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Alteration of the AT motif binding factor-1 expression in α-fetoprotein producing gastric cancer: Is it an event for differentiation and proliferation of the tumors?

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Abstract. α-fetoprotein producing gastric cancer (AFP-GC) rarely occurs, but it is classified as a special subtype of gastric cancer (GC). This tumor, as represented by the production of AFP, exhibits not only specific function but also different histology compared with ordinary-GC (O-GC). Clinically, it is likely to metastasize to the liver and, as a consequence, poor prognosis is recognized as one of the features. Recently, AT motif binding factor-1 (ATBF1) was identified as a modulator of AFP production by hepatocellular carcinoma, and the decreased expression of the protein was also reported in AFP-GC. However, little is known about the biological significance of the decreased expression. In this study, to clarify the biological characteristics of AFP-GC, antibody was raised against ATBF1 and immunohistochemistry was carried out. The antibody specifically recognized ATBF1, and the degree of expression could be characterized by immunohistochemistry. ATBF1 was expressed in O-GC and the area of tubular adenocarcinoma components of AFP-GC. On the other hand, the expression pattern varied in the hepatoid carcinoma components of AFP-GC, and AFP was expressed in the area without ATBF1 expression. Taken together, these results corroborated the previous reports that ATBF1 regulated AFP expression and inhibited transcription. Furthermore, in terms of differentiation induction, ATBF1 expression was decreased in the areas with little glandular formation. This may suggest that aberrant expression of ATBF1 induces the expression of various factors that are otherwise suppressed, and that this somehow determines the biological features of AFP-GC.

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Key words: AT motif binding factor-1 (ATBF1), α-fetoprotein producing gastric cancer, immunohistochemistry

Introduction

α-fetoprotein producing gastric cancer (AFP-GC) is said to account for 2-6% of all gastric cancers (GCs), and it belongs to a special subtype of GCs that literally produces α-fetoprotein (AFP) (1-4). This tumor is characteristic not only functionally but also histologically. As a biological feature, it often metastasizes to the liver and, because of that, is recognized as a tumor with an extremely poor prognosis with a 5-year survival rate of less than 10% (2-4). Nevertheless, to date, the mechanism of its likely metastasis to the liver is poorly understood. According to previous reports, AFP-GC is histologically classified into three subtypes: hepatoid adenocarcinoma, the yolk sac type, and the intestinal type. Among them, hepatoid adenocarcinoma is the most typical histological subtype of AFP-GC, and there are a number of reports that demonstrate the morphological and functional similarity with hepatocytes (1). The similarity with hepatocytes is not only in morphology but also includes bile production, protein and mRNA synthesis of AFP, albumin, and transferrin, and PIVKA-II. It should be noted that many questions remain to be answered in regard to how AFP-GC harbors the functions of hepatocytes. In general, AFP is produced in fetal organs, especially in the liver, but the production decreases rapidly after birth and is not observed in healthy adult tissue. In contrast, it is characteristic in hepatocellular carcinoma (HCC) that the AFP production is upregulated, and a variety of studies have been done to clarify the mechanism (5). Thus, several factors that modulate the AFP gene transcription in HCC have been found recently. Hepatocyte nuclear factor-1 (HNF1) was identified as a stimulator of AFP transcription, while AT motif binding factor-1 (ATBF1) was found to be a transcription suppressor. Both factors competitively bind the AT motif and regulate AFP transcription (6-8).

ATBF1 is one of the biggest transcription factors and characteristically has as many as 23 Zn fingers and four homeoboxes that serve as DNA binding domains (7). The mechanisms to regulate ATBF1 expression are poorly understood, but it is at least unlikely that the expression is regulated by the methylation of the promoter (9). ATBF1 is intimately involved in cell differentiation, which includes cells in the

gastrointestinal tract (10,11). Furthermore, it is related to growth factors, and it inhibits *c-Myb* expression (12). Recently, Kataoka *et al* reported the decreased expression of ATBF1 in AFP-GC with a reference to the studies on HCC (9). They employed an RNase protection assay to show that HNF1 was not directly involved in AFP production and demonstrated that a specific decrease in the expression of ATBF1, a competitor of HNF1, stimulated AFP production in AFP-GC. In addition, they showed that downregulation of ATBF1 corresponded with the site of AFP production. These results imply that the expression of the AFP gene is originally suppressed in GC, and that it exhibits transcriptional activity by the release from inhibition.

However, it largely remains unclear how GC, which does not originally produce AFP, acquires the ability to produce it in the process of carcinogenesis. In this study, in an attempt to clarify the biological features of AFP-GC, we prepared specific antibody against ATBF1 and compared the expression of ATBF1 in AFP-GC and non-AFP-producing GC by immunohistochemistry.

Materials and methods

Patients. A total of 14 surgically resected GCs with adjacent non-tumorous tissues were used. There were 4 cases with AFP-GC, in which AFP was detected preoperatively in peripheral blood. As a control, there were 10 cases with GC that did not produce AFP, and in which AFP was not detected in peripheral blood either preoperatively or postoperatively.

Cell culture. KATO III cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% (v/v) fetal calf serum (Bio-Whittaker, Walkersville, MD), 100 μg/ml streptomycin, 10 U/ml penicillin (Life Technologies, Inc.), and 0.25 μg/ml amphotericin B (Life Technologies, Inc.) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Peptide synthesis. Based on the amino acid sequence of ATBF1 registered in GenBank under Accession No. ATBF1, component peptides of ATBF1 were synthesized. Regions that contained hydrophilic domains and were putatively located at the membrane surface based on the protein structure were selected from the conserved regions specific to ATBF1. To prevent non-specific reaction of antibody, homology with other regions was searched, and regions that would not cross-react with other amino acid sequences were selected. Peptides were synthesized based on five amino acid sequences meeting these criteria in the ATBF1 gene. The following amino acid sequences were selected: 1017-HIKEGGKANEWRLK-1030 (ATBF1 No. 2), 1200-GSSESOLSSKROKT-1213 (ATBF1 No. 3), 1582-TGQPEPTSSPDNK-1594 (ATBF1 No. 4), 2082-NARAKEKKSKLSMA-2095 (ATBF1 No. 5), and 3657-TDDYSEESDLSQ-3670 (ATBF1 No. 6). These peptides were prepared by the solid phase Fmoc (9-fluorenylmethyloxycarbonyl) synthesis method by using the Pioneer peptide synthesizer (Applied Biosystems).

Immunization. Two rabbits (female, Japanese white rabbits) were inoculated subcutaneously with each of the five KLH-conjugated synthetic peptides as immunogens. Thus a total of 10 rabbits were used. They were immunized with 200 μg of each immunogen for the first time and then with 100 μg of each once a week for four weeks. One month later, antibody production was measured by ELISA, and 100 μg of each of the same immunogens was given as a booster. Whole blood was collected 49 days after the first immunization, and serum with a significantly high antibody titer was used as antiserum. Collected serum was precipitated with ammonium sulfate, freeze-dried, and stored at -4°C.

Western blotting. KATO III cells were harvested on ice with a rubber policeman. The cells were lysed in 10 mM 2[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (pH 7.4), 150 mM NaCl, 0.2% sodium dodecyl sulfate, 0.5% Na deoxycholate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 U/ml aprotinin, 2 mM ethylenediaminetetraacetic acid, 400 μM Na_3VO_4 and 10 mM NaF. The cell lysates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins on the gels were transfer. electrophoretically to a nitrocellulose membrane (Bio-Rad, Hercules, CA) at 50 V for 4 h. The nitrocellulose membrane was blocked with 5% skim milk in 100 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 0.1% Tween 20. Amersham ECL kit (Amersham, Pharmacia Biotec, Uppsala, Sweden) after it was reacted with serially diluted antiserum against ATBF1 and then anti-rabbit serum antibody labeled with horseradish peroxidase as the secondary antibody which was 1,000-fold diluted with blocking solution.

Immunohistochemistry. Primary foci and liver metastatic lesions were fixed with 15% buffered-formalin, paraffin-embedded, and sliced to prepare 5-μm-thick sections. The sections were deparaffinized in xylene and rehydrated in a descending series. The slides were then heated twice in a microwave oven (MI-77, Azumaya, Tokyo) in citrate-buffered solution 10 mmol/l) at pH 6.0 for 15 min to retrieve the epitope. They were cooled down to room temperature, and blocking of endogenous peroxidase activity was perfor. with 0.03% hydrogen peroxidase in phosphate buffered solution (10 mmol/l). Then, they were reacted with a blocking solution (Dako, X0909) for 30 min at room temperature to avoid non-specific reactions, followed by the reaction with serially diluted antisera against ATBF1 for 2 h at room temperature. They were incubated for 30 min at room temperature with biotin-labeled goat anti-rabbit immunoglobulin (Dako, K1015), and reacted for 30 min at room temperature with peroxidase-labeled streptavidin solution (Dako, K1016). Lastly, under microscopic examination, color was developed with diaminobenzidine containing 0.003% H_2O_2 (Dako, K3466) as a chromogen. Nuclei were counterstained with hematoxylin, and the slides were dehydrated and mounted with cover slides for microscopic examination. Immunostaining was performed on AFP with anti-human AFP (α-1-fetoprotein) rabbit antibody (Dako, N1501) in the same way as that used for ATBF1.

