登録された腹膜透析患者は386名（男性263名；女性123名）、平均年齢は年齢60.0±14.7歳（男性60.2±14.2歳；女性59.6±15.8歳）、PD歴（治療継続月数）の中央値は10か月（四分位範囲5–20か月）であった。追跡開始時の基礎データについて予後因子として検討を行うため、当初より血液透析を併用している患者を除外し、3か月以上追跡可能である患者302名を対象とし、最長5年間（60か月）の観察研究を行った。

2. 解析

上記解析対象患者の追跡期間中央値は25.5か月（四分位範囲12-42か月）であった。観察期間中腹膜透析を継続し得た患者は148名、血液透析(HD)へ完全移行した患者96名、死亡31名、腎移植13名、転院14名であった。これらの対象患者の総死亡リスク・PD離脱（HD完全移行）リスクについて検討を行った。一部予後の詳細なデータ（死因・PD離脱原因）については2012年末に行った追加調査のデータを参照した。

C. 研究結果

60か月時点における患者生存率は81.0%、PD継続率は49.4%であり、海外や国内の他のレジストリと比較して良好な値であった（学会発表1）。PD離脱の原因として腹膜炎、溢水、透析不全が多くを占め、死因は虚血性心疾患と悪性腫瘍が最多であった（学会発表2）。65歳以上のPD患者はより若年の患者と比較して、患者生存（P=0.0093）、PD継続（P=0.0005）とも有意に予後不良であったが、前者は後者に比較して有意に合併症スコアが高く拡張期血圧が低値、低アルブミン・低リンであった一方、尿量や除水量、腹膜透過性（4時間D/Pクレアチニン値）、透析効率には有意な差は認められなかった。65歳以上の死亡患者の死因は虚血性心疾患と悪性腫瘍が多い（65歳以上 vs. 65歳未満にてそれぞれ4/86 vs. 0/138、3/86 vs. 1/138）。一方でPD関連合併症は差がなく（0/86 vs. 1/138）、離脱の原因についても、PD管理上問題となりやすい溢水（65歳以上 vs. 65歳未満にてそれぞれ3/86 vs. 6/138）・透析不全（3/86 vs. 6/138）・EPSおよびその疑い（0/86 vs. 1/138）・他のPD関連合併症（0/86 vs. 1/138）、いずれも高齢者と若年者で明らかな傾向を認めなかった。しかし、PD離脱の原因において、PD関連再発性腹膜炎（7/86 vs. 4/138）および社会的理由（7/86 vs. 1/138）においては明らかに65歳以上で高頻度であった。腹膜炎年間発症率0.67回以上/患者年（国際腹膜透析会議のガイドライン推奨管理目標18患者月/回未満を逸脱する頻度に相当）の患者においてはPD継続率の明らかな低下が見られた（60ヶ月継続率58.7% vs. 5.2%, P<0.0001）。

D. 考察

海外のレジストリにおいて、PD患者の5年生存率は最近のもので5割～6割程度であり、本研究における予後データはそれらを凌駕するものであるが、導入患者背景の違いや離脱原因の違い（ANZDATAにおいては社会的理由等によるPD中断による患者死亡が約3割を占める）があり一概に結論できない。また海外の予後は経年的に改善しつつある。腹膜炎は本研究の結果からはPD離脱の明らかな予後不良因子であり、PD継続率向上のためには腹膜炎の予防管理が極めて重要である。本邦における透析患者の高齢化はPD患者も例外ではなく、在宅医療の推進に向けて高齢患者のPD治療マネジメントが今後大きな課題となろう。PD離脱の原因におけるPD関連再発性腹膜炎および社会的理由の頻度が高いという解析結果からは、高齢PD患者における手技の補助や見守り、社会的サポートの必要性が示唆される。

E. 結論

国内多施設合同PDレジストリからの予後データは海外や国内の他のレジストリと比較して良好であったが、腹膜炎発症率は明らかにPD離脱のリスクファクターであった。また高齢PD患者において腹膜炎と社会的理由によるPD離脱が目立ち、在宅医療としてのPD継続に必要な施策・因子・介入（リハビリなど）について検討していく必要がある。

F. 研究発表

1. 論文発表

なし

2. 学会発表

森永　裕士、杉山　斉：第21回日本腹膜透析医学会学術集会・総会　教育セミナー『PD up-to-date：Evidence-based overview　患者予後～海外と日本の違い』2015年11月28日　仙台国際センター（仙台市）

杉山　斉、森永　裕士：第21回日本腹膜透析医学会学術集会・総会　レジストリの成果『PDR-CS：多施設合同PDレジストリからの知見』2015年11月28日　仙台国際センター（仙台市）

G. 知的財産権の出願・登録状況

なし

厚生労働科学研究費補助金
（障害者対策総合研究事業（障害者政策総合研究事業（身体・知的等障害分野）））
「腎機能障害者の生活活動性を維持するための
安全で効果的な腹膜透析法の普及のための対策」

研究成果の刊行に関する一覧表レイアウト

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
北村　温美	重炭酸/乳酸緩衝PD液に期待されること	新田孝作	最新透析医療　先端技術との融合	医薬ジャーナル社	大阪市	2016年	274-279

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yumi Sei, Masashi Mizuno, Yasuhiro Suzuki, Masaki Imai, Keiko Higashide, Claire L Harris, Fumiko Sakata, Daiki Iguchi, Michitaka Fujiwara, Yasuhiro Kodera, Shoichi Maruyama, Seiichi Matsuo, Yasuhiko Ito	Expression of membrane complement regulators, CD46, CD55 and CD59, in mesothelial cells of patients on peritoneal dialysis therapy	Molecular Immunology	Jun:65(2)	302-309	2015年
Chieko Hamada, Kazuho Honda, Kunio Kawanishi, Hirotake Nakamoto, Yasuhiko Ito, Tsutomu Sakurada, Yudo Tanno, Toru Mizumasa, Masanobu Miyazaki, Misaki Moriishi, Masaaki Nakayama	Morphological caracteristics in peritoneum in patients with neutral peritoneal dialysis solution	J Artif Organs	18(3)	243-250	2015年

