milieu as 3 major bands shown by IEF analysis; Band I (free or unbound to VWF), Band II (not featured), and Band III (bound to high-molecular-weight VWF) as shown in normal plasma (NP). However, our aTTP patients in both patients with good ADAMTS13:AC responders (upper panel) and poor ADAMTS13:AC responders (lower panel) had IEF patterns totally different from those of normal individuals. Among 52 our aTTP patients, 3 poor ADAMTS13:AC responders (Patient 22, 26, and 47) had undetectably low plasma level of ADAMTS13:AG (<0.1% of normal). Thus, we chose Patient 26 as a representative to analyze an interaction between ADAMTS13:AG and ADAMTS13:INH (IgG) in plasma milieu using IEF as shown in Fig. 3.

Table S1. Clinical and laboratory findings in 20 TTP patients; good ADAMTS13:AC responders to PE therapy (ADAMTS13:AC ≥10% on 14th day after PE).

Table S2. Clinical and laboratory findings in 32 TTP patients; poor ADAMTS13:AC responders to PE therapy (ADAMTS13:AC <10% on 14th day after PE).

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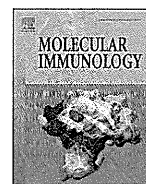

Expression of membrane complement regulators, CD46, CD55 and CD59, in mesothelial cells of patients on peritoneal dialysis therapy

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ABSTRACT

We investigated the expression of membrane complement regulators (CRegs), CD46, CD55 and CD59 in human mesothelial cells, and correlated with clinical background and level of complement (C) activation products in peritoneal dialysis (PD) fluids (PDF) to clarify influence of the C activation system in PD patients. Expression of CRegs was assessed on primary cultures of mesothelial cells (HPMC) harvested from PD fluid of 31 PD patients. Because expression of CD55 but not CD46 and CD59 in mesothelial cells was significantly correlated to value of dialysate-to-plasma creatinine concentration ratio (D/P Cre) ($p < 0.005$) as an indicator of peritoneal function, we focused on analysis of CD55 expression of HPMCs in comparison with levels of C activation products in the PDF of the PD patients, and their background factors. When comparing expression of the CRegs between systemic neutrophils and HPMC, no correlation was observed, supporting that change of CRegs' expression in HPMC was independently occurring in the peritoneum. Expression of CD55 protein in HPMC was closely correlated with expression at the mRNA level ($p < 0.0001$) and was inversely correlated with levels of sC5b-9 ($p < 0.05$), but not C3, C4, IL6 and CA125 in the PDF. Complications of diabetes, usage of icodextrin and residual renal function were not correlated with change of CD55 expression in HPMCs.

Our data show that the process of PD therapy modifies expression of CD55 on peritoneal mesothelium and triggers local C activation. These findings support efforts to modify PD therapy to limit effects on activation and regulation of the C system.

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Abbreviations: Adjusted sC5b-9, C3, C4, IL-6 or CA 125, levels of sC5b-9, C3, C4, IL-6 or CA125 adjusted by protein levels; αSMA, α-smooth muscle actin; Average MFI, value of MFI as the mean MFI of three independent experiments; β2MG, β2-microglobulin; BMI, body mass index; CA-125, carcinogenic antigen 125; CCre, renal clearance of creatinine; C, complement; CReg, membrane complement regulator; CKD, chronic kidney disease; DM, diabetes; DAPI, 49-6-diamino-2-phenylindole-2 HCl; D/P Cre, dialysate-to-plasma creatinine concentration ratio; ELISA, enzyme-linked immunosorbent assay; ESRD, end-stage renal disease; FACS, fluorescence-activated cells sorting; FITC, fluorescein isothiocyanate; HOMC, primary culture of mesothelial cells harvested from human omentum; HPMC, primary cultures of mesothelial cells harvested from PD fluid; HS, normal human serum; Kt/V urea, renal clearance of urea; mAb, monoclonal antibody; MFI, mean fluorescence intensity; pc, polyclonal; PD, peritoneal dialysis; PDF, PD fluid; RRT, renal replacement therapy; ZO-1, zonula occludens-1.

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1. Introduction

Peritoneal dialysis (PD) therapy is an important renal replacement therapy (RRT) across the globe and has many advantages compared with hemodialysis. However, long-term peritoneal dialysis (PD) therapy can be problematic because of impairment of peritoneal function. Peritoneal injuries resulting in failure can be caused by several physical stresses inherent in PD therapy, including exposure to peritoneal dialysis fluid (PDF), catheter trauma and peritonitis.

The complement (C) system plays an important role in the innate immune system and is now recognized as a multi-functional system with roles not only in defence against bacteria but also in viral infection, fertilization and modulation of acquired immunity (Ricklin et al., 2010). It is also clear that pathogenesis of various diseases is related to dysregulation of the C system (Mizuno and Morgan, 2004). In the peritoneum, there is little knowledge about the local C system and its regulation. Recently, we showed that local inhibition of membrane C regulators (CRegs) could trigger peritoneal inflammation and that dysregulated C activation induced severe peritoneal injuries in animal models (Mizuno et al., 2009, 2011, 2012). We also showed that exposure to PDF decreased CRegs expression in rat peritoneum and induced dysregulation of C activation (Mizuno et al., 2013). Although there were a few reports that levels of C proteins and activation products were increased both in serum and in the peritoneal cavity of PD patients and evidence suggests that some C proteins were produced in peritoneum (Dulaney and Hetch, 1984; Tang et al., 2004; Young et al., 1993), there was little information relating to dysregulation of the C system and expression of CRegs in peritoneum of PD patients; a single report examined expression of CD59 (Barbano et al., 1999). We hypothesise that uncontrolled C activation occurs in PD and is associated with development of peritoneal injury; we set out to test whether dysregulation of the C system could be demonstrated in peritoneal mesothelium of PD patients.

In the present study, we explored effects of PD therapy on local C activation/regulation. First, we observed that levels of sC5b-9 were correlated to dialysate-to-plasma creatinine concentration ratio (D/P Cre) as a measure of peritoneal function. To clarify the influence of C regulation in peritoneum of PD patients, we investigated the expression of CRegs on mesothelial cells of end-stage renal disease (ESRD) patients on PD, levels of sC5b-9 as an end-product of C activation and levels of C3 and C4 as C activation components in PDF; the findings clarify influence of PD therapy on the C activation system in PD patients.

2. Materials and methods

2.1. Antibodies

Mouse monoclonal antibodies (mAb), anti-human CD55 (clone 1C6) and anti-human CD59 (clone 1F5), were characterized as previously reported (Hideshima et al., 1990; Shibuya et al., 1992). MAb anti-human CD46 (clone J4-48) was purchased from Abnova (Taipei, Taiwan). MAb anti-human CD55 (clone 1C6) was kindly gifted by Dr. T. Fujita (Fukushima, Japan). Mouse anti-rat CD46 (mAb MM.1) which is expressed only in rat male genital tissues was used as a control IgG1 for immunohistochemistry (Mizuno et al., 2004) and purified mouse IgG (Life technologies, Camarillo, CA) was used as a control IgG1 for fluorescence-activated cells sorting (FACS) analysis. Fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse IgG was purchased from Invitrogen (Camarillo, CA). To characterize primary cultures of mesothelial cells, mouse anti-human cytokeratin 18 (Dako, Glostrup, Denmark), rabbit anti- zonula occludens-1 (ZO-1) (ZYMED Laboratories, South San Francisco, CA), mouse anti-human CD68 (PG-M1; Dako), mouse anti-human CD31 (JC/70A; Dako) and mouse anti-αSMA (Dako) were used. As secondary antibodies, FITC-labeled polyclonal (pc) rabbit anti-mouse IgG and FITC-labeled pc goat anti-rabbit IgG (Life technologies, Camarillo, CA) were used. For detection of C3b deposition, FITC-labeled polyclonal rabbit anti-human C3c was purchased from Dako. For flow cytometry characterisation of neutrophils, PE-labeled mouse anti-human CD16b (IgG2aκ) and an isotype-matched control were used (BD pharmingen).

Table 1
Background of peritoneal dialysis (PD) patients to analyze expression of membrane complement regulators.

		n (%)
Age (years old)	55.6 ± 12.7	31
PD history (months)	32.6 ± 23.7	31
Gender	Male	21 (67.7)
	Female	10 (32.3)
Background disease	DM*	11 (35.5)
	Glomerulonephritis	11 (35.5)
	Nephrosclerosis	5 (16.1)
	Polycystic kidney	1 (3.2)
	Malignant hypertension	1 (3.2)
	Others	2 (6.5)
With or without usage of icodextrin	With	16 (51.6)
	Without	15 (48.4)
Serum C3 level (mg/dL)	90.3 ± 17.6	31
Serum C4 level (mg/dL)	28.7 ± 7.9	31

* Diabetes.

2.2. Profiles of ESRD patients on PD therapy and demographic data

The present experiments were approved by the ethics committees from each Institute of Nagoya University and Daiyukai Daiichi Hospital and Nagoya Kyoritsu Hospital. All patients consented for use of their laboratory data and biological materials. Primary cell cultures were derived from human mesothelial cells harvested from PDF in PD patients or from omentum in non-chronic kidney disease (CKD) patients during laparoscopic operations performed for medical reasons. These patients demonstrated no significant proteinuria; estimated glomerular filtration rate, 80.3 ± 12 mL/min/1.73m^2; and serum creatinine level, 0.73 ± 0.18 mg/dL.

