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

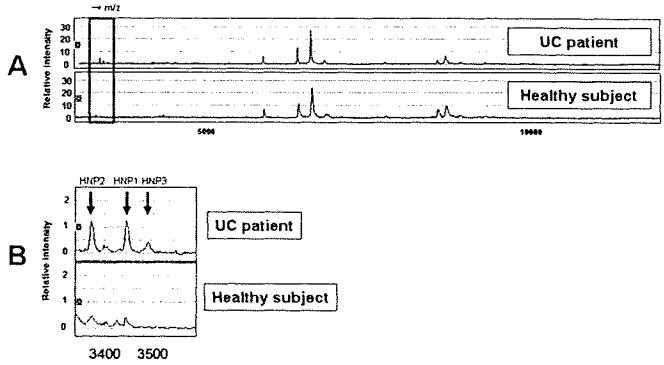

FIGURE 1. Serum proteomics of UC patients and healthy controls using SELDI-TOF/MS. (A) Spectra representing the serum proteins of a patient with UC and a healthy volunteer. The horizontal axis shows a range from 3000 to 10,000 m/z. Significant differences in peak intensities between patients with UC 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.

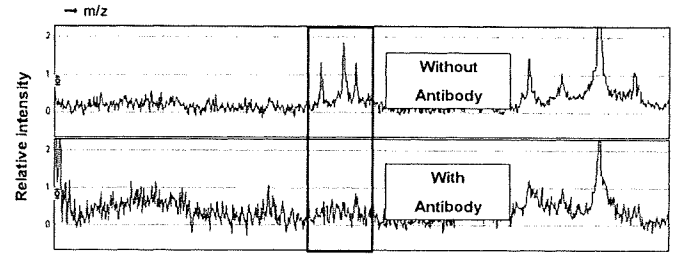

FIGURE 2. Immunodepletion assay using Protein A beads. Anti-HNP 1–3 antibodies (Hycult Biotechnology) were bound to 30 μL of Protein A beads. Sera from UC patients were incubated with these beads. After clearing by centrifugation, 3 μL of each supernatant was analyzed using an NP20 ProteinChip array.

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 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.

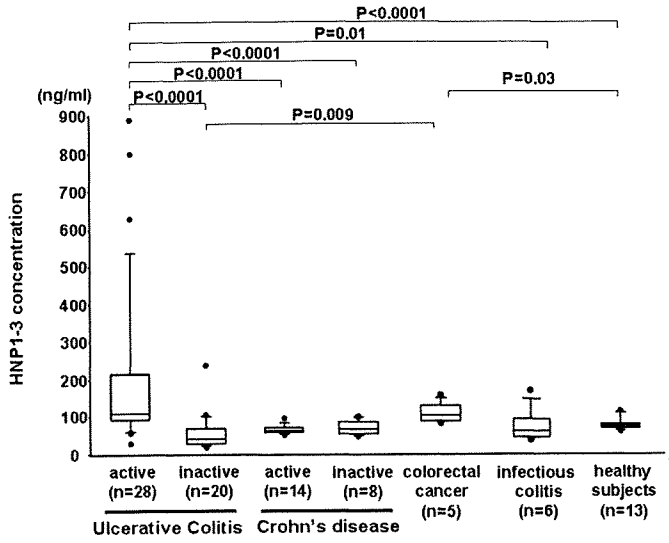

FIGURE 3. Concentrations of HNP 1–3 in the plasma of patients with UC, CD, colorectal cancer, infectious colitis, and healthy controls. Boxes indicate the median ± 25th percentile. The lower bar indicates the 10th percentile and the upper bar indicates the 90th percentile.

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,

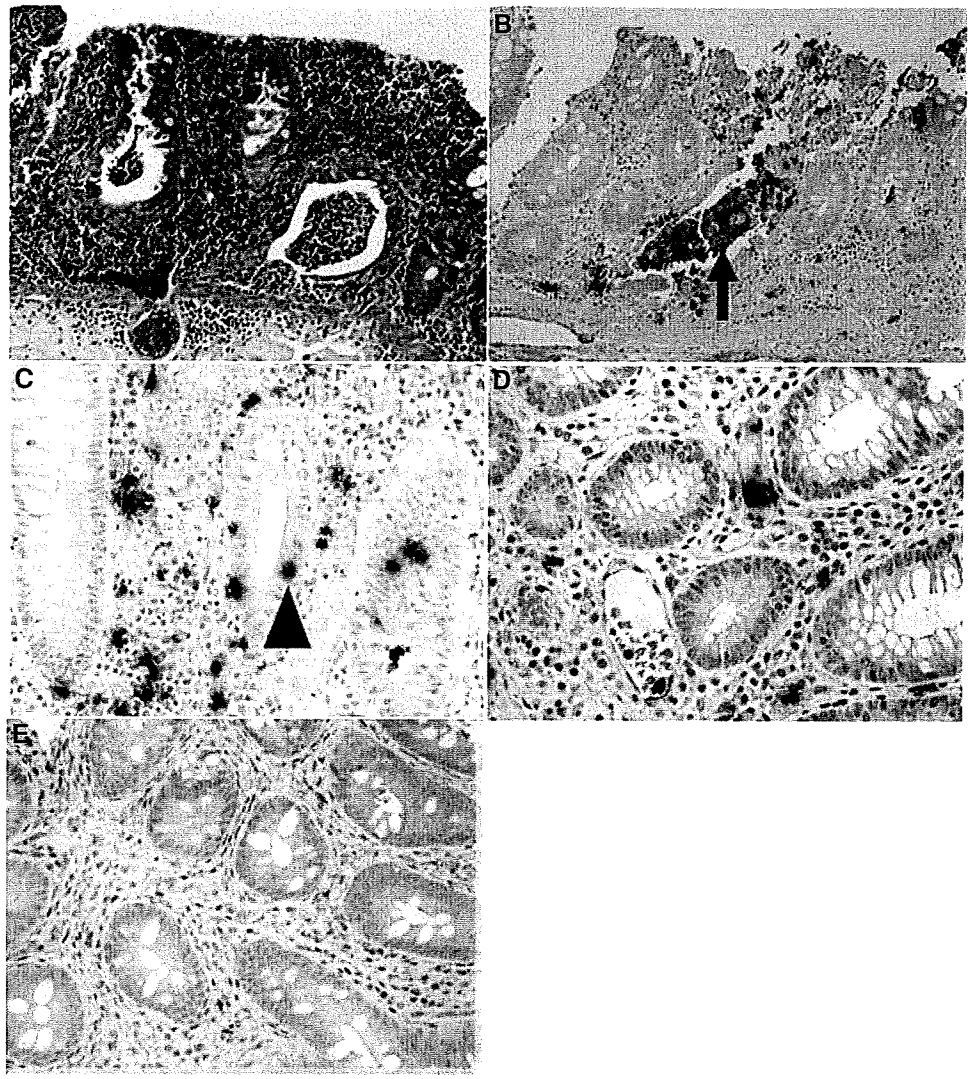

FIGURE 4. Expression of HNP 1–3 in the tissue of patients with active UC or CD and in normal colon tissue. (A) HE staining of colon tissues from patients with UC. (B,C) Immunohistochemical staining demonstrated extensive HNP 1–3 expression in the colon tissues of patients with UC. Many HNP 1–3-positive 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.