Results

Characteristics of the antisera. A remarkable elevation in antibody titer was recognized in sera from the 10 rabbits.

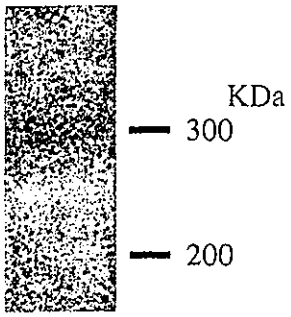

Figure 1. Western blotting for ATBF1 protein. Weak expression of 300 kDa protein in KATO III.

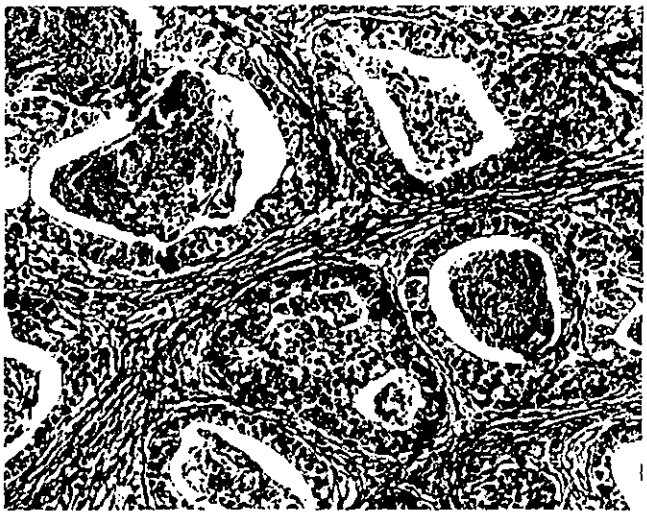

Figure 2. Tubular adenocarcinoma component in AFP-GC. Tumor cells showing dilated glandular appearance filled with necrotic debris.

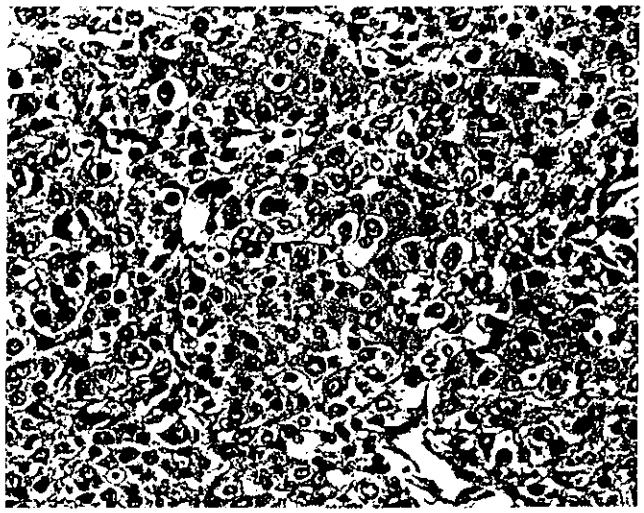

Figure 3. Hepatoid carcinoma component in AFP-GC. Polygonal uniform tumor cells arranged in a solid nest with frequent mitosis (arrow).

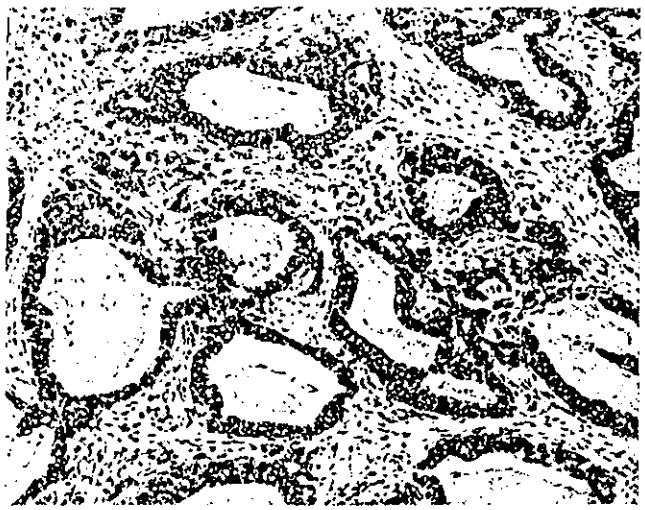

Figure 4. Immunohistochemical localization of ATBF1 in AFP-GC. Most of tumor cells diffusely expressed ATBF1 in the tubular adenocarcinoma component.

Western blotting revealed a band at the molecular weight position of 300 kDa (Fig. 1). To obtain the most appropriate concentration of the antisera, serially diluted antisera were used for immunostaining of GC tissue. In the normal gastric tissue, some pyloric glands were stained positively whereas foveolar epithelium and fundic glands were not. The most suitable antiserum and its appropriate concentration were determined based on the criterion that stromal connective tissue and space between tissues were stained negatively while a variety of cells, including normal cells, were stained positively. It was the diluted ATBF1 No. 3 serum (x100) that showed least non-specific reaction and could identify the localization.

ATBF1 expression in AFP-GC. Four cases with AFP-GC patho-histologically examined comprised a tubular adenocarcinoma component (Fig. 2) and a hepatoid carcinoma component (Fig. 3). ATBF1 was diffusely positive in the nucleus and cytoplasm of the tumor cells in a tubular adenocarcinoma component that comprised part of the tumor (Fig. 4). On the other hand, the hepatoid carcinoma components were stained in various ways. Some parts showed diffuse expression, while other lesions were completely negative or heterogeneous. The area negative for ATBF1 mainly consisted of a hepatoid carcinoma component (Fig. 5). When the localization was compared between AFP and ATBF1, hepatoid carcinoma with positive AFP expression were completely negative for ATBF1 (Fig. 6). Furthermore, in the area without ATBF1 expression, mitotic cells were frequently observed.

ATBF1 expression in non-AFP-producing GC. Positive staining for ATBF1 was diffusely observed in the nucleus and cytoplasm of non-AFP-producing GC similar to the case with tubular adenocarcinoma components of AFP-GC. ATBF1 was expressed diffusely and homogeneously in the lesions of well differentiated, tubular adenocarcinoma components to the same degree as in the poorly differentiated solid components. It should be noted that both tumors were expressed homogeneously and that no remarkably different expression was observed within a tumor, such as at the surface and invasive front.

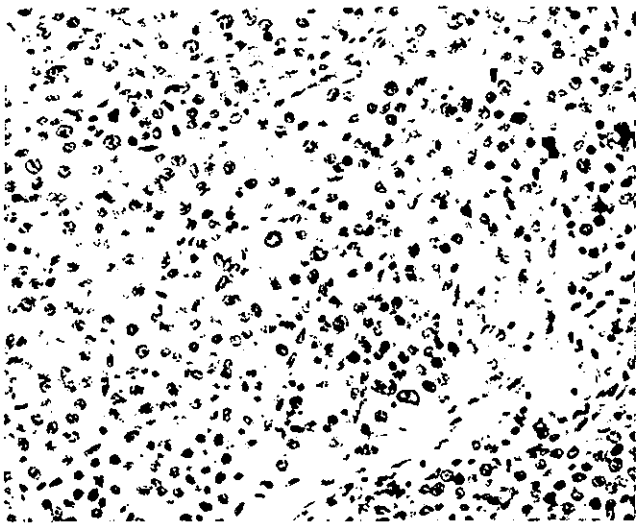

Figure 5. No tumor cells were positive for ATBF1 in hepatoid carcinoma component of AFP-GC.

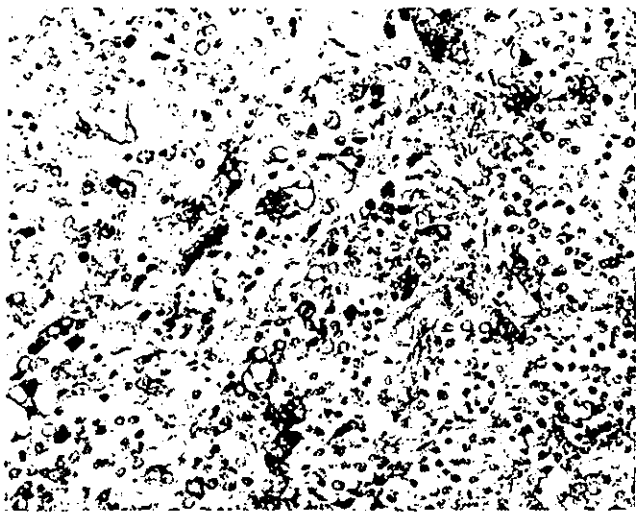

Figure 6. AFP positive cells scattered in same area as Fig. 5.