Marina Asano, Makoto Mizutani, Yasuko Nagahara, Koji Inagaki, Tetsuyoshi Kariya, Daijiro Masamoto, Makoto Urai, Yukihiro Kaneko, Hideaki Ohno, Yoshitsugu Miyazaki, Masashi Mizuno, Yasuhiko Ito	Successful treatment of Cryptococcus laurentii peritonitis in a patient on peritoneal dialysis	Internal Medicine	54(8)	941-944	2015年
Akihito Tanaka, Masashi Mizuno, Yasuhiro Suzuki, H Oshima, Fumiko Sakata, Hideaki Ishikawa, Saori Tsukushi, Yasuhiko Ito	Calcified amorphous tumor in the left atrium of a patient on long-term peritoneal dialysis.	Internal Medicine	54(5)	481-485	2015年
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伊藤恭彦、鈴木聡	腎代替療法（透析・移植）の適応と療法選択	日本医師会雑誌	143巻　第11号	2364-2369	2015年
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INTERNAL MEDICINE

□ CASE REPORT □

Successful Treatment of *Cryptococcus laurentii* Peritonitis in a Patient on Peritoneal Dialysis

Marina Asano[1], Makoto Mizutani[1], Yasuko Nagahara[1], Koji Inagaki[1], Tetsuyoshi Kariya[1], Daijiro Masamoto[1], Makoto Urai[2], Yukihiro Kaneko[2,3], Hideaki Ohno[2], Yoshitsugu Miyazaki[2], Masashi Mizuno[4] and Yasuhiko Ito[4]

Abstract

A 32-year-old man on peritoneal dialysis (PD) was hospitalized for seven days due to fever. A diagnosis of yeast-like fungal peritonitis was made by Gram staining. The patient was started on intravenous micafungin and oral fluconazole therapy following removal of the PD catheter. A fungal pathogen was isolated from the peritoneal fluid and identified as *Cryptococcus* species. Based on antifungal susceptibility testing, the treatment was changed to voriconazole and continued for 3 months. A genetic analysis identified the isolate as *Cryptococcus laurentii* (*C. laurentii*). This patient was diagnosed with *C. laurentii* PD-related peritonitis and was successfully treated with voriconazole and removal of the PD catheter.

Key words: *Cryptococcus laurentii*, peritoneal dialysis, voriconazole, catheter removal

(Intern Med 54: 941-944, 2015)
(DOI: 10.2169/internalmedicine.54.3586)

Introduction

Peritonitis is a major and potentially serious complication of peritoneal dialysis (PD) therapy and is one of the important reasons for withdrawal from PD (1). Although the most commonly identified pathogens are bacteria, fungal peritonitis is very serious and catheter removal is strongly recommended immediately following fungal identification by microscopy or culture (2). *Candida* species are reportedly the most frequently isolated fungi in PD-related peritonitis patients (3). Cryptococcal peritonitis is unusual and, to our knowledge, only 12 cases have been reported previously (4-7).

Cryptococcus neoformans infection in immunocompromised hosts is the most common of the cryptococcal infections. Other *Cryptococcus* species have traditionally been considered to be non-pathogenic (8). However, the incidence of infection due to these organisms, such as *Cryptococcus laurentii* (*C. laurentii*) and *Cryptococcus albidus* (*C. albidus*), has increased over the past four decades (8, 9). We know of two reported cases of peritonitis in PD patients caused by *C. laurentii* (10, 11). We herein report a case of peritonitis caused by *C. laurentii* in an adult patient on PD. To our knowledge, this is the first reported case of *C. laurentii* PD-related peritonitis that was successfully treated by voriconazole and the removal of the catheter.

Case Report

A 32-year-old man with IgA nephropathy had been on continuous ambulatory peritoneal dialysis (CAPD) since age 29. At age 30, CAPD was changed to combination PD and hemodialysis (HD) therapy. The PD bag exchange protocol was as follows: 2.5% glucose-based solution (Dianeal-N PD-2 2.5%™; Baxter, Tokyo, Japan) 2 L×3/daytime and icodextrin solution (Extraneal™; Baxter, Tokyo, Japan) 2 L/overnight. To change PD fluid (PDF) bags, the patient used a sterile connecting UV flash device (Clean Flash®; Baxter). He was not treated with any immunosuppressive therapies,

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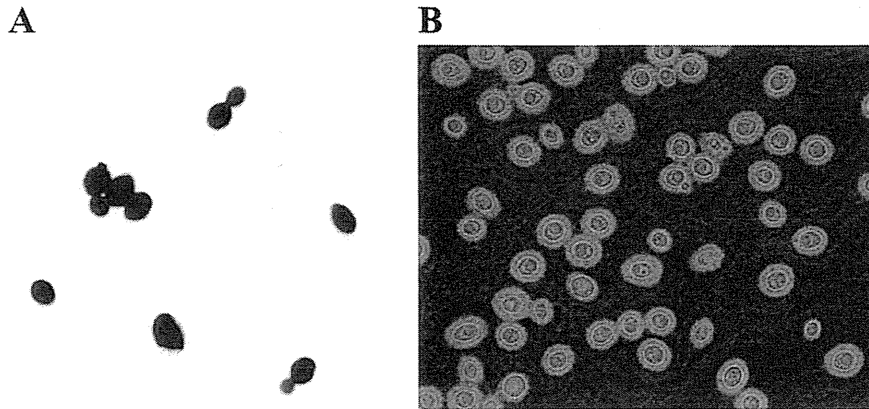

Figure 1. Gram stain (A) and India ink stain (B) of centrifuged sediment of the peritoneal effluent. (A) Yeast cells with large Gram-positive budding structures were identified in the peritoneal dialysis fluid by microscopy. (B) India ink staining of the isolate showed capsule formation around the cells.

Table. Antifungal Susceptibility Testing Results of *C. laurentii* Isolated from Peritoneal Fluid

Antifungal agent	MIC
Amphotericin B	0.25
Flucytosine	>64
Fluconazole	8
Itraconazole	0.25
Miconazole	1
Micafungin	>16
Voriconazole	0.25

The Testing was Performed according to the CLSI-M27-A3 Guidelines

and a serological test for human immunodeficiency virus (HIV) was negative. The patient had never previously experienced peritonitis before this episode and had not taken any antibiotics for more than six months.

