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Investigation of endocan level in Rheumatoid Arthritis patients' serum, and its relationship with disease activity

Inflammation & rheumatoid arthritis

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Abstract
Aim: Rheumatoid arthritis (RA) is a common chronic rheumatic disease. Endocan is a human endothelial cell-specific molecule. The aim of this study was to investigate the endocan level in RA patients' serum, and its relationship with disease activity.
Material and Methods: We enrolled 56 patients with RA, 55 patients with fibromyalgia (FMS), and 56 healthy volunteers in this cross-sectional study. Endocan was analyzed with an enzyme-linked immunosorbent assay (ELISA). The Kruskal-Wallis variance analysis was used to compare independent group differences. Chi-square analysis was used for comparison of categorical variables and Spearman correlation analysis was used to examine the relationship between continuous variables. P values <0.05 were considered statistically significant.
Results: The mean age was 44.03±10.79 years among RA patients. The mean disease duration was 64.44±53.82 months in RA patients. Serum endocan levels were similar between the groups including RA, FMS patients, and healthy subjects without any statistical significance (28.27 pg/ml in RA, 29.38 pg/ml in FMS, and 30.12 pg/ml in healthy subjects, $p > 0.05$). Endocan levels in the serum were also at similar titers in active and inactive RA patients (27.9 pg/ml, 28.64 pg/ml, respectively).
Discussion: We did not find increased levels of serum endocan in rheumatoid arthritis patients compared with fibromyalgia and healthy subjects. New studies with a higher number of patients are necessary for a more clear outcome.

Keywords
Rheumatoid Arthritis, Endocan, Fibromyalgia, DAS-28

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Introduction

Rheumatoid arthritis (RA) is a common chronic rheumatic disease that is characterized by synovial inflammation. RA commonly affects middle-aged women with a female/male ratio of 2-5/1. Multiple environmental and genetic factors play a role in underlying etiopathogenetic mechanisms [1]. Although there are various biomarkers that are used for evaluating treatment responses (ESR, CRP), for predicting risky subjects with progressive disease and/or severe disease activity, and for making therapeutic choices (RF, anti-CCP), new biomarkers (such as miR-146a and miR155) are also under investigation in this area [2]. It is also thought that histological patterns of synovial tissue biopsies will guide the choice of treatment modalities in the near future [3]. Thus different searches continue for new biomarkers. Currently, we know that altered blood vessel density is present in inflammatory synovial tissue and angiogenesis occurs via a release of vascular endothelial growth factor (VEGF) as a result of hypoxia. Basic fibroblast growth factor (FGF), angiopoietins and hypoxia-inducible factor-1 (HIF-1) are also associated pro-angiogenic molecules in RA, in addition to proliferative retinopathies and malignancies [4]. Angiogenesis facilitates the accumulation of various inflammatory cells and production of many pro-inflammatory cytokines. Synovial fibroblasts (SFs) and macrophage-like cells play a main role in the destruction of joints in RA patients and recent studies revealed active transmigration of SFs to other joints [5]. Interleukin-1β (IL-1β), (nuclear factor-kB-ligand) RANKL and tumor necrosis factor-alpha (TNF-α) have a destructive effect on affected joints [6]. Ephedrine was noted as one of the possible prospective therapeutic candidate agents due to an inhibitory effect on expressions of prostaglandin E2 (PGE2) and interleukin-6 in RA patients [7]. Ultimately, pannus formation occurs. Active cellular pannus is also a culprit factor in joint destruction. According to present studies, pannus tissue is a granulation tissue that has a proliferative capacity similar to a tumor-like mass [8]. Inhibitors of angiogenesis may have therapeutic effects in treating this proliferative synovitis. Endocan, also known as endothelial cell specific molecule-1 (ESM-1), is a chondroitin/dermatan sulphate proteoglycan that is expressed on the surface of vascular endothelium. Its expression, synthesis or secretion is affected by pro-inflammatory molecules such as TNF-α and VEGF [9]. Besides, endocan expression was induced by adiponectin more powerfully than IL-1β in synovial cells [10]. Endocan regulates cell adhesion in malignant and inflammatory diseases. ESM-1 adheres to lymphocytes through leukocyte function-associated antigen-1 (LFA-1). It could influence the accumulation of lymphocytes to the inflammation site. This proteoglycan also demonstrates Hepatocyte Growth Factor/Scatter Factor (HGF/SF) mediated mitogenic activity in tumor formation and tissue repair [11]. Kim KS et al investigated endocan expression in tissues of patients with RA and osteoarthritis (OA). Endocan was highly expressed in RA synovial tissues and in some OA tissues with concomitant severe inflammation [10]. In this study, we aimed to investigate endocan levels in RA patients' serum and its possible association with the disease activity.