Discussion

With reference to the protein sequence of ATBF1 registered in GenBank, the most appropriate peptides were synthesized and used as immunogens to prepare antisera against ATBF1. There are two known isoforms of ATBF1: ATBF1-A and ATBF-B. The sequences for synthetic peptides were selected from those mainly encoding DNA binding domains. Antiserum raised against one of the synthetic peptides was applicable for immunohistochemistry. In general, for various reasons it is often difficult to obtain satisfactory results with antiserum raised against a synthetic peptide. The reasons include antigen inactivation by formalin fixation, inability to recognize the target, and cross-reaction with other substances. However, the antiserum used in this study had high specificity in normal tissue, AFP-GC and non-AFP-producing GC, and could identify the morphological localization of the expression. Probably, this capability was made possible by antigen retrieval with microwave treatment as well as by measures to avoid non-specific reactions. We presume that this antiserum will be also applicable for usage other than immunohistochemistry.

AFP-GC is derived from gastric mucosa. It is not considered a tumor with an ectopic germ cell origin but has somatic cell origin, with the exception of yolk sac type tumors. Most hepatoid adenocarcinomas form tumors with the transition of a hepatoid component and a well differentiated adenocarcinoma component, suggesting the unlikely possibility that two types of cancers merged. Accordingly, it can be speculated that tumors originating in mucosal epithelium acquire the phenotype in the process of development. In other words, this suggests that tumor cells acquire a phenotype similar to that of hepatocytes, i.e., the ability to produce AFP, during the process of dedifferentiation (1).

AFP-GC seemed to have high proliferative activity because it frequently showed mitotic cells in some parts of hepatoid carcinoma components without ATBF1 expression, which confirms earlier reports that AFP-GC has high proliferative activity (4). With the downstream COX-2 and Bcl-2 taken into account, transcriptional suppression of ATBF1 also affects *c-Myb* expression, and accordingly ATBF1 downregulation, activating these factors, leads to cell cycle stimulation (12-14). It can be speculated that the cell cycle is stimulated via this pathway in AFP-GC. There is a possibility that ATBF1 may downregulate a variety of factors determining grades of malignancy in addition to those already reported.

It has been reported that ATBF1 induces differentiation (10,11). ATBF1 expression changes during differentiation not only in nerve cells but also in various other types of cells, and ATBF1 mRNA increases during the differentiation induced by DMSO treatment of human promyelocytic leukemia cells. Likewise, ATBF1 is not expressed in premature early lineage cells such as stem cells, but is upregulated along with differentiation. While ATBF1 expression decreased in the solid-cystic component, it was expressed diffusely in the areas with glandular formation. This suggests the possibility that differentiation of GC may be recognized during glandular formation and that ATBF1 induces differentiation, supporting the hypothesis that ATBF1 is involved in expression of cell morphology and differentiation. Based on these results, solid lesions where ATBF1 is downregulated may comprise a subpopulation of immature cells. If ATBF1 can be overexpressed in AFP-GC cells with poor glandular formation, glandular formation as well as decreased proliferative activity may be found. Should a treatment with differentiation induction be considered in GC, especially AFP-GC, ATBF1 could be one of the candidate targets.

It remains unclear how ATBF1 is downregulated in AFP-GC. Abnormalities of ATBF1 regulating genes are suspected, but at least downregulation of ATBF1 is not ascribable to hypermethylation of the promoter regions of the transcription-starting site (9). On the other hand, ATBF1 is encoded in 16q22.3-23.1 (15), and loss of heterozygosity (LOH) has been reported in this region in breast cancer (16) and HCC (17). It is possible that ATBF1 is downregulated in AFP-GC due to LOH. As ATBF1 was expressed heterogeneously in a tumor, it seems possible that LOH will be found heterogeneously with tumor growth. To investigate the functional

abnormality of ATBF1, it is necessary to examine LOH in this region. It is extremely rare to find AFP-GC at an early stage, and most of the cases are found at an advanced stage (2-4). This suggests the possibility that if there exist a variety of pathways in the multi-step gastric carcinogenesis, AFP-GC at first emerges similarly to commonly found GC, but ATBF1 can be downregulated at a later stage via different pathways.

Many questions still remain concerning AFP-GC's likely metastasis to the liver. Normal hepatocytes produce serum protein and are able to constantly monitor protein in the blood. It might be hypothesized that because HCC is likely to grow in vessels such as the portal vein, AFP-GC with similar characteristics frequently infiltrates to vessels and metastasizes to the liver, with the histology of the metastatic lesions showing hepatoid adenocarcinoma. In terms of high invasiveness, a variety of proteinases are generally produced in the invasive tumor front to facilitate tissue destruction. In the present case, however, ATBF1 downregulation was not specifically found at the front of tumor invasion, and there may be different mechanisms for the invasion of AFP-GC.

Taken together, abnormalities in ATBF1 expression may be one of the factors that determine functional and morphological features of AFP-GC.

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Adsorptive granulocyte and monocyte apheresis for refractory Crohn's disease: an open multicenter prospective study

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Background. Active Crohn's disease (CD) is often associated with elevated levels of platelets, granulocytes, and monocytes that are activated and resistant to apoptosis. The level of neutrophils in the intestinal mucosa has been quantitatively related to the severity of intestinal inflammation in CD. We postulated that patients with CD that is refractory to conventional medications might respond to a reduction of granulocytes and monocytes by adsorptive apheresis. ***Methods.*** Twenty-one patients with a CD activity index (CDAI) of 200–399 and unresponsive to standard medication, which included nutritional intervention, received granulocyte and monocyte adsorptive apheresis (GCAP) as an adjunct to their ongoing medication. GCAP was performed with an Adacolumn, which adsorbs granulocytes, monocytes, and a small fraction of lymphocytes (FcγR and complement receptor-bearing leucocytes). Patients received one GCAP session/week for 5 consecutive weeks. CDAI, International Organization for the Study of Inflammatory Bowel Disease (IOIBD), and IBD questionnaire (IBDQ) scores were evaluated. ***Results.*** During the initial conventional/nutritional therapy, no significant improvement was seen in any patient. However, at week 7 of GCAP therapy, significant improvements in CDAI, IOIBD, and IBDQ scores were observed. The CDAI, IOIBD, and IBDQ scores before GCAP were 275.6 ± 54.2, 3.4 ± 1.4, and 152 ± 22, respectively. The corresponding values after GCAP were 214.8 ± 89.2 ($P = 0.0005$), 2.54 ± 1.5 ($P = 0.0224$), and 165 ± 29 ($P = 0.0327$), respectively. ***Conclusions.*** GCAP could be effective for inducing remission and improving quality of life in patients with active CD that is refractory to conventional therapy.

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Key words: Crohn's disease · nutritional therapy · granulocytes and monocytes · adsorptive apheresis · IBD questionnaire · IOIBD

Introduction

Crohn's disease (CD) is a chronic recurrent inflammatory bowel disorder with variable disease expressions, giving rise to a multitude of complications, including fever, abdominal discomfort, diarrhea, anemia, and weight loss. Although ulcerative colitis is primarily confined to the colon and the rectum, CD can affect any part of the gut, from the mouth to the perianal region; up to 65% of CD patients may have CD affecting the small intestine.[1] Such patients are likely to develop serious nutritional complications, with the need for nutritional support with elemental diets or total parenteral nutrition.[1–5]

Although genetic background may be associated with the onset of CD, and dietary antigens are thought to have an important role in the exacerbation of CD,[6–9] it is true to say that factors which initiate and perpetuate CD are not well characterized at present. However, because a relapse may be triggered by an inflammatory response to dietary antigens in the intestinal wall,[6–9] nutritional therapy is thought to minimize the contribution of the normal diet to disease activity, and to promote remission.[2–5] In line with this assertion, a response rate of up to 77% has been reported following a course of nutritional therapy.[2,5,10,11]

Alternative therapies for patients who do not respond to a salicylate (5-acetyl salicylic acid [ASA] sulfasalazine) plus elemental diets or total parenteral nutrition include high-dose corticosteroids[12] and, more recently, anti-tumor necrosis factor (TNF-α) antibod-

.es.[13] However, both corticosteroids and anti-TNF-α antibody are associated with frequent adverse side effects.[14,15] Indeed, in Japan, most of our patients are reluctant to receive corticosteroid therapy for fear of the steroid-related adverse effects.

A potential target for the treatment of inflammatory bowel disease could be granulocytes and monocytes/macrophages. We thought that patients with active CD who had not responded to standard conventional therapy, including nutrition therapy with an elemental diet (ED) or total parenteral nutrition (TPN), might respond to selective granulocyte and monocyte adsorptive apheresis as an adjunct to their ongoing medication. A major role for these leucocytes in the clinicopathological features of CD (tissue injury and symptoms) is indicated by several lines of evidence.[16–20] First, in both CD and ulcerative colitis, peripheral blood neutrophils and monocytes/macrophages are elevated, showing activation and increased survival time.[16–22] Second, biopsy specimens from CD lesions reveal a high density of neutrophils, macrophages, and other inflammatory leucocytes within the inflamed tissues.[1,23] Third, the level of neutrophils in the intestinal mucosa was quantitatively related to the severity of intestinal inflammation and clinical relapse in both CD and ulcerative colitis.[24,25] This indicates that, during clinical remission, neutrophils infiltrate the intestinal mucosa and have a major role in mucosal inflammation, tissue injury, and CD relapse.[24–26]

The device we used for the apheresis was an Adacolumn.[21] The volume of this apheresis column is 335 ml, and the column is filled with cellulose acetate beads (carriers), 2 mm in diameter, that are bathed in sterile saline. The carriers adsorb granulocytes and monocytes/macrophages that bear FcγR and complement receptors.[21,27] Blood cell counts from the column inflow and outflow points show that the carriers typically adsorb about 65% of granulocytes, 55% of monocytes, and a small fraction of lymphocytes from the blood in the column.[21,28] Preliminary studies in patients with rheumatoid arthritis,[21,29] ulcerative colitis,[21,22,26] and CD[30] showed that granulocyte and monocyte adsorptive apheresis (GCAP) was associated with the alleviation of clinical symptoms and marked reductions in the levels of various inflammatory cytokines which are produced by leucocytes.[21,22,29] These observations indicated that the clinical efficacy of GCAP might not be due to its effect on the level of peripheral blood granulocytes and monocytes per se. The present study was carried out to investigate the efficacy of GCAP in patients with CD who were refractory to conventional medication.

