Role of Sulfated O-Glycans Expressed by High Endothelial Venule-Like Vessels in Pathogenesis of Chronic Inflammatory Gastrointestinal Diseases

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Lymphocyte homing is mediated by a cascade of adhesive interactions between circulating lymphocytes and specialized endothelial cells comprising high endothelial venules (HEVs). Sulfated O-glycans expressed on HEVs, collectively called peripheral lymph node addressin (PNAd), interact with L-selectin expressed on lymphocytes, contributing to the initial step of the lymphocyte homing. In chronic inflammatory states, PNAd is induced on HEV-like vessels but absent in non-lymphoid tissues under normal conditions. Such HEV-like vessels have been observed in various chronic inflammatory diseases including rheumatoid arthritis, lymphocytic thyroiditis, Helicobacter pylori-associated chronic gastritis, and inflammatory bowel disease (IBD), and implicated in lymphocyte recruitment in those diseases. In H. pylori-associated chronic gastritis, PNAd-expressing HEV-like vessels are induced, and the progression of chronic inflammation is highly correlated with appearance of these vessels. Furthermore, eradication of H. pylori by antibiotics resulted in disappearance of PNAd. These results indicate that inhibition of PNAd formation could have therapeutic effect by attenuating lymphocyte recruitment. In ulcerative colitis (UC), PNAd-expressing HEV-like vessels are induced, preferentially in the active phase, and T cells, particularly CD4+ T cells, are closely associated with these vessels, suggesting that T cell recruitment via PNAd-expressing HEV-like vessels plays at least a partial role in UC pathogenesis. Additionally, N-acetylgalactosamine-6-O-sulfotransferase 1 (GlcNAc6ST-1) is suggested to be a candidate to regulate PNAd induction on HEV-like vessels.

1. INTRODUCTION

Circulating lymphocytes enter the secondary lymphoid organs such as lymph nodes and Peyer’s patches to encounter foreign antigens by interacting with antigen presenting cells.1) This lymphocyte homing is mediated by a cascade of adhesive interactions between circulating lymphocytes and specialized endothelial cells comprising high endothelial venules (HEVs).2) HEV-composing endothelial cells have a characteristic cuboidal morphology and a prominent Golgi complex where unique sulfated O-glycans are synthesized.3) The sulfated O-glycans, collectively called peripheral lymph node addressin (PNAd),4) interact with L-selectin expressed on lymphocytes, contributing to the initial step of lymphocyte homing (tethering and rolling), which is further elaborated by lymphocyte chemokine-dependent activation, integrin-mediated firm attachment to the endothelium, and transmigration across blood vessels.5)

PNAd has been detected by a monoclonal antibody MECA-79,5) whose epitope has been shown to be 6-sulfo N-acetyllactosamine attached to extended core 1 O-glycans, Galβ1→4(SO3→6)GlcNAcβ1→3Galβ1→3GlcNAcα1→Ser/Thr (Fig. 1).6) Structural studies also show that 6-sulfo sialyl Lewis X on core 2 branched O-glycans, sialic acidα2→3Galβ1→4[Fucα1→3(SO3→6)]GlcNAcβ1→6Galβ1→3GlcNAcα1→Ser/Thr (Fig. 1), is present as a major L-selectin ligand on HEVs.7)

In chronic inflammatory states, PNAd is induced on HEV-like vessels but absent in non-lymphoid tissues under normal conditions.8,9) Such HEV-like vessels have been observed in various chronic inflammatory diseases including rheumatoid arthritis,10) lymphocytic thyroiditis,11) Helicobacter pylori-associated chronic gastritis,12) and inflammatory bowel disease (IBD),13,14) and implicated in lymphocyte recruitment in such diseases.

In this review, we discuss the function of sulfated O-glycans on HEV-like vessels induced in H. pylori-associated chronic gastritis and ulcerative colitis focusing on our previously published works.

