ESC HEART FAILURE
ESC Heart Failure 2021; **8**: 3156–3167
Published online 14 May 2021 in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/ehf2.13423

ORIGINAL RESEARCH ARTICLE

Involvement of chronic inflammation via monocyte chemoattractant protein-1 in uraemic cardiomyopathy: a human biopsy study

Tomoya Nakano[1,2], Kenji Onoue[1*], Ayako Seno[1], Satomi Ishihara[1], Yasuki Nakada[1], Hitoshi Nakagawa[1], Tomoya Ueda[1], Taku Nishida[1], Tsunenari Soeda[1], Makoto Watanabe[1], Rika Kawakami[1], Kinta Hatakeyama[3,4], Yasuhiro Sakaguchi[1], Chiho Ohbayashi[4] and Yoshihiko Saito[1]

[1]*Department of Cardiovascular Medicine, Nara Medical University, Kashihara, Nara, Japan;* [2]*Department of Internal Medicine, Yamato-Takada Municipal Hospital, Yamato-Takada, Nara, Japan;* [3]*Department of Pathology, National Cerebral and Cardiovascular Center, Suita, Osaka, Japan; and* [4]*Department of Diagnostic Pathology, Nara Medical University, Kashihara, Nara, Japan*

Abstract

Aims Patients undergoing dialysis, even those without coronary artery disease or valvular abnormalities, sometimes present with reduced heart function, which resembles dilated cardiomyopathy (DCM). This condition is known as uraemic cardiomyopathy (UCM). The mechanisms of UCM development are not fully understood. Previous studies demonstrated that the balance between placental growth factor (PlGF) and fms-like tyrosine kinase-1 (Flt-1) is correlated with renal function, and PlGF/Flt-1 signalling is involved in the development of cardiovascular diseases in patients with chronic kidney disease. This study was conducted to evaluate the pathogenesis of UCM and clarify the differences in the mechanisms of UCM and DCM by using human endomyocardial biopsy and blood samples.

Methods and results The clinical and pathological features of 30 patients on dialysis with reduced cardiac function [left ventricular ejection fraction (LVEF) ≤50%] (UCM group; mean age: 58.5 ± 9.4 years and LVEF: 39.1 ± 7.2%), 196 DCM patients (DCM group; mean age: 62.7 ± 14.0 years and LVEF: 33.5 ± 8.8%) as controls with reduced cardiac function (LVEF ≤ 45%), and 21 patients as controls with normal cardiac function (control group; mean age: 56.2 ± 19.3 years and LVEF: 67.5 ± 6.7%) were analysed. The percentage of the interstitial fibrosis area in the UCM group was greater than that in the DCM group ($P = 0.045$). In UCM patients, the percentage of the interstitial fibrosis area was positively correlated with the duration of renal replacement therapy ($P < 0.001$). The number of infiltrated CD68-positive macrophages in the myocardium and expression of monocyte chemoattractant protein-1 (MCP-1) in cardiomyocytes were significantly greater in the UCM group than in the other groups ($P < 0.001$, respectively). Furthermore, while the serum level of soluble form of Flt-1, an endogenous inhibitor of PlGF, in the UCM group was lower compared with that in the DCM group ($P < 0.001$), the serum levels of PlGF and PlGF/soluble form of Flt-1 ratio and plasma level of MCP-1 in the UCM group were higher than those in the DCM group ($P < 0.001$, respectively).

Conclusions These results suggest that activated PlGF/Flt-1 signalling and subsequent macrophage-mediated chronic non-infectious inflammation via MCP-1 in the myocardium are involved in the pathogenesis of UCM.

Keywords Human heart tissue; Immunohistochemistry; Monocyte chemoattractant protein-1; Placental growth factor; Uraemic cardiomyopathy

Received: 24 November 2020; Revised: 12 April 2021; Accepted: 2 May 2021
**Correspondence to: Kenji Onoue, Department of Cardiovascular Medicine, Nara Medical University, Kashihara, Nara, Japan. Tel: +81-744-22-3051; Fax: +81-744-22-9726. Email: konoue@naramed-u.ac.jp*

Introduction

Chronic kidney disease (CKD) is an independent risk factor for cardiovascular events.[1,2] Particularly, the mortality from cardiovascular disease of patients with CKD [estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m^2] is substantially higher than that of patients without CKD (eGFR ≥ 60 mL/min/1.73 m^2).[2–4] Congestive heart failure is

well known as the major cause of mortality among patients with CKD.[3,4] Despite the absence of ischaemic heart disease and valvular disease, patients with end-stage renal disease (ESRD) can develop left ventricular (LV) systolic dysfunction, LV hypertrophy, and LV dilatation, which is known as uraemic cardiomyopathy (UCM).[1,4,5] The pathological features of the UCM heart are diffuse interstitial fibrosis and cardiomyocyte hypertrophy.[5–7] The clinical features of the UCM are similar to those of dilated cardiomyopathy (DCM). However, the pathogenesis of these functional and phenotypical features in UCM, as well as whether the pathogenesis of UCM and DCM hearts is the same, remains unclear.

In patients with CKD, cardiovascular events are known to be caused by several factors such as uraemic toxins, inflammation, oxidative stress, and placental growth factor (PlGF).[4,5,8] Previous studies demonstrated that the balance between PlGF and fms-like tyrosine kinase-1 (Flt-1) is strongly correlated with renal function. PlGF is a member of the vascular endothelial growth factor (VEGF) family and PlGF selectively and specifically binds to Flt-1, also known as VEGF receptor-1. PlGF/Flt-1 signalling accelerates both vasculogenesis and atherosclerosis by enhancing chronic non-infectious inflammation, which enhances the progression of cardiovascular events.[8–11] A soluble form of Flt-1 (sFlt-1) lacks the transmembrane and intracellular domains of Flt-1 and regulates the availability of PlGF in the peripheral circulation.[9] Because the expression of PlGF is increased and that of sFlt-1 is decreased with decreases in eGFR, PlGF and sFlt-1 are important mediators of the pathogenesis of cardiovascular diseases in patients with CKD.[8,12,13] Our group also reported that sFlt-1 knockout mice showed persistent stimulation of PlGF/Flt-1 signalling in a pressure overload model, as well as presented with LV systolic dysfunction, diffuse interstitial fibrosis, and overexpression of monocyte chemoattractant protein-1 (MCP-1) in cardiomyocytes with macrophage infiltration in the myocardium.[14]

To investigate the mechanism of LV dysfunction in UCM patients and explore the difference between UCM and DCM hearts, we evaluated the involvement of inflammatory cells and expression of MCP-1 in cardiomyocytes using human LV endomyocardial biopsy and blood samples.

Methods

Patient population

The UCM group consisted of 30 consecutive patients with Stage 5 CKD on dialysis admitted to our hospital to investigate the aetiology of LV systolic dysfunction [LV ejection fraction (LVEF) ≤50%] between January 2007 and December 2016. They underwent transthoracic echocardiography and coronary angiography to exclude coronary artery disease and valvular disease. They also underwent LV endomyocardial biopsy to rule out acute myocarditis or secondary myocardial diseases, including cardiac amyloidosis. After excluding ischaemic or valvular heart disease and secondary myocardial diseases, they were diagnosed with UCM and enrolled in this study (UCM group). Patients already diagnosed with primary or secondary cardiomyopathy, including DCM, myocarditis, or cardiac amyloidosis, were excluded from the UCM group. As controls with normal LV function, 21 consecutive patients with conduction disorder who had all the following conditions between January 2007 and December 2016 were enrolled (control group): (i) patients who were excluded having coronary artery disease or LV dysfunction by coronary angiography and left ventriculography; (ii) patients who were excluded having any myocardial diseases including cardiac amyloidosis or myocarditis by LV endomyocardial biopsy; and (iii) patients who had not been diagnosed as primary or secondary cardiomyopathy. As controls with impaired LV function, 196 consecutive patients diagnosed clinically with DCM between January 2007 and December 2016 and underwent LV endomyocardial biopsy to exclude secondary myocardial diseases were enrolled (DCM group). DCM was defined as (i) LV end-diastolic diameter (LVEDD) index ≥33 mm/m^2 (men) or ≥32 mm/m^2 (women); (ii) LVEF ≤ 45%; and (iii) the absence of coronary artery disease by coronary angiography and absence of valvular disease by echocardiography.[15] In the three groups, patients with a history of recent infection or any other active systemic disease were excluded.

