Biochimica et Biophysica Acta 1863 (2017) 569–584

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

GPR4 deficiency alleviates intestinal inflammation in a mouse model of acute experimental colitis☆

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ARTICLE INFO

Article history:
Received 6 July 2016
Received in revised form 13 November 2016
Accepted 6 December 2016
Available online 07 December 2016

Keywords:
GPR4
Inflammatory bowel disease (IBD)
Acidosis
Endothelial cell
Inflammation

ABSTRACT

GPR4 is a proton-sensing G protein-coupled receptor that can be activated by extracellular acidosis. It has recently been demonstrated that activation of GPR4 by acidosis increases the expression of numerous inflammatory and stress response genes in vascular endothelial cells (ECs) and also augments EC-leukocyte adhesion. Inhibition of GPR4 by siRNA or small molecule inhibitors reduces endothelial cell inflammation. As acidotic tissue microenvironments exist in many types of inflammatory disorders, including inflammatory bowel disease (IBD), we examined the role of GPR4 in intestinal inflammation using a dextran sulfate sodium (DSS)-induced acute colitis mouse model. We observed that GPR4 mRNA expression was increased in mouse and human IBD tissues when compared to control intestinal tissues. To determine the function of GPR4 in intestinal inflammation, wild-type and GPR4-deficient mice were treated with 3% DSS for 7 days to induce acute colitis. Our results showed that the severity of colitis was decreased in GPR4-deficient DSS-treated mice in comparison to wild-type DSS-treated mice. Clinical parameters, macroscopic disease indicators, and histopathological features were less severe in the DSS-treated GPR4-deficient mice than the DSS-treated wild-type mice. Endothelial adhesion molecule expression, leukocyte infiltration, and isolated lymphoid follicle (ILF) formation were reduced in intestinal tissues of DSS-treated GPR4-null mice. Collectively, our results suggest GPR4 provides a pro-inflammatory role in the inflamed gut as the absence of GPR4 ameliorates intestinal inflammation in the acute experimental colitis mouse model.

1. Introduction

The pH-sensing G protein-coupled receptors (GPCRs) have emerged as a new class of receptors that are involved in sensing both local and systemic pH changes. Subsequently, these receptors have been implicated in various disease states associated with dysregulated pH homeostasis such as cancer, ischemia, metabolic acidosis, and inflammation [1–3]. Family members of the pH-sensing GPCRs include GPR4, TDAG8 (GPR65), and OGR1 (GPR68) [1–9]. These receptors are capable of sensing protons in the extracellular milieu by the protonation of several histidine residues on their extracellular domains [5,8,10]. GPR65 and GPR68 are predominately, though not exclusively, expressed on leukocytes and provide various roles in the exacerbation or amelioration of a diverse set of diseases associated with inflammation and acidosis [1,11]. GPR4, reciprocally, is highly expressed in vascular endothelial cells (ECs) and blood vessel rich tissues such as the lung, kidney, heart, and liver [9,12–14]. Recently, GPR4 has been shown to mediate EC inflammatory responses to acidosis and is central for leukocyte-endothelium interaction [15,16].

In response to extracellular acidosis (increased extracellular proton concentration), GPR4 has been reported as a pro-inflammatory mediator in a variety of ECs [15,16]. Both isocapnic and hypercapnic acidosis have been demonstrated to activate GPR4 and induce an inflammatory response in three types of primary endothelial cells, including human umbilical vein endothelial cells (HUVECs), human pulmonary artery

☆ Note: A part of the results described in this manuscript was presented as a poster at the 2015 Innovations Symposium of Kenneth Rainin Foundation "Wound healing, Repair and IBD", San Francisco, California, July 20-21, 2015. A recent version of this manuscript has been deposited into the preprint sever bioRxiv (doi: http://dx.doi.org/10.1101/059014).

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http://dx.doi.org/10.1016/j.bbadis.2016.12.005
0925-4439/

endothelial cells (HPAECs), and human lung microvascular endothelial cells (HMVEC-Ls) [15,16]. The GPR4 mediated inflammatory response to acidosis encompasses the induction of adhesion molecules such as *E*-selectin (SELE), vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1) in ECs and subsequently increases the functional adhesion of leukocytes *in vitro* [15,16]. In addition to adhesion molecules, GPR4 activation in ECs increases the expression of chemokines such as CCL20, CXCL2, and IL-8 (CXCL8) involved in the recruitment and activation of leukocytes [15,16]. Furthermore, GPR4 activity stimulates the induction of COX-2, NF-κB pathway genes, and stress responsive genes in ECs under acidic conditions. These results collectively describe GPR4 as pro-inflammatory through increasing leukocyte-EC adhesion and subsequent extravasation into inflamed tissues [15,16]. Therefore, GPR4 could potentially provide a role in the inflammatory response for host defense and the removal of pathogens or apoptotic cells in various tissues by the recruitment of leukocytes. If inflammation is not properly resolved, however, GPR4 could exacerbate inflammatory disorders.

Recently, a family of imidazo pyridine derivatives has been identified as exhibiting anti-inflammatory functions in ECs by reducing pro-inflammatory cytokine secretion, adhesion molecule expression, and leukocyte-EC adhesion through the inhibition of GPR4 [15,17,18]. In addition to chemical antagonists of GPR4, similar results were observed with use of siRNA inhibitors specifically targeting GPR4 expression [16]. Moreover, it has been shown that the expression of the GPR4 gene can be stimulated in ECs by inflammatory stresses such as cytokines (TNF-α) and reactive oxygen species (H_2O_2) [13], which commonly exist in inflammatory bowel disease.