2. INDUCTION OF HEV-LIKE VESSELS IN H. PYLORI-ASSOCIATED CHRONIC GASTRITIS

2.1. Pathophysiology of H. pylori-Associated Chronic Gastritis

H. pylori is a Gram-negative microaerophilic bacterium that infects over 50% of the world’s population.15) If untreated, this infection leads to chronic active gastritis and develops pyloric gland atrophy and intestinal metaplasia,
which are regarded as a condition that predispose to gastric adenocarcinoma.\(^{15,16}\)

The host responds to \(H.\) pylori infection primarily by mounting a strong neutrophilic response. Such a response contributes to gastric epithelial damage and is followed by a chronic inflammatory cell infiltrate composed of lymphocytes and plasma cells, forming mucosa-associated lymphoid tissue (MALT).\(^{17}\)

### 2.2. Induction of HEV-Like Vessels in \(H.\) pylori-Associated Chronic Gastritis

Because it has been reported that \textit{de novo} formation of HEV-like vessels, which express PNA\(D\), is associated with various chronic inflammatory diseases, we determined whether chronic inflammation caused by \(H.\) pylori infection is associated with formation of HEV-like vessels.\(^ {13}\)

To do so, gastric mucosa from patients infected with \(H.\) pylori was immunostained with monoclonal antibodies MECA-79 and HECA-452 which reacts equally well with sialyl Lewis X and 6-sulfo sialyl Lewis X capped structure on extended core 1 and core 2 branches (Fig. 1).\(^ {13}\)

Gastric mucosa derived from \(H.\) pylori-infected patients displayed HEV-like vessels expressing MECA-79 and HECA-452 antigens as well as CD31 and CD34, which are markers of vascular endothelial cells. These HEV-like vessels can potentially recruit L-selectin-expressing lymphocytes, because L-selectin: IgM chimeric protein bound to the same vessels in a functional manner.\(^ {13}\) These results indicate that \(H.\) pylori-induced inflammation is associated with formation of PNA\(D\) present on HEV-like vessels.

These results demonstrate that 6-sulfo sialyl Lewis X attached to extended core 1 O-glycans is present on HEV-like vessels, based on positive staining by MECA-79 and HECA-452 antibodies. To elaborate further the chemical nature of L-selectin ligands on these vessels, the NCC-ST-439 monoclonal antibody was used. NCC-ST-439 antibody binding has been verified for sialyl Lewis X-capped structure on Galβ1→4GlcNAcβ1→6GalNAcα1→R but not on natural core 2 branched O-glycan Galβ1→4GlcNAcβ1→6(Galβ1→3)Galα→4GlcNAcβ1→6GalNAcα1→R.\(^ {19}\) Moreover, it has not been determined whether 6-sulfo sialyl Lewis X is also recognized by this antibody. To test these possibilities, we made Chinese hamster ovary (CHO) cells expressing various types of O-glycans and stained cells with NCC-ST-439 antibody.\(^ {13}\) NCC-ST-439 antibody binds to CHO cells expressing non-sulfated and 6-sulfo sialyl Lewis X on core 2 branched O-glycans but barely to CHO cells expressing those capped structures on extended core 1 O-glycans. NCC-ST-439 antibody can also stain HEV-like vessels formed in the gastric mucosa. These combined results suggest that PNA\(D\) induced by \(H.\) pylori infection expresses 6-sulfo sialyl Lewis X on both extended core 1 and core 2 branched structures in the same manner as PNA\(D\) expressed in secondary lymphoid organs.\(^ {6}\)

#### 2.3. Increased Formation of HEV-Like Vessels Is Correlated with Progression of Inflammation

Based on the updated Sydney system,\(^ {20}\) progression of inflammation initiated by \(H.\) pylori infection is ranked in four stages from least to most severe: normal, mild, moderate, and marked. In moderate and marked stages, intestinal metaplasia frequently occurs, indicating an advanced stage of the disease. In the marked stage of inflammation (Fig. 2A, lower panels), recruitment of mononuclear cells obscures proper glands in the gastric mucosa, which contrasts with glands visible in mucosa at the mild stage (Fig. 2A, upper panels). This observation demonstrates that lymphocyte infiltration is more prominent when HEV-like vessels are more abundant.

