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ORIGINAL ARTICLE

Local application of hepatocyte growth factor using gelatin hydrogels attenuates noise-induced hearing loss in guinea pigs

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Abstract

Conclusion: Local application of hepatocyte growth factor using biodegradable gelatin hydrogels attenuates noise-induced hearing loss in guinea pigs. *Objectives:* To develop an inner ear drug delivery system using gelatin hydrogels that is capable of a sustained delivery of growth factors to the cochlea. We examined the efficacy of the local application of gelatin hydrogels containing hepatocyte growth factor (HGF) in protecting cochlear hair cells from noise-induced damage. *Materials and methods:* A piece of gelatin hydrogel previously immersed in either HGF or saline was placed on the round window membrane of a guinea pig 1 h after noise exposure (4 kHz octave band noise at 120 dB sound pressure level for 3 h). Auditory function was monitored using auditory brainstem responses (ABRs), and the loss of hair cells was evaluated quantitatively. *Results:* Local HGF treatment significantly reduced the noise exposure-caused ABR threshold shifts and the loss of outer hair cells in the basal portion of the cochleae.

Keywords: *Cochlea, drug delivery, growth factor, protection, hair cell*

Introduction

Sensorineural hearing loss (SNHL) is one of the most common disabilities. However, available therapeutic options are limited to hearing aids and cochlear implants. Therefore, many investigations have concentrated on finding novel therapeutic molecules that could possibly be used in the treatment of SNHL. These studies have discovered several agents that exhibit therapeutic activity against SNHL. Despite such basic research progress, the translation of these basic findings into useful therapeutic clinical agents has yet to be achieved. One considerable obstacle to the development of such clinical applications revolves around the current lack of a safe and effective method for drug delivery to the cochlea. As a way of resolving this, we have developed a new method for local inner ear treatment that uses gelatin hydrogel as the inner ear drug delivery system [1]. Biodegradable gelatin hydrogel has been used previously for the sustained release of proteins or peptides, including growth and trophic factors [2]. We have previously demonstrated the efficacy of gelatin hydrogels in the sustained delivery of brain-derived neurotrophic factor [3] and insulin-like growth factor 1 (IGF-1) [4,5] in animal experiments. In addition, we are currently performing a clinical trial designed to examine local IGF-1 therapy that uses gelatin hydrogels for treating acute SNHL (http://www.kuhp.kyoto-u.ac.jp/~ent/ClinicalTrial/Gel_Eng.html).

Hepatocyte growth factor (HGF) was originally identified as the protein that is responsible for stimulating hepatocyte proliferation [6]. It is present in various cells and is a paracrine cellular growth and morphogenetic factor [7,8]. Hearing impairment caused by aminoglycosides is ameliorated after the transfer of the HGF gene to the inner ear via an

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intrathecal injection of the viral vector [9]. The HGF gene transfer for the treatment of SNHL has been published and patented (US Patent 7390482). Thus, local, sustained application of rhHGF might be effective for the treatment of SNHL and could potentially be approved for clinical applications in the near future.

Previous reports have documented the potential use of gelatin hydrogel for a sustained release of HGF [2,10]. Therefore, based on the previous reported data, we designed the current study to examine the efficacy of using gelatin hydrogels for local rhHGF application to treat noise-induced hearing loss (NIHL) in guinea pigs.

Materials and methods

Experimental animals

A total of 18 male 4-week-old adult Hartley guinea pigs weighing 300–350 g (Japan SLC, Hamamatsu, Japan) served as the experimental animals. Animal care was conducted under the supervision of the Institute of Laboratory Animals at the Kyoto University Graduate School of Medicine. All experimental procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Biodegradable gelatin hydrogels

The biodegradable hydrogels were prepared as described previously [3–5]. Since other studies have analyzed the in vitro HGF release profiles from hydrogels and demonstrated that a hydrogel made with 10 mM glutaraldehyde allows for optimal HGF delivery [2,10], we designed the present study to use the same type of hydrogel.

Noise exposure and drug application

Baseline auditory brainstem response (ABR) thresholds were measured just before the noise exposure. Animals were then exposed to a 4 kHz octave band noise at 120 dB sound pressure level for 3 h in a ventilated sound exposure chamber. Sound levels were monitored and calibrated at multiple locations within the sound chamber to ensure stimulus uniformity.

A 2 mm^3 piece of hydrogel was immersed in 20 µl physiological saline that contained either 1.0 µg/µl rhHGF or physiologic saline alone (control). Under general anesthesia using midazolam (2 mg/kg, intramuscular; Astellas, Tokyo, Japan) and xylazine (2 mg/kg, intramuscular; Bayer, Tokyo, Japan), the piece of hydrogel was then placed on the round window membrane in the left ear of the animals 1 h after the noise exposure ($n = 6$ for each group).

