FIGURE 7. Flat lesion in a 40-year-old female patient with a 15-year history of extensive colitis. Colonoscopy revealed a visible red flat lesion in the rectum. Histological diagnosis of the resected specimen was adenocarcinoma that had invaded the subserosa: left, conventional endoscopy; right, H&E staining.

conventional colonoscopy using the dye spraying contrast-enhanced method. In flat or slightly elevated lesions, it is occasionally difficult to identify the lesions or to differentiate lesion borders with conventional endoscopy in the absence of dye spraying. Kiesslich et al[10,16] reported that dye-contrast-enhanced magnifying endoscopy using modified Kudo's criteria to differentiate neoplastic from nonneoplastic lesions can be applied in cancer surveillance endoscopy for ulcerative colitis. However, this could be difficult to use for all patients undergoing surveillance colonoscopy in every institution. Because substantial time would be required to train colonoscopists to specialize in this technique so that they would understand the procedures and findings, conventional endoscopy assisted with dye spraying can still serve as a suitable, albeit not perfect, solution for identifying neoplastic lesions. These lesions are good candidates for sampling in biopsies that are used for pathologic diagnosis. These findings are consistent with the reports of Sada et al[17] and Hata et al,[18–20] who described dye spraying being useful in the identification of lesions and explained that dysplasia and early cancer were characterized by granular or nodular protruding

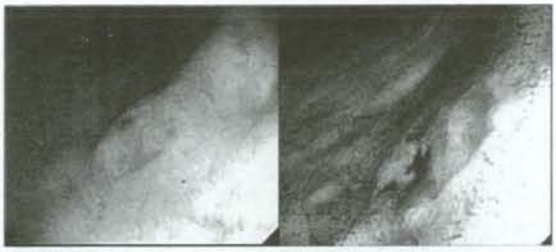

FIGURE 9. Depressed lesion in a 30-year-old female patient with a 4-year history of extensive colitis. Colonoscopy revealed a depressed lesion in the transverse colon. Histological diagnosis of the resected specimen was moderately differentiated adenocarcinoma extending into the muscularis propria: left, conventional endoscopy; right, chromoendoscopy.

mucosa or by lowly protruding or flat mucosa, often associated with the redness observed with dye-spraying endoscopy.[21]

As also reported by Kudo et al and Sada et al,[17] some neoplastic lesions associated with ulcerative colitis have a neoplastic pit pattern that can be observed with dye-spraying endoscopy; magnifying endoscopy of 10 protruded lesions of dysplasia (7 patients) and 5 early cancers (4 patients) showed IIIS- to IIIL-type pits or IV-type pits. However, Hata et al[18,19] reported they did not observe III- or IV-type pit patterns in some dysplastic lesions.

Although such neoplastic pit patterns are observed in some cases with elevated lesions, coexisting chronic inflammatory changes may modify the pit patterns, particularly in flat lesions. Some of the neoplastic lesions included in the present study were accompanied by long-lasting active inflammation. Therefore, in the diagnosis of dysplasia, it is not always easy to identify neoplastic pit pattern in the inflammatory mucosa as some of the pit patterns observed in areas of neoplastic histology or area with post-inflammatory

FIGURE 8. Flat lesion in a 43-year-old male patient with a 7-year history of extensive colitis. Colonoscopy revealed a visible flat lesion in the sigmoid colon with a small erosion. Chromoendoscopy revealed a relatively large granular pattern of the mucosa. Surrounding mucosa showed regenerative vascular pattern with mucosal atrophy. Histological diagnosis of the biopsy specimen was low-grade dysplasia: left, conventional endoscopy; right, chromoendoscopy.

FIGURE 10. Mixed-type lesion in a 50-year-old male patient with a 24-years history of extensive colitis. Colonoscopy revealed a protruding lesion with adjacent slightly elevated lesion in the rectum. Slightly elevated lesion showed a large granular pattern. Surrounding mucosa was almost normal in appearance. Histological diagnosis of the resected specimen was high-grade dysplasia: left, conventional endoscopy; right, chromoendoscopy.

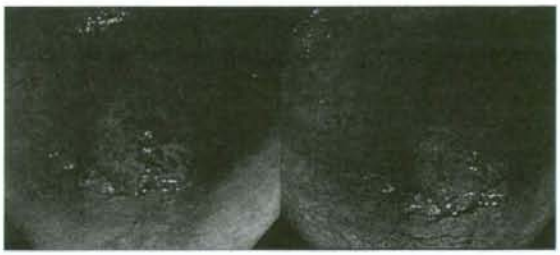

FIGURE 11. Mixed-type lesion in a 60-year-old male patient with a 22-year history of left-sided colitis. Colonoscopy revealed a protruding lesion in the sigmoid colon. Histological diagnosis of the resected specimen was well-differentiated adenocarcinoma extending into the submucosa: left, conventional endoscopy; right, chromoendoscopy.

changes in the background mucosa in patients with ulcerative colitis show similar findings (Fig. 12). Taken together, further studies are required to establish a pathologic diagnosis based on the actively inflamed mucosa of patients with long-lasting inflammation such as the chronic continuous type of ulcerative colitis. Further, novel criteria to differentiate ulcerative colitis-associated neoplastic lesions should be determined.

Future Perspectives in Surveillance Colonoscopy for Detecting Neoplastic Lesions Associated with Ulcerative Colitis

To improve the yield and ease of detecting neoplastic lesions, several novel modalities such as narrow-band imaging and autofluorescence endoscopy have been used.[22,23] The narrow-band imaging technique is useful for enhancing vascular images without dye spraying; therefore, it is easy to use in routine endoscopy. However, in long-standing cases of ulcerative colitis, hyperemic mucosa is frequently observed in surveillance colonoscopy. Because narrow-band imaging is based on differences in the absorption profiles of hemoglobin, it is sometimes difficult to obtain enhanced images of the small lesions related to dysplasia. However, in certain situations, narrow-band imaging could be a useful additional

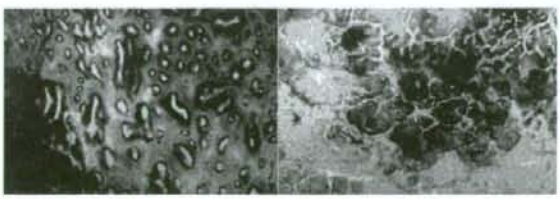

FIGURE 12. Pit pattern in long-standing ulcerative colitis was sometimes indefinite for fitting into a current category proposed by Kudo et al[12]: left, small but uneven pit pattern observed in the background mucosa; right, uneven pit pattern observed in the area with low-grade dysplasia.

method, as reported by East et al[24]; however, large-scale clinical trial data are required. Although autofluorescence imaging in endoscopy has been reported to be effective for the detection of esophageal cancer,[25] further studies are necessary to consider its applications in surveillance colonoscopy for colorectal cancer in ulcerative colitis. Although histological changes in the mucosa are key factors in diagnosing dysplasia in ulcerative colitis, endomicroscopy could be considered as an alternative technique.[26] Because it is important to detect candidate lesions that should be considered for endomicroscopy, close observation using conventional endoscopy and chromoendoscopy could play important roles in routine surveillance endoscopy in ulcerative colitis. Further investigations would be required to apply these new approaches to routine clinical endoscopy.

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Human Neutrophil Peptides 1–3 Are Useful Biomarkers in Patients with Active Ulcerative Colitis

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Background: A specific useful biomarker for diagnosing ulcerative colitis (UC) has not yet been described. This study employed proteomics to identify serum protein biomarkers for UC.

Methods: Ninety-four blood samples were isolated from patients and controls (including 48 UC, 22 Crohn's disease [CD], 5 colorectal cancer, and 6 infectious colitis patients and 13 healthy subjects). Serum samples were analyzed using the SELDI-TOF/MS ProteinChip system. After applying the samples to ProteinChip arrays, we assessed differences in the proteomes using Ciphergen ProteinChip software and identified candidate proteins, which were then characterized in immunoassays.

Results: Preliminary analysis using the ProteinChip system revealed significant peak-intensity differences for 27 serum proteins between 11 patients with UC and 7 healthy subjects. Among these proteins, 3 proteins (with mass/charge ratios of approximately 3400) were identified as human neutrophil peptides 1–3 (HNP 1–3). The presence of HNP 1–3 in the patient sera was confirmed using immunoassays. Enzyme-linked immunosorbent assays demonstrated that the mean plasma concentration of HNP 1–3 was significantly higher in patients with active UC ($n = 28$) than in patients whose UC was in remission ($n = 20$) or patients with CD ($n = 22$), infectious colitis, or healthy subjects, and tended to be higher than in patients with colon cancer. In addition, the plasma concentration of HNP 1–3 in patients that responded to corticosteroids-based therapy decreased after treatment, whereas it was not changed in nonresponders.

Conclusions: HNP 1–3 is a novel biomarker that may be useful for diagnosing patients with active UC and predicting treatment outcomes.

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Key Words: biomarkers, inflammatory bowel disease, ulcerative colitis, human neutrophil peptides 1–3, SELDI-TOF/MS, proteomics

Genetic and environmental factors contribute to the disease process of inflammatory bowel disease (IBD), including ulcerative colitis (UC).[1,2] The presence of active inflammation of the gut in patients with UC is associated with an acute-phase reaction and the migration of leukocytes to the gut. This, in turn, promotes the production of a large number of proteins.[3] Determination of inflammatory activity is important for the comprehensive assessment of patients with UC and for the tailoring of therapy.[4] Many clinical activity indices are used to stratify patients with UC. For example, the UC Disease Activity Index (UCDAI)[5] is a widely used measure of clinical parameters of disease activity. These indices, however, only provide indirect assessments of disease activity. Whereas albumin, hemoglobin, the erythrocyte sedimentation rate (ESR), and acute-phase protein levels are commonly used biological parameters for assessing UC, there are no accurate markers to assess the inflammatory activity observed with histopathologic or endoscopic analyses.[6]

Proteomic array technology, in which a ProteinChip system is coupled with surface-enhanced laser desorption ionization/time-of-flight/mass spectrometry (SELDI-TOF/MS) for the profiling of serum or plasma, is a powerful tool that allows the identification of new biomarkers for malignant tumors and autoimmune diseases.[7,8] This technology is a rapid and sensitive technique, in which the detected peak intensities for some proteins correlate with concentrations determined using enzyme-linked immunosorbent assay (ELISA). Novel blood biomarkers which are identified by this proteomics, may provide clinicians with more accurate parameters to assess inflammatory activity in UC.

