Roles of the Gel-Forming MUC2 Mucin and Its O-Glycosylation in the Protection against Colitis and Colorectal Cancer

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Received May 6, 2012

MUC2 is the major gel-forming colonic mucin that forms the two mucus layers. Recent studies using gene-targeted mice have revealed the physiological functions of Muc2, the mouse counterpart of human MUC2, and its O-glycosylation in the colon. Muc2-deficient mice spontaneously developed colitis and colorectal cancer. As for the O-glycosylation of Muc2, conditional core 1-derived O-glycan-deficient mice in the intestines exhibited a breached inner mucus layer and spontaneously developed colitis. Similarly, core 3-derived O-glycan-deficient mice exhibited an increased susceptibility to colitis and colorectal cancer, suggesting that both core 1- and core 3-derived O-glycans on Muc2 are required for colonic protection. Mice deficient in core 2-branched O-glycans synthesized after the formation of core 1 O-glycans also exhibited increased experimental colitis. Furthermore, our recent studies using gene-targeted mice deficient in N-acetylgalactosamin-6-O-sulfoyltransferase (GlcNAc6ST)-2 revealed that sulfation of the core 2-branched O-glycans of the colonic mucins by GlcNAc6ST-2 is required for the protection against experimental colitis. Taken together, these findings demonstrate the critical roles of the MUC2 mucin and its various O-glycans in the protection against colitis and colorectal cancer. Consistently, various alterations in the expression of mucins and their O-glycosylation have been noted in clinical samples of colorectal cancer. This review focuses on the roles of the MUC2 core protein and its O-glycosylation in health and disease.

Key words MUC2 mucin; glycosyltransferase; sulfotransferase; O-glycan; colitis; colorectal cancer

1. TWO MUCUS LAYERS OF THE COLON

The luminal surfaces of the gastrointestinal tract are covered by a mucus layer composed mainly of heavily glycosylated, high molecular weight glycoproteins called mucins. In the colon, the mucus layer is mainly composed of a large gel-forming mucin, MUC2. The mucus layer in the colon comprises two layers, the firm and loose mucus layers. The thickness of the firm and loose mucus layers are 100 and 700 μm in the rat,3 respectively, and 50 and 100 μm in the mouse,25 respectively. Interestingly, the commensal bacteria in the colon exclusively reside in the loose mucus layer and cannot penetrate through the inner firm mucus layer.25 Both of the mucus layers are known to be mainly composed of Muc2, the mouse counterpart of human MUC2, but the molecular mechanism of the transition from the firm to the loose mucus layer has not been clarified.

2. ROLE OF Muc2 IN COLITIS

Histological examinations of the thickness of the adherent mucus gel on the surface of colonic mucosa using surgical specimens of ulcerative colitis (UC) and Crohn’s disease (CD), two of the most important inflammatory bowel diseases (IBD), indicated that UC but not CD is often associated with reduced mucus thickness, suggesting the possible role of mucus in the pathogenesis of IBD.3,4 The pathophysiological function of the mucus layer was assessed using Muc2-deficient mice.5 The Muc2-deficient mice developed spontaneous colitis,6 which provides solid evidence that Muc2 is critically important for the colonic protection against colitis. Interestingly, commensal bacteria make direct contact with the epithelial surface of the colon in the Muc2-deficient mice, indicating that Muc2 is required for the formation of the abovementioned firm mucus layer.25 Dextran sulfate sodium (DSS) is widely used for the induction of experimental colitis in rodents.7 In typical experiments, DSS is added to the drinking water of rodents, and the extent of colitis is assessed by the body weight, extent of bloody diarrhea and histological scores. The symptoms observed in this model resemble UC, and thus it is regarded as an UC animal model. Although the precise molecular mechanisms by which DSS induces the experimental colitis are not known, the role of commensal bacteria has been widely recognized. Indeed, a recent report showed that bacteria penetrate the inner mucus layer before inflammation in the DSS animal model, suggesting that the disruption of the inner mucus layer composed of Muc2 is the initial event occurring in the development of colitis.8

3. ROLE OF Muc2 IN COLORECTAL CANCER

Several studies have shown that expression of MUC2 is decreased in colorectal adenocarcinoma but preserved in mucinous carcinoma.9,10 Consistently, Muc2-deficient mice also spontaneously developed adenomas, first in the small intestine, which progressed to invasive adenocarcinoma, and

The author declares no conflict of interest.
in the rectum. Somatic mutations in the *adenomatous polyposis coli* (*APC*) gene cause familial adenomatous polyposis (FAP) and 80% of sporadic colon cancers. Muc2-deficient mice did not show any alterations in the WNT/β-catenin/TCF4 signaling pathway characteristic of the somatic mutation in the *APC* gene. To dissect the complex interaction between Muc2 and APC in intestinal tumorigenesis, Muc2-deficient mice were crossed with Apc<sup>1638N/−</sup> and Apc<sup>Min/+</sup> mice carrying inactivated Apc alleles. As a result, transformation initiated by the *Apc* mutation was greatly exacerbated and the tumor development was significantly shifted toward the colon as a function of mutant Muc2 gene dosage, resembling the phenotype of Apc<sup>Min/−</sup> mice treated with DSS. Moreover, the intestinal epithelial cells of the flat mucosa of Muc2-deficient mice showed signs of subclinical chronic inflammation. Therefore, continuously transmitted inflammatory stimuli in Muc2-deficient mice most likely modulated the mutant *Apc*-initiated transformation through an inflammation-related pathway.