Material and Methods

This is a cross-sectional comparative study from a single tertiary health center. All individuals included in this study were evaluated by the same rheumatology team at one time. Between September 2015 and December 2016, a total of 76 RA patients admitted to the outpatient rheumatology clinic. We enrolled 56 patients with rheumatoid arthritis who meet the study inclusion and/or exclusion criteria, 55 patients with fibromyalgia (FMS) and 56 healthy volunteers who matched in terms of age and gender at the same time period. All of the RA patients fulfilled the 2010 American College of Rheumatology (ACR) classification criteria [12]. Patients with rheumatoid arthritis were subgrouped as active or inactive based on disease activity score-28 (DAS-28). DAS28 is one of the measurements of RA disease activity calculated on the basis of total information from general health, tender and swollen joints, and acute phase response. Baseline DAS-28 was evaluated in each patient in accordance with EULAR/ACR collaborative recommendations [13]. DAS-28 was calculated with the following formula: (0.56 × √number of tender joints) + (0.28 × √number of swollen joints) + (0.7 × erythrocyte sedimentation rate (ESR) + (0.014 × visual pain scale). The disease with upper values of DAS 28 above 3.2 (≥ 3.2) was accepted as active disease, whereas others with inactive disease. According to current knowledge, FMS is a non-inflammatory biopsychological disease that may be compared with inflammatory rheumatic diseases in clinical trials. Herein, 2010 ACR diagnostic criteria were used to diagnose patients with FMS [14]. All were diagnosed in our tertiary health center at Pamukkale University Faculty of Medicine.

Inclusion criteria were defined as follows: patients older than 18 years, and patients who fulfill each classification criteria for RA and FMS, patients having initial diagnosis by the same rheumatology team in our tertiary health center, and being volunteer for healthy individuals. Exclusion criteria were defined as follows: presence of alcohol use or smoking, having any co-morbidities including hypertension, diabetes mellitus, left ventricle dysfunction, heart valve pathologies, acute coronary syndrome, hypo-hyperthyroidism, liver and/or kidney dysfunction, malignancy or other systemic inflammatory diseases, or having local and/or systemic infectious disease in the recent three months, and RA patients treated with biological agents.

Coagulated blood samples were collected at 8-10 am after 12 hours of fasting. After appropriate centrifugation, all samples were stored at –80 °C until testing. Samples for endocan were examined with an enzyme-linked immunosorbent assay (ELISA) using a special kit (BOOSTER, Pleasanton, CA 94566) after incubating for 1.5 hours at 37C. Biotinylated anti-human ESM-1/Endocan antibody solution was added to each sample. The procedure is based on sandwich ELISA principle. Firstly, a mixture of samples has been made with a target antigen capture antibody, and than unbound parts were removed by washing. After washing, staining with tetramethylbenzidine (TMB) was applied. The final absorbance values of the samples were read using a Kayto RT – 2100c microplate reader at 450 nanometers (nm). Endocan concentrations were reported as

pg/mL. C-reactive protein (CRP), ESR, rheumatoid factor (RF) and total leukocyte count were also analyzed with standardized biochemical assessments to determine inflammatory state of the subjects.

This study was approved by the local ethics committee of the tertiary health center (2015/35-1) in accordance with the Helsinki Declaration. All participants approved a patient consent form.

Statistical Analysis

The statistical program SPSS version 23.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis using descriptive statistics. Continuous variables are given as mean ± standard deviation and categorical variables as numbers and percentages. The Kruskal- Wallis is a kind of nonparametric variance analysis that has been used to compare several independent group differences. Chi-square analysis was used for comparison of categorical variables and Spearman's correlation analysis was used to examine relationships between continuous variables. P-values <0.05 were considered statistically significant.

