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ORIGINAL ARTICLE

Significance of serum levels of angiopoietin-2 and its relationship to Doppler ultrasonographic findings in rheumatoid arthritis patients

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Abstract *Background:* Angiopoietin-2 (Ang-2) is connected to angiogenesis in synovial regions, but the significance of its levels in patients with rheumatoid arthritis (RA) is still unclear.

Aim of the work: To evaluate the significance of serum levels of Ang-2 in patients with RA. Also, to determine Ang-2 relationship to the findings of joints Doppler ultrasonographic findings.

Patients and methods: This study included 40 patients with RA, and 25 matched healthy controls. All patients were subjected to assessment of pain using visual analogue scale (VAS), assessment of personal activity using the Health Assessment Questionnaire (HAQ) score, and calculation of disease activity score (DAS 28). Laboratory assays of complete blood count (CBC), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), rheumatoid factor (RF) titre, and measurement of serum levels of Ang-2 by ELISA. Doppler ultrasonography (US) assessment for eight joints, with calculation of synovial thickness and total signal score (TSS), was done.

Results: Serum Ang-2 levels were significantly higher among patients (3191.3 ± 594.9 pg/ml) than controls (1771.7 ± 103.1 pg/ml) ($p < 0.001$). Serum Ang-2 levels were significantly correlated with ESR, CRP, DAS28, and duration of morning stiffness ($p < 0.001$, $p < 0.001$, $p < 0.001$, and $p = 0.025$, respectively). There was a significant correlation between serum Ang-2 levels and findings of US, regarding joint synovial thickness, and TSS ($p < 0.001$, for both).

Conclusion: Patients with RA had significantly higher levels of serum Ang-2 versus controls. In those patients, serum Ang-2 levels were significantly correlated with disease activity markers (ESR,

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CRP), DAS28, and duration of morning stiffness. Moreover, these levels were significantly correlated with synovial thickness, and TSS. The role of Ang-2 in RA pathogenesis might open the door to the development of new therapeutic strategies, particularly which target angiogenesis.

1. Introduction

Rheumatoid arthritis (RA) is a disease characterized by the chronic inflammation of joint synovial tissues. Inflammatory synovial tissue is called pannus. At such sites, many newly formed vessels are observed [1,2]. Angiogenesis is the formation of new capillaries from pre-existing blood vessels. Angiogenesis has been associated with inflammation and chronic inflammatory diseases, including RA [3,4].

Angiopoietin-2 (Ang-2), which is 478 amino acids in length with a molecular weight of 70 KDa, is a glycoprotein, and an angiogenic factor, plays an important role in the angiogenesis of pannus [5]. It is expressed in endothelial cells, stored in vesicles, and is rapidly released in response to specific stimuli at sites of vascular remodeling [6]. Ang-2 acts by binding to endothelium specific receptor Tyrosine kinase-2 (Tie-2) and the Ang/Tie system tightly controls the endothelial phenotype during angiogenesis and vascular inflammation in a unique fashion [7].

Doppler ultrasonography (US), which directly visualizes the synovial-membrane vessels, provides very early information on changes in synovitis activity during the course of inflammatory joint disease [8,9]. Doppler US can assess the synovial pannus and vascular tissues along with the detection of low-velocity blood flow at the microvascular level [10,11]. US is far more sensitive than physical examination for detecting rheumatoid synovitis. Also, it has similar sensitivity to magnetic resonance imaging, but is both far easier to use and considerably less expensive [12,13].

Therefore, the aim of the current study was to estimate serum levels of Ang-2 in patients with RA. Also, to correlate these levels with various clinical and Doppler US parameters.

2. Patients and methods

This study included forty RA patients [36 (90%) were females, and 4 (10%) were males], and their mean age was 43.9 ± 6.6 years. In addition, twenty five, age and sex matched healthy volunteers, were included. They included 20 (80%) females, and 5 (20%) males, and their mean age was 44.2 ± 6.8 years.