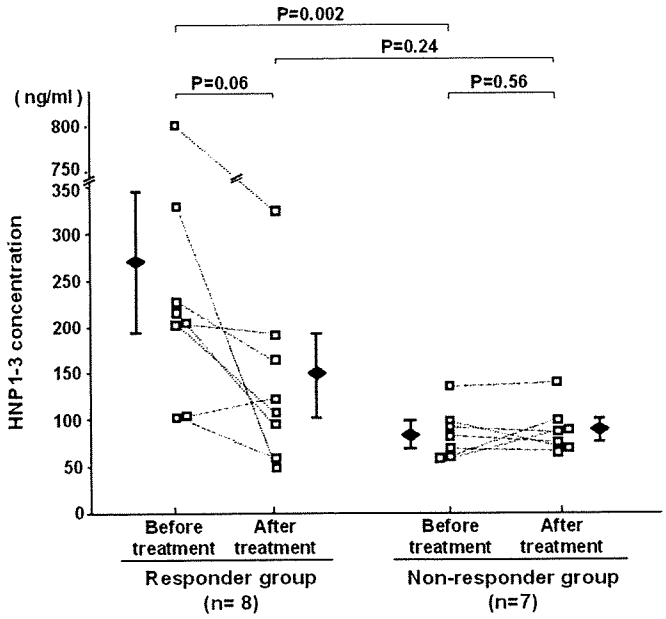

FIGURE 5. HNP 1–3 levels in the responder and nonresponder groups before treatment predicted therapeutic outcomes in UC patients; changes in the HNP 1–3 levels in UC patients in response to treatment are presented. The mean concentration of HNP 1–3 in the responder group before treatment was significantly 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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Induction of hepatocyte growth factor production in human dermal fibroblasts and their proliferation by the extract of bitter melon pulp

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ABSTRACT

Hepatocyte growth factor (HGF) is useful as a potential therapeutic agent for hepatic and renal fibrosis and cardiovascular diseases through inducing proliferation of epithelial and endothelial cells. HGF inducers may also be useful as therapeutic agents for these diseases. However, there have been no reports on induction of HGF production by plant extracts or juices. An extract of bitter melon (*Momordica charantia* L.) pulp markedly induced HGF production. There was a time lag of 72 h before induction of HGF production after the extract addition. Its stimulatory effect was accompanied by upregulation of HGF gene expression. Increases in mitogen-activated protein kinases (MAPKs) were observed from 72 h after the extract addition. Inhibitors of MAPKs suppressed the extract-induced HGF production. The extract also stimulated cell proliferation. Both activities for induction of HGF production and cell proliferation were eluted together in a single peak with 14,000 Da on gel filtration. The results indicate that bitter melon pulp extract induced HGF production and cell proliferation of human dermal fibroblasts and suggest that activation of MAPKs is involved in the HGF induction. Our findings suggest potential usefulness of the extract for tissue regeneration and provide an insight into the molecular mechanism underlying the wound-healing property of bitter melon.

1. Introduction

Hepatocyte growth factor (HGF), also known as scatter factor, was originally discovered as a mitogenic factor of rat hepatocytes in primary culture [1–5]. HGF is now recognized as a pleiotropic factor that functions as a mitogen, motogen, morphogen, anti-apoptotic factor and angiogenic factor acting on various types of cells [6,7]. Based on these actions, HGF has been shown to play critical roles in developmental and regenerative events of the liver and other tissues. Deficiency of HGF or its receptor, c-met, causes lethality at embryonic days 13.5–16.5 characterized by failure to develop a normal liver, muscle and placenta [8–10]. In addition, liver regeneration is impaired by pretreatment with an anti-HGF monoclonal antibody or in conditional HGF or c-met mutant mice [11,12]. Treatment with HGF stimulates liver growth in normal and partially hepatectomized animals, and injection of HGF is effective for treating animal models of chronic hepatic and renal diseases such as hepatic and renal fibrosis and liver cirrhosis [13–15]. Recent studies have also demonstrated the potential application of HGF for treating cardiovascular diseases such as peripheral vascular disease, myocardial infarction and cerebrovascular disease [16]. Moreover, HGF is capable of stimulating migration and proliferation of keratinocytes and thus has been suggested to be involved in cutaneous physiology and wound-healing [17,18]. HGF inducers may also be useful as therapeutic agents for these diseases.

HGF is mainly produced from mesenchymal cells such as fibroblasts and smooth muscle cells [19,20]. HGF production is induced in response to activation of PKA- and PKC-mediated pathways and is also induced by interleukin-1, tumor necrosis factor-α, interferon-γ, oncostatin-M, heparin, norepinephrine, staurosporine, a scatter factor-inducing factor, and growth factors such as epider-

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mal growth factor (EGF) and platelet-derived growth factor (PDGF) [21–30]. To date, however, there have been no reports on the effects of plant extracts or juices on HGF production.

Bitter melon (*Momordica charantia* L.), a member of the Cucurbitaceae family, is a plant that is cultivated throughout the world for use as a vegetable. The unripe fruit has also been used in developing countries as traditional medicine for microbial infections, sluggish digestion and intestinal gas, inflammation, fever reduction and wound-healing [31]. Recent studies on its pharmacological properties have shown several biological activities of bitter melon, including antidiabetic, antilipidemic, antibacterial, antiviral and anticancer activities [32–36].

Immunosuppressive and immunostimulating activities of bitter melon or its constituents have also been reported [37]. Prompted