Functional analysis

ABRs were measured to assess the auditory function, with the ABR threshold measurements performed at the 4, 8, and 16 kHz frequencies. ABRs were obtained before and after exposure to the noise, and on days 3, 7, 14, and 21 after the drug application. Animals were anesthetized using midazolam and xylazine and kept warm using a heating pad. Generation of acoustic stimuli and the recordings of the evoked potentials were performed using a PowerLab/4sp (AD Instruments, Castle Hill, Australia). Acoustic stimuli, consisting of tone-burst stimuli (0.1 ms $\cos^2$ rise/fall with a 1 ms plateau), were delivered monaurally through a speaker (ES1spc; Bioresearch Center, Nagoya, Japan) that was connected to a funnel fitted to the external auditory meatus. To record bioelectrical potentials, subdermal stainless steel needle electrodes were inserted at the vertex (ground), ventrolateral to the measured ear (active) and contralateral to the measured ear (reference). Stimuli were calibrated against a ¼-inch free-field microphone (ACO-7016; ACO Pacific, Belmont, CA, USA) connected to an oscilloscope (DS-8812 DS-538; Iwatsu Electric, Tokyo, Japan) or a sound level meter (LA-5111; Ono Sokki, Yokohama, Japan). Responses between the vertex and mastoid subcutaneous electrodes were amplified using a digital amplifier (MA2; Tucker-Davis Technologies, Alachua, FL, USA). Thresholds were determined from a set of responses at varying intensities with 5 dB SPL intervals. Electrical signals were averaged for 1024 repetitions. Thresholds at each frequency were verified at least twice.

Histological analysis

On day 21 after the drug application, animals were deeply anesthetized with midazolam and xylazine and the cochleae were exposed. After removal of otic vesicles, 4% paraformaldehyde in 0.01 mol/l phosphate-buffered saline (PBS) at pH 7.4 was gently introduced into the perilymphatic space of the cochleae. Temporal bones were then excised and immersed in the same fixative at 4°C for 4 h. After rinsing with PBS, cochleae were dissected from temporal bones and subjected to histological analysis in whole mounts. To quantitatively assess the hair cell loss, we examined three regions of the cochlear sensory epithelia that were at a distance of 40–60%, 60–80% or 80–100% from the apex.

Immunohistochemistry for myosin VIIa and F-actin labeling by phalloidin were performed to label the surviving inner hair cells (IHCs) and outer hair cells (OHCs). Anti-myosin VIIa rabbit polyclonal antibody (1:500; Proteus Bioscience, Ramona, CA, USA) was used as the primary antibody, and Alexa-546-conjugated anti-rabbit goat IgG (1:500; Molecular Probe, Eugene, OR, USA) was used as the secondary antibody. Following immunostaining for myosin VIIa, specimens were then stained with FITC-conjugated phalloidin (1:300; Molecular Probe). Specimens were viewed under a confocal microscope (TCS SP2; Leica Microsystems, Wetzlar, Germany). To test the non-specific labeling, the primary antibody was omitted from the staining procedures. Three authors (T.I., T.N., and Y.S.K.) counted the numbers of IHCs and OHCs in 0.2 mm long regions of the apical, middle or basal portions of the cochleae. The average of the values was used as the data for each animal.

Statistical analysis

Overall effects of rhHGF application on ABR threshold shifts were examined using a two-way factorial analysis of variance. When interactions were significant, multiple comparisons with Fisher's protected least significant difference (PLSD) were used for pairwise comparisons. Differences in the IHC and OHC numbers for each region of the cochlea between the rhHGF- and saline-treated cochleae groups were examined using a Student's t test. Values of $p<0.05$ were considered statistically significant. Values are expressed as the mean $\pm$ the standard error.

Results

Auditory function

Time courses of the alterations in the ABR threshold shifts at 4, 8, and 16 kHz after the application of rhHGF or saline are shown in Figure 1. Local application of rhHGF showed a significant effect on the reduction of the ABR threshold shifts at the 16 kHz frequency ($p=0.030$). There was also a significant difference in threshold shifts on day 21 between the rhHGF- and saline-treated animals, as shown by the Fisher's PLSD test ($p=0.045$). No significant differences were found for the threshold shifts between the two groups at 4 or 8 kHz.

Histological protection

Immunostaining for myosin VIIa and phalloidin staining demonstrated partial degeneration of the OHCs in the 60–80% distance regions from the apex

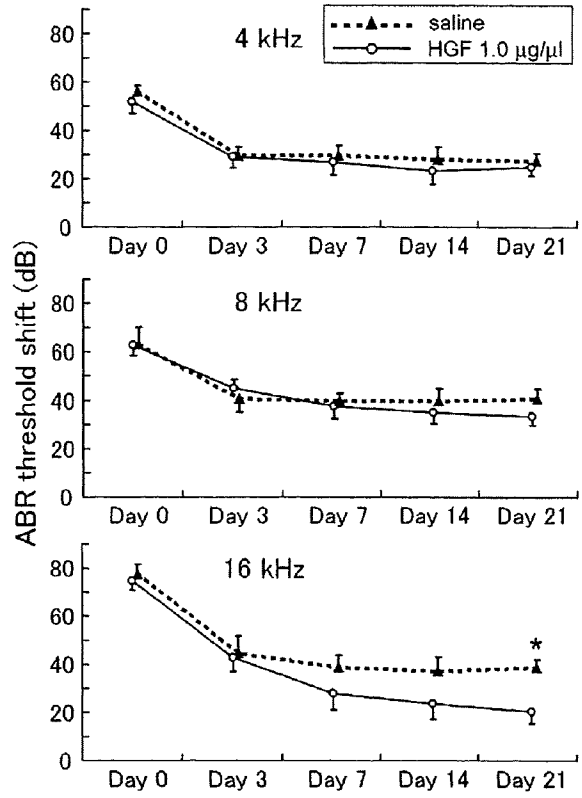

Figure 1. ABR threshold shifts after noise exposure in saline- and HGF-treated animals. An overall effect of HGF application is significant at 16 kHz (two factorial ANOVA, $p=0.030$), not at 4 or 8 kHz. The difference in threshold shifts between saline- and HGF-treated animals is significant on day 21 at 16 kHz. *$p=0.045$, Fisher's PLSD.

in the saline-treated cochleae (Figure 2A). The same region for the 1.0 μg/μl rhHGF-treated cochleae exhibited almost normal morphology (Figure 2B). In both experimental groups, OHC loss was not apparent in the 40–60% or 80–100% distance regions from the apex. IHCs were well maintained in every region of the cochleae in both groups. Quantitative assessments revealed a significant difference in OHC numbers in the 60–80% distance region from the apex between the saline- and rhHGF-treated cochleae (Figure 3, $p=0.003$). No significant differences in OHC numbers were observed in the 40–60% or 80–100% distance regions. There were also no significant differences in the IHC numbers noted in any of the cochleae regions between the two experimental groups.

