The Colonic Tissue Levels of TLR2, TLR4 and Nitric Oxide in Patients with Irritable Bowel Syndrome

Erdem Koçak¹, Erdem Akbal², Seyfettin Köklü³, Bilal Ergül⁴ and Murat Can⁵

Abstract

Objective Irritable bowel syndrome (IBS) is a highly prevalent and debilitating functional disorder. The toll-like receptors (TLRs) are a family of pathogen-recognition receptors in the innate immune system. In the present study we aimed to investigate the TLR2, TLR4 and nitric oxide (NO) levels in patients with IBS.

Methods Fifty-one IBS patients and 15 healthy controls were included in the present study. Colonic tissue levels of TLR2, TLR4 and NO were detected using an enzyme-linked immunosorbent assays (ELISA) and through biochemical methods.

Results The colonic tissue levels of TLR4 and NO were significantly higher in IBS patients than in healthy controls. A subgroup analysis, which was based on the presence of diarrhea and constipation, showed that TLR2 levels were significantly higher among individuals with diarrhea-predominant IBS than among constipation-predominant IBS patients and healthy controls. The TLR4 levels were significantly higher in the diarrhea-predominant IBS patients and constipation-predominant IBS patients than in comparison healthy controls. The colonic tissue levels of NO were higher in the constipation-predominant IBS patients than in the diarrhea-predominant IBS patients and healthy controls.

Conclusion In the present study we found that the colonic tissue levels of TLR and NO were elevated in IBS patients. Our results support the presence of a degree of immune dysregulation and oxidative stress in patients with IBS.

Key words: irritable bowel syndrome, toll-like receptors, innate immunity, nitric oxide

(Intern Med 55: 1043-1048, 2016)  
(DOI: 10.2169/internalmedicine.55.5716)

Introduction

Irritable bowel syndrome (IBS), which is characterized by abdominal pain, discomfort, bloating and the alteration of bowel habits, is one of the most common disorders of the gastrointestinal tract (1). The quality of life and social functioning are negatively affected in patients with IBS. In most countries, IBS affects 5-11% of individuals the population from 20 to 45 years of age, with a female predominance. The prevalence of IBS varies according to country and based on the criteria used to define IBS (2). At present, the exact pathogenesis and etiological factors of IBS are not clearly understood. Although investigations into the pathogenesis of IBS have focused on alterations in gastrointestinal motility and visceral hypersensitivity, no predominant pattern of motor activity has emerged as a marker for IBS. Recently, some authors have shown the presence of low-grade inflammation in patients with IBS.

The toll-like receptors (TLRs) are a recently discovered family of pattern recognition receptors, which show homology with the Drosophila Toll protein and the human interleukin-1 receptor family. The TLRs are members of the pattern recognition receptor (PRR) family and play a central role in the innate immune response in the mucosa (3). These receptors recognize pathogen-associated molecular patterns (PAMPs) and transduce the signals that are required for an effective innate immune response. The ability of the TLRs
to recognize diverse microbial molecules enables the host to rapidly detect the presence of pathogens, before the dissemination of infection (4). Increasing evidence shows that TLR expression and activation is specially regulated in the gastrointestinal (GI) tract and that this mechanism may lead to the continuous presence of physiological microflora in the gut.

Nitric oxide (NO) is a free radical which is produced from the oxidation of the terminal guanidine nitrogen of arginine, by a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzyme, NO synthase (NOS). NO has many biological functions including the regulation of vascular tone, platelet activation, and its action as a neurotransmitter in non-adrenergic, non-cholinergic innervations (5). GI exhibits smooth muscle responses to stimulation of the nonadrenergic noncholinergic inhibitory nerves and some authors have recently suggested that NO acts as an inhibitory neurotransmitter in this system (6).

A review of the current literature revealed that there is limited information on the pathophysiological role of TLRs and NO in patients with IBS. Furthermore, in most of the studies, the TLRs were evaluated in patients with IBS without a subgroup analysis. In the present study, we aimed to evaluate the colonic tissue levels of TLR2, TLR4, and NO in patients with IBS and IBS subgroups and to compare the levels with healthy subjects.

### Materials and Methods

The aim and the content of the study were explained to all of the patients and a written consent document was obtained. The study was approved by the clinical research ethical committee of Ankara Education and Research Hospital and was performed in accordance with the National Institute of Health guidelines. Fifteen healthy controls and 51 IBS patients (age: 18-65 years) who satisfied the Rome III criteria for the diagnosis of IBS were enrolled in the present study. The control group was selected from healthy subjects who underwent colonoscopy due to a family history of colon cancer. Laboratory samples were obtained on the morning of the colonoscopy procedure. The following parameters were included in the laboratory examination: white blood count (WBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and thyroid stimulation hormone (TSH). The exclusion criteria were: <18 or >65 years of age, post-infectious IBS, mixed IBS (alternating diarrhea and constipation), or a diagnosis of inflammatory bowel disease, celiac disease, lactose intolerance, thyroid disease, or diabetes mellitus. Clinical information was obtained from patient records and personal interviews. The following parameters were evaluated: age, sex, height, weight and body mass index (BMI).

