Upregulation of thrombospondin-1(TSP-1) expression in synovial tissues and plasma of rheumatoid arthritis (RA): Role of transforming growth factor-β1 toward fibroblast-like synovial cells

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Abstract

Objectives: To investigate the role of TSP-1 in RA.

Methods: Expression of TSP-1 in synovial tissues was determined by immunohistochemistry. Expression of TSP-1 in rheumatoid fibroblast-like synovial cells (FLS) was investigated by quantitative real-time PCR and ELISA. Correlations among the plasma TSP-1 and other parameters in RA patients were examined.

Results: Expression of TSP-1 was increased in rheumatoid synovial tissues. TGF-β1 clearly increased TSP-1 expression in FLS on both mRNA and protein level. Changes in plasma TSP-1 were associated with those in DAS28-ESR and plasma TGF-β1.

Conclusions: TSP-1 might be critically involved in the disease process of RA through TGF-β1/TSP-1 axis.

Introduction

Our recent investigations using circulating immune complexes (CICs) analysis revealed that CIC-associated TSP-1 is frequently found in serum of RA but not in other rheumatic diseases and healthy controls [1]. TSP-1 is a multifunctional glycoprotein expressed in cells from multiple lineages [2]. Although the role of TSP-1 in inflammation remains obscure, recent studies have shown that TSP-1 acts as a pro-inflammatory protein. For example, TSP-1 binds to specific receptors on polymorphonuclear leukocytes and stimulates their motility [3]. Other study showed that TSP-1 activates the macrophages through toll-like receptor 4 pathway [4].

In the present study, to establish the role of TSP-1 in RA both *in vitro* and in clinical practice, we used three approaches. First, we investigated the expression of TSP-1 in synovial tissues. Second, we investigated whether the expression of TSP-1 in fibroblast-like synovial cells (FLS) from RA patients is influenced by cytokines and growth factors. Finally, we analyzed the correlation between plasma levels of TSP-1 and clinical parameters of RA. By using these approaches, we show that TSP-1 is, at least in part, associated with the pathogenesis of RA.

Materials and Methods

Each patient provided a signed consent form to participate in the study, which was approved by the Institutional Review Board of Nagasaki University. All RA patients fulfilled the classification criteria for RA [5]. We obtained synovial tissues from patients with RA or osteoarthritis (OA) at the time of orthopedic surgery. FLS from RA patients were isolated from synovial tissues as described previously [6].

Reagents and stimulation assays

FLS were stimulated for 24 hours with transforming growth factor beta 1 (TGF-β1) (5 ng/ml), interleukin-1β (IL-1β) (10 ng/ml), IL-6 (100 ng/ml) with soluble IL-6 receptor (100 ng/ml), interferon-γ (IFN-γ) (10 ng/ml) (all from R&D Systems), tumor necrosis factor-α (TNF-α) (100 ng/ml) (Millipore). In another subset of stimulation experiments, FLS were stimulated with various concentrations of recombinant TSP-1 (R&D Systems) for various time periods (24, 48, or 96 hours).

RNA isolation and quantitative real-time PCR analysis

RNA was isolated with Trizol reagent (Life Technologies) and reverse-transcribed. Quantification of TSP-1 mRNA was performed by SYBR Green Real-time PCR as previously described [7]. The following primers were designed: TSP-1:5'-GGAGACAAAGACTGGCTTCTGGAC-3'(forward), 5'-GGCCACTGCAGGTGATGAGTAA-3'(reverse); β-actin:5'-AGCCTCGCCTTTGCCGA-3'(forward), 5'-CTGGTGCCTGGGGCG-3'(reverse). Expression of β-actin was used as endogenous control. For relative quantification, the comparative threshold cycle method was used.

Immunohistochemistry

Synovial tissues were stained using the labeled streptavidin-biotin method. We used TSP-1 antibody at 1:25 dilution (Thermo Scientific) or with mouse IgG (Jackson Immunoresearch Laboratories Inc.) as a negative control. Staining was visualized with diaminobenzidine using a peroxidase substrate kit then the area of TSP-1-positive staining was randomly quantified in one field per section by imaging software as we

previously described (Winroof; Mitani Corp.) [8].