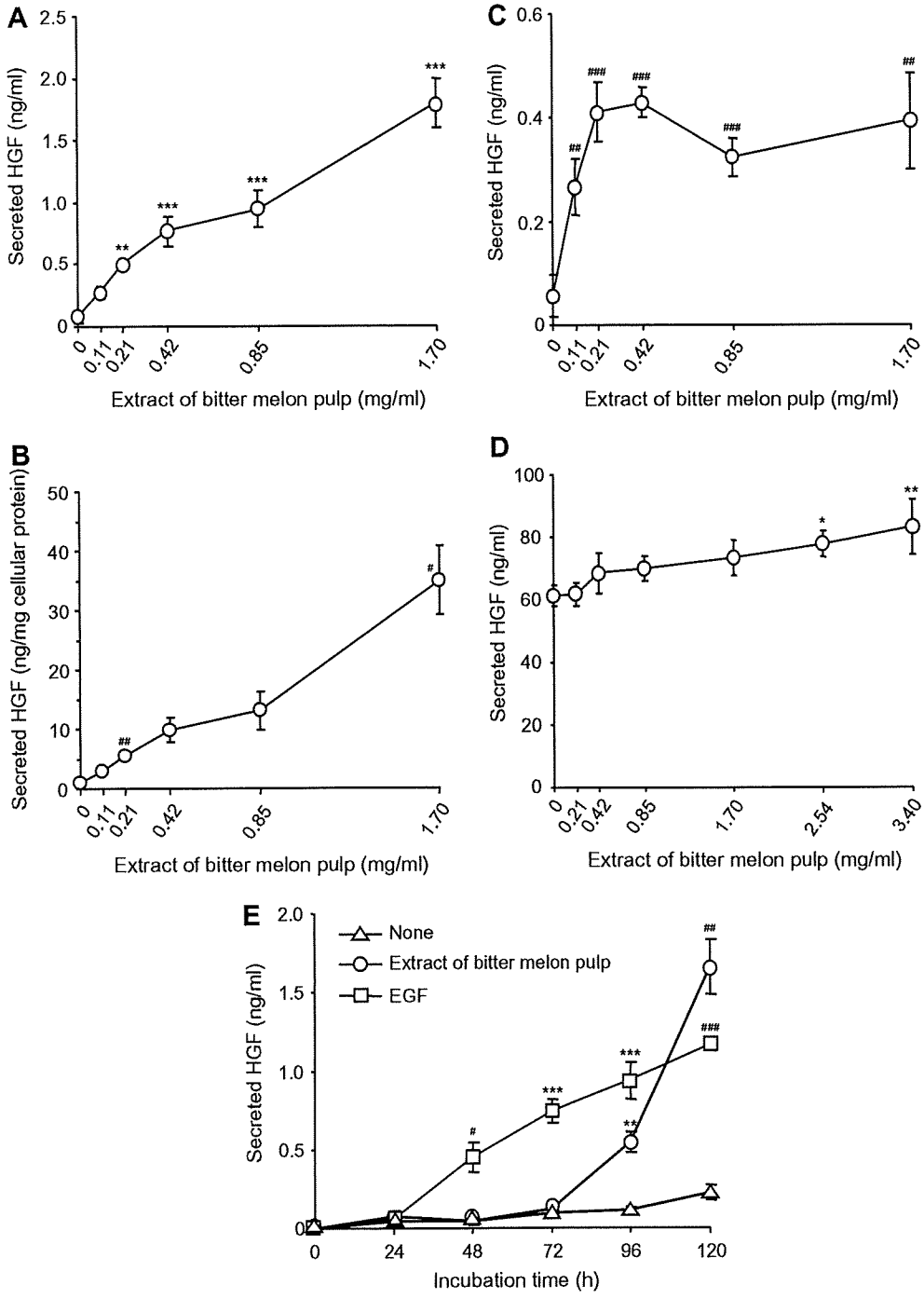

Fig. 1. Dose-response and time course for HGF production induced by the extract of bitter melon pulp. (A–D) Confluent dermal fibroblasts from neonatal donors (A, B) and a 3-day-old baby (C) and MRC-5 cells (D) were incubated for 120, 120 and 96 h, respectively, with the indicated concentrations of bitter melon pulp extract. (E) Confluent dermal fibroblasts from neonatal donors were incubated for the indicated periods with or without 0.42 mg/ml of bitter melon pulp extract and 3 ng/ml of EGF. The amount of HGF secreted into the medium was measured by an ELISA. The data are means ± SD of three (A, B, D and E) or five (C) independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs control (Dunnett's *t*-test); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs control (Dunnett's T3 test).

by the results of studies showing that the bitter melon extract augmentated cytokine secretion, we examined effects of the extract on HGF production. Here we describe that the extract of bitter melon pulp potently promoted HGF production and proliferation of human dermal fibroblasts. Our results suggest that the effect on HGF production is mediated through activation of mitogen-activated protein kinases (MAPKs), especially extracellular signal-regulated kinase (ERK).

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). GF109203X, PD98059 and SB203580 were purchased from Calbiochem (La Jolla, CA, USA). SP600125 and wortmannin were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA) and Wako Pure Chemical Industries (Osaka, Japan), respectively. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies against phospho-ERK, phospho-c-Jun N-terminal kinase (JNK) and phospho-p38 were purchased from Cell Signaling Technology (Danvers, MA, USA), and antibodies against ERK, JNK and p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human HGF cDNA (BamHI/KpnI fragment, 2.2 kbp) was derived from plasmids obtained from Dr. Naomi Kitamura (Tokyo Institute of Technology, Yokohama, Japan). Other reagents were obtained as described previously [26].

2.2. Cell culture

Human dermal fibroblasts derived from 200 individual neonatal donors (Cell Systems, Kirkland, WA, USA) and from a 3-day-old male baby (The Riken Cell Bank, Tsukuba, Japan) and the human embryonic lung fibroblast cell line MRC-5 (The American Type Culture Collection, Rockville, MD, USA) were cultured in DMEM supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (DMEM-10) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air as described previously [21].

2.3. Preparation of bitter melon extract

Unripe bitter melons (Sadowara Shironaga) were cultivated in Miyazaki Agricultural Research Institute (Miyazaki, Japan). The pulp of bitter melons was sliced, freeze-dried and powdered. The pulp powder was mixed with 10 vol. of phosphate-buffered saline (PBS) for 1.5 h under continuous stirring at 40 °C followed by centrifugation. The supernatant was collected by decantation, and the resulting precipitate was mixed again with 5 vol. of PBS for another 1.5 h under continuous stirring at 40 °C. After centrifugation, the supernatants were combined, filtered through a membrane filter and stored at −80 °C until use. The concentration (w/v) of the extract was calculated by subtracting the weight of lyophilized PBS of the same volume from the weight of the lyophilized PBS extract.

2.4. Gel filtration of bitter melon pulp extract

The extract of bitter melon pulp (2 ml) was applied to a column of Sephadex G-50 (1.5 × 30 cm) equilibrated with PBS and eluted with the same buffer at 4 °C. Fractions of 1 ml were collected.

2.5. Determination of HGF production

The medium of confluent human dermal fibroblasts and MRC-5 cells cultured in 96-well plates (Nunc, Roskilde, Denmark) was replaced with a fresh medium (DMEM-10) containing various amounts of the bitter melon pulp extract. The conditioned medium was collected after being incubated for various periods and was frozen at −30 °C for a human HGF ELISA. The sandwich ELISA for human HGF was performed at room temperature as described previously [38], with slight modification [39]. HGF levels were expressed as ng/ml or ng/mg of cellular protein as described previously [26].

2.6. MTT assay

Confluent human dermal fibroblasts grown in 96-well plates (Nunc) were incubated for 120 h with the extract of bitter melon pulp as described in the previous section. The medium was then replaced with 100 μl of the fresh medium (DMEM-10), and the cultures were incubated for 1 h. MTT assay was then performed as described previously [40].

2.7. Northern blot analysis

The medium of confluent human dermal fibroblasts grown in 90-mm dishes (Nunc) was replaced with the fresh medium (DMEM-10) containing the extract of bitter melon pulp, and the cells were incubated for 88 h. Total RNA was then isolated from the cells using RNA-Bee (TEL-TEST, Friendswoods, TX, USA). Northern blotting was performed as described previously [26]. The signal intensity of the 6.4-kb HGF mRNA band in the autoradiograms was normalized to the fluorescence intensity of the 28S rRNA band.

2.8. Western blot analysis

The medium of confluent human dermal fibroblasts grown in 24-well plates (Nunc) was replaced with the same fresh medium (DMEM-10), and the cells were incubated for about 18 h. The extract of bitter melon pulp was added without a medium change. After being incubated for an appropriate period, the cells were

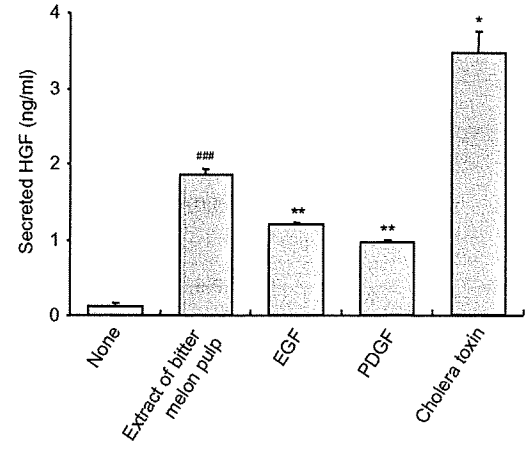

Fig. 2. Comparison of HGF production induced by the extract of bitter melon pulp with HGF production promoted by other inducers. Confluent dermal fibroblasts from neonatal donors were incubated for 120 h with or without 0.42 mg/ml of bitter melon pulp extract, 3 ng/ml of EGF, 10 ng/ml of PDGF, and 1 pM cholera toxin. The amount of HGF secreted into the medium was measured by an ELISA. The data are means of three independent experiments. Bars indicate SD. $^{###}p < 0.001$ vs control (Student's *t*-test); $^{*}p < 0.05$ and $^{**}p < 0.01$ vs bitter melon pulp extract (Dunnett's T3 test).

washed four times with ice-cold PBS and lysed by adding 40 μl of sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% Bromophenol Blue). Lysates were boiled for 10 min, briefly sonicated, and centrifuged. Protein in extracts was determined by a modification of the method of Lowry et al. [41]. Equivalent protein aliquots were separated by 10% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred electrophoretically to Immobilon-P membranes (Millipore, Billerica, MA, USA) as reported previously [42]. Blots were probed with various antibodies, incubated with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulins (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and detected with ECL Plus Western blotting detection reagents (GE Healthcare Bio-Sciences Corp.).

