Research Paper

N-acetyl-seryl-aspartyl-lysyl-proline: A new potential serum biomarker of rheumatoid arthritis

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Abstract **Background.** Angiogenic factors play an important role in the pathogenesis of rheumatoid arthritis (RA) through microvessel proliferation. *N*-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) is a tetrapeptide cleaved from the N-terminal of thymosin β4, known as a stimulator of angiogenesis. The objective of this study was to investigate the expression of sera and synovial fluid levels of AcSDKP relative to clinical manifestations in patients with RA.

Methods. The concentration of AcSDKP in sera and synovial fluids was measured using a high throughput method based on on-line solid-phase extraction liquid chromatography tandem mass spectrometry (SPE-LC-MS/MS). Serum or synovial fluid samples were collected from 52 patients with RA, 22 patients with osteoarthritis (OA), and 5 healthy controls. We included serum samples from 22 patients with RA treated with disease modifying antirheumatic drugs (DMARDs) at the beginning and after 12 weeks of treatment.

Results. Median serum levels of AcSDKP were 2.64 (ng/mL), 1.65 (ng/mL) and 0.69 (ng/mL) in patients with RA, patients with OA and healthy controls, respectively. There was positive correlation between serum AcSDKP and serum CRP in patients with RA. However there were no correlations between serum levels of AcSDKP and serum matrix metalloproteinase (MMP)-3, and ESR. Serum levels of AcSDKP and DAS28 [ESR] reflected improvements in the disease activity following treatment with DMARDs.

Conclusions. This is the first report demonstrating that the serum levels of AcSDKP might serve as a useful clinical marker of RA.

Key words: *N*-acetyl-seryl-aspartyl-lysyl-proline, biomarker, synovial fluid, rheumatoid arthritis, On-line solid-phase extraction liquid chromatography tandem mass spectrometry

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Received: October 29, 2020. Accepted: March 15, 2021.
Epub April 23, 2021.
DOI: 10.24508/mms.2021.06.004

Introduction

Rheumatoid arthritis (RA) is a progressive, autoimmune disease, characterized by chronic synovial inflammation and consistent degradation of articular cartilage and bone[1,2]. Angiogenesis is also known to play a key role in RA, and thus the ability to induce and sustain angiogenesis seems to be acquired in prolonged synovitis[3]. The modulation of angiogenesis is suggested to be reasonable strategy

in treatment of RA, because a lot of angiogenic factors such as vascular endothelial growth factor (VEGF), gliostatin/thymidine phosphorylase and fibroblast growth factor are overexpression[4–8].

Thymosin β4 (Tβ4) is the major actin-sequestering molecule in all eukaryotic cells, and a potent regulator of actin polymerization in mammals[9]. Recent studies have provided evidence that Tβ4 might have a role in the pathogenesis of RA. The level of Tβ4 has been shown to significantly increase in the synovial fluid (SF) and serum of patients with RA[10,11].

N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) is a tetrapeptide cleaved from the N-terminal of Tβ4[12]. This tetrapeptide is involved angiogenesis beside the regulation of the immune response and has been recognized to be an angiogenic activity. It inhibits the proliferation of human hematopoietic progenitors, and it has been reported as an angiogenic activity[13,14].

We hypothesized that AcSDKP may play an important, pathogenic role in the proinflammatory cascade during the course of RA. The aim of this study was to investigate the distribution of AcSDKP between the serum and SF in patients with RA and osteoarthritis (OA), and the serum of healthy controls. Our present study identified that serum AcSDKP could serve as a useful biomarker of RA.

