Serum levels of soluble CD93 in patients with chronic renal failure

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Abstract

In this clinical study, we examined the serum levels of soluble CD93 (sCD93) in patients with chronic renal failure (CRF) by an originally constructed enzyme-linked immunoassay (EIA) using a CD93 monoclonal antibody (mAb) (mNI-11) established in our laboratories. The serum concentrations of sCD93 in patients with CRF were significantly higher ($P < 0.001$) than those in normal healthy controls. Furthermore, the serum levels of interleukin-6 (IL-6) were also significantly higher in the patients with CRF ($P < 0.05$) than in the normal healthy controls. In addition, the serum levels of sCD93 were strongly correlated with the blood urea nitrogen (BUN) ($r = 0.797$, $P < 0.001$), serum creatinine ($r = 0.784$, $P < 0.001$) and serum cystatin C ($r = 0.913$, $P < 0.001$). Taken together, these findings suggest that the serum sCD93 may serve as a new biomarker for analyzing or measuring renal function.

Key words : biomarker, chronic renal failure, serum cystatin C, soluble CD93

Introduction

Human CD93, with a molecular weight of about 90-100 kDa, is a heavily *O*-glycosylated type I transmembrane protein consisting of unique C-type lectin domains. CD93 is selectively expressed on myeloid cell lineages (monocytes and granulocytes),endothelial cells and hematopoietic stem cells[1-5].In addition, it is also co-expressed on naive T lymphocytes ($CD4^+CD45RA^+$cells) of neonatal umbilical cord blood cells (UCBCs), but not on normal adult peripheral blood cells (PBCs)[6, 7]. In relation to its biological functions, CD93 is reported to play critical roles in the exclusion of apoptotic cells[8] and in angiogenesis[9,10].

CD93 has been demonstrated to be shed/released from the surface of activated human monocytes and granulocytes (mainly neutrophils) *in vitro*, and is therefore detected in a soluble form in the serum (sCD93)[11]. In particular, sCD93 shedding/release has been shown *in vitro* to occur in response to inflammatory mediators such as tumor-necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-8, endotoxin lipopolysaccharide (LPS) and the protein kinase C (PKC) activator phorbol myristate acetate (PMA)[11,12]. Furthermore, sCD93 has also been commonly detected in human serum/plasma of normal healthy adults. Enhanced sCD93 shedding/release, with elevation of the serum or synovial fluid concentrations of sCD93, has also been demonstrated in certain clinical conditions [5,13,14].

Recently, existence of a correlation between the serum sCD93 and serum cystatin C (cysteine protease inhibitor) concentrations was reported in both normal healthy adults and patients with myocardial infarction[15]. On the other hand, it has been reported that as cystatin C is filtered out of the blood by the kidneys, it may be a better marker of the glomerular filtration rate than the blood urea nitrogen (BUN) or serum creatinine[16,17]. However, the correlations of the serum concentrations

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of sCD93 with those of serum cystatin C or other common renal function markers such as the BUN and serum creatinine in patients with chronic renal failure (CRF) remain very poorly understood. CRF, also known as chronic kidney disease (CKD), is associated with a progressive loss of renal function over a period of months or years. Often, CRF is diagnosed as a result of screening of people with some risk factors for kidney damage, such as high blood pressure or diabetes mellitus[18]. In particular, this disease is often identified after it has led to one of its complications, such as cardiovascular disease, anemia or pericarditis[19]. Therefore, a new biomarker that would allow a precise and timely diagnosis of CRF is desired.

As described above, in this clinical study, to analyze the significance of sCD93 in renal function impairment, we examined the serum concentrations of sCD93 in patients with CRF as compared with those in normal healthy controls by an originally constructed enzyme-linked immunoassay (EIA) using a CD93 monoclonal antibody (mAb) (mNI-11) established in our laboratories[2,6,7,20].

Materials and Methods

Antibodies and reagents

A CD93 monoclonal antibody (mAb) (mNI-11; mouse IgG1)[2,6,7,20] was established in our laboratories. Enzyme immunoassay (EIA) kits for interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) were purchased from MBL Co. (Nagoya, Japan) and IBL Co. (Gunma, Japan), respectively. A biotin-conjugated CD93 mAb (X-2; mouse IgG1) and horseradish peroxidase (HRPOD)-conjugated streptavidin were purchased from Cosmo Bio Co. (Tokyo, Japan). Recombinant CD93 protein (TP306980) was purchased from OriGene Technologies, Inc. (USA). An EIA kit for cystatin C was purchased from R&D Systems (Minneapolis, MN, USA).