2.9. Determination of DNA synthesis

The medium of confluent human dermal fibroblasts cultured in 96-well plates (Nunc) was replaced with the fresh medium (DMEM-10) containing the extract of bitter melon pulp, and the cells were incubated for 78 h. The cells were then pulse-labeled with [^{3}H]thymidine (0.5 μCi/well, 2.5 Ci/mmol) for 24 h, trypsinized, and harvested on glass-fiber filters using a cell harvester as described previously [43]. The amount of [^{3}H]thymidine incorporated was measured in a liquid scintillation counter.

2.10. Statistical analysis

Results are expressed as means and SD of several independent experiments. Data in two groups were analyzed by Student's *t*-test or Welch's *t*-test. Multiple comparisons of the data were done by ANOVA followed by Dunnett's *t*-test, Dunnett's T3 test or Tukey's test. *P* values less than 0.05 were regarded as significant.

3. Results

3.1. Induction of HGF production by the extract of bitter melon pulp

Human dermal fibroblasts derived from 200 neonatal donors were incubated for 5 days with the extract of bitter melon pulp,

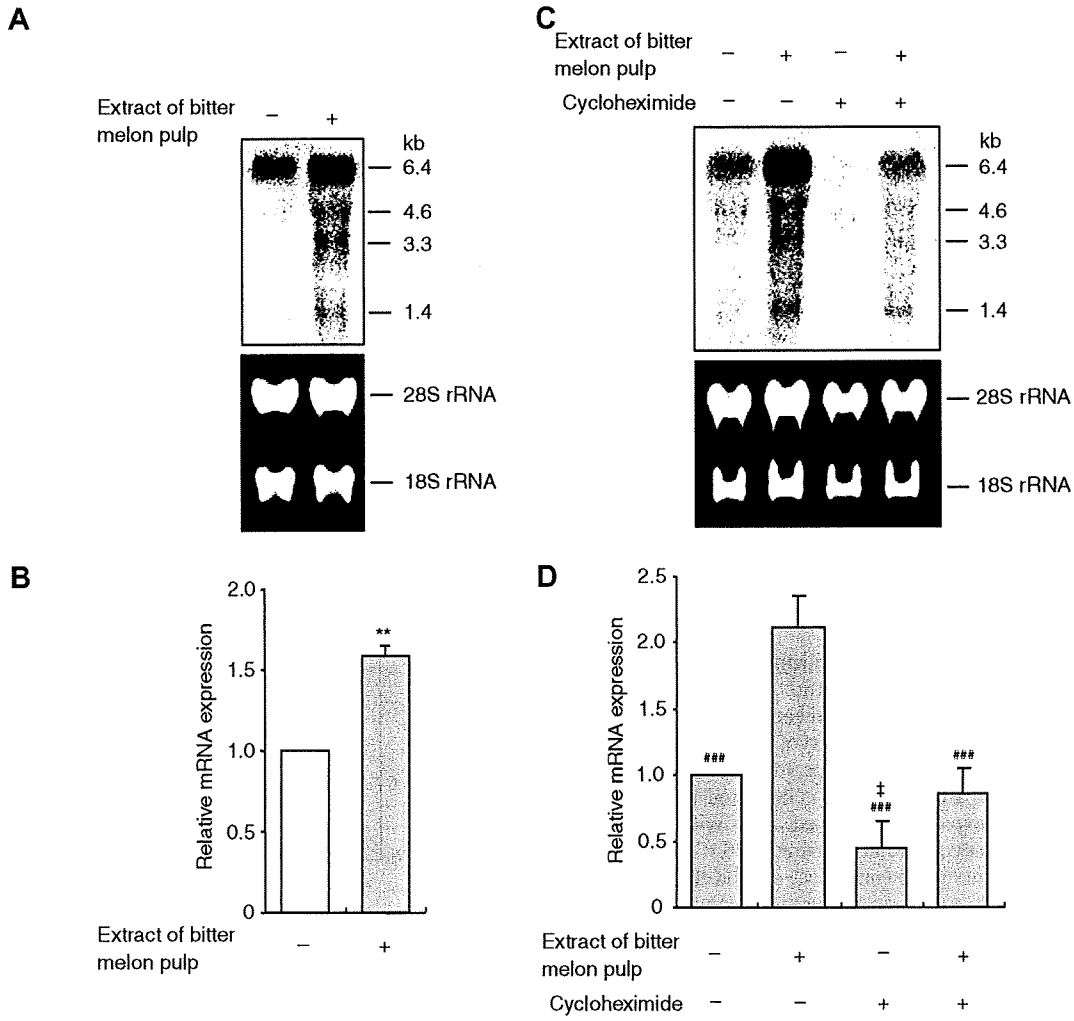

Fig. 3. Upregulation of HGF gene expression by the extract of bitter melon pulp and inhibition of the extract-induced HGF mRNA expression by cycloheximide. (A, B) Confluent dermal fibroblasts from neonatal donors were incubated for 88 h with or without 0.85 mg/ml of bitter melon pulp extract. (C, D) Confluent dermal fibroblasts were incubated for 88 h with or without 0.85 mg/ml of bitter melon pulp extract. During the last 24 h some cultures were incubated in the presence of cycloheximide (1 μg/ml). Total RNA was isolated and Northern blot analysis was performed using a ^{32}P-labeled cDNA probe for human HGF. The signal intensity of the 6.4-kb HGF mRNA band in the autoradiograms was normalized to the fluorescence intensity of the 28S rRNA band and expressed as fold-change relative to the control. Autoradiograms and fluorescence photographs (A, C) are representative of three independent experiments with similar results. The data (B, D) are means of three independent experiments. Bars indicate SD. $^{**}p < 0.01$ vs control (Welch's *t*-test); $^{\#\#\#}p < 0.001$ vs bitter melon pulp extract alone (Tukey's test); $^{\ddagger}p < 0.05$ vs medium alone (Tukey's test).

and HGF secreted from the cells was then determined by an HGF ELISA. The extract dose-dependently enhanced HGF production expressed as either ng/ml (Fig. 1A) or ng/mg of cellular protein (Fig. 1B). The HGF-inducing activity of the extracts prepared from some lots of bitter melon pulp decreased at concentrations of more than 0.85 mg/ml, but the extract exhibited dose-dependent HGF induction similar to that shown in Fig. 1A after being incubated for 10 min at 70 °C (data not shown, see below). HGF production in human dermal fibroblasts derived from a 3-day-old baby and in the human embryonic lung fibroblast cell line MRC-5, which produces a large amount of HGF, was also significantly increased by the extract (Fig. 1C and D). The time course of HGF production induced by the extract of bitter melon pulp is shown in Fig. 1E. There was a time lag of at least 72 h before upregulation of HGF production after the addition of the extract, whereas the time lag before EGF-induced HGF production was 24 h. These results suggest that the extract increased HGF production through an indirect mechanism.

Next, we compared the effect of the extract of bitter melon pulp with the effects of other agents known to induce HGF production in human dermal fibroblasts [25,26]. The effect of the extract of bitter melon pulp was stronger than the effects of growth factors such as EGF (3 ng/ml) and PDGF (10 ng/ml) but weaker than the effect of cholera toxin (1 pM) (Fig. 2).

3.2. Upregulation of HGF mRNA expression by the extract of bitter melon pulp

To investigate whether the extract of bitter melon pulp affects HGF mRNA expression, total RNA was isolated from human dermal fibroblasts incubated for 88 h with or without the extract, and Northern blot analysis was performed. As shown in Fig. 3A and B, the level of HGF mRNA expression in cells treated with the extract increased by 60% compared to that of the control.

Since induction of HGF production by the extract of bitter melon pulp was slow, we examined the requirement of *de novo* protein synthesis in the extract-induced HGF gene expression using the protein synthesis inhibitor cycloheximide. Confluent cells were incubated for 88 h with or without the extract, and some cultures were also treated with cycloheximide during the last 24 h. The level of HGF mRNA expression was then determined by Northern blot analysis. Treatment with cycloheximide completely inhibited HGF mRNA upregulation induced by the extract (Fig. 3C and D).

