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Authors
Padua, David
Vu, John P
Germano, Patrizia M
et al.

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The Role of Neuropeptides in Mouse Models of Colitis

David Padua, M.D., Ph.D., **John P. Vu, B.S.**, **Patrizia M. Germano, M.D.**, and **Joseph R. Pisegna, M.D.**

Division of Digestive Diseases, Department of Medicine, David Geffen School of Medicine, David Geffen School of Medicine and Divisions of Pulmonary and Gastroenterology, Hepatology and Parenteral Nutrition, Department of Veterans Affairs, VA Greater Los Angeles Healthcare System Los Angeles, CA 90073

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IBD and mouse models of colitis

Inflammatory bowel disease (IBD) is a gastrointestinal disorder that includes ulcerative colitis (UC) and Crohn's disease (CD) [1]. Studies have shown that IBD is a multifactorial set of diseases that results from an unregulated inflammatory response to intestinal microbes and foreign antigens in genetically susceptible hosts [2]. Patients with IBD develop intestinal inflammation that often manifests as abdominal pain, bloody diarrhea, fevers and weight loss. Histologically, the inflamed intestinal mucosa of IBD patients shows immune cell infiltration, bowel wall edema, loss of goblet cells, ulcer formation and diffuse erosions. For CD, these inflammatory lesions typically involve the whole intestinal wall and can occur throughout the entire gastrointestinal tract. UC, on the other hand, is restricted to the colon and rectum and the inflammatory changes seen in tissue specimens are typically limited to the mucosal layer [1]. Research has shown that the key factors implicated in IBD pathogenesis include tumor necrosis factor alpha (TNF-a), IL-6, IL-23, and IL-12. Therapies directed against these cytokines have been partially successful in treating aspects of IBD [3, 4]. However, up to 40% of CD patients do not have a clinically relevant response to TNF-a inhibitors, but for UC patient, it may be as high as 50%. Additionally, during 12 months of continuous treatment, two-thirds of CD patients do not have a sustained clinical response to the treatment [2, 5]. Given the significant morbidity associated with IBD, researchers are working on unraveling the pathogenesis of the disease with the hope of identifying new and effective therapies. One important tool in deciphering the pathogenesis of IBD has been the research use of animal models of colitis in which new pathogenic hypotheses can be tested and new therapeutics can be investigated.

Corresponding Author: Joseph R. Pisegna, M.D., Chief, Division of Gastroenterology, Hepatology and Parenteral Nutrition, Department of Medicine, VA Greater Los Angeles Healthcare System, Professor of Medicine, Molecular, Cellular and Integrative Physiology, David Geffen School of Medicine at UCLA, Tel: 310-268-3578, Fax: 310-268-4096, ; Email: jpisegna@mednet.ucla.edu

Several varieties of mouse models of colitis have been generated to investigate the etiology of intestinal mucosa inflammatory diseases. These models can be categorized into four broad groups: genetically engineered, immune manipulated, spontaneous and erosive/chemically induced models. Each mouse model possesses unique strengths and weaknesses in the study of intestinal inflammatory diseases with no mouse model completely replicating the characteristic IBD clinical pathology. Prior to 1993, most animal models relied on chemical induction of inflammation, originally described in the 1950s and 1960s [6-8]. However, in 1993, several studies were published showing colitis models in genetically engineered mice in the absence of exogenous chemicals or erosive agents [9-11]. Over the past several decades, these mouse models have helped to reveal important details regarding the biology of intestinal inflammation.

Chemically Induced Mouse Models of Colitis

Dextran sulfate sodium (DSS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) models of colitis in mice are among the most commonly used models to study intestinal inflammation. The ease of application and the relatively low cost make these models ideal for studying a vast array of inflammatory processes.