The study protocols were approved by the Nara Medical University Ethics Committee, 1176-5, and followed the *Declaration of Helsinki guidelines*. Written informed consent or an opt-out-approach for approval for the publication of patient information was applied in this study to obtain consent in all cases from either the patient or his/her family members.

Transthoracic echocardiography and laboratory data

Data from transthoracic echocardiograms and laboratory tests on admission were assessed. LVEF was calculated using the modified Simpson method. Interventricular septum thickness, posterior wall thickness, LVEDD, and LV end-systolic diameter (LVESD) were measured by M-mode echocardiography. eGFR was calculated according to the published equation for Japanese subjects: 194 × serum creatinine$^{-1.094}$ × age$^{-0.287}$ (× 0.739 for women). In some UCM and DCM patients, the serum levels of sFlt-1, PlGF, and VEGF-A and the plasma level of MCP-1 were measured using a sandwich enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA). The plasma and serum were collected after a small amount of heparin injection

at the time of endomyocardial biopsy procedure, and those were frozen at −80°C until analysis.

Myocardial tissue samples and immunohistochemical analysis

All LV endomyocardial biopsy samples were obtained from a standardized location at the apical posterior wall of the left ventricle. Formalin-fixed paraffin-embedded tissues were cut into 4-μm-thick sections using a microtome. The specimens were then processed for haematoxylin–eosin (HE) and Masson's trichrome (MT) staining and immunohistochemistry analysis. The cardiomyocyte diameter was measured at the nuclear level in at least 30 randomly selected cardiomyocytes from HE staining at a high-power field using a BZ-X710 microscope and BZ-X analyzer software system (Keyence, Osaka, Japan). Interstitial fibrosis was quantified as a percentage of the entire obtained myocardial tissue by MT staining. To cover the entire myocardium area at the high-power field, multiple images were captured. These high-resolution images were jointed using the BZ-X analyzer software. For immunohistochemistry analysis, anti-CD45 (ab8216, 1:500 dilution; Abcam, Cambridge, UK), anti-CD68 (ab125047, 1:100 dilution; Abcam), and anti-MCP-1 (ab9669, 1:200 dilution; Abcam) antibodies were used. The detailed methods are described in Supporting Information, *Text S1* and *S3*. The immunohistochemistry of CD45 and CD68 was performed using the serial section of biopsy samples used for MT staining. The number of CD45-positive lymphocytes and CD68-positive macrophages in the entire myocardial area was counted per square millimetre using the BZ-X analyzer software. MCP-1-positive areas in cardiomyocytes were assessed as a percentage of the entire cardiomyocytes. The endocardium, vascular structure, and interstitium were excluded in calculating the entire cardiomyocytes area.

To confirm the specificity of the polyclonal antibody used for MCP-1 immunostaining, we performed western blotting and immunohistochemistry analyses using autopsied human samples. The details of western blotting analysis and the peptide competition assay are available in Supporting Information, *Text S2* and *S3* and *Figure S1*.

Statistical analysis

Among the UCM, control, and DCM groups, continuous variables were expressed as the means and standard deviations or medians and inter-quartile ranges as appropriate, and categorical variables were expressed as percentages. The statistical significance of differences in continuous variables between the three groups was assessed by one-way analysis of variance or Kruskal–Wallis test, followed by Tukey's honest significant difference test or Mann–Whitney *U* test, as appropriate. The statistical significance of differences in categorical variables was assessed by χ^2 analysis. The Spearman correlation test was used to determine the relationship between the two variables. All comparisons with $P < 0.05$ were considered as statistically significant. JMP software (SAS Institute, Cary, NC, USA) was used to analyse the data.

Results

Baseline characteristics

The baseline clinical characteristics of the three patient groups, including cardiovascular risk factors and medications on admission, are presented in *Table 1*. The proportion of patients with hypertension was higher in the UCM group than in the control and DCM groups. While the duration of hypertension was similar between the three groups ($P = 0.67$), the systolic and diastolic pressure in the UCM group was significantly higher than the other groups. The proportion of atrial fibrillation was lower in the UCM group than in the DCM group. This is thought to be caused by the difference in left atrium volume. New York Heart Association functional class was similar between the UCM and DCM groups ($P = 0.11$). The time from symptom onset and the diagnosis was not significantly different between the UCM and DCM groups ($P = 0.86$). The aetiology and dialysis method of patients in the UCM group are presented in Supporting Information, *Table S1*. Twenty-six patients were on haemodialysis, and four were on peritoneal dialysis. The median duration of renal replacement therapy (RRT) was 2.3 (0.6–10.2) years. In the DCM group, the percentage of patients with CKD Stage 4, defined as an eGFR from 15 to 29 mL/min/1.73 m^2, was 6.1% and CKD Stage 5, defined as an eGFR below 14 mL/min/1.73 m^2, was zero (Supporting Information, *Figure S2*).

Echocardiography and laboratory data

The parameters of transthoracic echocardiography on admission are presented in *Table 1*. LV wall thickness in the UCM group was more hypertrophied than that in the other groups. The LVEDD index, LVESD index, and LVEF in the UCM group showed values between those of the control and DCM groups. E/e′ ratio (septal) had no difference between the UCM and DCM groups ($P = 0.27$). Thus, the echocardiographic characteristics in the UCM group were LV dilatation, and LV systolic and diastolic dysfunction associated with LV hypertrophy.

Laboratory data on admission for the three groups are presented in *Table 1*. Haemoglobin, uric acid, and sodium levels in the UCM group were the lowest among the three groups. Brain natriuretic peptide and potassium levels in the UCM group were significantly higher than those in the control and DCM groups.

