Synovial Fluid and Serum Levels of sE-Selectin, IL-1β and TNF-α in Rheumatoid Arthritis Patients

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Abstract. This work aims at determining the difference of pro-inflammatory cytokines, adhesion molecules in synovial fluid, serum of rheumatoid arthritis, osteoarthritis patients Synovial fluid and serum were obtained from rheumatoid arthritis (n = 21), osteoarthritis (n = 11) patients, serum of healthy individuals (n = 12). Interleukin-1β, tumor necrosis factor-alpha, sE-selectin levels were measured by Enzyme-Linked Immunosorbent Assay Test. Compared to controls, S-sE-selectin was significantly elevated in rheumatoid arthritis while S-IL-1β, S-TNF-α were elevated in rheumatoid arthritis and osteoarthritis patients. S-sE-selectin, SF-IL-1β and SF-TNF-α were significantly elevated in rheumatoid arthritis versus osteoarthritis. In rheumatoid arthritis, SF-sE-selectin was significantly lower while SF-TNF-α was higher than serum in rheumatoid arthritis and osteoarthritis. In rheumatoid arthritis, positive correlation between SF-E-selectin with serum – C-reactive Protein and S-sE-selectin; between S-sE-selectin with disease duration and S-TNF-α; between S-IL1-β with SF-IL1-β and disease activity while negative correlationbetween S-TNF-α with grip strength were found. A degree of vascular endothelial activation reflected by increased sE-selectin, is associated with disease activity, duration in rheumatoid arthritis. The positive association between IL-1β, disease activity and negative association between TNF-α, grip strength indicated that serial estimation of these cytokines may be used to monitor disease progression. Elevated TNF-α in synovial fluid than in serum of rheumatoid arthritis, osteoarthritis patients indicates its local formation in the synovium.

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Introduction

Rheumatoid arthritis (RA) is a chronic systemic disease characterized by an inflammatory erosive synovitis. Early changes in synovium are marked by neovascularization, inflammatory cell infiltration and associated synoviocyte hyperplasia, which produce a pannus of inflammatory vascular tissue. The pannus covers and erodes articular cartilage, eventually leading to erosive joint destruction[1]. In contrast, pathogenic basis of osteoarthritis (OA) is less clear-cut. It has been proposed that OA is a result of both mechanical and biological events that destabilize normal coupling of degradation and synthesis of articular cartilage and subchondral bone. Biomechanical stresses affecting articular cartilage and subchondral bone, biomechanical changes in articular cartilage and synovial membrane, and genetic factors are all important in its pathogenesis[2]. However, synovial tissue from patients with OA presents a thickening of the lining layer, increased vascularity and variable degrees of inflammatory mononuclear cell infiltration. The inflammatory process at onset of OA may be a primary cause or secondary to cartilage destruction[3]. Moreover, in both pathologies many factors are involved in synovial inflammation, where cytokines have emerged as regulatory factors of particular importance[4].

An early event in the pathogenesis of RA is infiltration of synovial membrane by large numbers of highly activated macrophages[5] which produced pro-inflammatory cytokines, such as Tumor Necrosis Factor-alpha (TNF-α) and interleukin (IL-1-β) that are present at high concentration in synovial fluid (SF) and pannus of rheumatoid joints[6] and seems to be controlled by lymphocytes[7]. Over expression of IL-1α, IL-1β, IL-6, TNF-α, granulocyte macrophage colony stimulating factor (GMCSF) and epidermal growth factor (EGF) has been described in OA synovium[2]. In experimental models, IL-1 increases cell migration into inflamed synovium by up-regulation of Cellular-adhesion Molecules (CAMs), stimulates the production of prostaglandins and metalloproteinases, inhibits collagen and proteoglycan synthesis and stimulates osteoclastic bone resorption[8].

Selectin family of adhesion molecules are responsible for tethering of leukocytes to vessel surface, permitting rolling of cell in direction of flow. This phase occurs before integrin-mediated arrest of leukocyte motion, firm adhesion, and transmigration[9]. E-selectin is a member of selectin family of adhesion molecules and appears on vascular luminal cell surface of endothelial cells. It is considered as a marker of endothelial cell activation. The establishment of a chronic synovitis involves traffic of circulating inflammatory cells into and through synovial membrane[10], regulated by cell adhesion molecules which are in turn regulated by pro-inflammatory cytokines[2]. The aberrant production or activation of soluble mediators have an important role in infiltration of rheumatoid synovium with mononuclear cells and seem to play a part in pathophysiology of the disease[11].