To investigate whether peritoneal function was related to levels of the C activation product sC5b-9 in PDF, we measured levels of sC5b-9 in 50 samples of overnight dwelling PDF from 38 patients on PD therapy (randomly selected from approximately 600 samples stored during past 2.5 years) and correlated with clinical parameters such as dialysate-to-plasma creatinine concentration ratio (D/P Cre).

We investigated expression of CRegs in primary mesothelial cells cultured from omentum or from PDF. Medical background of 31 PD patients in Nagoya University Hospital, Daiyukai Daiichi Hospital and Nagoya Kyoritsu Hospital are shown in Table 1. Medical records provided laboratory data (such as body mass index (BMI), serum levels of albumin, C3, C4, CH50 and β2-microglobulin (β2MG)),and also estimates of renal function (weekly residual renal clearance of creatinine (CCre) and weekly residual renal clearance of urea (Kt/V urea)). Dialysate-to-plasma creatinine concentration ratio (D/P Cre) was obtained by peritoneal equivalent test to evaluate peritoneal function (Twardowski, 1990).

2.3. Cell culture of a human mesothelial cell line

A human mesothelial cell line (Met-5A), derived from pRSV-T-transfection of cells from pleural fluid of noncancerous patients,

was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained according to ATCC guidelines.

2.4. Harvest of omentum specimens and primary culture

Normal mesothelial cells were prepared from omentum tissue harvested from unaffected sites in 3 CKD patients who had early stage (stage I) gastric tumors resected by laparotomy or laparoscopic surgery (Japanese Gastric Cancer Association, 2011). Human omentum mesothelial cell (HOMC) culture was performed as previously reported (Stylianou et al., 1990) with small modifications. Dissected omentum was digested twice sequentially for 15 min at 37 °C with trypsin (0.25%)/EDTA (1 mM) (Gibco®, Thermo Fisher Scientific, Waltham, MA). Released cells were harvested and washed once; cells were resuspended in 4 mL M199 medium with 15% FBS (Sigma-Aldrich), plated into type 1 collagen coated 60 mm dishes (IWAKI, Tokyo, Japan) and incubated at 37 C° for 7 days. We characterized primary cultures of human mesothelial cells by staining with relevant antibodies (Supplementary Fig. A). All in vitro experiments used passage 2 human mesothelial cells except for preliminary studies in Supplementary Figures.

2.5. Primary culture mesothelial cells in ESRD-patients on PD from the dwelling lavages

Human peritoneal mesothelial cells (HPMC) were obtained by centrifugation of overnight-dwell PD fluid taken randomly from clinically stable patients using methods described previously (Bot et al., 2003; Connel and Rheinwald, 1983; Mizutani et al., 2010). These PDF samples were harvested from PD patients who had not suffered with infectious peritonitis for at least 3 months prior to harvesting PDF. Briefly, HPMC were isolated using low speed (200 × *g*) centrifugation, washed with RPMI 1640 (Sigma-Aldrich) and then cultured in RPMI 1640 containing L-glutamine (Sigma-Aldrich) supplemented with 15% FBS (Sigma-Aldrich), insulin/transferrin/selenium A (Invitrogen, Tokyo, Japan), 10^{-5} M 2-mercaptoethanol (Wako, Osaka, Japan) and 400 μg/L Hydrocortisone (Sigma-Aldrich) in humidified air with 5% CO_2 at 37 °C. The cells reached confluence in 7–10 days, and were split two to three times and second passage cells were used for the following experiments.

2.6. Immunohistochemistry for characterization of primary cell cultures and for detection of CRegs in peritoneal tissues

Primary cultured mesothelial cells fixed with Methanol/acetone (v/v = 1/1) (Ito et al., 2000) were stained with mouse anti-cytokeratin, mouse anti-CD68, mouse anti-α-smooth muscle actin (αSMA) or mouse anti-human CD31, followed by incubation with FITC-labeled rabbit anti-mouse Ig. ZO-1 was detected using rabbit anti-ZO-1 followed by incubation with FITC-labeled goat anti-rabbit IgG mixed with DAPI (49-6-diamino-2-phenylindole-2 HCl; 100 ng/mL final concentration; Sigma-Aldrich) for counterstaining of nuclei.

To observe CReg expression in peritoneum, we used de-paraffinated peritoneal sections with well-preserved mesothelial cell layer from a male patient on short term PD therapy. Briefly, paraffin-embedded sections were de-waxed in xylene, re-hydrated and washed in PBS. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide in PBS, and nonspecific protein-binding sites were blocked with normal goat serum. Sections were then incubated with anti-human CD46, CD55 or CD59, followed by polyclonal goat anti-mouse IgG antibody, and horseradish peroxidase-labeled polymer (Histofine® Simple Stain Max-PO (M), Nichirei Biosciences, Tokyo, Japan). Enzyme activity was detected using 3,3′-diaminobenzidine tetrahydrochloride liquid system. Counterstaining was performed with hematoxylin.

2.7. Fluorescence-activated cell sorting (FACS) analysis for expression of CRegs in human peritoneal mesothelial cells and circulating neutrophils

To detect the expression of CD46, CD55 or CD59 in human mesothelial cells, the primary culture cells were firstly incubated with mAb J4-48, 1C6 or 1F5, respectively, or mouse IgG1 as an isotype-matched control, followed by incubation with FITC-anti mouse IgG.

To analyze expression of CRegs in peripheral neutrophils of PD patients, blood of 23 consented patients were taken into EDTA-2K (BD Vacutainer™ blood collection tubes, Becton, Dickinson and Company, Franklin Lakes, MJ) and hemolysis was performed with ACK lysing buffer (GIBCO®) according to the manufacturer's instruction. Briefly, 500 μL of harvested blood and 6 mL of ACK lysing buffer were mixed and left at room temperature for 5 min, then centrifuged at 1300 rpm for 5 min. After removal of the supernatant, the pellet, which mainly contained white blood cells, was suspended in 4 mL of PBS with 0.5% bovine serum albumin (v/v). After another centrifugation at 1300 rpm for 5 min at room temperature, the pellet was adjusted as 0.4×10^6 mL^{-1} and incubated with anti-human CD46, CD55 or CD59 mAb, followed by incubation with FITC-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grobe, PA). To detect neutrophils, samples were incubated with PE-labeled anti-human CD16b or PE-mouse IgG2aκ as a control antibody (data not shown).

To eliminate the effects of dead cells, cells were stained with 2.5 μL of 7-aminoactinomycin D (7-AAD; BD pharmingen™, San Jose, CA) and incubated for 10 min on ice in the dark. The neutrophil populations were obtained by gating based on a plot of FSC/SSC versus a plot of SSC/CD16 (Gopinath and Nutman, 1997). The mean fluorescence intensity (MFI) of CReg was evaluated under FACS analysis on a BD FACS-Canto™ II (Becton Dickinson, San Jose, CA). The experiments were repeated 3 times using independent HMPC for each patient within 8 weeks. The mean fluorescence intensity (MFI) of each CReg expression was evaluated by FACS analysis. Each value of MFI (average MFI) was the mean MFI of three independent experiments for each patient. For reproducibility, we calibrated the cytometer with the Spherotech 8 Peak Validation Beads (BD phramingen™, BD biosciences).

2.8. Polymerase chain reaction and expression of mRNA of CD55 in human mesothelial cells

RNA was prepared from cultured cells using the RNeasy Mini Kit (Qiagen), and first-strand cDNA was synthesized using the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA), according to the manufacturers' instructions. Total RNA (1 μg) was then reverse transcribed. To validate gene expression changes, quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed with an Applied Biosystems Prism 7500HT sequence detection system using Taq-Man gene expression assays for human CD55 (Hs00167090-m1), human C3 (Hs00163811-m1) and 18S ribosomal RNA (4319413E) according to the manufacturer's specifications (Applied Biosystems, Foster City, CA). The thermal cycler conditions were as follows: hold for 10 min at 95 °C, followed by two-step PCR for 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were performed in duplicate. Amplification data were analyzed with Applied Biosystems Sequence Detection Software version 1.4.1 (Applied Biosystems). 18S ribosomal RNA was used as an endogenous control.

2.9. Enzyme-linked immunosorbent assay (ELISA) for measurements of human sC5b-9, C3, C4, IL-6 and carcinogenic antigen 125 (CA-125) in PDF

To measure levels of human sC5b-9 in PDF, MicroVue™ SC5b-9 plus EIA kit (Quidel Co, San Diego, CA) was used. Levels of human C3 and C4 in PDF were measured by Assay Max ELISA kits (Assaypro LLC, St. Charles, MO) and levels of IL-6 and CA-125 in PDF were measured by Quantikine® ELISA (R&D systems®, Minneapolis, MN), respectively. Total protein amounts in PDF were also measured by BCA protein assay reagent (Thermo Fisher Scientific). All ELISA assays and measurement of protein amounts were performed according to the manufacturer's instructions. All samples were measured in duplicate and the mean value was used. Each value was adjusted for total protein level in the PDF according to the following formula:

Adjusted level of sC5b-9 (pg/mg), C3 (μg/mg), C4 (μg/mg), IL-6 (U/mg) or CA125 (pg/mg) in PDF = {(level of sC5b-9 (pg/mL), C3 (μg/mL), C4 (μg/mL), IL-6 (U/mL) or CA125 (pg/mL) in PDF)/(protein level in PDF (μg/mL))} × 1000.