Table 1 Patient characteristics

	Control group (n = 21)	Uraemic cardiomyopathy group (n = 30)	Dilated cardiomyopathy group (n = 196)	P-value (ANOVA)
Age (years)	56.2 ± 19.3	58.5 ± 9.4	62.7 ± 14.0	0.05
Male sex, n (%)	17 (81.0%)	20 (66.7%)	129 (65.8%)	0.37
Body mass index (kg/m^2)	23.1 ± 3.2	20.7 ± 3.3	23.6 ± 4.7	0.003
Systolic blood pressure (mmHg)	125.1 ± 12.0	149.1 ± 25.4	129.7 ± 26.3	<0.001
Diastolic blood pressure (mmHg)	68.7 ± 9.7	84.5 ± 16.4	79.7 ± 19.6	0.004
Heart rate (b.p.m.)	57.6 ± 15.8	86.5 ± 14.6	89.6 ± 27.3	<0.001
Duration from symptom onset to diagnosis (days)	23.0 (3.0–77.0)	44.5 (20.3–105.3)	50.0 (19.5–116.8)	0.06
NYHA functional class				
I, n (%)	6 (28.6%)	0	12 (6.1%)	<0.001
II, n (%)	15 (71.4%)	13 (43.3%)	53 (27.0%)	<0.001
III, n (%)	0	15 (50.0%)	98 (50.0%)	<0.001
IV, n (%)	0	2 (6.7%)	33 (16.8%)	0.05
Previous history				
Hypertension, n (%)	7 (33.3%)	21 (70.0%)	94 (48.0%)	0.02
Duration of hypertension (years)	9.4 ± 6.8	13.7 ± 9.7	13.6 ± 11.1	0.67
Diabetes mellitus, n (%)	4 (19.1%)	12 (40.0%)	53 (27.0%)	0.21
Dyslipidaemia, n (%)	6 (28.6%)	7 (23.3%)	54 (27.6%)	0.88
Smoker, n (%)	11 (52.4%)	17 (56.7%)	117 (59.7%)	0.79
Atrial fibrillation, n (%)	0 (0.0%)	1 (3.3%)	53 (27.0%)	<0.001
Medical treatment on admission				
ACEi/ARB, n (%)	6 (28.6%)	14 (46.7%)	84 (42.9%)	0.39
Beta-blocker, n (%)	2 (9.5%)	10 (33.3%)	34 (17.4%)	0.06
Aldosterone blocker, n (%)	1 (4.8%)	1 (3.3%)	31 (15.8%)	0.08
Laboratory data on admission				
Haemoglobin (g/dL)	14.1 ± 1.8	10.9 ± 2.0	14.0 ± 2.0	<0.001
Uric acid (mg/dL)	6.5 ± 1.9	5.9 ± 1.9	7.2 ± 2.2	0.004
Blood urea nitrogen (mg/dL)	18.9 ± 11.2	53.2 ± 23.2	19.9 ± 8.7	<0.001
eGFR (mL/min/1.73 m^2)	69.3 ± 26.8	6.1 ± 3.1	62.5 ± 21.5	<0.001
Sodium (mEq/L)	140.7 ± 2.2	138.3 ± 3.7	140.0 ± 3.5	0.01
Potassium (mEq/L)	4.2 ± 0.3	4.6 ± 0.7	4.2 ± 0.5	0.01
BNP (pg/mL)	36.9 (16.6–118.8)	1750.0 (351.5–2961.6)	460.4 (216.0–858.4)	<0.001
PRA (μg/mL/h)	2.0 (1.1–3.4)	2.3 (0.9–4.9)	1.8 (0.8–4.5)	0.72
PAC (pg/mL)	118.1 (91.0–184.1)	139.9 (72.5–365.7)	104.5 (64.1–164.2)	0.11
TTE parameters on admission				
IVS thickness (mm)	10.1 ± 1.6	11.4 ± 2.1	9.7 ± 1.5	<0.001
PW thickness (mm)	9.8 ± 1.3	11.6 ± 2.3	9.7 ± 1.5	<0.001
LVEDD index (mm/m^2)[a]	28.3 ± 3.1	37.4 ± 4.8	38.7 ± 5.1	<0.001
LVESD index (mm/m^2)[a]	17.5 ± 3.0	30.1 ± 4.9	32.3 ± 5.2	<0.001
LVEF (%)	67.5 ± 6.7	39.1 ± 7.2	33.5 ± 8.8	<0.001
E/e′ ratio (septal)	8.4 ± 3.4	21.7 ± 18.3	16.5 ± 8.4	0.001
LAD index (mm/m^2)[a]	21.6 ± 3.4	26.5 ± 4.1	27.6 ± 4.8	<0.001

ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; BNP, brain natriuretic peptide; E/e′ ratio, the early mitral filling velocity (E)/early diastolic mitral annular velocity (e′) ratio; eGFR, estimated glomerular filtration rate; IVS, intraventricular septal; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association; PAC, plasma aldosterone concentration; PRA, plasma renin activity; PW, posterior wall; TTE, transthoracic echocardiography.

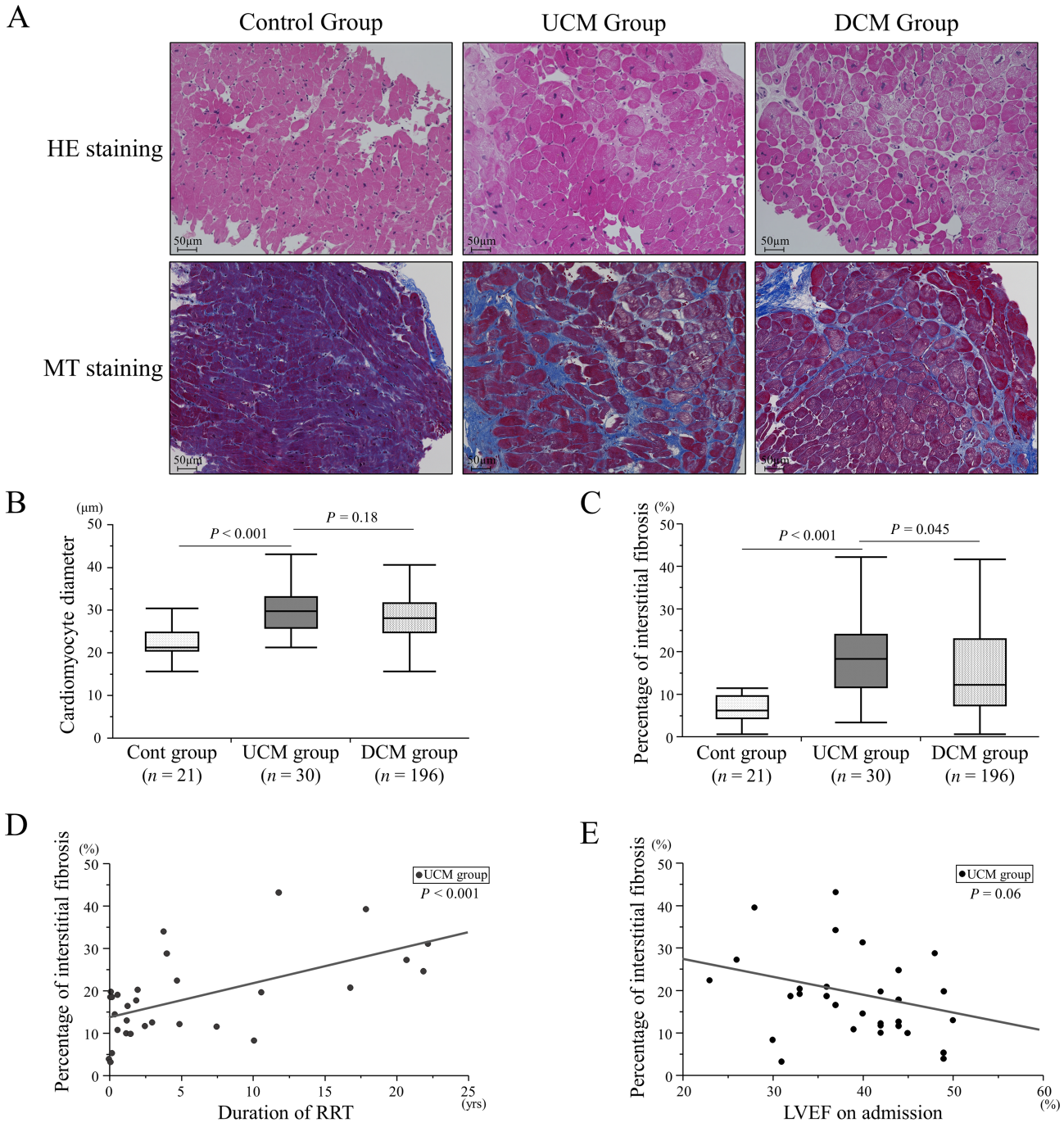
[a]Left ventricular end-diastolic dimension index [LVEDD index = LVEDD body surface area (BSA)], left ventricular end-systolic dimension index (LVESD index = LVESD BSA), and left atrial dimension index (LAD index = LAD BSA) were calculated by two-dimensional echocardiography.

Pathological features in left ventricular biopsy samples

We assessed the LV biopsy specimens by HE and MT staining. The cardiomyocyte diameter was significantly larger in the UCM group than in the control group, but there was no difference between the UCM and DCM groups (control, 22.2 ± 3.3 μm; UCM, 30.1 ± 5.9 μm; and DCM, 28.5 ± 5.4 μm, $P < 0.001$) [*Figure 1A* (upper panel) and *Figure 1B*]. The degree of interstitial fibrosis in the UCM group was significantly higher than that in the control group but similar to that in the DCM group (control, 7.3 ± 5.0%; UCM, 18.4 ± 9.9%; and DCM, 15.4 ± 11.6%, $P < 0.001$) [*Figure 1A* (bottom panel) and *Figure 1C*]. In the UCM group, the percentage of interstitial fibrosis was positively correlated with the duration of RRT ($P < 0.001$) (*Figure 1D*) and showed a tendency to be negatively correlated with LVEF on admission ($P = 0.06$) (*Figure 1E*).

