Seroprevalence of Fusobacterium varium in ulcerative colitis patients in Japan

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Abstract

The etiology of ulcerative colitis (UC) is unknown, while an exacerbating factor of this disease is associated with infectious agents. Recently, Fusobacterium varium has been found in the mucosa of a significant number of patients with UC. The aim of this study was to estimate the prevalence of F. varium infection based on serology, evaluate the relationship between F. varium seropositivity and UC, and determine the clinical characteristics of infected UC individuals. Seropositive patients were determined by immunoblotting with F. varium ATCC 8501 antigen. We also identified cross-reactive protein spots by peptide mass mapping analysis. These protein spots showed putative caseinolytic protease protein, putative translation elongation factor G, and putative enolase. Immunoblotting with F. varium antigen revealed signals with sera from 45 (40.2%) of the 112 UC patients and 20 (15.6%) of the 128 healthy controls, respectively (P < 0.01). In terms of disease activity, seropositive UC patients were more likely to have clinically severe disease than seronegative UC patients. Disease location in seropositive patients was more extensive than the seronegative patients. In conclusion, F. varium is a predominant infection in the UC population and is a potential pathogen of UC.

Introduction

Ulcerative colitis (UC) is a chronic inflammatory disease that affects the colon and rectum (Podolsky, 2002). Although the etiology of UC is unknown, the disease shares histological features with colitis caused by infectious agents (Rubin & Present, 1995). The mucosal microbial community may play an important role in exacerbating UC, as infections usually start with the adherence of microorganisms to the host cells (Burke & Axon, 1988). Patients with UC have abnormally high numbers of facultative anaerobic bacteria (van der Wiel-Korstanje & Winkler, 1975). Adhesive Escherichia coli have also been implicated in the pathogenesis of UC (Burke & Axon, 1988).

Recently, Fusobacterium varium has been found in the mucosa of a significant number of patients with UC (Ohkusa et al., 2002). On immunochemical examination of biopsy specimens from active UC cases, it was observed that F. varium invaded the mucus and mucosa, and lived in the crypts (Ohkusa et al., 2002). Similarly, Helicobacter pylori, found in the gastric mucosa of most patients with chronic gastritis or peptic ulcers, inhabited the mucous layer or invaded the epithelium (Marshall & Warren, 1984). The efficacy of antibiotic combination therapy for UC (amoxicillin, tetracycline, and metronidazole were selected on the basis of susceptibility tests with F. varium) has also been reported (Nomura et al., 2005).

Fusobacterium varium infection in UC can be diagnosed by a number of methods, including endoscopic biopsy culture of the colon mucosa, stool culture assays, and serological tests (Ohkusa et al., 2002). However, F. varium is difficult to culture because it is only recovered from the anaerobic bottle. Although serological testing, which is noninvasive and does not involve culture, is potentially useful for the diagnosis of F. varium infection, it has not been established widely for F. varium. Thus, there have been no previous F. varium prevalence studies or sero-epidemiological studies for UC.

In this study, we estimated the prevalence of F. varium infection based on serology by immunoblotting, evaluated...
the relationship between *F. varium* seropositivity and UC, and determined the clinical characteristics of infected UC individuals.

**Materials and methods**

**Study population**

UC patients were recruited consecutively in the department of gastroenterology at Nagoya University Hospital, Nagoya First Red Cross Hospital, the National Hospital of Nagoya, and Toyota Memorial Hospital from 1996 to 2005. Sera were obtained from 112 patients with active and relapsing UC. These patients had not received any antibiotics for at least 4 weeks before obtaining sera. Control sera were obtained from 128 non-UC hospital personnel who were not complaining of lower abdominal pain or hematochezia-like UC. All sera samples were frozen at −80°C before analysis. They were obtained with informed consent in accordance with the Helsinki Declaration.

**Serological study of patients with UC**

*Fusobacterium varium* ATCC 8501, JCM 3721, JCM 3722, and JCM 3723 were obtained from the American Tissue Culture Center and the Japan Collection of Microorganisms at the Riken BioResource Center (Wako, Japan). *Fusobacterium varium* were cultured on Brain Heart Infusion agar (Becton Dickinson and Company, Sparks, MD) plates at 37°C for 2 days under anaerobic conditions (AnaeroPack; Mitsubishi Gas Chemistry, Tokyo). *Bacteroides vulgatus*, *Fusobacterium nucleatum*, *Enterococcus faecalis*, *E. coli* (EPEC O18), *Clostridium perfringens*, *Bifidobacterium adolescentis*, *Lactobacillus paracasei*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Proteus mirabilis* were isolated from Nagoya University Hospital and Nagoya City University Hospital.