He presented to our hospital complaining of fever for the past seven days. His vital signs were as follows: blood pressure, 137/82 mmHg; pulse, 78/min; respiratory rate, 16/min; and temperature, 37.3℃. On physical examination, no abdominal tenderness was found. The clinical laboratory data at admission were as follows: peripheral white blood cell (WBC) count, 4,400 cells/mm^3 (neutrophils, 69%; lymphocytes, 12.7%; eosinophils, 8.8%); blood hemoglobin level, 14.2 g/dL; and C-reactive protein level, 4.0 mg/dL. The serum cryptococcal antigen test (Eiken-Latex agglutination test; Sero direct®, Tokyo, Japan) was negative. A Tenckhoff catheter was in place in the left lower quadrant and did not appear to be infected. When PDF exchange was performed at the hospital, the peritoneal fluid was noted to be cloudy. The WBC count in the first cloudy PDF was 3,500 cells/mm^3 (neutrophils, 60%; lymphocytes, 26%; eosinophils, 14%), indicating peritonitis. Gram staining of the centrifuged PDF sediment was performed, and yeast cells with large Gram-positive budding structures were identified by microscopy (Fig. 1A). Blood and sputum cultures collected at admission were negative. These findings suggested fungal peritonitis, and administration of intravenous micafungin and oral fluconazole was initiated. Although the number of WBCs in the effluent tended to decrease with antifungal treatment, the WBC counts remained steady at 1,000/mm^3. The peritoneal catheter was removed on admission day 7 according to the recommendation of the International Society for Peritoneal Dialysis (ISPD) guidelines, although there was no sign of infection around the cuffs and cultures of the tip of the catheter were negative. A fungal pathogen grew from the initial PDF, and the isolate was identified as a *Cryptococcus* species by a commercial laboratory 10 days after admission. In addition, India ink staining of the isolate showed capsule formation around the cells, consistent with the identification of a *Cryptococcal* species (Fig. 1B). Based on the antifungal susceptibility testing results (Table), the treatment was altered to the oral administration of voriconazole at a dose of 400 mg/day. The trough level of voriconazole was 0.35 μg/mL, which is lower than the recommended dose in the guidelines (12); therefore, we increased the dose of voriconazole to 600 mg/day. The patient's CRP levels improved and he was discharged on admission day 18. He has been in good condition for more than one year after completing 3 months of antibiotic therapy. Further identification of the isolate was subsequently performed by the National Institute of Infectious Diseases (Tokyo, Japan), and the isolate was identified as *C. laurentii* by sequencing the internal transcribed spacer (ITS) and D1/D2 regions of the ribosomal RNA, as previously described (13). The clinical course is summarized in Fig. 2.

Discussion

Fungal peritonitis in PD patients is a serious complication, leading to death in approximately 25% of episodes (14, 15). Although the majority of cryptococcal infections are due to *C. neoformans* (16), the incidence of infection due to other *Cryptococcus* species, such as *C. laurentii*

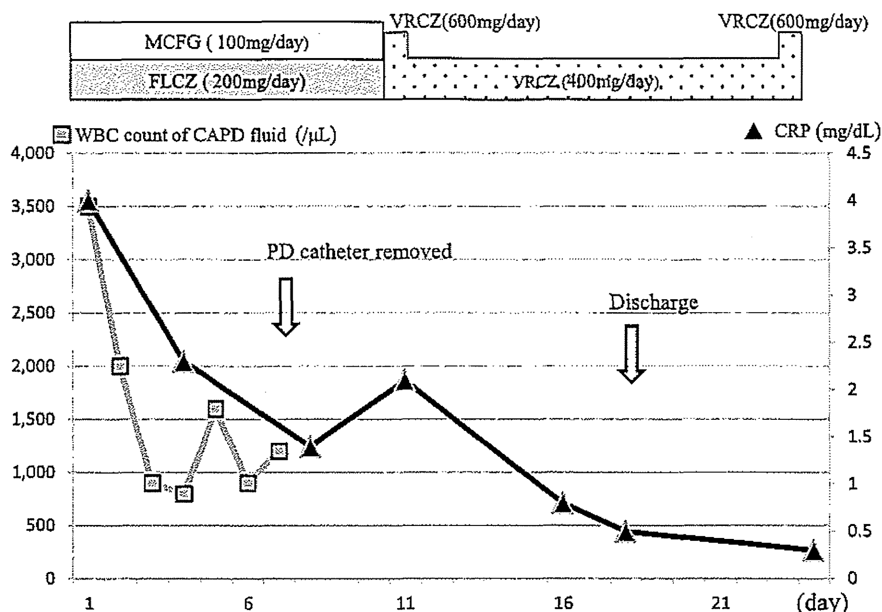

Figure 2. The patient's clinical course. The left Y axis indicates the WBC count in the CAPD fluid (/μL) and the right Y axis indicates the CRP level (mg/dL). MCFG: micafungin, FLCZ: fluconazole, VRCZ: voriconazole

and *C. albidus*, has increased over the past four decades (8, 9). There have been reports of lung, eye and cutaneous infections, meningitis, and fungemia caused by *C. laurentii* and *C. albidus* (8, 9).

A review of the cryptococcal PD-related peritonitis literature was performed by Yinnon et al. (4, 5). This report included 10 cases of cryptococcal peritonitis during CAPD, and 2 of these 10 patients died. Cultures of samples yielded *C. laurentii* in 2 cases, and *C. neoformans* in 6 cases, but the *Cryptococcus* species of the remaining 2 cases were not stated. Therefore, to the best of our knowledge, only 2 cases of peritonitis in PD patients caused by *C. laurentii* have been reported (10, 11). The PD catheters were removed in these 2 cases, resulting in successful treatment and survival (10, 11). Both were adolescent females, and neither was on immunosuppressive therapy. The natural habitats of *C. laurentii* are unknown (8, 9), and therefore we could not precisely identify the infection route for the current patient. However, these previous reports and our present case suggest that an indwelling PD catheter may be a risk factor even without significant immunosuppressive therapy.

Primary resistance to fluconazole and flucytosine has been reported in *Cryptococcus* species other than *C. neoformans* (17), although the interpretative clinical minimum inhibitory concentration (MIC) breakpoints in *C. laurentii* have not been determined. In the current case, the isolated *C. laurentii* had low MICs for itraconazole, amphotericin B and voriconazole, while the MIC for fluconazole was relatively high and the MIC for flucytosine was over the upper limit (Table). As far as we could determine, there is no previous report of the use of voriconazole to treat cryptococcal peritonitis in general, or specifically *C. laurentii* infection. In the previous reports, *C. laurentii* infections were successfully treated by combinations of amphotericin B, fluconazole and flucytosine, or intravenous amphotericin B alone (8). However, the use of intravenous amphotericin B results in poor peritoneal bioavailability (18). The intraperitoneal administration of amphotericin B causes chemical peritonitis associated with severe pain (18). Conversely, dose adjustment was not necessary when voriconazole was administered to patients on PD therapy (19). In addition, *in vitro* activities of voriconazole, posaconazole and fluconazole against 237 *C. neoformans* isolates were assessed by Pfaller et al. (20) who reported that voriconazole was more active than fluconazole. Furthermore, the ISPD guideline states that voriconazole is effective against fungal peritonitis, but there is no statement indicating that itraconazole is effective (2). Considered together, this information suggests that voriconazole is an effective agent for the treatment of cryptococcal peritonitis. Based on these data and information, we selected voriconazole to treat the current patient.