2.10. Functional assay of C activation on human mesothelial cells (HPMC)

To confirm whether a decrease of human mesothelial CRegs expression altered susceptibility to complement attack, we investigated the degree of C activation by normal human serum (HS) on HPMC isolated from both low- and high-expressing patients. Briefly, 2×10^5 HPMCs were plated on type I collagen-coated cover glass (12 mm diameter, Iwaki, AGC techno glass, Shizuoka, Japan) and cultured in 12 well plastic plates (BD Falcon™, BD biosciences, Bedford, MA). HPMC-coated cover glasses were incubated in RPMI 1640 medium with HS or with heat-inactivated HS (56 °C for 30 min) for 60 min at 37 °C (Mizuno et al., 2004, 2013). To detect C3 fragment deposition on mesothelial cells, the cover glasses were fixed with acetone at room temperature and incubated with FITC-labelled pc rabbit anti-human C3c. Cells were double-stained with DAPI (Sigma-Aldrich) to facilitate cell counting. The concentration of HS was adjusted from 0 to 7.5%; the same amount of heat-inactive HS was used as a negative control. Total and C3b-coated cells were counted in 10 randomly selected fields under ×200 magnification, and the C3b deposition was calculated according to the following formula:

$$\text{Deposition of C3b (\%)} = \text{number of C3b-coated cells/total number of cells} \times 100$$

The experiments above were independently repeated three times from different sources of HPMCs.

2.11. Statistical analysis

All values are expressed as the mean ± SD. Statistical analysis was performed by one-factor ANOVA. Correlations were assessed by linear regression. When significant differences were present, data were further analyzed using the Wilcoxon test between two groups. Comparisons between two groups for differences of CRegs expression of HPMC passages, of dwell times, of kinds of PDF, of collection timing of PDF and of C3b deposition were performed using paired Student's *t*-test. Correlation coefficients were calculated by Pearson correlation analysis. *p* Values of <0.05 (5%) were considered to be statistically significant. All analyses were performed using IBM SPSS Statistic 20 software (Armonk, NY).

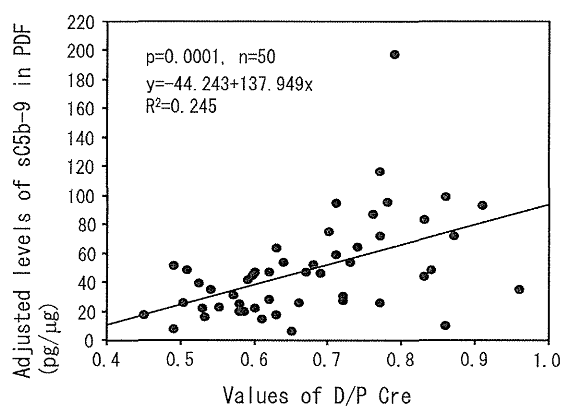

Fig. 1. *Levels of sC5b-9 in peritoneal dialysate (PDF) were correlated with values of dialysate-to-plasma creatinine concentration ratio (D/P Cre) in the PD patients.* In fifty PDF samples, levels of sC5b-9, adjusted for protein levels, were significantly correlated with D/P Cre values.

3. Results

3.1. Correlation between levels of sC5b-9 in PD fluids and D/P Cr in PD patients

We measured levels of sC5b-9, adjusted by protein levels (adjusted sC5b-9), in 50 overnight dwell PDF samples from 38 PD patients randomly selected to investigate correlations of their clinical status. Levels of adjusted sC5b-9 were significantly correlated with values of D/P Cre (Fig. 1), but not with age, gender, diabetes (DM)/non DM and usage of icodextrin (data not shown).

3.2. Expression of CRegs, CD46, CD55 and CD59 is abundant in human mesothelial cells in peritoneum

Expression of CD46, CD55 and CD59 was strongly observed in mesothelium along peritoneal surface and vessels in sub-peritoneal tissues (Fig. 2). The distribution pattern of CD55 and CD59 was similar to that observed in rat peritoneum (Mizuno et al., 2009). Of note, CD46 was not present in rat peritoneum as expression is restricted to male reproductive tissues (Mizuno et al., 2004).

CReg expression was evaluated in human mesothelial cell line (Met-5A cells) and primary cultured mesothelial cells from non-ESRD omentum (HOMCs) of 3 non-CKD patients and from PD fluid (HPMCs) of 31 PD patients. Both HOMCs and HPMCs were positively stained by anti-cytokeratin and anti-ZO-1, but not by anti-CD31, anti-CD68 and αSMA, supporting characteristics of mesothelial cells (Supplementary Fig. A). CRegs CD46, CD55 and CD59, were abundantly expressed in HPMCs, HOMCs and the mesothelial cell line Met-5A cells (Fig. 2), supporting strong expression of CRegs in human peritoneum. Comparison of expression of CRegs between HPMCs and HOMCs showed that expression of CD55 and CD59 in HPMCs was lower than in HOMCs, but expression of CD46 in HPMCs was slightly higher than in HOMCs (Fig. 2H).

3.3. Expression of mesothelial CRegs is independent of dwell time, PD fluid composition or passage number

To investigate stability of CRegs' expression in HPMCs, we compared expression of CRegs on HPMCs harvested from 4-h and overnight PDF from selected ESRD patients; there was no significant difference between these two groups (Supplementary Fig. B3). HPMCs expression of CRegs were not different between neutral dextrose PD fluid and icodextrin PD fluid (Supplementary Fig. B2). HPMCs expression of CRegs was stable between primary, second

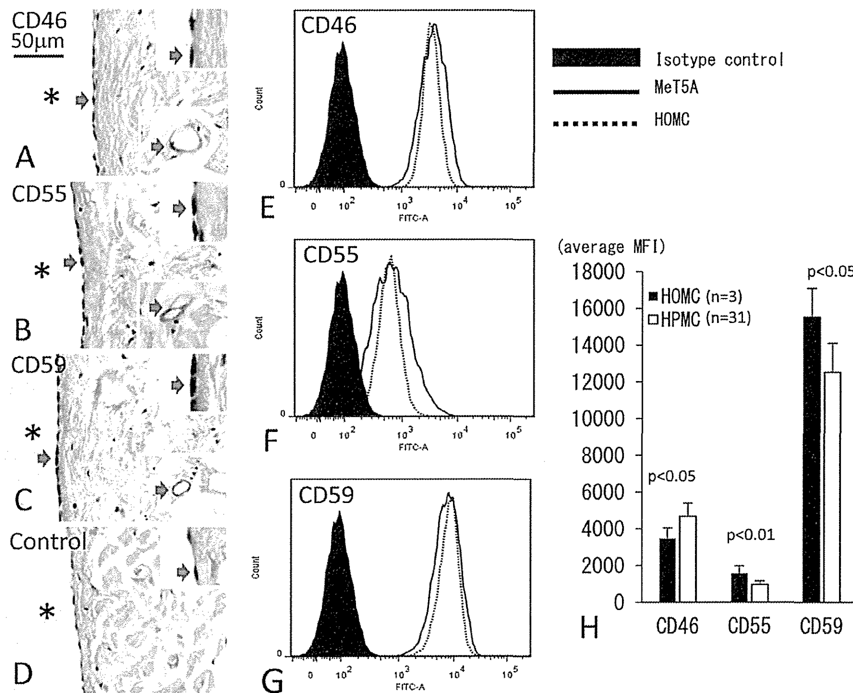

Fig. 2. *Membrane complement regulators CD46, CD55 and CD59, are abundantly distributed in human peritoneal tissues, Met-5A cells and on mesothelial cells from omentum (HOMC) of non-end stage renal disease (ESRD) patients.* Expression of CD46, CD55 or CD59 was analysed on 31 human primary culture of mesothelial cells (HPMCs) of ESRD patients on PD therapy and 3 HOMCs. Frames A, B and C show staining by anti-CD46, anti-CD55 and anti-CD59, respectively. CD46, CD55 and CD59, were abundantly observed in mesothelial cells along the peritoneal surface. Distribution of these CRegs in vessels was as observed in other tissues. Frame D shows negative control stained by iso-type-matched mAb. Blue arrows indicates positive-staining and * shows side of peritoneal surface. Original magnifications are ×630, and scale bar is the upper left corner of (A). Two-times magnified mesothelial cells from the original photos are shown on the upper right side of A, B, C and D. Insets (A, B, C) of the lower right side shows distribution of CRegs in vessels. Frames E, F and G show histograms of expression, CD46, CD55 and CD59 by flow cytometric analysis in Met-5A cells (solid line) and primary cultured mesothelial cells from non-ESRD omentum (HOMC, dotted line), respectively. Expression of CD46, CD55 and CD59 are similar between on Met-5A cells and HOMC. Graph H shows comparisons of CRegs between HOMCs and HPMCs.

passage and third passage cells from 5 selected PD patients (Supplementary Fig. B1). CRegs expression was also consistent at two different time points; the first harvest and a second harvest 6 or 8 weeks later (5 selected PD patients; Supplementary Fig. B4).

3.4. Expression of mesothelial CRegs did not correlate with any clinical laboratory data other than expression of CD55 which inversely correlated with D/P Cre values

There were no statistically significant differences in expression of CD46, CD55 and CD59 in HPMCs between patients grouped according to gender, DM status or PDF type. Expression did not correlate with age, duration of PD therapy, BMI and serum level of albumin. Serum creatinine levels, residual weekly Kt/V and residual weekly CCre as measures of residual renal functions were not correlated with expression of CD55 and CD59 in mesothelial cells. Expression of CD46 in HPMC was weakly correlated with serum level of CCre ($p = 0.038$) and residual weekly Kt/V ($p = 0.041$). There was no significant relationship between serum CH50 or C3 and C4 levels and expression of CD46, CD55 and CD59 in HPMC.