Results

Three patient groups were similar in terms of age, gender and body mass index (Table 1). The mean age of RA patients was 44.03±10.79 years. The mean disease duration in RA patients was 64.44±53.82 months. Forty-one (n=41, 73.2%) patients with RA were female. RA patients were subgrouped into active or inactive disease. Thirty-one RA patients had active disease, while the rest patients had inactive disease (n=25). Active RA patients had higher ESR and CRP values than inactive patients. Whereas, RF positivity and total leukocyte count were similar between the two RA patient subgroups. 78.6% (n=44) of RA patients were on treatment with methotrexate, followed by 76.8% (n=43) with glucocorticoids, 58.9% (n=33) with sulfasalazine, 17.8% (n=10) with antimalarials and 14.3% (n=8) with leflunomide. Serum endocan levels were similar between the groups including RA, FMS patients and healthy subjects without any statistical significance (28.27 pg/ml in RA, 29.38 pg/ml in FMS, and 30.12 pg/ml in healthy subjects, p >0.05) (Table 1). Values of ESR and CRP were significantly higher in active RA patients compared with inactive patients (Table 2). Serum endocan levels were at similar titers in active and inactive RA patients (27.9 pg/ml, 28.64 pg/ml, respectively) (Table 3). Serum endocan levels had a negative association with ESR, CRP and DAS-28 without statistical significance (r= -0.217, p=0.107 vs r= -0.170, p=0.209 and r= -0.076, p=0.575, respectively) (Table 3).

Table 1. Comparison of demographic features and baseline blood pressure between the study groups.

Variables	RA (n=56)	FMS (n=55)	Healthy controls (n=56)	P value
Age (mean ± SD)	44.03±10.79	41.76±8.1	41.48±8.49	(p>0.05)
BMI (kg/m²) (mean ± SD)	26.73±4.37	24.39±4.13	26.18±4.46	(p>0.05)
Gender (female) (n, %)	41 (73.2%)	44 (80%)	35 (62.5%)	(p>0.05)
Endocan (pg/ml) median (min-max)	28.27 (17.15-67.24)	29.3 (13.3-64.3)	30.1 (17.8-50.5)	0.807
CRP (mg/dl) median (min-max)	0.61 (0.008-23)	0.2 (0.03-2.0)	0.15 (0.01-1.4)	p<0.05*
ESR (mm/h) median (min-max)	30.5 (4-91)	21 (5-56)	13.5 (2-38)	p<0.05*

RA: rheumatoid arthritis, FMS: fibromyalgia syndrome, BMI: body mass index, CRP: c-reactive protein, ESR: erythrocyte sedimentation rate. *; statistical significance between RA & FMS and RA & healthy controls.

Table 2. Comparison of variables between active and inactive rheumatoid arthritis patients

Variables	Active RA (n=31) median (min-max)	Inactive RA (n=25) median (min-max)	P
Endocan (pg/ml)	27.9 (17.5-67.2)	28.64 (17.1-57.7)	0.525
CRP (mg/dl)	1.13 (0.05-23)	0.4 (0.01-7.1)	0.017
ESR (mm/h)	35 (4-91)	29 (6-67)	0.076
WBC (k/uL)	7900 (4370-17530)	7759 (4180-11710)	1.000
RF (IU/ml)	31 (46-229)	28.3 (1-203)	0.735

RA: rheumatoid arthritis, min-max: minimum-maximum, CRP: c-reactive protein, ESR: erythrocyte sedimentation rate, WBC: white blood cell count, RF: rheumatoid factor.

Table 3. Correlation analysis of endocan levels with age, disease duration, CRP, ESR and DAS-28

Endocan & Variables	r	p
Age	0.008	0.95
Disease duration	0.149	0.27
CRP (mg/dl)	-170	0.21
ESR (mm/h)	-0.217	0.11
DAS28	-0.076	0.58

CRP: c-reactive protein, ESR: erythrocyte sedimentation rate, RF: rheumatoid factor, DAS28: disease activity score 28.

