Abstract. The present study aimed to explore the correlation between cytokine expression of tumor necrosis factor α (TNF-α), interleukin (IL)-8 and IL-10 with occludin production, abdominal symptoms and psychological factors in patients with irritable bowel syndrome-associated diarrhea (IBS-D). A total of 42 IBS-D patients and 20 healthy controls were included, which were recruited from QiLu Hospital in China. ELISA and immunohistochemical analysis were performed for evaluating the cytokines (TNF-α, IL-8 and IL-10) and occludin protein levels in the peripheral blood mononuclear cells (PBMCs) of all subjects. In addition, the abdominal symptoms and psychological status were assessed in IBS-D patients. Levels of TNF-α and IL-8 in the PBMCs of patients with IBS-D were significantly higher than those in the controls (P<0.001 and P=0.007, respectively), while IL-10 levels were significantly reduced in patients with IBS-D (P=0.047). Occludin production was significantly reduced in patients with IBS-D as compared with that in the controls (P<0.001). In patients with IBS-D, levels of TNF-α and IL-8 were negatively correlated with occludin levels (r=-0.34, P=0.028; r=-0.52, P<0.001, respectively). IL-10 showed a negative correlation with occludin production (r=0.05, P=0.748). Furthermore, TNF-α, IL-8 and IL-10 levels were significantly correlated with symptoms scores (r=0.74, P<0.001; r=0.55, P<0.001; r=-0.80, P<0.001, respectively) in patients with IBS-D. Within the IBS-D group, TNF-α expression was significantly increased in patients with a self-rating depression scale (SDS) score ≥50 (P=0.016, P=0.008, respectively) as compared with that in patients scoring <50. In conclusion, the results of the present study suggested that in IBS-D, an imbalance of cytokine production evoked colonic epithelial barrier dysfunction, abdominal symptoms and psychological disorders.

Introduction

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder with symptoms including abdominal pain, frequent diarrhea, constipation and altered bowel habits (1). Based on the predominant symptoms, IBS is classified as IBS-associated diarrhea (IBS-D), constipation-predominant IBS (IBS-C) and IBS with alternating stool pattern (IBS-A) (2). IBS is a common clinical problem and has affected 8 to 20% of the general population worldwide (3). To date, great progress has been made in exploring the mechanism underlying IBS. One mechanism underlying the pathophysiology of IBS is based on changes in immune activity. The imbalance of pro- and anti-inflammatory cytokines contributes to the development of IBS (4). An accumulation of the pro-inflammatory cytokine tumor necrosis factor α (TNF-α) and a decreased production of interleukin 10 (IL-10) have been reported in IBS patients (4,5). Interleukin 8 (IL-8) is a modulator in recruitment and activation of polymorphonuclear neutrophils. The protein levels of IL-8 were found to be elevated during the development of IBS (6). In addition, proteasome-mediated protein degradation may be another factor leading to IBS. Furthermore, increasing occludin degradation was shown to contribute to the development of IBS (7). However, few studies have focused on the association between systemic cytokine expression and tight junction proteins in IBS.

The role of psychological factors in IBS is being increasingly recognized. IBS patients commonly show psychological symptoms, including anxiety, depression and fatigue (8,9). In one study, patients with IBS with increased fatigue and depression were shown to have an elevated mass of mast cells in the caecal mucosa (10). A significant association was made between the severity of psychological symptoms and the cellularity of the lamina propria in patients with IBS. To date, the association between cytokine expression and psychological factors has not been clearly determined.
The present study investigated the expression of the cytokines TNF-α, IL-8 and IL-10, as well as occludin in IBS-D patients and healthy controls. In addition, patients with IBS-D completed a self-report questionnaire and psychological assessment, which were used for the correlation of cytokine profiles with occludin protein production as well as abdominal and psychological symptoms in patients with IBS-D.

Materials and methods

Study population. The present study was approved by the Clinical Research Ethics Committee of Qilu Hospital of Shandong University in China and all participants or their parents signed informed consent forms prior to enrolment in the study.