Materials and Methods

Patients and controls

52 patients who met the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) criteria for the diagnosis of RA[15] were recruited from Nagoya City University Hospital for this study. These patients had not any evidence of infectious diseases in the last month prior to data collection and any history of malignancy and secondary Sjögren's syndrome. No cases had been taking angiotensin converting enzyme (ACE) inhibitors. Demographical (age and gender) and disease-related parameters of all patients were recorded at the time of blood sampling. With respect to disease specific parameters, disease duration, tender joint count, swollen joint count, and a patient global assessment were evaluated. In addition, we measured the serum levels of C-reactive protein (CRP) and matrix metalloproteinase-3 (MMP-3), the erythrocyte sedimentation rate (ESR), and the presence of the rheumatoid factor (RF) as well as anti-cyclic citrullinated peptide antibody (ACPA). Disease activity of RA patients was evaluated with the DAS28 [ESR] score[16] and was calculated at the time of blood collection during the clinical appointment. Anti-rheumatic therapy and any other concomitant treatments were recorded at the same time. The characteristics of the 52 patients with RA are presented in Table 1. Most patients were positive for RF and ACPA and had an erosive form of RA. Remission of RA according to DAS28 ($<$2.6) was observed in 10 patients (8 females and 2 males). At the time of examination, all patients were treated with at least one conventional synthetic disease modifying anti-rheumatic drugs (csDMARDs): methotrexate (MTX) (44 patients), sulfasalazine (4 patients), and tacrolimus (15 patients). Biological DMARDs (bDMARDs) were used in 11 patients (21.1%) (adalimumab in 4, etanercept in 3, and tocilizumab in 4 cases, respectively). Concomitantly, low-dose prednisone ($\leq$ 10 mg/d) was used in 12 patients (23.0%).

Table 1. Characteristics of 52 patients with RA

Variable	Results
Demographic variables	
Gender (F/M)	42/10
Age (years)	59 (25–83)
RA related variables	
Disease duration (years)	10.5 (0.5–49)
CRP (mg/dL)	0.7 (0.03–13.6)
ESR (mm/h)	30.5 (2–108)
MMP-3 (ng/mL)	84.2 (10–1752)
RF or ACPA positive/negative	37/15
DAS 28 [ESR]	3.72 (1.11–6.66)
Current MTX usage (number, %)	44 (84.6)
Current GC usage (number, %)	12 (23.0)
Current biologic therapy (number, %)	11 (21.1)

Values are median (range) or numbers (%).
CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; RF: rheumatoid factor; ACPA: anticitrullinated protein antibodies; DAS: disease activity score; MTX: methotrexate; GC: glucocorticoid.

Consecutive pairs of sera were collected at the time of change DMARDs and after 12 weeks in 22 patients with RA who did not respond to previous treatments. The clinical details of these patients are shown in Table 2. Serum samples were obtained from 22 patients with OA (18 females and 4 males) who met the ACR criteria for the classification of OA[17]. The median age of the patients was 73.5 years, with an age range of 50–82 years. Five normal serum samples were obtained from healthy individuals who vis-

Table 2. Characteristics of 22 patients with RA at the baseline

Laboratory variable		
No. of patients (F/M)	22	(16/6)
Age (years)	58.5	(26−81)
Disease duration (years)	9.1	(0.1−40.0)
CRP (mg/dL)	2.65	(0.03−13.57)
ESR (mm/h)	42	(2−88)
MMP-3 (ng/mL)	289	(18.9−934.5)
DAS28 [ESR]	4.22	(2.32−6.66)
Current MTX usage (number, %)	14	(63.6)
Current GC usage (number, %)	6	(27.2)
Current biologic therapy (number, %)	14	(63.6)

Values are median (range) or numbers (%).
CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; RF: rheumatoid factor; ACPA: anticitrullinated protein antibodies; DAS: disease activity score; MTX: methotrexate; GC: glucocorticoid.

ited our hospital for routine health tests and were without prior history of chronic inflammation or any form of arthritis, as evidenced by normal levels of CRP and ESR.

SF samples were obtained from 10 patients with RA (9 females and 1 male) and OA (7 females and 3 males) prior to total knee arthroplasty, respectively.

This study was approved by the Nagoya City University Ethics Committee (# 671), and informed consents were obtained from all patients.

