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Nerve Fiber Outgrowth Is Increased in the Intestinal Mucosa of Patients With Irritable Bowel Syndrome

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BACKGROUND & AIMS: Mediators released by the intestinal mucosa of patients with irritable bowel syndrome (IBS) affect the function of enteric and extrinsic sensory nerves, which can contribute to the development of symptoms. Little is known about the effects of mucosal mediators on intestinal neuroplasticity. We investigated how these mediators affect the phenotypes of colonic mucosa nerve fibers, neuron differentiation, and fiber outgrowth. **METHODS:** We analyzed mucosal biopsy samples collected from 101 patients with IBS and 23 asymptomatic healthy individuals (controls). We measured levels of neuronal-specific enolase, growth-associated protein 43, nerve growth factor (NGF), and tyrosine kinase receptor A (NTRK1) by immunohistochemistry and enzyme-linked immunosorbent assay. Primary rat enteric neurons and human SH-SY5Y cells were incubated with supernatants from the mucosal biopsies and analyzed by morphometric and polymerase chain reaction analyses. **RESULTS:** Compared with mucosal tissues of controls, mucosa from patients with IBS had a significant increase in the area of lamina propria occupied by neuronal-specific enolase-positive (57.7% increase) and growth-associated protein 43−positive fibers (56.1% increase) and staining density of NGF (89.3% increase) ($P < .05$ for all). Levels of NGF protein were also increased in tissues from patients with IBS vs controls (18% increase; $P = .16$) along with levels of NTRK1 (64% increase; $P < .05$). Mucosal supernatants from tissues of patients with IBS induced higher levels of neuritogenesis in primary culture of enteric neurons, compared with controls, and more NGF-dependent neuronal sprouting in SH-SY5Y cells. **CONCLUSIONS:** Nerve fiber density and sprouting, as well as expression of NGF and NTRK1, are significantly increased in mucosal tissues of patients with IBS. Mucosal mediators participate to these neuroplastic changes.

Keywords: Mast Cells; Enteric Nerves; Nerve Growth Factor; Growth-Associated Protein 43.

BASIC AND TRANSLATIONAL AT

The irritable bowel syndrome (IBS) is characterized by abdominal pain/discomfort and bowel habit changes occurring in the absence of an organic cause. IBS represents one of the most common gastrointestinal disorders, with an overall prevalence estimated around 10%−15% in Europe and the United States.[1] The pathogenesis of IBS remains poorly understood, but data suggest that symptoms can arise as a variable combination of psychological factors (eg, anxiety and depression), changes in intestinal motor function, and increased visceral perception.[2] There has been recent remarkable advancement in the understanding of the basic mechanisms underlying bowel dysfunction in patients with IBS.[2] The fact that a subgroup of patients develop IBS after an acute episode of bacterial or viral gastroenteritis strongly supports the involvement of microbiological/organic factors in the pathogenesis of IBS.[3] Attention has also been directed to the complex interplay among gut microbiota,[4] mucosal barrier,[5] enteroendocrine cells,[6] immunocytes, and enteric nerves.[7,8] Abnormal release of bioactive factors in the intestinal milieu, including serotonin,[9] histamine, and mast cell tryptase,[10,11] has been shown to have an impact on gut nerve intrinsic/extrinsic activity in a receptor-dependent manner. Mucosal extracts obtained from the colon of IBS patients adoptively transferred to naïve animals or human tissues increased intestinal submucous neuron excitability,[12] mesenteric sensory nerve activity,[11] and visceral or somatic sensitivity.[13,14] The long-term effect of these and other neuroactive substances on the enteric nervous system has yet to be studied, but might induce nerve plasticity with potential consequences for bowel physiology and symptom generation. A previous study by Akbar et al[15] showed that the overall density of mucosal innervation as well as substance P and transient receptor potential vanilloid type-1 (TRPV-1) nerve staining was increased in patients with IBS compared with controls. Interestingly, TRPV-1 staining was significantly correlated with abdominal pain perception.[15] A previous study supports the concept that neurotrophic factors contribute to visceral hypersensitivity in IBS.[16] Nerve growth factor (NGF) evokes nerve fiber growth and pain transmission through its interaction with the

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Abbreviations used in this paper: **IBS, irritable bowel syndrome; IBS-C, irritable bowel syndrome with constipation; IBS-D, irritable bowel syndrome with diarrhea; IBS-M, irritable bowel syndrome with mix of constipation and diarrhea; NGF, nerve growth factor; GAP43, growth-associated protein 43; NSE, neuronal-specific enolase; RA, retinoic acid; TRPV-1, transient receptor potential vanilloid type-1; NTRK1, tyrosine kinase receptor A.**

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preferred receptor tyrosine kinase receptor A (NTRK1).[17] Mucosal mast cells might be key players in this scenario because their increased vicinity to nerve terminals correlates with the severity and frequency of abdominal pain in IBS.[10] In addition, mast cells are proficient natural producers of factors affecting nerve function and growth, including NGF and brain-derived neurotrophic factor (BDNF).[18–20] In addition, mucosal mast cell infiltration in the colon was associated with an NGF-dependent increase in nerve fiber density and synaptogenesis in the neonatal maternal deprivation model of IBS.[21]

We hypothesized that increased tissue infiltration of mast cells in the colonic mucosa of IBS patients induces neuroplastic changes and neuronal sprouting via the release of NGF. In order to test this hypothesis, we assessed nerve fiber density, nerve sprouting (as assessed by the quantification of mucosal growth-associated protein 43 [GAP43]), NGF amount, and the preferential NGF receptor NTRK1 expression in patients with IBS and controls. We also evaluated the impact of IBS mucosal mediators and NGF on neuronal differentiation in a human neuroblastoma cell line and in primary cultures of rat enteric nervous system.