A total of 42 patients with IBS-D were recruited from gastroenterology clinics at Qilu Hospital, including 15 males (mean age, 36.4±6.9 years; range, 20-57 years and 27 females (mean age, 38.1±5.9 years; range, 18-56 years) (Table I). All patients had IBS symptoms corresponding to the Rome III criteria, including abdominal discomfort or pain (11). Symptoms were required to be present for the previous six months and persist for at least three months. IBS patients undergoing more than three bowel movements per day and loose stool forms were diagnosed with IBS-D based on the Bristol Stool Form Scale (12). Patients with organic gastrointestinal disorders and acute gastrointestinal infection and organic disease (including diabetes mellitus, hepatic or renal dysfunction, and thyroid disease) were excluded. The patient cohort was selected following positive diagnosis using colonoscopy, biopsy, abdominal ultrasonography and other clinical or laboratorial tests, including blood and stool analyses followed by assessment of erythrocyte sedimentation rate, C-reactive protein, liver and renal function, thyroid function, fasting plasma glucose, calcium and electrolytes.

In addition, 20 healthy asymptomatic controls were recruited from the Department of Health Examination of Qilu Hospital, including eight males (mean age, 37.9±7.8 years; range, 21-59 years) and 12 females (mean age, 35.1±8.1 years; range, 19-60 years) (Table I). The controls were not on any medication for gastrointestinal disease and had a normal body mass index.

Isolation and culture of peripheral blood mononuclear cells. A amount of 5 ml peripheral blood were extracted from the healthy individuals and IBS-D patients following fasting for 12 h. Isolation of peripheral blood mononuclear cells (PBMCs) was performed by density gradient centrifugation at 2,000 × g for 20 min. Blood was collected into sterile heparinized tubes and mixed with 5 ml RPMI 1640 (Gibco-BRL, Invitrogen Life Technologies, Carlsbad, CA, USA). The diluted blood was treated with 5 ml Ficoll-Paque (Sigma-Aldrich, St Louis, MO, USA) and centrifuged at 2,000 × g for 20 min. The PBMCs were then washed twice with phosphate-buffered saline (PBS) and RPMI 1640 media (Gibco-BRL). The viability of PBMCs was detected by trypan blue (Gibco-BRL) staining.

Cell culture and ELISA. PBMCs were resuspended to achieve a density of 1x10⁶ cells/ml in RPMI 1640 medium with 10% fetal bovine serum, 100 U/ml penicillin (Beyotime Institute of Biotechnology, Haimen, China) and 100 µg/ml streptomycin (Beyotime Institute of Biotechnology). PBMCs were cultured at 37°C in a humidified 5% CO₂ atmosphere for 24 h. Cell-free supernatants were obtained following centrifugation at 3,000 × g for 10 min (4°C) and stored at -80°C for further investigation. The expression of TNF-α, IL-8 and IL-10 in PBMCs was measured using ELISA kits (Peprotech, Rocky Hill, NJ, USA) according to the manufacturer's instructions. Optical density (OD) was measured at a wavelength of 450 nm and the reference wavelength was corrected to 590 nm, using an automated ELISA reader (ELx800; BioTek, Winooski, VA, USA). The limit of sensitivity of the assays was 2 pg/ml.

Immunohistochemical analysis of occludin. Tissue samples from the rectosigmoid colon were obtained from all participants. Sections of colonic biopsies were fixed in 4% paraformaldehyde (Damao Chemical Reagent Factory, Tianjin, China) for 2 h at room temperature, embedded in optimum cutting temperature (OCT) compound (Fisher Thermo Scientific, Waltham, MA, USA) and subsequently frozen at -80°C. The sections of frozen colonic tissues (4 µm) were mounted on glass slides and air-dried for 30 min, then fixed with acetone (Big Alum Chemical Reagent Factory) (10 min, 4°C) and washed in PBS. Sections were treated with 3% H₂O₂ in methanol (Big Alum Chemical Reagent Factory) for 15 min and non-specific binding was blocked with 5% goat serum (Intergen, Purchase, NY, USA) for 4 h at room temperature. Then sections were incubated with rabbit monoclonal anti-occludin antibody (1:100 dilution; Zymed, Invitrogen Life Technologies) overnight at 4°C and horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (IgG) antibody (1:5,000; ZSIIQ-BIO, Beijing, China) for 2 h at room temperature. The slides were washed in PBS and stained with diaminobenzidine (DAB; Amresco LLC, Solon, OH, USA). The microphotographs were examined under a microscope (BX-51; Olympus Corp., Tokyo, Japan).