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Host defense processes, which rely on both innate and adaptive immune mechanisms, are critical for the development of IBD.[1,2] Innate immunity participates in the activation of antigen-specific adaptive immune responses, including the production of antimicrobial peptides/proteins. In mammals, defensins, a class of antimicrobial peptides, can be divided into 2 major groups: α-defensins and β-defensins.[9] Six types of α-defensins have been identified, 4 of which are produced predominantly by neutrophils and phagocytes and stored in the granules of these cell types (denoted human neutrophil peptides 1–4; HNP 1–4). The remaining 2 α-defensins are localized in Paneth cell granules (denoted human α-defensins 5 and 6; HD 5 and 6). Although the amino-acid sequences of HNP 1, HNP 2, and HNP 3 are very similar, the sequence of HNP 4 is different than those of HNP 1–3. HD 5 is expressed by metaplastic Paneth cells in the colons of patients with UC or CD. The expression levels of HD 5 in blood, however, have not been examined; there are currently no data evaluating HNP 1–3 expression in patients with IBD.

In this study we clearly demonstrate serum profiling with increased levels of HNP 1–3 in the sera of patients with UC using a proteomics-based approach. We also compared the protein levels of HNP 1–3 in plasma samples from patients with UC and Crohn's disease (CD), before and after treatment for UC, and in patients in which treatment was effective or not effective. These analyses will contribute to our understanding of the pathogenesis of UC and aid in the discovery novel biomarkers to assess disease activity and therapeutic responses.

MATERIALS AND METHODS

Patients

After obtaining written informed consent, we analyzed a total of 94 blood samples from patients with IBD, colorectal cancer (CRC), infectious colitis, and control subjects. Forty-eight patients were diagnosed with UC (20 females and 28 males; median age, 39 years; age range, 12–72 years) and 22 with CD (11 females and 11 males; 29 years; 16–57 years). The control group contained 13 healthy subjects (5 females and 8 males; median age 30 years; age range, 24–34 years) and 5 with CRC (1 female and 4 males; median age 62 years; age range, 52–80 years) and 6 with infectious colitis (3 females and 3 males; median age 42 years; age range, 17–77 years). The study protocol was approved by the Ethics Committee of the Kagoshima University Graduate School of Medical and Dental Sciences (Kagoshima, Japan) and the Faculty of Medicine at the University of Miyazaki (Miyazaki, Japan). All IBD patients were diagnosed using established endoscopic, radiological, histological, and clinical criteria. The inactive or remission phase of UC was defined as a UCDAI score less than or equal to 2, whereas the active phase was defined as a UCDAI score greater than or equal to 3.[5] Twenty and 28 patients with UC were identified as inactive-phase and active-phase patients, respectively. All of the patients with active-phase UC were treated with oral corticosteroids, whereas 23 received leukocytapheresis therapy (LCAP) (Table 1). Furthermore, 4 of the active UC patients did not respond to treatment and eventually underwent a total colectomy. Fourteen patients with CD had high disease activities based on an International Organization for the Study of Inflammatory Bowel Disease (IOIBD) score of 2 or greater[10] and were regarded as active-phase patients. Eight patients that had lower IOIBD scores (0 or 1) were defined as inactive-phase patients. All 5 CRC patients were diagnosed with Duke's A group cancers by endoscopic, radiological, and histological examinations. All 6 patients with infectious colitis had diarrhea and fever, and were diagnosed based on clinical findings.

SELDI-TOF/MS

We used chips with cationic surfaces for analysis (CM10; Bio-Rad Laboratories, Hercules, CA). Serum samples were denatured in urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% dithiothreitol, and 2% ampholites), and then diluted 1:9 in binding/washing buffer (50 mM sodium acetate, pH 4). After washing the chip twice in binding/washing buffer, we applied 100 μL of diluted serum to each chip spot. Samples were incubated for 40 minutes and washed 3 times. After rinsing the chips once in water, 0.5 μL CHCA (α-cyano-4-hydoroxycinnamic acid; Nacalai Tesque, Kyoto, Japan) was applied twice to each spot and allowed to air-dry. Arrays were analyzed using a ProteinChip Reader (ProteinChip Biology System II; Bio-Rad Laboratories). TOF spectra were generated with laser shots collected in positive mode. The laser intensity ranged from 190 to 195 with a detector sensitivity of 6. On average, 65 laser shots per spectrum were used. A mixture of standard mass calibrant proteins (All-in-one Peptide Standard; Bio-Rad Laboratories) in 500 nL was used to calibrate the system for mass accuracy. The standards were applied to a single spot of the normal phase chip array (NP20; Bio-Rad Laboratories), after which two 1.0-μL samples of saturated CHCA were applied. TOF values were compared to the molecular masses of the standard proteins; calibration was performed according to the manufacturer's instructions.[7]

Immunodepletion Assay

Initially, 6 μL of anti-HNP 1–3 antibody solution (120 ng; Hycult Biotechnology, Netherlands) was bound to 30 μL of Protein A–agarose (Sigma Chemical, St. Louis, MO) for 15 minutes on ice. The postcentrifugation supernatant was discarded and the pellet was washed twice in buffer containing 20 mM HEPES (pH 7.8), 25 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.05% NP40. Then 15 μL of sera from each patient with UC was incubated with a pellet for 45 minutes on ice. As a negative control, samples were incubated with

TABLE 1. Characteristics of Patients with UC or CD

	UC		CD	
Disease activity[a]	Active	Inactive	Active	Inactive
Number	28	20	14	8
Gender (M/F)	19/9	9/11	10/4	6/2
Age (range), yr	41 ± 16 (14–68)	31 ± 16 (12–72)	32 ± 13 (16–57)	28 ± 7 (18–40)
Disease duration (range), yr	5.6 ± 4.8 (1–19)	5.2 ± 4.3 (1–18)	9.4 ± 7.4 (3–22)	6.0 ± 3.8 (1–13)
Treatment[b]				
5-aminosalicylic acid	28	19	14	8
Corticosteroid	28	7	10	2
Leukocytapheresis	23	0	0	0
Type of UC				
Left-side colitis	4	8	—	—
Pancolitis	24	12	—	—
Type of CD				
Ileal	—	—	4	2
Ileocolonic	—	—	9	5
Colonic	—	—	1	1

UC, ulcerative colitis; CD, Crohn's disease. Data are shown as the means ± SD or range.
[a]Active UC is defined as an Ulcerative Colitis Disease Activity Index score equal to or greater than 3, and active CD is defined as an International Organization for the Study of Inflammatory Bowel Disease score equal to or greater than 2.
[b]Includes the overlap treatment.

Protein A–agarose in the absence of a specific antibody. After incubation, samples were cleared by centrifugation; 3 μL of each supernatant was analyzed on NP20 ProteinChip arrays using a PBS II reader.[11]

ELISA

We determined the HNP 1–3 (P59665, P59666) concentrations in plasma using a human HNP 1–3 ELISA kit (Hycult Biotechnology) according to the manufacturer's instructions. Samples were analyzed in duplicate using a plate reader (Bio-Rad Laboratories) at 450 nm. The concentration of each protein in the plasma was calculated according to a standard curve.

Immunohistochemical Studies

HNP 1–3 expression in colon tissue was evaluated using immunohistochemistry. Abnormal colon tissues were obtained by total colectomy in patients with UC, whereas normal colon tissues were isolated in surgical resections for colon cancer by taking surrounding normal tissue without malignant cells. Colon tissues were fixed in 10% formalin and embedded in paraffin. For histological examination, 5-μm slices were stained with hematoxylin and eosin (HE). The anti-HNP 1–3 monoclonal antibodies (BMA Biochemicals, Augst, Switzerland) was diluted to a final concentration of 0.5% (w/v) in phosphate-buffered saline (PBS) supplemented with 1% fetal bovine serum (FBS). Immunohistochemical analysis of paraffin-embedded sections using antibodies against HNP 1–3 was performed as described.[12] EnVision plus horseradish peroxidase (Dako, Carpinteria, CA) was applied to samples; chromatin 3′,3′-diaminobenzidine was used to detect bound antibody.

Statistical Analysis

Values shown are the means ± SD. Statistical significance, including that for differences in laboratory data and individual peaks in SELDI-TOF/MS, was determined using Mann–Whitney *U*- and paired *t*-tests. *P*-values < 0.05 were considered to be statistically significant. The discriminatory power for each putative marker was described via the area under the curve (AUC) from receiver operating characteristic (ROC) analysis. The statistical analyses were performed using StatView 4.5 software (Abacus Concepts, Berkeley, CA), SPSS software (SPSS, Chicago, IL), and Ciphergen ProteinChip Software (Fremont, CA) v. 3.0.2.