This study aimed to measure concentration of cytokines (IL-1β and TNF-α) and adhesion molecules (sE-selectin) in serum and SF, from patients with RA and compared to OA patients which served as diseased controls and serum of healthy controls in order to determine whether certain cytokine and adhesion molecule patterns are specifically related to RA. Relation between serum and SF IL-1β, TNF-α and sE-selectin levels and disease activity, severity of joint inflammation and destruction and functional status was also examined.

Patients and Methods

In this cross sectional study, serum and SF specimens were obtained from 21 patients with RA (20 females and one male), from 11 patients with knee OA (9 females and 2 males) as well as from serum of 12 age and sex matched healthy controls (10 females and 2 males). All patients fulfilled criteria of American College of Rheumatology (ACR) for diagnosis of RA[12] and OA[13]. All patients were selected from outpatient and inpatient clinics of the Rheumatology and Rehabilitation and the Neurology Departments, Assiut University Hospital, Assiut, Egypt (December 2003 - March 2004). All subjects were presenting with synovial effusion of knee joints (unilateral or bilateral). None of the patients received intra-articular corticosteroids within 3 months before joint fluid aspiration. Approval was granted from the local ethics committee for this study and written informed consent was obtained from all participants. The mean age of RA patients was (mean ± SD; 42.52 ±

8.02 years); OA patients (49.27 ± 10.19 years); and controls (44.33 ± 5.76 years); with a mean disease duration of (5.91 ± 3.63 and 8.55 ± 7.01 years) for RA and OA groups, respectively. At the time of sample collection, 5 RA patients were treated with Nonsteroidal Anti-Inflammatory Drugs (NSAIDS); 9 with methotrexate (MTX) and NSAIDS; 4 with NSAIDS and steroids; 1 with MTX and steroids; 1 with steroids only; and 1 with MTX only. All OA patients were treated with NSAIDS.

Clinical assessment of RA patients included: tender joints numbers (Ritchie articular index)[14], swollen joints numbers, and a visual analogue scale (VAS) (scale 0-10) for pain severity. Duration of morning stiffness was registered in terms of minutes. Hand grip strength was measured by standardized dynamometers according to McRae[15]. Assessment of mean disease grading activity (MDGA) using multivariate analysis according to Mallya and Mace[16], 4 mean grades were calculated. Functional status was assessed and graded according to Hochberg *et al.*[17]. Radiographs of other affected joints and knee joints, from which SFs were aspirated, were taken with standard film at study entry. The radiographic findings were scored using Larsen's method[18], grading changes from 0 (normal); I (slight periarticular osteoporosis); II (marked abnormalities); III (severe abnormalities); IV (mutilating abnormality); and V (maximal damage).

The OA was diagnosed clinically and all patients had correlative radiological changes of the affected joint with negative SF examinations for microbial organisms. All patients had a weight bearing anteroposterior and lateral plain X-ray for knee joints and the severity was assessed according to Kellgren and Lawrence (KL)[19]. According to radiological grading, OA patients were divided into 4 groups, where grade 0: normal; I: minute osteophytes; II: definite osteophytes = mild; III: narrowing of joint space = moderate; IV: greatly reduced joint space and subchondral bone sclerosis = severe. No evidence of calcifications was observed in X-rays of OA patients.

Blood samples were clotted for 30 minutes and then centrifuged for 10 minutes at 1,000 gravity. Serum samples were sent to a central laboratory for routine examination and part of the serum aliquots was frozen at –70°C immediately after collection.

All patients underwent diagnostic or therapeutic arthrocentesis. SF samples were treated with 10 µg hyaluronidase (Sigma Chemical Co., St Louis, MO, USA)/ml SF and incubated at 37°C for 45 minutes. The hospital laboratory determined WBC counts derived from patient SF at the time of arthrocentesis after SF treatment with hyaluronidase and counts were used as diagnostic or therapeutic information in the patient's medical record. The level of polymorph nuclear cells in SF was expressed as a percentage of the overall number of WBCs. Thereafter, SF was centrifuged at 1,000 g for 10 minute to remove cell debris. The samples were kept in small aliquots at ─80°C until use. Before use, the SF samples were recentrifuged at 1,000 g to remove precipitated material by freezing and thawing.

