tumorigenesis independent of Tn antigen expression. Furthermore, we found that both colitis and colonic tumorigenesis are mediated by epithelial-derived mucosal inflammasomes.

Materials and Methods

Generation of Mice

Mice lacking core 3–derived *O*-glycans (*C3GnT*$^{-/-}$), or intestinal core 1–derived *O*-glycans (*C1galt1*$^{f/f}$;*VillinCre* or IEC *C1galt1*$^{-/-}$) were generated as described.[7,8] Mice lacking both types of *O*-glycans (DKO) were generated through crossing IEC *C1galt1*$^{-/-}$ with *C3GnT*$^{-/-}$ mice. DKO mice were crossed with mice lacking both caspase 1 and 11 (*Casp1/11*$^{-/-}$) to generate DKO mice lacking major canonical and noncanonical inflammasomes (DKO$^{\Delta infl}$). Mixed sexes were used throughout the studies because phenotypes did not show gender dependency. Broad-spectrum antibiotics to deplete the microbiota were administered as described.[8]

Tissue Preparation and Staining

Fresh tissues were harvested from mice, fixed, and processed (paraffin embedding, cryopreservation) for histology and immunostaining as described.[8] Immunohistochemical and/or epifluorescent staining was performed as described for all antibodies.[8]

Bacterial Analysis

Bacterial analysis in antibiotic-treated or nontreated mice was performed by fluorescence in situ hybridization using the universal EUB338 probe, and by qPCR of *16S ribosomal RNA* gene from genomic DNA extracted from fecal materials.[6]

Protein Extraction and Western Blot

Cell or tissue lysates were quantified, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and probed with given antibodies. Bands were detected by standard chemiluminescence and imaged for densitometry using ImageJ software (National Institutes of Health, Bethesda, MD).

RNA Extraction and Quantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from tissues, and complementary DNA was synthesized for quantitative polymerase chain reaction (qPCR) assays. Supplementary Table 1 lists the primer sequences and reaction conditions for all genes analyzed.

Organ Culture and Enzyme-Linked Immunosorbent Assay

Colon tissue pieces were incubated in media for 24 hours, and supernatants of tissues were analyzed by enzyme-linked immunosorbent assay.

Bone Marrow Chimera Studies

Bone marrow (BM) cells were collected from wild-type (WT), DKO, *Casp1/11*$^{-/-}$, and DKO$^{\Delta infl}$ mice and equal numbers were injected into the appropriate pre-irradiated groups. Mice were euthanized 9 weeks later for analysis.

Statistics

All data represent the means ± SEM unless otherwise indicated. An unpaired 2-tailed or Student *t* test or Mann–Whitney *U* test was used to assess the statistical significance of differences between 2 groups. One-way analysis of variance followed by the Bonferroni post-test was used to analyze the significance of differences among 3 or more groups. The Fisher exact test was used to determine the significance of differences in the percentage of incidence among groups.