The patients took 90 mL of the phospho-soda on the night before the colonoscopy procedure. A doctor, who was experienced in colonoscopy, performed a full colonoscopy procedure in all of the IBS patients and controls. In total, six biopsies were taken from the normal-appearing mucosa in the sigmoid colon and rectum. The biopsy was performed with a single-use 2.8 mm biopsy forceps (Endojaw). Tissue samples were homogenized with phosphate buffered saline using a glass Teflon homogenizer (Ultra Turrax IKA T18 Basic) after cutting the tissues into small pieces with scissors (for 2 min at 5,000 rpm). The homogenate was then centrifuged at 5,000×g for 15 min. The supernatant was used for the analysis. The protein concentrations of the supernatants were determined by the methods of Lowry et al. (7). The tissue NO levels (nitrite+nitrate) were measured by a spectrophotometer at 545 (Shimadzu, Tokyo, Japan) after the conversion of nitrate to nitrite by copperized cadmium granules (8). The tissue levels of TLR2 and TLR4 were measured by solid phase sandwich enzyme-linked immunosorbent assays (ELISA) using ELISA kits (Cusabio, Wuhan, China) in accordance with the manufacturer’s protocol. The standard ranges of TLR2 and TLR4 are 0.32-20 ng/mL and 0.156-10 ng/mL, respectively. The sensitivity of the TLR2 and TLR4 assay for the samples was 0.08 ng/mL and 0.04 ng/mL, respectively.

The statistical analyses were performed using the SPSS 15.0 software program. Continuous variables are shown as the mean ± standard deviation or the median (range), while the categorical variables are expressed as a percentage. The Shapiro-Wilk test was used to investigate whether or not the continuous variables showed a parametric distribution. Student’s t test or the Mann-Whitney test were used to assess whether the significance of the differences. The significance of the linear correlation between the continuous variables was evaluated with the Spearman correlation test. The chi-square test was used for the categorical comparisons. A p value of <0.05 was considered to be statistically significant.

### Results

Table 1 shows the main demographic and laboratory data for all of the subjects. There were no significant differences between the healthy controls and the IBS patients with respect to age, gender, height, weight, BMI and laboratory test results, including the serum levels of WBCs, ESR, CRP and TSH (Table 1).

In the IBS patients, the colonic tissue levels of TLR4 were significantly higher than in healthy controls (IBS...
Figure 1. Colonic tissue TLR-4 and NO levels of patients with IBS were significantly higher than control group (p<0.01). Colonic tissue TLR-2 levels were not different between healthy control and patients with IBS.

Table 2. Comparison of Demographic and Laboratory Data of Healthy Control and IBS Subgroups.

<table>
<thead>
<tr>
<th></th>
<th>IBS-diarrhea (n: 20)</th>
<th>IBS-constipation (n:31)</th>
<th>Control group (n: 15)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>38.85 ± 11.11</td>
<td>38.93 ± 10.14</td>
<td>37.90 ± 10.47</td>
<td>0.78</td>
</tr>
<tr>
<td>Gender</td>
<td>14 F / 6 M</td>
<td>29 F / 2 M</td>
<td>11 F / 4 M</td>
<td>0.21</td>
</tr>
<tr>
<td>Height</td>
<td>161.95 ± 9.4</td>
<td>158.13 ± 6.6</td>
<td>165.20 ± 10.15</td>
<td>0.13</td>
</tr>
<tr>
<td>Weight</td>
<td>70.50 ± 8.9</td>
<td>65.80 ± 8.6</td>
<td>70.50 ± 5.16</td>
<td>0.18</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.93 ± 2.6</td>
<td>26.53 ± 3.2</td>
<td>25.96 ± 2.05</td>
<td>0.36</td>
</tr>
<tr>
<td>WBC</td>
<td>10,121 ± 1,381</td>
<td>7,632 ± 1,800</td>
<td>7,300 ± 2,152</td>
<td>0.36</td>
</tr>
<tr>
<td>ESR</td>
<td>14.84 ± 12.71</td>
<td>19.12 ± 13.42</td>
<td>14.75 ± 6.2</td>
<td>0.38</td>
</tr>
<tr>
<td>CRP (mg/dL)</td>
<td>0.45 ± 0.29</td>
<td>0.43 ± 0.31</td>
<td>0.47 ± 0.2</td>
<td>0.87</td>
</tr>
<tr>
<td>TSH (u/mL)</td>
<td>1.41 ± 0.65</td>
<td>2.14 ± 2.09</td>
<td>2.08 ± 1.2</td>
<td>0.72</td>
</tr>
</tbody>
</table>