Discussion

In the present study, we hypothesized that serum endocan may be a biomarker of endothelial cell activation and angiogenesis associated with inflammation. Our findings showed that serum endocan was at similar titers in RA and FMS patients, and healthy controls. Absence of significant difference between FMS and healthy controls also ensured that endocan was not associated with inflammation. FMS was selected due to beinig non-inflammatory rheumatic disease. Today, endocan is started to be mentioned as a useful biomarker at vascular dysfunction of hypertension, renal disease and advanced cardiovascular disease [15]. Although effects of gender/sex on cardiovascular diseases are not clearly determined and social determinants of biological sex were reviewed comprehensively, cardiovascular diseases are thought to be mostly men diseases [16]. In this context, the similar endocan titers in RA patients and healthy controls may be explained by that the vast majority of the patient group were females.

Paul Balanescu et al showed higher endocan levels in systemic sclerosis (SSc) patients compared with sex- and age-matched healthy controls. They noted that endocan may have a discriminative and predictive role in differentiating diffuse and limited forms and in developing microvascular complications [17]. Ilknur Balta et al also detected increased circulating serum endocan levels in Behçet disease (mean serum levels of endocan were 1.29 ± 0.60 ng/mL) compared with healthy subjects. According to their investigation, serum endocan was

higher in patients with ocular symptoms and arthritis. Endocan was correlated with acute phase reactants including ESR, CRP and disease activity [18]. Serum circulating endocan levels were detected at high titers in psoriasis (PSo) patients and correlated with Psoriasis Area and Severity Index (PASI), and high sensitive CRP as an indicator of endothelial dysfunction. The authors suggest its use as a surrogate marker in endothelium-associated pathological diseases [19].

In addition, Göksel Tuzcu et al investigated this molecule in RA patients. They showed that serum endocan was significantly higher in RA patients than healthy controls. In their study, endocan was positively correlated with age, DAS-28 in RA patients and carotid intima-media thickness in the evaluation of pre-atherosclerotic lesions [20]. These are inconsistent with our study results. This may be explained by difference in the mean age of RA patients between the two studies. The patients in their study were older than our RA patients. Besides, they had a much smaller patient group compared with ours, and treatment modalities of the RA patients were not reported in their study. In our study, a vast majority of RA patients were on various treatment modalities. Whereas, various effective treatment modalities and/or medical conditions are related to variations in endocan levels in different diseases. For instance, strict glycemic control decreases serum endocan levels in type 2 diabetes mellitus [21]. The other example is ultraviolet-B (UVB) treatment in patients with psoriasis [22]. Turgay Celik et al also showed that amlodipine and valsartan decreased serum endocan levels in newly diagnosed hypertensive patients. This result was attributed to the anti-inflammatory effects of these two drugs [23]. All of the therapeutic effects mentioned above were explained by the reversal of endothelial dysfunction in each disease. Thus, the absence of a significant correlation between endocan levels and inflammatory markers and/or disease activity score may be attributed to the possible effect of using glucocorticoids, anti-inflammatory drugs and/or immune-modulatory drugs.

Fibromyalgia is the most common widespread pain syndrome in the general population with a prevalence of 2% to 8% and it frequently accompanies various inflammatory diseases [14]. Negative effects of pain and stress on endothelial function were shown in FMS patients [24]. Serum endocan levels were also higher in fibromyalgia patients than healthy controls in the recent literature [25]. In our study, serum endocan levels were similar between fibromyalgia patients and healthy controls. The FMS patients had neither other systemic diseases nor older age compared with literature. Similar endocan levels may be attributed to this condition.

There are several limitations of our study. Firstly, the vast majority of RA patients had ongoing variable treatment modalities including anti-inflammatory and immune-modulatory drugs that could affect endocan levels of serum. Having a heterogeneous patient group with various drugs is also problematic for comparison of subgroups. Patients treated with the same drug or without any drug may be included. But this time, achieving this sample size would be difficult in the same time period. Secondly, concurrent pro-inflammatory and/or pro-angiogenic cytokine analysis was not obtained simultaneously. Although patients who had no comorbidities and any smoking addiction were included in the study in order to decrease the confounding effects of these factors, a relatively small-sized patient groups was another limitation of the study. Absence of a power analysis for determination of the sample size is another limitation.

Conclusion

Serum endocan levels were similar between RA patients, FMS patients, and healthy subjects. Further studies with more homogeneous and large-sized patient groups are needed to validate these results. Also, early-RA patients who are not treated with any conventional/biological agents can provide more accurate knowledge.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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