Expression of CD55, but not CD46 and CD59, was significantly inversely correlated with D/P Cre (Fig. 3A–C), therefore we focused on CD55 in the following analysis.

3.5. Levels of sC5b-9, C3, and C4 in PDF inversely correlate with expression of CD55 at the protein and mRNA levels in human mesothelial cells

Levels in PDF of sC5b-9, but not C3 or C4, were significantly correlated with mesothelial cell expression of CD55 protein in the 31 ESRD patients on PD therapy (Fig. 4A–C).

Levels of IL-6 and CA125 in PDF were not correlated with expression of CD55 in mesothelial cells in the 31 ESRD patients on PD therapy (Fig. 4D and E). Of note, the level of C3 in PDF was not

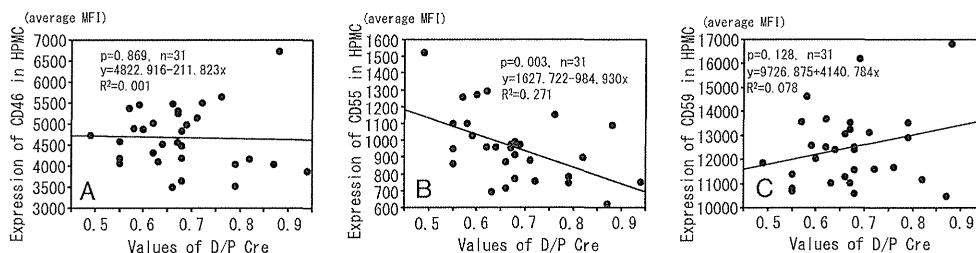

Fig. 3. *Correlation between expression of membrane complement regulators, CD46, CD55 and CD59 and dialysate-to-plasma creatinine concentration ration (D/P Cre).* Graphs A, B and C show the correlation between value of D/P Cre and expression of CD46, CD55 and CD59. D/P Cre and expression of CD55 are significantly correlated.

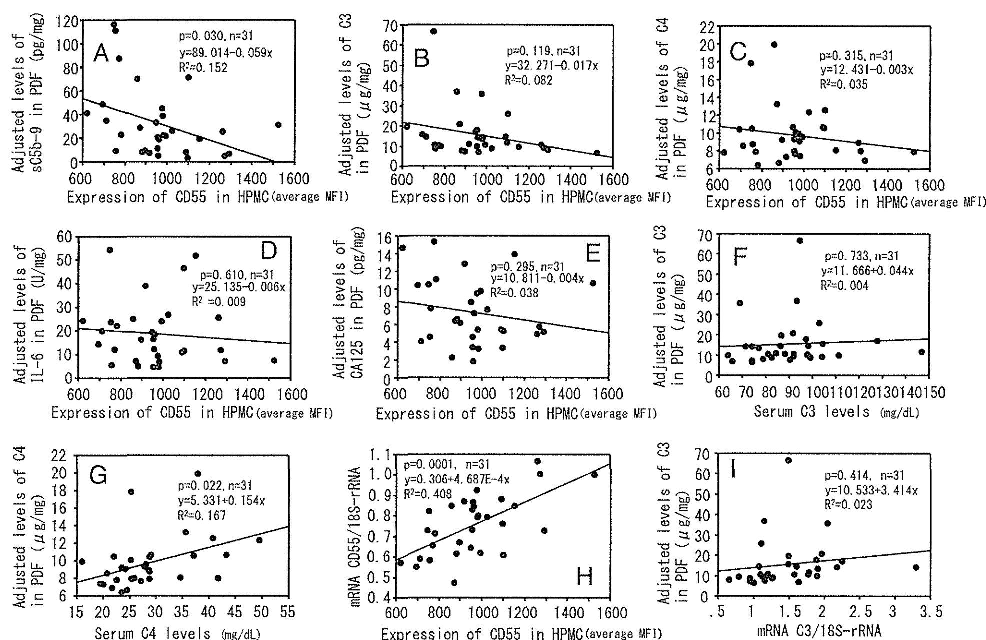

Fig. 4. *Mesothelial expression of CD55 in HMPCs correlated with levels of sC5b-9 in PDF, but not levels of C3, C4, IL-6 and CA-125 in PDF; protein level of CD55 correlated with mRNA levels in HMPCs.* Graphs A, B, C, D and E show correlations of CD55 expression in HMPC for levels of sC5b-9, C3, C4, IL-6 and CA125 in peritoneal dialysate (PDF), respectively. Expression of CD55 in HMPC was inversely correlated with levels of sC5b-9 in the PDF (A). The others were not correlated with expression of CD55 in mesothelium. The correlation between serum and PDF of C3 was not observed (F), but there was a weak correlation between serum and PDF C4 (G). Significant correlation of CD55 expression between protein and mRNA is observed in HMPC (H). Protein level of C3 in PDF did not correlate with mRNA level

correlated with serum C3 level (Fig. 4F) although PDF level of C4 weakly correlated with serum C4 level (Fig. 4G).

When we compared protein expression of CD55 with the mRNA in HPMCs of 31 ESRD patients on PD therapy using RT-PCR, expression of CD55 protein on HPMCs was strongly correlated with level of the mRNA of CD55 ($p < 0.0001$, Fig. 4H). The mRNA level of C3 in HMPCs did not correlate with levels of C3 in PDF (Fig. 4I) or with CD55 expression in HMPCs (data not shown).

3.6. Expression of CD55 on neutrophils (CD16b-positive cells) in circulating blood was not correlated with mesothelial cell expression

To investigate systemic expression of CD46, CD55 and CD59, peripheral leukocytes were harvested from 23 of 31 PD patients and neutrophils were analysed by gating based on a plot of FSC/SSC versus a plot of SSC/CD16b. While CD55 levels on mesothelial cells correlated with D/P Cre, CReg expression on neutrophils from the same patients did not correlate with CReg expression on HPMC (Supplementary Fig. C).

3.7. HPMCs with lower CD55 expression are more susceptible to complement activation

We selected a PD patient with lower HPMCs expression of CD55 (patient 1) and another with high expression of CD55 (patient 2) (Fig. 5). Significantly more C3b deposition was observed on HPMC from the patient with lower expression of CD55 on incubation with 3.5% and 7.5% HS (Fig. 5D).

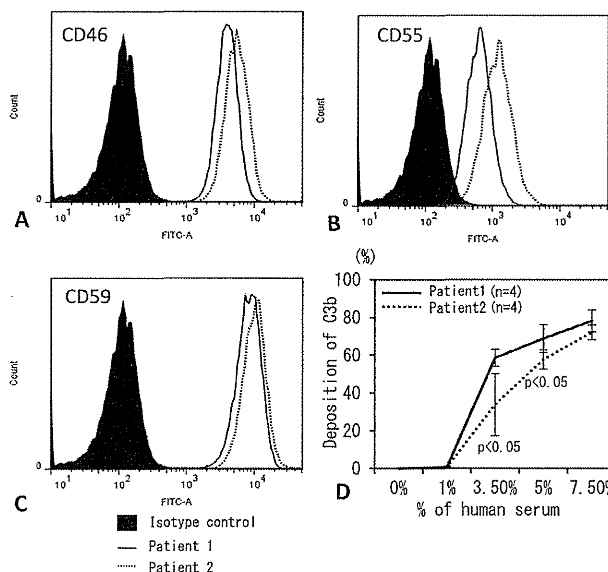

Fig. 5. *Comparison of complement activation in primary cultured mesothelial cells (HPMCs) expressing low and high levels of CD55.* The histograms A, B and C show expression of CD46, CD55 and CD59 in HPMCs, respectively. Graph D shows C3b deposition following incubation with normal human serum and with the heat-inactivated serum using HPMC with lower CD55 expression (patient 1; D/P Cre 0.59) and higher CD55 expression (patient 2; D/P Cre 0.87). In the histograms A, B and C and graph D, solid and dotted lines show patient 1 and patient 2, respectively.

4. Discussion

Because we observed significant correlation between levels of the C activation product, sC5b-9, in PDF and D/P Cre values as a peritoneal function, we hypothesized that peritoneal modifications affecting CReg expression might occur in PD patients. We first confirmed that three CRegs, CD46, CD55 and CD59, are abundantly expressed by mesothelial cells of human peritoneum. Mesothelial expression of CD55, but not CD46 and CD59, correlated with D/P Cre as a predictor of peritoneal function in ESRD patients on PD therapy. Expression of CD55 in circulating neutrophils was not correlated

with D/P Cre, suggesting a local effect in the peritoneum independent of systemic expression of CD55. In mesothelial cells, levels of CD55 protein were strongly correlated with mRNA level of CD55. In a functional study we observed increased C3b deposition on HPMC with lower expression of CD55, supporting the concept that altered CD55 expression might be associated with impairment of regulation of C activation in peritoneal cells. These results suggested that PD therapy might affect expression of the CD55 in peritoneal mesothelium of PD patients accompanied with regulation at the mRNA level, thus the C system might be more easily activated in peritoneum due to decreased control.

Although levels of sC5b-9 in PDF were inversely correlated with expression of CD55 in HPMCs, levels of C3 or C4 in PDF were not. A previous report demonstrating that mesothelial cells produce C3 and C4 in vitro (Tang et al., 2004) supports our observation that C3 and C4 present in the peritoneum might be mainly delivered from mesothelial cells. The large molecular size (>1000 kDa) of sC5b-9 also indicates that it is unlikely to be delivered from the host circulation (Greenstein et al., 1995; Mizutani et al., 2010; Young et al., 1993). These findings suggest that sC5b-9 detected in PDF is produced in the peritoneal cavity and is retained there. Of note, C3 levels in PDF were not correlated with serum levels, although C4 levels weakly correlated in serum/PDF, suggesting the alternative pathway might be the dominant activation route in the peritoneal cavity. Our data indicate that there is no direct correlation between CD55 levels and C3 mRNA levels.