After examining over 140 human specimens, we found that the number of HEV-like vessels, as detected by MECA-79 or HECA-452 antibodies, correlates positively with the progression of inflammation (Fig. 2B), and that more patients display HEV-like vessels as inflammation progresses (Fig. 2C). \(H.\) pylori was detected in 0%, 21%, 82% and 87% of patients in normal, mild, moderate, and marked stages of inflammation, respectively. Overall, HEV-like vessels were found in 79.2% of \(H.\) pylori infected patients.

#### 2.4. Formation of HEV-Like Vessels Requires Continuous \(H.\) pylori Infection

To determine whether formation of HEV-like vessels is correlated with \(H.\) pylori infection, gastric biopsies were obtained from 17 patients with chronic active gastritis before and after eradication of \(H.\) pylori by treatment with antibiotics and a proton pump inhibitor. Patients with moderate inflammation displayed both \(H.\) pylori and HEV-like vessels detected by MECA-79 and HECA-452 antibodies (Fig. 3A). After eradication of \(H.\) pylori, the gastric mucosa of all patients no longer displayed HEV-like vessels as assessed by MECA-79 and HECA-452 staining and showed minimum lymphocyte infiltration (Fig. 3B). These results indicate that continuous infection of \(H.\) pylori is necessary for formation and maintenance of HEV-like vessels expressing PNA\(D\). It is tempting to speculate that bacterial components such as LPS acting through Toll-like receptor-dependent pathways in the gastric epithelium, stimulate the release of cytokines, i.e., lymphotoxin (LT) α.\(^ {21}\) This effect might in turn modulate gene expression in post-capillary venules in ways that could cause their biochemical, functional, and morphological transformation by up-regulating chemokines, such as CCL19 and CCL21 that act on CCR7 receptors.\(^ {21}\)
3. INDUCTION OF HEV-LIKE VESSELS IN ACTIVE ULCERATIVE COLITIS

3.1. Pathophysiology of Ulcerative Colitis Ulcerative colitis (UC) is a chronic, relapsing inflammatory disorder affecting the colonic mucosa. Although its etiopathogenesis has not been definitively elucidated, it is currently considered an abnormal inflammatory response to intestinal microbial flora with or without components of autoimmunity.23

In UC, in addition to cryptitis/crypt abscesses, a diffuse chronic mononuclear inflammatory cell infiltrate composed mainly of lymphocytes and plasma cells in the lamina propria is almost universally present.17 It is widely accepted that these inflammatory cells use the same extravasation mechanisms operating in normal conditions, e.g. lymphocyte homing in a chronic state of so-called "physiological inflammation", but in an exaggerated and uncontrolled manner.24

3.2. Induction of HEV-Like Vessels in Colonic Mucosa with UC To evaluate the formation of HEV-like vessels in UC,13 we first examined hematoxylin and eosin (HE)-stained tissue sections of colonic mucosa obtained from patients with UC. Indeed, HEV-like vessels morphologically identical to HEVs in secondary lymphoid organs were observed within a diffuse lymphoplasmacytic infiltrate throughout the lamina propria of the colonic mucosa with UC (Figs. 4A, B), particularly in the active phase, and several lymphocytes in the lumen were attached to the luminal surface of these vessels (Fig. 4C). Moreover, in severe cases, numbers of neutrophils in addition to lymphocytes were seen attached to the luminal surface of these vessels (Fig. 4D). In addition, HEV-like vessels were detected in the T cell zone of lymphoid follicles that were occasionally observed in UC tissues; however, such vessels were more abundant in the area of active lymphoplasmacytic infiltrate in the lamina propria.