Immunostaining for CD45 and CD68 was performed to assess the infiltration of inflammatory cells (*Figure 2A*). Although the number of CD45-positive lymphocytes in the myocardium was similar among the three groups [control,

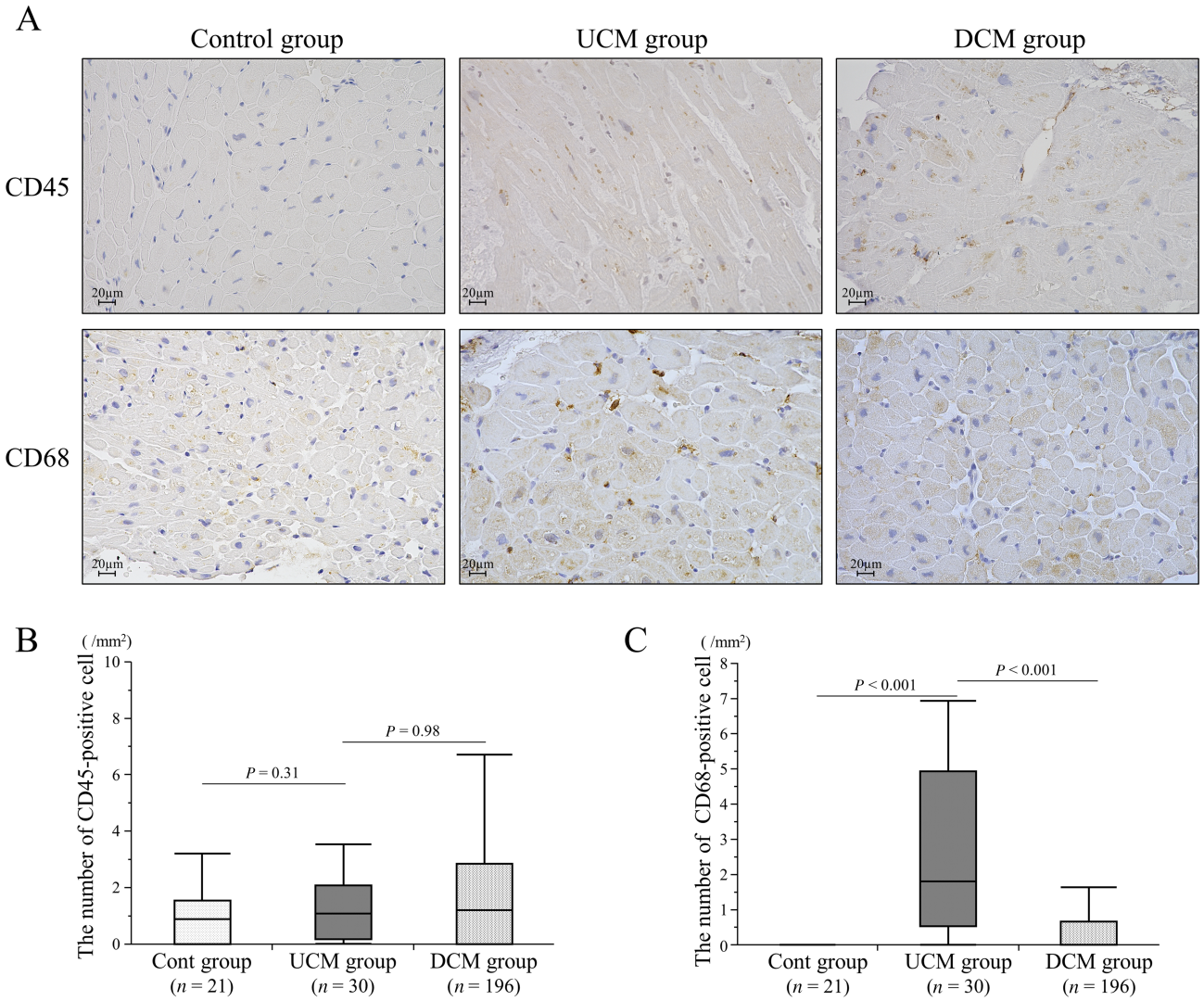
Figure 1 Degree of cardiomyocyte diameter and interstitial fibrosis. (A) Upper micrographs show haematoxylin–eosin (HE) staining, and bottom micrographs show Masson's trichrome (MT) staining. Bar graph shows (B) the cardiomyocyte diameter based on HE staining and (C) percentage of interstitial fibrosis based on MT staining. In the uraemic cardiomyopathy (UCM) group, the relationship between the percentage of interstitial fibrosis and (D) duration of renal replacement time (RRT), and (E) left ventricular ejection fraction (LVEF) on admission were significantly correlated. DCM, dilated cardiomyopathy.



0.9 (0.0–1.6) cells/mm^2; UCM, 1.1 (0.2–2.1) cells/mm^2; and DCM, 1.2 (0.0–2.8) cells/mm^2, P = 0.52] [*Figure 2A* (upper panel) and *Figure 2B*], the number of CD68-positive macrophages in the myocardium was significantly greater in the UCM group than in the other two groups [control, 0.0 (0.0–0.0) cells/mm^2; UCM, 1.8 (0.5–4.9) cells/mm^2; and DCM, 0.0 (0.0–0.7) cells/mm^2, $P < 0.001$] [*Figure 2A* (bottom panel) and *Figure 2C*]. Thus, macrophages showed

DOI: 10.1002/ehf2.13423

Figure 2 Immunostaining of CD45-positive and CD68-positive cells. (A) Immunohistochemistry staining for CD45 and CD68, visualized by diaminobenzidine (brown), to assess the infiltration of inflammatory cells in myocardium. Bar graph shows quantification of (B) CD45-positive lymphocytes and (C) CD68-positive macrophages in the myocardium. DCM, dilated cardiomyopathy; UCM, uraemic cardiomyopathy.



greater infiltration in the UCM group than in the other groups.

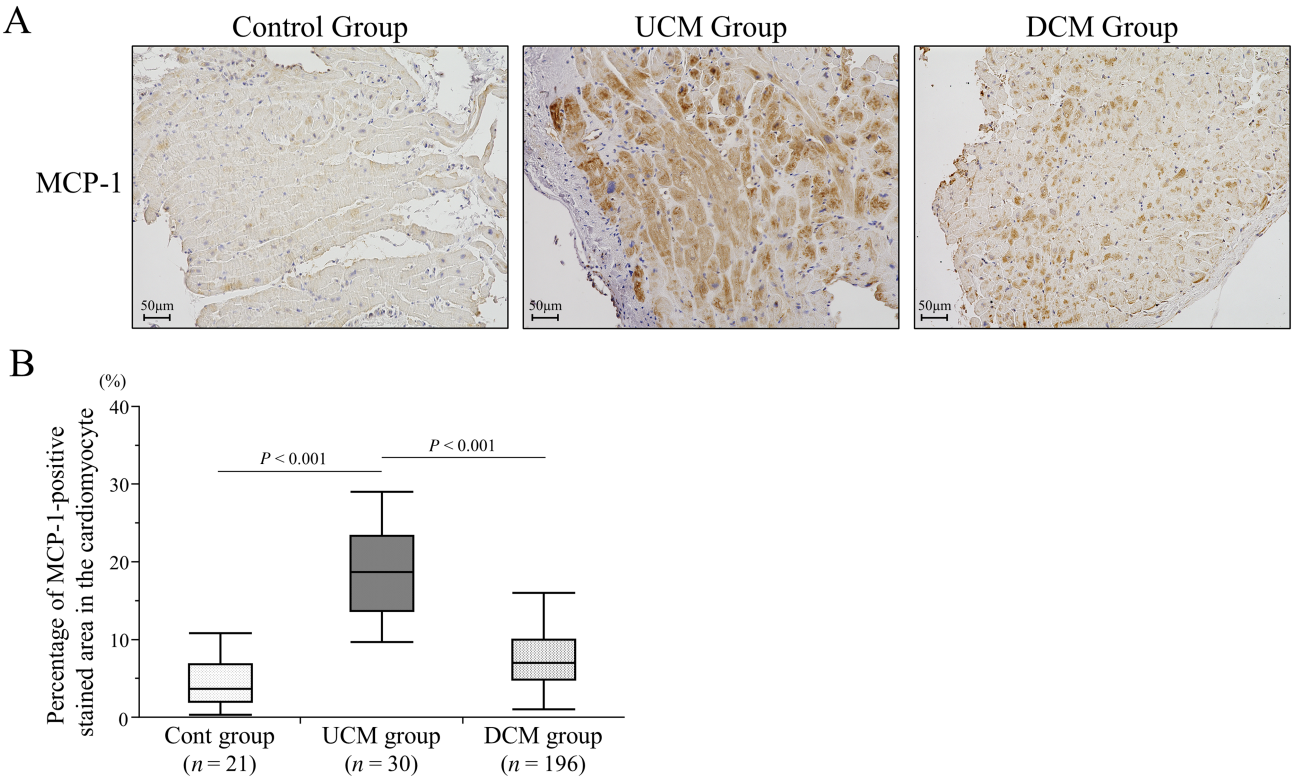
Expression of monocyte chemoattractant protein-1 in left ventricular biopsy