ELISA

Proteins were detected by ELISA using ELISA kits specific for TSP-1, IL-6, TGF-β1, TNF-α and vascular endothelial growth factor (VEGF) according to the manufacturer's instructions (R&D Systems).

Clinical evaluation of the patients with RA

The present study included 16 patients with active RA (Detailed characteristics of the patients, as well as additional information concerning the methodology used in these studies, are available upon request from the authors). Disease activity score (DAS) 28-ESR, plasma or serum concentrations of TSP-1, TGF-β1, IL-6 and VEGF were examined at baseline and after introduction of DMARD therapy (from 3 months to 15 months).

Statistical analysis

GraphPad Prism software was used for statistical analysis. Normal distribution of the data was confirmed using the Kolmogorov-Smirnov test. For related data, statistical significance was evaluated by Student's paired *t*-test (parametric data) or by Wilcoxon matched-pairs signed rank test (non-parametric data). Student's unpaired *t*-test (parametric data) or Mann-Whitney U test (non-parametric) was used for unrelated data. The strength of the correlation was judged by Spearman's rank correlation coefficient. All data were expressed as the mean and standard deviation (SD). Values of $p < 0.05$ were considered statistically significant.

Results

Elevated expression of TSP-1 in RA synovial tissues

Pronounced expression of TSP-1 was found in the synovial lining and sublining layers in RA synovial tissues, but also perivascular areas as compared with that of osteoarthritis patients (Figure1A). The quantification analysis using Winroof software confirmed these results (Figure1B).

TSP-1 production from FLS is induced by TGF-β1

Among inflammatory cytokines and growth factors, TGF-β1 most clearly increased TSP-1 expression in FLS on an mRNA level (Figure2A). To confirm this, we analyzed the expression of TSP-1 on the protein level after stimulation with TGF-β1 by ELISA (Figure2B). Similar to mRNA levels, TGF-β1 markedly induced TSP-1 protein, indicating that TGF-β1 stimulates TSP-1 production at both the transcriptional and protein levels. However, other stimuli, including IL-1β, did not induce TSP-1 production at the protein level [representative results for TSP-1 in the culture supernatants of each stimulated FLS were 1.45 ng/ml (TNF-α), 0.97 ng/ml (IL-1β), 2.34 ng/ml (IL-6), 1.38 ng/ml (IFN-γ), 59.73 ng/ml (TGF-β1) and control (3.46 ng/ml)].

TSP-1 did not induce production of TGF-β1, IL-6 or TNF-α

We stimulated FLS with TSP-1 for various time periods (24, 48, 96 hours) and at different concentrations. Both TGF-β1 and TNF-α were not detected after TSP-1 stimulation. IL-6 could be detected, but not increased by TSP-1 [The representative

mean values of IL-6 after TSP-1 stimulation (for 24 hours) were 105.6 pg/ml (control), 107.0 pg/ml (10ng/ml), 94.7 pg/ml (100ng/ml), 88.0 pg/ml (1000ng/ml)].

Plasma levels of TSP-1 correlated with disease activity of RA

Following DMARDS were used as new treatment in our study: methotrexate (12 patients), salazosulfapyridine (1 patient), etanercept (2 patients), infliximab (1 patient). Overall, DAS28-ESR and serum VEGF (5.18±1.30, 724.8±647.1 pg/ml at baseline, respectively) were significantly decreased after introduction of treatment (3.51±2.03, $P<0.05$, 514.3±411.2 pg/ml, $P<0.05$), whereas other parameters were no changed [Baseline: TSP-1 4.79±5.59 μg/ml, TGF-β1 18.47±8.63 pg/ml, IL-6 17.84±26.01 pg/ml. After induction of new treatment: TSP-1 5.16±5.49 μg/ml ($P=0.86$), TGF-β1 15.81±8.21 pg/ml ($P=0.28$), IL-6 8.63±12.04 pg/ml ($P=0.25$)].

There was large variability in the value of each parameter among each case, therefore we adopted to compare the amount of change. We investigated the correlations between plasma levels of TSP-1 and other parameters. The result was shown in Figure3. The changes (Δvalues) in TSP-1 significantly correlated with that in DAS28-ESR. Since it was found *in vitro* that TGF-β1 stimulates the production of TSP-1 in FLS, there was a clear correlation between the ΔTSP-1 and Δ TGF-β1 during DMARD therapies. Similar correlations were found between TSP-1 and IL-6, VEGF.