Western blot analysis. For western blot analysis, biopsy specimens from the rectosigmoid colon were immediately stored at -80°C. Protein was extracted from 100-mg colonic samples by homogenization in ice-cold lysate buffer (20 mmol/l Tris-HCl, pH 7.5; 100 mmol/l NaCl; 0.5 mmol/l EDTA; 1 mmol/l PMSF; 1 mmol/l DTT; 2 mmol/l sodium orthovanadate; 0.5% Nonider P40; and 0.1% protease inhibitor cocktail; all from Sigma-Aldrich) for 30 min at 4°C and centrifugation at 15,000 × g for 15 min at 4°C The protein content was determined by Pierce bicinchoninic acid (BCA) protein assay kit (Fisher Thermo Scientific). Protein samples (25 mg) were separated using 10% SDS-PAGE and transferred to nitrocellulose membranes (Pall Corporation, Dreieich, Germany). Membranes were blocked in 5% nonfat milk (BD Biosciences, San Jose, CA, USA) for 1 h at room temperature. The membranes were incubated with rabbit monoclonal anti-occludin (1:50 dilution; Zymed) and monoclonal anti-β-actin (1:1,000; Kangtai Biological Products Co., Ltd., Shanghai, China) overnight at 4°C. The membranes were then incubated with HRP-conjugated goat anti-rabbit IgG antibody (1:5,000; Biokangtai) for 1 h at room temperature. The expression intensity was determined by calculating the ratio of integrated optical density (IOD) between occludin and β-actin (Santa Cruz Biotechnology, Dallas, TX, USA).
Symptom questionnaire. In order to evaluate the severity of the symptoms, a self-validated report questionnaire based on the Rome III criteria was developed for the IBS-D patients according to previous studies (13,14). The questionnaire items mainly focused on the intensity and frequency of abdominal symptoms, including abdominal pain, incomplete emptying following bowel movement, three or more bowel movements per day, loose, mushy or watery stools, and urgency to move bowels. The intensity of abdominal symptoms was graded according to the criteria as follows: 0, Absence of symptoms; 1, mild symptoms; 2, moderate symptoms, which do not affect day-to-day activities; and 3, severe symptoms, which impaired day-to-day activities. The frequency scoring criterion was 0, no onset; 1, one to three times per week; 2, four to six times per week; and 3, every day. The total scores ranging from 0 to 15 were calculated as the sum of each item score of abdominal symptoms.

Psychological assessment. The Zung self-rating anxiety scale (SAS) and the self-rating depression scale (SDS) were applied to IBS-D patients according to the previous studies with slight modifications (15,16). For SDS or SAS rating, questionnaires including 20 items were filled in by the patients to rate their perceived moods and feelings. The scoring for each item was graded according to the four quantitative terms: 1, a percentage of the time; 2, some of the time; 3, a good part of the time; 4, most of the time. The scoring for SAS or SDS was performed using a multiple choice questionnaire with positive and negative answering options. Initial scores were calculated as follows: Scores obtained from the 20 items of the SDS or SAS questionnaires were summarized and multiplied by 1.25. The final score was rounded to the nearest whole score. A score < 50 was considered to be normal, a score of 50-74 was defined to indicate a mild disorder, a score of 75-87 indicated a moderate disorder and a score > 88 indicated a severe disorder. In the present study a score ≥ 50 on the anxiety or depression scale was regarded to indicate psychological distress.

Statistical analysis. Data were analyzed by SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and values were expressed as the mean ± standard deviation. The independent-sample t-test was applied for assessing the difference in cytokine expression, IOD of occludin, abdominal symptoms scores and psychological scores between IBS-D patients and normal controls. The correlation among the variables was evaluated by Pearson correlation. P<0.05 was considered to indicate statistically significant differences between values.

### Table I. Basic characteristics of patients and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
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<tbody>
<tr>
<td>Number</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Gender</td>
<td>15</td>
<td>27</td>
</tr>
<tr>
<td>Age (years)</td>
<td>36.4±6.9</td>
<td>38.1±5.9</td>
</tr>
<tr>
<td>Range (years)</td>
<td>20-57</td>
<td>18-56</td>
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</table>

*Expressed as the mean ± standard deviation.

Results

**Comparison of cytokine levels in the IBS-D and control groups.** Overall, the TNF-α and IL-8 levels (81.51±22.18 vs. 41.77±10.74 pg/ml, P<0.001; 152.21±38.10 vs. 126.19±24.12 pg/ml, P=0.007) were significantly higher in IBS-D patients than those in healthy controls (Fig. 1). However, IBS-D patients showed decreased IL-10 production compared with that in controls (87.11±22.46 vs. 98.54±16.43 pg/ml, P=0.047) (Fig. 1).