Methods

Study objectives

Our primary objective was to evaluate the efficacy of GCAP as an adjunct to therapy in patients with active CD that was refractory to standard Japanese conventional therapy for active CD (see "Conventional therapy" section below). The efficacy of the GCAP treatment was based on changes in the Crohn's disease activity index (CDAI) during or at the end of the therapy relative to the entry values, without a major focus on endoscopic or radiographic changes. Treatment safety and changes in the International Organization for the Study of Inflammatory Bowel Disease (IOIBD) score, according to de Dombal,[31] and changes in the inflammatory bowel disease (IBD) questionnaire (IBDQ) score were evaluated as our secondary objectives. The IBDQ has been reported to be a reliable indicator of quality of life in patients with IBD.[32]

Patients

The demography of the 21 patients recruited for this study is presented in Table 1. All patients had a previous diagnosis of Crohn's disease (colitis or ileocolitis). To qualify for entry to the study, patients had to have a CDAI score in the range of 200 to 399, and to have been classified as refractory to conventional therapy (including nutritional intervention) if no significant improvement in CDAI was observed (CDAI > 200) during at least 2 weeks of therapy (see below).

Table 1. Demography of 21 patients with refractory Crohn's disease at entry who were recruited for granulocyte and monocyte adsorptive apheresis therapy with the Adacolumn

Demography	Number of patients
Male/Female	14/7
Age (years)	27.4 ± 9.0 (12–57)[a]
Duration of disease (months)	80.1 ± 53.5 (3–180)[a]
Location of lesions	
Ileocolitis	13
Colitis	8
Previous bowel resection	8
Nutritional therapy (30–40 kcal/kg per day)[c]	21
Stenosis/stricture	5
Anal lesions	13
Ongoing medications	
Prednisolone	6 (5–17 mg)[b]
5-ASA/Sulfasalazine	17 (2.25–4.0 g)[b]
Metronidazole	2 (500 mg)[b]
Crohn's disease activity index	275.6 ± 54.2
C-reactive protein (mg/dl)	2.7 ± 4.1 (0.14–17.59)[a]

[a] Values are means ± SD (range)
[b] Dose
[c] Nutritional therapy: elemental diet, 17; total parenteral nutrition, 4

Conventional therapy

In Japan, the Ministry of Health has approved standard guidelines of conventional therapy for active CD (first-line therapy) that comprises an aminosalicylate (5-ASA or sulfasalazine) plus nutritional therapy.[2] This regimen can alleviate the disease in the majority of patients within 2 weeks,[2,10] and those who do not respond are classified as having refractory CD.[30] In this study, nutritional therapy consisted of an elemental diet (ED; n = 17) or total parenteral nutrition (TPN; n = 4). ED treatment was done by using Elental (Ajinomoto, Tokyo, Japan), while TPN (30–40 kcal/kg per day) was done by using products from reputable suppliers in Japan. All products were brands approved by the Ministry of Health. Table 1 shows that 17 of the 21 patients were on aminosalicylates (2.25–4 g/day); 6 of these patients were on prednisolone (5–17 mg/day) as well, and 2 were receiving metronidazole (500 mg/day).

Adsorptive granulocyte and monocyte apheresis

The procedure for GCAP was essentially based on earlier studies in patients with rheumatoid arthritis and ulcerative colitis.[21,22,26,28,29] Briefly, the apheresis column (Adacolumn) and circuit lines were provided by Japan Immunoresearch Laboratories (Takasaki, Japan). The column has a capacity of 335 ml and is filled with cellulose acetate beads, 2 mm in diameter, as the column adsorptive carriers. Differential leucocyte counts have shown that the carriers adsorb granulocytes, monocytes/macrophages, and a small fraction of lymphocytes from the blood in the column.[21,27] This device has been CE marked (validated) by TUV (Notified Body) and approved by the Japan Ministry of Health for the treatment of active ulcerative colitis. The column was placed in an extracorporeal setting, with a perfusion rate of 30 ml/min; the duration of one GCAP treatment session was 60 min.

Study design and assessment of response to therapy

The study design is outlined in Fig. 1. This was a prospective open-label study, carried out at 16 centers in 13 cities throughout Japan. Therefore, with a target patient number of 21, the number of patients per center was very small. Further, centers recruited their own patients, but with strict adherence to the study protocol. During the first 2 weeks, patients were screened, and information about their disease course and drug therapy was compiled. As indicated above, the protocol specified that patients must have been on conventional therapy for at least 2 weeks prior to the initiation of GCAP therapy. Likewise, for patients who were on corticosteroid, 5-ASA or sulfasalazine therapy, the drug

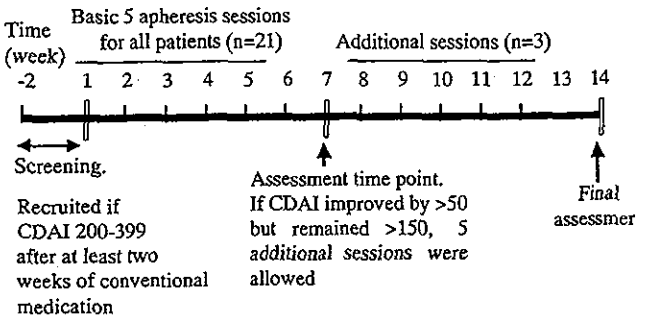

Fig. 1. Study design, patient screening, and treatment and efficacy assessment time points. *CDAI*, Crohn's disease activity index

therapy should have been continued at a stable dose during the previous 2 weeks for the corticosteroid and during the previous 8 weeks for salicylates. No change in conventional therapy was expected during the 5 weeks of GCAP treatment. Each patient was to receive one GCAP session per week for 5 consecutive weeks. Week 7 was the first efficacy assessment time point. Patients in whom the CDAI decreased by more than 50 points, but remained above 150, could have five further GCAP sessions, at the discretion of the attending physician. However, only 3 patients did have an additional GCAP session, and of these, just 1 patient reached week 14, which was the final assessment time point for patients with more than five sessions.

In accord with the principle of intention to treat, if a patient was withdrawn from the study, for whatever reason, assessment was done at that time point, and the data were included in the assessment at week 7. Clinical remission was assumed when the CDAI improved to below 150, while clinical response was defined as a decrease of the CDAI by more than 50 points. Likewise, if the CDAI had increased by more than 50 points, the patient's CD was considered to have worsened; otherwise, the patient's CD was considered unchanged. After the GCAP treatment course, patients who had active disease could continue with their conventional medication.

Exclusion criteria

Because leucocytapheresis is a non-drug therapy, that involves blood flow, its efficacy is likely to be affected by poor or intermittent blood flow. To minimize this possibility, patients who appeared to present difficulties in achieving blood access were excluded. Pregnancy, age less than 12 years or over 76 years, granulocyte count of less than 2000/μl, and hemoglobin less than 8 g/dl were other main exclusion criteria. Likewise, any patient who had an obvious need for surgery was not recruited.

Ethics

Leucocytapheresis with the Adacolumn is an approved therapy in Japan. Nonetheless, the final version of the study protocol was submitted to the Japan Ministry of Health and approval was obtained. Likewise, the institutional review board of each hospital approved the study protocol. Further, all patients provided written informed consent after they were informed of the purpose of the study and the nature of the procedures involved. In under age patients (less than 20 years), consent was obtained from the patient and one of the patient's parents. Patients were told that they could withdraw from the study at any time without jeopardizing their future treatment.

Statistical analysis

Where appropriate, data values are presented as means ± SEM, or as mean ± SD values, and comparisons were made by using the *t*-test, unless indicated otherwise. A significance level of 0.05 was used for all statistical tests, and two-tailed tests were applied when appropriate.

Results

Patient compliance

A total of six patients withdrew before week 7. Of these, one received only two sessions and was withdrawn due to a more than expected drop in the patient's leucocyte count. One patient withdrew after receiving three sessions, and another two patients withdrew after receiving four sessions, due to lack of efficacy. A further two patients withdrew after receiving five sessions. The outcome is presented in Fig. 2.