3.3. Involvement of MAPKs in HGF production induced by the extract of bitter melon pulp

To reveal what signaling pathway is involved in the extract-induced HGF production, we investigated the effects of selective signal transduction inhibitors on induction of HGF production by the extract. As shown in Fig. 4, the ERK kinase inhibitor PD98059, which inhibits phosphorylation of ERK, almost completely suppressed the induction of HGF production. The p38 inhibitor SB203580 and the JNK inhibitor SP600125 also potently inhibited the induction of HGF production. HGF production induced by the extract was moderately suppressed by the PKC inhibitor GF109203X but was not significantly influenced by the phosphatidylinositol 3-kinase inhibitor wortmannin.

3.4. Effects of the extract of bitter melon pulp on phosphorylation of MAPKs

Since it was suggested that ERK phosphorylation plays a critical role in HGF production induced by the extract of bitter melon pulp, we investigated the effect of the extract on ERK phosphorylation by Western blotting. When the cells were stimulated with growth factors (e.g., EGF) or phorbol 12-myristate 13-acetate, a PKC activator, ERK phosphorylation transiently occurred within a few minutes and thereafter rapidly declined to the control level (data not shown). In contrast, the levels of ERK phosphorylation in cells treated with the extract did not increase within 6 h after addition of the extract (data not shown). Thus, we examined whether long-term treatment with the extract of bitter melon pulp could induce ERK phosphorylation. As shown in Fig. 5A, ERK phosphorylation

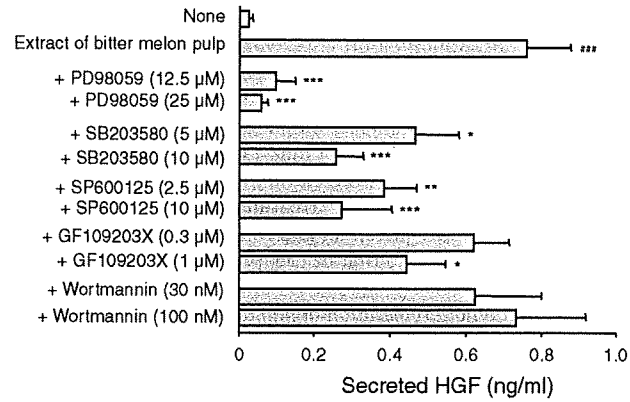

Fig. 4. Suppression of bitter melon-pulp-extract-induced HGF production by inhibitors of MAPKs. Confluent dermal fibroblasts from neonatal donors were preincubated with or without inhibitors of MAPKs and other protein kinases for 2 h and then incubated for 120 h with or without 0.42 mg/ml of bitter melon pulp extract in the presence or absence of the inhibitors. The inhibitors of MAPKs and other protein kinases were dissolved in dimethyl sulfoxide, and the final concentration of dimethyl sulfoxide in all cultures, including the control, was 0.1%. The amount of HGF secreted into the medium was measured by an ELISA. The data are means of four independent experiments. Bars indicate SD. $^{###}p < 0.001$ vs control (Welch's *t*-test); $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs bitter melon pulp extract alone (Tukey's test).

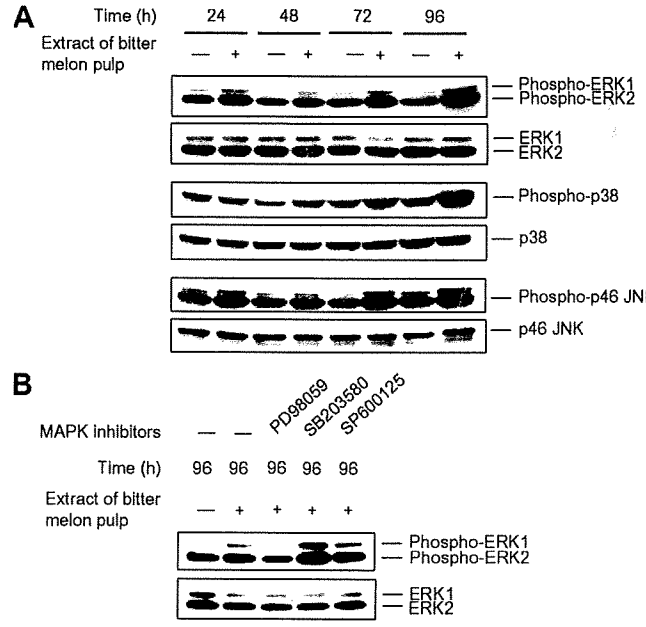

Fig. 5. Upregulation of phosphorylation of ERK, p38 and JNK by the extract of bitter melon pulp. Confluent dermal fibroblasts from neonatal donors were incubated for the indicated periods with or without 0.85 mg/ml of bitter melon pulp extract in the presence or absence of MAPK inhibitors (25 μM PD98059, 10 μM SB203580 or 10 μM SP600125). Equal amounts of cell extracts (10 μg) were subjected to SDS–PAGE and immunoblotted with specific antibodies against phosphorylated and total ERK, p38 and JNK. Immunoblots are representative of three independent experiments with similar results.

gradually increased from 72 h after addition of the extract. Further increase in phosphorylation level was observed at 96 h, whereas the cells cultured with the medium alone showed no change at any times examined. Upregulation of ERK phosphorylation induced by the extract was completely inhibited by PD98059, while neither SB203580 nor SP60012 had an inhibitory effect (Fig. 5B). We also investigated the effects of the extract on phosphorylation of other kinds of MAPK, p38 and JNK. Treatment with the extract significantly increased the phosphorylation levels of p38 and JNK with a time course similar to that of ERK phosphorylation (Fig. 5A).

3.5. Stimulation of proliferation of human dermal fibroblasts by the extract of bitter melon pulp

Upregulation of MAPKs suggests a potential stimulation of cell proliferation induced by the extract of bitter melon pulp. To investigate an effect of the extract on cell proliferation, confluent human dermal fibroblasts were incubated with or without the extract of bitter melon pulp, and the viable cell number was determined by the MTT assay. The extract significantly increased the cell number at doses of 0.21 and 0.42 mg/ml (Fig. 6A). Fig. 6B shows the effect of the extract on DNA synthesis in the fibroblasts. The extract markedly enhanced [^{3}H]thymidine incorporation. The time course of cell proliferation induced by the extract of bitter melon pulp is shown in Fig. 6C. There was a time lag of at least 48 h before enhancement of cell proliferation after the addition of the extract.

3.6. Properties of an active substance(s)

The extract was subjected to gel filtration to determine the molecular mass of a substance with activity for induction of HGF production and that of a substance with growth-promoting activity. Both activities were eluted in a single peak with almost the same molecular mass (Fig. 7A). The apparent molecular mass was about 14,000 Da, if the active substance(s) is a polypeptide. The HGF production-inducing activity in the extract of bitter melon pulp was stable for 10 min incubation up to 70 °C (data not shown), but the activity was completely inactivated by being incubated for 10 min at 80 °C (Fig. 7B). The activity in the extract was not inactivated by being treated with 0.01% trypsin for 30 min at 37 °C (data not shown). When the extract was precipitated with ethanol, the HGF production-inducing activity was retained in the supernatant of 70% ethanol (data not shown).

4. Discussion

The data presented in this report show that the extract of bitter melon pulp markedly induced HGF production from human dermal fibroblasts. The extract also stimulated HGF production from the human lung fibroblast line MRC-5, suggesting that the effect is not specific for cell type. To our knowledge, this is the first report on the stimulatory effect of a vegetable extract on HGF production. HGF is mitogenic to hepatocytes, other epithelial cells and endothelial cells [6,7]. Injection of HGF or HGF gene has been effective for treating animal models of chronic hepatic and renal diseases such as hepatic and renal fibrosis and liver cirrhosis and of cardiovascular diseases [14–16]. Thus, HGF inducers may also be useful as therapeutic agents for these diseases. Studies are under way in our laboratory to determine the effect of the bitter melon pulp extract on HGF production in vivo.