**Detection of *F. varium* in patients’ sera**

The bacteria DNA was extracted from patients’ sera using the Qiagen DNA extraction kit (Qiagen, Hilden, Germany). The bacterial 16S rRNA gene was amplified by PCR as described previously (Hashikawa et al., 2004). We also cultured the patients’ sera to detect *F. varium*. Briefly, 100 μL of patients’ sera was directly spotted on Brain Heart Infusion agar plates and cultured under anaerobic conditions up to 1 week.

**One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of *F. varium* proteins**

*Fusobacterium varium* were usually cultured in 3 mL of Brain Heart Infusion broth (Becton Dickinson and Company) under anaerobic conditions. After 18 h of bacterial culture, when the bacteria were in the late stationary phase of growth, 1 mL of the 3 mL culture was precipitated with trichloroacetic acid (final concentration, 10%). After an acetone wash, the precipitate was dissolved in 50 μL of SDS-PAGE buffer. Each 20 μL of the bacterial protein samples were electrophoresed. The gels were stained with Coomassie brilliant blue.

**Western blotting assay**

Western blotting assay was performed as described previously (Ohkusa et al., 2002). Briefly, after the total bacterial proteins of cultured bacteria were isolated and separated by molecular size using SDS-PAGE, they were transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes. The diluted sera (1:500) were then incubated for 16 h at room temperature with the antigen-containing membranes. Antigen–antibody complexes were detected with horseradish peroxidase-conjugated goat anti-human immunoglobulins (IgG, IgA, and IgM; Acris antibodies GmbH, Hiddenhausen, Germany), which were used as secondary antibodies at 1000 × dilution. Sera in which all four *F. varium* antigens were detected were defined as seropositive for *F. varium* infection.

**Peptide mass mapping analysis**

Sample protein mixtures were prepared as described previously (Tanaka et al., 2005). In brief, protein spots were excised from the gels after SDS-PAGE and the peptide mixtures were extracted from the excised gel pieces with acetonitrile solution containing 0.1% (v/v) trifluoroacetic acid. The extracts were dried and stored at 4°C before mass analysis. Nanoelectrospray tandem mass analysis was performed as described previously (Tanaka et al., 2005). The proteins in the spots were identified by their conformation to the description on the Matrix Science website (http://www.matrixscience.com/).

**Competitive adsorption assay**

Female BALB/c mice (SLC, Shizuoka, Japan) aged 7 weeks were used in this study. Mouse *F. varium* antisera were raised against *F. varium* ATCC 8501. *Fusobacterium varium* ATCC 8501 bacterial protein samples were subjected to SDS-PAGE and transferred onto a PVDF membrane. After transfer, the membrane was incubated with or without 1:10 diluted mouse *F. varium* antisera for 3 h. After washing the membrane, each strip of the membrane was incubated with three human *F. varium*-positive sera and two human *F. varium*-negative sera for 16 h. Three human *F. varium*-positive sera were from two UC patients (No. 1 and No. 2) and one from a non-UC hospital personnel (No. 3). Two human
F. varium-negative sera were from one UC patient (No. 4) and one non-UC hospital personnel (No. 5). These sera were selected randomly from sera collected in this study. After that, antigen–antibody complexes were detected with horse-radish peroxidase-conjugated goat anti-human immunoglobulins according to the Western blotting assay.

**Statistical analysis**

The significant difference between the groups was assessed by Fisher exact test. *P* values of < 0.01 were considered statistically significant.

**Results**

**Detection of F. varium in patients’ sera**

We cultured patients’ sera to confirm *F. varium* under an anaerobic atmosphere; however, we could not detect any bacteria (data not shown). Furthermore, we extracted DNAs from *F. varium*-positive sera samples with and performed PCR assays. However, we could not find any PCR products from the sera samples (data not shown).

**Detection of F. varium antigen proteins**

Seropositivity for *F. varium* antigen was determined by immunoblotting as described before (Ohkusa *et al.*, 2002). After separation by SDS-PAGE and Western blotting assay with *F. varium* ATCC 8501 antigen, bands for IgG, IgA, and IgM were seen at 40–100 kDa. In particular, three strong bands were represented (Fig. 1). We also identified these protein spots by peptide mass mapping analysis. Band A showed a putative caseinolytic protease (ClpB) protein, putative mass 97.289 kDa. Band B showed a putative translation elongation factor G (EF-G), putative mass 75.962 kDa. Band C showed a putative enolase, putative mass 46.651 kDa. Although several bacterial proteins were reactive on other bacterial species, all the three antigen proteins (ClpB, EF-G, and enolase) reacted only with *F. varium* bacterial species (Fig. 1). Furthermore, we performed a competitive adsorption assay to confirm the *F. varium*-specific nature of human antibody response. Although we did not detect three *F. varium*-specific bands from the PVDF membrane incubated with mouse anti-*F. varium* serum, we found these bands without mouse anti-*F. varium* serum (Fig. 2). We also found these bands from all three *F. varium*-positive sera-treated membranes and did not find them from two *F. varium*-negative sera-treated membranes. We did not find any differences in band detection between UC patients and non-UC hospital personnel in the competitive adsorption assay.