**Comparison of occludin expression between IBS-D and control groups.** Immunohistochemical analysis of occludin is shown in Fig. 2. Occludin was primarily present on the surface of colonic epithelial cells. In healthy controls, the staining of occludin was intense and continuous, while faint and discontinuous staining was observed in the colon tissue of IBS-D patients (Fig. 2A). Western blot analysis showed a protein band at ~65 kDa for occludin. Relative occludin expression in the rectosigmoid colon was lower in patients with IBS-D compared with that in healthy controls (0.38±0.14 vs. 1.24±0.39, P<0.001) (Fig. 2B).

**Correlation between cytokine production and occludin expression in IBS-D patients.** In patients with IBS-D, occludin
Correlation between cytokine production and abdominal symptoms score in patients with IBS-D. The mean abdominal symptoms score was 11.17±5.06 for IBS-D patients and was positively correlated with TNF-α and IL-8 production (r=0.74, P<0.001; r=0.55, P<0.001, respectively); however, it was negatively correlated with IL-10 production (r=-0.80, P<0.001) (Fig. 4A-C).

Psychological distress and correlation with cytokine levels in IBS-D patients. Among the 42 patients, 20 (66.7%) with an SDS score ≥50 were diagnosed with depression and 17 (40.4%) had an SAS score ≥50 and were considered to have anxiety. IBS-D patients with an SDS score ≥50 had significantly higher TNF-α (P=0.004) and IL-8 levels (P=0.008) than those with an SDS score <50. IBS-D patients with an SAS score ≥50 showed significantly higher IL-8 expression compared with those with an SAS score <50 (P=0.016). However, there was no statistical difference in IL-10 expression between patients with SAS or SDS score ≥50 and those with lower scores (P=0.385; P=0.275) (Table II).

Discussion

IBS-D, a subgroup of IBS, is common in males and females (17). Evidence showed that immune activation was an important event in the development of IBS (18). PBMCs, including lymphocytes, leukocytes and macrophages, have key roles in immune activation and have been used to investigate the specific cytokines that can indicate the disease stages (19). In the present study, levels of the pro-inflammatory cytokines TNF-α and IL-8 and those of the anti-inflammatory cytokine IL-10 were assessed in PBMCs from IBS-D patients. The results showed that TNF-α and IL-8 production were significantly greater in PBMCs from IBS-D patients than those in healthy controls, while IL-10 production was significantly decreased in IBS-D patients. These findings were consistent with the widely accepted mechanism of IBS, namely that the imbalance of cytokines involved in inflammatory response results in persisting inflammation (5). A previous study showed that the frequency of the high-producer genotype for IL-10 was significantly lower in patients with IBS compared with that in healthy ones, which may contribute to the progression of inflammation in IBS (5).

Table II. Association of cytokine production in peripheral blood mononuclear cells of patients with diarrheic irritable bowel syndrome and anxiety as well as depression scores.

<table>
<thead>
<tr>
<th>Score</th>
<th>TNF-α (pg/ml)</th>
<th>IL-8 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
</tr>
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<tbody>
<tr>
<td>SAS</td>
<td></td>
<td></td>
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<tr>
<td>≥50 (n=17)</td>
<td>86.1±23.4</td>
<td>169.3±30.7</td>
<td>82.6±25.7</td>
</tr>
<tr>
<td>&lt;50 (n=25)</td>
<td>80.0±21.1</td>
<td>142.0±36.7&quot;</td>
<td>89.2±24.3</td>
</tr>
<tr>
<td>SDS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥50 (n=20)</td>
<td>93.3±22.0</td>
<td>168.5±35.2</td>
<td>83.5±25.3</td>
</tr>
<tr>
<td>&lt;50 (n=22)</td>
<td>73.6±18.7&quot;</td>
<td>139.1±32.4&quot;</td>
<td>91.2±18.7</td>
</tr>
</tbody>
</table>

Values are presented as the mean ± standard deviation. *P<0.05 compared with SAS score ≥50; †P<0.05 compared with SAS ≥50. SAS, self-rating anxiety scale; SDS, self-rating depression scale; IL, interleukin; TNF, tumor necrosis factor.