Discussion

In the present study we found that TSP-1 expression in synovial tissues was much higher in RA than OA. This result is consistent with previous reports [9,10]. The published data on the effects of TSP-1 in RA and inflammation have been controversial

[11,12]. But considering the result that expression of TSP-1 is evident in the lining and sublining layers of rheumatoid synovial tissues where active inflammation is found, TSP-1 might be involved in rheumatoid synovitis.

Here we could show that TGF-β1 significantly augmented TSP-1 synthesis from FLS. This is the first observation to describe the role of TGF-β1 as an activator of TSP-1 in FLS. Although TGF-β1 is known as a paradoxical regulator for inflammation [13], TGF-β1 has the competence to inhibit Fas-mediated apoptosis of FLS [14], activates the pathway of NF-kappaB coordinating with interleukin-1 and TNF-α and induces synovial lining hyperplasia [15,16]. The close interplay between TSP-1 and TGF-β1 has been well established. TSP-1 is known as an activating factor for a latent form of TGF-β1 [17]. We have found that production of TSP-1 from FLS is increased by the stimulation of TGF-β1, whereas TSP-1 does not induce production of TGF-β1. Our findings suggested TSP-1 does not directly act on cytokines and growth factors production. Further studies will be needed to better understand the role of the TGF-β1/TSP-1 axis in RA synovial tissues.

The Δvalues of DAS28, plasma TGF-β1, serum IL-6 and VEGF were significantly correlated with Δplasma TSP-1. Although TSP-1 showed no interaction with IL-6 *in vitro* study, Δplasma TSP-1 significantly correlated with Δserum IL-6. It might come from the effect not through synovial tissues or it might reflect the disease activity individually. Correlation between Δplasma TSP-1 and ΔDAS28 indicates that TSP-1 may be implicated in active disease of RA and could become a novel biomarker of RA as well as IL-6.

There are some limitations in this study. First we must refer to small sample size especially in clinical evaluations. It is apparent that larger number of samples is

appropriated to estimate the more accurate results. Second, in clinical evaluation, we enrolled only active RA patients. However, previous study reported that plasma concentrations of TSP-1 are elevated in RA patients as compared with healthy controls [11]. Therefore, we suspected that TSP-1 particularly involves in RA pathological condition.

In conclusion, our study showed that TSP-1, strongly expressed in RA synovial tissues, is induced by TGF-β1. Furthermore, the change of plasma TSP-1 by therapeutic intervention significantly correlated with the changes in disease activity. Taken together, these finding indicate that TSP-1 might be critically involved in the disease process of RA, and considered as a useful biomarker not only for diagnostic purposes but also for the evaluation of disease activity.

All authors declared non conflict of interest.

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Figure legends

Figure1

TSP-1 expression in synovial tissues from patients with RA and OA. **A**, Representative sections of synovial tissues stained for TSP-1 or control IgG. Positive staining of TSP-1 appears as a light brown color. Expression is seen in the synovial lining and sublining layers and perivascular areas. **B**, The quantification analysis of TSP-1 staining in RA (n=4) and OA (n=4) synovial tissues was performed using Winroof software. Expression of TSP-1 was determined relative to OA synovial tissue, which was defined as 1. Values are presented as the means.

Figure2

A, Increment of TSP-1 mRNA expression by TGF-β1. RA-FLS (n=4) was stimulated with TGF-β1 (5 ng/ml), IL-1β (10 ng/ml), IL-6 (100 ng/ml) with sIL-6R (100 ng/ml), TNF-α (100 ng/ml) or IFN- γ (10 ng/ml) for 24 h. TSP-1 expression was determined by SYBR Green real-time PCR and was stated relative to the control, which was defined as 1. Values are presented as the means ± SD. *$p<0.05$, **$p<0.01$ versus the controls (no stimulation).

B, Increment of TSP-1 protein production in culture supernatants from RA-FLS by TGF-β1. RA-FLS (n=6) was stimulated with TGF-β1 (5 ng/ml) for 24 hours. TSP-1 protein production in the supernatants was examined by ELISA. Values are presented as the means ± SD. *$p<0.05$ versus the controls (no stimulation).