The Erythrocyte Sedimentation rate (ESR) (Westergren method), serum C-reactive Protein (CRP) concentration, rheumatoid factor (RF) by latex agglutination slide test and complete blood count (CBC) using coulter T 600 cell counter were determined at the time of clinical evaluation by routine methods. Commercially available Enzyme-Linked Immunosorbent Assay Test (ELISA) kits were used to determine concentrations of IL-1β, TNF-α and sE-selectin (KHC0011, KHC3011, KHS2011, BioSource Europe S.A., Nivelles, Belgium) in the serum and SF. The detection limit of the assay for of IL-1β, TNF-α and sE-selectin were < 2 pg/mL, < 3 pg/mL, < 0.1 ng/mL, respectively. Intra- and interassay coefficients of variation were 5.45 and 6.0%, respectively for sE-selectin; 4.4 and 6.7%, respectively for IL-1β; 4.4 and 7.4%, respectively for TNF-α. All assays were duplicated according to the instructions from the manufacturer.

Statistical analysis

Statistical analysis was made using SPSS program version 12 (Chicago, USA). Data were expressed as mean ± SD. One way analysis of variant (ANOVA) and "Student's" *t* test was used to compare means between groups. Spearman's correlation was used to test variance between factors. P value less than 0.05 were regarded significant.

Results

The clinical and laboratory findings of patients with RA or OA are summarized in Table 1. The 1st and 2nd hour ESR and CRP

concentrations were higher in RA than OA ($p < 0.01$, $p < 0.01$, $p < 0.05$). There were significant elevation in SF WBCs and polymorphonuclear leucocytes (PMNLs) in RA patients *vs.* OA ($p < 0.0001$ for both).

Table 1. The demographic, clinical and laboratory features of patients with rheumatoid arthritis (RA) and Osteoarthritis (OA). Data presented as means (SD).

	Rheumatoid Arthritis (n=21)	Osteoarthritis (n=11)	Control (n=12)
Women / Men	20/1	9/2	10/2
Age (yrs)	42.52 ±8.016 [1]P>0.05 [2]P>0.05	49.27 ±10.19 [1]P>0.05	44.33 ±5.76
Disease Duration (yrs)	5.91 ±3.63 [2]P>0.05	8.55±7.01	
Duration of Morning Stiffness (min)	76.19 ±50.05	39.86±13.14	
Subcutaneous Nodules (Positive/Negative)	17/4	—	
Pain scale(VAS) (cm)	6.52 ±2.67	5.44±3.12	
Functional Capacity I II III IV	5 8 6 2	—	
Ritchie Articular Index	28.33 ±11.98		
Swollen Joints (n)	3.00 ±1.61	2.00 ±0.6	
Grip Strength	50.71 ±40.57	—	
MDGA	3.24 ±0.44	—	
ESR (mm/ h) 1st	65.19 ±34.31 [2]**P<0.01**	30.09 ±16.31	
2nd	91.10 ±33.33 [2]**P<0.01**	52.45 ±21.87	
CRP (mg/l)	17.71 ±18.05 [2]**P<0.05**	3.82 ±7.72	
Radiological grading I II III IV V	8 7 2 4 0	1 3 3 4 —	
RF (positive/negative)	9/12	—	
WBCs (X10^9/l)			
Blood	8.66 ±2.90 [2]P>0.05	7.56 ±2.06	
Synovial Fluid	4.23 ± 1.54 [2]P>0.0001	0.95 ± 0.46	

Table 1. Contd.

	Rheumatoid Arthritis (n=21)	Osteoarthritis (n=11)	Control (n=12)
Blood Lymphocytic Count (X10^9/l)	3.39 ±1.17 [2]P>0.05	2.90 ±1.67	
Synovial Fluid Polymorphonuclear Cells (PMNLs)	55.38 ±4.49 [2]P<0.0001	19.82 ±3.28	

[1]P: Significance versus Serum of Controls.
[2]P: Significance versus Osteoarthritis.
ESR: Erythrocytic Sedimentation Rate; CRP: C-reactive protein; MCV: Mean Corpuscular Volume.

S-sE-selectin level was significantly higher in RA patients compared to OA and controls (p<0.05, p<0.001). In RA patients, SF-sE-selectin concentration was significantly lower than its corresponding serum level (p<0.0001). S-IL-1β and S-TNF-α levels were significantly elevated in both RA and OA patients compared with controls (p<0.001 for all). SF-IL-1β and SF-TNF-α concentrations were significantly elevated in RA compared to OA patients (p<0.05, p<0.001). SF-TNF-α levels were significantly elevated compared to its corresponding serum levels in both RA and OA patients (p<0.001, p<0.05) (Table 2).