Discussion

Our findings indicate that local application of rhHGF using biodegradable gelatin hydrogels is effective in the attenuation of OHC damage due to noise trauma, resulting in the reduction of ABR

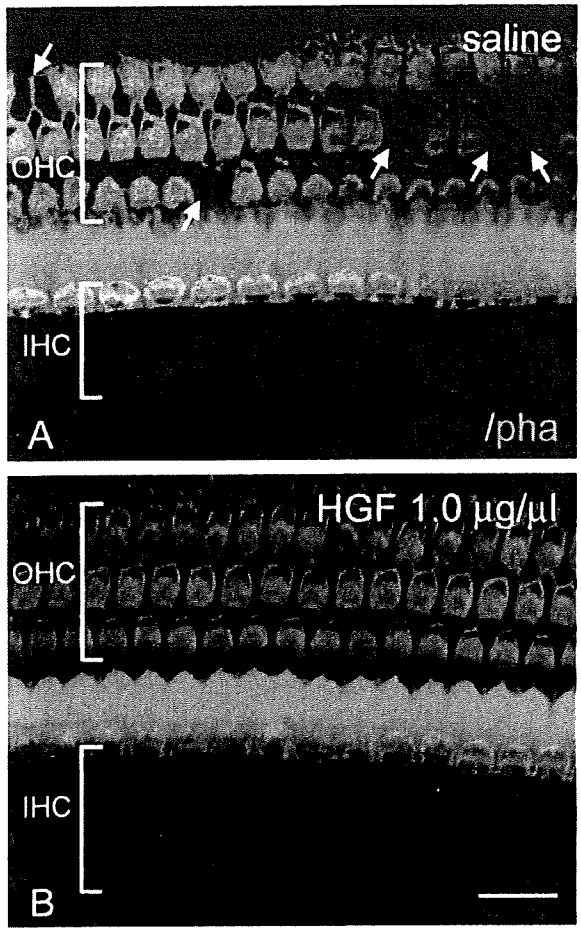

Figure 2. Immunostaining for myosin VIIa (myo) and phalloidin staining (pha) demonstrated loss of outer hair cells (OHC) in the upper basal portion of the saline-treated cochlea (A) and preservation of OHC in that of the HGF-treated cochlea (B). Arrows indicate loss of OHC. IHC, inner hair cells. Scale bar represents 20 μm.

thresholds. ABR measurements demonstrated that post-traumatic local application of rhHGF via gelatin hydrogels had a significant effect on the attenuation of threshold shifts at 16 kHz. Histological analyses demonstrated significant protection of the OHCs in the 60–80% distance from the apex, which is the region responsible for the 10–20 kHz hearing range [11].

Our previous study using IGF-1 indicated that there was a significant reduction of ABR threshold shifts at 4 or 8 kHz [9]. The present findings demonstrated that local HGF treatment caused significant effects at 16 kHz. The spread of the growth factors from the base to the apex of the cochlea occurred by diffusion. Thus, the molecular weights of growth factors could influence the distribution of these factors within the cochlea. The molecular weight of HGF is 69 kDa for the α-subunit and 34 kDa for the β-subunit, while that for

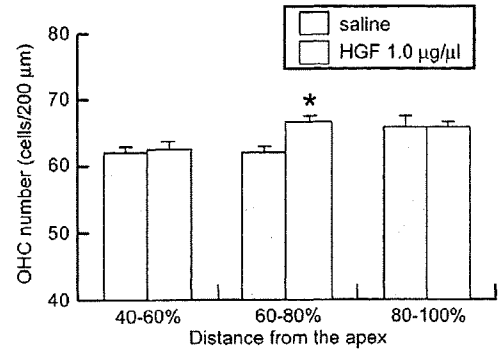

Figure 3. Means of numbers of surviving outer hair cells (OHCs) in saline- and HGF-treated cochleae. In the 60–80% distance region from the apex, the value of HGF-treated cochleae is significantly higher than that of saline-treated cochleae. $*p = 0.003$, t test. Bars represent standard errors.

IGF-1 is 7.6 kDa. Therefore, HGF may be abundantly distributed in the more basal portions of the cochlea as compared with that seen for the IGF-1 distribution.