Experience regarding the optimal duration of treatment for *C. laurentii* infections is limited due to the limited number of reported cases and lack of controlled trial data. However, it was reported that a PD patient with *C. neoformans* peritonitis died after nine weeks of treatment with antifungal drugs, including amphotericin B; at the postmortem examination, cryptococcosis in the lung, spleen and brain was revealed (5). Our patient was successfully treated by 3 months of voriconazole. Further accumulation of data from successfully-treated cases will inform future decisions regarding selection of the most suitable antifungal agents and the optimal duration of treatment for *C. laurentii* PD-related peritonitis.

The authors state that they have no Conflict of Interest (COI).

Acknowledgement

We would like to thank A. Okawara, M. Nagi, and Y. Dantsuji for their valuable technical assistance. This work was partly supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Ministry of Health, Labour and Welfare of Japan.

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INTERNAL MEDICINE

□ CASE REPORT □

Calcified Amorphous Tumor in the Left Atrium in a Patient on Long-term Peritoneal Dialysis

Akihito Tanaka[1], Masashi Mizuno[1,2], Yasuhiro Suzuki[1,2], Hideki Oshima[3], Fumiko Sakata[1,2], Hideaki Ishikawa[4], Saori Tsukushi[4] and Yasuhiko Ito[1,2]

Abstract

A 66-year-old woman with an 11-year history of peritoneal dialysis (PD) for diabetic nephropathy and renal failure exhibited a movable tumor in the left atrium on echocardiography. Tumor resection was performed due to the difficulty in diagnosing the tumor and the future risk of heart failure and embolization. Light microscopy showed a calcified amorphous tumor (CAT), a rare intracardiac mass characterized by the presence of a pedicle and diffuse calcification. An increased calcium-phosphate product level was suspected as an etiology, although degeneration, inflammation and/or mineral balance disorders may also induce the development of CAT. We herein report the first known case of CAT in a PD patient.

Key words: calcified amorphous tumor, peritoneal dialysis, bone mineral disease

(Intern Med 54: 481-485, 2015)
(DOI: 10.2169/internalmedicine.54.2967)

Introduction

A calcified amorphous tumor (CAT) is a non-neoplastic cardiac tumor characterized by the presence of a pedicle and diffuse calcification that was originally described in 1997 (1). The pathological features of CAT include acellular/hypocellular tumor components with abundant fibrous tissue and nodular calcium deposits (1). Although the etiology remains unclear, degeneration, focal chronic inflammation and/or mineral balance disorders may be involved. Since its original description, a few cases of CAT have been reported in patients with end-stage renal disease (ESRD) under hemodialysis (HD) (2-6). However, no reports have previously described CAT in patients on peritoneal dialysis (PD). We herein report the first case of CAT in a PD patient.

Case Report

A 66-year-old woman with ESRD secondary to diabetic nephropathy was referred to our hospital. She had been maintained on PD for the past 11 years and had since experienced two episodes of PD-related peritonitis, at nine years and three months prior to the current admission. Other than these episodes, no marked infectious events had been noted, including periodontitis, dental caries or episodes of bacteremia. The patient had started PD with a prescription comprising 1.5 L of 1.5% glucose solution (Dianeal-N PD-2 1.5%™, Baxter, Tokyo, Japan; Table 1) three times in the daytime and once overnight, with good control of calcium and phosphate metabolism as well as body fluids. In 2007, a decreased urine volume resulted in volume overload, and the PD recipe was changed three times (Fig. 1). Finally, the PD regimen was as follows: 1.5 L of 2.5% glucose solution (Dianeal-N PD-2 2.5%™, Baxter) three times in the daytime and 1.5 L of icodextrin solution overnight. The dialysate-to-plasma creatinine concentration ratio (D/P creatinine) on peritoneal equilibration tests and the efficacy of dialysis (weekly creatinine clearance and Kt/V) are summarized in Table 2. Despite our best efforts, it was difficult to control the phosphate level, and the amount of oral phosphate binder, including calcium carbonate, was increased until 2010 (Fig. 1). In January 2009, the administration of ci-

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Received for publication March 26, 2014; Accepted for publication July 14, 2014
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Table 1. Prescription Medication and Ionic Concentration of Peritoneal Dialysis (PD) Solution on the Day of Admission

Medication		
Cinacalcet hydrochloride		25 mg/2 days
Alfacalcidol		0.25 μg/day
Calcium carbonate		1,500 mg/day
Sevelamer hydrochloride		2,250 mg/day
Nifedipine		40 mg/day
Telmisartan		80 mg/day
Vildagliptin		50 mg/day
PD solution	Dianeal-N PD-2 (glucose solution)	Extraneal (icodextrin solution)
Na^+ (mEq/L)	132	132
Ca^{2+} (mEq/L)	3.5	3.5
Mg^{2+} (mEq/L)	0.5	0.5
Cl^- (mEq/L)	96	96
Lactate (mEq/L)	40	40

nacalcet hydrochloride was started due to a persistently high serum level of intact parathyroid hormone (iPTH). In 2008, echocardiography was performed to assess the cardiac function (white arrow in Fig. 1), which was found to be within the normal range, with no apparent intracardiac masses. However, high serum levels of corrected calcium and phosphate continued for approximately three years, from 2007 to 2010 (Fig. 1). The corrected calcium-phosphate product level was largely maintained at less than 50 mg^2/dL^2 until 2007 then subsequently increased, reaching >70 mg^2/dL^2 in 2009 and 2010. This exacerbation appeared to be caused by a decrease in the urine volume as well as poor control of the patient's diet. In 2011, a routine echocardiography scan (black arrow in Fig. 1) disclosed a hyperechoic movable mass adhering to the mitral valve. We recommended surgical treatment based on a perceived risk of embolism (7, 8); however, the patient initially declined to undergo surgical intervention since she remained asymptomatic. Following our repeated recommendations, she finally consented to surgery and was referred to our hospital. Due to her long history of PD, an arteriovenous fistula on the left forearm was created prior to referral to our hospital. However, the patient had no other past medial history or history of anticoagulant or antiplatelet treatment. The hemoglobin A1c level [National glycohemoglobin standardization program (NGSP) value] had been largely controlled between 6.0% and 7.0% until a few months before admission for resection of the cardiac tumor. The details of the medications prescribed on referral to our hospital are shown in Table 1.