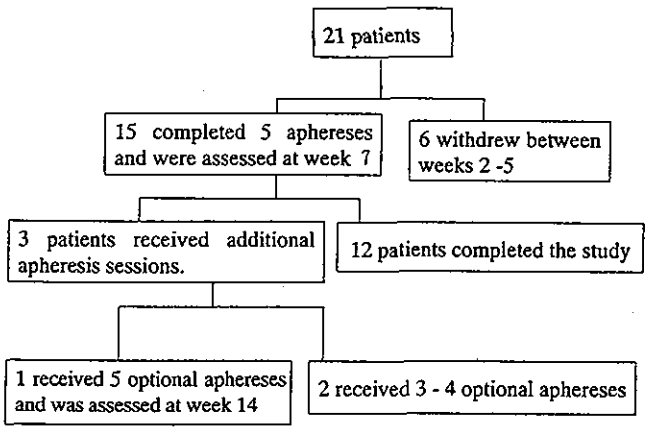

Fig. 2. Flow chart, showing the number of leucocytapheresis sessions dispensed during the 14 weeks of the study (design, shown in *Fig. 1*)

Changes in leucocyte counts during the apheresis procedure

Figure 3 shows changes in leucocyte counts at several time points during the 60-min apheresis therapy and then at 24 h. All counts, except for the values at 24 h, correspond to the column inflow and represent peripheral blood level. The total leucocyte count showed a drop of 49% at 30 min relative to the count at time 0. However, despite apheresis being continued, the inflow count at 60 min had markedly increased, showing only a 9% fall relative to the count at time 0. This reflects an influx of CD10-negative neutrophils from the bone marrow into the peripheral circulation.[24] Looking at the three main leucocyte populations, the inflow counts at the 15-min and 30-min time points relative to the counts at time 0 showed a significant drop for all three leucocyte populations. The fall in the inflow lymphocyte count during apheresis was unexpected, because the column carriers do not significantly adsorb lymphocytes.[21,27,28]

Changes in CDAI, IOIBD, and IBDQ following leucocytapheresis

Figure 4 shows significant improvements in the CDAI, IOIBD, and IBDQ scores after GCAP. The CDAI, IOIBD, and IBDQ scores (mean ± SD) at baseline were 275.6 ± 54.2, 3.4 ± 1.4, and 152 ± 22, respectively. The corresponding values after GCAP therapy were 214.8 ± 89.2 ($P = 0.0005$), 2.4 ± 1.5 ($P = 0.0224$) and 165 ± 29 ($P = 0.0327$), respectively, showing impressive alleviation of symptoms and improvement in quality of life. When we considered only the 11 responders, the

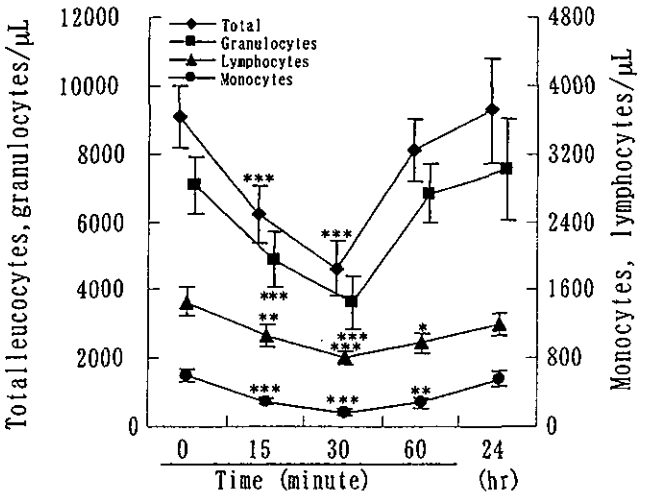

Fig. 3. Changes in peripheral blood leucocytes during and 24 h after Adacolumn leucocytapheresis therapy in 21 patients with refractory Crohn's disease. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, vs time 0

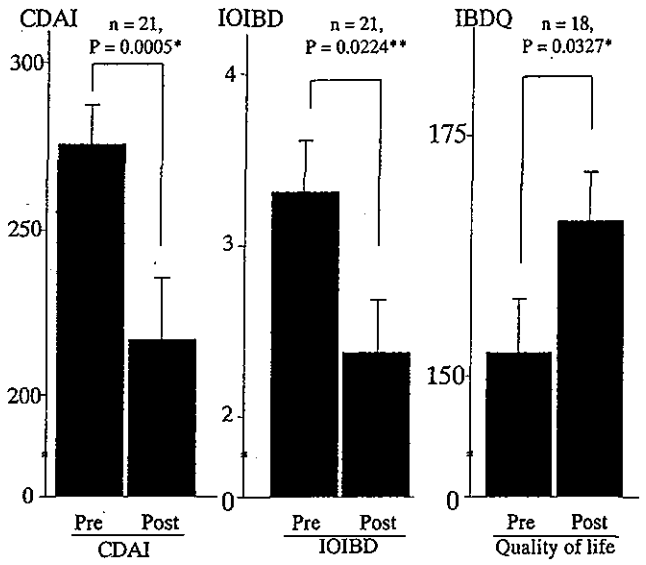

Fig. 4. Changes in the CDAI, International Organization for the Study of Inflammatory Bowel Disease (*IOIBD*), and IBD questionnaire (*IBDQ*) scores (mean ± SEM) during leucocytapheresis (GCAP) therapy in 21 patients with Crohn's disease unresponsive to conventional medications, including nutritional therapy. *By paired *t*-test; **by Wilcoxon signed rank test

above scores were as follows: 267 ± 38 ($P = 0.0001$), 3.6 ± 1.2, and 155 ± 25, and the corresponding values after GCAP therapy were 151 ± 49 ($P = 0.0001$), 1.6 ± 1.0 ($P = 0.002$), and 178 ± 26 ($P = 0.0212$), respectively.

Overall response to therapy

Eleven of the 21 patients (52.4%) responded to the therapy, 6 with clinical remission and 5 with significant improvement. The remaining 10 patients (non-responders) remained unchanged, and according to the protocol definitions, no patient worsened. It should be mentioned that 4 of the non-responders were not on 5-ASA prior to entry or beyond. There was no other significant difference in patient background between responders and non-responders to GCAP. Regarding the sustainability of the response, up to 6 months after the end of GCAP therapy, 2 of the 11 responders were hospitalized for alternative therapy due to the worsening of CD symptoms. One patient showed worsening of CD symptoms within 3 months after the last GCAP therapy, the other within 5 months.

Unexpectedly, there was no change in the mean C-reactive protein (CRP) level at week 7. The CRP value at entry was 2.7 ± 4.1 mg/dl (mean ± SD; range, 0.14–17.6 mg/dl). The values at week 5 and post GCAP were 1.9 ± 2.6 mg/dl (range, 0.11–11.6 mg/dl) and 2.7 ± 2.8 mg/dl (range, 0.33–11.6 mg/dl), respectively. The corresponding values for the 11 responders were 1.9 ± 2.3 mg/dl (range, 0.14–7.44 mg/dl), 0.9 ± 0.6 mg/dl (range, 0.11–2.01 mg/dl), and 1.9 ± 1.4 mg/dl (range, 0.33–4.66 mg/dl), respectively, showing no statistically significant improvement. However, in patients who had anal lesions and in whom the mean entry CRP was 2.7 mg/dl or more the CRP level decreased significantly, from 7.7 ± 5.8 mg/dl to 5.1 ± 4.0 mg/dl ($P = 0.0483$; $n = 5$).

Study safety

During GCAP therapy, a total of ten non-severe side effects in six patients were reported. These were: palpitation ($n = 1$), headache ($n = 3$), congested nostrils ($n = 1$), dizziness ($n = 1$), feeling of weariness in one patient ($n = 2$ occasions), rash on the legs ($n = 1$), and pelvic pain ($n = 1$). However, no patient discontinued GCAP therapy due to these side effects, except for the patient with the pelvic pain, which resolved within 24 h. Further, there was no evidence of opportunistic infection in any patient during or after GCAP therapy.

Discussion

In earlier studies, we found that the majority of patients with active CD responded to conventional medication that included nutritional therapy, without corticosteroids,[2,10] but a minority of them remained refractory.[30] The present study was initiated in June 2001 and was completed in April 2003. The major time-consuming factor was the difficulty in finding patients who did not readily respond to conventional therapy (a salicylate plus nutritional therapy) and who could therefore be classified as refractory and meet our protocol inclusion criteria. Given that all 21 patients in the present study were found to be unresponsive to conventional medications, GCAP was considered to be a rescue therapy for these patients.

In Japan, the initial first-line medication for active CD is 5-ASA or sulfasalazine, together with nutritional therapy with elemental diets (EDs) or TPN.[2,5,10,11,33] Nutritional therapy, in addition to providing energy, protein, and essential nutrients, has two other major benefits: (a) it ameliorates the diarrhea and abdominal discomfort associated with a normal diet; and (b) it appears to minimize intestinal inflammation provoked by dietary antigens.[6–9] Based on the experience in Japan, nutritional therapy can induce remission in up to 77% of patients during 1 month of therapy.[11] Nonetheless, it may be argued that, although all of the 21 patients we recruited were refractory to nutritional therapy, some of them might have responded to corticosteroids. While this argument should not be denied, most of the 15 patients who were not on corticosteroids

at entry to GCAP therapy had received corticosteroid treatment at some stage in the past and either had become refractory to steroids or were reluctant to receive steroids.