The IBS patients were divided into two subgroups according to their bowel habits: 39.2% of the patients had IBS with diarrhea (IBS-D; female, n=14; male, n=6), while 60.8% of the patients had IBS with constipation (IBS-C; female, n=29 female; male, n=2). There were no significant differences between the subgroups of the IBS patients and the healthy controls in terms of age, gender, height, weight, BMI and laboratory test results (Table 2).

The colonic tissue levels of TLR4 in the IBS-D and IBS-C subgroups were significantly higher than those in the control group (IBS-D, 3.01±2.12; IBS-C, 2.44±2.4; Control group, 1.38±0.54) (Fig. 2). There was no significant difference between the IBS-D and IBS-C subgroup in the tissue levels of TLR4. In the IBS-D subgroup, the colonic tissue levels of TLR2 were significantly higher than in patients of the IBS-C subgroup and the healthy controls (IBS-D group, 6.89±5.19; IBS-C group, 4.10±2.93; Control group, 4.02±2.09) (Fig. 2).

Before the subgroup analysis, we found that the colonic tissue levels of NO were significantly higher in IBS patients than in healthy controls. Interestingly, different results were obtained after the subgroup analysis. The colonic tissue levels of NO were found to be significantly higher in the pa-
tients of the IBS-C subgroup than in the IBS-D subgroup and the healthy controls (IBS-C, 61.40±35.18; IBS-D, 45.15±22.30; Control group, 38.44±14.19) (Fig. 2). These results indicate that there was an association between high tissue levels of NO and constipation.

Discussion

In the present study, the colonic tissue levels of TLR4 and NO were found to be significantly higher in IBS patients than in healthy subjects. Interestingly, a subgroup analysis revealed different results for patients with constipation-dominant and diarrhea-dominant IBS. The levels of TLR4 and NO levels were higher in the patients with constipation-dominant IBS than in healthy controls. In addition we found that patients with diarrhea-dominant IBS had significantly higher tissue levels of TLR2 and TLR4 in comparison to healthy controls.

Contrary to popular belief, IBS is a functional bowel disease, not a psychosomatic disorder. It has been widely accepted that the bowel dysfunction in patients with IBS is caused by changes in the nerves and muscle that control the sensation and motility of the bowel. However, the exact mechanism involved in the pathophysiology of IBS is not clearly understood. Increasing evidence shows the presence of low-grade immune activation in IBS patients (9). A Swedish study showed the presentation of B-cell antigens in IBS patients was associated with an altered capacity for providing co-stimulation to T cells (10). In another study from the same center, Ohman et al. suggested that the level of T cell activation increased in patients with IBS due to the presence of low-grade immune activation and that this may be also be associated with IBS symptoms (11). In patients with IBS, the over-activation of the hypothalamic-pituitary-adrenal axis and the elevation of pro-inflammatory cytokines (including IL-6 and IL-8) were demonstrated (12). In light of these studies most of the authors suggested that alterations of the immune system and the elevation of the pro-inflammatory cytokines have an important role in the pathogenesis of IBS.

The activation of the TLRs has been suggested to play a critical role in cytokine production. The colonic microbiota is one of the most diverse communities in the gut and it is a major source of immune stimulation. Kassinen et al. showed that the fecal microbiota of patients with IBS differed significantly to that of healthy subjects (13). Alterations of the fecal microbiota in IBS patients may cause the expression and activation of the TLRs. Furthermore, some studies addressed the presence of low-grade inflammation in the colonic mucosa of IBS patients (14-16).

To date, only a small number studies have investigated the relationship between the TLRs and IBS. The first study, in which the author suggested that the upregulation of TLR4 in IBS patients may contribute to occurrence of IBS, was published in 2008 (17). In another study from the same center, the author evaluated the role of the TLR4 and the TLR4 signal transduction pathway in the pathogenesis of diarrhea dominant IBS. They concluded that the upregulation of