Previous studies have demonstrated that several agents ameliorate NIHL when they are applied before noise exposure; however, only limited agents including IGF-1 [5] show protective effects by post-exposure administration. Local application of $_D$-Jun N-terminal kinase-1 ($_D$-JNK-1) peptide, an inhibitor of c-Jun N-terminal kinase, 12 h after noise exposure attenuates NIHL [12]. The efficacy of $_D$-JNK-1 peptide has been demonstrated by application via an osmotic mini-pump or a hyaluronic acid gel. In the current study, we used the gelatin hydrogel for sustained delivery of rhHGF into the cochlea. This system may also be utilized for local delivery of $_D$-JNK-1 peptide, because the gelatin hydrogel is suitable for sustained delivery of peptides [1,2]. The efficacy of local $_D$-JNK-1 peptide application via gelatin hydrogels will be evaluated in the near future. Post-exposure administration of edaravone, a free radical scavenger, also rescues cochleae from NIHL [13]. Locally applied edaravone via an osmotic mini-pump can rescue OHCs even when it is applied 21 h after noise exposure. Edaravone is clinically available; however, how to deliver edaravone into the cochlea continuously is an obstacle for clinical use. Gelatin hydrogels are not suitable for sustained delivery of edaravone, because edaravone is not soluble in water [1,2]. Therefore, drug delivery systems that fit for edaravone should be developed before clinical application of local edaravone treatment.

The mechanisms of cochlear hair cell protection by HGF are not well understood. The cochlear hair cells are degraded through the process of apoptosis after exposure to intense noise [14]. Exposure to intense sound causes production of hydroxyl radicals

in the cochlear hair cells [15], which leads to peroxidation of the mitochondrial membrane and the release of cytochrome *c* from the mitochondria to the cytosol. The Bcl-2 family proteins, Bcl-xL and Bak, are produced in the hair cells following noise exposure, and it is the balance of these two proteins that is responsible for the regulation of this process [16]. Predominance of Bcl-xL, which is an anti-apoptotic member of the Bcl-2 family, results in the suppression of the cytochrome *c* release, whereas a predominance of the pro-apoptotic member, Bak, leads to the promotion of the cytochrome *c* release. HGF is known to up-regulate Bcl-xL, which is mediated by the phosphorylation of STAT3 [17]. Therefore, OHCs might be protected against noise through the same pathway. HGF also has anti-oxidant activity [18], which contributes to the protection of cells from apoptosis. This mechanism could possibly involve the same mechanism of protection provided by HGF for the OHCs. In the mechanisms of NIHL, disruption of afferent dendrites attached to IHCs is also involved [19]. Therefore, a regrowth of the nerve fibers and a re-afferentiation of the IHC is important for recovery of hearing after noise trauma. After spinal cord injury, HGF promotes axonal regrowth resulting in functional recovery [18]. This mechanism could also be involved in the significant reduction of ABR threshold shifts observed in the present study. In order to be able to elucidate the HGF distinct mechanism for the protection of auditory systems, further investigations are required.

In conclusion, the present findings suggest that HGF potentially has a role as a protector of OHCs from noise trauma. We are currently in the process of developing a clinical treatment for SNHL that administers local IGF-1 via gelatin hydrogels. Present results strongly suggest that HGF is the next therapeutic candidate that can be used as a local treatment agent via gelatin hydrogels in SNHL clinical trials.

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ORIGINAL ARTICLE

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

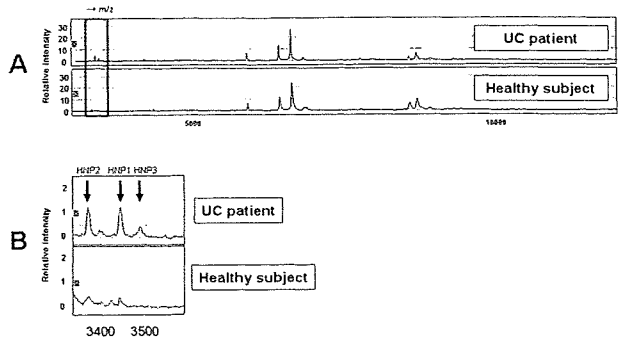

FIGURE 1. Serum proteomics of UC patients and healthy controls using SELDI-TOF/MS. (A) Spectra representing the serum proteins of a patient with UC and a healthy volunteer. The horizontal axis shows a range from 3000 to 10,000 m/z. Significant differences in peak intensities between patients with UC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

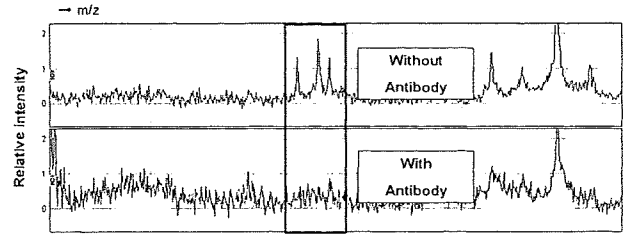

FIGURE 2. Immunodepletion assay using Protein A beads. Anti-HNP 1–3 antibodies (Hycult Biotechnology) were bound to 30 μL of Protein A beads. Sera from UC patients were incubated with these beads. After clearing by centrifugation, 3 μL of each supernatant was analyzed using an NP20 ProteinChip array.

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.