The most significant outcome of the leucocyte reduction therapy in this study was the marked improvement in CDAI, IOIBD, and IBDQ scores, with an overall response rate of 52.4%, in patients who had failed to respond to conventional therapy. The therapy was well tolerated and no severe adverse effects were observed. This is similar to the experience during GCAP therapy in patients with rheumatoid arthritis,[21,29] and those with ulcerative colitis,[26] in trials that reported adverse events such as mild headache or light-headedness in a small number of patients.[26] The safety, tolerability, and efficacy of leucocytapheresis with the Adacolumn suggest that, in contrast to steroids, with this therapy, unpleasant side effects are unlikely. Further, the outcome of this study should stimulate further initiatives to directly target granulocytes and monocytes/macrophages in the therapy of CD. However, a major factor that could strengthen the impact of our results would be the inclusion of a control group, which was missing in this study. With this in mind, we believe that a future study, using a larger cohort of patients together with the inclusion of a control group, could enhance our understanding of the full efficacy of adsorptive granulocyte and monocyte apheresis therapy in CD.

Although the column carriers adsorbed a large fraction of granulocytes and monocytes from the blood in the column, the number of these leucocytes in the patients' systemic circulation did not fall below the normal range under the conditions used in this study. Instead, flow cytometry indicated a net influx of immature neutrophils (CD10-negative neutrophils) into the circulation from marginal pools, including the bone marrow, and these cells would be less inflammatory than those removed.[24] Furthermore, in patients with rheumatoid arthritis and those with ulcerative colitis, after adsorptive leucocytapheresis therapy, the production of proinflammatory cytokines (TNF-α, interleukin [IL]-1β, IL-6, and IL-8) by peripheral blood leucocytes was markedly suppressed, together with the downmodulation of L selectin, which has a key role in the initiation of leucocyte extravasation.[21,22,29,34] Hence, the overall effect of granulocyte and monocyte reduction therapy should be reduced levels of activated leucocytes and inflammatory cytokines, and diminished leucocyte infiltration of the mucosa.

In conclusion, it appears that the reduction of monocytes/macrophages and neutrophils is associated with improvements and remission in some patients with active refractory CD. Alternatively, it could be said that granulocytes and monocytes have an active role in mucosal inflammation and the exacerbation of CD. Granulocyte and monocyte adsorptive apheresis should serve as a non-pharmacological adjunct to therapy in CD when conventional medications fail.

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ORIGINAL ARTICLE

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Association analysis of *SLC22A4*, *SLC22A5* and *DLG5* in Japanese patients with Crohn disease

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Abstract Crohn disease (CD) is an inflammatory bowel disease characterized by chronic transmural, segmental, and typically granulomatous inflammation of the gut. Recently, two novel candidate gene loci associated with CD, *SLC22A4* and *SLC22A5* on chromosome 5 known as *IBD5* and *DLG5* on chromosome 10, were identified through association analysis of Caucasian CD patients. We validated these candidate genes in Japanese patients with CD and found a weak but possible association with both *SLC22A4* ($P=0.028$) and *DLG5* ($P=0.023$). However, the reported genetic variants that were indicated to be causative in the Caucasian population were completely absent in or were not associated with Japanese CD patients. These findings imply significant differences in genetic background with CD susceptibility among different ethnic groups and further indicate some difficulty of population-based studies.

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Introduction

Inflammatory bowel diseases (IBDs), which are usually classified into two clinical entities—Crohn disease (CD; MIM 266600) and ulcerative colitis (UC)—are chronic conditions characterized by remitting and relapsing inflammation of the small and/or large intestines. Familial aggregation and twin studies indicate a presence of genetic factors susceptible to this condition. Genome-wide linkage analyses have localized genes conferring susceptibility to IBD to several possible candidate loci on chromosomes 1, 3, 5, 6, 7, 10, 12, 14, 16, 19, and 22 (Hugot et al. 1996; Satsangi et al. 1996; Cho et al. 1998; Duerr et al. 2000; Hampe et al. 1999; Rioux et al. 2000, 2001).

Among the several candidate loci, susceptible genes at two distinct loci were recently identified through the evidences of strong association with the CD phenotype. In the *IBD5* locus on chromosome 5, single-nucleotide polymorphisms (SNPs) in two candidate genes, *SLC22A4* and *SLC22A5*, both of which encode organic cation transporters, revealed significant associations with CD (Peltekova et al. 2004). A C1672T substitution in exon 9 of the *SLC22A4* gene and a G-207C in the *SLC22A5* promoter region were indicated as functional and causative mutations to increase susceptibility to CD. The other gene identified from chromosome 10 was the *DLG5* gene, encoding a scaffolding protein involved in the maintenance of epithelial integrity. Risk-associated variants, including a G113A substitution in exon 3 of the *DLG5* gene, constructed two distinct haplotypes with a replicable distortion in transmission (Stoll et al. 2004).

To investigate a possible role of these candidate gene loci, one corresponding to *SLC22A4* and *SLC22A5* and another corresponding to *DLG5*, in the pathogenesis of

CD in Japanese, we examined SNPs of these three genes in a large number of clinical samples. We here report an absence of DNA substitutions or lack of association for the candidate-causative SNPs, which were indicated in the previous reports, in the Japanese CD patients. However, we observed a weak association of other genetic substitutions in these genes of Japanese patients with CD. Our results indicate that the reported substitutions in the three genes are unlikely to be causative to Japanese CD patients, but the candidacy of these two loci for Japanese CD cannot be totally excluded.

Materials and methods

Subjects and DNAs

Japanese blood samples were obtained with written informed consent from 484 CD patients at the Social Insurance Central General Hospital and from 345 unaffected control individuals belonging to the Osaka-Midosuji Rotary Club. All CD cases were diagnosed at the Inflammatory Bowel Unit of the Social Insurance Hospital by clinical, radiological, endoscopic, and histological findings according to the Lennard-Jones' criteria (Lennard-Jones 1989). Patients with indeterminate colitis were excluded. DNAs were prepared from these samples according to standard protocols.

DNA sequencing

To search genetic variations in these candidate loci including the five reported variants, C1672T in exon 9 of *SLC22A4* and G-207C in the *SLC22A5* promoter, as well as G113A in exon 3, C4136A in exon 23 and 35delA in intron 26 of *DLG5*, we carried out direct sequencing of those regions in 48 individuals with confirmed diagnosis of CD by means of the BigDye Terminator RR Mix (Applied Biosystems, USA) with ABI 3700 sequencers using the primers listed in Table 1.

Markers

SNPs in the *SLC22A4* and *SLC22A5* genes were screened according to methods described previously (Saito et al. 2002). We selected 17 SNPs, including six in *SLC22A4* (SLC22A4 1–6), three in *SLC22A5* (SLC22A5 1–3), and eight in *DLG5* (DLG5 1–8) (Table 2). Information for each SNP in the *SLC22A4*, *SLC22A5*, and *DLG5* chosen for this study was obtained from the Japanese SNP (JSNP) database (http://snp.ims.u-tokyo.ac.jp) (Hirakawa et al. 2002; Haga et al. 2002).

SNP analysis and genotyping

We amplified multiple genomic fragments using 20 ng of genomic DNA for each polymerase chain reaction (PCR), as described previously (Ohnishi et al. 2001). We genotyped all participants for a total of 17 SNPs indicated in Table 2 by means of the Invader assay (Mein et al. 2000). The C4136A in exon 23 and 35delA in intron 26 (deletion of an adenine at the 35th nucleotide in intron 26) of *DLG5* were examined by direct sequencing using the same primers.

Statistical analysis

Genotype distributions and allele frequencies of each SNP were compared, respectively, between the cases and the controls, as described elsewhere (Yamada et al. 2001). Haplotype frequencies were estimated using the expectation-maximization algorithm (Ott 1977).