Next, we performed an *F. varium* seroprevalence study for UC. A total of 240 patients were admitted, with a mean age of 37 years (range: 18–68). Based on clinical and endoscopic findings, 112 patients were suffering from UC and 128 were controls. Using *F. varium* ATCC 8501, strong signals were evident with sera from 45 (40.2%) of the 112 patients with active UC and 20 (15.6%) of the 128 healthy controls (Table 1). Moreover, these signal bands were also detected using the other three *F. varium* strain antigens (data not shown). There was no significant difference in immunoreactivity to the same human sera among the four *F. varium* strain antigens. Based on the *F. varium* seropositivity, which is defined as the coincidence of all four *F. varium* strain antigens, 45 of the 112 UC patients and 20 of the 128 controls were identified as infected.
Fusobacterium varium seropositivity in UC patients and non-UC patients

Patients with F. varium seropositivity were divided into a UC patient group and a control patient group. The Odds ratio of F. varium seropositivity between UC and controls was 4.15. The two groups were compared with regard to age and sex. Table 1 shows the clinical details of the UC and control patients with F. varium seropositivity. There was a significant difference between UC patients and controls (P < 1.23e–5). The seropositivity rate in UC was about three times higher than the controls. There was no significant difference in sex or age between the two groups.

Characteristics of F. varium-seropositive and seronegative UC patients

Patients with UC were divided into F. varium-seropositive and -seronegative groups. The two groups were compared with regard to: (1) number, (2) sex, (3) age, (4) disease activity, (5) disease location, and (6) medical treatment. Table 2 shows the clinical details of patients with UC with or without F. varium seropositivity. The F. varium seropositivity rate in all the UC patients was lower than the seronegativity rate. Fusobacterium varium-seropositive and -seronegative UC patients were similar in age and sex.

Discussion

This study showed a strong association between disease severity and F. varium infection, and our results support our hypothesis that F. varium is related to UC. These results may also support the effectiveness of F. varium eradication therapy (Nomura et al., 2005).

Table 1. Clinical details of ulcerative colitis patients and control patients with Fusobacterium varium infection

<table>
<thead>
<tr>
<th></th>
<th>UC (n = 112)</th>
<th>Control (n = 128)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients (%)</td>
<td>45 (40.2)</td>
<td>20 (15.6)</td>
<td>1.23e–5</td>
</tr>
<tr>
<td>M/F</td>
<td>27/18</td>
<td>12/8</td>
<td>NS</td>
</tr>
<tr>
<td>Mean age</td>
<td>41.9</td>
<td>43.25</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS, not significant.

Table 2. Clinical details of patients with ulcerative colitis infected with or without Fusobacterium varium

<table>
<thead>
<tr>
<th></th>
<th>Fusobacterium varium (+) (n = 45)</th>
<th>Fusobacterium varium (–) (n = 67)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>M/F</td>
<td>27/18</td>
<td>46/21</td>
<td>NS</td>
</tr>
<tr>
<td>Mean age</td>
<td>38/48</td>
<td>35/33</td>
<td>NS</td>
</tr>
<tr>
<td>Disease activity</td>
<td>Mild/moderate/severe</td>
<td>9/22/14</td>
<td>8.16e–10</td>
</tr>
<tr>
<td>Disease location</td>
<td>Rectum/left side/total colitis</td>
<td>51/10/68</td>
<td>4.47e–10</td>
</tr>
<tr>
<td>Medical treatment</td>
<td>SASP/steroid</td>
<td>32/13</td>
<td>40/27</td>
</tr>
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NS, not significant; SASP, salazosulfapyridine.