On-line solid-phase extraction liquid chromatography tandem mass spectrometry (SPE-LC-MS/MS) analysis of AcSDKP

Synthetic AcSDKP was obtained from Peptide Institute Inc. (Osaka, Japan). The AcSDKP-$^{13}C_6$, $^{15}N_2$ was synthesized based on the previous report[18]. AcSDKP was measured using an SPE-LC-MS/MS system as previously described[19]. The stock solutions (0.1 mg/mL) for Ac-SDKP and Ac-SDKP-$^{13}C_6$, $^{15}N_2$ were prepared by dissolving the appropriate amount of standard in pure water. Calibration standards were prepared by diluting an aliquot of the stock solution in pure water.

The lower limit of quantification (LLOQ) in human plasma sample was detected as the concentration of the lowest calibration by on-line SPE-LC-MS/MS. The LLOQ was 0.1 ng/mL based on signal per noise (plasma control sample) of 10[18].

Statistical analysis

All data were entered into an electronic database and analysed using GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA). All statistical tests were performed at a significance level of α=0.05, that is, all p-values ≤ 0.05 were considered significant. All confidence intervals were computed using a confidence level of 95%. Comparisons of patient groups were done with analysis of the variance (ANOVA) and Bonferroni test if the distribution was normal, if not with the Kruskal−Wallis with Dunn's post-hoc test was used to determine differences across groups for AcSDKP. All data were presented as median and interquartile (IQR). Pearson correlation test was used to determine the association between log-transformed AcSDKP and clinical and laboratory variables. The statistical significance of the differences in the levels of the indicated parameters between baseline and the indicated treatment time points was examined using the Wilcoxon signed rank test.

Results

Calibration curve and multiple reaction-monitoring (MRM) chromatogram of Ac-SDKP and internal standard in human plasma for recovery test

Standard solutions of Ac-SDKP were prepared in pure water and added to a fixed concentration of Ac-SDKP-$^{13}C_6$, $^{15}N_2$ (1 μg/mL) for a calibration curve covering the concentration range from 0.1 to 75 ng/mL for human plasma samples (Fig. 1a). We proposed the method to evaluate endogenous Ac-SDKP levels in human plasma samples. Quantitative analysis was performed using multiple reaction-monitoring (MRM) mode in order to maximize sensitivity of quantitative ion, and ratio of sample/internal standard (Fig. 1b).

Serum levels of AcSDKP in healthy controls and in patients with RA and OA

We compared the levels of AcSDKP in the serum of patient groups and healthy controls. We found that in healthy controls, AcSDKP was present only at low levels (a median level of 0.69 ng/mL) in the circulation. Patients with OA had a median serum level of AcSDKP of 1.65 ng/mL, whereas patients with RA had a median level of 2.64 ng/mL, respectively. The Serum levels of AcSDKP were significantly increased in the RA patients group when compared to either healthy control or OA group (p<0.05; Fig. 2). There was no statistically significant difference in