Materials and Methods

Subjects and Patients

IBS patients were diagnosed according to Rome III criteria.[1] Controls were recruited by public advertisement or from asymptomatic patients undergoing colonoscopy for colorectal cancer screening or follow-up of polyposis. All patients and subjects included in the study were seen at the Department of Medical and Surgical Sciences of the University of Bologna. We enrolled a total of 23 controls (12 were female; mean age was 42.9 years; range, 20–70 years) and 101 Rome III IBS patients (67 were female; mean age was 40 years; range, 18–69 years). All the experiments involved samples from at least 5 controls and 10 IBS patients. To reduce the number of experimental conditions and to identify a clear-cut bowel habit phenotype, we included mainly patients with IBS with diarrhea (IBS-D) (n = 54) and with constipation (IBS-C) (n = 40). For the experiments with primary cultures of rat myenteric neurons (see Primary Cultures of Rat Myenteric Neurons and SH-SY5Y and Supplementary Material), we also included 7 IBS patients with a mixture of constipation and diarrhea (IBS-M). Exclusion criteria included the use of nonsteroidal anti-inflammatory drugs, corticosteroids and mast cell stabilizers, tricyclic antidepressant, or serotonin selective reuptake inhibitors, major abdominal surgery, celiac disease (excluded by detection of anti-transglutaminase and anti-endomysial antibodies), allergic diseases (family and personal history and specific anti-IgE antibodies), asthma and other organic or severe psychiatric disorders as assessed by history taking and appropriate consultations and laboratory tests. Patients and controls gave written informed consent and the study protocol was approved by the local Ethic Committee and was conducted in accordance with the Declaration of Helsinki.

In all cases, we obtained 10 mucosal biopsies from the proximal descending colon during colonoscopy. Two biopsies were fixed in buffered 10% formalin and processed for H&E histology to exclude microscopic colitis and to perform immunohistochemistry (see Immunohistochemistry). Four biopsies were used to obtain mucosal mediators (see Mucosal Mediator Release) and 4 biopsies were used for protein extraction.

Mucosal Mediator Release

Spontaneous release of mucosal mediators was obtained using a method described previously.[10] Briefly, upon removal, 4 mucosal biopsies were rapidly immersed in hard plastic tubes containing 1 mL Hank's solution (Gibco BRL-Life Technologies, Carlsbad, CA) heated at 37°C, and continuously oxygenated with a mixture of 95% O_2 and 5% CO_2. After 25 minutes incubation, samples were centrifuged at 200*g* for 10 minutes and 400–600 μL of the bathing solution were collected, filtered with a 0.22-μm syringe filter and stored at −20°C. At the end of the release experiment, biopsies were blotted and weighed.

Nerve Growth Factor Protein Assay

NGF content was quantified in homogenized colonic biopsies using an enzyme immunoassay kit according to manufacturer's instructions (Emax Immuno Assay System, Promega, Milano, Italy). The NGF ImmunoAssay System is designed for sensitive and specific detection of NGF in an antibody sandwich format (<3% cross-reactivity with other neurotrophic factors). For the NGF assay, biopsy specimens from 17 controls and 46 patients (14 IBS-C and 32 IBS-D) with IBS were homogenized using tissue protein extraction reagent with the addition of a protease inhibitor cocktail (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions and stored at −80°C until assay. Protein content was measured using a Coomassie blue–based colorimetric assay (Bradford assay; Bio-Rad Laboratories, Hercules, CA).

Immunohistochemistry

Immunohistochemistry was performed by application of validated protocols in use in our laboratory (see Supplementary Material). The quantification of immunoreactivity was performed by 2 operators in a blind fashion using a Leitz (Weitzlar, Germany) Orthoplan microscope (25× objective) equipped with a computer-assisted analysis system (Cytometrica software; C&V, Bologna, Italy), by slight modifications of a method published previously[10] (Supplementary Material).

Western Blot Analyses

Protein expression of GAP43 was evaluated by Western blot analysis in rat primary cultures of myenteric neurons exposed to controls or IBS supernatants (Supplementary Material).

Primary Cultures of Rat Myenteric Neurons and SH-SY5Y

Primary cultures of rat enteric nervous system were obtained using previously validated methods (Supplementary Material). For morphometric analyses on primary cultures, cells were plated on glass coverslips and were maintained for 24 hours in Neurobasal-B27 medium containing biopsy supernatants from controls or IBS patients diluted 1/2. Cells were then fixed in 4% paraformaldehyde, immunostained for class III beta-tubulin (Tuj1), and analyzed for neuronal morphometry. For protein expression analyses, cells were plated in 24-well dishes and maintained in culture at 37°C in a humidified chamber with 95% O_2:5% CO_2 for 14 days. Half of the medium was replaced every