Figure3

Correlations between Δvalues in plasma TSP-1 and those in DAS28-ESR (A), plasma TGF-β1 (B), serum IL-6 (C), and serum VEGF (D) after introduction of DMARD therapies (n=16). The Spearman's rank correlation coefficient and the corresponding p value are shown above each scatter plot.

Figure1

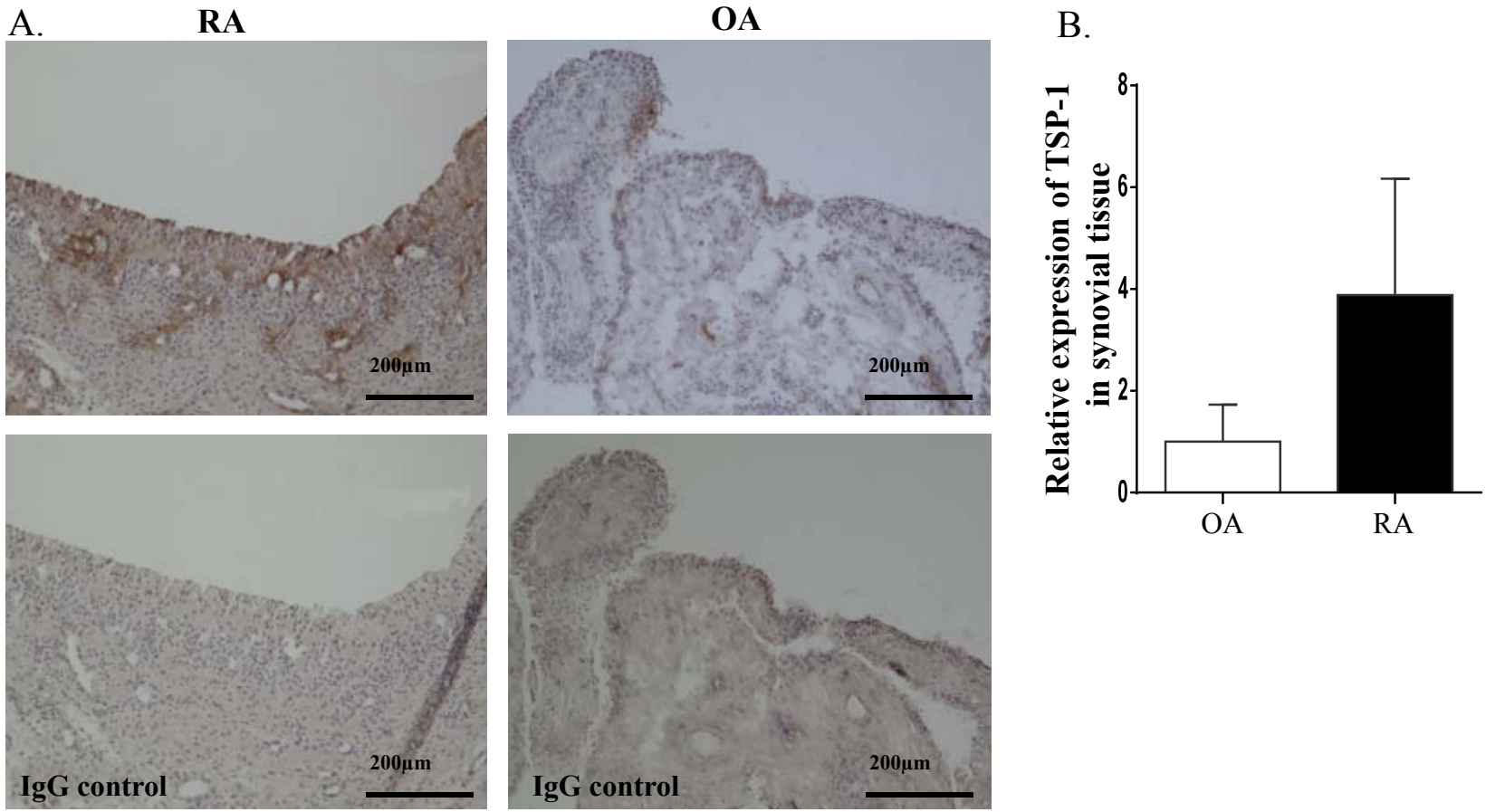

A.
RA
OA
200μm
200μm
IgG control
200μm
IgG control
200μm
B.
Relative expression of TSP-1 in synovial tissue
0
2
4
6
8
OA
RA