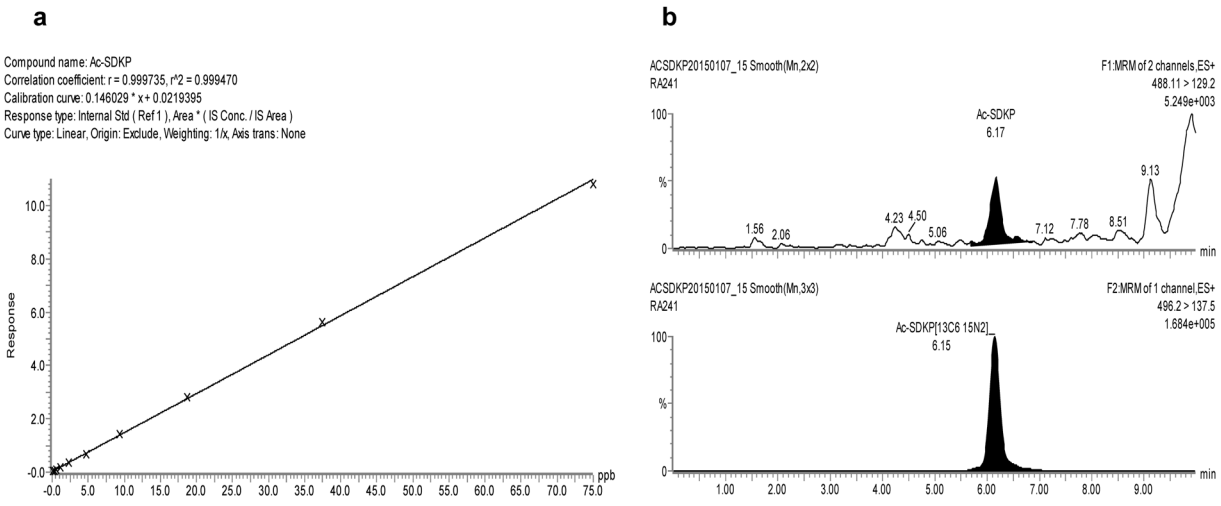

Fig. 1.

(a) Calibration standards were prepared by diluting an aliquot of the stock solution in pure water. Standard solutions of Ac-SDKP were prepared in pure water and added to a fixed concentration of Ac-SDKP-$^{13}C_6$, $^{15}N_2$ for a calibration curve covering the concentration range from 0.1 to 75 ng/mL for human plasma samples. (b) Representative multiple reaction-monitoring (MRM) chromatogram of AcSDKP. Lower graph indicates 1 ng/mL of AcSDKP-$^{13}C_6$, $^{15}N_2$ and the upper graph shows serum sample from RA patients.

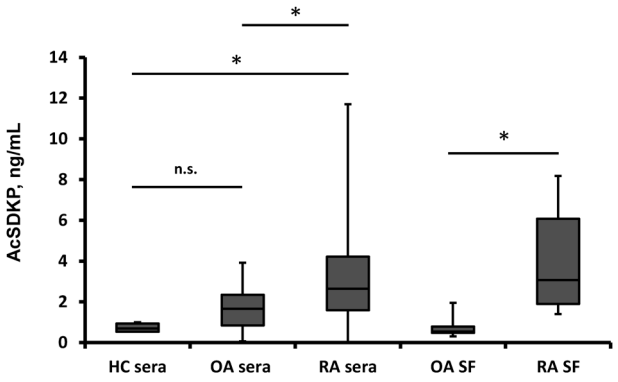

Fig. 2. Serum and synovial fluid levels of AcSDKP in patients with RA, OA, and healthy controls.

Box and whisker plots denote maximum and minimum, IQR and median. $^*p<0.05$ was considered significant.

AcSDKP levels between healthy controls and OA patients. These data suggest that AcSDKP levels are elevated in RA patients compared to OA and healthy individuals.

RA disease activity is categorized as being in remission (DAS 28 [ESR]<2.6), low (DAS 28 [ESR]<3.2), moderate (DAS 28 [ESR]≥3.2), or high (DAS 28 [ESR]>5.1) based on specific DAS 28 [ESR] cut-off scores. Because the vast majority of patients with RA in our study were undergoing treatment at the time of study, and most patients were diagnosed with low to moderate disease activity, we divided patients with RA into two groups based on the DAS28 [ESR] score to evaluate the ability of AcSDKP to monitor disease severity and activity: patients who were in remission or low disease activity (DAS28 [ESR]<3.2) and patients with moderate to high disease activity (DAS28 [ESR] ≥ 3.2). The RA group with DAS28 [ESR] lower than 3.2 (n=23) had a median serum level of AcSDKP of 3.42 ng/mL and the RA group with DAS28 [ESR] higher than 3.2 (n=29) had a median level of 2.34 ng/mL. There was no statistically significant difference between these groups (data was not shown).

Synovial fluid levels of AcSDKP in patients with RA and OA

AcSDKP was detected in the SF of patients with RA and OA. The median SF levels of AcSDKP in patients with OA were shown to be 0.54 ng/mL, whereas patients with RA exhibited a median level of 3.07 ng/mL. AcSDKP levels in the RA SF were shown to be significantly higher than in OA SF (Fig. 2).

Correlation between serum levels of AcSDKP and clinical features

Pearson correlation analysis confirmed the correlation between serum levels of log AcSDKP in RA patients and clinical features. Serum levels of log AcSDKP showed slightly positive correlation with CRP (r=0.23, p=0.95), however there was no statistically significant difference. Serum levels of log AcSDKP had no correlation in DAS28 [ESR], MMP-3, ESR. In addition, log AcSDKP exhibited a negative trend that did not reach statistical significance with RF (Fig. 3).