RESULTS

Profiling Serum Proteins in Patients with UC

We performed differential profiling of serum proteins in 11 patients with UC and 7 normal healthy controls using the SELDI ProteinChip system. Peaks were automatically detected using Ciphergen ProteinChip Software 3.0.2.[7,13] Twenty-seven serum peaks in the 3000–10,000 m/z range

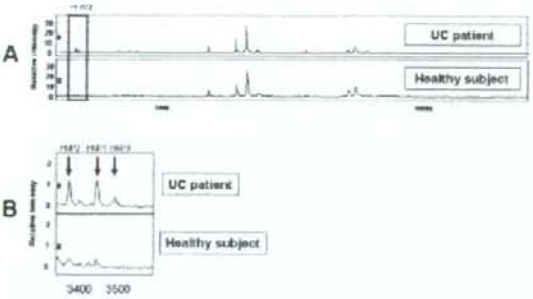

Figure 1. Serum proteomics of UC patients and healthy controls using SELDI-TOF/MS. (A) Spectra representing the serum proteins of a patient with UC and a healthy volunteer. The horizontal axis shows a range from 3000 to 10,000 m/z. Significant differences in peak intensities between patients with UC and healthy volunteers were found for 27 peaks. (B) The intensities of the protein peaks are shown for the range between 3300 and 3600 m/z. Protein peaks with m/z values of 3371, 3443, and 3486 represent HNP 2, HNP 1, and HNP 3, respectively.

were significantly different between the 2 patient groups (Fig. 1). Sixteen peaks resulted in *P*-values less than 0.01 (Table 2). The most dramatic difference was detected for a 3371 m/z protein, the level of which was increased in the sera of UC patients compared with healthy controls.

Identification of HNP 1–3

A previous study of colon tumor tissue identified a similarly increased signal at 3371 m/z using ProteinChip

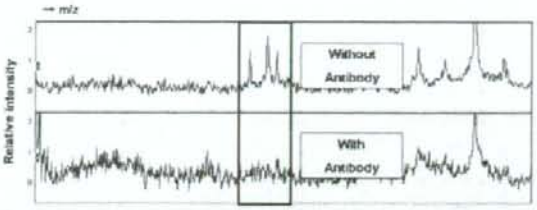

Figure 2. Immunodepletion assay using Protein A beads. Anti-HNP 1–3 antibodies (Hycult Biotechnology) were bound to 30 μL of Protein A beads. Sera from UC patients were incubated with these beads. After clearing by centrifugation, 3 μL of each supernatant was analyzed using an NP20 ProteinChip array.

arrays.[12,14] The peak was confirmed to correspond to HNP 2 with an immunodepletion assay. Peaks at 3443 and 3486 m/z, reported to correspond to HNP 1 and HNP 3 in the previous report, were also found to be significantly increased in analyses of the sera of UC patients compared to results observed for control samples. HNP 1, 2, and 3 have similar structures consisting of 30, 29, and 30 amino acids, respectively; 29 of the amino acids are identical among the peptides.[12,15] We also subjected the serum samples to immunodepletion assays using monoclonal antibodies against HNP 1–3 and found that the 3371, 3443, and 3486 m/z protein peaks in the SELDI-TOF MS spectra were no longer observed for the sera from patients with UC (Fig. 2). These peaks were clearly observed for negative control samples, which underwent immunodepletion assays in the absence of specific antibodies. These results indicate that the 3371, 3443, and 3486 m/z

TABLE 2. Discriminatory Peaks and Mean Values in Samples from Patients with Ulcerative Colitis and Healthy Volunteers

Mass to Charge (m/z)	Ulcerative Colitis (n = 11)	Healthy Subject (n = 7)	P-value
3371	1.42 ± 0.66	0.40 ± 0.10	4.8×10^{-4}
4789	0.51 ± 0.82	0.05 ± 0.03	4.8×10^{-4}
5421	0.34 ± 0.24	0.09 ± 0.02	4.8×10^{-4}
8688	0.65 ± 0.41	1.70 ± 0.38	6.8×10^{-4}
5838	0.79 ± 0.85	0.21 ± 0.05	9.4×10^{-4}
4351	0.82 ± 0.62	2.21 ± 0.56	1.3×10^{-3}
5620	0.11 ± 0.05	0.39 ± 0.23	1.7×10^{-3}
6881	1.00 ± 0.59	2.24 ± 0.46	1.7×10^{-3}
9358	0.17 ± 0.06	0.80 ± 0.52	1.7×10^{-3}
7023	0.12 ± 0.07	0.66 ± 0.46	2.4×10^{-3}
4469	3.31 ± 2.16	1.02 ± 0.59	3.2×10^{-3}
4542	0.39 ± 0.17	0.16 ± 0.02	4.3×10^{-3}
4590	0.86 ± 0.45	1.63 ± 0.26	4.3×10^{-3}
4287	0.68 ± 0.37	1.26 ± 0.39	5.7×10^{-3}
2900	0.18 ± 0.12	0.37 ± 0.14	9.8×10^{-3}
2979	1.00 ± 0.88	0.26 ± 0.15	9.8×10^{-3}

Statistical significance was determined using a Mann-Whitney *U*-test.

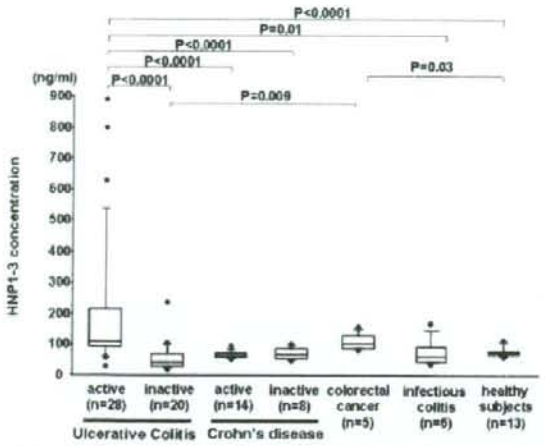

Figure 3. Concentrations of HNP 1–3 in the plasma of patients with UC, CD, colorectal cancer, infectious colitis, and healthy controls. Boxes indicate the median ± 25th percentile. The lower bar indicates the 10th percentile and the upper bar indicates the 90th percentile.

protein peaks, which were larger in the spectra for sera of UC patients, corresponded to HNP 1–3.

Concentrations of HNP 1–3 in Plasma

It was not possible to determine the individual concentrations of HNP 1, 2, or 3 using commercially available ELISA kits; therefore, we evaluated the total concentration of HNP 1, 2, and 3 in plasma. We found that there was a clear correlation between the serum HNP 1–3 peak intensities determined using the SELDI system and the plasma HNP 1–3 concentration measured using ELISAs in 11 patients with UC and 7 normal controls ($r = 0.68$, $P < 0.01$). We then determined the plasma concentrations of HNP 1–3 in 48 UC patients, 22 CD patients (Table 1), 5 CRC patients, 6 infectious colitis patients, and 13 healthy controls (Fig. 3). The plasma concentrations of HNP 1–3 were significantly higher in patients with active UC (203.1 ± 215.5 ng/mL) than in patients with inactive UC (58.3 ± 49.5 ng/mL), CD (active; 65.5 ± 11.2 ng/mL, inactive; 70.4 ± 20.0 ng/mL), infectious colitis (72.2 ± 16.5 ng/mL), or the healthy controls (77.5 ± 16.4 ng/mL). In addition, HNP 1–3 concentrations in patients with active UC tended to be higher in patients with CRC at Duke's stage A (100.8 ± 27.6 ng/mL), but not significantly. HNP 1–3 concentrations in CRC patients were also higher than those in patients with inactive UC and healthy controls.

Expression of HNP 1–3 in Intestinal Tissue

We examined the localization of HNP 1–3 in normal tissues and those from patients with active-phase CD or UC using immunohistochemistry. The colonic mucosa, lamina propria, muscle layer, and crypt abscesses of patients with active UC exhibited strong staining with anti-HNP 1–3 antibodies (Fig. 4). These sections contained a number of infiltrating neutrophils (Fig. 4B,C), which may provide a source of the secreted HNP 1–3 near the colonic epithelium. Positive staining for neutrophils, however, was seen in the blood vessels of both normal and abnormal colon tissues. In addition, small numbers of neutrophils with positive staining were seen in submucosal tissue of patients with CD (Fig. 4D). Epithelial cells in colon samples from patients with inflamed CD or from normal healthy subjects did not exhibit staining with anti-HNP 1–3 antibodies (Fig. 4D,E).

HNP 1–3 as a Biomarker in UC Patients

We investigated the association between the HNP 1–3 concentration and the clinical course of UC. We determined the HNP 1–3 concentrations in pairs of plasma samples from 15 patients with active UC obtained before and after induction therapy with corticosteroids (Table 3). Eight UC patients in the responder group were successfully treated by induction therapy. The elevated HNP 1–3 levels of UC patients in the responder group were reduced after induction therapy (Fig. 5). In contrast, 7 patients in the nonresponder group, 2 of whom had a total colectomy and 5 who quickly relapsed, were not effectively treated. The HNP 1–3 levels of patients in the nonresponder group before treatment were lower than those in the responder group and were not changed after treatment (Fig. 5). Additionally, although plasma HNP 1–3 levels (means ± SD) of responder active UC patients (273.0 ± 224.8 ng/mL) were higher than those with active CD (65.5 ± 11.2 ng/mL) ($P < 0.001$), those with nonresponder active UC (84.6 ± 26.5 ng/mL) were similar to those with active CD. These results indicate that patients with active UC and low HNP 1–3 levels do not respond well to treatment.