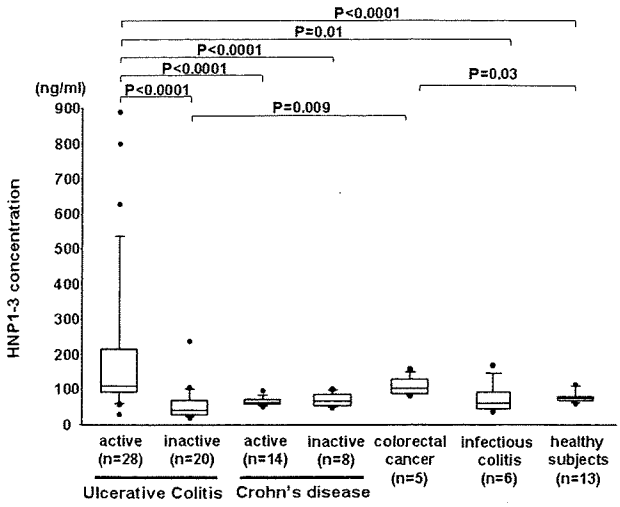

FIGURE 3. Concentrations of HNP 1–3 in the plasma of patients with UC, CD, colorectal cancer, infectious colitis, and healthy controls. Boxes indicate the median ± 25th percentile. The lower bar indicates the 10th percentile and the upper bar indicates the 90th percentile.

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,

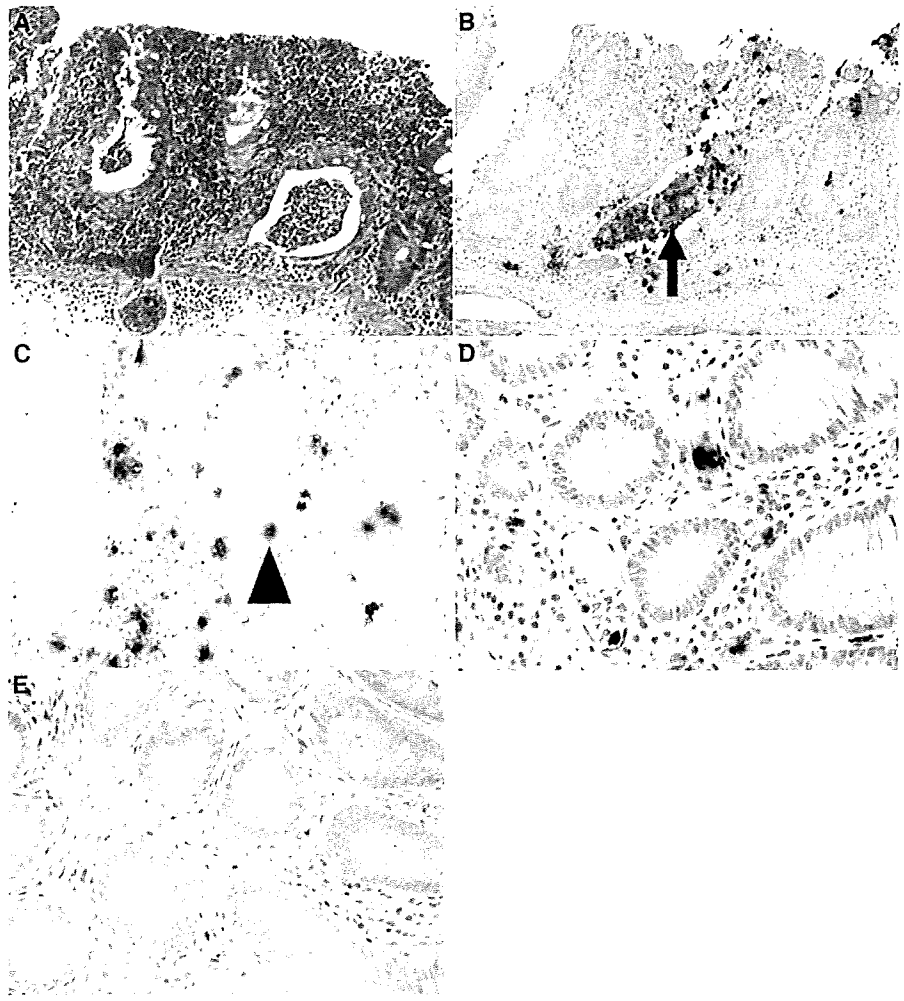

FIGURE 4. Expression of HNP 1–3 in the tissue of patients with active UC or CD and in normal colon tissue. (A) HE staining of colon tissues from patients with UC. (B,C) Immunohistochemical staining demonstrated extensive HNP 1–3 expression in the colon tissues of patients with UC. Many HNP 1–3-positive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

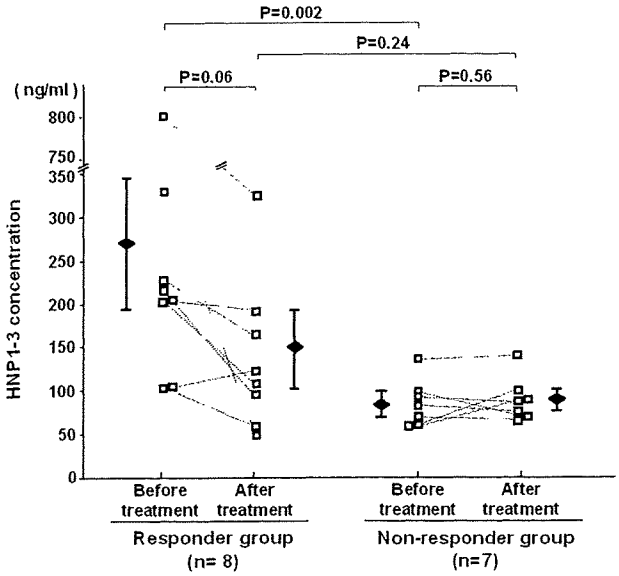

FIGURE 5. HNP 1–3 levels in the responder and nonresponder groups before treatment predicted therapeutic outcomes in UC patients; changes in the HNP 1–3 levels in UC patients in response to treatment are presented. The mean concentration of HNP 1–3 in the responder group before treatment was significantly 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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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Clinical significance of alanine aminotransferase levels and the effect of ursodeoxycholic acid in hemodialysis patients with chronic hepatitis C