Basic clinical data and residual renal function did not impact mesothelial expression of CD55 and CD59 in peritoneum of PD patients. Levels of IL-6 and CA125 in the PDF did not correlate with expression of CD55 in the HPMC although these parameters in PDF are well-known as valuable markers of mesothelial cells associated with inflammation and cellular activity in PD patients (Hurst et al., 2001; Szeto et al., 2007).

Expression of CRegs is important to maintain homeostasis in the host (Mizuno and Morgan, 2004). Imbalance of activation and suppression of C regulation can trigger pathogenesis in various tissues (Mizuno and Morgan, 2004). Altered CRegs expression has been reported in some injured tissues such as glomeruli (Mizuno et al., 2007) and in tumors such as breast cancer and gastrointestinal tumor (Ikeda et al., 2008; Li et al., 2001; Schmitt et al., 1999). In the peritoneum during PD therapy, it was reported that production of C components was enhanced in mesothelial cells and some modification of CD59 expression was found (Barbano et al., 1999). We previously demonstrated that CRegs played key roles to regulate C activation in normal and injured peritoneum in rats and that decreased expression of CRegs induced under low pH condition was a factor in damage caused by bio-incompatible PDF (Mizuno et al., 2009, 2011, 2012). Current and past reports show that modification of CD55 expression in mesothelial cells is induced by PD therapy, PDF and/or exposure to PD catheters, peritonitis and uremic toxins.

Roles of the C system in the peritoneum may include opsonization to scavenge microorganisms and cell fragments in order to maintain host homeostasis; impairment of C-mediated opsonization exacerbates infectious peritonitis (Celik et al., 2001; Drevets and Campbell, 1991; Leendertse et al., 2010; Tang et al., 2004). Exposure to PDF may impair opsonization as a peritoneal defense mechanism (Kazancioglu, 2009). Possibly, decreased expression of CD55 in mesothelial cells of PD patient is a host defense response to facilitate C activation and opsonization in the peritoneal cavity. Lower expression of CD55 was accompanied by higher peritoneal levels of sC5b-9, a product of C activation in peritoneum. In contrast, expression of another C3 level CReg, CD46, and of the terminal pathway regulator CD59, were not correlated to D/P Cre.

Our results suggest that PD therapy modifies expression of CD55 in mesothelial cells of peritoneum and that CD55 levels are indicative of peritoneal function. Limitations in the present study include the relatively small sample size, focus on stable PD cases and the use of a cross-sectional protocol. Moreover, it remains unclear whether HPMCs directly reflect the status of resident peritoneal mesothelium; we analyzed expression of CRegs in the primary cultured mesothelium because we could not directly estimate CRegs expression in human peritoneum. We could not clarify the causes of altered expression of CD55; we plan future studies that will analyze larger populations to support results and to investigate changes of CRegs according to the time course of PD. In addition, it will be important to clarify which PD factors affect expression of CRegs in mesothelial cells in PD patients.

Conflict of interest statement

CL Harris has an employment contract with GlaxoSmithKline.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2015.02.005.

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ORIGINAL ARTICLE

Efficacy and safety of eculizumab in childhood atypical hemolytic uremic syndrome in Japan

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Abstract
Background Atypical hemolytic uremic syndrome (aHUS) is a severe life-threatening disease with frequent progression to end-stage renal disease (ESRD). Eculizumab, a humanized anti-C5 monoclonal antibody targeting the activated complement pathway, has recently been introduced as a novel therapy against aHUS. We, therefore, investigated the efficacy and safety of eculizumab in Japanese pediatric patients.
Methods We retrospectively analyzed clinical course and laboratory data of the first ten children with aHUS treated with eculizumab nationwide.
Results Seven patients were resistant to plasma therapy and three were dependent on it. Causative gene mutations were found in five patients. Two patients had anti-complement factor H autoantibody. Three patients had a family history of thrombotic microangiopathy (TMA). After initiation of eculizumab, all patients immediately achieved hematological remission and could successfully discontinue plasma therapy. The median periods to normalization of platelet count, lactate dehydrogenase levels and disappearance of schistocytes were 5.5, 17 and 12 days, respectively. Nine patients recovered their renal function and the median period to terminate renal replacement therapy (RRT) was 3 days. However, two patients progressed to ESRD and required chronic RRT at the last observation. No patients had a relapse of TMA under regular eculizumab therapy. No serious adverse events occurred during the follow-up period.
Conclusions Eculizumab is efficacious and well-tolerated therapy for children with aHUS. Although pathogenic mutations could not be detected in five patients, all patients showed immediate normalization of hematological abnormalities, strongly suggesting complement-related aHUS. This prompt hematological amelioration can become an indicator for therapeutic efficacy of eculizumab. However, appropriate indications and optimal duration of the treatment remain unclear.

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Introduction

Hemolytic uremic syndrome (HUS) is defined by microangiopathic hemolytic anemia, thrombocytopenia and acute kidney injury. In children, more than 90 % of cases with HUS are caused by Shiga toxin-producing *Escherichia coli* (STEC) infection. In contrast, atypical HUS (aHUS), accounting for less than 10 % of HUS, is rare and mainly caused by unregulated complement activation via the alternative pathway due to genetic abnormalities. aHUS is known as a severe life-threatening disease which often develops multiple organ damage, and has a very poor prognosis with high mortality rate, frequent progression to end-stage renal disease (ESRD) and high recurrence even after kidney transplantation [1, 2].

In approximately 50 % of patients with aHUS, genetic abnormalities in complement regulatory factors are identified, which include complement factor H (CFH), complement factor I (CFI), complement factor H-related proteins (CFHR), membrane cofactor protein (MCP), complement factor B (CFB), complement 3 (C3), and thrombomodulin (THBD) [1–6]. Unregulated complement activation is often triggered by infection, pregnancy, drugs and surgery in aHUS [1, 2]. For decades, plasma therapy (plasma infusion and exchange) has been the only, first-line and standard treatment against aHUS. In spite of plasma therapy, the disease frequently developed ESRD associated with a high fatality rate [7–9].

In recent years, eculizumab has been used as a successful treatment for both adult and pediatric aHUS patients [9–12]. Eculizumab is a recombinant, fully humanized hybrid IgG_2/IgG_4 monoclonal antibody directly blocking human complement component C5. This drug terminates the activated complement pathway by binding C5 and inhibiting the generation of pro-inflammatory C5a and the lytic C5b-9 membrane attack complex [9]. To date, some reports have demonstrated that eculizumab is effective in pediatric aHUS patients [9, 12–16]. However, the efficacy and safety of eculizumab for Japanese pediatric aHUS patients remain unclear.

In Japan, eculizumab received approval for the indication of aHUS in September 2013. Some Japanese pediatric aHUS patients had already been treated with eculizumab provided by Alexion Pharmaceuticals, Inc on compassionate grounds before the approval. In this nationwide study, we retrospectively investigated the indication, efficacy, adverse events and outcomes of this new therapy for the treated pediatric aHUS patients in Japan.

Materials and methods

Patients

Japanese pediatric aHUS patients less than 18 years of age treated with eculizumab (Alexion Pharmaceuticals, Chesire, CT, USA) between April 2011 and November 2013 were eligible for this study. We defined aHUS according to the diagnostic criteria proposed by the joint committee of the Japanese Society of Nephrology and the Japan Pediatric Society; microangiopathic hemolytic anemia (hemoglobin < 10 g/dL), thrombocytopenia (platelet count <150,000/μL) and acute kidney injury (increased serum creatinine to a level 1.5-fold higher than reference values) with the exclusion of STEC infection and thrombotic thrombocytopenic purpura (TTP) diagnosed with markedly reduced ADAMTS13 activity [17]. During the study period, eculizumab was administered to 13 pediatric aHUS patients in Japan. We excluded three patients from this study owing to bone marrow transplantation-associated aHUS, and analyzed ten patients at seven institutions based on medical records retrospectively. This study was conducted following the principles established in the Declaration of Helsinki and approved by the Institutional Review Board/Ethics Committee of Kyushu University (approval number 25-241).

Evaluation of eculizumab therapy

On the indications of eculizumab therapy, we defined as follows: "refractory to" as failing to achieve thrombotic microangiopathy (TMA) remission after two or more sessions of plasma therapy, and "dependent on" as having any episode of relapsing TMA after the pause or the cessation of plasma therapy. The clinical efficacy of eculizumab was evaluated by the achievement of hematological remission and of the withdrawal from plasma therapy and renal replacement therapy (RRT). Hematological remission of aHUS was defined as normalization of platelet count (>150,000/μL), normalization of lactate dehydrogenase (LDH) levels (under reference values at each institution) and disappearance of schistocytes. Estimated glomerular filtration rate (eGFR) was calculated using the polynomial formula in Japanese children for patients between the ages of 2 and 13 years [18], and the classical Schwartz formula for patients under the age of 2 years [19]. Adverse events associated with eculizumab, outcomes including relapse of TMA and renal injury at the last observation were also evaluated.

Statistical analysis

All data were analyzed with GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Paired analysis was performed using Wilcoxon test. P values <0.05 were considered to be statistically significant.