Previous studies showed that inflammatory cytokines, such as MCP-1, participate in the recruitment and activation of monocytes/macrophages. Therefore, we investigated whether MCP-1 was expressed in UCM hearts by immunostaining. MCP-1 expression was observed in cardiomyocytes. The degree of the positively stained area by the anti-MCP-1 antibody in the cardiomyocyte was significantly higher in the UCM group than in the control and DCM groups (control, 5.1 ± 4.0%; UCM, 23.6 ± 7.4%; and DCM, 9.8 ± 5.6%, $P < 0.001$) (*Figure 3A* and *3B*).

Blood levels of placental growth factor, soluble form of fms-like tyrosine kinase-1, and monocyte chemoattractant protein-1

To investigate the differences in pathological features between groups, we measured the serum levels of PlGF, sFlt-1, and VEGF-A and the plasma level of MCP-1 in the

Figure 3 Immunostaining of monocyte chemoattractant protein-1 (MCP-1). (A) Immunohistochemistry staining for MCP-1 using the specific antibody. MCP-1 was visualized by diaminobenzidine and found to be expressed in cardiomyocytes. (B) Quantification for MCP-1-positive-stained area in cardiomyocytes. DCM, dilated cardiomyopathy; UCM, uraemic cardiomyopathy.



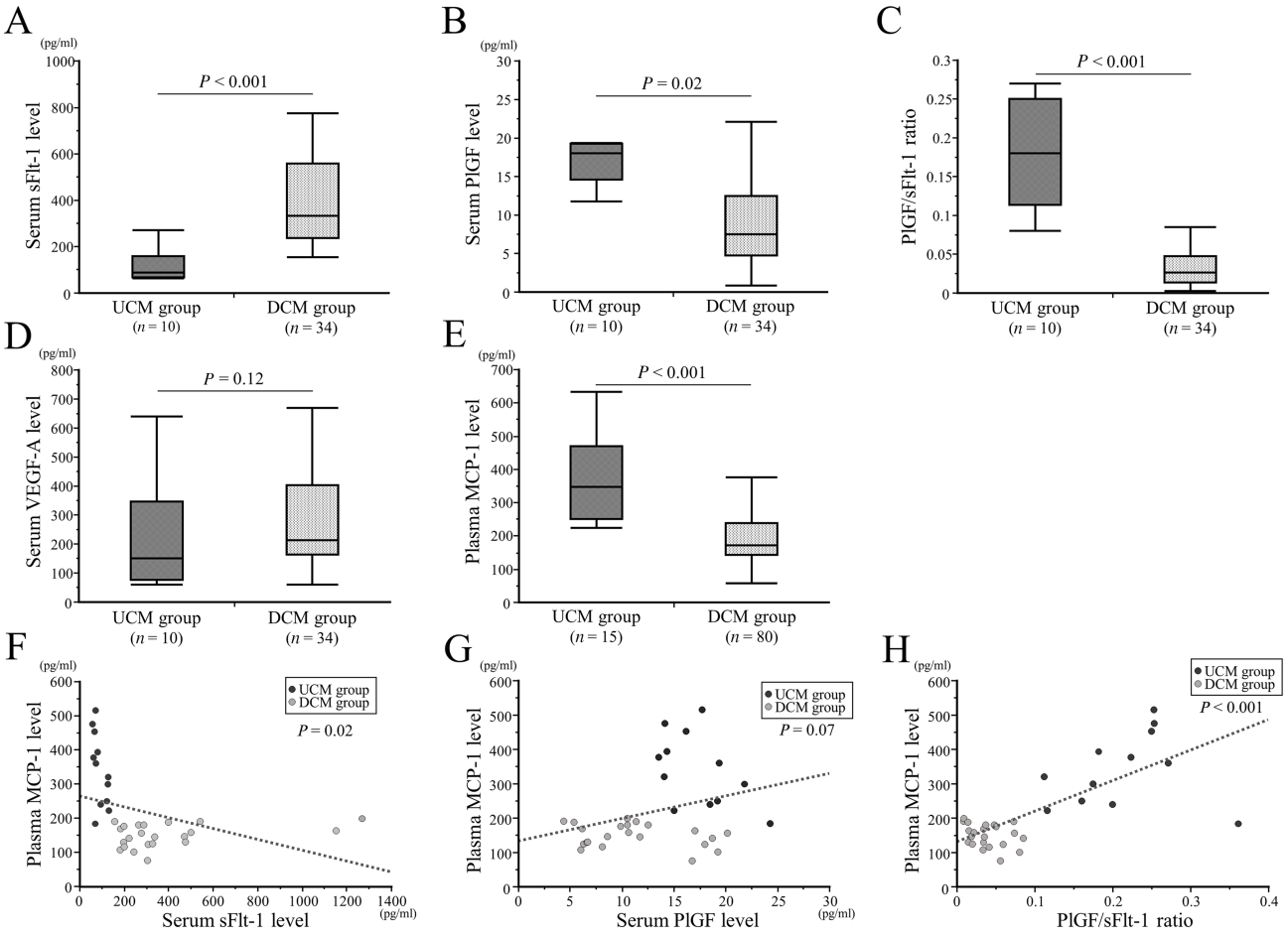
UCM and DCM groups. In the UCM group, as compared with the DCM group, the serum level of sFlt-1 was significantly lower (UCM, 120.4 ± 75.2 pg/mL; DCM, 430.8 ± 264.5 pg/mL, $P < 0.001$) (*Figure 4A*), the serum level of PlGF was significantly higher (UCM, 18.0 ± 4.9 pg/mL; DCM, 10.4 ± 5.7 pg/mL, $P < 0.001$) (*Figure 4B*), and PlGF/sFlt-1 ratio was significantly higher (UCM, 0.18 ± 0.07; DCM, 0.03 ± 0.02, $P < 0.001$) (*Figure 4C*). The serum level of VEGF-A, which also binds to Flt-1 in the same manner as PlGF, was comparable between the UCM and DCM groups (UCM, 221.0 ± 199.7 pg/mL; DCM, 295.0 ± 180.5 pg/mL, $P = 0.39$) (*Figure 4D*). The serum levels of sFlt-1 in the UCM and DCM groups were positively correlated with eGFR ($P = 0.01$) (Supporting Information, *Figure S3A*). In contrast, the serum levels of PlGF and PlGF/sFlt-1 ratio were negatively correlated with eGFR ($P = 0.01$ and $P < 0.001$, respectively) (Supporting Information, *Figure S3B* and *S3C*). The serum levels of VEGF-A in the UCM and DCM groups were not correlated with eGFR ($P = 0.57$) (Supporting Information, *Figure S3D*).