We evaluated the relationship between the HNP 1–3 levels and the clinical activity of UC. There was a significant correlation between the HNP 1–3 levels and the UCDAI score or the white blood cell count (WBC) of UC patients ($r = 0.54$, $P < 0.01$; $r = 0.55$, $P < 0.01$, respectively), although no correlation between the HNP 1–3 levels and the C-reactive protein (CRP) levels was noted ($r = 0.24$). In addition, ROC analysis was performed to estimate the efficiency of induction therapy for patients with active-phase UC; we calculated the sensitivity and specificity of HNP 1–3 levels for discriminating responder UC patients from nonresponders. We obtained a sensitivity of 89% and a specificity of 80% using a cutoff value of 100 ng/mL HNP 1–3; the ROC AUC was 0.89 between the responder and nonresponder groups of UC patients. For evaluations of the activity of UC, we compared such inflammatory markers as the CRP level and the WBC to the HNP 1–3 level in patients with UC. ROC AUC of the CRP level and WBC were 0.76 and 0.56, respectively. Thus,

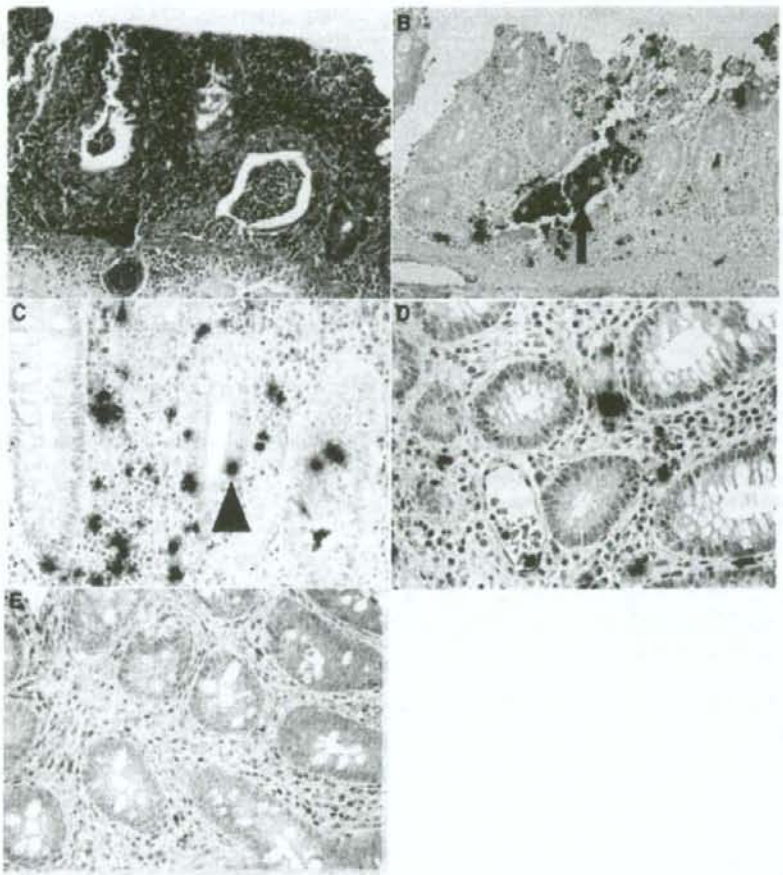

Figure 4. Expression of HNP 1–3 in the tissue of patients with active UC or CD and in normal colon tissue. (A) HE staining of colon tissues from patients with UC. (B,C) Immunohistochemical staining demonstrated extensive HNP 1–3 expression in the colon tissues of patients with UC. Many HNP 1–3-positive cells were observed in the crypt abscesses (B: arrow) and in neutrophils that had migrated into the epithelial layers (C: arrowhead). In addition, an ulcer lesion observed in the colon sample stained positive for HNP 1–3. (D,E) Although small numbers of neutrophils in the blood vessels and submucosal tissues were positive for HNP 1–3, epithelial cells in colon samples from patients with inflamed CD or normal subjects were not positive for HNP 1–3. Original magnification: 100× (A,B) and 200× (C–E).

the level of HNP 1–3 had a high discriminatory power for estimating the efficacy of treatment in patients with UC.

DISCUSSION

We identified 27 proteins that showed significant differences in the serum protein profiles of patients with UC compared with those of healthy controls using SELDI-TOF/MS analysis. Of these proteins, 3 signals around 3400 m/z were confirmed to correspond to HNP 1, 2, and 3. In addition, we observed an increase in HNP 1–3 plasma levels in patients with active-phase UC compared with that seen in patients with remission-phase UC or CD; these levels were higher in the plasma of UC patients who showed better therapeutic outcomes than in samples from nonresponder patients.

Several studies have suggested that the development of IBD requires the interaction of genetic factors with both specific luminal bacterial antigens and environmental triggers that break the mucosal barrier.[16-18] Although the principle treatment for IBD is the suppression of inflammation, treatment strategies for the 2 diseases, UC and CD, are somewhat different. Whereas these differences may address the different biomarkers of the 2 conditions, a specific biomarker for IBD remains unknown. To discover a biomarker of UC, we

TABLE 3. Characteristics of Patients with Active UC in the Responder Group and Nonresponder Group

	Responder	Nonresponder	*P*-value
Number	8	7	
Gender (M/F)	5/3	5/2	0.7
Age (yr)	33.5 ± 13.8 [14–50]	42.3 ± 19.8 [16–68]	0.4
CRP (mg/dl)	1.7 ± 1.7	3.3 ± 4.5	0.4
WBC (cells/ul)	12714 ± 4604	7657 ± 3423	*0.04*
Platelets × 10^4/ul	40.4 ± 7.4	36.2 ± 11.1	0.3
HNP 1–3 (ng/ml)	273.0 ± 224.8	84.6 ± 26.5	*0.002*
Type of UC			
Pancolitis/Left-side colitis	7/1	5/2	0.6
UCDAI score	9.4 ± 4.6	8.6 ± 1.9	0.7
Duration	6.7 ± 6.5 [1–19]	5.7 ± 5.1 [2–16]	0.8

Data are shown as the means ± SD [ranges]. Statistical significance was determined using a Mann-Whitney *U*-test or Fisher's exact test, as appropriate. UC, ulcerative colitis; UCDAI, Ulcerative Colitis Disease Activity Index.

employed ProteinChip technology. The likelihood of finding reliable tumor markers by analyzing tissue may be higher than in analyses of serum[12]; malignant cells may produce proteins that are useful biomarkers. In nonmalignant diseases, such as UC, protein profiling of serum or plasma may be more informative than that of tissue samples. Additionally, fluid samples, such as serum, are easier to obtain than tissue samples. Thus, we used serum samples to identify new biomarkers for UC.

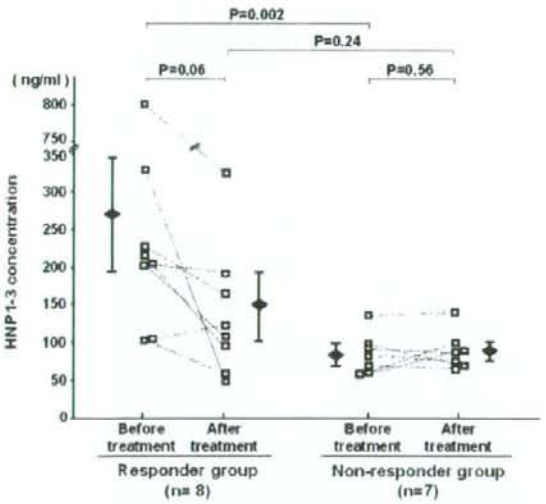

Figure 5. HNP 1–3 levels in the responder and nonresponder groups before treatment predicted therapeutic outcomes in UC patients; changes in the HNP 1–3 levels in UC patients in response to treatment are presented. The mean concentration of HNP 1–3 in the responder group before treatment was significantly higher than that seen in the nonresponder group, which indicates that HNP 1–3 levels may be an effective predictor of therapeutic outcomes. HNP 1–3 levels tended to decrease after treatment in the responder group, whereas no changes were observed for the nonresponder group. Patients whose plasma was not obtained after treatment were excluded from analysis.

Defensins are one of the most extensive peptide families of naturally occurring antibiotics. These peptides exhibit microbicidal activities against Gram-positive and Gram-negative bacteria, mycobacteria, fungi, and certain enveloped viruses. HNP 1–3 are part of the α-defensin family and components of the innate immune response. HNP 1–3 are synthesized by neutrophil precursor cells and released at inflammatory sites by mature circulating neutrophils.[9,19] The expression of HNP 1–3 has been observed in epithelial cells of the ileum and colon in patients with active UC or CD.[20] Whether neutrophils within inflamed colon tissue express HNP 1–3 in IBDs, however, is not known. In this study, we demonstrated that the colon mucosal tissue of patients with active UC or CD displayed minimal immunoreactivity for HNP 1–3, whereas the infiltrating neutrophils were stained strongly. These results indicate that HNP 1–3 were secreted from neutrophils, leading to increased plasma levels in patients with UC. High concentrations of HNP 1–3 can be cytotoxic for epithelial cells due to cytolysis and can induce apical conduction in Cl^- secretory epithelia.[21,22] Thus, whereas HNP 1–3 have antibacterial activities in the early phase of UC, they also may injure the colon if they are overexpressed by infiltrating neutrophils. High concentrations of HNP 1–3 may adversely affect colon tissues in UC patients, potentially contributing to diarrhea.[23] HNP 1–3 are secreted from the azurophilic granules of neutrophils following stimulation with IL-8.[24] Epithelial-derived IL-8 is thought to mediate neutrophil migration and infiltration during the inflammatory process of UC.[25,26] IL-8 mRNA levels are

significantly higher in UC patients with crypt abscesses.[27] Although HNP 1–3 have been reported to be expressed by surface enterocytes in the mucosa of patients with active IBD,[28] we observed only minimal staining of the colonic surface mucosa from patients with active UC using anti-HNP 1–3 antibodies. Moreover, Caco-2 and HT-29 cells, 2 colon epithelial cell lines, do not express HNP 1–3 (data not shown). Therefore, we hypothesized that HNP 1–3 are expressed by neutrophils following stimulation with IL-8, which suggested a correlation between the IL-8 and HNP 1–3 levels. We did not, however, observe a correlation between the IL-8 and HNP 1–3 levels in the plasma from active UC patients, and there was no association between the disease activity score and plasma IL-8 concentrations (data not shown). These results indicate that HNP 1–3 expression may be affected by other factors and HNP 1–3 values appear to be more useful to measure clinical UC disease activity than IL-8 levels.