Results

Patient characteristics

Table 1 shows the characteristics of the ten patients (four males, six females) at aHUS onset. The median age at the onset was 0.95 (range 0.1–13.8) years. Three patients had a family history of HUS. No patients had a history of parental consanguinity. Four patients had episodes of probable triggers of aHUS including respiratory infection, gastroenteritis and vaccination. Serum C3 levels were low (<70 mg/dL) in three patients. The result of hemolytic assay for CFH activity evaluation by incubating patient plasma with sheep red blood cells [20] was enhanced in four patients and mildly enhanced in another three patients. In two patients, anti-CFH antibody was detected. In all patients, the genes responsible for aHUS, which include CFH, CFI, MCP, C3, CFB and THBD, were investigated [20]. Five patients carried potentially causative mutations: previously reported mutations in two (Patients 1, 8) and novel ones in three (Patients 5, 9, 10). Renal biopsy was performed in six patients and all biopsy specimens revealed evidence of TMA histologically.

Post-aHUS onset, prior to eculizumab administration

The baseline characteristics and parameters, and therapies of ten patients before the administration of eculizumab are shown in Table 2. During the course of disease before the administration of eculizumab, plasma therapy had been performed in all patients and eight patients had received RRT. The major indications for eculizumab were critically "refractory to ($n = 7$)" or "dependent on ($n = 3$)" plasma therapy for TMA remission. The median period from aHUS onset to administration of eculizumab was 22 days (range 3–1591 days). All patients received meningococcal vaccine before or after the administration of eculizumab according to the appended paper. When the patients were vaccinated after or less than two weeks before the initiation of eculizumab, they were given temporary antibiotic prophylaxis such as ampicillin [21].

Efficacy and outcomes of eculizumab therapy

The efficacy of eculizumab therapy in ten patients is summarized in Table 2. After the first infusion of eculizumab, the median periods to normalization of platelet count, LDH levels, and disappearance of schistocytes were 5.5 days (range 4–14), 17 days (range 1–93) and 12 days

Table 1 Patient characteristics at aHUS onset

Patient no.	Sex	Age at onset (years)	Probable trigger	Family history	Low C3 levels (<70 mg/dL)	Hemolytic assay[a]	Anti-CFH antibody	Mutation of candidate genes[b]	Renal biopsy
1	F	1.4	URI, flu vaccination	HUS	(−)	(+)	nd	CFH	TMA
2	F	7.6	(−)	CKD	(+)	(+)	(+)	(−)	TMA
3	M	0.4	(−)	Cerebral infarction	(+)	(−)	(−)	(−)	TMA
4	F	10.3	(−)	(−)	(−)	(+)	(+)	(−)	TMA
5	M	3.6	(−)	(−)	(−)	(±)	(−)	MCP	TMA
6	M	0.5	(−)	HUS	(−)	(±)	(−)	(−)	nd
7	F	0.3	Gastroenteritis	(−)	(+)	(−)	(−)	(−)	TMA
8	M	13.8	*Campylobacter jejuni* infection	HUS	(−)	(±)	(−)	C3, CFH	nd
9	F	0.1	*Bordetella pertussis* infection	(−)	(−)	(−)	(−)	THBD	nd
10	F	0.4	(−)	(−)	(−)	(+)	(−)	C3	nd

aHUS atypical hemolytic uremic syndrome, *M* male, *F* female, *URI* upper respiratory tract infection, *C3* complement 3, *CFH* complement factor H, *MCP* membrane cofactor protein, *THBD* thrombomodulin, *TMA* thrombotic microangiopathy, *nd* not done, *CKD* chronic kidney disease

[a] Hemolytic assay; (+) for enhanced hemolytic activity (±) for mildly enhanced hemolytic activity

[b] CFH, CFI, MCP, C3, CFB and THBD were investigated as the candidate genes for aHUS

Table 2 Summary of the eculizumab therapy in ten patients

Treatment before eculizumab therapy	
Plasma therapy; PI and/or PE	10/10 (100 %)
RRT; HD and/or PD	8/10 (80 %)
Antihypertensive agent	8/10 (80 %)
Anticoagulant therapy; rTM	2/10 (20 %)
At the administration of eculizumab	
Indication for eculizumab therapy	
Refractory to plasma therapy	7/10 (70 %)
Dependent on plasma therapy	3/10 (30 %)
Median period from onset to administration	22 days (range 3–1591)
Median age	2.2 years (range 0.2–13.8)
Laboratory data: median	
Hemoglobin	8.3 g/dL (range 4.0–11.6)
Platelet count	10.6 × 10^4/μL (range 1.4–68.6)
LDH	478 U/L (range 216–2695)
Serum creatinine ($n = 5$)[a]	1.10 mg/dL (range 0.28–6.06)
eGFR ($n = 5$)[a]	45.0 mL/min/1.73 m^2 (range 14.2–59.1)
UP/UCr ($n = 7$)	9.8 mg/mgCr (range 2.0–44.6)
Efficacy of eculizumab therapy	
Achievement of hematological remission	10/10 (100 %)
Median period to normalization or improvement	
Platelet count; >15 × 10^4/μL ($n = 6$)	5.5 days (range 4–14)
LDH ($n = 8$)	17 days (range 1–93)
Hemoglobin; ≥11 g/dL ($n = 6$)	49 days (range 6–112)
eGFR; ≥60 mL/min/1.73 m^2 ($n = 8$)	25.5 days (range 7–82)
Median period to disappearance of schistocytes ($n = 9$)	12 days (range 4–49)
Achievement of withdrawal	
Plasma therapy ($n = 9$)	9/9 (100 %)
RRT ($n = 6$)	5/6 (83 %)
Median period to withdrawal	
Plasma therapy ($n = 9$)	0 days (range 0–10)
RRT ($n = 5$)	3 days (range 2–487)
Adverse events	2/10 (20 %)[b]
At the last observation	
Median follow-up period after administration of eculizumab	202 days (range 28–958)
Laboratory data: median	
Hemoglobin	11.8 g/dL (range 9.0–15.6)
Platelet count	28.6 × 10^4/μL (range 17.7–60.4)
LDH	247 U/L (range 144–308)
Serum creatinine ($n = 8$)[a]	0.34 mg/dL (range 0.20–0.47)
eGFR ($n = 8$)[a]	78.6 mL/min/1.73 m^2 (range 63.8–149.6)
ESRD on PD	2/10 (20 %)
Hematuria and proteinuria	3/9 (33 %)[c]
Medication of antihypertensive agent	6/10 (60 %)
Relapse of TMA during eculizumab therapy	0/10 (0 %)
Eculizumab continuation	10/10 (100 %)

PI plasma infusion, *PE* plasma exchange, *RRT* renal replacement therapy, *HD* hemodialysis, *PD* peritoneal dialysis, *rTM* recombinant thrombomodulin, *LDH* lactate dehydrogenase, *eGFR* estimated glomerular filtration rate, *UP/UCr* urinary protein-creatinine ratio, *ESRD* end-stage renal disease, *TMA* thrombotic microangiopathy

[a] Data of patients on dialysis are excluded

[b] Fever, nausea, hair loss and headache ($n = 1$). Rash, wheezing and hypotension ($n = 1$)

[c] One patient was in anuria

(range 4–49), respectively. Plasma therapy was successfully discontinued in all patients, although one patient could not discontinue RRT (Patient 7). Regarding the patients who achieved the cessation of plasma therapy and RRT, the median periods to the withdrawal from plasma therapy and RRT were 0 days (range 0–10) and 3 days (range 2–487), respectively. Figure 1 shows the improvement in parameters of both TMA and renal function after the administration of eculizumab therapy. At the last observation, two patients (Patients 1, 7) had ESRD and were on chronic peritoneal dialysis and three patients (Patients 1, 2, 5) had hematuria and proteinuria. No patients relapsed with TMA after the administration of eculizumab and the regular infusion of eculizumab had been continued for all patients until the last observation.

Adverse events

Adverse events indicating causality in eculizumab infusions were observed in two patients: fever, nausea, hair loss and headache in Patient 2; rash, wheezing and hypotension in Patient 7. As these symptoms were transient and not serious, regular eculizumab therapy was continued. No patients suffered from severe infections including meningococcal infection during the follow-up period.

Discussion

The findings in this nationwide study indicate that eculizumab therapy was effective and well tolerated for Japanese pediatric aHUS patients who were refractory to or dependent on plasma therapy. Our results mostly correspond with the results of previous case reports and clinical trials of eculizumab for aHUS patients [9–16].