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Abstract
Background The natural history of hepatitis C virus (HCV) carriers and the effect of ursodeoxycholic acid (UDCA) have not been fully elucidated among hemodialysis (HD) patients.
Methods Eighty-four anti-HCV antibody- and HCV RNA-positive and 154 anti-HCV antibody-negative HD patients who were retrospectively observed for at least 3 years were analyzed. We investigated the factors associated with thrombocytopenia ($< 1.3 \times 10^5/\mu L$) and decreased platelet count (PLT) (more than 20% decrease during the follow-up period), which were considered to be indicators of hepatic fibrosis. In addition, another 16 HD patients with HCV who received 300 mg/day UDCA orally for at least 6 months were investigated. Changes in alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT) and PLT were assessed.
Results After the 60.3-months mean follow-up period, HCV infection was independently associated with both thrombocytopenia [odds ratio (OR) 2.589] and decreased PLT (OR 2.339) in 238 HD patients. In 84 HD patients with HCV, the average ALT levels ($\geq$ 15 IU/L) during the follow-up period was associated with thrombocytopenia (OR 3.882) and decreased PLT (OR 4.470). In addition, ALT, AST and GGT significantly decreased at 6 months after starting UDCA, but PLT did not change in 16 HD patients with HCV.
Conclusions These results indicate that HCV infection is a risk for thrombocytopenia which should be associated with hepatic fibrosis in HD patients. In addition, the clinical course of ALT levels predicts the progression of thrombocytopenia, and UDCA may effectively lower ALT levels in HD patients with HCV.

Keywords Hemodialysis · HCV · Thrombocytopenia · ALT · Ursodeoxycholic acid

Introduction

Chronic kidney disease (CKD) patients who are on hemodialysis (HD) continue to have a higher prevalence of hepatitis C virus (HCV) infection than the general population [1–4]. The prevalence of anti-HCV seropositivity among patients undergoing regular dialysis in developed countries ranges between 7 and 40% [5–8].

HCV infection in HD patients is usually recognized as asymptomatic and cirrhosis is infrequent in this population [9]. One of the reasons for these findings is that the clinical course of chronic hepatitis C extends over decades and dialysis patients generally have higher morbidity and mortality rates than the general population, making the long-term consequences of HCV infection with HD difficult to establish [6]. However, more recently, the prognosis of HD patients has been improving, so addressing HCV infection in these patients is becoming more important [10].

The strong association between serum alanine aminotransferase (ALT) levels and the fibrosis progression rate or occurrence of hepatocellular carcinoma has been well documented in HCV carriers without HD [11–13]. HD

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patients with persistent HCV infection also have higher ALT levels than those patients without HCV, and ALT values may predict the outcome of HCV infection in patients with HD [14]. In contrast, ALT values are still typically within the normal range in HCV carriers with HD and ALT values are lower in HCV carriers with HD than those without HD. Recently, the risk of liver disease-related deaths is higher in chronic hepatitis C patients with ALT levels closer to the upper limit of the normal range (ULN) (20–29 IU/L) compared to patients with lower ALT levels (< 20 IU/L) [15, 16]. In addition, it has been proposed that the cut-off for serum ALT levels should be reduced by half to screen for hepatic damage in HCV carriers with HD [17]. However, the association between serum ALT levels in those patients with HCV and fibrosis progression has not been fully elucidated.

Platelet count (PLT) is a simple biomarker of hepatic fibrosis in HCV carriers [18]. PLT is also lower in HCV RNA-positive HD patients than in HD patients with HCV RNA-negative serum [16]. In addition, severe hepatic fibrosis is independently associated with thrombocytopenia ($< 1.3 \times 10^5/\mu L$) in HCV carriers with end-stage renal disease [19]. This study evaluated the association of ALT status over a long period and changes in PLT, which was considered an indicator of hepatic fibrosis, in HD patients.

Several trials have examined the efficacy of interferon monotherapy or interferon plus ribavirin combination therapy in HD patients with HCV, and some of these patients obtained a sustained virological response [20]. However, the virological response was limited and side effects may occur more frequently in patients with HD than in those without HD [21, 22]. Therefore, other therapies should be considered for these patients. For chronic hepatitis C patients with or without HD, ursodeoxycholic acid (UDCA) has already been used up to 150 mg/day as routine care in Japan. In addition, the effect of UDCA up to 900 mg/day in HCV carriers who are not undergoing HD was investigated [23], and the use of UDCA up to 900 mg/day was approved for use by chronic hepatitis C patients after April 2007 in Japan. However, the effect of UDCA was not fully elucidated in HCV carriers with HD. Therefore, in this retrospective study we investigated the clinical significance of biochemical markers in the natural course of disease with particular emphasis on PLT and assessed the effect of oral UDCA on serum biomarkers in those patients with HCV.