Neutrophils are critical cellular mediators of the inflammation observed in UC. Neutrophils increase in number and display augmented activation during active-phase UC, but not inactive-phase UC.[28] Neutrophils extensively infiltrate colon tissue in patients with UC, and can be detected in the inflamed mucosa during even the early stages of inflammation.[29,30] Platelets are also important in the pathophysiology of UC.[31] Cytapheresis therapy (including LCAP) in combination with steroid therapy can be an effective treatment option for patients with active UC.[32] LCAP may remove and modulate both leukocytes and platelets, thereby altering the expression of proinflammatory cytokines.[33,34] The effect of LCAP on HNP 1–3 levels, however, has not been examined, and further studies are needed to determine whether HNP 1–3 levels decrease in response to LCAP. In addition, we showed that HNP 1–3 levels in the plasma were higher in patients with active UC than in those with infectious colitis, and HNP 1–3 levels were similar between patients with infectious colitis and healthy controls. In contrast, it was reported that HNP 1–3 levels in patients with severe infectious diseases, such as sepsis, were higher than those in healthy controls.[35] The disease severity of the enrolled patients with infectious colitis in this study may have affected our results. Cytapheresis therapy, however, may not be effective for severe infectious diseases, including infectious colitis, and high concentrations of HNP 1–3 in patients with active UC may be associated with disease characteristics. Further examination, including cases of infectious colitis with sepsis, will be necessary.

As previously reported, we found that several inflammatory makers, including the CRP level, WBC, and platelet count, decreased after treatment. Changes in these inflammatory markers did not predict the treatment outcome of patients with UC, whereas plasma levels of HNP 1–3 correlated with UC disease activity and predicted the therapeutic outcome. There were no correlations between plasma HNP 1–3 levels and inflammatory markers, such as platelet counts and CRP levels. These results may suggest that high levels of HNP 1–3 independently indicate the activity of disease and the feasible treatment outcome in patients with UC. However, there is a limitation in the use of HNP 1–3 measurement as a biomarker; low levels of HNP 1–3 in colitis patients did not diagnose whether they had nonresponder UC or active CD. Therefore, low levels of HNP 1–3 in colitis patients should be assessed by clinical symptoms, stool for bacterial examination, and endoscopic and radiographic examination of the gastrointestinal tract for diagnosis. Other proteins and peptides that were detected by SELDI/TOF-MS in this study are now under investigation and may serve as additional biomarkers for the assessment of IBD, especially in nonresponder UC patients.

The levels of HNP 1–3 in tumor tissue and serum were reported to increase in patients with CRC.[12] It was also reported that plasma HNP 1–3 concentrations determined using ELISA increased in Duke's stages C and D, but not in A or B compared to healthy controls.[14] In contrast, we showed that HNP 1–3 concentrations in CRC patients at Duke's stage A were higher than those seen in patients with inactive UC and healthy controls. Although HNP 1–3 concentrations in CRC patients at Duke's stage A seem to be similar between our study and a previous study[14] (100.8 ± 27.6 versus 105.4± 80.6 ng/mL, respectively), the concentrations in the healthy controls were different between the 2 studies (77.5 ±16.5 versus 96.6 ± 36.2 ng/mL). In addition, Albrethsen et al[14] mentioned that in addition to Duke's C and D, HNP 1–3 expression in CRC tissues at Duke's A and B was higher than in normal tissue by SELDI ProteinChip. It is controversial whether the increased HNP 1–3 in tumors is localized to cancer cells or to neutrophilic leukocytes. There is the possibility that the plasma HNP 1–3 levels will increase in patients with CRC at Duke's stage A and that HNP 1–3 concentration is a potential marker for the assessment of CRC patients with advanced disease.[12,14] In addition, these results indicate that HNP 1–3 levels may not be able to distinguish between active UC and colon cancer. In the clinical setting, however, UC can typically be distinguished from colon cancer by various clinical features, such as diarrhea, fever, and colonoscopic findings. On the other hand, colon cancer commonly occurs in patients with UC, especially those who have suffered from the disease for a long period of time; such colon cancers are difficult to detect using colonoscopy. HNP 1–3 levels may help to signal the occurrence of colon cancer in UC patients when high concentrations of HNP 1–3 are detected in the absence of active colitis; these patients should be extensively examined, including total colonoscopy and random biopsies.

In conclusion, we used SELDI-TOF/MS to perform serum protein profiling and determined that HNP 1–3 levels increase in patients with active-phase of UC. We also con-

firmed that HNP 1–3 are predictive markers for UC treatment outcomes. Although these markers may not distinguish UC from CRC, HNP 1–3 are useful markers for the differential diagnosis of patients with IBD.

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IgG Oligosaccharide Alterations Are a Novel Diagnostic Marker for Disease Activity and the Clinical Course of Inflammatory Bowel Disease

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BACKGROUND AND AIMS: Patients with inflammatory bowel disease (IBD) share several immunologic similarities with rheumatoid arthritis (RA). Patients with RA have significantly increased levels of serum agalactosyl immunoglobulin G (IgG). Our aim was to investigate the clinical significance of analyzing the oligosaccharide structure of serum IgG in patients with IBD.

METHODS: Serum IgG oligosaccharide structures were analyzed using high-performance liquid chromatography in 60 patients with Crohn's disease (CD), 58 patients with ulcerative colitis (UC), 27 healthy volunteers (HV), and 15 disease controls (DC). The activity and mRNA level of beta-1,4-galactosyltransferase (Beta4GalT) in antibody-secreting cells were investigated in these subjects.

RESULTS: The agalactosyl fraction of the fucosylated IgG oligosaccharides (G0F/G2F) in CD and UC was significantly greater than that in HV and DC ($P < 0.001$). The percentage of subjects with a high G0F/G2F in CD, UC, HV, and DC was 72%, 33%, 0%, and 0%, respectively. G0F/G2F, which is significantly correlated with disease severity in both CD and UC, had higher sensitivity to diagnose IBD compared with anti-*Saccharomyces cerevisiae* antibody. Moreover, G0F/G2F was significantly correlated with the prognosis of UC patients: patients with a high G0F/G2F did not maintain long-term remission. The activity and mRNA level of Beta4GalT were significantly elevated in UC but not in CD.

CONCLUSIONS: G0F/G2F is a potentially effective diagnostic marker of disease activity in both CD and UC, and of the clinical course in UC. A pathophysiologic difference between CD and UC was also demonstrated.

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INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is characterized as a chronic relapsing inflammatory process of the digestive tract. Although the precise etiology of IBD remains unknown, both genetic susceptibility (1) and dysregulation of the mucosal immune responses against enteric host flora (2) have pivotal roles in its pathogenesis. Several serologic markers related to immune responses were developed for the diagnosis of IBD (3–8). The prevalence of perinuclear antineutrophil cytoplasmic antibodies in the UC population is between 45% and 82% (3). Approximately 2–28% of patients with CD, however, also express this antibody (3). Anti-*Saccharomyces cerevisiae* antibody (ASCA), directed against oligomannan, is expressed in 48–69% of patients with CD and approximately 5–15% of patients with UC (3). Although several other antibodies have been investigated to aid in the diagnosis of IBD (3–8), the sensitivities and specificities of these antibodies are not high enough to have an essential role in the current diagnostic algorithm for IBD.

Immunoglobulin (Ig) G carries *N*-linked oligosaccharides at the Cgamma2 domain of the Fc fragment at asparagine 297 (9), all of which are biantennary complex type with or without bisecting *N*-acetylglucosamine (GlcNAc), corefucose, galactose, and sialic acid residues (10–12). There are increased levels of agalactosyl IgG, which lacks terminal galactose in the IgG oligosaccharide, in the sera of patients with rheumatoid arthritis (RA) (13) and other chronic inflammatory diseases, including systemic lupus erythematosus, Sjögren's syndrome, and tuberculosis (14, 15). The oligosaccharide structure of IgG or its relationship to disease activity and prognosis in IBD patients, however, has not yet been investigated. In addition, the significance or incidence of anti-agalactosyl IgG autoantibodies has not been examined in IBD, although the existence of such autoantibodies has been reported in certain autoimmune diseases, including RA (16, 17).

In the present study, we analyzed the oligosaccharide structures of IgG using high-performance liquid chromatography (HPLC) and investigated the serum anti-agalactosyl IgG antibody levels. Our comprehensive oligosaccharide analysis revealed that the agalactosyl fraction in the fucosylated oligosaccharides was significantly higher in CD and UC patients than in healthy volunteers (HV) and disease controls (DC). Agalactosyl IgG was observed in UC patients with an aggressive disease phenotype. Agalactosyl IgG levels were closely correlated with disease activity and the clinical course of IBD, and had a significantly higher sensitivity to diagnose IBD compared with ASCA. Finally, investigation of the expression and activity of glycosyltransferases and glycosidases revealed a pathophysiologic difference between CD and UC.

MATERIALS AND METHODS

Subjects

Serum samples were collected from 60 patients with CD, 58 patients with UC, 27 age/gender-matched unrelated HV, and 15 patients with colonic inflammation including appendicitis, diverticulitis, and ischemic colitis (disease control, DC). All participants were Japanese, recruited at the Department of Gastroenterology and Hepatology, Osaka University Hospital (Suita, Osaka, Japan), the Department of Gastroenterology, Osaka Rosai Hospital (Sakai, Osaka, Japan), and the Department of Surgery, Rinku General Medical Center (Izumisano, Osaka, Japan). The ethics committee at each hospital approved the study protocol and written informed consent was obtained from each participant. Patients were diagnosed with CD or UC according to endoscopic, radiologic, histologic, and clinical criteria provided by the Council for International Organizations of Medical Sciences in the World Health Organization and the International Organization for the Study of Inflammatory Bowel Disease (18–20). Disease location and behavior in CD were determined based on the Vienna classification (21). Clinical activities were determined using the Crohn's Disease Activity Index (CDAI) for CD (22) or the Clinical Activity Index (CAI) for UC (23). Clinical remission was defined as CDAI of <150 in CD and CAI of <6 in UC (22, 24). Detailed patient characteristics are presented in Table 1.

