Molecular cloning reveals nearly half of patients with Crohn’s disease have an antibody to peroxiredoxin 6-like protein

Masahiro Iizuka,*† Osamu Nakagomi,¶ Hiroshi Nanjo,‡ Mitsuro Chiba,§ Tsuneo Fukushima,** Akira Sugita,†† Shiho Sagara,* Yasuo Horie† and Sumio Watanabe‡‡

*Akita Health Care Center, Akita Red Cross Hospital, †Department of Internal Medicine, Akita University School of Medicine, ¶Division of Clinical Pathology, Akita University School of Medicine and ‡Department of Gastroenterology, Nakadori General Hospital, Akita, ‡Division of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, and Global Center of Excellence, Nagasaki University, Nagasaki, **Department of Surgery, Matsushima Hospital, Yokohama and ††Department of Surgery, Yokohama Municipal Hospital, Yokohama, and ‡‡Department of Internal Medicine, Juntendo University School of Medicine, Tokyo, Japan

Abstract

Background and Aim: Crohn’s disease (CD) is a chronic inflammatory bowel disease (IBD) of unknown etiology. We aimed to identify the etiological agent of CD using a molecular cloning strategy that was particularly focused on identifying agents causing immune abnormalities and infectious agents.

Methods: We constructed a cDNA library derived from the inflamed intestinal tissue of a CD patient, and screened 1.5 million clones in this library with the serum from another typical CD patient. The expressed cDNA clones that positively reacted with the serum were then expressed as fusion proteins with glutathione S-transferase, and western blotting was performed using the sera of 22 CD, 13 ulcerative colitis (UC), and 16 non-IBD patients.

Results: We identified nine positive clones that did not contain any viral or bacterial genomic DNA. Of these, we selected one clone (clone 50) with which the typical CD patient’s serum most strongly reacted. Clone 50 is highly homologous to the antioxidant protein peroxiredoxin 6. In western blotting, the sera of 47.6% CD patients (small intestine type 80%, large and small intestine type 43%, large intestine type 0%) showed strong reactivity to clone 50, none of the UC patients were reactive to clone 50, and 18.8% of non-IBD patients were very weakly reactive to it. We also found that the expression of peroxiredoxin 6 was significantly increased in inflamed intestinal epithelia of CD.

Conclusion: The present study first showed that some CD patients have an antibody against peroxiredoxin 6-like protein, which may be involved in the pathogenesis of CD.

Key words

Accepted for publication 27 March 2012.

Correspondence
Dr Masahiro Iizuka, Akita Health Care Center, Akita Red Cross Hospital, 3-4-23 Nakadori, Akita 010-0001, Japan. Email: maiizuka@woody.ocn.ne.jp

Introduction

Crohn’s disease (CD) is a chronic intractable inflammatory bowel disease (IBD) that can affect the entire gastrointestinal tract, from the mouth to the anus. Although several factors, such as genetic susceptibility, infectious agents, enteric microflora, oxidative stress, and immune abnormalities, have been suggested to be involved in the etiology of CD, the definite causative pathogens for this disease are yet to be identified.1-4 Regarding the immune abnormality associated with CD, several antibodies, such as antibodies againstSaccharomyces cerevisiae (ASCA) andPseudomonas fluorescens, are frequently observed in patients with CD. However, the roles of these antibodies in disease pathogenesis remain unclear.5-8

Young and Davis9 developed a molecular cloning strategy for the cloning and isolation of genes that encode proteins responding to probe antibodies by using an expression vector, λgt11. The hepatitis C virus was first identified using this approach.9-10 Furthermore, molecular cloning has been used to identify autoantibodies generated in the body during various disease conditions.11,12 We previously used this approach to identify the protein present in gut mucosa to which an antibody against the measles virus responds.13

In the present study, we aimed to identify the etiological agents of CD using a molecular cloning strategy that was particularly focused on identifying agents causing immune abnormalities, such as autoantibodies, and infectious agents.