Figure 2. Expression of tight junction protein occludin in the rectosigmoid colonal tissues of HCs and IBS-D patients. (A) Immunostaining of the tight junction protein occludin in the rectosigmoid colonal tissues of HCs and IBS-D patients (magnification, x200). Brown and yellow staining indicate positivity for occludin. (B) Western blot analysis of tight junction protein occludin in the rectosigmoid colonal tissues of HCs and IBS-D patients. Analysis showed a protein band at ~65 kDa for the tight junction protein occludin. The same experiment was repeated three times. Relative occludin expression was quantified by integral optical density is expressed as the mean ± standard deviation in HCs and IBS-D patients. *P<0.01. IBS-D, irritable bowel syndrome with diarrhea; HC, healthy control.

expression was negatively correlated with TNF-α and IL-8 production (r=-0.34, P=0.028; r=-0.52, P<0.001, respectively) (Fig. 3A and B). However, there was no correlation between occludin and IL-10 production (r=0.05, P=0.748) (Fig. 3C).
Although a previous study reported that the increasing activity of faecal serine protease from patients with HBS-D was able to induce a marked internalization of tight junction proteins (20), the present study showed that occludin was restricted to the surface of colonic epithelial cells in HCs and IBS-D patients without intensive internalization. The present study also observed the dysfunction of the tight junction protein occludin in IBS-D patients by immunohistochemistry.
and western blot analysis. The immunohistochemical staining of occludin was faint and discontinuous in IBS-D patients and occludin protein was significantly decreased compared with that in healthy controls. Recent evidence also suggested that occludin expression was decreased in IBS-D patients and the levels of occludin were negatively correlated with the intensity of symptoms (21). Occludin is one of the tight junction proteins, and an important component of intestinal mucosa. Patients with occludin disruption also present with impaired intestinal permeability and increased stool frequency (22). In the present study, reduced occludin levels indicated that the tight junction was disrupted in IBS-D patients. Therefore, although the occludin distribution appeared normal under colonoscopy and conventional histology, the intestinal mucosal barrier of the IBS-D patients had become disrupted.

It is well documented that IBS patients show an increased number of immunocompetent cells in the mucosal immune response (23). Thus, it was hypothesized that the increased mucosal immunocytes, along with elevated levels of systemic cytokines, including TNF-α and IL-8, contributed to mucosal barrier disruption. The findings of the present study indicated that occludin expression was negatively correlated with TNF-α and IL-8 production, but was not correlated with IL-10 production. In addition, the present study indicated that the mean symptom score of IBS-D patients was positively correlated with TNF-α and IL-8 production, but was negatively correlated with IL-10 production. The peripheral inflammation is characterized by elevated pain sensitivity, which is mediated by the release of inflammatory cytokines and growth factors (24). TNF-α was reported to contribute to inflammatory sensory hypersensitivity (24). Therefore, elevated levels of cytokines may be sufficient to sensitize sensory nerve terminals, which may have resulted in a decreased threshold for visceral sensitivity in IBS patients and explain the high abdominal symptoms score in patients with IBS-D.

In addition, the present study also found significant associations between psychological distress and cytokine production. IBS patients with psychological distress trended to show higher production of pro-inflammatory cytokines (TNF-α and IL-8) and lower levels of the anti-inflammatory cytokine IL-10. Similar results were reported in a recent study that indicated that the levels of pro-inflammatory cytokine IL-1β were significantly higher in IBS-D patients with anxiety and depression and that the IL-10 levels were significantly lower compared with those in IBS-D patients without anxiety and depression ones (25). These results suggested that psychological distress may cause changes in pro-inflammatory and anti-inflammatory cytokine expression in IBS-D patients, which may contribute to the occurrence or aggravation of IBS.

Psychiatric and psychological factors are closely associated with the mechanism of IBS. The effect of psychological factors on the symptoms and outcome of IBS is becoming increasingly recognized. Studies have indicated that psychiatric and psychological factors are independent risks for developing IBS (26). Psychological stress can contribute to the initiation and reactivation of experimental gastrointestinal inflammation and increase the intestinal permeability (27,28). Piche et al (10) also found that the number of IBS mast cells was significantly correlated with the Fatigue Impact Scale score and the Beck Depression Inventory score. The results of the present study demonstrated that psychological factors may alter immunological function and lead to the increased secretion of pro-inflammatory cytokines.

However, it must be pointed out that the present study has certain limitations: Due to the small cohort size used in the present study, further studies using a large number of samples are required to confirm the results. Furthermore, different subgroups of IBS and additional cytokines should be studied to better elucidate the pathological mechanisms of IBS.

In conclusion, the present study demonstrated that the balance of cytokine production was impaired in IBS-D patients. The imbalanced cytokines expression evoked colonic epithelial barrier dysfunction and may be associated with abdominal symptoms and psychological disorders.

Acknowledgements

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