Figure 2. Colonic tissue Toll-like 4 levels of patients with IBS-D and IBS-C group were significantly higher than control group. Patients with IBS-D, colonic tissue Toll-like 2 levels were significantly higher than IBS-C and healthy control. Colonic tissue NO levels of patients with IBS-C were significantly higher than IBS-D and healthy control.
TLR4 might contribute to occurrence of diarrhea dominant IBS (18). Three recent clinical studies evaluated the role of the TLRs in the pathogenesis of IBS. The first study evaluated the expression of TLRs in colonic biopsy samples of IBS patients. Furthermore, they showed increased levels of TLR4 and TLR5 and decreased levels of TLR7 and TLR8 in IBS patients (19). In the second study the authors investigated the activity of various TLRs on the peripheral blood cells. In the present study, the authors demonstrated that TLR2 and TLR4 induced the production of TNF-alpha in IBS patients (20). In the most recent study, which was published by Belmonte et al., the authors demonstrated the significant upregulation of TLR2 and TLR4 in the colonic mucosa of IBS patients (21). Interestingly within these trials, only one study performed a subgroup analysis of IBS patients. Similarly to the last study, we also performed a subgroup analysis. In our study, the colonic TLR4 levels were elevated in both the IBS-C and IBS-D subgroups; however, the TLR2 levels were only elevated in the IBS-D subgroup.

The association between TLR2 and TLR4 and colonic inflammation has previously been reported in patients with inflammatory bowel disease (22, 23). The ability of TLRs to recognize a broad spectrum of microbial molecules enables the host to rapidly detect the presence of pathogens (4). TLR2 and TLR4 appear to recognize several different microbial molecules. It has been shown that some virulence factors, including the presence of fimbriae and enterotoxins activated TLR2 and TLR4 (19-23). Stress has been shown to increase the motility and sensation of the colon to a greater degree in IBS patients than in healthy individuals without IBS. In the literature, some authors have suggested that up to two thirds of IBS patients have a psychiatric disorder (24, 25). In addition it has been shown that chronic stress modulates the immune system through TLR4-mediated phosphoinositide 3-kinase/Akt signaling (26). Alterations of fecal microbiota and chronic stress may cause the expression and activation of TLR4 in patients with IBS.

TLR2 recognizes a wide array of microbial molecules representing broad groups of species such as Gram-positive and Gram-negative bacteria. Ozdil et al. showed that microscopic colitis and focal active colitis was significantly increased in patients with diarrhea-dominant IBS (27). Moreover prospective studies have shown that 3-36% of enteric infections lead to post-infectious IBS and that it usually manifests in the diarrhea-dominant form. Although the exact pathogenesis of post-infectious IBS is unknown, several factors have been implicated, including persistent changes in the gastrointestinal microbiota, mucosal immunocytes and enterochromaffin cells (28). The elevated tissue levels of TLR2 in the patients of the IBS-D subgroup may lead to persistent, low-grade colonic inflammation. Regarding our results, high TLR2 levels could be used as a potential biomarker for diarrhea-dominant IBS and might reflect a different pathogenesis. In addition the elevated TLR4 levels of both subgroups supports the presence of low-grade inflammation in IBS patients.

The finding of elevated tissue levels of NO in IBS patients was the second important result of our study. After the subgroup analysis we found that it was related with the high tissue levels of NO in the IBS-C subgroup. NO, which is generated by the oxidation of L-arginine by inducible NOS (iNOS), is an important effector molecule in the host defense mounted by the immune system. During inflammation, NO is elevated due to the upregulation of iNOS (29). The nonadrenergic-noncholinergic (NANC) inhibitory nerves are responsible for most of the nerve induced relaxations of gastrointestinal muscle. NO has recently been shown to be a neurotransmitter in the NANC inhibitory nerves in the gastrointestinal tract. Furthermore, some authors have suggested that NO might have an inhibitor role in the NANC system (30, 31).

In one study from Japan, the authors showed that the colons of patients with slow-transit constipation were more strongly innervated by nitric oxide. The authors concluded that the elevation of NO levels mediated the NANC inhibitory nerves and played an important role in the pathogenesis of patients with slow-transit constipation (32). Visceral hypersensitivity may be a major potential pathophysiological factor in irritable bowel syndrome. Kuiken et al. evaluated the effect of NO synthase inhibitor NG-monomethyl-L-arginine in patients with IBS and suggested that NO significantly increased the threshold for discomfort/pain and that it may be involved in the pathophysiology of the visceral hypersensitivity of IBS patients (33). At present, the relationship between IBS and NO is not clear. However, published studies have shown that elevated levels of NO can cause colonic dismotility, pain and discomfort in patients with IBS. In our study we found that elevated levels of NO in the colonic tissue of patients with IBS-C. According to our results we suggested that in patients with IBS-C, high levels of NO might be lead to colonic dismotility, discomfort and constipation.

In conclusion, our findings support the presence of immune dysregulation and oxidative stress in patients with IBS. In the future, investigators may focus on the role of TLRs and oxidative stress in the pathogenesis of IBS. In addition larger studies with subgroup analyses are required to reveal the pathological differences between the IBS subtypes.

The authors state that they have no Conflict of Interest (COI).

References