Methods

Construction and screening of the cDNA expression library. The cDNA expression library was constructed under informed consent using the Lambda gt-11/EcoRI/CIAP-treated vector kit (Stratagene, La Jolla, CA, USA) from the
inflamed large intestinal tissue that was surgically excised from a 38-year-old patient with CD of large and small intestine type. In the present study, we diagnosed patients as having CD based on the clinical, endoscopic, and histological criteria. The 1.5 million clones that were deposited in the cDNA library were expressed as β-galactosidase fusion proteins in the Escherichia coli Y1090 strain and then screened with the serum of a 42-year-old patient with typical CD of small intestine type. For immunological screening, we used the patient’s serum in the active stage of the disease. Briefly, 0.1 mL of the bacteria to be transformed was mixed with 0.1 mL of SM buffer containing approximately 3 × 10^6 pfu of the bacteriophage λgt11 plated on 90 mm plates. The infected plates were incubated at 42°C for 3.5 h, overlaid with isopropylthio-β-D-galactoside (IPTG)-impregnated nitrocellulose filters (Amer sham Pharmacia Biotech, Buckinghamshire, UK) and incubated at 37°C for at least 4 h. The filters were peeled off from the plates and washed in TNT buffer (10 mM Tris Cl [pH 8.0], 150 mM NaCl, 0.05% Tween 20). After blocking the filter for 30 min in 5% skimmed milk in TNT buffer, the filter was incubated with blocking buffer containing the patient’s serum (1 : 50 dilution) and E. coli lysate for 2–4 h at room temperature. Positive clones expressing proteins that the serum antibodies bound were visualized with a horseradish peroxidase (HRP)-conjugated anti-human immunoglobulin secondary antibody (Dako, Copenhagen, Denmark) (1 : 1000 dilution) and with 4-chloro-1-naphthol (Wako, Osaka, Japan) as the developing solution.

cDNAs were isolated from the positive λgt11 vector gene clones and amplified by polymerase chain reaction (PCR) using the λgt11 forward primer (5′-GGTGCGAGACTCTGGAGCCG-3′) and the λgt11 reverse primer (5′-TTGACACGAGACACACTG GTAATG-3′). The PCR-amplified DNA fragments were then inserted into the TA cloning vector (Invitrogen, San Diego, CA, USA) and the nucleotide sequence of the positive clone was determined by the DyeDeoxy Terminator Cycle Sequencing method (Applied Biosystems, Perkin-Elmer Corporation, Cleveland, OH, USA). After confirming the nucleotide sequences, we performed genetic analysis of the positive clones.

**Screening of the positive clone with the serum of a patient with Crohn’s disease.** The protein encoded by the cDNA of the positive clone was subsequently expressed as a glutathione S-transferase (GST)-fusion protein by using the GST Gene Fusion System (Amersham Pharmacia Biotech). The double-stranded DNA insert of the positive clone was ligated with the pGEX-4T-2 vector containing the GST open reading frame. Competent E. coli BL21 cells (Stratagene) were transformed with the ligation product and the transformed bacteria were used to express the fusion protein in the presence of 0.1 mM IPTG. The fusion protein was purified from bacterial lysates by affinity chromatography using a glutathione-Sepharose 4B column and eluted in glutathione elution buffer containing 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0). The purified recombinant protein was then screened by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and western blotting by using the patient’s serum that was used in the screening of the cDNA clone, as the primary antibody.

**Western blotting.** To determine whether CD patients specifically possess the antibody to the positive clone, we performed western blotting with the fusion protein from the positive clone and sera from various patients with CD, ulcerative colitis (UC), non-inflammatory bowel disease (non-IBD). We collected sera during the active stage of the disease under informed consents of 22 patients with CD (males 13, females 9; mean age, 25.0 ± 7.4 years [SD]), 13 patients with UC (males 4, females 9; mean age, 30.3 ± 7.0 years), and 16 patients with non-IBD (five patients with gastritis; four acute enterocolitis; two colon polyps; one irritable bowel syndrome; one gastric ulcer; one gallbladder polyp; one indeterminate enteritis; one endometriosis) (males 8, females 8; mean age, 31.3 ± 12.1 years). Fusion proteins were heated (at 90°C, for 3 min), resolved on SDS-PAGE gels (approximately 1.5 μg per lane), and then transferred onto polyvinylidene difluoride membrane (Millipore, Yonezawa, Japan). The membranes were then immunoblotted with patients’ sera diluted 1 : 4000 times and with E. coli lysates. The blots were treated with an HRP-conjugated anti-human immunoglobulin secondary antibody (Dako) (1 : 20 000 dilution), and bands were visualized using the enhanced chemiluminescence (ECL)-system (Amersham Pharmacia Biotech). The intensity of the positive bands in the western blotting was evaluated by using “Image J,” which is a public domain Java image processing inspired by the US National Institutes of Health’s (NIH) Image.

We then compared various clinical characteristics of CD patients whose sera were positive for the antibody against the recombinant protein with those of patients whose sera were negative in western blotting.

