POSSIBLE ROLE OF SERUM LEVELS OF VASCULAR ENDOTHELIAL GROWTH FACTOR AND ENDOSTATIN IN RHEUMATOID ARTHRITIS ANGIogenesIs IMBALANCE

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KEY WORDS: VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF), ENDOSTATIN, RA

ABSTRACT

Objective: To measure serum levels of the main angiogenic inducer marker (VEGF) and the main angiogenic inhibitor marker (endostatin) in rheumatoid arthritis patients. Also, to study their correlation to clinical and laboratory variables of the disease in an attempt to provide more insight regarding their possible role in the angiogenesis imbalance and pathogenesis of RA.

Methodology: Twenty RA patients and fifteen age and sex matched healthy persons served as a control group underwent full history taking, thorough clinical examination, and routine rheumatological profile. Measurement of serum VEGF and endostatin levels were done using enzyme linked immunosorbent assay (ELISA) in rheumatoid arthritis patients and compared with controls. Comparison between patients with or without systemic involvement regarding serum level of VEGF was done. Correlations between serum levels of VEGF and signs of disease activity were also done.

Results: A highly significant increase in the mean values of serum VEGF was found in RA patients compared to control subjects (t=11.83, p<0.001), while there was no statistically significant difference between both RA and control groups regarding mean values of endostatin (t=0.06, p>0.05). In addition a highly significant increase in the mean values of serum VEGF was found in RA Patients with extra-articular manifestation (EAM) compared to Patients without EAM (t=2.98, p<0.01). Serum VEGF was positively correlated with ESR, DAS, and CRP (r =8.48, p<0.01; r = 0.542, p< 0.5; and r = 0.49, p< 0.5) respectively.

Conclusion: We found an imbalance between the production of angiogenic growth factors and angiogenic inhibitors in RA. This may play an important role in the angiogenesis imbalance and pathogenesis of RA. In addition we conclude that VEGF level is related to disease activity and extra-articular manifestation of RA, so it can be considered a good indicator for evaluation of disease activity, systemic organ involvement and planning treatment strategies.

INTRODUCTION

Rheumatoid arthritis is a systemic autoimmune disease characterized by persistent synovial inflammation and joint destruction. Erosive joint damage is associated with extensive growth of the synovial pannus and its invasion to cartilage and subchondral bone. The expansion of synovial tissue is due to proliferation and infiltration of cells of
lympho-hemopoietic origin, and formation of new micro-vessels from the preexisting vasculature, a process known as angiogenesis, which is essential for maintaining and nourishing the synovial tissue mass, it facilitates recruitment of inflammatory cells to the synovium (Ballara et al., 2001).

Angiogenesis is a complex multistep process that is tightly controlled by angiogenesis inducers and inhibitors. Under normal conditions, the levels of angiogenesis inducers and inhibitors are balanced (Koch, 2000). Controlled Angiogenesis is essential during tissue repair, fetal development, and female reproductive cycle. In contrast, uncontrolled angiogenesis promotes tumors, retinopathies and inflammatory angiogenic diseases including RA (Pandya et al., 2006).

It is thought that synovial angiogenesis in RA is driven by a combination of tissue hypoxia, up regulation of endothelial growth factors, and down regulation of angiogenesis inhibitors (Paleolog & Fava, 1998). Although new blood vessels deliver oxygen to the augmented inflammatory cell mass, the neo-vascular network is dysfunctional and fails to restore tissue oxygen homeostasis, so that the rheumatoid joint remains a markedly hypoxic environment (Taylor & Sivakumar, 2005).

Vascular endothelial growth factor (VEGF) is a heparin–binding glycoprotein. It is the most specific growth factor for endothelial cells. It is involved in several steps in pathological angiogenesis including proliferation and migration of endothelial cells to form new vessels; it also increases the penetration and extravasation of plasma molecules from blood vessels. It was initially named vascular permeability factor (Brown et al., 1997).

Expression of VEGF is increased by hypoxia and consequently, it is up regulated in many angiogenesis-dependant diseases associated with perfusion insufficiency and increased metabolic demand including RA (Ballara et al., 2001). It is expressed in response to soluble mediators such as cytokines (IL-1, TNF, IL-6) and growth factors. Its receptors are the best-characterized system in the angiogenesis regulation of rheumatoid joints (Maruotti et al., 2006). It is produced locally by activated synovial monocytes, macrophages, fibroblasts and synoviocytes (Berchmans et al., 2005).