4. ROLE OF THE *O*-GLYCAN CORE STRUCTURES OF MUC2 IN COLITIS AND COLORECTAL CANCER

The contributions of the carbohydrate moieties of the mucins to their barrier function against colitis and, in some cases, against colorectal cancer have also been recently reported. In those reports, each one of the three types of mucin-type *O*-glycan core structures (Fig. 1) has been deleted. The systemic deletion of core 1 β,1,3-galactosyltransferase (C1GalT1, also known as T-synthase) causes embryonic lethality. To determine the role of core 1-derived *O*-glycans in the intestinal epithelium, C1GalT1 conditional knockout (KO) mice were generated by crossing mice withloxP sites flanking the C1galT1 gene with intestinal epithelium-specific Cre-expressing transgenic mice, the Villin-Cre mice. The C1GalT1 conditional KO mice lacking core 1-derived *O*-glycans developed spontaneous colitis that resembled human UC. Interestingly, the same paper identified somatic mutations in the X-linked gene encoding C1GalT1-specific chaperon 1 (C1GalT1C1, also known as Cosmc) in the colonic epithelium of a subset of UC patients. Core 3 β,1,3-N-acetylgalcosaminyltransferase (C3GnT)-deficient mice lacking core 3-derived *O*-glycans were highly susceptible to experimental triggers of colitis and colorectal adenocarcinoma, which was attributed to a colon-specific reduction in the Muc2 protein. Furthermore, core 2 β,1,6-N-acetylgalcosaminyltransferase-2 (C2GnT2)-deficient mice lacking intestinal core 2-branched *O*-glycans showed an increased susceptibility to colitis without a reduction in the Muc2 core proteins, indicating that the core 2-branched *O*-glycans are required to protect the animal against colitis.

5. SULFATION OF MOUSE COLONIC MUCINS BY GLCNAC6ST-2

Colonial mucins composed mainly of MUC2 are highly sulfated in the normal mucosa but much less abundantly in colorectal cancers. To assess the physiological function of Muc2 sulfation, we examined sulfotransferases in the mouse colon. We found that the sulfotransferase GlcNAc6ST-2, which is known to be involved in the sulfation of *l*-selectin ligands in lymph node high endothelial venules (HEVs), is highly expressed in the mouse colon. We thus sought to determine whether GlcNAc6ST-2 is involved in the sulfation of colonic mucins using GlcNAc6ST-2-deficient mice. To assess the sulfation of mucin, serial sections were stained with Alcian blue at pH 1.0 because Alcian blue selectively binds to sulfated carbohydrates under this condition. In the GlcNAc6ST-2-deficient mice, the Alcian blue staining of the colon was significantly diminished, while the staining intensity with the anti-Muc2 antibody did not differ compared with that observed in the WT mice. No obvious further reduction of the staining intensity with Alcian blue was observed in the GlcNAc6ST-1 and GlcNAc6ST-2 double-deficient mice, suggesting that GlcNAc6ST-1, another sulfotransferase involved in the sulfation of *l*-selectin ligands in HEVs, is not involved in the sulfation of colonic mucins. These results indicate that the sulfation of mucins is largely mediated by GlcNAc6ST-2 in the mouse colon.

6. STRUCTURAL ANALYSIS OF SULFATED *O*-GLYCANS ATTACHED TO MOUSE COLONIC MUCINS

We next performed a carbohydrate structural analysis of the neutral and sulfated *O*-glycans of mouse colonic mucins using liquid chromatography coupled to electrospray ionization tandem mass (LC-ESI-MS/MS) spectrometry. GlcNAc6-ST-2-defined oligosaccharides were completely absent from the *O*-glycans obtained from the GlcNAc6ST-2-deficient mice. Two isomeric sulfated oligosaccharides at *m/z* 975 were found in the LC-ESI-MS/MS analysis. One of the oligosaccharides, which contains GlcNAc-sulfate, was completely absent in the oligosaccharide fraction from the GlcNAc6ST-2-deficient mice, whereas the other oligosaccharide, which contains Gal-sulfate, was present in the GlcNAc6ST-2-deficient mice at a level comparable with that in the WT mice. These results indicate that GlcNAc-6-*O*-sulfation is the predominant sulfate modification of the mouse colonic mucins and that GlcNAc6ST-2 is essential for this carbohydrate modification.
7. PROTECTIVE ROLE OF SULFATION OF COLONIC MUCINS AGAINST COLITIS