All RA patients were attendants of the Rheumatology inpatient or outpatient Department, Faculty of Medicine, Menoufiya University Hospital, in the period from September 2012 to May 2013.

Diagnosis of RA was made according to the American Rheumatism Association (ARA) criteria of American College of Rheumatology [14]. Patients with other suspected or known collagenic disease, liver disease, or renal disease were excluded from this study. The study was approved by our ethics committee of the faculty of medicine, and an informed consent was taken from all subjects.

All patients and controls were subjected to full history taking especially for duration of morning stiffness, fatigue and HAQ score assessment; and clinical examination, particularly for number of swollen joints, number of tender joints, and patient's assessment of pain using visual analogue scale (VAS). Moreover, disease activity score (DAS 28), was calculated with assessment of swollen and tender joints using 28-Joint counts [15].

Laboratory investigations included assays of complete blood count (CBC), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), rheumatoid factor (RF) titre, and serum levels of Ang-2.

2.1. Sample collection and assay

7 ml of venous blood samples were collected from all subjects under complete aseptic condition by clean venipuncture then dispensed into three tubes: 3 ml into plain tubes for chemical analysis of the previously mentioned parameters, 2 ml was transferred into EDTA tubes for complete blood count and 1.6 ml of blood was transferred to a tube with 0.4 ml citrate for ESR measurement. The samples in plane tubes, let to stand to clot and serum was separated in aliquots after centrifugation and stored at −70° until analysis of the following materials. CRP was determined using immunoassay [16]. RF concentration was assessed using RF Latex which was a slide agglutination test for the qualitative and semiquantitative detection of RF in human serum. Latex particles coated with human gammaglobulin were agglutinated when mixed with samples which contain RF [17]. ESR was determined according to Westergren method, and CBC was determined using a pentra-80 automated blood counter (ABX-France, Montpellier, France).

2.2. Ang-2 sample assay

It was assessed by The RayBio-Human Ang-2 Enzyme-Linked Immunosorbent Assay (ELISA) kit, which was in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human Ang-2 in the serum. This assay employed an antibody-specific for human Ang-2 coated on a 96-well plate. Standards and samples were pipetted into the wells and Ang-2 present in a sample was bound to the wells by the immobilized antibody. The wells were washed and biotinylated anti-human Ang-2 antibody was added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted to the wells. The wells were again washed, a TMB substrate solution is added to the wells and colour develops in proportion to the amount of Ang-2 bound. The Stop solution changes the colour from blue to yellow, and the intensity of the colour is measured at 450 nm.

2.3. US assessment of joints

A Hitachi ultrasound system (Hitachi medical systems, Japan) was used for the US examinations using a 15 MHz linear array transducer. The target joint was scanned at predefined transducer positions for that particular joint and the synovial membrane was examined longitudinally and transversally. Blood flow in the synovial membrane was visualized with colour Doppler US. The colour Doppler settings were the same for all joints and all patients with a gain setting just below the noise level using our set up for low flow-Nyquist limit +/− 0.014 m/s and 7 MHz Doppler frequency. With this set up all the colour pixels in the image correspond to motion, that is, blood flow. Quantitative estimation of the vascularization in the synovial membrane was performed using the selected colour Doppler image (CDI) with maximum colour activity. At present the colour Doppler is as sensitive as the power Doppler on the Hitachi. The digitally stored CDI in DICOM format was transferred to a processing programme. The synovium was traced, indicating the region of interest (ROI). On the US image the synovium appeared as a predominantly hypoechoic mass covering the bony surfaces adjacent to the joint. By using a colour recognition function the number of colour pixels as well as the total number of pixels in the ROI were counted [18]. The number of colour pixels was then expressed in relation to the total amount of pixels in the ROI as the colour fraction. The total number of pixels (=synovial area) was also noted separately, as an estimate of the synovial membrane volume.