**Immunohistochemistry.** Distributions of peroxiredoxin 6 protein in intestinal mucosa were immunohistochemically examined. We examined normal intestinal mucosa from non-IBD patients (colon and ileum) and inflamed intestinal mucosa from CD patients (colon and ileum). Methods of immunohistochemistry are shortly described as follows. After 24 h of fixation in formalin, the samples embedded in paraffin, and sections were cut at 3 μm thickness using a microtome, and mounted on MAS-coated slides. Nonspecific binding was blocked by incubating the slides with Dako cytometry protein block (X0909) (Dako) for 5 min at room temperature. The sections were then incubated with primary antibody (anti-Peroxiredoxin 6 polyclonal antibody [ab73350]) (Abcam, Cambridge, MA, USA) diluted 1 : 800 for 30 min at room temperature. After incubation, unbound antibodies were washed in PBS-Tween for 5 min, and then incubated with 3% hydrogen peroxide in methanol for 15 min to block endogenous peroxidase activity. The sections were washed and incubated with secondary antibody (Histone Simple Stain goat MAX-PO MULTI [rabbit and mouse]) (Nichirei Biosciences, Tokyo, Japan) for 30 min at room temperature. Unbound antibodies were washed in PBS and the bound antibodies were visualized using DAB as chromogen substrate reagent Liquid DAB + Substrate (K3468) (Dako). Negative controls for normal rabbit serum incubated with secondary antibodies were also processed and revealed no signal.

**Functional assays for peroxiredoxin 6-like protein.** To elucidate the function of peroxiredoxin 6-like protein, we performed western blot for detecting the activation of the signal...
peptides involving anti-oxidative or cytoprotective function using intestinal epithelial cells. Confluent IEC-6 monolayers grown on 6-well dishes maintained for 24 h in serum-deprived DMEM were treated for 5, 15, 30, and 60 min with 0.1 μg/mL of recombinant peroxiredoxin 6-like protein. Preparation of IEC cells was performed as described before. Proteins from the resulting supernatant (10 μg per lane) were heated (90°C, 3 min), subjected to SDS-PAGE, and then transferred onto a polyvinylidene difluoride membrane followed by immunoblotting with polyclonal antibodies specific to p44/42 (extracellular signal-regulated kinase [ERK] 1/2) mitogen-activated protein kinase (MAPK), phosphorylated p44/42 MAPK (Thr202/Tyr204), IκB-α, or serine-phosphorylated IκB-α (Cell Signaling, Beverly, MA, USA) (1 : 1000 dilution). Positive bands were visualized with the secondary HRP-conjugated antibody (Dako) (1 : 2000 dilution) with the ECL-system.

Statistical analysis was performed using the χ² test or analysis of variance (ANOVA), followed by multiple comparison testing using Fisher’s protected least significant difference test and Bonferroni/Dunn test, and a P-value of < 0.05 was considered statistically significant.

This study was approved by the Ethical Committee of the Faculty of Medicine, Akita University School of Medicine (No. 120).

Results

Detection of positive clones by immunological screening. We detected 63 positive plaques (clones) by immunologically screening. A typical positive plaque is shown in Figure 1a. Sequencing analysis of these 63 positive clones revealed that several clones were identical; therefore, the number of positive clones was eventually reduced to 9. Among these nine clones, we chose one clone, i.e. clone 50, which strongly reacted with the patient’s serum. This clone was 462 nucleotides long (Gene Bank accession number, DQ480128). The deduced amino acid sequence of this clone is shown in Figure 2. The expressed form of this clone consisted of two peptides (the first peptide consisted of 39 amino acids [residues 1–39], the latter peptide consisted of 114 amino acids [residues 41–154], and the two were separated by a stop codon [residue 40]). A homology search of the positive clone performed using the Gene Bank data base revealed that the amino acid sequence of the first peptide exhibited 63% similarity with human peroxiredoxin 6 (gi:1718024). The nucleotide sequence of the first peptide has 97% similarity with human peroxiredoxin 6 (gi:28559000). The latter peptide exhibited 100% similarity with human cathepsin K precursor (gi:1168793).