Before the era of eculizumab, plasma therapy was the first line and the only therapy for aHUS. Plasma therapy was assumed to be effective by providing normal

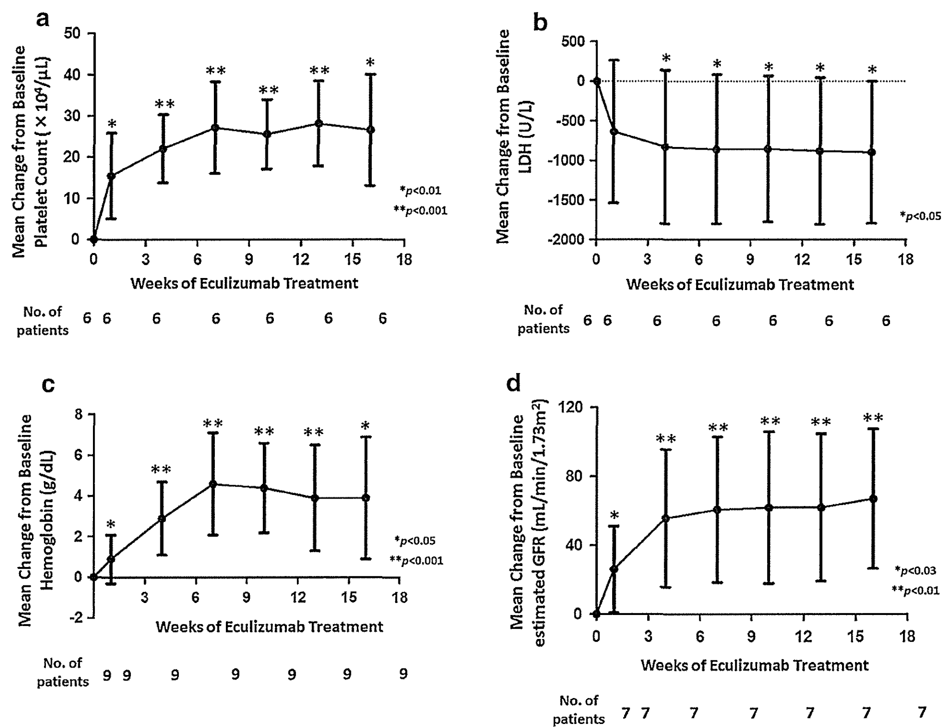

Fig. 1 The improvement of blood and renal parameters in aHUS after the administration of eculizumab therapy. **a** Platelet count ($n = 6$), **b** lactate dehydrogenase concentration ($n = 6$), **c** hemoglobin concentration ($n = 9$) and **d** estimated glomerular filtration rate ($n = 7$)

complement proteins and removing mutant abnormal proteins or auto-antibodies [7–9]. According to the guideline published by the European Paediatric Study Group for HUS in 2009, plasma therapy was recommended to be started as soon as possible (within 24 h) after the diagnosis of aHUS [22]. Nevertheless, it was reported that 29 % of children with aHUS still died or reached ESRD within one year [6]. It was also described that plasma therapy induced complete or partial remission of 63, 25, 57, 88 and 75 % in aHUS patients with CFH, CFI, C3, THBD mutations or anti-CFH autoantibodies, respectively [4]. The analysis of European pediatric aHUS patients mainly treated with plasma therapy in 2009 demonstrated that 11 % of the patients failed to achieve hematological remission and 17 % remained dialysis-dependent at day 33 [23]. These data indicate that the efficacy of plasma therapy in aHUS is limited. Moreover, the incidence of complications in plasma therapy related to vascular access in children is higher than in adults [9].

Eculizumab has become the alternative therapeutic option for aHUS since 2009. This new drug received approval for the indication of aHUS in the United States and Europe in 2012, and in Japan in 2013. The previous case series and the results of clinical trials demonstrated that eculizumab was significantly more effective than conventional plasma therapy for aHUS patients [9–16]. Zuber et al. [9] reviewed the case series of aHUS patients who were treated with eculizumab as a first-line therapy or a rescue therapy. In these cases, all the patients could achieve hematological remission, and 80 % of children and 31 % of adults showed a full recovery to baseline renal function. In a prospective phase 2 clinical trial of eculizumab for patient with aHUS over 12 years of age, who were resistant to or dependent on plasma therapy, eculizumab therapy resulted in improvement of both thrombocytopenia and renal impairment [10].

In our study, after the administration of eculizumab, hematological remission was quickly achieved and plasma therapy was also immediately discontinued in all patients. Notably, platelet count was normalized within several days after the first infusion of eculizumab in most patients. This is one of the most distinctive features of the efficacy of eculizumab [10, 11]. This quick response in the resolution of hematological abnormality strongly suggests that all patients were affected by complement-related aHUS, and it can be the appropriate indicator for therapeutic efficacy of eculizumab. It may also indicate that this therapy can rapidly stop further intravascular thrombus formation by direct blockade of downstream of abnormally activated complement cascade.

On renal outcome, renal impairment was also improved in most patients, although one patient (Patient 7) could not discontinue dialysis and two patients (Patient 1, 7) had developed ESRD at the last observation. Previous literature

suggested that earlier initiation of eculizumab therapy was associated with better improvement of renal function [9–11], although some cases experienced a significant improvement in renal function after RRT of several months [9, 24, 25]. In our study, two of the eight patients who required RRT during the course of the disease (Patients 1, 3) started eculizumab more than one month after the onset of aHUS. Patient 3 could successfully withdraw from RRT 69 days after the administration of eculizumab. Although there is not enough evidence to elucidate the mechanism of such late improvement in renal function as several months after chronic RRT, the extent of renal damage in capillary endothelial cells and interstitium before eculizumab therapy might influence the outcome of renal function. In addition, pediatric patients may have a greater potential to recover renal function than adult patients because each nephron is still in developing during the early stages of life.

There are also other advantages to this new therapy. As eculizumab blocks the downstream complement cascade at the late component C5, it preserves the early components of the cascade which play important roles in phagocytosis and opsonization of microorganisms [9]. In addition, eculizumab is a fully humanized recombinant monoclonal antibody with minimal immunogenicity. Therefore, serious infusion reactions and the development of neutralizing antibodies against the drug are rare [26]. Eculizumab has changed the strategy of management of aHUS dramatically, and it has become not only the substitute for plasma therapy but also the frontline therapy.

However, eculizumab therapy also has some serious adverse events and critical disadvantages. One of the serious adverse events is an increased susceptibility to meningococcal infection. As eculizumab causes late complement pathway deficiencies and inhibits the membrane attack complex formation, patients are at risk of severe encapsulated bacterial infections, especially by *Neisseria meningitidis* [2, 7, 9]. It is well known that individuals deficient in components of the terminal complement pathway are highly predisposed to invasive, often recurrent meningococcal infections [27]. In patients treated with eculizumab for paroxysmal nocturnal hemoglobinuria, the meningococcal infection rate was 0.42 per 100 patients per year [26]. Although annual incidence of this infection, such as bacterial meningitis or sepsis, is only approximately ten patients per year in Japan [28], vaccination against *Neisseria meningococcus* is mandatory for all the patients treated with eculizumab at least two weeks prior to the initiation of the therapy. In this study, all patients were provided with meningococcal vaccine, but they were unapproved and imported because there were no approved vaccines in Japan at that time [29].

If the meningococcal vaccine cannot be given, patients should receive antibiotic prophylaxis until at least two weeks after the vaccination [21]. However, limited-time antibiotic prophylaxis cannot be sufficient to prevent from this critical infection. Tetravalent vaccines against serotypes A, C, Y and W135 are commonly available, but they are not effective for serotype B which is major type found in Japan [30]. Moreover, serum antibody titer acquired by the vaccination can be decreased in several years. It means that we have to keep in mind the risk of meningococcal infection for all the patients treated with eculizumab even after the vaccination.

Furthermore, as the optimal duration of eculizumab treatment has not yet been established, life-long therapy is recommended to prevent relapses at this time in spite of very high-cost therapy [9]. Zuber et al. [9] reviewed five patients treated with a single infusion of eculizumab who experienced relapse mostly within one year and progressed to ESRD. Legendre et al. [10] described that five of the 18 patients who received inappropriate eculizumab doses had subsequent severe complications of TMA. However, it remains unclear whether all patients with aHUS require chronic life-long eculizumab therapy. Ardissino et al. [31] reported that seven of the ten patients could discontinue eculizumab under home monitoring of urine hemoglobin, although three patients with CFH-related aHUS relapsed after discontinuation. Therefore, the risk of relapse in aHUS patients after discontinuation of eculizumab therapy could depend on the genetic background. Better understanding of the genetic correlation and establishing useful biomarkers for monitoring aHUS disease activity are needed to address the optimal duration of this extremely expensive therapy.

Unfortunately, a portion of aHUS patients are resistant to eculizumab therapy. The lack of efficacy of eculizumab may come from a delay in starting eculizumab therapy [9–11], the pathogenesis of aHUS with non-complement-dependent mechanisms such as DGKE mutations [32], or host genetic C5 variants which inhibit the binding of eculizumab to the target epitope [33]. However, there are still some patients in whom the reason of the ineffectiveness is not clear. In this study, Patient 7 could achieve hematological remission but did not have any improvement of renal impairment even though she was administered eculizumab as early as 17 days after aHUS onset. In such cases, unknown factors or mechanisms other than complement activation may be involved in severe renal injury.

An increasing number of children with aHUS will receive this revolutionary treatment in the future. The French Study Group for aHUS/C3G suggested that eculizumab may be considered as a first-line therapy in children with a first episode of aHUS [9]. We should give careful consideration to the indication for eculizumab because some critical issues remain with this new therapy.

However, delayed initiation of eculizumab could also lead to not only irreversible renal impairment but also severe systemic multiple organ damage caused by TMA. As complement investigations take a long time and complement-related mutations have been found only in 50–70 % of patients with aHUS [1–7], identification of a genetic mutation is not necessary for clinical diagnosis and treatment initiation [9, 10]. Therefore, empirical eculizumab therapy for aHUS may be a choice in the early phase of disease after excluding STEC infection and TTP. If patients achieve hematological remission promptly, they could quite likely have aHUS related to abnormalities of complement regulatory factors.

There were some limitations to this study. Firstly, it was a retrospective study and the number of patients enrolled was small. Secondly, most patients were observed for very short periods after the administration of eculizumab. Therefore, we need to confirm the long-term efficacy and outcome of eculizumab therapy in pediatric aHUS. Finally, although immediate and definite efficacy of eculizumab was confirmed in all the patients, genetic mutations of complement-related proteins could not be detected in half of them. Even for the detected mutations, novel ones were not evaluated whether they were definitely causative or not. In fact, Patient 5 carried a novel MCP mutation, but also revealed mildly enhanced hemolytic assay suggesting aberrant CFH function. Patient 10 demonstrated enhanced hemolytic assay, but no CFH-related mutation was detected. In these patients, interacting with some other mutations or SNPs of the complement-related proteins may contribute to the onset of aHUS. We need further investigation to elucidate these discrepancies.