Table 2. Serum and synovial fluid levels of interleukin-1β (IL-1β) and sE-selectin in-patients with rheumatic arthritis and controls. Data were expressed as mean (SD).

	Rheumatoid Arthritis	Osteoarthritis	Control
Serum sE-selectin (ng/ml)	21.89±11.32 [1]P<0.001 [2]P<0.05	14.06±2.50 [1]P>0.05	6.76 ± 3.15
Synovial Fluid sE-selectin (ng/ml)	11.25±5.38 [2]P>0.05 [3]P<0.0001	8.74±3.08 [3]P>0.05	—
Serum IL-1β (pg/ml)	23.61±7.95 [1]P<0.001 [2]P>0.05	27.35±6.77 [1]P<0.001	5.63±1.61
Synovial Fluid IL-1β (pg/ml)	29.03±12.22 [2]P<0.05 [3]P>0.05	19.58±5.24 [3]P>0.05	—
Serum TNF-α (ng/ml)	45.26±6.37 [1]P<0.001 [2]P>0.05	47.44±5.06 [1]P<0.001	6.93±1.31
Synovial Fluid TNF- α (ng/ml)	228.60±86.54 [2]P<0.001 [3]P<0.001	107.60±23.86 [3]P<0.05	—

[1]P: significance versus serum of controls.
[2]P: significance versus osteoarthritis.
[3]P: significance serum versus corresponding synovial fluid.

In RA patients, there was a significant positive correlation (r) between SF-sE-selectin with CRP (r = 0.52, p < 0.05), serum sE-selectin (r = 0.69, p<0.0001) (Fig. 1). Serum sE-selectin was positively correlated with duration of disease (r=0.55, p<0.01) and S-TNF-α (r=0.45, p<0.05) (Fig. 2). S-IL1-β was positively correlated with MDGA (r=0.45, p<0.05) and SF-IL1-β (r=0.60, p<0.01), between blood-WBCs with SF-WBC (r=0.98, p<0.0001) and SF-PMNLs (r=0.71, p<0.001). S-TNF-α was negatively correlated with grip strength (r=−0.44, p<0.05). In OA patients, there was a significant positive correlation between SF-sE-selectin with S-sE-selectin (r=0.70, p<0.05).
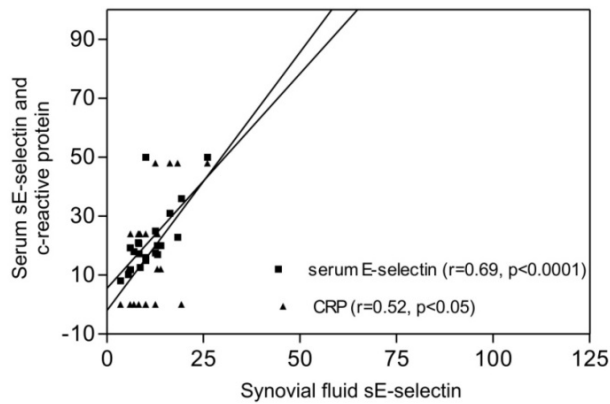

Fig. 1. Correlation and linear regression between synovial fluid sE-selection with serum sE-selectin and c-reactive protein in rheumatoid arthritis patients.
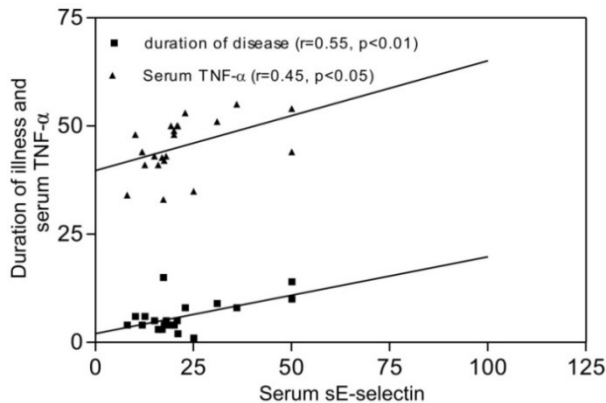

Fig. 2. Correlation and linear regression between serum sE-selectin with duration of disease and serum TNF-α in rheumatoid arthritis patients.

Discussion

Cytokines are local protein mediators, known to be involved in almost all important biological processes, including cell growth activation, inflammation, immunity, and differentiation[20]. IL-1 and TNF-α are considered to be key pro-inflammatory cytokines in development of RA[21].