Inflammatory bowel disease (IBD) is characterized by chronic, aberrant mucosal inflammation of the gastrointestinal tract [19]. There are two distinct disease subsets in which IBD can take form, namely, Crohn's disease (CrD) and ulcerative colitis (UC). The exact etiology of IBD is unknown, but a complex interaction between immunologic, environmental, microbiome, and genetic constituents is believed to contribute to the disease onset and continued progression. Both CrD and UC have distinct, yet overlapping clinical and histopathological features that are a result of altered mucosal homeostasis. The production of cellular metabolic byproducts contributes to an acidic inflammatory mucosal loci in IBD [20]. Indeed, an acidic inflammatory microenvironment is a hallmark of chronically inflamed tissue as numerous studies have shown that local tissue pH below 7.0, and sometimes even below 6.0, is detected in inflammatory diseases and alters cellular functions [20–25]. In addition to tissue acidosis in the gut, reports indicate that the lumen of the colon is more acidic in patients with IBD than patients without IBD [26–29]. As a result, host vasculature, leukocyte infiltrates, and stromal cells often function within an acidic tissue microenvironment and can in turn modulate the inflammatory response [20].

Inflammation in IBD is a conglomerate of gut associated pathologies, but one particular pathological hallmark is a hyper-dysregulated vascular inflammatory response in the gut [30]. Host vasculature is critical in mediating the extent of inflammation and subsequent tissue damage resulting from chronic inflammation. The inflammatory response requires the active passage of leukocytes such as neutrophils, monocytes, and lymphocytes to the site of inflammation through host vasculature. EC adhesion molecules and chemokines facilitate leukocyte complementary binding for firm adhesion and subsequent extravasation from the blood vessel wall into tissue. The endothelium therefore functions as a gate; either barring or allowing the passage of inflammatory cells into inflamed tissue. Modulating the passage of leukocytes into tissue is an ideal target for IBD therapy. Currently, anti-adhesion biologics such as natalizumab and vedolizumab are used in the clinic for IBD patients [31,32]. Even though anti-adhesion therapies have proven efficacious in the clinical remission of IBD, there have been some limitations reported. For example, cases of progressive multifocal leukoencephalopathy have been observed in patients treated with natalizumab [33,34].

We hypothesize that endothelial GPR4 expression functions as a "gatekeeper" in regulating the extent of leukocyte infiltration into the inflamed colon. In this study, we observed that GPR4 mRNA expression was increased in the inflamed colon of human IBD samples as well as in a DSS-induced experimental colitis mouse model. GPR4 deficiency reduced the overall inflammation parameters used to gauge the extent of disease severity in the acute colitis mouse model. Altogether, our study has identified GPR4 as a potential regulator of intestinal inflammation and suggests that molecular responses to the acidic microenvironment in inflamed intestinal tissues may be a novel mechanism involved in IBD pathogenesis. A similar mechanism may also exist in other inflammatory disorders.

2. Materials and methods

2.1. Dextran sulfate sodium (DSS)-induced acute experimental colitis mouse model

All experiments were carried out in 9 week old male and female wild-type and GPR4-deficient mice. GPR4 deficient mice and wild-type littermates were generated as previously described [9] and were backcrossed to C57BL/6 background for 11 generations. The mice were maintained specific pathogen-free of exogenous murine viruses, ectoparasites, endoparasites, and *Helicobacter*. Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility under environmental conditions of a 12:12 light/dark cycle, temperature maintenance at 22 ± 1 °C and relative humidity range of 30-70%. Mice were group housed in microisolator caging on corncob bedding and provided autoclaved tap water and pelleted diet (ProLab 2000, Purina Mills, St. Louis, MO) *ad libitum*. Colitis was induced by the addition of 3% (w/v) Dextran Sulfate Sodium Salt (DSS) [36,000-50,000 M.Wt, Lot# Q1408, MP Biomedical, Solon, OH] to autoclaved drinking water. Mice were treated with 3% DSS or water for seven consecutive days, with a replenishment of 3% DSS or water every two days. Mouse body weight and clinical phenotypic scores were assessed daily during the treatment period and tissue was collected at the end of the treatment period. Animal studies were performed according to the randomized block experimental designs that can increase the power and reproducibility [35]. All animal experiments were approved by the Institutional Animal Care & Use Committee of East Carolina University, Greenville, North Carolina and were in accordance with the *Guide for the Care and Use of Laboratory Animals* administered by the Office of Laboratory Animal Welfare, NIH.

2.2. Clinical scoring

Colitis severity was quantified using the clinical phenotype parameters of weight loss and fecal score which were determined daily for each mouse. The fecal score was determined using the parameters of stool consistency and fecal blood (0 = normal, firm, dry; 1 = formed soft pellet, negative hemoccult test; 2 = formed soft pellet with positive hemoccult test; 3 = formed soft pellet with visual blood; 4 = liquid feces with visual blood; 5 = no feces, only bloody mucus or empty colon upon necropsy). Presence of fecal blood was determined by the use of the Hemoccult Single Slides screening test (Beckman Coulter, Brea, CA).

2.3. Collection of tissue for histology and molecular analysis

After the seven day treatment of DSS, mice were euthanized and the entire gastrointestinal tract was removed. The colon length was measured from anus to ileocecal junction, then detached from the cecum. The colon was then washed with phosphate buffered saline (PBS) to remove fecal matter. Six, two-millimeter segments of the colon were resected commencing from the anus and moving toward the cecum and promptly snap frozen in liquid nitrogen for storage at a −80 °C