Materials and methods

Study population

The patients in this study were retrospectively recruited. This study was approved by the Kagoshima University Graduate School of Medical and Dental Sciences. The study population consisted of patients who were on HD in August 2008 and whose data were obtained at least 3 years before August 2008 at 17 HD facilities in Kagoshima, Japan. Their alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), total cholesterol (TC) and PLT were monitored once or twice each month. In 2539 patients, 243 patients were positive for anti-HCV, 143 patients were excluded because they were positive for hepatitis B virus surface (HBs) antigen, they were positive for anti-HCV antibody (anti-HCV) but were not examined for HCV RNA, they had received antiviral treatment or they had hepatocellular carcinoma (HCC). The final population enrolled in this study consisted of 100 patients. Among this cohort of 100 HD patients who were both anti-HCV- and HCV RNA-positive, 84 subjects had not received UDCA and were enrolled in study 1 (HD + HCV Group) and 16 subjects had already received 300 mg/day UDCA for at least 3 months after April 2007 when UDCA up to 900 mg/day was approved for use by chronic hepatitis C patients in Japan and were enrolled in study 2 (UDCA Group). The control subjects in study 1 were 154 HD patients who were anti-HCV-negative (HD Group), and the controls in study 2 were the 84 HD patients among the study 1 population who were both anti-HCV- and HCV RNA-positive but had not received previous treatments including UDCA and were observed until August 2008 (non-UDCA Group). Of the 84 HD patients who were controls in study 2, 2 patients died before November 2008. Blood samples were obtained before routine HD procedures and then were used to assay for ALT, AST, GGT, TC and PLT. The relationship of these markers to PLT and the percent change in PLT were examined, and the percent change in PLT was calculated according to the formula: $\Delta\%PLT = [PLT \text{ (at the end of study)} - PLT \text{ (at enrollment)}]/PLT \text{ (at enrollment)} \times 100$.

Serum HCV markers

Serum anti-HCV and HBsAg were determined using a commercially available third-generation enzyme-linked immunosorbent assay and anti-HBs assay, respectively. For anti-HCV antibody-positive patients, HCV RNA was quantified using the COBAS TaqMan HCV kit (COBAS AmpliPrep/COBAS TaqMan HCV assay, Roche Diagnostics, Tokyo, Japan) during the follow-up period. The serologically defined HCV genotype (HCV serotype) was also determined with a serological genotyping assay kit (Immunocheck F-HCV Grouping, International Reagents Co., Tokyo, Japan). In some patients, the HCV genotype was examined (HCV Core Genotype, SRL, Tokyo, Japan). HCV genotype 1b was included with serotype I, and genotypes 2a and 2b with serotype II. No other HCV genotype was detected in this study population.

Study 1

The HD + HCV Group, which contained 84 HD patients with HCV, was compared to the HD Group, which contained 154 HD anti-HCV-negative patients. We compared the basal characteristics at enrollment and the changes in PLT during the follow-up period between the two groups. In addition, we divided the HD + HCV patients into the following four groups according to the average ALT level of all available ALT levels during the follow-up period: Group A, ALT < 15; Group B, 15 ≤ ALT < 20; Group C, 20 ≤ ALT < 30; and Group D, 30 ≤ ALT Clinical characteristics at baseline or average ALT levels and change in PLT during the follow-up period were compared between these four groups.

Study 2

Sixteen patients with HD and HCV had been treated with 300 mg/day UDCA orally for at least 3 months after April 2007, when UDCA up to 900 mg/day was approved for chronic hepatitis C patients, until August 2008 (UDCA Group). These patients were observed every month for at least 6 months before the administration of UDCA and then monitored for the efficacy of UDCA for more than 3 months until August 2008. Then, these patients were observed for a total of at least 6 months until November 2008. We compared the basal characteristics between the UDCA Group just before UDCA treatment and the non-UDCA Group in May 2008. In addition, the changes in ALT, AST, GGT and PLT during the follow-up period were compared between the two groups. For example, the percent of ALT was calculated according to the formula: %ALT = [ALT (−6, 0, 1, 2, 3 or 6 M]/ALT[0 M] × 100).

Statistical analysis

When appropriate, χ^2 test, Fisher's exact test, Student's *t* test and Mann–Whitney *U* test were used to compare the frequencies or means. Logistic regression models were used for calculating the odds ratios (ORs), 95% confidential intervals (CIs) and *P* values. Statistical analyses were performed using STATVIEW (version 5.0; Abacus Concepts, Berkeley, CA), or SPSS (SPSS Inc., Chicago, IL) software programs. A *P* value less than 0.05 was considered statistically significant.

Results

Demographic characteristics of study 1 subjects

As shown in Table 1, 84 HD patients among the anti-HCV-positive patients were HCV carriers (positive for HCV RNA). One hundred fifty-four HD patients were anti-HCV-negative. On average, the frequency of males, levels of AST, ALT and GGT were higher and TC and PLT were lower at baseline in patients with HCV than those in patients without HCV The follow-up period was also shorter in patients with HCV than those in patients without HCV In contrast, there were no significant differences between the two groups with respect to age and duration of dialysis.

Table 1 Baseline characteristics of hemodialysis patients

	HCV (+)[a]	HCV (−)[b]	*P* value
Number	84	154	
Age (year)	64.4 ± 10.3	62.2 ± 12.5	0.165
Sex (male/female)	54/30	77/77	0.034
Duration of HD (years)	13.5 ± 9.6	11.8 ± 7 4	0.669
Follow-up period (months)	56.8 ± 15.8	62.2 ± 7.9	0.039
HCV RNA (Log IU/mL)[c]	4.9 ± 1.4	–	
Serotype (I/II/undetermined)[c]	59/21/4	–	
AST (IU/L)	19.7 ± 8.5	14.9 ± 6.7	<0.001
ALT (IU/L)	18.5 ± 9.3	13.2 ± 7.1	<0.001
GGT (IU/L)	41.5 ± 43.0	30.1 ± 42.1	0.002
TC (mg/dl)	153.7 ± 41.0	167.1 ± 35.0	0.003
PLT (× 10^5/μl)	1.59 ± 0.53	1.93 ± 0.73	<0.001