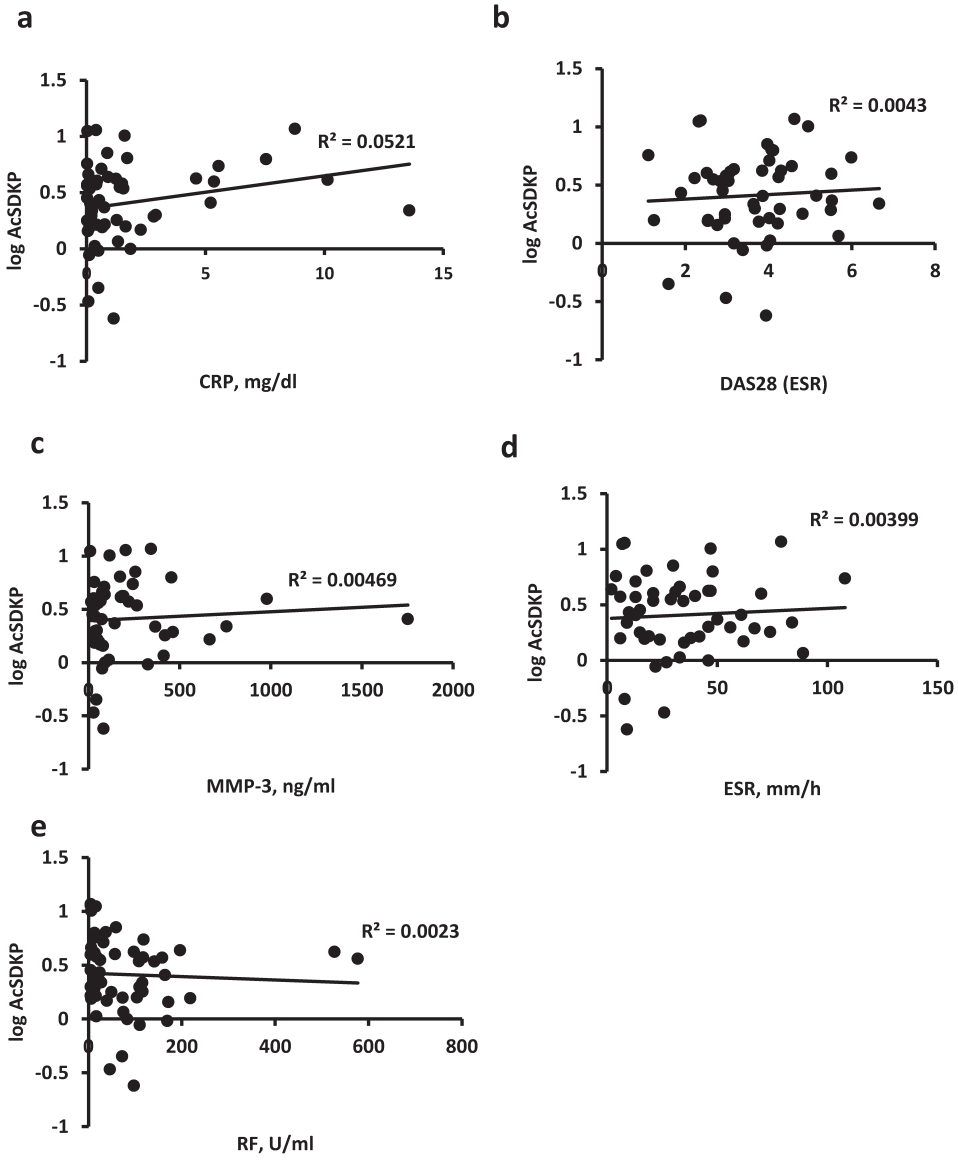

Fig. 3. Correlation between serum levels of log AcSDKP and indicated clinical measures in patients with RA.
The *x*-axis reflects the concentration of CRP in mg/dL, the DAS28 [ESR] score, the concentration of MMP-3 in ng/mL, the measurement of ESR in mm/h, and the concentration of RF in U/mL. The *y*-axis reflects the plasma concentration of log AcSDKP in ng/mL. Scatter plot graphs showing the correlation between (a) CRP and AcSDKP, (b) DAS28 [ESR] and AcSDKP, (c) MMP-3 and AcSDKP, (d) ESR and AcSDKP, and (e) RF and AcSDKP.
The relationship between these variables was evaluated using the Pearson correlation test. Trend lines indicate linear correlation.

Consecutive 22 pairs of serum AcSDKP at the time of changing DMARDs and after 12 weeks

The mean DAS 28 [ESR] score of 22 patients was 4.22 and decreased to 2.79 after 12 weeks of treatment ($p<$ 0.01). Clinical and laboratory parameters decreased significantly after 12 weeks of treatment in all patients: AcSDKP levels from 3.74 to 1.98 ng/mL (mean values), CRP levels from 2.65 to 0.25 mg/dL (mean values), MMP-3 levels from 289.0 to 107.3 ng/mL (mean values), and ESR levels from 42 mm/h to 18 mm/h (mean values) at the time of changing DMARDs and after 12 weeks, respectively (Fig. 4).

Discussion

Recent progress in the treatment of RA has been made through introduction of MTX, bDMRADs and Janus kinase inhibitors. A delay in initiating therapy against RA could adversely affect treatment outcomes, such as disease activity, remission, functional ability, and radiological progression. Therefore, early diagnosis and effective treatment is very important[20,21]. There is an unmet need for specific and easy-to-measure biomarkers for the diagnosis of RA and identification of patients who are at increased risk of developing erosive, joint destructive disease. We focused on