Ethics statement

The study protocol was approved by the institutional review board (IRB) of Kyushu University of Health and Welfare and IRB number was 14-009. Informed consent was obtained from all the donors prior to their participation in this study.

Processing of the blood and serum samples

Blood samples of patients with chronic renal failure (CRF) (5 males and 9 females; age 71.7 ± 11.5 yr) were obtained from Ogawa Clinic of Urology (Nobeoka-city, Miyazaki). The diagnosis of CRF was established by conventional, clinical and histological criteria. Normal healthy control blood samples (5 males and 5 females; age 41.0 ± 11.3 yr) were obtained from normal healthy volunteers. All serum samples were prepared using the standard method and the sera were stored at -80℃ until use.

Measurement of the soluble form of CD93 (sCD93) in the serum

EIA plates (Sumitomo Co., Tokyo) were coated with 500 ng/mL of CD93 mAb (mNI-11) in carbonate-bicarbonate buffer (0.01 M $NaCO_3$, 0.035 M $NaHCO_3$, pH9.6) for 24 hr at 4℃ . The wells were washed four times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST) and blocked with PBST containing 2% BSA (BSA-PBST) for 60 min at room temperature. Thereafter, the wells were washed three times with PBST. Sera (x 100 dilution with 0.1% BSA-PBST; 50 μL) and biotin-conjugated CD93 mAb (X-2) (x 2,500 dilution with 0.1% BSA-PBST; 50 μL) were then added to each well, followed by incubation for 60 min at room temperature with shaking. A recombinant protein of the CD93 molecule (TP306980) (OriGene Technologies, Inc.) was added to each well as a positive control or as a CD93 standard. The wells were washed eight times with PBST, followed by addition of HRPOD-conjugated streptavidin (x 5,000 dilution with 0.1% BSA-PBST; 100 μL) to each well and incubation of the wells for 30 min at room temperature with shaking. The wells were then washed 10 times with PBST, followed by addition of the substrate-chromogen (TMB; Cosmo Bio Co.; 100 μL) to each well and incubation for 10 min at room temperature with gentle shaking. The reaction was stopped by the addition of 0.5 M-HCl (100 μL), and the optical density (O.D.) was read at 450 nm using a multichannel EIA-microplate reader (TOSHO Co.). The

experiment was repeated five times.

Measurement of IL-6, VEGF and cystatin C in the serum

The measurement of IL-6, VEGF and cystatin C in the serum samples was performed using an IL-6 detection EIA kit, VEGF detection kit and cystatin C detection kit, respectively. Each of the experiments was repeated three times.

Measurement of BUN and serum creatinine

Measurement of BUN and creatinine in the serum samples was performed by outsourcing the laboratory examination to a Special Reference Laboratory (SRL). Each experiment was repeated three times.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). The statistical analysis was performed using an unpaired Student' s *t*-test. Pearson' s correlation was used for studying the correlations of the serum sCD93 concentrations with the BUN, serum creatinine and serum cystatin C. Differences at $P < 0.05$ were considered to indicate significance.

Results and Discussion

First of all, we examined the concentrations of the well-known renal function markers such as BUN and serum creatinine in patients with CRF and normal healthy controls. As expected and shown in Figure 1, the levels of these biomarkers were significantly higher ($P < 0.05$) in the patients with CRF than in the normal healthy controls (BUN: 32.20 ± 21.30 vs. 14.96 ± 2.63 mg/dL; serum creatinine: 2.18 ± 1.77 vs. 0.72 ± 0.12 mg/dL). In addition, the serum levels of IL-6 were also significantly higher ($P < 0.05$) in the patients with CRF than in the normal healthy controls (6.69 ± 6.40 vs. 1.83 ± 1.06 pg/mL) (Fig.2), suggesting elevated inflammatory responses in CRF patients. However, the serum level of VEGF was not significantly different ($P = 0.115$) between the patients with CRF and the normal healthy controls (38.84 ± 30.50 vs. 22.44 ± 8.94 pg/mL) (Fig.2).