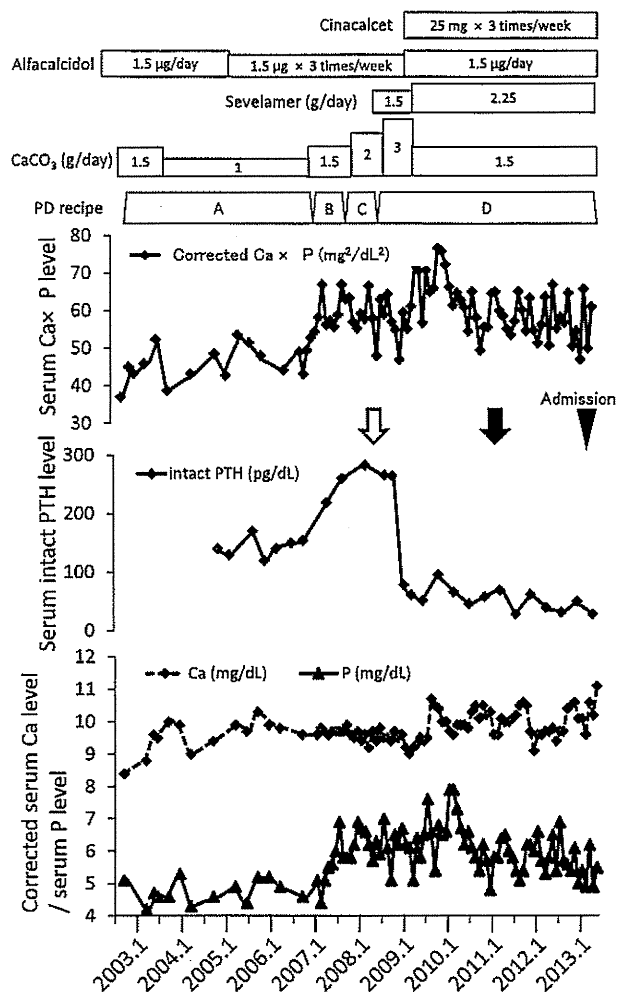

Figure 1. Trends in the serum levels of calcium (Ca), phosphate (P) and intact parathyroid hormone (iPTH) following the administration of phosphate binders and the peritoneal dialysate regimen. This figure shows the clinical course and laboratory data for calcium and phosphate metabolism starting in 2002, the beginning of peritoneal dialysis (PD) therapy. The administration of cinacalcet hydrochloride (cinacalcet), alfacalcidol, phosphate binders, such as calcium carbonate ($CaCO_3$) and sevelamer hydrochloride (sevelamer), is shown in the top bars of the figure. The doses are indicated in the frames. The arrows show the timing of the echocardiographic examinations and the arrowhead indicates the timing of admission for surgery. The PD recipe was changed as follows:A) 1.5 L of 1.5% ×3 times in the daytime and once overnight. B) 1.5 L of 1.5% ×4 times in the daytime and once overnight. C) 1.5 L of 1.5% or 2.5% ×4 times in the daytime and 1.5 L of icodextrin solution overnight. D) 1.5 L of 2.5% ×3 times in the daytime and 1.5 L of icodextrin solution overnight.

At the time of referral to our hospital, the patient remained asymptomatic. On admission, a physical examination revealed clear respiratory sounds and a slight cardiac murmur during systole on chest auscultation (Levine II/IV around the intersection of the midclavicular line and fifth intercostal space). The abdomen was soft with exit of the PD catheter placed in the lower right abdomen. The exit site was clear. The patient's body temperature was 36.6°C, with a blood pressure of 137/66 mmHg and heart rate of 60 beats/min with a sinus rhythm. The clinical laboratory data for the blood and serum were as follows: white blood cell count, $4.6\times10^3/\mu L$; red blood cell count, $340\times10^4/\mu L$; hemoglobin, 9.0 g/dL; hematocrit, 27.9%; platelet count, $15.2\times10^4/\mu L$; C-reactive protein, 0.08 mg/dL; total protein, 6.2 g/

Table 2. Summary of D/P Creatinine of Peritoneal Equilibration Test and of Dialysis Efficacies

Year	D/P Creatinine	Kt/V (L/week)	Creatinine clearance(L/week)
2004	0.60	1.58(Dia)+1.37(Res)	34.23(Dia)+47.15(Res)
2011	0.63	2.04(Dia)	45.16(Dia)
2012	0.65	2.12(Dia)	43.53(Dia)

D/P: dialysate-to-plasma creatinine concentration ratio, Dia: dialysis, Res: residual renal function. Residual renal function was lost in 2011 and 2012.

dL; albumin, 3.3 g/dL; glutamic oxaloacetic transaminase, 10 IU/L; glutamic pyruvic transaminase, 6 IU/L; γ glutamyl transpeptidase, 20 IU/L; total cholesterol, 114 mg/dL; high-density lipoprotein cholesterol, 39 mg/dL; low-density lipoprotein cholesterol, 51 mg/dL; choline esterase, 271 IU/L; total bilirubin, 0.3 mg/dL; lactate dehydrogenase, 294 IU/L; alkaline phosphatase, 702 IU/L; creatinine kinese, 121 IU/L; blood urea nitrogen, 76.0 mg/dL; creatinine, 9.83 mg/dL; sodium, 131 mEq/L; potassium 3.4 mEq/L; uric acid 7.6 mg/dL; corrected calcium, 10.82 mg/dL; phosphate, 4.3 mg/dL; glycosylated hemoglobin (NGSP), 8.2%; iPTH, 28 pg/mL; activated partial thromboplastin time, 34 seconds (97.7% compared to healthy controls); and prothrombin time (PT), 12.9 seconds (103.0% compared to healthy controls). The data obtained from a blood gas analysis (room air) were as follows: pH, 7.397; pO_2, 68.5 mmHg; pCO_2, 40.9 mmHg; $cHCO_3$, 24.7 mmHg; and actual base excess, 0.4 mmol/L. The electrocardiographic findings remained within the normal limits. Chest radiography showed enlargement of the heart, with a cardiothoracic ratio of approximately 60%. On admission, simple thoracoabdominal computed tomography (CT) showed severe calcification of the aorta far from the level of bifurcation of the renal and coronary arteries; however, no significant ectopic calcification was noted around the subcutaneous soft tissues or joints. In addition, echocardiography confirmed a hyperechoic movable mass adherent to the mitral valve (Fig. 2a, b). The detailed echocardiographic results are shown in Table 3. On day 3 after admission, excision of the intracardiac tumor was performed. Intraoperatively, the tumor was seen to arise from a calcified area on the posterior wall of the left atrium, with gelatin-like material adhering around the tumor (Fig. 2c). The maximum diameter of the resected tumor was approximately 1 cm (Fig. 2d). A culture of the gelatin-like material yielded negative results, while a pathological examination of the tumor revealed a hypocellular, fibrous mass without evidence of malignancy, although the lesion was accompanied by severely calcified lesions (Fig. 2e, f). The final diagnosis was therefore CAT. The patient was subsequently maintained on HD in order to accurately control her body fluids for approximately one week after the operation, after which she quickly recovered. She was returned to PD therapy in accordance with her strong request on day 12 after admission and was discharged from the hospital on day 20.