In conclusion, eculizumab is an efficacious and well-tolerated therapy in Japanese pediatric aHUS patients. The appropriate indication for use of this breakthrough therapy and the proper period of the treatment have yet to be determined.

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RESEARCH ARTICLE

von Willebrand Factor-Rich Platelet Thrombi in the Liver Cause Sinusoidal Obstruction Syndrome following Oxaliplatin-Based Chemotherapy

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Abstract

Oxaliplatin-based chemotherapy is widely used to treat advanced colorectal cancer (CRC). Sinusoidal obstruction syndrome (SOS) due to oxaliplatin is a serious type of chemotherapy-associated liver injury (CALI) in CRC patients. SOS is thought to be caused by the sinusoidal endothelial cell damage, which results in the release of unusually-large von Willebrand factor multimers (UL-VWFMs) from endothelial cells. To investigate the pathophysiology of CALI after oxaliplatin-based chemotherapy, we analyzed plasma concentration of von Willebrand factor (VWF) and the distribution of VWFMs in CRC patients. Twenty-three patients with advanced CRC who received oxaliplatin-based chemotherapy with (n = 6) and without (n = 17) bevacizumab were analyzed. CALI (n = 6) and splenomegaly (n = 9) were found only in patients who did not treated with bevacizumab. Plasma VWF antigen (VWF:Ag) and serum aspartate aminotransferase (AST) levels increased after chemotherapy only in patients without bevacizumab. VWFM analysis in patients who did not receive bevacizumab showed the presence of UL-VWFMs and absence of high molecular weight VWFMs during chemotherapy, especially in those with CALI. In addition, plasma VWF:Ag and AST levels increased after chemotherapy in patients with splenomegaly (n = 9), but not in patients without splenomegaly (n = 14). Histological findings in the liver tissue of patients who did not receive bevacizumab included sinusoidal dilatation and microthrombi in the sinusoids. Many microthrombi were positive for both anti-IIb/IIIa and anti-VWF antibodies. Plasma UL-VWFM levels might be increased by damage to endothelial cells as a result of oxaliplatin-based chemotherapy. Bevacizumab could prevent CALI and splenomegaly through inhibition of VWF-rich platelet thrombus formation.

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Introduction

Colorectal cancer (CRC) is the second most common cancer and fourth most common cause of cancer-related death worldwide [1]. Systemic chemotherapy for CRC with modern chemotherapeutic and biological agents has undergone dramatic advances over the last decade; the tumor response rate is now as high as 80%. The most commonly used systemic chemotherapy for advanced CRC is a combination of 5-fluorouracil (5-FU), folinic acid, and either oxaliplatin (FOLFOX) or irinotecan (FOLFIRI) [2, 3]. Recently, monoclonal antibodies against vascular endothelial growth factor (VEGF-A) such as bevacizumab and epidermal growth factor receptor (EGFR-A) such as cetuximab and panitumumab have also contributed to improvements in tumor response rate and survival [4].

Although oxaliplatin-based chemotherapy has benefited patients with CRC, chemotherapy-associated liver injury (CALI), including sinusoidal obstruction syndrome (SOS), has been observed after cytotoxic therapy. Histopathological findings associated with oxaliplatin-induced sinusoidal injury are very similar to those seen in SOS [5]. Rubbia-Brandt et al [6]. reported that sinusoidal dilatation in post-chemotherapy liver resection specimens was strongly associated with oxaliplatin use. In previous studies, SOS following oxaliplatin-based chemotherapy caused splenomegaly and thrombocytopenia [7, 8]. One randomized clinical study showed that bevacizumab in addition to conventional chemotherapy prolonged survival in patients with metastatic CRC compared to conventional chemotherapy alone [9]. The combination of bevacizumab and FOLFOX was associated with a lower incidence of SOS and thrombocytopenia than for FOLFOX alone [10]. In addition, bevacizumab was reported to have a protective effect in the liver of patients treated with oxaliplatin-based chemotherapy for CRC [11].

We have previously reported that plasma ADAMTS13 activity (ADAMTS13:AC) was reduced in patients with SOS, formerly called hepatic veno-occlusive disease (VOD), after hematopoietic stem cell transplantation (SCT) [12]. Subsequently, we found that high molecular weight von Willebrand factor (VWF) multimers (H-VWFMs) were defected during the early post-SCT stage and the number of unusually large VWF multimers (UL-VWFMs) was elevated prior to SOS onset [13]. VWF is synthesized in vascular endothelial cells and released into the plasma as UL-VWFM, the most active form with respect to platelet interaction [14]. In the normal circulation, UL-VWFMs are rapidly degraded into smaller VWFMs by ADAMTS13 under high shear stress conditions [15]. ADAMTS13:AC deficiency increases plasma levels of UL-VWFMs, leading to platelet thrombi under high shear stress conditions and resulting in thrombotic thrombocytopenic purpura (TTP). ADAMTS13:AC is lower in individuals with congenital ADAMTS13 gene mutations (Upshaw-Schulman syndrome) and individuals with acquired autoantibodies against ADAMTS13.

In this study, we investigated plasma VWF in patients with advanced CRC receiving oxaliplatin-based chemotherapy to determine the pathophysiology of CALI. Furthermore, we confirmed the relationship between platelet thrombi and liver injury based on pathological and immunohistochemical findings in liver tissue. We also investigated the protective effect of bevacizumab against liver injury via VWF.

Patients, Materials and Methods

Patients

Twenty-nine patients with CRC who received oxaliplatin-based chemotherapy between February 2011 and August 2013 at Nara Medical University Hospital were included in this study. These patients were treated with the following 3 oxaliplatin-based regimens with or without

bevacizumab/panitumumab: 1) 5-FU and folinic acid plus oxaliplatin (FOLFOX6), 2) capecitabine plus oxaliplatin (CapeOX), or 3) S-1 plus oxaliplatin (SOX). These treatments comprised adjuvant chemotherapy after radical surgery in 14 patients, chemotherapy for unresectable metastatic CRC in 11 patients, and neoadjuvant chemotherapy to treat advanced CRC in 5 patients. Six patients were excluded from this study for the following reasons: tumor progression (n = 3), self-discontinuation (n = 2), and adverse effects of chemotherapy (n = 1).

Ultimately, 23 CRC patients were analyzed in this study (Table 1): 12 patients who received adjuvant chemotherapy, 6 patients who received chemotherapy for unresectable metastatic CRC, and 5 patients who received neoadjuvant chemotherapy. Of these, 13 were treated with an oxaliplatin-based regimen only, 4 were treated with an oxaliplatin-based regimen plus panitumumab, and 6 were treated with an oxaliplatin-based regimen plus bevacizumab (Table 1). CALI was diagnosed based on the following 2 criteria: 1) serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), or total bilirubin (T-Bil) more than 2 times the upper limit of normal range at our hospital (ALT >70 IU/mL, AST >70 IU/mL, T-Bil 2.0 mg/dL) and 2) no other explanation for CALI.

This study was performed with the permission of the ethics committee of Nara Medical University and complied with the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from each patient.

Blood sampling

Plasma samples were collected from CRC patients at 5 or 6 time points during chemotherapy every month, starting at baseline before chemotherapy (month 0) for 4 or 5 months after the initiation of chemotherapy. Chemotherapy was suspended when the patients underwent liver section for metastatic liver cancer. These samples were taken before the starting chemotherapy. Blood was collected in plastic tubes containing 1/10 volume of 3.8% sodium citrate. The plasma was separated by centrifugation at 3,000 g for 15 minutes at 4°C. Aliquots were stored at -80°C until use.

Assays of VWF antigen, activity, and ADAMTS13 activity

Plasma VWF:Ag levels were measured by sandwich ELISA using a rabbit anti-human VWF polyclonal antiserum (DAKO, Denmark) [16]. The value obtained from normal individuals (n = 20) in this assay was 102 ± 33% [16]. To determine the VWF activity, the collagen binding activity of plasma VWF (VWF:CB) was measured using a commercially available kit (VWF-CBA ELISA, PROGEN Biotechnik GmbH, Heiderberg, Germany) according to the manufactures's instructions. ADAMTS13:AC was determined using a commercially available chromogenic act-ELISA kit (Kainos Laboratories Inc., Japan) [17]. The value obtained for normal individuals (n = 55) in the act-ELISA was 99 ± 22%.17 The value of 100% was defined as the amount of VWF:Ag and ADAMTS13:AC in pooled normal human plasma (NP). To evaluate the both levels of VWF and ADAMTS13, the ratio of VWF:CB to ADAMTS13:AC was calculated in this study.

VWF multimer analysis

Multimer analysis of plasma VWF was essentially performed according to the method of Ruggeri and Zimmerman [18], with modifications as reported by Warren et al [19]. The lower gel consisted of 1% agarose (SeaKem® Gold Agarose, Lonza, Rockland, ME, USA) and 10% glycerol dissolved in 50 mmol/L phosphate buffer (pH 8.8) with 0.1% sodium dodecyl sulfate (SDS). The upper gel was prepared with 0.8% agarose (SeaKem® HGT Agarose, Cambrex, Rockland) dissolved in 370 mmol/L phosphate buffer (pH 6.8) with 0.1% SDS. The electrophoresis buffer consisted of 50 mmol/L Tris-glycine buffer (pH 8.3) containing 0.1% SDS. The