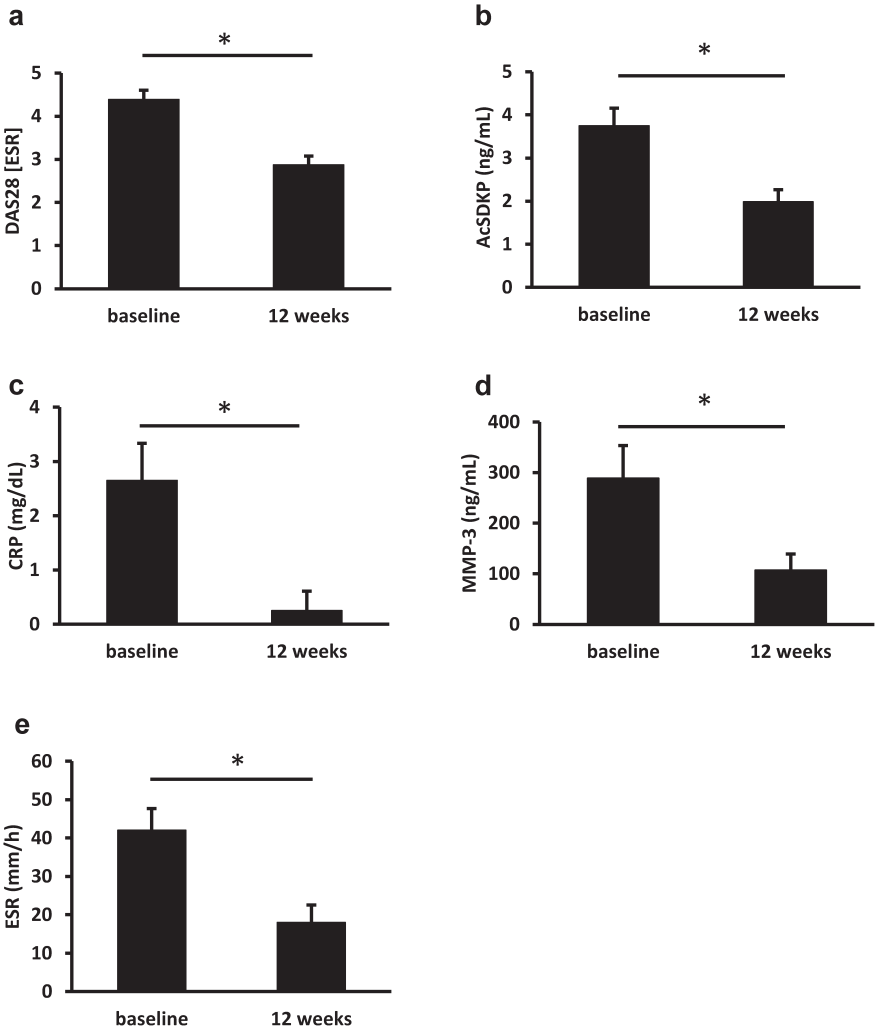

Fig. 4. Serum samples were collected from 22 patients with RA at baseline and after 12 weeks of treatment with DMARDs. DAS28 [ESR] (a), serum levels of AcSDKP (b), CRP (c), MMP-3 (d) and ESR (e) were significantly down-regulated at 12 weeks after treatment compared with baseline. Results are presented as the mean±SEM. The mean differences in the clinical measures between baseline and 12 weeks later were evaluated with the Wilcoxon signed rank test. $^{*}p<0.05$ was considered significant.

AcSDKP and investigated whether this peptide was an RA biomarker.

Osteoarthritis (OA) is a joint disease in which articular cartilage degeneration and collapse, as well as osseous proliferation, are progressively impaired in joint function. Pain is also strong, leading to significant deterioration in activities of daily living and quality of life. Compared to RA, OA has generally been described as a non-inflammatory joint disease. In recent years, it has become clear that various inflammatory cytokines are involved in the development of OA, however the expression levels are extremely low compared to RA. In this study, sera from healthy subjects and OA patients were used as controls.

According to our results, the level of AcSDKP in the serum of patients of RA was 3-fold higher than in healthy controls, and 1.5-fold higher than in patients with OA. Importantly, the serum levels of AcSDKP were low or undetectable in healthy controls, as well as in patients with OA. Similarly, we found that the levels of AcSDKP in the synovial fluid of patients with RA were significantly higher than in patients with OA.

It is known that AcSDKP is physiologically degraded by ACE and partially eliminated in urine. Therefore, patients receiving ACE inhibitor treatments displayed higher levels of AcSDKP in both their plasma and urine[22,23]. In our study, none of our tested subjects were prescribed ACE inhibitors.

The expression of AcSDKP was originally considered a reflection of angiogenesis. Indeed, AcSDKP and its precursor peptide, Tβ4, enhanced angiogenesis and exerted antifibrotic effects associated with the normalization of organ function[24,25]. High levels of Tβ4 and AcSDKP have been