Next, we examined the serum concentrations of sCD93 and cystatin C in the patients with CRF and normal healthy controls. As shown in Figure 3, the serum concentrations of sCD93 and cystatin C were significantly higher ($P < 0.01$) in the patients with CRF than in the normal healthy controls (sCD93: 1.61 ± 0.60 vs. 0.66 ± 0.08 ng/mL; cystatin C: 2.06 ± 1.19 vs. 0.75 ± 0.08 ng/mL).

Finally, we analyzed the correlations between the serum sCD93 concentrations and the BUN, serum creatinine, and serum cystatin C. As shown in Figure 4, the serum concentrations of sCD93 were strongly correlated with the BUN ($r = 0.797$, $P < 0.001$), serum creatinine ($r = 0.784$, $P < 0.001$) and serum cystatin C ($r = 0.913$, $P < 0.001$); interestingly, the strongest correlation was observed for the serum cystatin C.

Cystatin C, with a molecular weight of about 13 kDa, is a housekeeping enzyme belonging to the cystatin superfamily. Cystatin C is produced in all cells and tissues and is present in all biological fluids, and is an important extracellular cysteine protease inhibitor that functions to protect various cells throughout the body against the onslaught of multiple foreign antigens[21,22]. Meanwhile, its low molecular weight allows it to be freely filtered by the glomerular membrane in the kidney and its concentration in the blood has been reported to be correlated with the glomerular filtration rate[16]. The serum level of cystatin C is independent of the weight and height, muscle mass, age and sex. Thus, serum cystatin C may be a better marker of the glomerular filtration rate and kidney function than the serum creatinine, which is currently the most commonly measured parameter of kidney function[17,18]. The strong correlation between the serum sCD93 and cystatin C concentrations may suggest that the serum sCD93 level may be a better marker of the glomerular filtration rate than the BUN or serum creatinine. Therefore, these findings suggest that the measurement of sCD93 may be useful for analyzing the renal function.

The detailed mechanism(s) underlying the elevated concentrations of sCD93 in the sera of patients with CRF remains unclear at the present time. However, we

suggest some possible reasons, as follows; there may be more vigorous shedding/release of sCD93 from the cell surface membrane in response to some stimulating factor(s) in patients with CRF than in the normal healthy controls. However, the possible factors responsible for enhancing sCD93 shedding/release in patients with CRF remain unknown, studies indirectly suggest that patients with CRF have elevated inflammatory responses. Lending support to the above contention, enhanced sCD93 shedding in response to inflammatory mediators such as TNF-α, IL-6 and IL-8 has been demonstrated *in vitro*[11]. Furthermore, enhanced sCD93 shedding/release into the synovial fluid or serum has also been reported in patients with some autoimmune diseases[5,14]. In fact, we also found elevated serum levels of IL-6, which is involved in multiple immune responses, in the sera of patients with CRF in this study. Thus, the high concentrations of sCD93 in the sera of patients with CRF may be influenced by the actions of IL-6.

Another proposed mechanism for the enhanced shedding/release of CD93 into the serum of patients with CRF is surface expression of unglycosylated of CD93, as glycosylation of CD93 has been reported to be a very important factor for the stability of expression of this molecule on the cell membrane[23]. Although the precise reason(s) for the high concentrations of sCD93 in the sera of patients with CRF still remains to be identified, we are vigorously attempting to analyze the detailed mechanism(s) of shedding/release of CD93 into the serum in patients with CRF.

We have reported, for the first time, the serum concentrations of sCD93 in patients with CRF. The definition of chronic kidney disease differs from that of acute kidney disease in that the reduction in kidney function must have been present for over three months[24,25]. Thus, a new biomarker is required for a precise and timely diagnosis of CRF. In this clinical study, we found that the serum concentrations of sCD93 in patients with CRF may provide some useful information. In particular, based on the finding of a strong correlation between the serum sCD93 concentration and serum cystatin C concentration, it appears that sCD93 might be a better marker of the glomerular filtration rate than the BUN or serum creatinine. Further analyses are needed to identify the precise mechanism(s) of these findings at the cellular and molecular levels.

Disclosure

No authors have any conflict of interest.

