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Thrombospondin-1 and Transforming Growth Factor Beta Are Pro-Inflammatory Molecules in Rheumatoid Arthritis

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INTRODUCTION

The pathophysiology of rheumatoid arthritis (RA) has been extensively studied and multiple factors have been implicated in the onset and perpetuation of the disease.¹ The molecules that had been identified to date include but are not limited to cytokines and growth factors. Thrombospondin-1 (TSP1) is one of the proteins involved in RA.^{2–6} TSP1 is a multifunctional glycoprotein present in multiple cells including platelets, leukocytes and endothelial cells.⁷ TSP1 promotes thrombin generation on the cellular surface of leukocytes with a subsequent action on endothelial cells that upregulates the expression of TSP1. In addition, TSP1 is an effective activator of transforming growth factor beta (TGFβ).⁸ TGFβ is a pleiotropic immunoregulatory cytokine found up-regulated in RA.⁵ Even though TGFβ is considered as an anti-inflammatory molecule, in some pathologic conditions TGFβ may act as a pro-inflammatory cytokine.⁹ In RA in particular, elevated TGFβ levels in plasma and synovial fluid have been reported,¹⁰ however its role in this disease remains obscure.

This study determined the signature expression pattern in the cytokine network and its potential interface with TSP1 and TGFβ in RA by the use of protein profiling rolling- circle amplification (RCA). RCA is a useful alternative to measure multiple cytokine levels utilizing a single small sample volume of 50 μL. Among the advantages of the RCA method is that is more sensitive and specific when compared to conventional ELISA techniques. The specificity of the technique is due to its principle in which amplified signals remain localized at each of the microarray spots. Global patterns of cytokine expression are more likely to yield biological relevance and clinically useful information than assays of a single cytokine.

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METHODS

Study Design and Patient Characteristics

Patients affected with RA (n=20), diagnosed according to the 1987 American College of Rheumatology criteria were recruited at the time of their first visit from the Rheumatology Unit at Temple University Hospital. Patients were untreated for RA at the time of their first visit. Healthy volunteers (n=13) were selected from the Sol Sherry Thrombosis Research Center of Temple University. The entire research activities were carried out in accordance with the Declaration of Helsinki. Twenty milliliters of whole blood were collected in tubes containing EDTA for plasma isolation.

The average age of the RA patients was 51 ± 8.16 years. Ninety five percent were female. Patient's race/ethnicity was recorded as follows: 45% (n=9) African-American, 25% (n=5) White non-Hispanic and 30% (n=6) Hispanic. Average weight of the patients was 82.27 ± 38.7 kg with a body mass index average of 33.7 ± 11.3 . Concomitant hypertension was found in 35% and smoking was an associated factor in 25% of the patients. Active and mildly active disease was present in 35% and 65% of the patients respectively.

Circulating levels of TSP1 and TGF β by ELISA

TSP1 and TGF β were determined using commercially available quantitative sandwich enzyme immunoassays (ChemiKine™ Human TSP1/THBS1 EIA Kit, Chemicon International, Inc., Temecula, CA, USA and Quantikine® TGF β , R&D Systems, Inc., Minneapolis, MN, USA). Protocols, procedures and equipment were used accordingly to the manufactures specifications.

Multiplexed cytokine protein profiling on microarray by rolling-circle amplification (RCA)

Plasma samples were analyzed by Allied Biotech, Inc. (Ijamsville, MD) using the protocol previously described.¹¹ Briefly, teflon-coated glass slides containing 16 circular areas or "subarrays" were incubated with thiolsilane and γ -maleimidobutyryloxy succinimide ester. Monoclonal antibodies were placed in quadruplicate onto the slides using a pin-tool type microarrayer. Subsequently, slides were blocked and 10 microliters of plasma were applied to each subarray. After incubation, the biotinylated secondary antibodies were placed into each subarray. Previously prepared mouse monoclonal anti-biotin IgG conjugated to an amine-modified oligonucleotide was annealed with another oligonucleotide that had been circularized and applied to each subarray. The RCA reaction was carried out with a reagent containing T7 native DNA polymerase in the presence of Cy5 detector probe. Cytokines measured are listed in table I.

Statistical Analysis

Statistical evaluation was carried out by non parametric Mann Whitney test and multiple comparisons by one (group)-way multivariate analysis of variance. Follow-up univariate analyses of variance were performed as needed. Statistical significance was defined as $p < 0.05$.

RESULTS

Plasma levels of TSP1 and TGF β

Circulating plasma levels of TSP1 ($p=0.001$) were significantly elevated in the RA patients when compared with the control group. TGF β circulating plasma levels were elevated in the RA group, but the levels only showed a statistical trend ($p=0.1$) when compared with control (Table I).

Signature expression pattern for cytokines measured

Plasma circulating levels of pro-inflammatory cytokines/chemokines namely IL12, CXCL10/IP10 and CCL4/MIP1 β were found significantly elevated in the RA patients when compared to control group (Table I). The circulating plasma levels of IFN γ were found elevated but did not reach statistical significance when compared with the control group. In addition, two anti-inflammatory cytokines, namely IL4 and IL5 circulating plasma levels were found significantly elevated in patients afflicted with RA when compared to the control group. We found also statistical trends among other cytokines including IL12B and IL6 which were elevated in the RA group compared to the control group.