Furthermore, the plasma level of MCP-1 was higher in the UCM group than in the DCM group (UCM, 370.3 ± 131.4 pg/mL; DCM, 195.2 ± 78.1 pg/mL, $P < 0.001$) (*Figure 4E*). The plasma level of MCP-1 in the UCM and DCM groups was negatively correlated with eGFR on admission ($P < 0.001$) (Supporting Information, *Figure S3E*) and the serum level of sFlt-1 ($P = 0.03$) (*Figure 4F*). Although not significant, the plasma level of MCP-1 tended to be positively correlated with the serum level of PlGF ($P = 0.07$) (*Figure 4G*). The plasma level of MCP-1 showed a positive correlation with the PlGF/sFlt-1 ratio ($P < 0.001$) (*Figure 4H*).

Discussion

The present study demonstrated that cardiomyocytes' overexpression of MCP-1, the main chemokine responsible for recruiting macrophages, and infiltration of macrophages into the myocardium were associated with the progression of interstitial fibrosis and cardiac dysfunction in human LV biopsy samples from UCM patients. This suggests that macrophage-mediated chronic non-infectious inflammation via pro-inflammatory cytokines is involved in the development of UCM.[11,16]

Figure 4 Serum level of soluble isoform of fms-like tyrosine kinase-1 (sFlt-1), placental growth factor (PlGF), PlGF/sFlt-1 ratio, and vascular endothelial growth factor-A (VEGF-A) and plasma level of monocyte chemoattractant protein-1 (MCP-1). Serum levels of (A) sFlt-1, (B) PlGF, (C) PlGF/sFlt-1 ratio, and (D) VEGF-A and (E) plasma level of MCP-1 between the uraemic cardiomyopathy (UCM) and dilated cardiomyopathy (DCM) groups. Relationship between plasma level of MCP-1 and (F) serum level of sFlt-1, (G) serum level of PlGF, and (H) PlGF/sFlt-1 ratio.



Clinical and pathological features of the uraemic cardiomyopathy heart

As reported previously,[7,17,18] the UCM heart in this study clinically displayed a more hypertrophied LV wall, more dilated LV, worse LV systolic and diastolic function, pathologically larger LV cardiomyocyte size, and higher degree of interstitial fibrosis compared with the control heart. The degree of interstitial fibrosis increased with the duration of RRT in the UCM patients, which agrees with a previous study showing that the degree of interstitial fibrosis increased over time in patients with hemodialysis.[19] Several factors of the pathogenesis of the UCM hearts have been reported, such as uraemic toxins, inflammation, and oxidative stress.[1,4,5] These findings suggest that the development of interstitial fibrosis might be associated with exposure time of uraemic retention compounds with harmful or biochemical activity in the UCM heart. Furthermore, the increased formation of interstitial fibrosis and subsequent decrease in number of cardiomyocytes might contribute to LV systolic dysfunction.[20,21]

Activated placental growth factor/fms-like tyrosine kinase-1 signal pathway in uraemic cardiomyopathy heart

Our group demonstrated that the balance between PlGF and sFlt-1, which functions as an endogenous inhibitor of PlGF, is strongly related to renal function,[8,11,12] and impairment of renal function and subsequent accumulation of uraemic toxins are key factors in the activation of the PlGF/Flt-1 signal.[12,22] In this study, both the serum PlGF level and PlGF/sFlt-1 ratio were higher in the UCM group than in the DCM group, while the serum VEGF-A level was similar between both groups. Furthermore, unlike the serum VEGF-A level, the serum PlGF level and PlGF/sFlt-1 ratio increased with worsening eGFR

(Supporting Information, *Figure S3*). Previous reports indicated that both PlGF and VEGF-A levels in circulation were increased as a result of impaired myocardial perfusion and worked as the trigger of neoangiogenesis.[23,24] At the same time, the PlGF production from endothelial cells was reported to be activated under severe CKD, which was not seen in VEGF-A.[12] In this study, the serum VEGF-A level was not increased in UCM; however, the serum PlGF level was increased. Those findings suggested that the activation of PlGF production from endothelial cells rather than impaired myocardial perfusion is dominant as a cause of PlGF increase in the ESRD patients.[8,11–13]

Macrophage-mediated chronic inflammation in uraemic cardiomyopathy heart

We previously reported that the overexpression of MCP-1 in cardiomyocyte and subsequent infiltration of macrophage in myocardium caused the interstitial fibrosis and LV dysfunction under persistently activated PlGF/Flt-1 signalling.[14]

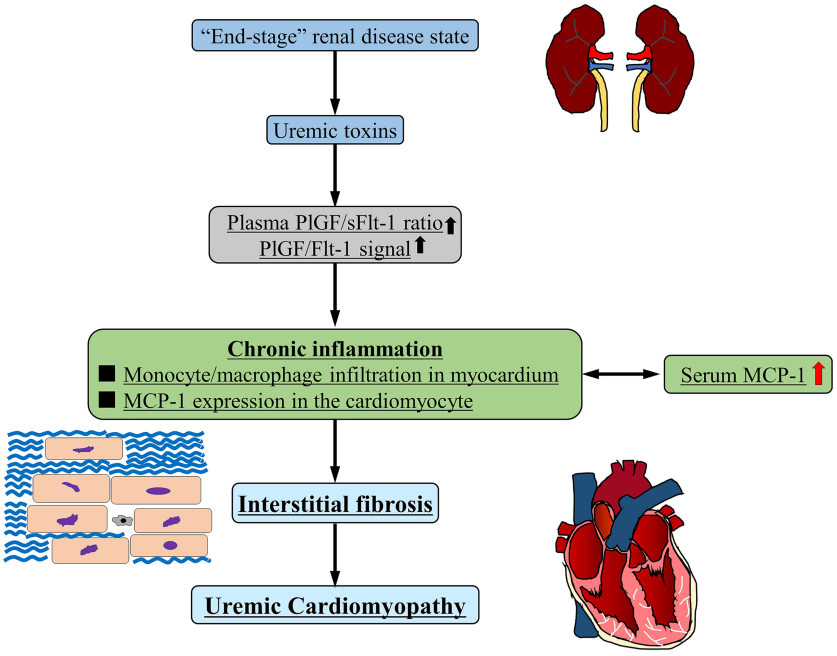
In this study, UCM hearts demonstrated more degree of interstitial fibrosis and macrophage infiltration, as previously reported. Macrophage can be broadly classified into two different subtypes: classically activated (M1) and alternatively activated (M2) macrophages.[25,26] In this study, as M2 macrophages were rarely observed in the UCM heart (data not shown), the majority of macrophages were thought to be M1 macrophages. This finding suggested that M1 macrophages might be polarized by a uraemic toxin[27] and the activation of PlGF/Flt-1 signalling, and the sustained pro-inflammatory environment lead to the development of interstitial fibrosis,[14,16,25,26] which is consistent with the degree of interstitial fibrosis to be higher with a longer duration of RRT.