Since the extract of bitter melon pulp upregulated phosphorylation of ERK, p38 and JNK and the extract-induced HGF production was potently inhibited by PD98059, SB203580 and SP600125, which specifically block ERK, p38 and JNK signaling pathways, respectively, it seems likely that MAPKs are important molecules responsible for the HGF production-inducing effect of the extract.

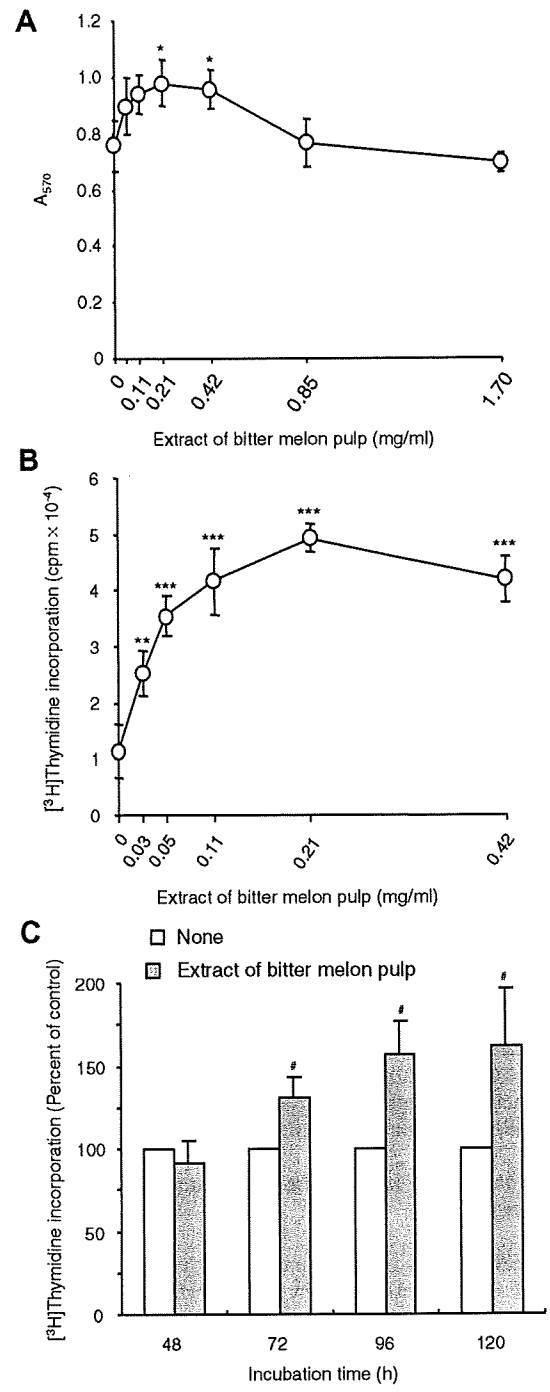

Fig. 6. Stimulation of proliferation of dermal fibroblasts by the extract of bitter melon pulp. Confluent dermal fibroblasts from neonatal donors were incubated for 120 h (A), 102 h (B) and the indicated periods (C) with or without the indicated concentrations (A, B) and 0.21 mg/ml (C) of bitter melon pulp extract. In the time course experiment (C), the medium of confluent cells was replaced with the same fresh medium (DMEM-10) 24 h before addition of the extract. The extract of bitter melon pulp was then added without a medium change. The number of viable cells and DNA synthesis were then determined by the MTT method (A) and [^{3}H]thymidine incorporation during the last 24 h (B, C), respectively. The data are means ± SD of three (A, B) or four (C) independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs control (Dunnett's *t*-test); $^{\#}p < 0.05$ vs control (Welch's *t*-test).

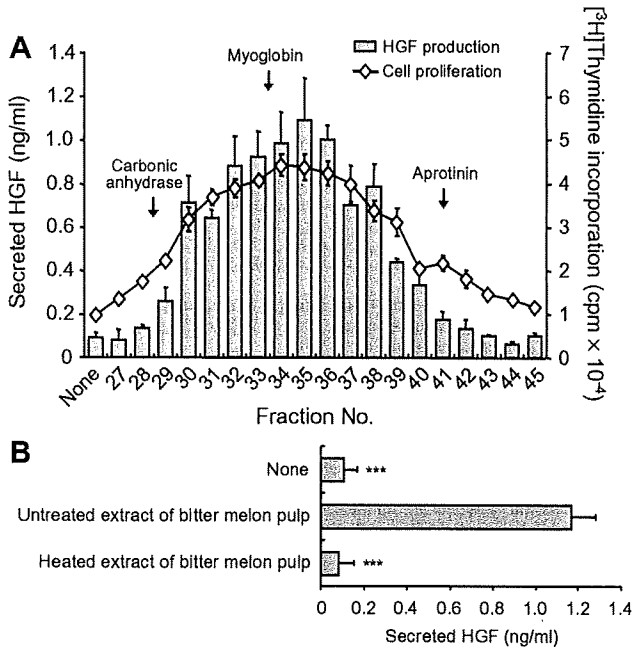

Fig. 7. Gel filtration profile of HGF production-inducing activity and cell growth-promoting activity of bitter melon pulp extract and inactivation of HGF production-inducing activity by heating. (A) Two milliliter of bitter melon pulp extract was applied to a Sephadex G-50 column, and 1-ml fractions were collected. For the assay of HGF production induction assay, confluent dermal fibroblasts from neonatal donors were incubated for 120 h with or without 5% of each fraction. The amount of HGF secreted into the medium was measured by an ELISA. For the assay of cell growth-promoting activity, confluent dermal fibroblasts from neonatal donors were incubated for 78 h with or without 2.5% of each fraction. The cells were then pulse-labeled with [^{3}H]thymidine for 24 h. The amount of [^{3}H]thymidine incorporated was measured in a liquid scintillation counter. Molecular weight markers used are carbonic anhydrase (29,000), myoglobin (17,000) and aprotinin (6500). The data are representative of three independent experiments with similar results and expressed as means + or ± SD of triplicate cultures. (B) The extract of bitter melon pulp (33.9 mg/ml) was incubated for 10 min at 80 °C and centrifuged. Confluent dermal fibroblasts were incubated for 120 h with 1.25% (v/v) of the supernatant. The amount of HGF secreted into the medium was measured by an ELISA. The data are means of three independent experiments. Bars indicate SD. $^{***}p < 0.001$ vs untreated extract of bitter melon pulp (Dunnett's *t*-test).

There was a time lag of at least 48 h before upregulation of the phosphorylation of MAPKs in the fibroblasts after addition of the extract, whereas upregulation of the phosphorylation of MAPKs occurred within 5 min after addition of EGF (our unpublished data). These results suggest that the extract increases phosphorylation levels of MAPKs indirectly through a mechanism by which an unidentified factor(s) is induced. There was also a time lag of at least 72 h before upregulation of HGF production after addition of the extract, whereas the time lag before EGF-induced HGF production was about 24 h. Thus, induction of HGF production occurs approximately 24 h later than upregulation of the phosphorylation of MAPKs. We have speculated that one of the most likely candidates is a growth factor such as EGF or PDGF. This notion is supported by the fact that the extract stimulated proliferation of the cells and the fact that growth factors such as EGF, PDGF and bFGF induce HGF production in fibroblasts [25]. The notion is also consistent with our results showing that the HGF production-inducing activity and the cell growth-promoting activity of the extract were not separated by gel filtration (Fig. 7A) and that HGF mRNA upregulation induced by the extract was inhibited by treatment with the protein synthesis inhibitor cycloheximide (Fig. 3C and D). Production of EGF and PDGF-AA from human dermal fibroblasts, however, was not induced by the extract of bitter melon pulp (our unpublished data). It has been reported that HGF also has growth-stimulating activity of human dermal fibroblasts [44]. The extract-induced augmentation of cell proliferation, however, may not be mediated through the induction of HGF production because the former occurred earlier than the latter. Thus, the mediator for induction of HGF production and cell proliferation remains to be determined.