Endostatin is a non-collagenic domain, 20 KD proteolytic fragment of collagen XVIII. It is an endogenously produced angiogenesis inhibitor (Dag et al., 2005). Studies had shown that endostatin inhibits the endothelial cell proliferation and migration (in-vitro) and tumor growth dependant on angiogenesis (in-vivo) (Kruger et al., 2000). The ability of endostatin to inhibit angiogenesis in tumors suggests that it may be useful for treatment of other pathological neo-angiogenic conditions such as RA. It has an inhibitory effect against immunocytes, and can produce apoptosis on RA synovia (Matsuno et al., 2002).

There are numerous clinical trials testing the hypothesis that inhibition of VEGF may have therapeutic value in RA (Pandya et al., 2006), and others suggest that anti-angiogenesis treatment using human recombinant endostatin represents a potential new therapeutic strategy for RA (Matsuno et al., 2002).

In our study, we are assessing the serum levels of VEGF and endostatin in RA patients and testing their possible correlation with the disease activity variables to focus the light on their possible rule in the angiogenesis imbalance in RA.
Aim of work:
To measure serum levels of the main angiogenic inducer marker (VEGF) and the main angiogenic inhibitor marker (endostatin) in rheumatoid arthritis patients. This is in an attempt to study their correlation to clinical and laboratory variables of the disease in an attempt to provide more insight regarding their possible role in the angiogenesis imbalance and pathogenesis of RA.

PATIENTS AND METHODS
Twenty patients fulfilling the American College of Rheumatology classification criteria for RA (Arnett et al., 1988) were included in this study. A group of 15 healthy subjects of matched age and sex served as a control group.

At the time of the study, all patients were subjected to the following:

1- Full history taking with special emphasis on: age, sex, disease duration, and duration of morning stiffness.

2- Thorough clinical examination including: assessment of extra-articular manifestations, and assessment of disease activity (which was carried out using modified disease activity score (DAS) calculated using ESR, 28 tender joint count, 28 swollen joint count, and VAS (Prevoo et al., 1995). The values were from 0 to 10, where 0 stood for the least active, and 10 stood for the severely active).

3- Laboratory investigations:
All patients and controls were subjected to:

a. Complete blood count (CBC) using Coulter Counter T660.

b. Erythrocyte sedimentation rate (ESR) using Westergren method.

c. C-reactive protein (CRP) using enzyme linked immunosorbent assay (ELISA) technique.

d. Rheumatoid factor (RF) by latex agglutination.

e. Serum VEGF using ELISA technique (Quantikine R & D system Inc., USA).

f. Serum endostatin using ELISA technique (Quantikine R & D system Inc., USA)

Measurement of serum VEGF:
Quantitative Sandwich Enzyme Immunoassay technique was used to measure serum VEGF. The system uses micro-plates with wells precoated with monoclonal antibody specific for VEGF. Standards and samples were pipette into wells. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for VEGF was added to the wells. Following a wash to remove unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of total VEGF bound in the initial step. The concentrations were reported as pg/ml.

Measurement of serum endostatin:
Serum level of endostatin was measured by a quantitative Enzyme Immunoassay technique. The system uses micro plates with wells precoated with rabbit anti-Human Endostatin polyclonal antibody. Standards and samples were pipette into wells. Fifty µl of the diluted streptavidin alkaline phosphatase was added into each well. After washing 200 µl of the prepared color reagent solution were dispersed into each well. Then the wells were incubated at room temperature for 20 minutes. The final reading was taken and reported as ng/ml.

Statistical analysis:
Data were collected, tabulated and analyzed using the (Scientific package of social statistics) program on an IBM compatible personal computer.

The mean and standard deviation for each parameter were determined and the statistical significance was calculated using
the student’s “t” test for paired data. A value of \( p < 0.05 \) was considered statistically significant.

Correlation matrix and correlation coefficient "r" for relationship of different variables were calculated using Pearson’s method. Correlation of qualitative variables was done using Spearman’s correlation.

**RESULTS**

Demographic and baseline clinical characteristics of patients and control group:

This study was carried out on 20 RA patients; 18 were females (90%) ranged in age from 30 to 49 years with a mean of 36.9±5.1 years. 18 patients (90%) had +ve Rheumatoid factor.

A group of 15 healthy subjects, 13 females and 2 males ranged in age from 29 to 50 years with a mean of 35.4±4.6 years, served as a control group.

