GASTROENTEROLOGY 2011;141:1334–1345

The PepT1–NOD2 Signaling Pathway Aggravates Induced Colitis in Mice

GUILLAUME DALMASSO,* HANG THI THU NGUYEN,* SARAH A. INGERSOLL,* SARAVANAN AYYADURAI,* HAMED LAROUI,* MOIZ A. CHARANIA,* YUTAO YAN,* SHANTHI V. SITARAMAN,* and DIDIER MERLIN*,‡

*Department of Medicine, Division of Digestive Diseases, Emory University, Atlanta, Georgia; ‡Veterans Affairs Medical Center, Decatur, Georgia

BACKGROUND & AIMS: The human di/tripeptide transporter human intestinal H-coupled oligonucleotide transporter (hPepT1) is abnormally expressed in colons of patients with inflammatory bowel disease, although its exact role in pathogenesis is unclear. We investigated the contribution of PepT1 to intestinal inflammation in mouse models of colitis and the involvement of the nucleotide-binding oligomerization domain 2 (NOD2) signaling pathway in the pathogenic activity of colonic epithelial hPepT1. **METHODS:** Transgenic mice were generated in which hPepT1 expression was regulated by the β-actin or villin promoters; colitis was induced using 2,4,6-trinitrobenzene sulfonic acid (TNBS) or dextran sodium sulfate (DSS) and the inflammatory responses were assessed. The effects of NOD2 deletion in the hPepT1 transgenic mice also was studied to determine the involvement of the PepT1–NOD2 signaling pathway. **RESULTS:** TNBS and DSS induced more severe levels of inflammation in β-actin–hPepT1 transgenic mice than wild-type littermates. Intestinal epithelial cell–specific hPepT1 overexpression in villin-hPepT1 transgenic mice increased the severity of inflammation induced by DSS, but not TNBS. Bone marrow transplantation studies showed that hPepT1 expression in intestinal epithelial cells and immune cells has an important role in the proinflammatory response. Antibiotics abolished the effect of hPepT1 overexpression on the inflammatory response in DSS-induced colitis in β-actin–hPepT1 and villin-hPepT1 transgenic mice, indicating that commensal bacteria are required to aggravate intestinal inflammation. *Nod2*−/−, β-actin–hPepT1 transgenic/*Nod2*−/−, and villin-hPepT1 transgenic/*Nod2*−/− littermates had similar levels of susceptibility to DSS-induced colitis, indicating that hPepT1 overexpression increased intestinal inflammation in a NOD2-dependent manner. **CONCLUSIONS: The PepT1–NOD2 signaling pathway is involved in aggravation of DSS-induced colitis in mice.**

Keywords: IBD; Mouse Model; Immune Response; Bacteria-Derived Peptides.

BASIC AND TRANSLATIONAL AT

PepT1 is a di/tripeptide transporter highly expressed in epithelial cells of the small intestine, but expressed only poorly or not at all in the colon.[1] Our group previously observed that colonic PepT1 expression is enhanced under conditions of chronic inflammation such as inflammatory bowel disease (IBD),[1] a finding that has been confirmed by other investigators.[2] One normal transport function of gut epithelial cells is the absorption of small peptides from the diet, mediated by peptide transport activity.[3,4] This is achieved by the action of an apical membrane protein termed *human intestinal H-coupled oligonucleotide transporter* (hPepT1) that co-transports peptides with H^+.[5,6] The specificity of hPepT1 is broad and includes many dipeptides and tripeptides in addition to various peptide-derived drugs.[7–13]