This study also demonstrated that the expression of MCP-1 in the cardiomyocyte and the plasma level of MCP-1 were increased in the UCM heart.[14,28,29] Previous reports also showed that MCP-1 was overexpressed in cardiomyocyte from patients' sample with DCM and ischaemic cardiomyopathy.[30,31] Considering that plasma MCP-1 level was increased with worsening eGFR,[32] overexpression of MCP-1 might be associated with activation of PlGF/Flt-1 signalling under severe CKD.[14]

Clinical and pathological comparison between the uraemic cardiomyopathy and dilated cardiomyopathy heart

Uraemic cardiomyopathy heart displayed LV dilatation and LV systolic and diastolic dysfunction, which resembles DCM phenotype. Comparison of the morphological and pathological findings revealed that the LV wall thickness, the extent of

Figure 5 Schematic representation of development of uraemic cardiomyopathy (UCM). In UCM patients, activation of placental growth factor (PlGF)/fms-like tyrosine kinase-1 (Flt-1) signal may be associated with uraemic toxins, and subsequent macrophage-mediated chronic non-infectious inflammation via overexpression of monocyte chemoattractant protein-1 (MCP-1) in the myocardium was involved in the mechanism of interstitial fibrosis and development of UCM hearts. sFlt-1, soluble isoform of fms-like tyrosine kinase-1.



interstitial fibrosis, macrophage infiltration, and MCP-1 expression in the UCM heart were greater than in the DCM heart. The pathological findings in UCM patients observed in this study, cardiomyocyte hypertrophy, and fibrosis could be caused merely by hypertension because the prevalence of hypertension was significantly higher in the UCM group. However, when we compared all UCM patients (n = 30) with DCM patients with hypertension (n = 94) (Supporting Information, *Table S2*), intraventricular septal and posterior wall in the UCM patients had more hypertrophy than those in the DCM patients with hypertension (UCM patients: 11.4 ± 2.1 and 11.6 ± 2.3 mm; DCM patients with hypertension: 10.1 ± 1.5 and 10.2 ± 1.6 mm, $P < 0.01$, respectively). The cardiomyocyte diameter in the UCM patients was similar to that of the DCM patients with hypertension (UCM patients: 30.1 ± 5.9 μm; DCM patients with hypertension: 29.3 ± 5.6 μm, $P = 0.51$), while the degree of interstitial fibrosis in the UCM patients was severer than that in the DCM patients with hypertension (UCM patients: 18.4 ± 9.9%; DCM patients with hypertension: 14.9 ± 10.8%, $P = 0.04$). These findings suggested that the LV wall hypertrophy mechanism specific to the UCM was caused by interstitial fibrosis, which might be affected not only by hypertension but also by other factors such as a uraemic toxin.

The mechanism of development of uraemic cardiomyopathy heart

Our findings suggested that activated PlGF/Flt-1 signalling by uraemic toxins and subsequent macrophage-mediated chronic inflammation via overexpression of MCP-1 play an important role in the development of UCM. Based on our findings, the predicted mechanism of UCM development is shown in *Figure 5*. However, our findings cannot fully explain this mechanism. Previous reports demonstrated that the interaction between blood and dialysis membrane in haemodialysis leads to increased cytokine production including MCP-1.[33] The cytokines produced during haemodialysis may also be associated with the development of UCM. We analysed the differences in each index between patients with haemodialysis (n = 26) and with peritoneal dialysis (n = 4) to assess the influence of dialysis membrane. As shown in Supporting Information, *Table S3*, there were no differences in indices regarding inflammation, such as the number of infiltrated inflammatory cells or the degree of interstitial fibrosis. We, therefore, believe the dialysis membrane has little effect on the development of UCM.

Limitations

This study had some limitations. First, the sample size was small, and all samples were obtained from a single hospital. Thus, a larger number of samples should be evaluated to clarify the mechanisms of UCM development. Second, DCM patients may have been included in the UCM groups. It was not possible to exclude cases in which patients who initially had DCM, regardless of renal function, reached ESRD. Third, blood sampling of the control group was not performed, although previous studies revealed lower levels of PlGF and MCP-1 in patients with normal renal and cardiac function.[8,34] Fourth, we conducted immunohistochemistry analysis to evaluate MCP-1 expression. Generally, it is better to combine western blotting or reverse transcription polymerase chain reaction to confirm the results of immunohistochemistry, which depends on the specificity and sensitivity of the antibodies used. However, in this human study, we could not obtain large samples enough to extract protein or mRNA. To determine the specificity of the anti-MCP-1 antibody, we performed western blot analysis and peptide competition assays using an autopsied human sample.

Conclusions

The activation of the PlGF/Flt-1 signalling by uraemic toxins and the subsequent macrophage-mediated chronic non-infectious inflammation via the overexpression of MCP-1 in the myocardium may be involved in the mechanism of cardiac hypertrophy, interstitial fibrosis, and progression of LV dysfunction in UCM hearts. Our data have the potential to provide the basis for the development of new therapeutic methods.

Acknowledgements

The authors thank Tomoko Ioka, Miyako Sakaida, and Tomoyo Adachi for providing technical assistance.

Conflict of interest

None declared.

Funding

This work was supported in part by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) KAKENHI Grant Number JP19155855 (Grants-in-Aid from the MEXT); Health Labour Sciences Research Grant Numbers 19189094 and 17933459 [Technology and the Ministry of Health, Labour and Welfare of Japan (Comprehensive Research on Life-Style Related Disease including Cardiovascular Disease

and Diabetes Mellitus)]; and the Japan Agency for Medical Research and Development (AMED) under Grant Numbers JP19ek0210080, JP19ek0210118, JP19ek0210121, and JP19ek0210115 (Practical Research Project for Life-Style related Diseases including Cardiovascular Diseases and Diabetes Mellitus), AMED under Grant Numbers JP19ek0109367 and JP19ek0109406 (Practical Research Project for Rare/Intractable Diseases), and AMED under Grant Number JP19km0405009 (Platform Program for Promotion of Genome Medicine).

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Immunohistochemistry protocol for CD45 and CD68 using human cardiac tissue specimens.
Data S2. Western blot analysis protocol using autopsied human cardiac sample.
Data S3. Immunohistochemistry and peptide competition assay protocol for MCP-1 using human cardiac tissue specimens.
Table S1. Patient characteristics in the UCM group.
Table S2. Clinical and pathological characteristics between the UCM and DCM with hypertension groups.
Table S3. Clinical and pathological characteristics between UCM patients on Haemodialysis and on Peritoneal dialysis.
Figure S1. Western blot analysis, immunohistochemistry, and peptide competition assay for anti-MCP-1 antibody specificity.
Figure S2. Renal function of DCM patients.
Figure S3. The association between the eGFR and the blood cytokine level in the UCM and DCM groups.