An increase in serum levels of IL-1β and TNF-α in both RA and OA patients compared to controls was found in this study. In SF, IL-1β and TNF-α levels were significantly elevated in RA compared to OA patients. Meanwhile, both RA and OA patients' levels of TNF-α were significantly elevated than its corresponding serum levels. Both animal models and clinical studies of RA have implicated IL-1β and TNF-α in pathogenesis of RA[22]. Other studies suggested that TNF-α stimulates IL-1 production and both act synergistically in RA patients[23]. Similarly, Nakamura *et al.*[24] reported elevated serum levels of IL-1β; while, Lettesjo *et al.*[23] reported elevated SF-IL-1β in patients with RA receiving slow acting anti-rheumatic drugs and/or corticosteroids, suggesting that these cytokines contribute to severe forms of arthritis. Similarly, Bertazzo *et al.*[25] reported elevated levels of SF-IL-1β in RA compared to OA patients. The elevated levels of synovial TNF-α compared to its corresponding serum levels could be explained by that in rheumatoid synovium, IL-1 and TNF-α are mainly produced by macrophages[26]. In this respect, Furuzawa-Carballeda and Alcocer-Varela[4] reported that RA synovial tissue is partially responsible for cytokines synthesized in joints during triggering and perpetuation of inflammatory response.

The positive correlations obtained by this study in RA patients between serum-IL1β with SF-IL1-β and with disease activity grading and negative correlation between serum-TNF-α with grip strength were expected. Evidence supports correlation of levels of disease activity in RA and progression of joint damage with levels of IL-1 and TNF-α was reported previously[27]. IL-1 plays a critical role in destruction of cartilage and bones[28]**.**

The ability of cells to adhere to other cells and extracellular matrix (ECM) through CAMs is central to tissue remodeling and inflammation[29]. It was shown that sE-selectin mediates endothelial cell

chemotaxis. Furthermore, sE-selectin expresses angiogenic activity which is potentially increasing the ingress of mononuclear cells into inflamed RA synovial tissue[30]. It is postulated that sE-selectin, which is expressed very early in rheumatoid process, may be important in triggering angiogenesis in the initial stages of RA[31]. In this study, the serum concentration of sE-selectin was significantly raised in RA patients compared to OA and healthy controls. Our finding was similar to those of other studies[11,32]. Meanwhile, in some studies the serum concentration of sE-selectin was increased in patients with RA relative to healthy controls, but those differences did not reach a significant level[33].

In this series, a serum level of sE-selectin in RA patients was significantly elevated compared to its corresponding SF levels. It had been reported that expression of sE-selectin molecules occurs in rheumatoid synovial tissue and seems to be associated with synovial inflammatory activity[34]. On the other hand, we found elevated levels of SF sE-selectin in RA patients compared to OA but this elevation did not reach significant levels. On the contrary, others[35] reported that SF sE-selectin were higher in RA than in non-inflammatory joint diseases. Johnson et al.,[36] also reported that expression of sE-selectin on synovial endothelium only increased in RA when compared with OA or normal synovium.

Patients included in this study were treated with MTX and/or steroids. MTX is a potent inhibitor of the enzyme dihydrofolate reductase (DHFR) that leads to the inhibition of de novo purine and pyrimidine synthesis. Low-dose MTX, which is administered in weekly doses of 0.1–0.3 mg/kg, has been shown to be very effective in the management of rheumatoid arthritis[37]. MTX can reduce inflammatory cell numbers in tissues by lowering both the concentration of soluble adhesion molecules[38] and the tissue expression of adhesion molecules such as E-selectin, ICAM-1, and VCAM-1[39]. Although patients included in this study were treated with MTX, the levels of sE–selectin were still high. In partial contrary, Cobankara et al.,[40] reported significantly higher sE-selectin levels in RA untreated patients compared to controls but, the difference returned to normal after MTX treatment. So, it should be expected that patients in this study may show higher levels of sE-selectin if they were left untreated.

In RA patients, a significant positive correlation between synovial sE-selectin with CRP and with serum sE-selectin was found in this study. Serum sE-selectin was positively correlated with duration of disease and with serum TNF-α. Meanwhile, a significant positive correlation between SF-sE-selectin with S-sE-selectin in OA patients was also reported in this study. One possible explanation for association between serum sE-selectin with serum TNF-α and with disease duration is that in chronic inflammatory disorders such as RA, expression of vascular endothelial selectins is prolonged[41], probably due to continued presence of pro-inflammatory mediators such as TNF-α which are expressed at high levels in synovium[22]. It was found that TNF-α and IL-1β regulates expression of sE-selectin on vascular endothelium[10].