To examine the role of the colonic mucin sulfation by GlcNAc6ST-2 under pathological conditions, we examined DSS-induced experimental colitis in GlcNAc6ST-2-deficient mice.23) Seven days after 5% DSS administration, a significant increase in the CD45$^+$ leukocyte infiltration into the colon was observed in the GlcNAc6ST-2-deficient mice compared with the WT mice. Staining of the sections with an anti-F4/80 monoclonal antibody (mAb), which is specific for macrophages, and anti-Gr-1 mAb, which is specific for granulocytes, showed that significantly more macrophages and granulocytes infiltrated the proximal colon of the GlcNAc6ST-2-deficient mice than that of the WT mice. This observation suggested a protective function of GlcNAc6ST-2 against leukocyte infiltration in experimental colitis in mice.

Mice lacking a sulfate transporter, NaS1, were reported to show a decrease in mucin sulfation accompanied by an enhanced susceptibility to experimental colitis.24) However, the NaS1-deficient mice showed only a partial reduction in mucin sulfation, and no structural data for the $O$-glycans attached to the colonic mucins were reported. In contrast, the results of our study23) described above provide evidence that the GlcNAc-6-$O$-sulfation of colonic mucins was completely eliminated in GlcNAc6ST-2-deficient mice and that this sulfation is important for the barrier function of colonic mucins to prevent DSS-induced colitis, indicating a clear relationship between the structure and function of the carbohydrate moieties of colonic mucins in mice. At present, it is uncertain how sulfation of colonic mucins protects against experimental colitis in mice. One possibility is that the mucus layer formation and the bacterial residence might be affected by the lack of sulfation. Further studies using GlcNAc6ST-2-deficient mice will be required to clarify this point.

Our carbohydrate structural analysis of mouse colonic mucins indicated that sulfation preferentially occurred on the C-6 of GlcNAc residues of the core 2-branched $O$-glycans. We also detected a small amount of galactose-sulfated oligosaccharides, consistent with the RT-PCR analysis that showed that the galactose sulfotransferases, KSST and Gal3STs, were expressed in the mouse colon.25) In humans, the sulfation of MUC2 was found more abundantly on galactose residues than on GlcNAc residues.25) In agreement, the mAb 91.9H, which

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Fig. 2. Structural Characterization and Relative Abundances of the $O$-Glycans on the Colonic Mucins from WT and GlcNAc6ST-2$^{-/-}$ Mice

The relative abundance was calculated by dividing the area of each peak by the total area of the peaks detected in the LC-ESI-MS analysis. ND, not detected.
was raised against human colonic sulfomucin, recognizes the HSO₃⁻Galβ1→3(Fucol→4)GlcNAc structure.²⁰) Interestingly, this mAb binds well to normal epithelial cells in the human colon but minimally to the adjacent colon carcinoma cells in clinical specimens.²⁷) In addition, the expression of the 6-sulfosialyl Lewis³ antigen containing GlcNAc-6-O-sulfate in the normal human colonic epithelial cells was also reported to be significantly decreased in colorectal cancers, accompanied by an increase in the unsulfated sialyl Lewis³ antigen.²⁰) Furthermore, studies using rectal biopsies taken at colonoscopy from patients with ulcerative colitis indicated that the sulfation of the mucins is significantly reduced in these patients.²⁹) These clinical data suggest that the sulfation of colonic mucins plays an important role in maintaining the normal physiological function of the colon. In this regard, the observation that the colonic mucins in both humans and mice are highly sulfated is notable.

8. CONCLUDING REMARKS

Recent studies using gene-targeted mice have revealed the protective functions of Muc2, the mouse counterpart of human MUC2, and its O-glycan core structures against colitis and colorectal cancers, as summarized in Table 1. Furthermore, our recent findings demonstrate that GlcNAc6ST-2 functions as a major sulfotransferase in the sulfation of colonic mucins in mice, which serve as a mucosal barrier against inflammatory stimuli in the intestinal tract. In agreement with the phenotypes of these gene-targeted mice, various alterations in the expression of mucins and their O-glycosylation have also been noted in clinical samples. Taken together, these findings provide new insights into the importance of both the MUC2 core protein and its carbohydrate moieties in health and disease.

Acknowledgements I would like to thank Drs. Yuki Tobisawa, Jotaro Hirakawa, Yasuyuki Imai, and Minoru Fukuda for their collaborations. This work was supported in part by a Grant-in-Aid for Scientific Research, Category (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (24390018).

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