3.3. Preferential Induction of PNAd-Expressing HEV-Like Vessels in the Active Phase of UC To further charac-
terize these HEV-like vessels, immunohistochemical studies with MECA-79 and HECA-452, both of which react with carbohydrate moieties that constitute PNAd (Fig. 1), were carried out. As shown in Fig. 5 (left panels), colonic mucosa of active phase UC tissue displayed MECA-79 + and HECA-452 + HEV-like vessels, which were also positive for CD34, a marker of vascular endothelial cells. On the other hand, in remission phase UC tissue, only a small fraction of vessels was positive for MECA-79 or HECA-452 (Fig. 5, right panels).

Quantitative analysis of immunostained sections with UC made up of active (n=32) and remission (n=12) phases divided based on the UC Disease Activity Index (UCDAI) showed that the percentage of MECA-79 + HEV-like vessels among CD34 + vessels in the active phase was greater than that seen in remission phase samples with statistical significance (Fig. 6). On the other hand, the percentage of HECA-452 + HEV-like vessels among CD34 + vessels did not differ between these two phases. These results suggest that preferential induction of the MECA-79 epitope on HEV-like vessels is associated with lymphocyte recruitment to the colonic mucosa in the active phase of UC.

Given that L-selectin-IgM chimeric protein binds HEV-like vessels formed in UC in a calcium-dependent manner, these results overall suggest that preferential induction of PNAd on HEV-like vessels in the active phase of UC results in increased influx of circulating L-selectin-expressing lymphocytes into the colonic mucosa, which in turn influences disease activity assessed by the UCDAI. These results are consistent with our previous study of H. pylori-induced chronic gastritis, which demonstrated that the occurrence of PNAd-expressing HEV-like vessels is highly correlated with progression of chronic inflammation as assessed by the updated Sydney system.

3.4. Increased Transcripts Encoding GlcNAc6ST-1 in the Active Phase of UC Several glycosyltransferases and sulfotransferases function in the biosynthesis of the MECA-79 epitope, namely, core 1 extending β1,3-N-acetyl-glucosaminyltransferase (Core1-β3GlcNAcT), N-acetylglucosamine-6-O-sulfotransferase 1 (GlcNAc6ST-1), and GlcNAc6ST-2. Core1-β3GlcNAcT is a unique enzyme that...
adds a GlcNAc residue to non-reducing Gal in a core 1 moiety through a β1,3-linkage to form an extended core 1 structure. On the other hand, the HEV-specific sulfotransferase GlcNAc6ST-2 is a more critical enzyme than GlcNAc6ST-1 in sulfating non-reducing GlcNAc at the C6-position in HEVs.

To determine whether transcripts encoding the above enzymes increase preferentially in the active phase of UC, RT-PCR was then carried out. Core1-β3GlcNAcT transcripts were detected in all cases examined, regardless of disease activity (Fig. 7). On the other hand, GlcNAc6ST-1 transcripts were preferentially increased in the active phase of UC. GlcNAc6ST-2 transcripts could not be detected. These results suggest that GlcNAc6ST-1 is a key enzyme responsible for PNAd biosynthesis in colonic mucosa in the active phase of UC.

Recent studies show that LT produced by T cells is the chemokine responsible for increasing GlcNAc6ST-2 transcripts. Drayton et al. propose a possible scenario that endothelial cells express the LTβ receptor and respond to LT; GlcNAc6ST-2 is then induced in those cells and modifies the oligosaccharide side chain of the core protein in the Golgi apparatus, giving rise to the MECA-79 epitope. It is tempting to speculate that a similar mechanism may function to regulate GlcNAc6ST-1 transcripts. Currently, such a mechanism remains to be clarified.