In partial agreement with results in this study, Kuuliala *et al*.[42] has found that serum sE-selectin level was significantly correlated with CRP, active joint count, progression of joint destruction and Health Assessment Questionnaire score, but not with extra-articular involvement. Given that CRP and sE-selectin are both markers of systemic inflammation, an hypothesis can be proposed that sE-selectin levels may also reflect smoldering inflammation. This study and others[42] found a correlation between serum and SF concentrations of sE-selectin. Other investigators[41] found an association between sE-selectin levels and joint damage process and our findings might therefore give some support to hypothesis that sE-selectin participates in the pathogenic mechanisms in rheumatoid joint. The observed association can be explained, at least in part, by proinflammatory properties of sE-selectin, which might promote joint inflammation and thereby compromise physical function of patient. In consistence with others[11] we did not find a significant association between serum sE-selectin level and disease activity indicators. An association between serum levels of sE-selectin and ESR[41] and joint damage was found by other studies[31]. In this study, there was no correlation between the sE-selectin levels and leukocyte count. This indicates that increased sE-selectin concentrations do not simply reflect a raised leukocyte population.

Conclusion

Activating effects on synovial cell proliferation, including macrophages and fibroblasts lead to more production of SF-IL-1ß, while

active endothelium leads to production of sE-selectin in synovial fluid. Activation/injury to endothelium through selectins may be important in rheumatoid arthritis. Measurement of sE-selectin may increase our understanding of the relationship between *in vivo* endothelial activation and inflammation involved in rheumatic diseases. The positive correlation between serum sE-selectin and disease duration and between synovial sE-selectin and CRP and serum sE-selectin in RA patients suggest that serum levels of sE-selectin may provide a useful additional marker for endothelial activity in RA patients. Future studies should emphasize on cytokines, their receptors and concomitant estimation of cytokine inhibitors. This study suggested that the future development of drug treatments for RA should focus on treatments which modulate or alter function of specific cell adhesion molecules, particularly E selectin.

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قياس مستويات الإسلكتين والإنترلوكين–١–بيتا وعامل النخر الورمى ألفا في السائل الزليلى ومصل مرضى الرثيان المفصلي

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المستخلص. تهدف هذه الدراسة إلـى استبيان الفـرق بـين السيتوكينات السابقة للالتهابات وجزيئات الالتـصاق فـي الـسائل الزليلى ومصل مرضى الرثيان المفصلي والفـصال العظمـى. اشتملت الدراسة على ٢١ مريضا مصابين بالرثيان المفصلي، ١١ بالفصال العظمى و ١٢ من الأصحاء كمجموعة ضابطة. تم قياس الإنترلوكين–١–بيتا، عامل النخر الورمي ألفا، الإسـلكتين بجهـاز المطياف الذرى. أوضحت الدراسة وجـود زيـادة فـي مـستوى الإسلكتين في مصل مرضى الرثيان المفصلي مقارنـة بالفـصال العظمى والمجموعة الضابطة. وانخفاض مستوى الإسـلكتين فـي السائل الزليلى مقارنة بالمصل في مرضى الرثيان المفصلي. زيادة مستويات الإنترلوكين–١–بيتا وعامل النخرالورمى ألفا في أمصال مرضى الرثيان المفصلي والفصال العظمى مقارنـة بالمجموعـة الضابطة، وارتفاع مستوياتهم في السائل الزليلى في مرضى الرثيان المفصلي مقارنة بمرضى الفصال العظمى ، وزيادة عامل النخـر الورمي في السائل الزليلى مقارنة بمـستواه فـي أمـصال كـلا المرضين. ووجدت علاقة موجبة بين الإسلكتين بالـسائل الزليلـى

والمصل وبين مستوى الإسلكتين في المصل وطول مدة المــرض وعامل النخر الورمي في مصل هــؤلاء المرضــى. وكــذا بــين الإنترلوكين في المصل ومستواه في السائل الزليلى ودرجة نــشاط المرض وعلاقة سلبية بين عامل النخر الورمي في المصل وقــوة قبضة اليد. نستخلص من الدراسة وجود دور واضح لنشاط البطانة الوعائية في مرض الرثيان المفصلي. ارتفاع مستوى عامل النخــر الورمي في السائل الزليلى للمرضى يرجح إنتاجه موضعيا بالزليل.