IgG Purification

Serum IgG was purified using protein G sepharose (Amersham Pharmacia Biotech, Buckinghamshire, UK). Briefly, half-diluted serum with phosphate-buffered saline (PBS) was loaded onto a protein G sepharose column. The column was subsequently washed with a minimum of 10 column volumes of PBS, followed by the same volume of 10 mM ammonium bicarbonate. Column-bound IgG was eluted using 0.1% trifluoroacetic acid.

Analysis of Pyridylaminated N-Linked Oligosaccharide of IgG by Reverse Phase HPLC

N-linked oligosaccharides were released from serum IgG and labeled with 2-aminopyridine, as described previously

Table 1. Patient Characteristics

	CD (N = 60)	UC (N = 58)	HV (N = 27)	DC (N = 15)
Male/female	44/16	32/26	17/10	6/9
Age, yr, mean (SD)	38 (14)	39 (15)	38 (11)	36 (17)
Age at diagnosis, yr, mean (SD)	29 (13)	34 (14)		
Bowel surgery (including appendectomy), N (%)	36 (60)*	2 (3)		
Extraintestinal manifestations, N (%)	5 (8)	2 (3)		
Treatment				
Salazosulfapyridine or mesalazine, N (%)	50 (83)	51 (88)		
Steroids, N (%)	4 (7)*	29 (50)		
Immunomodulators, N (%)	5 (8)	4 (7)		
Infliximab, N (%)	8 (13)	0 (0)		
Total parental nutrition or elemental diet, N (%)	39 (65)*	6 (10)		
Disease location (N)				
Small bowel/colon/both/unknown	11/11/37/1			
Extensive/left colon/rectum and sigmoid		31/18/9		
Disease behavior (N)				
Inflammatory/structuring/penetrating/unknown	18/22/16/4			
CRP, mg/dL, mean (SD)	1.6 (3.2)	1.7 (3.2)		
CDAI (CD) or CAI (UC), mean (SD)	197 (102)	5.9 (5.7)		

*$P < 0.001$ *versus* UC.

(12). Briefly, *N*-linked oligosaccharides were released from purified IgG samples by overnight incubation with 0.5 mU glycopeptidase F (Takara Bio Inc., Shiga, Japan) at 37°C. Oligosaccharides were further incubated with 50 mM ammonium acetate (pH 4.0) for 30 min, lyophilized, and labeled with 2-aminopyridine by GlycoTag (Takara Bio Inc.) following the manufacturer's instructions. Excess reagent was removed with a cellulose cartridge glycan preparation kit (Takara Bio Inc.) and then oligosaccharides were incubated with 2 M acetic acid at 80°C for 2 h to remove sialic acids. Pyridylamino (PA)-oligosaccharides from IgG were analyzed on reverse phase HPLC system (Waters Corp., Milford, MA) using PALPAK Type R-MB (Takara Bio Inc.) at a flow rate of 0.5 mL/min using 10 mM sodium phosphate (pH 4.4, solvent A) and the same buffer containing 0.5% 1-butanol (solvent B) at 40°C. The glycans were separated with a gradient of 0–50% solvent B for 30 min followed by 10 min of 50% solvent B. PA-oligosaccharides were detected using a fluorescence detector (Waters 2475, Waters Corp) at wavelengths of 320 nm for excitation and 400 nm for emission.

Analysis of Anti-Agalactosyl IgG Antibody

The anti-agalactosyl IgG antibody levels were measured using a lectin enzyme immunoassay kit (Eitest CARF, Eizai Co., Tokyo, Japan), according to the manufacturer's instructions (16). Briefly, diluted serum samples were added to plates precoated with human agalactosyl IgG. After rinsing with the washing buffer, biotinylated *Ricinus communis* agglutinin (RCA) 120, which recognizes the terminal galactose of anti-agalactosyl IgG antibody, was added to the plate and incubated for 1 h. After another rinse, horseradish peroxidase-conjugated streptavidin was added and the plate was incubated for 1 h. After a final rinse, the plate was incubated with a chromogen substrate solution. The reaction was stopped with 2 mM sodium azide after 30 min of incubation, and absorbance was measured at a wavelength of 405 nm with a microplate reader using a reference wavelength of 490 nm. Values greater than 6.0 AU/mL were defined as positive (16).

*Analysis of Anti-*Saccharomyces cerevisiae *Antibody*

Serum ASCA concentrations were examined using the ASCA IgG enzyme-linked immunosorbent assay kit (Genesis Diagnostics, Cambridge, UK), according to the manufacturer's instructions. Values over 10 U/mL were defined as positive.

Isolation of B cells and Plasma Cells From Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells were isolated from the heparinized venous blood of subjects by Ficoll–Hypaque density-gradient centrifugation. B cells and plasma cells were separated from peripheral blood mononuclear cells with a B cell isolation kit II and plasma cell isolation kit, respectively (Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer's instructions.

Real-Time Reverse Transcription-Polymerase Chain Reaction for Beta4GalT

Total cellular RNA was isolated using Isogen-LS (Wako Chemicals, Osaka, Japan), and complementary DNA was synthesized from 0.1 to 0.5 μg of total RNA using Superscript III first-strand system (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. For TaqMan real-time reverse transcription-polymerase chain reaction (RT-PCR), the reaction mixture was prepared by TaqMan Universal PCR Master Mix with predesigned and prelabeled TaqMan PCR primer and probe set for human beta-1,4-galactosyltransferase (Beta4GalT) I or human beta-actin endogenous control (Applied Biosystems, Foster City, CA). Real-time PCR was performed using an ABI PRISM 7900HT Sequence Detection System instrument and software (Applied Biosystems). Each sample was run in duplicate. The relative RNA amount was calculated with the $\Delta\Delta$Ct method (25) and normalized to internal control beta-actin.

Analysis of Beta4GalT Activity in Plasma Cells

Beta4GalT activity was measured as described previously (26, 27). Isolated plasma cells were dissolved in TNE buffer (25 mM Tris-HCl [pH 7.8], 1% Nonidet P-40 [NP-40], and 1 mM ethylenediaminetetraacetic acid [EDTA]) and the supernatant was collected. The cellular supernatant was mixed with uridine diphosphate (UDP)-galactose (Sigma-Aldrich, St. Louis, MO) and PA-agalactosyl *N*-linked oligosaccharides as acceptor substrates, which were generated as previously reported (28). The mixture was incubated at 37°C for 24 h and the reaction was terminated by boiling for 1 min. The samples were then centrifuged at 12,000 *g* for 10 min and 5 μL of 25 μL supernatants were analyzed by HPLC, as described above. Beta4GalT activity was calculated as follows: the area under the peak of galactosylated oligosaccharides was measured after the reaction, and the concentration was determined using a standard galactosyl biantennary PA-oligosaccharide. Beta4GalT activity was expressed as nmol/h by dividing the concentration of galactosyl oligosaccharides by the incubation time.

Statistical Analyses

Differences between measurements and groups were tested with Mann-Whitney U-test. Either the χ^2 test, χ^2 test with Yates' correction (when sample number was less than 10), or Fischer's exact test (when sample number was less than 4), where appropriate, was used for the comparison of frequencies. Sensitivity for each test result was defined as the probability of a positive test result in a patient with the disease under investigation. Specificity was defined as the probability of a negative test result in a patient without the disease under investigation. A receiver operated characteristic (ROC) curve was generated by plotting sensitivity *versus* 1 − specificity (29, 30). Area under the curve (AUC) was calculated by StatMate software (ATMS Co., Tokyo, Japan). A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Increased Agalactosyl Fraction of the Fucosylated IgG in IBD Patients

Normal oligosaccharide structures of human neutral IgG comprise 12 major structural variants (Fig. 1A) (12). We analyzed the profiles of IgG neutral oligosaccharides using HPLC in combination with fluorescent labeling of oligosaccharides. Representative profiles of HV and CD are shown in Figure 1B. We divided the oligosaccharides into three subgroups (groups I–III) according to the existence of core-fucose, bisecting GlcNAc, and both. Group II oligosaccharides, which have only core-fucose, were the major group, comprising approximately 80% of the total oligosaccharides. In fucosylated group II oligosaccharides of HV, the peak of the agalactosyl oligosaccharide (Fig. 1B) was usually lower than that of the fully galactosyl oligosaccharide (Fig. 1B). In contrast, in CD, the peak of the agalactosyl oligosaccharide (e) was higher than that of the fully galactosyl oligosaccharide (h) (Fig. 1B).

We defined the agalactosyl peak in oligosaccharides (a, e, and i) as G0 and the fully galactosyl peak (d, h, and l) as G2. The peak height ratio of G0 to G2 was calculated by dividing the peak height of G0 by that of G2 in each group. In fucosylated group II oligosaccharides, the peak height ratio of G0 to G2 in both CD and UC was significantly higher than that in HV ($P < 0.001$ for CD and UC) and the ratio in CD was significantly higher than that in UC ($P < 0.001$, Fig. 2). In order to exclude the possibility that the increase in the peak height ratio of G0 to G2 in group II is a common feature not only of IBD but also of other intestinal inflammation, we analyzed the ratio in DC. The ratio of G0 to G2 in group II in both CD and UC was also significantly higher than that in DC ($P < 0.001$ for CD and $P < 0.01$ for UC). In group III oligosaccharides, containing also core-fucose, a significant increase was also observed in the ratio of G0 to G2 in both CD and UC when compared with HV and DC. In the nonfucosylated group I oligosaccharides, however, the ratio of G0 to G2 was not increased in IBD (Fig. 2).