The HGF production-inducing activity in the extract of bitter melon pulp was not reduced by trypsin treatment. Although bitter melon contains protease inhibitors [45], the azocaseinolytic activity of trypsin was only slightly inhibited by the extract (our unpublished results). The results of gel filtration of the extract indicated that the HGF production-inducing activity was eluted in the fractions of high molecular weight. Therefore, the active substance in the extract seems to be a non-proteinous macromolecule. Most of the macromolecular inducers of HGF are proteins such as growth factors and cytokines [24,25,27,28,30]. Non-proteinous macromolecular HGF inducers reported so far is heparin which is effective in MRC-5 cells and others [23]. However, heparin does not upregulate HGF gene expression and does not effectively induce HGF production in human dermal fibroblasts [23]. Thus, it is likely that the active substance in the extract is a hitherto-unidentified inducer of HGF production.

Bitter melon has been shown to possess a wound-healing property [31,46]. Wound-healing is a complex process of inflammation, granulation tissue formation, angiogenesis, re-epithelialization and remodeling [47]. The complexity of this process is compounded by the fact that skin is comprised of two distinct compartments, the ectodermally derived epithelial epidermis and the mesodermally derived mesenchymal dermis, which are separated by a basement membrane barrier. To regenerate this organ, numerous cellular, hormonal, matrix and enzymatic activities in each compartment are required to act in a coordinated manner with those in the other compartment. If re-epithelialization occurs without sufficient underlying granulation tissue formation, which encompasses macrophage accumulation, fibroblast ingrowth, matrix deposition and angiogenesis, then an atrophic scar results. If granulation tissue formation proceeds without the requisite re-epithelialization, then a nonhealed wound with hypergranulation tissue is the outcome. The extract of bitter melon pulp was found to have both HGF production-inducing activity and fibroblast growth-promoting activity. HGF is capable of stimulating migration and proliferation of keratinocytes and thus has been suggested to be involved in cutaneous physiology and wound-healing [17]. The extract is expected to stimulate regeneration of the epithelial epidermis and mesenchymal dermis in a coordinated manner.

In conclusion, this study showed that the extract of bitter melon pulp significantly induced HGF expression in different cell types. The results suggested that the effect is mediated through indirect activation of MAPKs, especially ERK. To the best of our knowledge, this is the first report on the stimulatory effect of a vegetable extract on expression of HGF. This study also showed that the extract markedly stimulated the proliferation of fibroblasts. Our findings suggest potential usefulness of the extract for tissue regeneration and provide an insight into the molecular mechanism underlying the wound-healing property of bitter melon.

Acknowledgments

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Association of a genetic polymorphism in ectonucleotide pyrophosphatase/phosphodiesterase 1 with hepatitis C virus infection and hepatitis C virus core antigen levels in subjects in a hyperendemic area of Japan

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Background. The clinical course of chronic hepatitis C virus (HCV) infection is strongly associated with insulin resistance and obesity. The K121Q polymorphism in the ectonucleotide pyrophosphatase/phosphodiesterase (*ENPP*)-1 gene and the rs7566605 genotype located near insulin-induced gene 2 have been shown to be associated with insulin resistance and obesity. This study examined whether the K121Q polymorphism in *ENPP1* or the rs7566605 genotype is associated with the clinical course of HCV infection. ***Methods.*** The relationships between the clinical characteristics of 469 anti-HCV antibody-seropositive subjects (353 were positive for HCV core antigen or RNA, whereas 116 were negative for HCV RNA) and the polymorphisms were analyzed. ***Results.*** No significant differences in body mass index, plasma glucose level, serum insulin level, and other biochemical markers were observed between subgroups of subjects with different genotypes at the K121Q polymorphism or rs7566605. The frequency of the homozygous wild-type genotype at K121Q in HCV carriers, however, was significantly higher than that in subjects who were negative for HCV RNA (84.5% vs. 75.9%; $P < 0.05$). Moreover, in HCV carriers, HCV core antigen levels in subjects homozygous for the wild-type genotype at K121Q were significantly higher than in heterozygous carriers of K121Q (5358 fmol/l vs. 4002 fmol/l; $P = 0.04$). In contrast, the rs7566605 genotype was not associated with hepatitis C viremia or with the HCV core antigen level. ***Conclusions.*** The K121Q variant of *ENPP1* may be associated with hepatitis C viremia and core antigen levels in HCV carriers.

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Key words: hepatitis C virus, *ENPP1*, insulin resistance, viremia, single nucleotide polymorphism, HCV core antigen

Introduction

Hepatitis C virus (HCV) infection, a major cause of chronic hepatitis, may progress to cirrhosis or hepatocellular carcinoma (HCC). Persistent HCV infection can be detected in the sera of 50%–80% of subjects positive for anti-HCV antibodies; in contrast, 20%–50% of those subjects are consistently negative for HCV RNA, suggesting that they have successfully eliminated the HCV infection.[1] Factors such as ethnicity, icteric clinical presentation, absence of human immunodeficiency virus (HIV) infection, and specific HLA type II alleles have been shown to be associated with viral clearance.[2–4] Even in the absence of these factors, however, viral clearance may occur, suggesting the presence of other unidentified cofactors.

Being overweight or obese is an independent risk factor for hepatic steatosis, which accelerates the activity and progression of chronic hepatitis C (CHC).[5] Another risk factor for steatosis is insulin resistance, which is associated with advanced fibrosis and hyporesponsiveness to antiviral therapy.[6] Although obesity and insulin resistance are known to be caused by a combination of genetic and environmental factors, the impact of genetic factors on the clinical course of HCV infection or the severity of liver disease has not been fully elucidated.

A number of reports indicate that single nucleotide polymorphisms (SNPs) in the gene encoding the K121Q variant of ectonucleotide pyrophosphatase/phosphodi-

esterase 1 (*ENPP1*, also known as PC-1) influence insulin resistance, type 2 diabetes, and obesity.[7–11] Recently, the rs7566605 genotype, which is located near the gene encoding insulin-induced gene 2 (*INSIG2*), was also shown to be strongly associated with insulin resistance.[12] Other studies, however, have reported no significant associations between the K121Q variant and insulin resistance or type 2 diabetes,[13–15] and the association between the K121Q variant or rs7566605 genotype and the clinical features of patients with chronic HCV infection has not been fully evaluated.

We examined the natural history of HCV infections in an adult Japanese community-based population in an HCV hyperendemic area beginning in 1994.[16,17] Because movement of the residents in or out of this region is rare, this area provided an appropriate setting to investigate the effects of a genetic background on HCV infections. In this study, we sought to determine the prevalence of the rs7566605 genotype and polymorphisms of the *ENPP1* gene encoding the K121Q variant and to assess their relationship with body mass index (BMI), insulin resistance, and the clinical characteristics of subjects positive for anti-HCV antibodies in an HCV hyperendemic area in Japan.

Materials and methods

Study population

We evaluated 459 anti-HCV antibody-seropositive subjects. Among these subjects, 343 were positive for HCV RNA or HCV core antigen (HCV carrier group), and 116 were negative for both HCV RNA and HCV core antigen (HCV RNA-negative group). All the subjects were Japanese and lived in an HCV hyperendemic area (Town C).[16–18] The Town C HCV study is a cohort study examining the natural course of HCV infections in adult residents of a community in Miyazaki Prefecture, Japan. Residents who were identified as anti-HCV antibody positive at general health examinations were invited to participate in annual examinations for liver disease. No one in this study population had received interferon therapy or was positive for hepatitis B surface antigen. Informed consent was obtained from all participants at the time of enrollment. This study was approved by the human subjects committees of the University of Miyazaki (Faculty of Medicine, Japan), the Harvard School of Public Health, and the Boston University School of Public Health.