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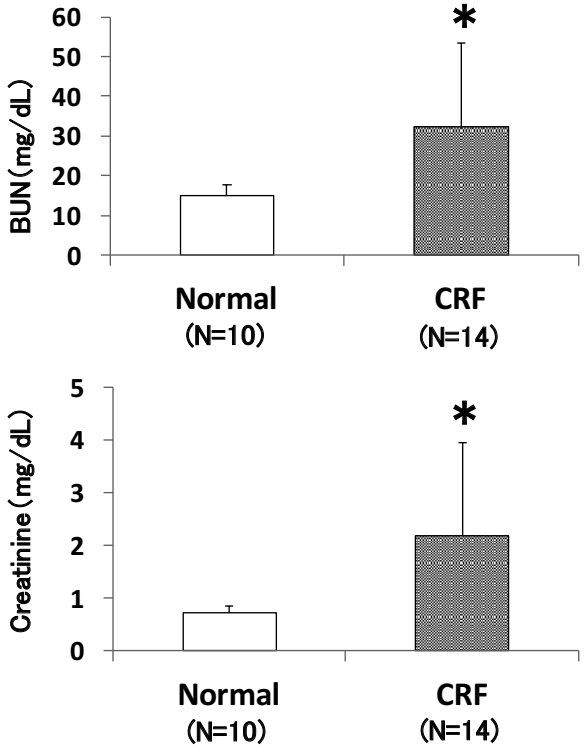

Figure 1. Concentrations of the BUN and serum creatinine in patients with CRF (n=14) and normal healthy controls (n=10). The measurements were performed by a routine procedure (outsourced laboratory examination). Each measurement was repeated three times. * P < 0.05 (Normal vs. CRF).

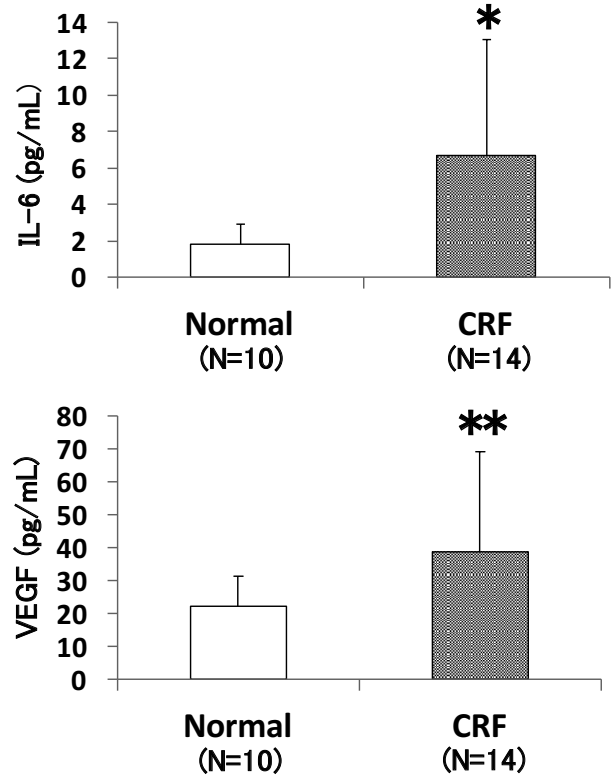

Figure 2. Concentrations of the serum IL-6 and serum VEGF in patients with CRF (n=14) and normal healthy controls (n=10). The measurements were performed using EIA kits (See Materials and Methods for details). Each measurement was repeated four times. *P < 0.05 (Normal vs. CRF). **P = 0.115 (Normal vs. CRF).

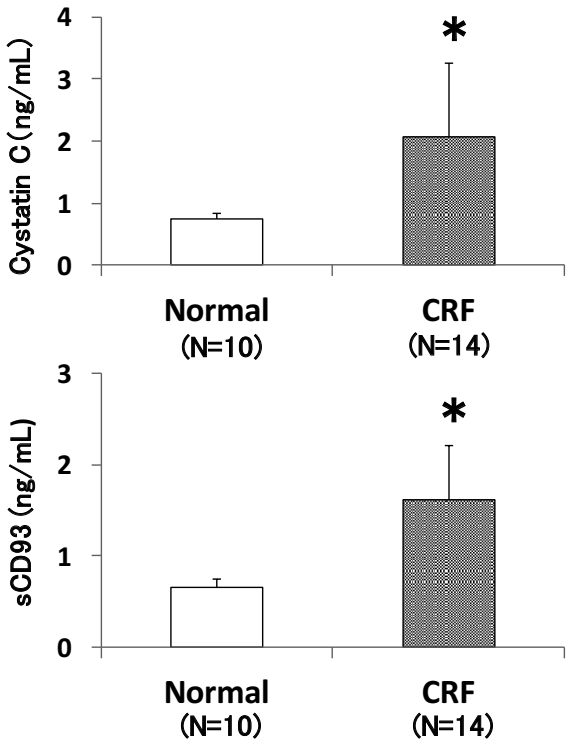

Figure 3. Concentrations of the serum sCD93 and serum cystatin C in patients with CRF (n=14) and normal healthy controls (n=10). The measurements were performed using EIA kits (See Materials and Methods for details). Each measurement was repeated four times. *P < 0.01 (Normal vs. CRF).