Correlation Between G0F/G2F and Disease Activity

Among the three subgroups of oligosaccharides, fucosylated group II was the major group, and most clearly reflected the oligosaccharide alterations in IBD. We defined the peak height ratio of G0 to G2 of the fucosylated group II as "G0F/G2F," and G0F/G2F was used for the following clinical analysis. Because it was controversial whether the prevalence of agalactosyl IgG in IBD correlates with C-reactive protein (CRP) and disease activity (31, 32), we investigated the correlation of G0F/G2F with clinical parameters. In CD, G0F/G2F in active patients (CDAI $\geq$150) was significantly higher than that in patients in remission (CDAI <150, $P < 0.01$, Fig. 3A). G0F/G2F was also significantly higher in CD patients with extensive disease where inflammation was not limited to the terminal ileum (category L2 and L3 in Vienna Classification) (21) than in patients with inflammation in the terminal ileum alone (category L1, $P < 0.05$, Fig. 3B). Similarly, G0F/G2F was significantly higher in active UC patients (CAI $\geq$6) than in patients in remission (CAI <6, $P < 0.01$, Fig. 3C). G0F/G2F was significantly higher in UC patients with extensive disease (total colitis) than in those with only

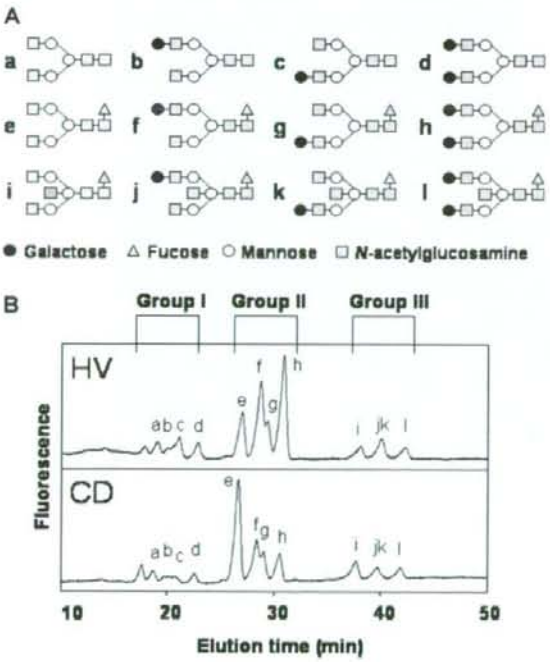

Figure 1. Structures of oligosaccharides attached to human IgG. (*A*) Structural patterns of *N*-linked neutral oligosaccharides. (*B*) Representative profiles of neutral IgG oligosaccharides purified from HV and CD patients.

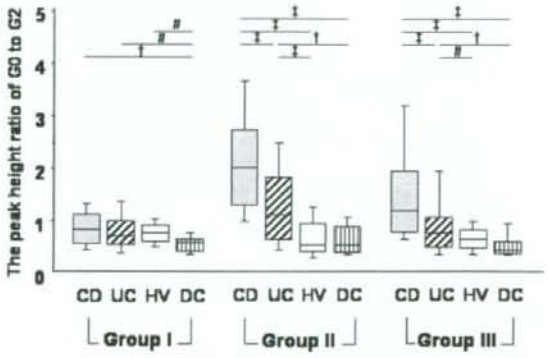

Figure 2. Increased fucosylated agalactosyl IgG in IBD patients. The peak height ratio of G0 to G2 in each subgroup was calculated for the subjects. Box plots show 50% of the relevant patient population. The line inside the box represents median value. Whiskers indicate the 90th and 10th percentiles. $^{\dagger}P < 0.001$, $^{\ddagger}P < 0.01$, $^{\#}P < 0.05$ by Mann-Whitney U-test.

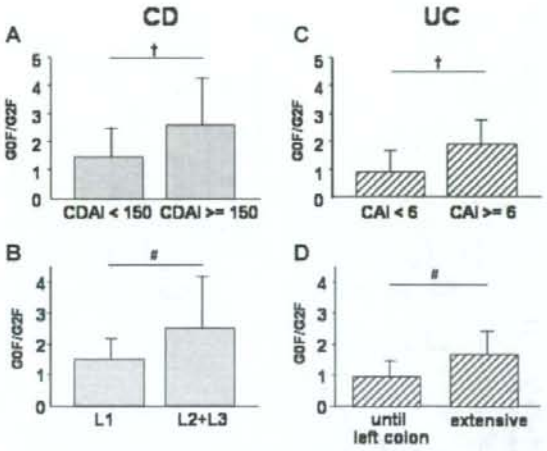

Figure 3. G0F/G2F and clinical manifestations. (*A*) G0F/G2F of CD patients in remission (CDAI <150) and in an active stage (CDAI ≥150). (*B*) G0F/G2F of CD patients with category L1 and L2 + L3. (*C*) G0F/G2F of UC patients in remission (CAI <6) and in an active stage (CAI ≥6). (*D*) G0F/G2F of UC patients with only left-side colon involvement and with extensive disease. Results are shown as mean ± SD. $^{\dagger}P < 0.01$, $^{\#}P < 0.05$ by Mann-Whitney U-test.

left-side colon involvement ($P < 0.05$, Fig. 3D). In contrast to a previous observation (32), we found no correlation between G0F/G2F and CRP level, age at onset, or disease duration (data not shown).

Infrequent Positive Rate of Anti-Agalactosyl IgG Antibody in IBD Patients

Anti-agalactosyl IgG antibody is used as an early diagnostic marker for RA, and 84% of patients with RA are positive for anti-agalactosyl IgG antibody (16, 17). Unexpectedly, only 1 of 49 patients with CD (2.0%) and 2 of 51 patients with UC (3.9%) were positive for anti-agalactosyl IgG antibody. In the 3 patients who were positive for anti-agalactosyl IgG antibody, there were no differences in the disease characteristics when compared with the patients negative for anti-agalactosyl IgG antibody (data not shown).

G0F/G2F as a Serologic Marker for IBD

We then investigated the effectiveness of the IgG oligosaccharide structure as a serologic marker for IBD. The condition in which G0F/G2F was equal to or higher than 1.4, which was the mean + 2 SD of G0F/G2F in HV, was defined as "G0F/G2F-positive." The G0F/G2F-positive rate in CD, UC, HV, and DC was 72%, 33%, 0%, and 0%, respectively (Fig. 4A). We then compared the sensitivity and specificity of G0F/G2F with those of ASCA for the discrimination of IBD by ROC curve and AUC. Both the sensitivity and specificity of G0F/G2F were higher than those of ASCA for the differentiation of CD and HV (AUC of G0F/G2F *vs* ASCA 0.926, 95% confidence interval [CI] 0.872–0.980 *vs* 0.815,

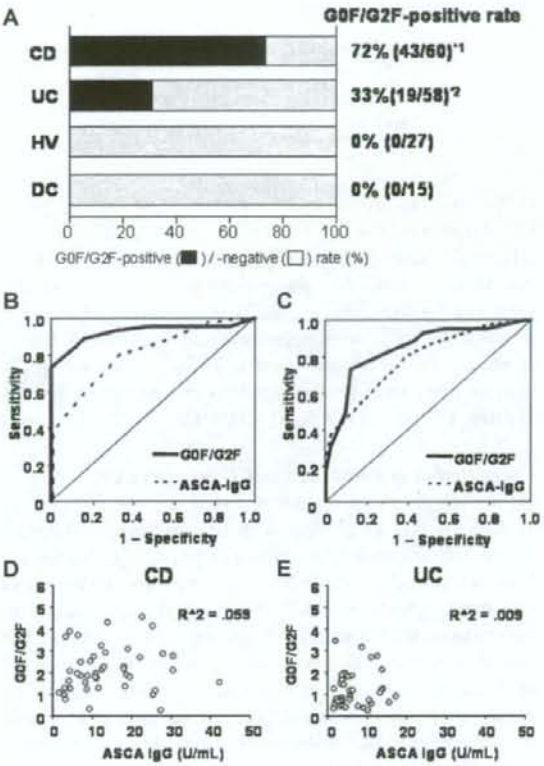

Figure 4. Effectiveness of G0F/G2F-positive rate as a serologic marker for IBD. (*A*) The G0F/G2F-positive rate in the subjects. The χ^2 test was used for the comparison between CD and UC and Fisher's exact test was used for the comparison between CD and HV or DC and between UC and HV or DC. $^{*1}P < 0.001$ to UC, HV, and DC, $^{*2}P < 0.001$ to HV, and $P < 0.010$ to DC. (B) The ROC curves for G0F/G2F and ASCA levels for the discrimination between CD and HV, or (*C*) between CD and UC. Sensitivity is represented on the *y*-axis and 1 − specificity on the *x*-axis. (*D,E*) The correlation between ASCA and G0F/G2F in CD and UC.

95% CI 0.732–0.897, Fig. 4B). Moreover, both the sensitivity and specificity of G0F/G2F were higher than those of ASCA for the differentiation of CD and UC (AUC of G0F/G2F *vs* ASCA 0.849, 95% CI 0.780–0.918 *vs* 0.792, 95% CI 0.714–0.869, Fig. 4C). There was no correlation between G0F/G2F and ASCA levels in CD and UC (Figs. 4D and E).

G0F/G2F as a Marker for Predicting the Clinical Course of IBD

We then investigated the correlation between G0F/G2F and the clinical background of IBD. When "clinical relapse-free" was defined as the condition in which patients maintain remission for more than 1 yr by taking either salazosulfapyridine or 5-aminosalicylic acid (without corticosteroid, antitumor necrosis factor [TNF]-α antibody, and immunomodulators), the clinical relapse-free rate of G0F/G2F-positive UC patients

Table 2. Clinical Relapse-Free Rate and G0F/G2F in Patients With CD and UC

	G0F/G2F-Negative	G0F/G2F-Positive	*P* Value
UC	77% (20/26)	11% (1/9)	<0.001
CD	50% (2/4)	6% (1/17)	0.08

(11%) was significantly lower than that of G0F/G2F-negative UC patients (77%, $P < 0.001$, Table 2). The clinical relapse-free rate of G0F/G2F-positive CD patients was lower than that of G0F/G2F-negative patients, although the difference was not significant (Table 2). Moreover, in UC patients whose CRP levels were negative at the time of blood sampling, the clinical relapse-free rate of G0F/G2F-positive UC patients (0%) was significantly lower than that of G0F/G2F-negative UC patients (90%, $P < 0.001$).