Tenosynovial and intrabursal pulsed Doppler (PD) signals were graded on a semi-quantitative scale from 0 to 3 (grade 0 = absence, no synovial flow; grade 1 = mild, less than or equal to 3 isolated signals; grade 2 = moderate, more than three isolated signals or confluent signals in less than half of the synovial area; grade 3 = marked, signals in more than half of the synovial area); these scores corresponded to the maximum score for PD signals obtained from any of the synovial sites evaluated at each joint, as documented by Naredo et al [19]. Also, the sum of the PD signals scores obtained at each joint was used as the PDUS scores. We have chosen eight synovial sites from eight joints, including bilateral wrists (dorsal recess), elbows, ankles and knee joints. The eight-joint (8j) PDUS score was the sum of the eight synovial sites [20].

The images were evaluated with the sequence and name of the patient ''blinded''. The synovium was noted as non-inflamed when no pixels were present in the ROI [21].

2.4. The Health Assessment Questionnaire (HAQ) score

It assessed the ability to perform 20 activities of daily living [22], with four response categories. HAQ score, was a well evaluated instrument for the assessment of personal activity [23]. The Arabic version of HAQ score was validated for use in RA [24].

Statistical analysis: Results were collected, tabulated, statistically analysed by IBM personal computer and statistical package SPSS version 16. Two types of statistics were done; descriptive, e.g., no, percentage (%), mean, standard deviation (SD) and range; and analytical, which included the following tests. Student's *t*-test, was used to collectively indicate the presence of any significant difference between two groups for a normally distributed quantitative variable. Fisher's exact test, was used to compare between two groups or more regarding one qualitative variable in 2×2 contingency table when the count of any of the expected cells less than 5. Pearson's correlation analysis, was used to show strength and direction of association between two quantitative variables. *P*-value, was considered non-significant if $p > 0.05$, significant difference if $p < 0.05$, and highly significant if $p < 0.01$ or $p < 0.001$.

3. Results

There was no significant difference between the studied RA and control groups regarding age and sex ($p = 0.861$ and $p = 0.288$, respectively). Serum Ang-2 levels were significantly higher among patients (3191.3 ± 594.9 pg/ml) than controls (1771.7 ± 103.1 pg/ml) ($p < 0.001$).

Clinical and laboratory characteristics of RA patients are given in Table 1. Correlation between Ang-2 and clinical, and laboratory data of RA patients, was shown in Table 2. Correlation between serum angiopoietin-2 and DAS 28 to Doppler ultrasound synovial thickness and score, for different joints among rheumatoid arthritis patients, is shown in Table 3.

Fig. 1, shows colour Doppler US of the right knee with four hot spots and synovial Doppler score equal to 2. In contrast, Fig. 2, shows colour Doppler US of the left wrist with synovial thickness equal to 5.6 mm, active pannus and five hot spots, with synovial Doppler score equal to 2.

4. Discussion

In our work, we noted an increase in the serum levels of Ang-2 in patients with RA, versus controls. This is compatible with the results of previous studies [25,26]. Also, serum levels of Ang-2 in patients with RA were positively correlated with markers of inflammation (ESR, and CRP), and DAS28. This is in accordance with recent studies [26,27]. In line with our work, Kumpers et al. [28], reported that Ang-2 was elevated in systemic lupus erythematosus patients with disease activity.

We noticed that Ang-2 levels were correlated with morning stiffness, RF titre, but not with duration of RA, or patients' age. Morning stiffness, which is characteristic of RA, usually persists more than one hour but often lasts several hours. Its duration is a useful gauge of the inflammatory activity of the

Table 1 Clinical and laboratory characteristics of rheumatoid arthritis patients.

Patients ($n = 40$)	Mean ± SD	Range
Disease duration (years)	9.0 ± 6.4	2–20
Duration of MS (minutes)	45.5 ± 22.6	5–60
Number of swollen joints	4.6 ± 3.8	1–17
Number of tender joints	6.3 ± 3.9	1–17
Serum RF titre (IU/ml)	60.6 ± 68.5	12–356
VAS (cm)	5.3 ± 1.1	4–8
DAS 28	4.8 ± 0.7	3.8–6.2
HAQ	6.0 ± 2.9	4–14

MS = Morning stiffness, RF = Rheumatoid factor, VAS = Visual analogue scale, DAS28 = Disease activity score, HAQ = Health Assessment Questionnaire.