Discussion

CAT is a rare cardiac tumor first proposed in 1997 by Reynolds et al. based on 11 cases (1). The background factors of CAT cases have been shown to involve various diseases, including malignancy, systemic lupus erythematosus and ischemic heart disease, with ESRD reported in one case (1).

Although the etiology of CAT remains unclear, a thrombotic state has been suggested to induce the development of CAT in association with hypercoagulation and/or degenerated thrombosis (1). Disorders of calcium and phosphate metabolism are also considered to be potential causes of CAT (2). Although a small number of cases of CAT have been reported in ESRD patients on HD (2-6), no cases involving ESRD patients on PD have been reported. We herein provided the first description of the onset of CAT in an ESRD patient on long-term PD.

ESRD patients usually exhibit bone mineral disorders, and mitral annulus calcification (MAC) and tumoral calcinosis have been reported to be complications of such patients, with increasing corrected calcium-phosphate product levels, hyperparathyroidism and low turnover of bone (9, 10). In the present case, the patient had received PD therapy for 11 years, and high calcium-phosphate product and serum iPTH levels were observed prior to the formation of CAT (Fig. 1). Therefore, the increased serum corrected calcium and phosphate levels and iPTH value may have facilitated the development of CAT. In addition, the serum phosphate level is consistently high in patients on PD, whereas it temporarily decreases during every HD session. Patients on PD may therefore be more affected by an increased calcium-phosphate product level than patients on HD. However, the relationships between CAT formation and the calcium-phosphate product level or duration of dialysis remain unclear. In PD patients with a high level of serum calcium, the use of a PD solution with a low calcium concentration appears to improve the calcium balance and may have been a suitable choice in the present case at the previous hospital when the serum level of corrected calcium was high. However, the improve in both the serum calcium and phosphate levels was not sufficient to persuade the patient to change from a PD solution with a standard calcium concentration to a solution with a low calcium concentration, according to a physician from the previous hospital. In addition, the patient

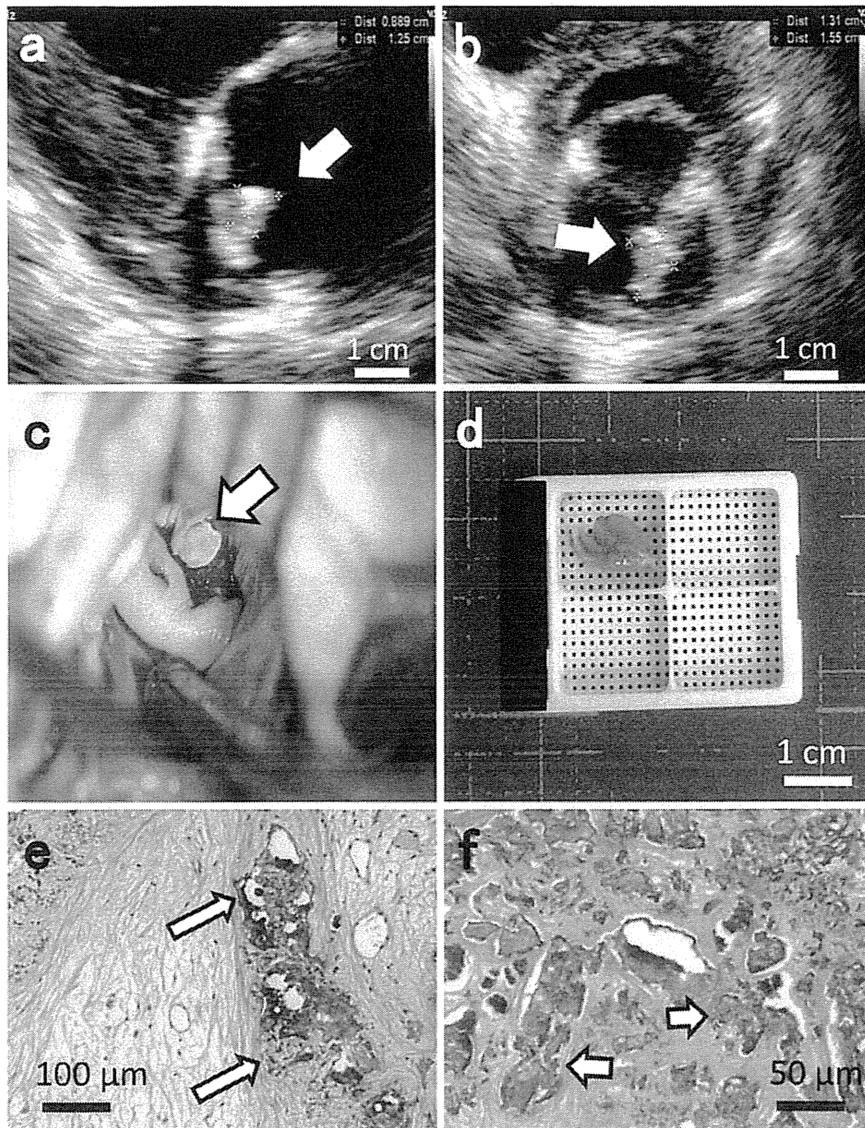

Figure 2. Echocardiography findings on admission, the intraoperative appearance of the tumor and light microscopy findings. Echocardiography performed in early 2013 upon admission to our hospital. a) Long-axis view. b) Short-axis view. The white arrows show a highly echoic, mobile mass adherent to the mitral valve. c) The arrow shows the tumor in the left atrium. d) The resected tumor. e, f) Findings of light microscopy with Hematoxylin and Eosin staining. The arrows indicate the calcified lesion [original magnification ×200 (e) and ×400 (f)].