Blood tests for hepatic fibrosis markers, anti-HCV antibodies, and HCV core antigen levels

Serum anti-HCV antibodies were detected using chemiluminescence enzyme immunoassays and a third-generation kit (Lumipulse Ortho II; Ortho-Clinical Diagnostics, Tokyo, Japan) at least once for each subject between 2001 and 2003. Additionally, 301 subjects in the HCV carrier group and 100 subjects in the HCV RNA-negative group were known to be positive for anti-HCV antibodies before 1996 as a result of second-generation enzyme immunoassay testing (Immunocheck F-HCV Ab; International Reagents, Kobe, Japan).[16–19] The presence of serum HCV RNA was determined using qualitative reverse transcription-polymerase chain reaction (RT-PCR) (Amplicore HCV; Nippon Roche, Tokyo, Japan). HCV core antigen levels were measured using immunoradiometric assays and a cutoff value for a positive result of 20 fmol/l (Ortho HCV Ag IRMA test; Ortho-Clinical Diagnostic). The levels of plasma glucose (normal range, 70–109 mg/dl), serum insulin (≤17 mU/ml), aspartate aminotransferase (AST) (10–40 IU/l), alanine aminotransferase (ALT) (5–40 IU/l), γ-glutamyl transpeptidase (GTP) (female: 7–30 IU/l; male: 7–70 IU/l), ferritin (female: 7–110 mg/dl; male: 24–286 mg/dl), and the platelet count (12.0–34.0 × 10^4 cells/μl) were examined in each patient. The HCV serotype of each subject was determined before 2001. If the HCV serotype was not determined, the HCV genotype was examined (HCV Core Genotype; SRL, Tokyo, Japan). HCV genotype 1b was considered to be serotype I and genotypes 2a and 2b were considered to be serotype II. No other HCV genotype was detected in this study. Insulin resistance was assessed using a homeostasis model assessment of insulin resistance (HOMA-IR). HOMA-IR values were calculated as follows: plasma glucose (mg/dl) × serum insulin (mU/ml)/405. Hyaluronic acid and type IV collagen 7S, which are known to be hepatic fibrosis markers, were examined using a latex bead agglutination assay (LPIA-ACE HA; Mitsubishi Kagaku Iatron, Tokyo, Japan; normal range: ≤50 ng/ml) and a radioimmunoassay (Type IV collagen 7S kit; Mitsubishi Kagaku Iatron; normal range: ≤6.0 ng/ml), respectively.

DNA extraction and real-time PCR allelic discrimination assays

DNA extraction and real-time PCR allelic discrimination assays were carried out as described previously.[19] Briefly, 10 μl whole blood was drawn into an ethylenediaminetetraacetic acid (EDTA)-containing Vacutainer by venipuncture. Genomic DNA was extracted from the buffy coat fraction, which was separated from the blood by centrifugation at 3000 rpm using Mag-Extractor System MFX-2000 (Toyobo, Osaka, Japan) according to the manufacturer's protocol. The *ENPP1* K121Q SNP was examined using PCR and sequence-specific primers. Real-time PCR allelic discrimination assays were designed using TaqMan SNP genotyping

assays (Applied Biosystems, Foster City, CA, USA). Assays were performed to genotype the A→C SNP corresponding to *ENPP1* K121Q using commercially available primers (dbSNP ID: rs1044498; TaqMan SNP genotyping assays ID: C_1207994_20). We also evaluated the rs7566605 genotype located near the *INSIG2* gene.[12] Genotyping of the G→C SNP (rs7566605) was performed with the primers rs7566605-F (AGTAGGGTGAGGAAACCAAATTCTC) and rs7566605-R (CATGACCCCTACCGTCTCTATTTT), and the probes rs7566605-VIC (ACAGAGATGTTA CATCAC labeled with the dye VIC) and rs7566605-FAM (CACAGAGATATTACATCAC labeled with the dye FAM) in a custom TaqMan genomic assay. Briefly, 5 ng DNA was mixed with TaqMan Universal PCR master mix (Applied Biosystems) and allelic discrimination assay mix (900 nM each primer and 200 nM each FAM or VIC-labeled probe). PCRs were carried out in a total volume of 6 or 10 µl in 96-well PCR plates. The PCR conditions were as follows: 50°C for 2 min for contamination control with AmpErase uracil-*N*-glycosylase and 95°C for 10 min to activate the AmpliTaq Gold enzyme, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. Genotypes were assessed using the TaqMan allele-specific assay method and an ABI Prism 7000 sequence detection system according to the manufacturer's protocol (Applied Biosystems). All genotypes were scored using the allelic discrimination program from the ABI software.

Statistical evaluation

The differences in mean values were assessed using Mann–Whitney *U* tests. Fisher's exact tests and χ^2 tests were used where appropriate. Univariate and multivariate logistic regression analyses were also used to determine the factors that significantly associated with viral clearance or viral load. All statistical analyses were performed using STATVIEW 4.5 software (Abacus Concepts, Berkeley, CA, USA) or SPSS version 11.01 statistical analysis software (SPSS, Chicago, IL, USA). *P* values less than 0.05 were considered statistically significant.

Results

Characteristics of the subjects

The clinical characteristics of the study population are shown in Table 1. In this study, 343 subjects were positive for anti-HCV antibodies and the presence of HCV RNA and/or HCV core antigen (HCV carrier group), whereas 116 subjects were positive for anti-HCV antibodies but were negative for both HCV RNA and HCV core antigen (HCV RNA-negative group). The mean age of the subjects was 70 years (range, 42–97 years old), and the mean BMI of the subjects positive for anti-HCV antibodies was 23 kg/m^2 (range, 15.6–33.5 kg/m^2). Although there were no differences in the distribu-

Table 1. Clinical characteristics of subjects positive for antihepatitis C virus (HCV), according to the presence of hepatitis C viremia

Characteristics	HCV carrier[a] (n = 343)	HCV RNA-negative[b] (n = 116)	P value[c]
Age (years)	70.7 ± 9.7	69.6 ± 11.2	0.67
Sex (male/female)	117/226	37/79	0.66
History of alcohol consumption (daily/occasionally/none)[d]	110/23/174	35/7/63	0.83
Past history of BT (yes/no)[d]	50/273	25/83	0.07
HCV core antigen	4871.6 ± 4869.4 (325)	–	–
HCV serotype (I/II)[e]	225/118	–	–
Body mass index	23.1/1/3.0 (286)	23.1 ± 3.3 (93)	0.73
AST (IU/l)	49.4 ± 32.9	26.4 ± 8.6	<0.001
ALT (IU/l)	44.9 ± 38.2	20 ± 10.1	<0.001
γ-GTP (IU/l)	35.0 ± 52.3 (248)	21.6 ± 26.4 (91)	<0.001
PLT (×10^4)	19.1 ± 6.2 (342)	23.8 ± 5.6	<0.001
Tryglyceride (mg/dl)	110.2 ± 57.2 (248)	123.2 ± 59.4 (93)	0.02
Total cholesterol (mg/dl)	170.3 ± 34.7 (248)	193.1 ± 30.8 (93)	<0.001
HbA1c (%)	5.3 ± 0.7 (248)	5.4 ± 1.0 (91)	0.12
Glucose (mg/dl)	97.3 ± 34.4 (273)	95.6 ± 23.6 (88)	0.86
Insulin (µU/ml)	11.4 ± 11.4 (273)	9.3 ± 13.7 (88)	<0.001

Data are shown as means ± SD (number of subjects examined)
BT, blood transfusion; AST, aspartate aminotransferase; ALT, alanine transferase; GTP, guanosine triphosphatase; PLT, platelet count
[a]Positive for HCV RNA or HCV core antigen
[b]Negative for HCV RNA and HCV core antigen
[c]Data were evaluated by χ^2 test, Fischer's exact test, or Mann–Whitney test, as appropriate
[d]Excluding subjects whose history was not available
[e]Including subjects whose HCV genotype was determined even if serotype was undetermined