The use of DSS dates back to 1985 with the publication of a hamster model to study UC [12]. DSS is a sulfated polysaccharide shown to induce acute, self-limiting colitis in multiple models including hamsters, rats, guinea pigs, and mice [13, 14]. Animals that are given DSS in their drinking water develop intestinal inflammation. Typically, they are given concentrations of 2-10% of DSS for brief time periods of 4-14 days [15]. The severity of colitis differs with the administration of different molecular weights of DSS such as 5kDa, 40kDa, and 500kDa [16]. This variable response may be dependent on factors related to the DSS concentration, molecular weight, duration of DSS exposure, and chemical batch, but they are related also to animal strain differences and colonic bacterial microflora. Interestingly, the DSS induced inflammation does not appear to require the presence of T cells or B cells and can even ben induced in immunodeficient animals [17, 18]. Mouse strains have shown different genetic susceptibilities to DSS-induced colitis in various studies, as Swiss Webster, C3H/HeJ are the most susceptible to DSS colitis [19], whereas, NOD/LtJ are more resistant. Melgar et al described that, in 5-day of exposure to DSS, C57BL/6 mice developed progressive chronic colitis, whereas BALB/c mice only an acute colitis which completely resolved after DSS removal [20].

The first changes related to DSS were observed after 1 day of DSS treatment and include the loss of tight junction complex and increased pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ, IL-10, and IL-12) in the colon [21]. The earliest histologic lesions observed in DSS-induced colitis are typically seen at day 3. At this time point, histology reveals loss of crypts, ulcerations and erosions, whereas, in the following days, mucosal edema, epithelial degradation, loss of goblet cells, mucin depletion, abscesses, and infiltration of neutrophils, macrophages, lymphocytes and plasma cells become recognizable [22]. On a macroscopic level, the DSS colitis model can induce weight loss, bloody diarrhea, anemia, and eventually death.

The mechanisms of action for DSS are thought to be direct toxicity to colonic epithelial cells and alteration of the epithelial barrier integrity. The DSS model is a model of injury-induced necrosis and inflammation rather than a lymphocyte-driven model of chronic disease. Additionally, an imbalance between epithelial apoptosis and proliferation has seen suggested to be implicated as an important factors in the pathogenesis of the enteric lesions [23]. As a result, colonic mucosal permeability increases and enables the penetration of large molecules as DSS. However, the manner in which DSS passes through the mucosal epithelial cell layers and induces colitis remains unclear. One hypothesis is that DSS-induced epithelial barrier dysfunction may allow the entry of luminal antigens and microorganisms into the mucosa resulting in a break of oral tolerance and an immune inflammatory response [16].

One of the advantages of DSS-induced mouse colitis model is that the histologic alterations are similar to the features of IBD in humans. As human UC, DSS colitis shows a rectal localization and marked mucosal ulcerations and edema. However, DSS colitis can also occasionally exhibit features of CD such as transmural inflammation, patchy distribution of inflammation, prominent lymphoid follicles, and fissuring ulcers [16]. The cytokine profile for DSS colitis shows the involvement of Th1, Th2, and Th17 cytokine pathways [17]. Additionally, this model can develop dysplastic lesions that resemble those observed in human UC. Furthermore, DSS-induced colitis is considered an acceptable model that can be used for translational studies investigating various therapeutic agents to treat human IBD [16]. However, the lack of a definitive role for the adaptive immune system and the intestinal microbiota in the pathogenesis of colitis has suggested that the DSS model might be more useful in investigating the mechanisms of mucosal wound repair rather than true colitis [24].

The intrarectal administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS) induces an erosive colitis. It was first used in rats and gained popularity with the use of this model to mice over the past 10-15 years [25, 26]. Neurath et al first described this procedure for inducing colitis in 2–4 months old BALB/c or SJL/J mice [27]. The mechanism of action is thought to be through the ethanol disruption of the mucosal barrier, that enables the translocation of the water soluble TNBS into the submucosal layer. TNBS, which is a hapten, is hypothesized to interact with some amino acid groups on intestinal mucosa and bacterial proteins, in such a way inducing a delayed-type hypersensitivity reaction [19]. TNBS treated animals develop diarrhea, rectal bleeding, rectal prolapse, and marked wasting. The clinical manifestations typically peak by 2-4 weeks and can be followed by partial recovery or death. Histologically, there are significant transmural immune inflammatory infiltrates, composed of macrophages, CD4 lymphocytes and neutrophils. Ulceration, edema, and loss of goblet cells can be seen by day 7. However, even at a 2 month time point, lymphoid aggregates were still present and early fibrosis can be detected [27].