Figure2

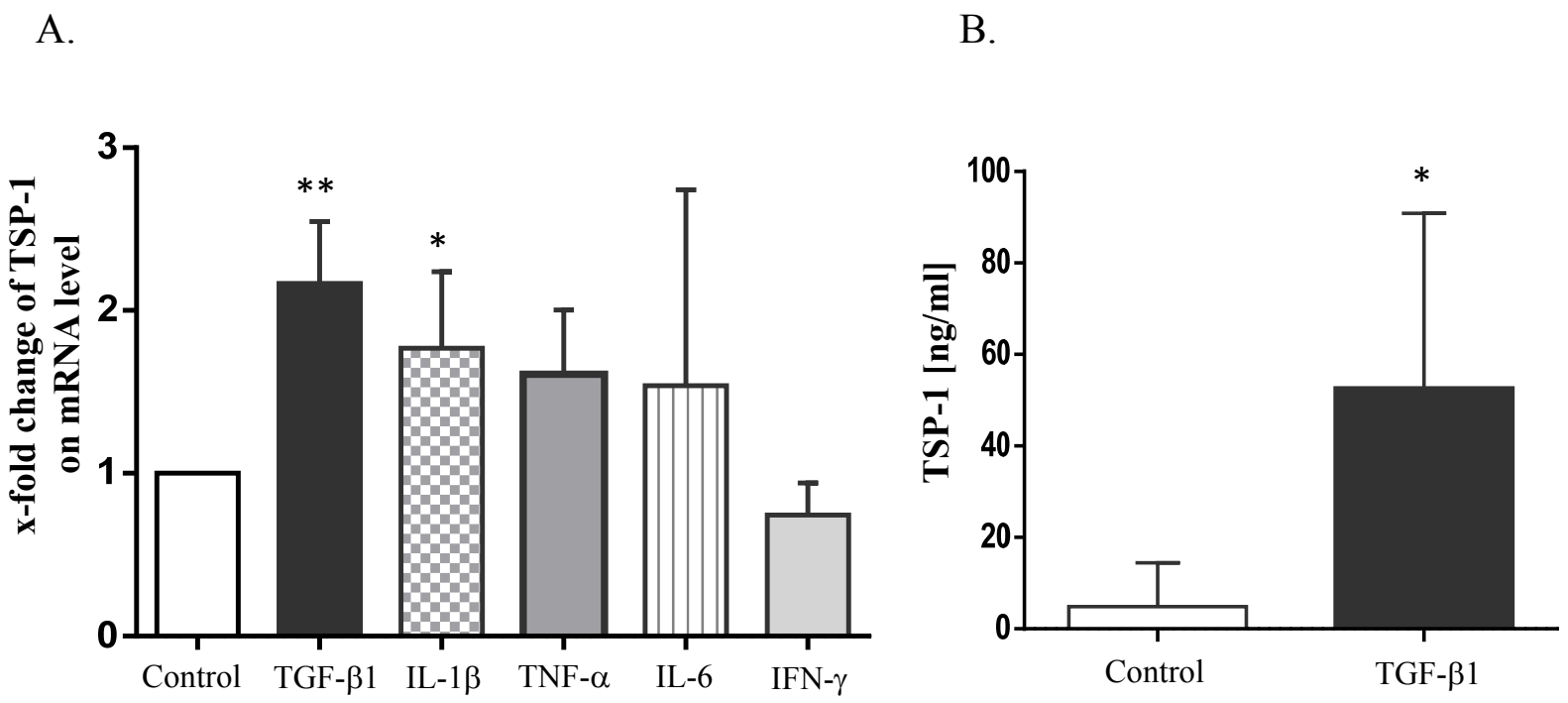
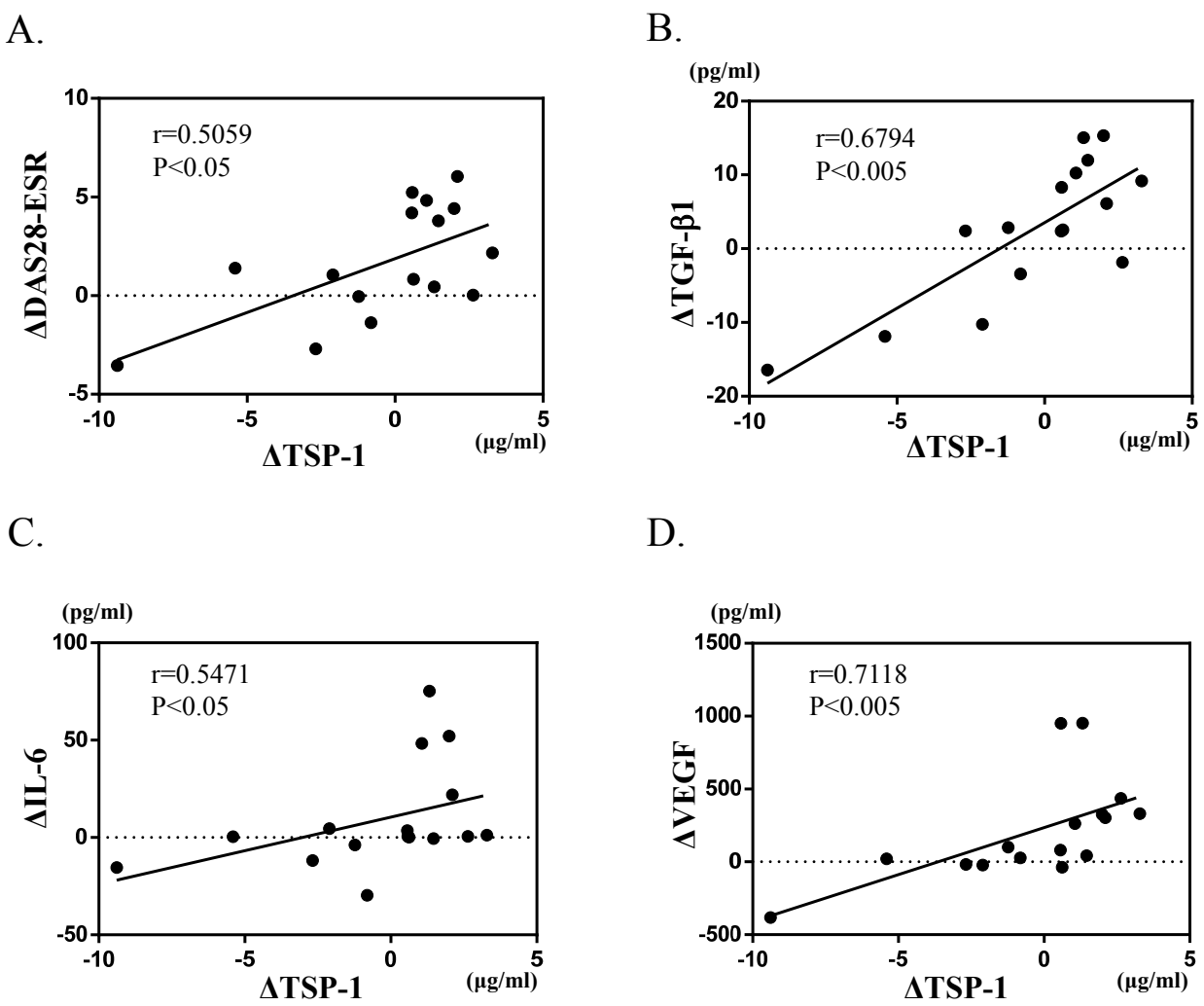

A.
B.
x-fold change of TSP-1 on mRNA level
3
2
1
0
**
*
Control
TGF-β1
IL-1β
TNF-α
IL-6
IFN-γ
TSP-1 [ng/ml]
100
80
60
40
20
0
*
Control
TGF-β1

Figure3


A.
r=0.5059
P<0.05
ΔDAS28-ESR
ΔTSP-1
(μg/ml)
B.
(pg/ml)
r=0.6794
P<0.005
ΔTGF-β1
ΔTSP-1
(μg/ml)
C.
(pg/ml)
r=0.5471
P<0.05
ΔIL-6
ΔTSP-1
(μg/ml)
D.
(pg/ml)
r=0.7118
P<0.005
ΔVEGF
ΔTSP-1
(μg/ml)