3.5. Close Association of T Cells, Particularly CD4+ T Cells, with PNAd-Expressing HEV-Like Vessels Formed in the Colonic Mucosa with UC

In order to determine which lymphocyte population closely associates with PNAd-expressing HEV-like vessels, we undertook triple immunostaining to observe HEV-like vessels and a certain pair of lymphocytes. We then quantified the number of each lymphocyte type attached to PNAd-expressing HEV-like vessels in the active and remission phases of UC.

Using triple immunostaining, we observed that both CD3+ T cells and CD20/CD79+ B cells were associated with MECA-79+ HEV-like vessels (Fig. 8A). The number of CD3+ T cells in the lumen attached to the luminal surface per PNAd-expressing HEV-like vessel is greater than CD20/CD79+ B cells with high statistical significance (Fig. 8D). The number of CD4+ T cells is significantly greater than CD8+ T cells (Fig. 8E). The number of CXCR3+ Th1 cells and ST2L+ Th2 cells do not differ significantly (Fig. 8F). Data are presented as means ± S.E.M. ∗∗p<0.01; NS, not significant. Adapted with permission from Suzawa et al. 2016.
lymphocyte subsets simultaneously (Figs. 8A—C).\textsuperscript{14} The number of respective CD3\textsuperscript{+} T and CD20/CD79\alpha\textsuperscript{+} B cells each, CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells each, and CXCRI\textsuperscript{3} Th1 and ST2L\textsuperscript{+} Th2 cells each in the lumen attached to the luminal surface of MECA-79\textsuperscript{+} HEV-like vessels was determined. As shown in Fig. 8D, the number of T cells was significantly greater than that of B cells, and among T cell subsets, the number of CD4\textsuperscript{+} T cells was significantly greater than CD8\textsuperscript{+} T cells (Fig. 8E). The number of Th1 and Th2 cells did not differ significantly (Fig. 8F). These results suggest that T cell populations, particularly CD4\textsuperscript{+} T cells, are preferentially recruited via PNAd-expressing HEV-like vessels formed in the colonic mucosa with UC.

It is widely accepted that activated memory/effector T cells that do not express L-selectin but do express α4β7 integrin home to MALT elaborating its counter receptor MAdCAM-1 in physiological conditions.\textsuperscript{20} Here, a significant proportion of T cells, particularly CD4\textsuperscript{+} T cells, associated with PNAd-expressing HEV-like vessels. Although it has not been formally proven, one possibility is that increased recruitment and subsequent activation of L-selectin-expressing naïve T cells in inflamed tissue via PNAd-expressing HEV-like vessels may play a role in UC pathogenesis.

4. FUTURE PERSPECTIVE

In this review, we have shown that the HEV-specific sulfated O-glycans expressed by HEV-like vessels play important roles on pathogenesis of inflammatory gastrointestinal diseases such as H. pylori-associated chronic gastritis and UC. Recently, anti-adhesive therapies have received attention as effective therapeutic strategies for various inflammatory diseases.\textsuperscript{30,31} Since MECA-79 blocks the L-selectin-mediated lymphocyte homing,\textsuperscript{32} this antibody could be applicable for anti-adhesive therapy for inflammatory diseases in clinical medicine. In fact, it has been reported that MECA-79 has a significant therapeutic effect in a sheep model of asthma.\textsuperscript{33} In addition, we revealed that knocking out both GlcNAc6ST-1 and GlcNAc6ST-2 results in disappearance of the MECA-79 epitope and subsequent impaired contact hypersensitivity.\textsuperscript{30,31} Thus, future studies are of great significance to develop novel therapeutics directed to PNAd for the treatment or management of chronic inflammatory diseases. Additionally, we recently demonstrated that 6-sulfo sialyl Lewis X on N-glycans has a critical function as an L-selectin ligand and plays a role in lymphocyte homing.\textsuperscript{34} It is of great interest to determine the role of 6-sulfo sialyl Lewis X-capped N-glycans in pathogenesis of chronic inflammatory diseases as well.

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