Commensal bacteria that colonize the human colon produce significant amounts of di/tripeptides. We previously reported that PepT1 transports the small formylated bacterial peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP).[14,15] We have since shown that other bacteria-derived peptides such as muramyl dipeptide (MDP) and L-Ala-γ-D-Glu-meso-DAP (Tri-DAP) also may be transported by hPepT1.[16,17] Small bacterial peptides occur at substantially lower levels in the small intestine compared with the colon. Interestingly, hPepT1 expression normally is restricted to the small intestine, a site in which small bacterial peptide concentrations are low, reflecting the sparse bacterial load of this tissue relative to that of the colon. Thus, the profile of hPepT1 expression along the normal human digestive tract is such that access of small bacterial peptides to hPepT1 is minimized, to reduce intracellular uptake of such peptides. We found that this normal expression pattern becomes altered in patients with chronic ulcerative colitis or Crohn's disease,[1] in whom expression of hPepT1 occurs in the colon. The transporter consequently mediates intracellular accumulation of small prokaryotic materials.

We have shown that intracellular accumulation of bacterial products such as Tri-DAP, fMLP, and MDP may trigger signals that lead to initiation of intestinal inflammatory responses.[14,16,17] Transport of fMLP into Caco2-BBE cells stimulates nuclear factor-κB and activator protein 1 (AP-1) activity, which may in turn initiate inflammatory responses in intestinal epithelial cells (IECs).[18,19] MDP and Tri-DAP also induce nuclear fac-

Abbreviations used in this paper: DSS, dextran sodium sulfate; fMLP, N-formyl-methionyl-leucyl-phenylalanine; hPepT1, human di/tripeptide transporter human intestinal H-coupled oligonucleotide transporter; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IFN, interferon; IL, interleukin; MDP, muramyl dipeptide; MPO, myeloperoxidase; mRNA, messenger RNA; NOD2, nucleotide-binding oligomerization domain 2; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNF, tumor necrosis factor; Tri-DAP, L-Ala-γ-D-Glu-meso-DAP; WT, wild-type.

0016-5085/$36.00
doi:10.1053/j.gastro.2011.06.080

tor-κB activation in Caco2-BBE cells, which is confirmed by secretion of the chemokine interleukin (IL)-8 and monocyte chemoattractant protein-1 (MCP-1).[16,17] Interestingly, mucosal IL-8 and monocyte chemoattractant protein-1 are highly expressed in intestinal regions affected by IBD. IL-8 and monocyte chemoattractant protein-1 are chemoattractants for neutrophils and monocytes, respectively. The finding that hPepT1-mediated transport of bacterial oligopeptides into IECs stimulates both IL-8 secretion[14,18] and increases neutrophil transepithelial migration[14,18] suggests a cascade of signaling events are activated upon bacterial peptide transport.

In addition to maintaining efficient physical and biological barriers, the intestinal epithelium plays a role in inducing innate and adaptive immunity. Sensing pathogens is the first step in mounting an effective immune response required for elimination of the invading organism and establishing protective immunity. Nucleotide-binding oligomerization domain (NOD)-like receptors, consisting of more than 20 related family members, are present in the cytosol and recognize intracellular ligands.[20–23] NOD1 is activated by peptides that contain a diaminophilic acid, such as the PepT1 substrate Tri-DAP, and NOD2 recognizes muramyl dipeptides including the PepT1 substrate MDP. Thus, it has been suggested that PepT1 transport activity plays an important role in determining the intracellular levels of ligands for NOD1 and NOD2, which in turn control the extent of activation of downstream inflammatory pathways.[20–23]

Based on our reports on the role played by hPepT1 in intestinal inflammation,[1,14–18,24–26] Zucchelli et al[27] tested *hPepT1* polymorphisms for association with IBD. They reported that a functional *hPepT1* single-nucleotide polymorphism (rs2297322) was associated with IBD in 2 cohorts of Swedish and Finnish patients.[27] In addition, they observed that the transport activity of hPepT1 single-nucleotide polymorphism (rs2297322) was higher compared with wild-type (WT) hPepT1.[27] Importantly, this new finding, together with previous data showing aberrant colonic PepT1 expression in IBD patients,[1,2] provides solid evidence of the pathologic relevance of intestinal PepT1.