Results

To examine a possible association of genetic substitutions in the two candidate loci—one including *SLC22A4* and *SLC22A5* and the other at *DLG5*—with susceptibility to CD in the Japanese population, we first examined DNA sequences from 48 CD patients in 438–976 bp genomic regions, including the five major genetic

Table 1 List of primers in this study

	Position	Primer		Product size
		Forward	Reverse	
Amplification for mutation analysis and genotyping				
SLC22A4	Ex9	AACTCTGGTAGGCAAAGAACTC	GTCCTACTTACCATTTCACTTTC	438
SLC22A5	Promoter	CTAGGATCGTTAATCGTGAAG	CTGAGCAGGAAGAAGATGAG	866
DLG5	Ex3	TCACTTTCAGTTCTACCTGCTAC	TCTAGGAGACAGTGGTAGGG	641
	Ex23	GAGACAGGATGCTCACAGCTTC	AACTCCTGAAGACCTGGTGTG	527
	Ex26	CTGATCGTGTTCCTTCTGTGCTG	AGGTCTCAAGGCTACATCTCCTC	976
Sequencing for mutation analysis and genotyping				
SLC22A4	Ex9	CATGCACAATGTCATCTGCC	ATAGGAGGACTCTCTGGGCAC	
SLC22A5	Promoter	GGACTCGGACCCCAAGGCCTC	AAGAAGATGAGGCGCTGGAAG	
DLG5	Ex3	ACTTTCAGTTCTACCTGCTACCG		
	Ex23	ATGCTCACAGCTTCCTGAGGTC	AAGACCTGGTGTGCGGCCTG	
	Ex26	CCTTCTGTGCTGTGGTCCAG	CGTTATGCCTTCTGACCCATC	

Table 2 List of genotyped single-nucleotide polymorphisms (SNPs) in *SLC22A4*, *SLC22A5* and *DLG5*

SNP No.	Contig No.	Contig position	Location	Position	Substitution	Major allele	Minor allele	IMS-JST ID	dbSNP ID
SLC22A4									
SLC22A4_1	NT_034772.5	34051988	Intron 1	6274		A	G	IMS-JST150334	rs3792874
SLC22A4_2	NT_034772.5	34052322	Intron 1	6608		C	T	IMS-JST150336	rs3792876
SLC22A4_3	NT_034772.5	34052415	Intron 1	6701		A	G	IMS-JST150337	rs3792877
SLC22A4_4	NT_034772.5	34062488	Intron 1	16774		G	A	IMS-JST190202	rs3828671
SLC22A4_5	NT_034772.5	34063419	Intron 2	450		T	C	IMS-JST000452	rs270608
SLC22A4_6	NT_034772.5	34066274	Intron 3	1801		A	G	IMS-JST150344	rs3792884
SLC22A5									
SLC22A5_1	NT_034772.5	34129422	Intron 2	237	L269L	T	C	IMS-JST175234	rs270608
SLC22A5_2	NT_034772.5	34136187	Exon 4	155		G	A	IMS-JST101643	rs274558
SLC22A5_3	NT_034772.5	34144702	Intron 9	187		T	C	IMS-JST001553	rs2074610
DLG5									
DLG5_1	NT_008583.16	28131940	Intron 15	56		C	T	IMS-JST111768	rs3758463
DLG5_2	NT_008583.16	28131859	Intron 15	137		C	T	IMS-JST111767	rs3758462
DLG5_3	NT_008583.16	28123048	Intron 21	8948		G	C	IMS-JST013817	rs1248625
DLG5_4	NT_008583.16	28116810	Intron 26	862		C	T	IMS-JST040839	rs2289311
DLG5_5	NT_008583.16	28107184	Intron 28	181		C	A	IMS-JST013818	rs2241831
DLG5_6	NT_008583.16	28106275	Intron 29	700		C	T	IMS-JST013820	rs2241833
DLG5_7	NT_008583.16	28103306	Exon 32	151		G	A	IMS-JST025913	rs1058202
DLG5_8	NT_008583.16	28102795	Exon 32	662		G	A	IMS-JST025916	rs2165047

variants—C1672T in exon 9 of *SLC22A4* and G-207C in the *SLC22A5* promoter, G113A in exon 3, C4136A in exon 23, and 35delA in intron 26 of *DLG5*—that were reported to have significant associations with CD in the Caucasian population (Table 1). Among these five genetic variations reported previously, we found that the three SNPs, C1672T, G-207C, and G113A, were completely absent in the Japanese CD cases. Since the C4136A and 35delA variations were observed in the Japanese population, we carried out genotyping of 484 Japanese CD patients for these variations and found no association of these two reported substitutions to CD in the Japanese population (Table 3).

To further verify whether these three genes can be excluded as candidates for Japanese CD, we performed case-control association studies by means of genotyping of 17 JSNPs located within the three genes at the two loci as shown in Table 2. The analyses using allelic, recessive, and dominant models for CD patients versus controls disclosed an association of two SNPs, one at SLC22A4_2 ($P=0.028$) by dominant model and the other at DLG5_2 ($P=0.023$) by recessive model, although the associations observed here were much weaker than those for the five genetic variations observed in Caucasian CD cases (Table 4). In addition, we constructed the haplotype structure using the 19 genotyped variations and examined its association with CD but found no significant association with CD (data not shown). Our studies have indicated that the five reported variants are unlikely to be disease causative, but we have not excluded a possibility that these genes may play some role in susceptibility to CD in the Japanese population.

Discussion

Genetic factors that affect susceptibility to CD have been disclosed through genetic linkage and population-based association studies although it is very far from complete understanding of the subject. *CARD15* was found to be associated with IBD by means of genome-wide sib-pair

Table 3 Association of major genetic variants in *DLG5* with Crohn disease (CD) in the case-control study

SNP No.	Case	Control	Allele 1[a] versus 2		Genotype 11 versus others		Genotype 11 + 12 versus others	
			χ^2 (P-value)	OR (95% CI)	χ^2 (P-value)	OR (95% CI)	χ^2 (P-value)	OR (95% CI)
4136C → A in exon 23								
1–1	334	221						
1–2	129	109	2.08	1.21	2.48	1.27	0.057	1.10
2–2	14	11	(0.15)	(0.93–1.56)	(0.12)	(0.94–1.71)	(0.81)	(0.49–2.46)
Sum	477	341						
35delA in intron 26								
1–1	31	18						
1–2	173	115	1.52	1.16	0.56	1.25	1.31	1.18
2–2	273	210	(0.22)	(0.92–1.46)	(0.46)	(0.69–2.28)	(0.25)	(0.89–1.57)
Sum	477	343						

[a]Allele 1 indicated as risk allele

Table 4 Association of *SLC22A4* and *DLG5* with Crohn disease (CD) in the case-control study

SNP No.	Case	Control	Allele 1[a] versus 2		Genotype 11 versus others		Genotype 11 + 12 versus others	
			χ^2 (*P*-value)	OR (95% CI)	χ^2 (*P*-value)	OR (95% CI)	χ^2 (*P*-value)	OR (95% CI)
SLC22A4_2								
1–1	49	37						
1–2	227	133	2.33	1.18	0.08	0.94	4.82	1.36
2–2	207	174	(0.13)	(0.95–1.45)	(0.78)	(0.60–1.47)	(0.028)*	(1.03–1.80)
Sum	483	344						
DLG5_2								
1–1	323	201						
1–2	140	126	3.33	1.25	5.14	1.39	0.09	0.89
2–2	19	12	(0.068)	(0.98–1.60)	(0.023)*	(1.05–1.86)	(0.76)	(0.43–1.87)
Sum	482	339						

[a]Allele 1 indicated as risk allele
*$P < 0.05$

analysis (Hampe et al. 2001; Hugot et al. 2001; Ogura et al. 2001). Through the candidate gene approach, various genes, such as mucin 3 (*MUC3*), tumor necrosis factor (*TNF*), and *HLA class II*, were identified as candidate genes susceptible to IBD in some populations (Nakajima et al. 1995; Kyo et al. 1999, 2001; Negoro et al. 1999). In addition, recent studies identified three candidate susceptibility genes at two loci, one was *SLC22A4* and *SLC22A5* on chromosome 5 corresponding to *IBD5* (Peltekova et al. 2004), and the other was *DLG5* on chromosome 10 (Stoll et al. 2004).

Our case-control study for *SLC22A4*, *SLCA22A5*, and *DLG5* showed no evidence of association between SNPs in the *SLC22A5* gene and CD and that there might be some associations with SNPs in the two gene loci, *SLC22A4* and *DLG5*, to the disease, if any. In addition, it is notable that the SNPs showing weak and possible associations in our study were different from ones reported previously; three variations, C1672T in exon 9 of *SLC22A4*, G-207C in the *SLC22A5* promoter region, and G113A in exon 3 of *DLG5*, that showed the strong associations in Caucasian CD were completely absent in Japanese. The two remaining candidate variants, C4136A of exon 23 and 35 delA in intron 26 of *DLG5*, were found to be polymorphic in Japanese, but no association between these SNPs and Japanese CD was observed.

Interestingly, the genetic variants that showed the strong association in Caucasian but were completely absent in Japanese CD were indicated to interact with other genetic variants of *CARD15* that was also indicated to be a candidate susceptible gene to CD. Three major polymorphisms in the *CARD15* gene—R702W, G908R, and 1007fs—were confirmed to be independently associated with susceptibility to Caucasian patients with CD (Ahmad et al. 2002; Cuthbert et al. 2002; Lesage et al. 2002). However, our extensive DNA sequence analysis of this gene in more than 400 Japanese CD patients failed to identify such genetic variations except for a single case, indicating no involvement of *CARD15* in pathogenesis of Japanese CD (Yamazaki et al. 2002). Ethnic differences in the genetic variations among Caucasian, Asian, and African populations were also shown by others (Bonen et al. 2002; Inoue et al. 2002; Croucher et al. 2003).