did not show good compliance with dietary advice related to calcium and phosphate metabolism, and an assessment of the serum level of glycosylated hemoglobin as a possible factor inducing ectopic calcification showed her blood sugar control to generally be adequate at the previous hospital, although poor glycemic control may have been related to the progression of vascular calcification (11). Furthermore, thrombosis may also be a cause of CAT formation, as ESRD patients may have coagulation abnormalities (12) and/or receive anticoagulant or antiplatelet agents (1). Previous reports of CAT in ESRD patients on HD therapy may have been associated with the regular use of anticoagulants during HD. Another possibility regarding the onset of CAT is the opportunity for bacteremic events, such as endocarditis, to induce the development of organized thrombi in ESRD patients, who are generally immunocompromised. However, the current patient had no history of treatment with anticoagulant or antiplatelet agents and had not experienced any bacteremic events other than peritonitis. Importantly, the asymptomatic CAT was only identified on routine echocardiography in this case. We thus consider routine echocardiography to be important for making an early diagnosis of CAT, as, in this and previous cases, CAT has been shown to develop rapidly unaccompanied by symptoms (6). CAT may originate in any of the four chambers of the heart (1), although the development of this condition in patients on HD has been reported to be related to MAC (3, 4, 6). However, diagnosing CAT based only on the

Table 3. Summary of Echocardiographic Findings in June 2008 and May 2013

	Normal range	June 2008	May 2013(on admission)
AOD (mm)	20-37	31.1	29.0
LAD (mm)	19-40	45.1	48.8
LVDd (mm)	40-55	49.4	49.7
LVDs (mm)	30-45	30.1	30.2
IVSTd (mm)	7-11	13.4	15.6
LVPWTd (mm)	7-11	12.2	10.4
EF (%)	>55	not determined	69.6
FS (%)	>28	39.1	39.2
Left ventricular wall motion		within normal limits	within normal limits
Mitral regurgitation		none	moderate
Aortic regurgitation		none	none
Tricuspid regurgitation		mild	moderate
Aortic stenosis		none	none
E/A ratio	>1	0.83	0.9
DcT (msec)	>150	168	174
Mass lesion in LA		not detected	9×13mm
Valve calcification		mild in aortic valve	mild in mitral valve

AOD: aortic orifice diameter, LAD: left atrial dimension, LVDd: left ventricular end-diastolic dimension, LVDs: left ventricular end-systolic diamesion, IVSTd: end-diastolic interventricular septal wall thickness, LVPWTd: left ventricular posterior wall end-diastolic thickness, EF: ejection fraction, FS: fractional shortening, E/A ratio: a ratio of peak mitral E wave velocity to peak mitral A wave velocity ratio, DcT: deceleration time, LA: left atrium

presence of MAC is difficult, as MAC is not an uncommon finding in ESRD patients. Furthermore, it can be very difficult to differentiate CAT from vegetation, myxoma and thrombosis with calcification in both in non-ESRD and ESRD patients, and surgical resection is necessary to achieve a definitive diagnosis and prevent embolism in such cases (7, 8).

The present study is the first report of CAT in a PD patient. Routine echocardiography is useful for screening to achieve an early diagnosis of CAT in ESRD patients and prevent unexpected complications. Although the mechanisms underlying the development of CAT are unclear, controlling the levels of calcium and phosphate is important for preventing the development of CAT in ESRD patients.

The authors state that they have no Conflict of Interest (COI).

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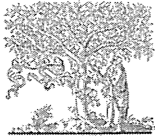

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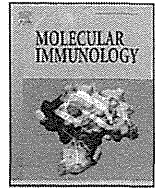

Expression of membrane complement regulators, CD46, CD55 and CD59, in mesothelial cells of patients on peritoneal dialysis therapy

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ARTICLE INFO

Article history:
Received 21 October 2014
Received in revised form 31 January 2015
Accepted 5 February 2015

Keywords:
Complement
Membrane complement regulators
Peritoneal dialysis
D/P creatinine
CD55

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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http://dx.doi.org/10.1016/j.molimm.2015.02.005
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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,

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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 TaqMan 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.

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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:

Deposition of C3b (%)
= number of C3b-coated cells/total number of cells × 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).

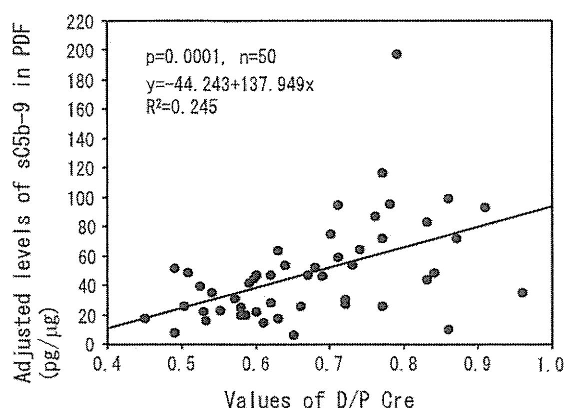

Fig. 1. *Levels of sC5b-9 in peritoneal dialysate (PDF) were correlated with values of dialysate-to-plasma creatinine concentration ratio (D/P Cre) in the PD patients.* In fifty PDF samples, levels of sC5b-9, adjusted for protein levels, were significantly correlated with D/P Cre values.

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

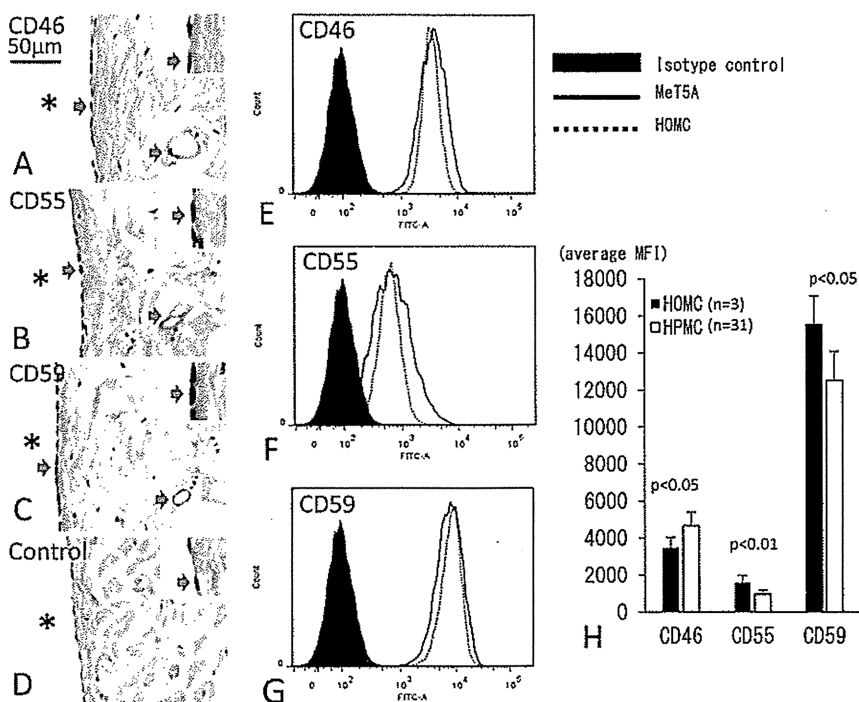

Fig. 2. *Membrane complement regulators CD46, CD55 and CD59, are abundantly distributed in human peritoneal tissues, Met-5A cells and on mesothelial cells from omentum (HOMC) of non-end stage renal disease (ESRD) patients.* Expression of CD46, CD55 or CD59 was analysed on 31 human primary culture of 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