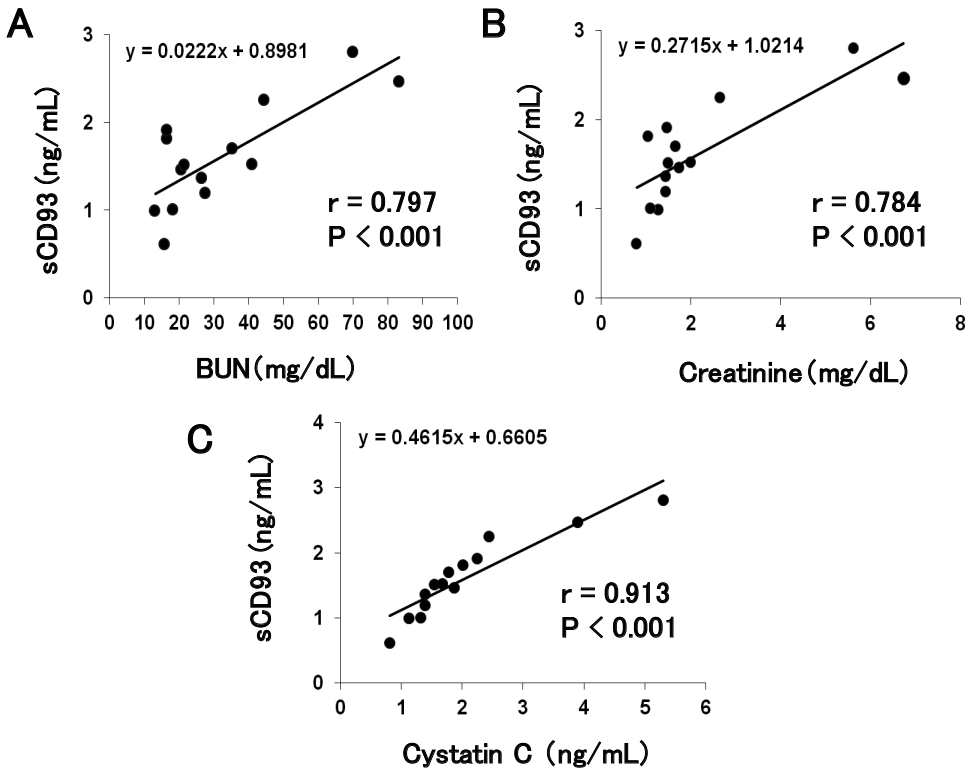

Figure 4. Analysis of the correlations of the serum concentrations of sCD93 and the BUN, serum creatinine, and serum cystatin C in patients with CRF. A: Correlation between serum sCD93 and BUN (r = 0.797, P < 0.001) in all CRF patients. B: Correlation between serum sCD93 and serum creatinine (r = 0.784, P < 0.001) in all CRF patients. C: Correlation between serum sCD93 and serum cystatin C (r = 0.913, P < 0.001) in all CRF patients.

慢性腎不全患者における可溶性CD93の血清レベル

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要　旨

慢性腎不全（CRF）患者における可溶性CD93（sCD93）の血清レベルを自主開発した抗ヒトCD93抗体（mNI-11）とオリジナル構築酵素抗体法（EIAキット）を用いて検討した。その結果、CRF患者血清中のsCD93は健康人と比較して有意に高値を示した。また、血清中インターロイキン-6（IL-6）もCRF患者は健康人に比較して有意に高値を示した。CRF患者血清中のsCD93と腎機能マーカーである尿素窒素、血清クレアチニン、血清シスタチンCとの相関を解析したところ、いずれも強い相関を示した。特に血清シスタチンCとはより強い相関を示した（r＝0.903、P＜0.001）。以上の結果から、CRF患者血清中のsCD93は、特に血清シスタチンCとの相関性がより強いことから、早期の腎障害を検出する新規のバイオマーカーになることがわかった。

キーワード：バイオマーカー，慢性腎不全，血清シスタチンC，可溶性CD93分子