Table 2 Correlation between serum angiopoietin-2 and clinical, and laboratory data of rheumatoid arthritis patients.

	Angiopoietin-2 (pg/ml)	
	r	*P* value
Age (years)	−0.273	0.088(NS)
Disease duration (years)	−0.210	0.193(NS)
Duration of MS (minutes)	0.354	0.025 (S)
Haemoglobin (g)	0.125	0.443(NS)
Leucocyte count (1000/ml)	0.410	0.009(HS)
Platelet count (1000/ml)	−0.242	0.133(NS)
ESR (mm/h)	0. 675	< 0.001(HS)
CRP (mg/dl)	0.666	< 0.001(HS)
RF titre (IU/ml)	0.462	0.003(HS)
VAS (cm)	−0.252	0.117(NS)
DAS 28	0.624	< 0.001(HS)
HAQ	0.131	0.421(NS)

MS = Morning stiffness, ESR = Erythrocyte sedimentation rate, CRP = C-reactive protein, RF = Rheumatoid factor, VAS = Visual analogue scale, DAS28 = Disease activity score, HAQ = Health Assessment Questionnaire, NS = Non-significant, S = Significant, HS = Highly significant.

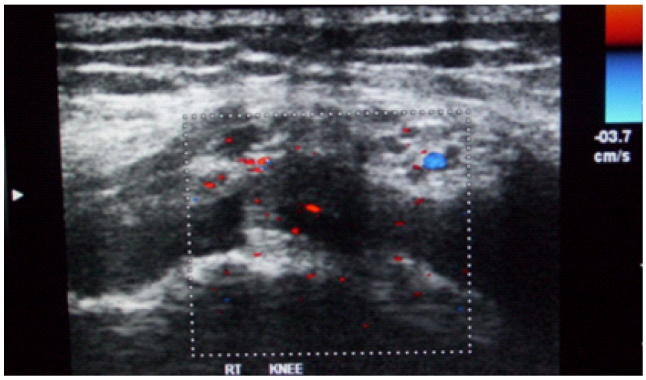

Figure 1 Colour Doppler ultrasound of the right knee showing four hot spots with synovial Doppler score equal to 2.

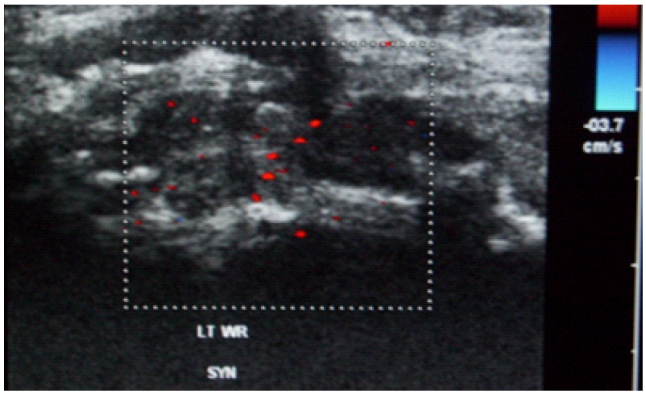

Figure 2 Colour Doppler ultrasound of the left wrist showing synovial thickness equal to 5.6 mm with active pannus and five hot spots synovial Doppler score equal to 2.

disease [29].Therefore, Ang-2 levels which are correlated with inflammatory markers and DAS28, are expected to correlate with morning stiffness duration. Contrary to our work, Westra et al. [26], found that Ang-2 levels were correlated positively with age. This difference may be due to difference in patient's characteristics such as age limits.

The finding that Ang-2 was correlated with leucocytes count is interesting. Ang-2 was considered to be a chronic inflammatory mediator [30]. It was reported that the activated synoviocytes-derived Ang-2 might play an important role in RA pathogenesis through promoting synovial inflammation [27].