We failed to confirm the association of the five candidate genetic variations in the *SLC22A4*, *SLCA22A5*, and *DLG5* genes in the previous reports to be susceptible to Japanese CD. However, we found a weak association of SNPs in the two genes, *SLC22A4* and *DLG5*, with Japanese CD. The results indicate a possibility that the five SNPs in the previous reports may not be causative, but the SNPs that we found to have possible association with or specific genetic substitutions having linkage disequilibrium with these SNPs in the region may play some role in Japanese CD. Nonetheless, combining the data that there is no association of *CARD15* with Japanese CD, it is apparent that there should be a presence of ethnic differences in susceptibility to CD. Further studies including both large-scale genomic and environmental analysis involving a large number of cases and controls are warranted to identify genes susceptible to CD on a worldwide scale, and such studies would eventually shed more light on the etiology of IBD.

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A Pilot Randomized Trial of a Human Anti-Interleukin-6 Receptor Monoclonal Antibody in Active Crohn's Disease

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Background & Aims: **Interleukin-6 (IL-6) regulates immune response and inflammation. We carried out a pilot placebo-controlled study to investigate the efficacy, pharmacokinetics, and safety of MRA, a humanized monoclonal antibody to IL-6 receptor, in patients with active Crohn's disease.** ***Methods:*** **Thirty-six patients with active Crohn's disease (Crohn's Disease Activity Index [CDAI] ≥150) were randomly assigned to receive biweekly intravenous infusion of either placebo, MRA, or MRA/placebo alternately for 12 weeks at a dose of 8 mg/kg. The study's primary end point was a clinical response rate that was defined as a reduction of CDAI ≥70.** ***Results:*** **At the final evaluation, 80% of the patients (8 of 10) given biweekly MRA had a clinical response as compared with 31% of the placebo-treated patients (4 of 13; $P = 0.019$). Twenty percent of the patients (2 of 10) on this regimen went into remission (CDAI <150), as compared with 0% of the placebo-treated patients (0 of 13). The clinical response rate of the every-4-week regimen was 42% (5 of 12). The serum concentrations of MRA were detected at 2 weeks after every infusion, at which time acute phase responses were completely suppressed; however, they were not suppressed at 4 weeks. Endoscopic and histologic examination showed no difference between MRA and placebo groups. The incidence of adverse events was similar in all the groups.** ***Conclusions:*** **This is the first clinical trial of humanized anti-IL-6 receptor monoclonal antibody in Crohn's disease. A biweekly 8 mg/kg infusion of MRA was well tolerated, normalized the acute-phase responses, and suggests a clinical effect in active Crohn's disease.**

Crohn's disease (CD) is a chronic granulomatous inflammation of the gastrointestinal tract. The incidence of CD is the greatest in early adult life, and increases year by year; therefore, effective therapy with long-term safety is earnestly desired. Although the exact cause of CD remains unclear, overproduction of proinflammatory cytokines has been repeatedly emphasized,[1] and these cytokines may be potential targets for the treatment.

Interleukin-6 (IL-6) is a pleiotropic cytokine with central roles in immune regulation and inflammation.[2] IL-6 can transduce its signal into the cells lacking IL-6 receptors (IL-6R) when it forms a complex with soluble IL-6R (sIL-6R).[3] The importance of IL-6 and sIL-6R in the physiopathology of CD has been well documented. Serum concentrations of sIL-6R were increased in patients with active CD, and serum IL-6 and sIL-6R concentrations correlated with C-reactive protein (CRP) levels.[4] The levels of IL-6 and sIL-6R in colonic organ cultures were elevated in patients with CD, especially in those with active inflammation.[5]

In the T-cell transfer murine colitis model, administration of rat anti-mouse IL-6R monoclonal antibody suppressed the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in the intestinal vascular endothelium. The treatment also reduced colonic expression of tumor ne-

Abbreviations used in this paper: CDEIS, Crohn's disease endoscopic index of severity; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IBDQ, Inflammatory Bowel Disease Questionnaire; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; mAb, monoclonal antibody; SAA, serum amyloid A; SAE, serious adverse event; sIL-6R, soluble interleukin-6 receptor; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α; VCAM-1, vascular cell adhesion molecule-1.

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crosis factor α (TNF-α), IL-1β, and interferon-γ mRNA without affecting the production of transforming growth factor β (TGF-β), IL-4, and IL-10.[6] Furthermore, anti-IL-6R mAb displayed therapeutic efficacy against established colitis through the induction of lamina propria T-cell apoptosis.[7] Therefore, blocking the IL-6 signaling pathway is considered a new therapeutic strategy for CD, and we carried out a pilot clinical trial of humanized anti-IL-6R mAb MRA for active CD.

Materials and Methods

Patients

Patients with CD, diagnosed in terms of history and radiologic or endoscopic intestinal appearance, who were at least 20 years of age were eligible for the study. Patients were to have a score on the Crohn's Disease Activity Index (CDAI)[8] ≥150, which indicates active CD, and abnormal serum levels of CRP. Patients were screened for eligibility at least 2 weeks before treatment. A total of 36 patients were screened, and all patients underwent randomization at 7 study centers in Japan between May 2001 and December 2001. Each center enrolled from 1 to 10 patients. The study was approved by the Institutional Review Board for each participating center, and all patients gave written informed consent. Patients were required to have previous treatments with mercaptopurine or azathioprine for at least 6 months before screening or corticosteroids (a maximum dosage of 60 mg/day of prednisone), mesalazine, salazosulfapyridine, metronidazole, or elemental diet for at least 4 weeks before screening. In addition, increasing the dose of mercaptopurine or azathioprine was prohibited from 8 weeks before screening; corticosteroids, mesalazine, salazosulfapyridine, or metronidazole from 2 weeks; and elemental diet from 4 weeks. Increasing the dose of those drugs was also prohibited throughout the study. Moreover, eligible patients had to have a white blood cell count ≥3500/mm^3 and platelet count ≥100,000/mm^3 at enrollment. Discontinuation of cyclosporin, methotrexate, or tacrolimus treatment before screening required a 12-week washout; surgical operation for CD or total parenteral nutrition before screening required a 4-week washout. Women were required to have a negative pregnancy test and to practice adequate birth control for the study duration. Breast-feeding women were also excluded. Patients with a history of the following were excluded from the study: medical history of serious allergic reaction; serious infections; significant cardiac, blood, respiratory system, neurologic, endocrine, renal, and hepatic diseases; and psychiatric disorders.

Study Design and Randomization

The study was a randomized, double-masked, placebo-controlled study. The central enrollment center assigned randomly eligible patients to 1 of 3 treatment groups according to a computer-generated randomization schedule. Each group received 6 intravenous infusions 2 weeks apart; MRA at a dose of 8 mg/kg (M2W), alternating infusions of 8 mg/kg of MRA and placebo (M4W), and placebo. The regimen was determined according to the previous study of rheumatoid arthritis.[9] Patients were given intravenously the study drug over a 1-hour period and were kept under careful observation for 1 hour after administration. Individual randomization concealment codes were held by the center for emergency use. Investigators, patients, and the trial's sponsor remained masked to the randomization codes until data analysis was completed.

The clinical response rate, defined as a decrease in the CDAI scores of 70 points or more, hypothesized for the placebo group was between 10% and 20% and that for the MRA groups was between 40% and 60%. Based on this hypothesis, we estimated that a sample size of 10 patients in each group would be needed to detect a significant difference in clinical response rates between the placebo and MRA groups (M2W and M4W) at a power of 80% and a 5% level of significance.

MRA (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) is a humanized anti-human IL-6R mAb that binds to both the membrane-bound form and the soluble form of human IL-6R with high affinity and specificity. It was constructed by grafting the complementarity-determining regions of the mouse anti-human IL-6R mAb into human IgG1 to re-create a properly functioning antigen-binding site in a reshaped human antibody.[10]

Study Procedures and End Points

Every 2 weeks from initial treatment to final visit at 12 weeks, the patient's CDAI scores and samples for clinical laboratory measurements were collected. The clinical response was defined by a decrease in the score ≥70 points from baseline, and a clinical remission was defined by the score <150. The health-related quality of life, as measured by the Inflammatory Bowel Disease Questionnaire (IBDQ),[11] was evaluated at baseline, 6, and 12 weeks.

A primary end point was the clinical response rate at the final evaluation. The secondary end points were the remission rates, changes from baseline of the IBDQ, erythrocyte sedimentation rate (ESR), CRP, serum amyloid A protein (SSA), and fibrinogen at each assessment time. An endoscopic examination was performed at the baseline and the last observation and CDEIS (Crohn's disease endoscopic index of severity)[12] was measured by using a 10-cm visual analog scale.

Safety evaluations including all adverse events reported by investigators, clinical laboratory tests, and vital signs were performed throughout the study. Clinical laboratory tests included complete blood count, coagulation test, blood chemistry test, ESR, antinuclear antibodies, anti-DNA antibodies, and urinalysis. The examination of antibodies to MRA and serum concentrations of MRA was performed by a central laboratory (SRL Inc., Tokyo, Japan), and the data were kept masked until code break except for positive results of anti-MRA antibodies that were to be reported for discontinuation of the treatment.

Serum levels of MRA were measured by an enzyme immunoassay using MT18 monoclonal antibody specific for another binding site on IL-6R than that detected by MRA in combi-