Unless otherwise indicated, data are given as the mean ± SD or number of patients

HD hemodialysis, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *GGT* gamma-glutamyl transpeptidase, *TC* total cholesterol, *PLT* platelet count

[a] HCV (+), both anti-HCV antibody and HCV RNA positive

[b] HCV (−); anti-HCV antibody negative

[c] HCV RNA and serotype were examined during follow-up period

Predictors of thrombocytopenia in HD patients

Table 2 summarizes the results of a univariate analysis of factors associated with thrombocytopenia (PLT < 1.3 × 10^5/μl) at the end of study 1 (August 2008) using 9 baseline characteristics in all HD patients with or without HCV. Older age, HCV viremia, elevated AST, ALT, and GGT levels were significantly associated with thrombocytopenia. In addition, a multivariate analysis revealed that HCV viremia was independently associated with thrombocytopenia (Table 2). Furthermore, after the 60.3-month mean follow-up period (mean of HD + HCV Group, 56.7 months; HD Group, 62.2 months), PLT in the HD + HCV Group had decreased (from 1.59 × 10^5/μL to 1.22 × 10^5/μL) significantly compared to that in the HD Group (from 1.93 × 10^5/μL to 1.77 × 10^5/μL) (average Δ%PLT in each patient: −22.4 vs. −5.3%, $P < 0.001$). Variables that were statistically significant by a univariate analysis were further analyzed to identify variables that

Table 2 Univariate and multivariate analyses of variables associated with thrombocytopenia (< 1.3 × 10^5/μl) in HD patients

Variables	Odds ratio	95% CI	*P* value
Univariate analysis			
Age (years)			
<60	1.0		
≥60	1.994	1.141–3.484	0.015
Sex			
Female	1.0		
Male	1.494	0.868–2.571	0.147
Duration of dialysis (years)			
<10	1.0		
≥10	1.065	0.624–1.818	0.816
Follow-up period (months)			
<55	1.0		
≥55	0.727	0.4–1.321	0.296
HCV			
(−)	1.0		
(+)	4.533	2.555–8.043	<0.0001
AST (IU/L)			
<30	1.0		
≥30	7.741	2.095–28.603	0.002
ALT (IU/L)			
<20	1.0		
≥20	3.793	2.017–7.133	<0.0001
GGT (IU/L)			
<50	1.0		
≥50	2.836	1.396–5.758	0.004
TC (mg/dl)			
<150	1.0		
≥150	0.58	0.296–1.135	0.112
Multivariate analysis			
Age (years)			
<60	1.0		
≥60	1.783	0.937–3.394	0.078
HCV			
(−)	1.0		
(+)	2.589	1.317–5.091	0.006
AST (IU/L)			
<30	1.0		
≥30	5.123	0.996–26.339	0.050
ALT (IU/L)			
<20	1.0		
≥20	1.75	0.786–3.896	0.171
GGT (IU/L)			
<50	1.0		
≥50	1.743	0.783–3.88	0.174

Abbreviations as in Table 1

were independently associated with a more than 20% decrease in PLT. As a result, male sex (OR 2.375; 95% CI, 1.319–4.278; $P = 0.004$) and HCV viremia (OR 2.339; 95% CI, 1.295–4.224; $P = 0.005$) were factors that were independently associated with more than a 20% decrease in PLT

Predictors of thrombocytopenia in HD patients with HCV

Table 3 summarizes the results of a univariate analysis of factors associated with thrombocytopenia (PLT < 1.3 × 10^5/μL) at the end of study 1 (August 2008) using 10 baseline characteristics in HD patients with HCV. The patients with HCV and thrombocytopenia had significantly higher frequencies of elevated ALT and GGT levels at baseline. However, age, sex, duration of HD, follow-up period, history of diabetes mellitus (DM), and elevated AST and TC levels were not significantly different between patients with and without thrombocytopenia. In addition, elevated ALT and GGT levels at baseline were not significantly associated with thrombocytopenia in patients with HCV by a multivariate analysis.

On the other hand, a univariate analysis that compared a decrease in PLT of more than 20% with a decrease less than 20% revealed that male sex and elevated ALT levels at baseline were associated with decreased PLT in patients with HCV. A multivariate analysis of two variables that were statistically significant by a univariate analysis also revealed that high ALT levels (ALT ≥ 20 IU/L) at baseline were independently associated with decreased PLT in patients with HCV (OR 3.318; 95% CI, 1.256-8.764; $P = 0.016$).

Furthermore, we divided patients with HCV into four groups according to average ALT levels during the follow-up period. As Table 4 shows, 30, 19, 18 and 17 patients were in Groups A, B, C and D, respectively. Age, duration of dialysis, follow-up period, HCV RNA levels, distribution of HCV serotype, frequency of diabetes mellitus, TC levels and PLT were not significantly different between the four groups. However, serum AST levels and ALT levels at baseline were significantly different, and these levels gradually increased from Group A to D. The distribution of sex was also significantly different and the frequency of males was higher in Groups B, C and D than in Group A. The decreasing rate of change in PLT was significantly higher in Groups B, C, and D compared to Group A (Fig. 1). In addition, the average ALT levels (≥ 15 IU/L) during the follow-up period were independently associated with thrombocytopenia (OR 3.882; 95% CI, 1.257–11.987;