The TNBS model has a characteristic cytokine profile consistent with Th1 and Th17 cells. Isolated lamina propria CD4+ T cells have been shown to produce high levels of IL-12, IL-17, TNF-alpha, IFN-gamma, IL-2, macrophage inhibitory protein-1, and lower levels of IL-4 [28]. Of note, one study showed that the severity of TNBS induced colitis can be decreased by antibodies to anti-IL-12 [27]. In TNBS chronic models of inflammation, continued increases of IL-12, IL-17 and macrophage inhibitory protein-1 were found, thus

suggesting the existence of an increased Th1/Th17 response, more consistent with a CD pathogenesis [28]. As in the DSS colitis models, different strains of mice have variable degrees of inflammation in the TNBS model as the SJL/J and BALB/c mice are highly susceptible, whereas, C57BL/6 and C57BL/10 mice are more resistant to its development [19]. Ultimately, the TNBS model has the advantage of being simple to use, inexpensive and relatively reproducible, while it mimics the CD pathophysiology.

Early Genetically Engineered Mouse Models

As noted, the genetically modified animal models of colitis have greatly expanded our understanding of gastrointestinal inflammation. One of the first models was the IL-10 knockout murine model. IL-10 was initially identified as a cytokine produced by T helper cell subset 2 (Th2) that would inhibit the activity of Th1 cells [29]. For example, *in vitro* studies found that IL-10 can suppress Th1 cytokine profile and growth of various inflammatory immune cells [30]. Defective IL-10 signaling pathway genes were found to be involved in both CD and UC, indicating that an impaired IL10 signaling is likely to be involved in the development of gastrointestinal inflammation [31-33]. In 1993, Kuhn et al published the first description of the IL-10 knockout mouse model and showed that IL10−/− mice raised in conventional housing environments are predisposed to developing spontaneous enterocolitis by 2-3 months of age [34]. The lack of evidence of intestinal inflammation in neonates suggests that the colitis is likely the result of an inappropriate response to enteric antigens [17]. Of note, IL-10 knockout mice raised in a germ-free environment did not develop colitis [35, 36]. Histologically, at 3 months the IL-10 knockout mice show multifocal lesions throughout the cecum, colon and rectum with much fewer lesions being present in the small bowel [37]. The inflammatory infiltrates were present in the mucosa and submucosa and were notable for epithelial hyperplasia, crypt abscesses and focal ulcers. By 6 months, the lesions became more severe and the majority of animals developed colorectal adenocarcinomas [37]. Clinically, the mice developed weight loss, leukocytosis, splenomegaly, and anemia. The mice were found to have higher levels of IL-1, IL-6, tumor necrosis factor-alpha, and interferon-gamma [35]. The IL10−/− murine model continues to be a powerful tool in the study of gastrointestinal inflammation.

A second genetically modified animal model was published a few years later targeting the gene for the G protein subunit Gai2 [38]. Deletion of this gene led to the development of a mucosal inflammatory process that is clinically and pathologically similar to ulcerative colitis in humans. These animals showed acute and chronic mucosal inflammation with ulcerations limited to the colon as well as the development of crypt abscesses and loss of goblet cells. Gai2 knockout mice had irregular dilatation of the colon that presented focal lesions with thickened and inflamed wall. Interestingly, one third of these knockout animals developed invasive, nonpolypoid adenocarcinomas as early as 12 weeks of age. Mechanistically, thymocytes and peripheral T cells from Gia2 knockout animals produced substantially more IL-2, IFN-gamma, and TNF-alpha [38].