In terms of disease activity, seropositive patients were more likely to have clinically severe disease than seronegative patients (mild/moderate/severe: seropositive, 9/22/14; seronegative, 51/10/68) (P < 8.16e–10). Disease location in seropositive patients was more extensive than that in seronegative patients (P < 4.47e–10). Thirty-two seropositive patients and 40 seronegative patients were taking salazosulfapyridine. Thirteen seropositive patients and 27 seronegative patients were taking steroid. There were no significant differences among medical treatments, including steroid therapy.
Fusobacteria are nonspore-forming Gram-negative anaerobic bacilli, and are normal commensals in the human oropharynx, gastrointestinal tract, and female genital tract (Brook, 1989). *Fusobacterium* species are considered to be important factors in mixed anaerobic infections (Bourgault et al., 1997). *Fusobacterium nucleatum* and *Fusobacterium necrophorum* species are most frequently isolated from clinical samples. However, reports of infection caused by *F. varium* are rare. The organism has mostly been isolated from intra-abdominal infections. In a study of fusobacterial infections in children, 243 strains of *Fusobacterium* species were recovered from 226 of 1399 (16.2%) specimens, out of which only five (2.2%) were *F. varium* (Brook, 1989). In a series of 40 patients with *Fusobacterium* bacteremia, *F. nucleatum* was isolated in 16 cases and *F. necrophorum* in eight cases, while *F. varium* was isolated only in three cases (Bourgault et al., 1997).

Although the diagnosis of infection is primarily based on culture findings, it is aided by serological tests such as immunoblotting or an enzyme-linked immunosorbent assay (ELISA) when culture is difficult (Jungblunt et al., 1999). In general, an ELISA should be performed for screening, and positive results should be confirmed by an immunoblotting assay (Centers for Disease Prevention and Control, 1995). For bacterial antigen analysis, the immunoblotting assay is widely used for serodiagnosis (Jungblunt et al., 1999). The bacterial seroprevalence criteria are simply defined to distinguish between positive and negative sera because bacterial protein antigens are visualized as bands according to their molecular mass. *Fusobacterium varium*-specific antigens had not been clarified before our study. From this study, we determined three protein antigens by peptide mass mapping analysis. We also clarified specific bacterial antigen reactions to patients’ sera by an immunoblotting assay because we had not purified these *F. varium*-specific antigen proteins for ELISA. These bacterial antigen proteins may be useful for detection of *F. varium* by ELISA in future.

Although a previous report showed that UC serum reacted only on the *F. varium* protein antigen (Ohkusa et al., 2002), our result demonstrated that UC serum reacted on several bacterial protein antigens. This is consistent with another previous report that patients with UC showed an increased immune response toward several enteric bacteria (Furrie et al., 2004). However, we confirmed the different bacterial antigen patterns to react on UC patients’ sera by the Western blotting assay and clearly distinguished from *F. varium* antigens to other bacterial antigens (Fig. 1).

In this study, we showed that the *F. varium* antigens, which react to UC serum, are three major proteins: ClpB protein, EF-G, and enolase. Cross-reactive *F. varium* antigens to four strains were common in our study. Although the complete genome sequence of *F. varium* has not been obtained, we annotated them from the genome of *F. nucleatum* and *Desulfovibrio vulgaris*, which are closely related to *F. varium*. ClpB protein, which belongs to the ClpATPase family of stress response proteins, is involved in protein degradation and disaggregation in both prokaryotic and eukaryotic cells (Schirmer et al., 1996; Turner et al., 1998). EF-G participates in the elongation phase of protein synthesis by translocating the peptidyl tRNA from the A-site to the P-site (Kaziro, 1978). EF-G also has a role after the termination phase of translation (Lauber et al., 2000). A glycolytic metalloenzyme, α-enolase (2-phospho-D-glyceraldehyde hydratase), catalyzes the stepwise dehydration of d-(C)-2-phosphoglyceric acid to phosphoenolpyruvate through an anti-β-elimination mechanism (Brewer, 1981; Reed et al., 1996). It is essential for the degradation of carbohydrates via glycolysis and other catabolic pathways, as well as for glucose synthesis via gluconeogenesis (Wold, 1971). The virulence of ClpB, EF-G, and α-enolase in fusobacteria has not been clearly elucidated before. Although the effect of these antigen proteins cross-linking in the host has not been well studied, they have been proposed as candidates for vaccines, because they have been shown to react with the patients’ sera (Noll et al., 1997). Thus, further investigation of these proteins is desirable.

The limitations of this study are as mentioned. In our study, we could not distinguish *F. varium* colonization of the colon from the *F. varium* colonization of other sites, including the oropharynx, by seroepidemiology. Our study revealed that almost 40% of UC patients had *F. varium*, but the other 60% of UC patients were not infected. Therefore, it may be difficult to assume that the situation in UC is the same as that with *H. pylori* in peptic ulcer, because the prevalence of *H. pylori* in peptic ulcer patients is > 80% (Graham, 1989). Although our immunoblotting studies revealed a statistical link between *F. varium* infection and UC, the exact pathogenic role of *F. varium* infection in UC is still a matter of debate, and the process that underlies this association is also unclear.

In conclusion, our data suggest that *F. varium* is a predominant factor for infection in UC patients. Further investigations with a larger number of patients and basic research may elucidate the relationship between *F. varium* and the pathogenesis of UC.

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