associated with tumor progression in hematologic malignancies, with the levels of AcSDKP being reported to be markedly elevated in solid neoplasms. An association between the levels of AcSDKP and tumor angiogenesis has been observed[14,26,27]. In rats after traumatic brain injury, administration of AcSDKP promoted neurovascular remodeling include angiogenesis and neurogenesis, as well as improves functional recovery[28]. In healthy condition, the major source of the AcSDPK have been Tβ4[12,29]. In recent study, AcSDPK content was not completely absent in mice lacking Tβ4 [25,30]. Nevertheless ACE activity in SF from patients with RA was significantly greater than that in SF from OA patients[31,32], the concentrations of AcSDKP were higher in RA-derived SF than in OA-derived SF. As the reason, the production of Tβ4, a precursor of AcSDKP, is abundant in RA[10,11], and there may be a source of AcSDKP different from healthy condition.

Vascular proliferation is known to be one of the earliest events in the development of RA and could be the causal process triggering RA, which has been described as an angiogenesis-dependent disease[33,34]. A number of angiogenic factors such as gliostatin and VEGF have been shown to be involved in the neovascularisation process in the RA joint. Measurements of the serum levels of gliostatin and VEGF have been useful methods for monitoring the disease activity of RA[6,8,35]. Therefore, we investigated whether the serum levels of AcSDKP were decreased following treatment with bDMARDs or MTX. AcSDKP may be instrumental as an easy-to-measure serum marker that can facilitate diagnosis of RA and discriminate RA from OA and potentially other forms of arthritis with an inflammatory component. In previous reports, AcSDKP had been measured using enzyme-linked immunosorbent assay. In search of more accurate measurements, we have adopted the method of SPE-LC-MS/MS analysis which is the quantitative determination of AcSDKP with relatively simple and highly sensitive[18,19]. Moreover, this analytical method is suitable for measuring large numbers of clinical samples.