TSP1 Interface with Cytokine Network and TGF β

TSP1 displayed statistical trends in the RA group (not present in the control group) that correlated with some pro-inflammatory molecules, namely CCL4/MIP1 β ($r=-0.388$, $p=0.083$), and TGF β ($r=-0.398$, $p=0.074$). Noteworthy to mention is that in all cases the inverse correlation between TSP1 and pro-inflammatory cytokines seen in patients affected with RA was due to elevated circulating levels in plasma of TSP1 that exceeded the increment observed in CCL4/MIP1 β and TGF β .

DISCUSSION

We now report the profile of a full spectrum of cytokines and chemokines within the context of TSP1 and TGF β in plasma of patients with RA. To our knowledge this is the first study in the literature documenting such correlations by the use of this novel technique. A recent study utilizing the same technology has documented elegantly the cytokine signature profile but in patients with juvenile idiopathic arthritis (JIA).¹² The authors found a significant proinflammatory cytokine signature in plasma and synovial fluid of patients with JIA, specifically during the active disease. In contrast to JIA, in adult RA different cytokines are relevant, thus our study documents the protein fingerprint for adult RA.

TSP1 is found in trace amounts in plasma under physiologic conditions, however during the inflammatory response, TSP1 is avidly secreted from multiple cells.⁷ In this study, TSP1 was elevated in the plasma of RA patients when compared to controls. Previous microarray studies using RNA of monocytes isolated from RA patients showed upregulation of the TSP1 gene in conjunction with the TNF α , IL1 β and IL6 genes which are well known key players in the pathophysiology of RA.¹³ Studies have demonstrated that purified TSP1 is capable to induce TNF α and IL1 β production in human monocytes using similar TSP1 concentrations found in plasma of patients with RA.¹⁴ In our study the cellular source responsible for the TSP1 plasma levels could not be specifically defined by the parameters measured. However since plasma levels of CCL4/MIP1 β , a marker of monocyte activation, were increased; one potential cellular source for circulating TSP1 may have arisen from activated monocytes.

Previous studies have established the notion that TSP1 is a major regulator of TGF β activation in vivo.⁸ TGF β mediates an array of biologic processes including growth and development, inflammation and host defense and tissue repair. TGF β is generated by inflammatory cells as part of the cytokine network. In its defensive role, TGF β facilitates resolution of inflammation and promotes tissue repair. However, as for the cytokine network, a critical balance of this growth factor is required for its defensive properties to take place in an orchestrated fashion. The literature indicates that a local or systemic excess of this growth factor is associated with unresolved inflammation and indeed, TGF β plasma levels are elevated in RA patients and constitutively upregulated in RA synoviocytes. These observations are in agreement with the present study and represent an opportunity to further explore the interface of the cytokine network including TGF β and TSP1.

This study provides the signature expression pattern of the cytokines and the fine balance in the cytokine network in normal individuals and documents the imbalance that occurs in RA. This expression pattern is of interest to expand our perspective on the roles played by cytokine in RA. In the RA patients a significant increase in the plasma levels of CXCL10/IP10 were documented, a protein secreted from a variety of cells including monocytes, endothelial cells, and fibroblasts in response to interferon gamma (IFN γ).¹⁵ IFN γ levels in the RA group showed a trend for higher levels but did not reach statistical significance. CXCL10/IP10 is known for its antiangiogenic properties, however this pathologic irony in RA may be explained by the ability of the CXCL10/IP10 receptor, chemokine CXCR3, to function also as a receptor for CXCL4 (platelet factor-4) a chemokine which has angiogenic properties.¹⁵ Although in our study CXCL4 levels were not measured, there is evidence in the literature for platelet activation in RA which in turn will lead to release CXCL4 and TSP1 from the alpha-platelet granules.

IL4 was significantly elevated in the RA patients when compared to control individuals. There is evidence in the literature that this cytokine may share common signaling pathways with IL13. Koch et al recently demonstrated inhibition of angiogenesis by both *interleukin-4* and *interleukin-13* gene therapy in an animal model of erosive arthritis.¹⁶ Clearly in humans affected by RA the elevated levels of protective angiostatic cytokines, namely IL4, IL13, IL12 and CXCL10/IP10 are not capable of counteracting the massive systemic imbalance observed in this autoimmune disease and therefore resulting in inflammation, angiogenesis and joint destruction. In summary, this study describes the signature expression profile of multiple cytokines in patients afflicted by RA. Future studies in our laboratory will focus on determining the specific mechanism by which TSP1 and TGF β contribute to the process of inflammation and angiogenesis in rheumatoid arthritis.

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Table 1
Plasma Levels of TSP1 and Cytokines in Patients with RA

Protein/Cytokine	Control (N=13)		RA (N=20)		Significance (P)
	Mean	SEM	Mean	SEM	
Coagulation related protein					
TSP1	25.45	7.36	315.04	86.69	0.001
Cytokine					
IL1 β	4.17	3.17	25.78	11.16	NS
IL4	150.84	71.73	1810.9	508.78	0.001
IL5	416.89	183.13	2338.8	503.59	0.025
IL6	24.37	17.94	182.78	50.23	0.005
IL12	38.33	22.33	609.38	135.51	0.002
IL12B	581.37	343.73	3356.34	875.39	0.039
IL13	41092.89	9557.77	61110.87	13404.17	NS
TNF α	155.47	71.14	452.46	134.72	0.067
IFN γ	2784.28	1204.62	21440.27	8525.82	0.032
TGF β	4571.01	634.58	10139.83	2072.27	0.1
Chemokine					
IL8	3501.98	553.05	4378.39	511.25	NS
CCL4/MIP1 β	77.74	29.90	219.33	29.65	0.014
CXCL10/IP10	2571.27	914.68	12765.00	2305.93	0.001

Italicized value represents statistical trends (when p value is between 0.1 and 0.05)