In our work, Ang-2 was found to be not correlated with HAQ score. This may be due to the characteristic of Ang-2 which acts as an inflammatory mediator [29], but not related to personal activity.

We found a positive correlation between Ang-2 levels, and synovial thickness as well as most joint signal scores. This is in agreement with the findings of similar studies [19,31], who reported that the TSS correlated with the level of Ang-2. The synovium is normally a relatively acellular structure with a delicate intimal lining. In contrast, the RA synovium is greatly ex-

Table 3 Correlation between serum angiopoietin-2 and DAS28 to Doppler ultrasound synovial thickness and score, for different joints among rheumatoid arthritis patients.

	Angiopoietin-2		DAS28	
	r	*P* value	*r*	*P* value
Synovial thickness (mm):				
Wrist joints	0.593	< 0.001 (HS)	0.139	0.393(NS)
Elbow joints	0.294	0.065 (NS)	0.254	0.114(NS)
Ankle joints	0.556	< 0.001 (HS)	0.020	0.903(NS)
Knee joints	0.634	< 0.001 (HS)	0.359	0.023(S)
Synovial signal score:				
Wrist joints	0.504	< 0.001 (HS)	0.525	0.001(HS)
Elbow joints	0.050	0.758 (NS)	0.241	0.134(NS)
Ankle joints	0.659	< 0.001 (HS)	0.483	0.002(S)
Knee joints	0.882	< 0.001 (HS)	0.509	0.001(HS)
Total signal score (TSS)	0.882	< 0.001 (HS)	0.195	0.228(NS)

DAS28 = Disease activity score, **NS** = Non-significant, **S** = Significant, **HS** = Highly significant.

panded, and many infiltrating cells, including not only inflammatory hematopoietic lineage cells but also vascular endothelial cells, exist in the RA synovium [32,33].

The increase in synovial blood flow signals has been observed histologically to be caused by an increase in the number of blood vessels in the synovial tissue, i.e., angiogenesis [34,35]. Increased blood flow signals on power Doppler US in the proliferated synovium (pannus) of patients with RA have been used to evaluate articular inflammation [36,37].

We assessed TSS in eight joints for every patient. However, other studies used different numbers of joints. Actually, Doppler US has been regarded as a valuable tool for globally examining the extent of synovitis in RA. However, it is presently difficult to determine a minimal number of joints to be included in a global US score. There was a lack of clear definition of synovitis as well as varying validity data with respect to the proposed scores. So, scoring systems included a wide range and number of joints [20,38]. Till present, further studies are required in order to achieve optimal US scoring systems for monitoring patients with RA in clinical trials and in clinical practice [39].

Implications of the study. Firstly, the identification of markers for disease activity in patients with RA is crucial. Ang-2 reflects the degree of inflammation in patients with RA. Furthermore, it might predict disease outcomes. Its use in everyday practice might help to identify the subset of patients who require aggressive treatment [40,41]. Secondly, using power Doppler US, might not only help to identify and monitor patients but also might provide an early assessment of the response to a specific drug treatment. In the future, the development of standard criteria will enable the widespread use of this method in individual patients seen in everyday clinical practice [8,42]. Finally, medications which specifically target angiogenesis have produced promising results in other fields. Their use in the treatment of RA will probably be considered in the near future, although adjustments for the specific features of RA will be needed. Angiogenesis markers can be expected to identify those patients most likely to benefit from anti-angiogenesis treatment options [4].

In conclusion, patients with RA had significantly higher levels of serum Ang-2 versus controls. In those patients, serum Ang-2 levels were significantly correlated with disease activity markers, DAS28, duration of morning stiffness, but not patients' age, duration of RA, or HAQ scores. Moreover, these levels were significantly correlated with US findings, namely joint synovial thickness, and TSS. Therefore, serum Ang-2 levels might be used as a useful clinical biomarker for RA activity.

Conflicts of interest

The authors declare no conflicts of interest.

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