References

1. Edwards NC, Moody WE, Chue CD, Ferro CJ, Townend JN, Steeds RP. Defining the natural history of uremic cardiomyopathy in chronic kidney disease: the role of cardiovascular magnetic resonance. *JACC Cardiovasc Imaging* 2014; **7**: 703–714.
2. Longenecker JC, Coresh J, Powe NR, Levey AS, Fink NE, Martin A, Klag MJ. Traditional cardiovascular disease risk factors in dialysis patients compared with the general population: the CHOICE Study. *J Am Soc Nephrol* 2002; **13**: 1918–1927.
3. Jablonski KL, Chonchol M. Recent advances in the management of hemodialysis patients: a focus on cardiovascular disease. *F1000Prime Rep* 2014; **6**: 72–81.
4. Lekawanvijit S. Cardiotoxicity of uremic toxins: a driver of cardiorenal syndrome. *Toxins* 2018; **10**: 352–380.
5. Alhaj E, Alhaj N, Rahman I, Niazi TO, Berkowitz R, Klapholz M. Uremic cardiomyopathy: an underdiagnosed disease. *Congest Heart Fail* 2013; **19**: E40–E45.
6. Edwards NC, Moody WE, Yuan M, Hayer MK, Ferro CJ, Townend JN, Steeds RP. Diffuse interstitial fibrosis and myocardial dysfunction in early chronic kidney disease. *Am J Cardiol* 2015; **115**: 1311–1317.
7. Yoshizawa S, Uto K, Nishikawa T, Hagiwara N, Oda H. Histological features of endomyocardial biopsies in patients undergoing hemodialysis: comparison with dilated cardiomyopathy and hypertensive heart disease. *Cardiovasc Pathol* 2020; **49**: 107256.
8. Onoue K, Uemura S, Takeda Y, Somekawa S, Iwama H, Imagawa K, Nishida T, Morikawa Y, Takemoto Y, Asai O, Soeda T, Okayama S, Ishigami K, Nakatani K, Kawata H, Horii M, Nakajima T, Akai Y, Iwano M, Saito Y. Reduction of circulating soluble fms-like tyrosine kinase-1 plays a significant role in renal dysfunction-associated aggravation of atherosclerosis. *Circulation* 2009; **120**: 2470–2477.
9. Shibuya M. Vascular endothelial growth factor receptor-1 (VEGFR-1/Flt-1): a dual regulator for angiogenesis. *Angiogenesis* 2006; **9**: 225–230.
10. Iwama H, Uemura S, Naya N, Imagawa K, Takemoto Y, Asai O, Onoue K, Okayama S, Somekawa S, Kida Y, Takeda Y, Nakatani K, Takaoka M, Kawata H, Horii M, Nakajima T, Doi N, Saito Y. Cardiac expression of placental growth factor predicts the improvement of chronic phase left ventricular function in patients with acute myocardial infarction. *J Am Coll Cardiol* 2006; **47**: 1559–1567.
11. Saito Y. The role of the PlGF/Flt-1 signaling pathway in the cardiorenal connection. *J Mol Cell Cardiol* 2021; **151**: 106–112.
12. Matsui M, Takeda Y, Uemura S, Matsumoto T, Seno A, Onoue K, Tsushima H, Morimoto K, Soeda T, Okayama S, Somekawa S, Samejima K, Kawata H, Kawakami R, Nakatani K, Iwano M, Saito Y. Suppressed soluble Fms-like tyrosine kinase-1 production aggravates atherosclerosis in chronic kidney disease. *Kidney Int* 2014; **85**: 393–403.
13. Matsui M, Uemura S, Takeda Y, Samejima K, Matsumoto T, Hasegawa A, Tsushima H, Hoshino E, Ueda T, Morimoto K, Okamoto K, Okada S, Onoue K, Okayama S, Kawata H, Kawakami R, Maruyama N, Akai Y, Iwano M, Shiiki H, Saito Y. Placental growth factor as a predictor of cardiovascular events in patients with CKD from the NARA-CKD study. *J Am Soc Nephrol* 2015; **26**: 2871–2881.
14. Seno A, Takeda Y, Matsui M, Okuda A, Nakano T, Nakada Y, Kumazawa T, Nakagawa H, Nishida T, Onoue K, Somekawa S, Watanabe M, Kawata H, Kawakami R, Okura H, Uemura S, Saito Y. Suppressed production of soluble Fms-like tyrosine kinase-1 contributes to myocardial remodeling and heart failure. *Hypertension* 2016; **68**: 678–687.
15. Kubanek M, Sramko M, Maluskova J, Kautznerova D, Weichet J, Lupinek P, Vrbska J, Malek I, Kautzner J. Novel predictors of left ventricular reverse remodeling in individuals with recent-onset dilated cardiomyopathy. *J Am Coll Cardiol* 2013; **61**: 54–63.
16. Wang B, Wang ZM, Ji JL, Gan W, Zhang A, Shi HJ, Wang H, Lv L, Li Z, Tang T, Du J, Wang XH, Liu BC. Macrophage-derived exosomal Mir-155 regulating cardiomyocyte pyroptosis and hypertrophy in uremic cardiomyopathy. *JACC Basic Transl Sci* 2020; **5**: 148–166.
17. Izumaru K, Hata J, Nakano T, Nakashima Y, Nagata M, Fukuhara M, Oda Y, Kitazono T, Ninomiya T. Reduced estimated GFR and cardiac remodeling: a population-based autopsy study. *Am J Kidney Dis* 2019; **74**: 373–381.
18. Tonelli M, Karumanchi SA, Thadhani R. Epidemiology and mechanisms of uremia-related cardiovascular disease. *Circulation* 2016; **133**: 518–536.

19. Gross ML, Ritz E. Hypertrophy and fibrosis in the cardiomyopathy of uremia—beyond coronary heart disease. *Semin Dial* 2008; **21**: 308–318.
20. Schwarz F, Mall G, Zebe H, Blickle J, Derks H, Manthey J, Kübler W. Quantitative morphologic findings of the myocardium in idiopathic dilated cardiomyopathy. *Am J Cardiol* 1983; **51**: 501–506.
21. Aoki T, Fukumoto Y, Sugimura K, Oikawa M, Satoh K, Nakano M, Nakayama M, Shimokawa H. Prognostic impact of myocardial interstitial fibrosis in non-ischemic heart failure—comparison between preserved and reduced ejection fraction heart failure. *Circ J* 2011; **75**: 2605–2613.
22. Zakiyanov O, Kalousová M, Zima T, Tesař V. Placental growth factor in patients with decreased renal function. *Ren Fail* 2011; **33**: 291–297.
23. Nakamura T, Funayama H, Kubo N, Yasu T, Kawakami M, Momomura S, Ishikawa SE. Elevation of plasma placental growth factor in the patients with ischemic cardiomyopathy. *Int J Cardiol* 2009; **131**: 186–191.
24. Iwasaki H, Kawamoto A, Tjwa M, Horii M, Hayashi S, Oyamada A, Matsumoto T, Suehiro S, Carmeliet P, Asahara T. PlGF repairs myocardial ischemia through mechanisms of angiogenesis, cardioprotection and recruitment of myo-angiogenic competent marrow progenitors. *PLoS ONE* 2011; **6**: e24872.
25. Guiteras R, Flaquer M, Cruzado JM. Macrophage in chronic kidney disease. *Clin Kidney J* 2016; **9**: 765–771.
26. Engel JE, Chade AR. Macrophage polarization in chronic kidney disease: a balancing act between renal recovery and decline? *Am J Physiol Renal Physiol* 2019; **317**: F1409–f1413.
27. Li Y, Yan J, Wang M, Lv J, Yan F, Chen J. Uremic toxin indoxyl sulfate promotes proinflammatory macrophage activation by regulation of β-catenin and YAP pathways. *J Mol Histol* 2021; **52**: 197–205.
28. Niu J, Azfer A, Deucher MF, Goldschmidt-Clermont PJ, Kolattukudy PE. Targeted cardiac expression of soluble Fas prevents the development of heart failure in mice with cardiac-specific expression of MCP-1. *J Mol Cell Cardiol* 2006; **40**: 810–820.
29. Niu J, Azfer A, Kolattukudy PE. Monocyte-specific Bcl-2 expression attenuates inflammation and heart failure in monocyte chemoattractant protein-1 (MCP-1)-induced cardiomyopathy. *Cardiovasc Res* 2006; **71**: 139–148.
30. Hanna A, Frangogiannis NG. Inflammatory cytokines and chemokines as therapeutic targets in heart failure. *Cardiovasc Drugs Ther* 2020; **34**: 849–863.
31. Kobayashi M, Nakamura K, Kusano KF, Nakamura Y, Ohta-Ogo K, Nagase S, Sakuragi S, Ohe T. Expression of monocyte chemoattractant protein-1 in idiopathic dilated cardiomyopathy. *Int J Cardiol* 2008; **126**: 427–429.
32. Stinghen AE, Gonçalves SM, Martines EG, Nakao LS, Riella MC, Aita CA, Pecoits-Filho R. Increased plasma and endothelial cell expression of chemokines and adhesion molecules in chronic kidney disease. *Nephron Clin Pract* 2009; **111**: c117–c126.
33. Pertosa G, Grandaliano G, Gesualdo L, Schena FP. Clinical relevance of cytokine production in hemodialysis. *Kidney Int* 2000; **76**: S104–S111.
34. Fukami A, Yamagishi S, Adachi H, Matsui T, Yoshikawa K, Ogata K, Kasahara A, Tsukagawa E, Yokoi K, Imaizumi T. High white blood cell count and low estimated glomerular filtration rate are independently associated with serum level of monocyte chemoattractant protein-1 in a general population. *Clin Cardiol* 2011; **34**: 189–194.