Additional mouse models that have been critical in expanding our understanding of inflammatory bowel disease include the adoptive T cell models. One particularly troublesome aspect of working on IL10 knockout and other genetically engineered models is

that the time to inflammation as well as the severity of inflammation is highly variable. In the adoptive T cell models, naïve (CD4+CD45RBhigh) T cells are transferred into immunodeficient animals and can induce reliable degrees of inflammation within the gut [11, 39]. One of the main immnudeficient mice used are the the recombinase activating gene-1-deficient (RAG-/-) mice. Adoptive T cell transfers into these animals can induce pancolitis including small bowel inflammation at 6-8 weeks, a feature that is unique among the various mouse models of IBD [39]. Histological evaluation of the intestines shows hallmarks of CD pathophysiology such as transmural inflammation, epithelial cell hyperplasia, mononuclear infiltration, and villous blunting. In the more severe cases, there was also loss of crypts to inflammatory infiltrates and the formation of crypt abscesses [40].

Neuropeptides Regulating Immune Function in the GI Tract

Neuropeptides such as substance P (SP), neurotensin, neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), and somatostatin are important signaling mediators that connect the nervous system to a variety of cell types within the intestines. Both immune and non-immune cells express neuropeptide receptors and have been shown to regulate a network of genes implicated in immune regulatory processes. Studies have revealed that neuropeptides are implicated in the interaction between epithelial and immune cells to initiate and maintain inflammation. Neuropeptides can modulate the innate immune response by affecting phagocytosis, release of oxygen and nitrogen radicals, and the production of pro- and anti-inflammatory molecules. They are typically synthesized as precursors and then packaged and processed to mature neuropeptides within the secretory granules [41]. Their role in the development of colitis, however, has yet to be fully characterized. This review will highlight some of the studies aimed at identifying the role of neuropeptides in the development of IBD.

Neurotensin and Colitis

Neurotensin is a 13 amino acid bioactive peptide mainly expressed in the brain and gastrointestinal tract [42]. Within the GI tract, it has been shown via immunohistochemistry to be localized into endocrine cells and neurons of the mucosa, submucosa and muscularis layers. In addition to its actions in the brain, neurotensin acts on the cardiovascular, gastrointestinal, and central nervous systems, particularly on chloride secretion, cellular motility, and cell growth. In the intestine, neurotensin has trophic effects on the small and large bowel, pancreas and stomach. It has been shown to inhibit gastric and small bowel motility while stimulating colonic motor activity. Animal studies have shown that intravenous administration of neurotensin can cause mast cell degranulation and increase in vascular permeability, histamine and leukotrienes levels [43, 44]. Neurotensin interacts on the cell surface of both immune and epithelial cells with specific receptors (NTR1 and NTR2) that belong to the seven trans-membrane G-protein-linked superfamily [45, 46]. Using the TNBS colitis rat model, Akcan et al described a reduction in inflammation in the setting of exogenous neurotensin administration. Neurotensin treated rats had lower colitis scores and lower inflammatory cytokines, as TNF-alpha and IL-6 [47]. However, additional studies, revealed an opposite effect for neurotensin in colitis. Using a *Clostridium difficile* toxin model of inflammation, Castagiuolo et al showed that intestinal inflammation could be

inhibited by the administration of a neurotensin receptor antagonist. Colonic explants exposed to either *Clostridium difficile* toxin or neurotensin had an increase in mast cell degranulation, which could be inhibited by the neurotensin receptor inhibitor [48]. Further evidence for neurotensin as a promoter of colitis comes from studies that used genetically modified animals. Koon et al induced TNBS colitis in neurotensin knockout mice and showed a reduced inflammatory response and lower levels of inflammatory cytokines. In this study, neurotensin signaling in mesenteric fat cells led to inflammatory cytokines secretion and macrophage infiltration [49]. Differences in neurotensin effects in colitis might be due to the specific experimental design. However, given its increase in animal models of colitis and in IBD patients, it appears that neurotensin could actively participate in the inflammatory process [50]. Further research will be necessary to determine whether neurotensin can serve as a theraupeutic target or biomarker for IBD treatment.