To investigate the in vivo pathogenic role of PepT1 in intestinal inflammation, we generated 2 transgenic mouse lines in which hPepT1 expression was driven either by a β-actin promoter, which results in hPepT1 expression in all tissues, or a villin promoter, which drives hPepT1 expression specifically in IECs, and assessed inflammatory responses using murine models of colitis. The effect of NOD2 deletion on such responses in the transgenic mice was studied further to explore the potential involvement of the PepT1/NOD2 signaling pathway in colitis.

Materials and Methods

Generation of hPepT1 Transgenic Mice

The hPepT1 full-length protein was cloned using the following primers: forward: 5'-CGC CAT GGG AAT GTC CAA ATC-3'; reverse: 5'-CCC CGG TTA AGT GTC TTT GTC TAC-3'. hPepT1 then was subcloned into the pβAct-3xHA vector (which contains the β-actin promoter, see map in Supplementary Figure 1) and the pBS KS Villin MES vector[28] (which contains the villin promoter, see map in Supplementary Figure 2). Transgenic founder mice (FVB/N background) generated by pronuclear injection (Emory transgenic mouse and gene targeting core facility) were identified by polymerase chain reaction using the following primers: β-actin forward: 5'-CTC CAC CAA ACG CAG ACA CA-3'; villin forward: 5'-TCC TGT GTG CTA TCA CAG CC; reverse: 5'-AGT TCG CCA GCC TAG TGG AT-3'. Homozygous mouse lines then were generated and their genotype was confirmed by crossing the selected homozygous transgenic hPepT1 mice with WT mice to generate offspring that were 100% heterozygous for hPepT1. Experiments were performed using homozygous mice. For transgenic experiments, 2 founder lines showing similar results were used. hPepT1 transgenic mice were back-crossed 10 times with WT C57/BL6 mice and then crossed with NOD2 KO mice (C57/BL6 background) obtained from Charles River (Wilmington, MA). Histologic examinations were performed independently by pathologists from Charles River. All animal procedures were approved by the Animal Care Committee of Emory University and were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* from the US Public Health Service.

Induction of Colitis

See Supplementary Materials and Methods section.

Colonoscopy

See Supplementary Materials and Methods section.

Bone Marrow Transplantation

Bone marrow transplantation was performed as described previously.[29] Briefly, the femur and tibia were removed and stripped off all muscle and sinew, and bone marrow cells were harvested by flushing the bone cavity with basal marrow medium (Iscove's medium; Cambrex; East Rutherford, NJ). After washing with phosphate-buffered saline, bone marrow cells were resuspended in basal marrow medium. Approximately 5×10^6 cells in 50 μL were transplanted retro-orbitally. Four treatment groups with 6 animals per group were used (WT→WT, WT→hPepT1$^{+/+}$, hPepT1$^{+/+}$→WT, and hPepT1$^{+/+}$→hPepT1$^{+/+}$). Mice were given neomycin at 2 mg/mL for the first week post-transplantation. Five weeks after transplantation, we induced colitis by 3% dextran sodium sulfate (DSS) and mice were assessed daily for rectal bleeding, weight loss, and diarrhea. At the end of the experimental period, mice were killed and engraftment was verified by genotyping bone marrow cells using the following specific primers: hPepT1 forward: 5'-ACC ATA CGT TTG TGG CTC TG-3'; hPepT1 reverse: 5'-GAG GTG ACT GCT TGT CCA ATT-3'.

Broad-Spectrum Antibiotic Treatment

Mice were treated for 4 weeks with ampicillin (1 g/L; Cellgro, Manassas, VA), vancomycin (500 mg/L; plantMedia, Dublin, OH), neomycin sulfate (1 g/L; Bioworld, Dublin, OH), and metronidazole (1 g/L; MP Biomedicals, LLC, Solon, OH) as described.[30]

Myeloperoxidase Activity in the Colon

See Supplementary Materials and Methods section.

Protein Extraction and Western Blot Analysis

See Supplementary Materials and Methods section.