Upregulation of Beta4GalT in UC but not in CD

To investigate the mechanism underlying the increase in agalactosyl IgG in patients with IBD, we analyzed serum beta-galactosidase activity, which is responsible for the release of terminal galactose from IgG oligosaccharides. Fresh sera from patients with CD and HV were incubated with pyridylaminated biantennary oligosaccharides with an outer arm of galactose, and these oligosaccharides were subjected to HPLC analysis. The terminal galactose was not depleted in the sera of patients with CD or HV, suggesting that there is no increase in beta-galactosidase activities in the sera of patients with either CD or HV (data not shown).

We next examined the possibility that IBD patients have compromised beta-galactosyltransferase enzyme activity in both plasma cells and B cells. In plasma cells prepared from UC patients, Beta4GalT I mRNA expression was significantly higher than that in CD ($P < 0.05$) or HV ($P < 0.01$, Fig. 5A) patients. Beta4GalT I mRNA expression in B cells of UC patients was also significantly higher than that of CD or HV ($P < 0.05$, Fig. 5B) patients. Furthermore, Beta4GalT activity in the plasma cells of UC patients was higher than that of CD or HV patients (Fig. 5C).

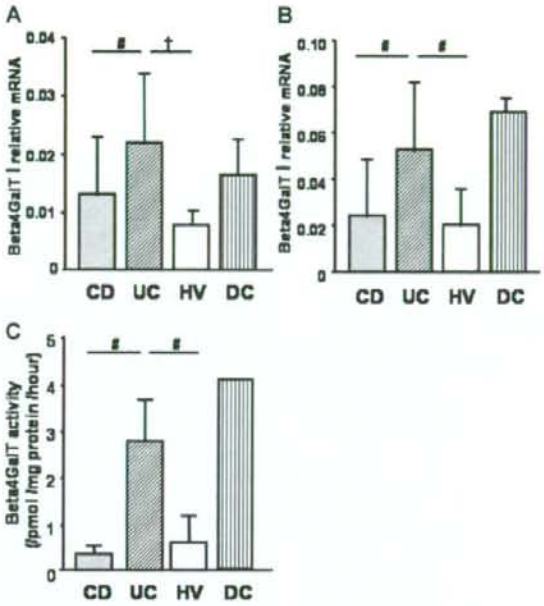

Figure 5. mRNA expression and enzyme activity of beta-1,4-galactosyltransferase (Beta4GalT). (*A*) Beta4GalT I mRNA expression in plasma cells of CD (N = 9), UC (N = 9), HV (N = 5), and DC (N = 3). (*B*) Beta4GalT I mRNA expression in B cells of CD (N = 8), UC (N = 5), HV (N = 5), and DC (N = 2). (*C*) Beta4GalT enzyme activity in plasma cells of CD (N = 3), UC (N = 3), HV (N = 3), and DC (N = 1). Results are shown as mean ± SD. $^{\dagger}P < 0.01$, $^{*}P < 0.05$.

DISCUSSION

The findings of the present study indicate that G0F/G2F, the extent of agalactosylation of fucosylated IgG, is a potential diagnostic marker for IBD. Among several serologic markers reported to have diagnostic value for IBD, ASCA is most suitable for detecting CD (3–8). Our results, however, demonstrate that G0F/G2F is a better marker than ASCA for the differentiation between CD and HV or CD and UC. In addition, G0F/G2F was not increased in patients with DC, suggesting that intestinal inflammation is not the direct cause of IgG agalactosylation. Furthermore, our results indicate that G0F/G2F reflects the clinical activity, severity, and clinical outcome of IBD. Especially in G0F/G2F-positive UC patients, the clinical relapse-free rate for a 1-yr period was significantly lower than that in the G0F/G2F-negative patients. Although this analytical method would not be useful for screening CD and UC patients in the general population, it will be useful to distinguish IBD from DC. Furthermore, it can be useful to distinguish aggressive disease from nonaggressive ones. The clinical outcome of not only UC but also CD patients for a longer period and with a larger sample size needs to be investigated and the project is currently ongoing in our laboratory. CD patients expressing an increased number of antibodies against anti-I2, antiouter membrane protein C, anti-CBir1 flagellin, and ASCA exhibit rapid disease progression (33). In addition to these reported antibodies, our study revealed that G0F/G2F has a predictive value of rapid disease phenotype in both CD and UC.

Although increased agalactosyl IgG levels are reported in approximately half of CD patients (32), the levels have not been so high, as demonstrated in the present study. Previously, anti-GlcNAc antibodies that detect galactose-uncovered terminal GlcNAc were used for measuring agalactosyl IgG. The specificity of anti-GlcNAc antibodies for the terminal agalactosylation is limited, however, because several subsets of oligosaccharides in IgG, including those with bisecting GlcNAc (Fig. 1A), can be recognized by the anti-GlcNAc antibody. In addition, the increase in

nonspecific glycosylation, such as Fab oligosaccharides or extra branches, will enhance the reactivity to this antibody. Thus, the degree of agalactosylation of IgG is likely to be underestimated. Our study clearly reevaluated the high prevalence of agalactosylation in fucosylated IgG oligosaccharides in IBD by comprehensive oligosaccharide analysis using HPLC.

Along with the increase in agalactosyl IgG, anti-agalactosyl IgG antibody is increased in several diseases, including RA (16). In contrast, the present study has revealed that anti-agalactosyl IgG antibody levels were not increased in IBD in spite of the increase in G0F/G2F. Thus, RA and IBD may be serologically distinguishable by anti-agalactosyl IgG antibody. There are several possible mechanisms to explain the absence of increased anti-agalactosyl IgG antibody levels in IBD: the first is that B cells and plasma cells in IBD are less responsive to agalactosyl IgG in terms of producing antibodies than those in other inflammatory diseases such as RA. The second is that agalactosyl IgG in IBD binds to other proteins to escape antibody production. The third is a problem of the methodology for measuring anti-agalactosyl IgG antibody with RCA120 lectin; the molecule(s) that bind to plate-coated agalactosyl IgG are not necessarily antibodies. Although the precise mechanisms are not clear, the measurement of anti-agalactosyl IgG antibody levels using the present method might not be useful for the diagnosis and treatment of IBD.

Beta4GalT has a role in conjugating galactose to the outer arm of GlcNAc in the *N*-linked oligosaccharides of IgG, and alterations in the Beta4GalT level are involved in IgG glycosylation (34). Thus, G2 oligosaccharides are generated from G0 oligosaccharides through an interim product with galactose in one of the outer branches of the oligosaccharide (G1). Therefore, the peak height ratio of G0 to G2, rather than G0 to G1 or G1 to G2, will mostly reflect the maturation status of glycosylation. However, it has not been fully proven how and where glycosylation is controlled. Furthermore, the function of agalactosyl IgG has not been clarified yet. Although Beta4GalT mRNA expression and its activity in B cells are often reported to be lower in RA, there are several conflicting reports (34, 35). In the present study, Beta4GalT I mRNA expression in B cells and plasma cells was upregulated in UC but not in CD, and Beta4GalT enzyme activity in plasma cells was increased in UC and DC but not in CD. The changes in agalactosylation observed in IBD are not clearly explained by the enzyme activities. However, elevated Beta4GalT may serve to conjugate galactose to the rapidly produced and consumed IgG during inflammation in UC and DC. In contrast, Beta4GalT was not elevated in CD in spite of the chronic inflammation. The mechanistic difference in the enzyme activity needs to be clarified. When seen from a different angle, lack of an increase in Beta4GalT in CD might be partially responsible for the increased agalactosyl IgG in CD. Besides the enzyme activity, there is another possibility that the extent of glycosylation alters the half-life of the protein. Furthermore, the half-life of glycosylated IgG might be affected by the extent of glycosylation in the inflammatory conditions, *e.g.*, UC and CD. It is important to clarify these issues and these projects are in progress.

In addition to the diagnostic value of G0F/G2F, the dramatic change in the oligosaccharide structure of IgG might be associated with the pathogenesis of IBD. In a murine collagen-induced arthritis model, agalactosyl IgG is pathogenic (36). Agalactosyl IgG either activates the complement pathway or directly activates macrophages after binding to either mannose-binding lectin or mannose receptors, respectively (37–39). Further studies are required to clarify the role of agalactosyl IgG in mucosal inflammation.

In conclusion, G0F/G2F is increased in IBD and reflects disease activity and clinical course. These findings suggest that G0F/G2F is a novel serologic marker for IBD, and a marker to predict the clinical course. Fucosylated agalactosyl IgG is a potential therapeutic target in IBD.

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STUDY HIGHLIGHTS

What Is Current Knowledge

- Patients with rheumatoid arthritis have significantly increased levels of serum agalactosyl immunoglobulin G (IgG) and anti-agalactosyl IgG antibody.
- The precise oligosaccharide structure of IgG and its relationship to disease activity and prognosis in inflammatory bowel disease (IBD) patients have not yet been investigated.
- Serologic markers for IBD, *e.g.*, anti-*Saccharomyces cerevisiae* antibody (ASCA), do not have an essential role in the current diagnostic algorithm for IBD.

What Is New Here

- The agalactosyl fraction of the fucosylated IgG oligosaccharides in IBD was significantly greater than that in healthy volunteers and a disease control.
- An infrequent positive rate of anti-agalactosyl IgG antibody was observed in IBD patients.
- Extent of agalactosylation of IgG correlated with disease activity of IBD and is a potentially effective diagnostic marker for IBD.
- mRNA expression and enzyme activity of galactosyltransferase were different between Crohn's disease and ulcerative colitis.

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