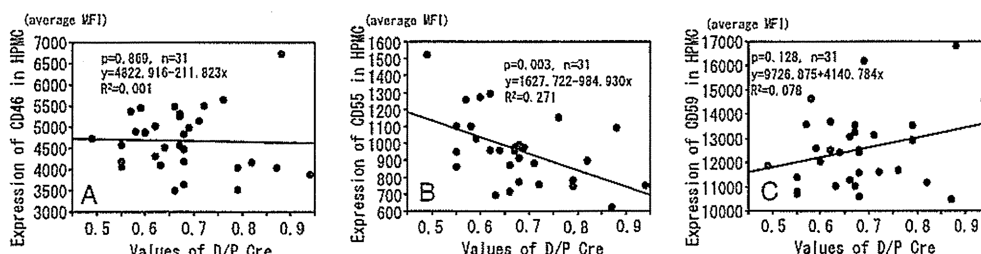

Fig. 3. *Correlation between expression of membrane complement regulators, CD46, CD55 and CD59 and dialysate-to-plasma creatinine concentration ration (D/P Cre).* Graphs A, B and C show the correlation between value of D/P Cre and expression of CD46, CD55 and CD59. D/P Cre and expression of CD55 are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

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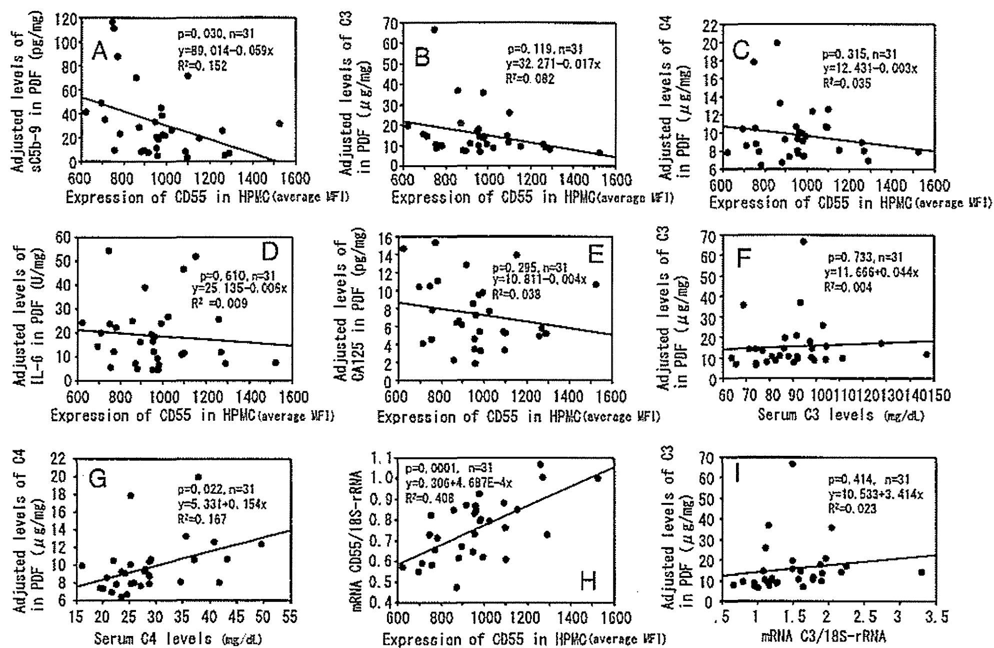

Fig. 4. *Mesothelial expression of CD55 in HMPCs correlated with levels of sC5b-9 in PDF, but not levels of C3, C4, IL-6 and CA-125 in PDF; protein level of CD55 correlated with mRNA levels in HMPCs.* Graphs A, B, C, D and E show correlations of CD55 expression in HMPC for levels of sC5b-9, C3, C4, IL-6 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).

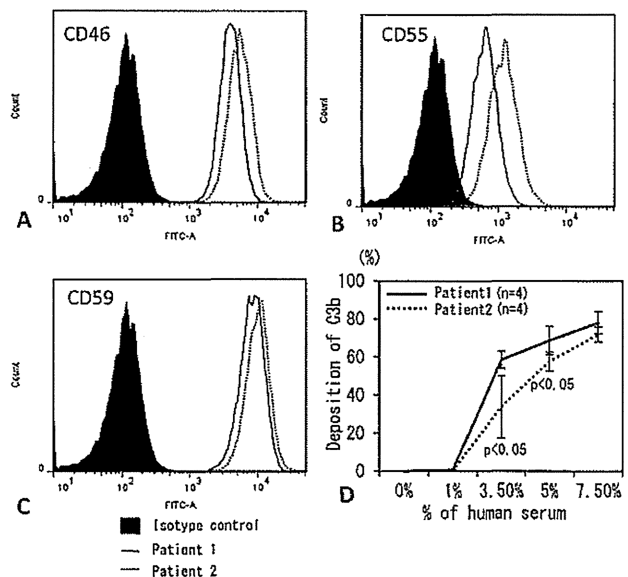

Fig. 5. *Comparison of complement activation in primary cultured mesothelial cells (HPMCs) expressing low and high levels of CD55.* The histograms A, B and C show expression of CD46, CD55 and CD59 in HPMCs, respectively. Graph D shows C3b deposition following incubation with normal human serum and with 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

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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.

Acknowledgements

The authors thank N. Suzuki, N. Asano, and Y. Sawa for technical help, B.P. Morgan (Cardiff University School of Medicine, Cardiff, UK) for editing the English in this manuscript and M. Horie (Daiyukai Daiichi Hospital) and H. Kasuga (Nagoya Kyoritsu Hospital) for helpful support.

This work was in part supported in part by a 2012 research grant from the Aichi Kidney Foundation, by the Ministry of Education, Science, and Culture of Japan a Grant-in-Aid 24591227 for Scientific Research, by the 2012 research grant from Japanese association of dialysis physician (JADP) Grant 2012-07, and by a Ministry of Health, Labour and Welfare of Japan grant-in-aid for Progressive Renal Diseases Research, Research on Rare and Intractable Disease.

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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