The expression of AcSDKP was determined locally in the inflamed joints and also systemically in circulation of patients with RA was measured extracellularly. Whether AcSDKP found extracellularly originates from synovial tissues or is a subject of active secretion is presently unknown. AcSDKP has a half-life of 4.5 min in the circulation, because it is hydrolyzed by ACE[36]. The half-life of CRP is 19 h[37], while the half-life of AcSDKP is as short as 3−4 h in sera[38]. It is suggested that biomarkers with a short half-life will more accurately indicate the disease activity at the time of measurement.

Pearson correlation analysis confirmed the correlation between serum AcSDKP levels in RA patients and clinical features. AcSDKP levels were not significantly correlated with clinical features. Serum AcSDKP levels showed slightly positive correlation with CRP, DAS28 [ESR], MMP-3 and ESR, but not with RF, which exhibited a negative trend that did not reach statistical significance. Overall, these findings show a statistically no significant association between AcSDKP and clinical features. However, this trend needs to be further investigated and validated using larger patient cohorts that cover the entire spectrum of RA disease activity.

From simply diagnostic point of view, the serum levels of Tβ4 were preferable to AcSDKP, because it was reported that serum levels of Tβ4 were approximately 10 fold higher in RA patients than in healthy controls. Although, from the view point of responsiveness to anti-rheumatic treatment, no significant difference was shown in the change in Tβ4 concentration before and after therapy[10].

In the present study, we investigated the effects of DMARDs on the serum levels of AcSDKP. Serial laboratory measurements revealed a reduction in the levels of AcSDKP following administration of DMARDs. As well as clinical features, such as DAS28 [ESR], CRP, MMP-3 and ESR, the levels of AcSDKP was also shown to have a high response to treatment with DMARDs. These results suggest that AcSDKP may be involved in the pathophysiology of RA and may have an effect in the chronic arthritis during the process of RA.

Our study has several limitations. First, since the study population was small, the result may not be valid for all patients with RA. Second, several baseline characteristics, such as concomitant use of corticosteroid, bDMARDs, and csDMARDs were different among our study population. Third, in real world RA therapy using the National Health Insurance medical examination and blood sampling every day or every week are difficult. Serial data pertaining to AcSDKP levels were obtained from some patients treated with DMARDs every 4 weeks. These patients were found to be responsive to therapy and serum AcSDKP, CRP, MMP-3 and DAS 28 [ESR] levels gradually decreased with course of treatment (data not shown).

Conclusions

In conclusion, we found that the levels of AcSDKP were increased in the serum and synovial fluid of patients with RA. Moreover, the serum levels of AcSDKP reflected improvement in the disease activity during the clinical course of patients with RA. Our results validate AcSDKP for future studies focusing on its potential as a biomarker for RA.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Nagoya City University Ethics Committee and Informed consent was obtained from all the patients upon their enrollment in the study.

Consent for Publication

Written informed consents were obtained from all the patients for publication of this report.

Acknowledgements

This research was supported, in part, by MEXT/JSPSKAKENHI Grant Number 18K09114, 19K18473 and 20K09465.

We would like to thank Editage (www.editage.com) for English language editing.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

YO, YK, KA, KI and YW-N designed the project, performed most of the experiments and analyzed the data. YO and YK also wrote the manuscript. HO, YJ, HY, KM, HA, GK and NT assisted with the interpretation of the data and the preparation of the manuscript. MK and MN provided the RA serums and participated in designing some of the experiments. YW-N, HM designed the study, analyzed the data, supervised the overall project and wrote the manuscript. All authors read and approved the final manuscript.

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