Vasoactive Intestinal Polypeptide (VIP) and Colitis

The neuropeptide vasoactive intestinal peptide (VIP) is a 28 amino acid peptide originally isolated from the small intestine of pigs in 1970 [51]. VIP, initially found in the lungs and gastrointestinal tract, is often associated with neurons and nerve fibers (48). Within the gastrointestinal tract, VIP is located in all the layers of the colonic mucosa with the highest concentration in the myenteric plexus [52, 53]. VIP is part of the secretin and glucagon family of peptides and has a 68% homology with the pituitary adenylyl cyclase-activating polypeptide (PACAP) neuropeptide. VIP and PACAP bind to their specific G protein-coupled receptors VPAC1 and VPAC2 [54]. Through these receptors, VIP is able to perform a broad array of functions such as regulating gastric acid and intestinal secretions, enzymes release from the exocrine and endocrine pancreas, cellular motility, vasodilation, and intestinal contractility [55, 56]. Additionally, VIP has been shown to modulate T cell and macrophage development as well as cytokine production. VIP can regulate the Th1/Th2 balance by altering their regulatory cytokines [57, 58]. Given its role in immune cell regulation, VIP has been investigated also in animal models as a potential modulator of colonic inflammation; however, the results of several studies have not been consistent. Abad et al in 2003, using a TNBS mouse model of colitis, reported that exogenous VIP administration reduced the clinical and histopathologic severity of the disease and mitigated weight loss, diarrhea, and intestinal inflammation [59]. However in 2005, Newman et al using the same TNBS murine model, demonstrated that prophylactic or therapeutic intraperitoneal treatment with VIP did not affect the clinical and histological inflammatory parameters [60]. More recently, the development of VIP and VPACs genetically engineered mouse models has allowed a better characterization of the endogenous VIP pathways in colitis. VIP knockout animals were described by Lelievre et al to have altered intestinal anatomy at baseline with distorted colonic crypts, cell proliferation anomalies, increased apoptosis, and altered permeability [61]. Yusta et al also reported their findings on VIP knockout animals and showed abnormal villous structure, increases in the crypt compartment and cell proliferation within the small bowel. Notably, these changes were not seen by antagonizing VIP in wildtype animals and surprisingly, they were not reversed by exogenous administration of VIP in the knockout animals [62]. These results suggest that VIP has a role in early development of the gut and in certain contexts is not easily reversible.

In 2011, Yadav et al analyzed the role of VPACs receptors using specific knockout mice and the DSS-induced colitis model. While VPAC2 receptor knockout mice on T-cells were shown to develop a worse course of DSS-induced colitis, VPAC1 knockout were seen to be resistant to colitis [63]. Recent studies by Vu et al [64], using the DSS colitis model, revealed that VIP knockout mice, and wild type mice treated with a VIP or VPAC1 antagonist, became resistance to colitis and had significantly reduced levels of colonic inflammation and expression of inflammatory cytokines. In 2015 Abad et al, using the TNBS model in VIP knockout mice, showed a resistance to colitis and observed lower levels of TNF-alpha and IL-6 inflammatory cytokines as compared to WT littermates [51]. Finally, in 2015 Wu et al evaluated a VIP knockout animal using dinitrobenzene sulfonic acid (DNBS) or DSS treatments and found that they developed more severe colitis. In an elegant study, the knockout animals were treated with exogenous VIP, which rescued the phenotype [65]. Overall, these seemingly controversial results highlight the complexity of VIP signaling and the context dependent signaling that is at play in each of these models.

Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) and Colitis

Immunological over-responsiveness to unknown stimuli has been listed as a possible cause of IBD. The key modulators in IBD are considered the mucosal, antigen-activated T cells that have a Th1 cytokine phenotype in CD and mainly a mixed Th1/Th2 phenotype in UC [1]. The colonic mucosa is abundantly innervated by fibers containing neuropeptides; they are known to play an important role in the digestive motility and secretory functions, but their effects on the gut immune responses are still obscure. Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) is the last discovered neuropeptide in the VIP, secretin, and glucagon family of peptide hormones. Three types of receptors have been identified for PACAP: VPAC1 and VPAC2, that are both coupled to only adenylyl cyclase (AC), whereas the other receptor, PAC1, has a 1000-fold selectively higher affinity for PACAP and can couple to both AC and phospholipase C (PLC) [66]. It was previously shown that PACAP and PAC1 are abundantly expressed in the myenteric plexus of normal colonic mucosa, and that PAC1 are expressed in normal human, rodent and Jurkat T cells (unpublished data), in which PACAP induces cAMP increase, inhibits tyrosine phosphorylation, IL-2 release and mitosis [67]. Also, it was recently shown that PAC1 can enhance the T cell receptor (TCR) intracellular signaling, via Gaq and PLCγ coupling, causing Ca^{2+} increase, IL-2 release and proliferation. PACAP potently regulates the immune system through the activation of PAC1 [68], however, the role of PACAP and its receptor, PAC1, in inflammatory colitis is unclear and deserves further investigation.

Discussion and Clinical Implications

Experimental models of colitis are critical in identifying new and promising therapies to tackle the course of IBD. Given the multifactorial nature of IBD's pathophysiology, researchers will continue to rely on complex model systems to understand the mechanisms behind IBD and develop better future therapies. Indeed, the development of the Smad7 antisense therapy, the latest in novel therapeutic approaches to treat IBD, has relied heavily on mouse models of colitis [69]. Smad7 is part of the TGF-beta signaling pathway and functions as an inhibitor which prevents the signaling to the nucleus from activated TGF-

beta receptors to initiate transcription of various downstream targets [70]. Researchers have found that IBD patients expressed high levels of TGF-beta, whose signaling pathway is known for inhibiting immune cell function [71]. This seemingly contradictory finding was explained by the finding that IBD patients also express higher levels of the inhibitory Smad7 [72]. The expression of high Smad7 levels inhibit the TGF-beta signaling and its immune suppressor functions. Researchers have then decreased the expression of Smad7 by using an antisense molecule [73]. This novel therapeutic approach was tested in a mouse TNBS colitis model and found to efficiently restore active TGF-beta signaling and reduce TNBS-induced colitis severity [73]. Recently, the results of a phase 2 clinical trial that were published, showed in active CD patients impressive results with a significant percentage of patients treated with Smad7 antisense therapy achieving clinical remission [74]. These results, along with many others, highlight the importance of experimental colitis models in IBD research.

As described above, neuropeptides play complex roles in immune cell regulation. Many studies highlight the context-dependent nature of neuropeptide signaling and its role in regulating the development and function of immune cells and their cytokine profiles. Future research will be necessary to elucidate the exact nature of neuropeptide signaling in IBD pathogenesis. As evidence mounts that neuropeptides are implicated in immune cell dysregulation, therapies targeting these neuropeptides may be called upon to regulate immune inflammatory pathways in IBD. Indeed, preclinical studies have already shown the efficacy of certain neuropeptide inhibitors in ameliorating colitis phenotypes. In colitis animal models, the neurotensin receptor inhibitor SR-48,692 was able to suppress the induced colonic secretion, mucosal permeability, and histologic changes associated with Clostridium difficile toxin A induced colitis [48]. In addition, VIP antagonists were similarly able to reduce colitis in animal models. DSS colitis induced C57Bl6 mice treated with VIPHyb, a broad spectrum VIP antagonist which inhibits mainly VPAC1, but also VPAC2 and PAC1 receptors, or with PG 97-269 which selective inhibits only VPAC1 receptors, were found to have significantly reduced inflammatory parameters of colitis and decreased levels of pro-inflammatory cytokines such as IL-1, TNF-alpha, and IL-6 [64]. Further studies are needed to better understand the nature of neuropeptide signaling in IBD. Animal models of colitis, neuropeptide knockout animals, and neuropeptide antagonists will be critical to this process. It is foreseeable that novel neuropeptide antagonists and possibly antisense molecules targeting specific neuropeptides might be developed as future IBD therapies.

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