Strong reactivity of sera from CD patients with clone 50. We then inserted the nucleotide sequence of clone 50 into a GST vector and expressed the encoded protein as a GST fusion protein. The fusion proteins bands before and after purification are shown in Figure 1b. We confirmed that the patient’s serum used in the screening of the positive clones also strongly reacted with the purified fusion protein of approximately 33 KDa size in western blotting (Fig. 1c). We further determined that the serum obtained from the patient in the remission stage of CD reacted equally strongly with the fusion protein and that the patient’s serum did not react with GST (data not shown).
We then performed western blotting for sera of 22 patients with CD, 13 patients with UC, and 16 patients with non-IBD by using the fusion protein (Fig. 3). The reactivity of the serum of one CD patient (lane 16) could not be determined because of very strong background. The sera of 10/21 (47.6%) CD patients, none of the 13 (0%) of UC patients, and 3/16 (18.8%) patients with non-IBD reacted with the recombinant fusion protein of the positive clone (Fig. 3). The reactivity of the sera of CD patients was significantly higher than that of the sera of non-CD patients ($P = 0.00028$). In addition, positive bands of the sera of the non-IBD patients appeared to be significantly weaker than those of the sera of CD patients. With regard to the extent of the lesions in CD patients, positive ratio of the western blotting tended to be higher in small intestine type (80%) compared with large and small intestine type (43%) and large intestine type (0%) (Fig. 4). In non-IBD patients, two cases probably had small intestinal lesions (indeterminate enteritis 1, acute enterocolitis 1), and one case was positive and the other was negative in western blotting. CD patients were divided into western blot-positive and western blot-negative groups, and the two groups were compared with regard to gender, age, age at onset, duration of disease, perianal disease status, the use of steroids, history of surgery, clinical type (perforating type vs non-perforating type), stricturing, and extent of the lesions (Table 1). With regard to gender, we compared the two groups (western blot-positive and negative groups) that included patients with CD, UC, and non-IBD (Table 1). Western blot-positive patients had a higher incidence of previous surgery ($P = 0.045$), but had no association with gender, age, age at onset, duration of disease, perianal disease, use of steroid, clinical type, and stricturing. With regard to the extent of lesions, more patients with small intestine type lesions tended to belong to the western blot-positive group (40%) rather than the western blot-negative group (9%); however, this tendency was not statistically significant.

Figure 3  Response of sera from Crohn’s disease (CD) patients to clone 50. Western blotting with the clone 50-glutathione S-transferase (GST)-fusion protein and sera from CD patients (Upper panel), ulcerative colitis (UC) patients (middle panel), and non-inflammatory bowel disease (IBD) patients (lower panel). Upper panel: lane 1, molecular weight marker (MM) and lanes 2–23, sera from patients 1–22 i.e. sera from the 22 CD patients. Positive bands were observed in patients 1–6, 9, 11, 13, and 22. Middle panel: lane 1, molecular weight marker (MM), lane 2, positive control (PC) i.e. serum from CD patient number 1; lanes 3–15, sera from patients 1–13 i.e. sera from the 13 UC patients. No positive bands are seen. Lower panel: lane 1, molecular weight marker (MM), lane 2, positive control (PC) i.e. serum from CD patient number 1; lanes 3–18, sera from patients 1–16 i.e. sera from the 16 patients with non-IBD. Weak positive bands are observed for sera of patients 5, 10, and 16.

Figure 4  Positive ratios of the western blotting in Crohn’s disease patients with each affected area. Positive ratio of the western blotting in Crohn’s disease patients with each affected area (small intestine type [black column], large and small intestine type [gray column], and large intestine type) is shown.
Results of immunohistochemistry. As shown in Figure 5, positively-stained cells, which included plasma cells, lymphocytes, macrophages, were observed in intestinal mucosa. In normal ileal mucosa, epithelial cells at crypt side were weakly stained (a). In inflamed ileal mucosa of CD patients, positive cells were significantly increased and most of the epithelial cells were strongly stained (b). In normal colonic mucosa, positive cells in lamina propria were increased compared with ileal mucosa and most of the epithelial cells were positively stained (c). In inflamed colonic mucosa of CD patients, it appears that positive cells were increased and epithelial cells were more intensely stained compared to normal colonic mucosa (d).

Results of functional assays for peroxiredoxin 6-like protein. Results of western blotting regarding the activation of ERK1/2 MAPK and IκB-α signaling pathways in intestinal epithelial cells after the treatment with recombinant peroxiredoxin 6-like protein are shown in Figure 6. The activation of ERK1/2 MAPK was observed, but phosphorylation of IκB-α, which is an excellent marker of nuclear factor-κB (NF-κB) activation, was not detected in this assay.

Discussion
In this study, we attempted to identify etiological agents of CD, with particular focus on immune abnormalities and infectious agents, by molecular cloning strategy. We screened 1.5 million clones deposited in the cDNA library that was derived from an inflamed intestinal tissue of a CD patient, with serum from a typical CD patient and obtained two novel results. First, we did not detect any bacterial or viral genome sequences by using this approach. This result does not support the possibility that persistent viral or bacterial infection causes CD. Second, we identified a
...of murine colitis model. It is notable that very recent study demonstrated that injury. It was also shown that peroxiredoxin 6 protects keratinocytes and lens cells from cellular oxidative stress. In addition, our study first shows that nearly half of CD patients have the antibody to human peroxiredoxin 6-like protein. We believe that further studies with a larger number of patients are warranted to confirm our observation.

Acknowledgments

This work was supported in part by a Grant-In-Aid for Scientific Research from the Japanese Society for the Promotion of Science and Health and Labour Sciences Research Grants for Research on...

References