Table (1): Demographic and clinical characteristics of patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age “years”</td>
<td>30-49</td>
<td>36.9</td>
<td>5.1</td>
</tr>
<tr>
<td>Disease Duration “years”</td>
<td>7-18</td>
<td>11</td>
<td>3.4</td>
</tr>
<tr>
<td>Visual analog scale</td>
<td>40-100</td>
<td>56.5</td>
<td>14.5</td>
</tr>
<tr>
<td>Morning stiffness “minutes”</td>
<td>60-120</td>
<td>75</td>
<td>11.5</td>
</tr>
<tr>
<td>Disease activity score</td>
<td>3.2-4.9</td>
<td>3.9</td>
<td>0.54</td>
</tr>
<tr>
<td>Erythrocyte sedimentation rate “mm in 1st hour”</td>
<td>40-110</td>
<td>65.84</td>
<td>21.30</td>
</tr>
<tr>
<td>C-reactive protein “mg/l”</td>
<td>7-52</td>
<td>12.9</td>
<td>6.2</td>
</tr>
<tr>
<td>Serum vascular endothelial growth factor “pg/ml”</td>
<td>380-650</td>
<td>510</td>
<td>104</td>
</tr>
<tr>
<td>Serum endostatin “ng/ml”</td>
<td>37.9-68.3</td>
<td>55.2</td>
<td>14.2</td>
</tr>
</tbody>
</table>

The mean values of serum VEGF, ESR, and CRP were significantly higher in RA patients compared to control subjects (\( t = 11.83, 14.77 \) and 8.73 respectively & \( p<0.001 \) for all). On the other hand the mean values of serum endostatin were not significantly different between RA patients and the control subjects (\( t=0.06, p>0.05 \) (table 2) (Fig 1, 2). Patients were divided in to two groups according to the presence or absence of extra-articular manifestations. Extra-articular manifestations were present in eight patients: rheumatoid nodules were present in six, skin signs of vasculitis were present in four, neural affection was present in five, and pulmonary manifestations were present in one.

Table (2): Statistical comparison between RA patients and controls regarding mean values of both serum VEGF and serum endostatin.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>patients</th>
<th>control</th>
<th>t</th>
<th>p</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum vascular endothelial growth factor. (pg/ml)</td>
<td>510 ± 104</td>
<td>160 ± 82</td>
<td>11.83</td>
<td>.000</td>
<td>HS</td>
</tr>
<tr>
<td>Serum endostatin (ng/ml)</td>
<td>55.2 ± 14.2</td>
<td>51.8 ± 12.0</td>
<td>0.42</td>
<td>.67</td>
<td>NS</td>
</tr>
</tbody>
</table>
On comparing serum VEGF and endostatin levels between patients with and without extra articular manifestations we found that the mean values of serum VEGF was significantly higher in RA Patients with extra articular manifestations in contrast to Patients without extra articular manifestations ($t=2.98$, $p=.000$), while no significant differences were found between both groups regarding serum endostatin levels ($t=0.31$, $p=.76$). (Table: 3) (Figs: 3 & 4).
Table (3): Comparison between RA Patients with extra articular manifestations and Patients without extra articular manifestations regarding mean values of both serum VEGF and endostatin.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients with extra-articular manifestation</th>
<th>Patients without extra-articular manifestation</th>
<th>t</th>
<th>p</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum VEGF (pg/ml)</td>
<td>570 ± 140</td>
<td>360 ± 120</td>
<td>5.42</td>
<td>.000</td>
<td>HS</td>
</tr>
<tr>
<td>Serum endostatin (ng/ml)</td>
<td>56.1 ± 12.56</td>
<td>52.9 ± 13.92</td>
<td>0.31</td>
<td>.76</td>
<td>NS</td>
</tr>
</tbody>
</table>

VEGF= vascular endothelial growth factor.

![Graph showing comparison between RA patients with extra-articular manifestation and Patients without extra articular manifestations regarding serum endostatin level](image)

On studying the correlation between serum VEGF level and various disease parameters (using Pearson's correlation coefficient for quantitative data and Spearman correlation coefficient for qualitative data) we found that serum VEGF level was positively correlated with ESR (r =8.48, p<0.01), DAS (r = 0.542, p<0.5) and CRP (r = 0.493, p< 0.5).

Table (4): correlation between vascular endothelial growth factor (VEGF) and disease parameters.

<table>
<thead>
<tr>
<th>Disease parameters</th>
<th>r</th>
<th>p</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>erythrocyte sedimentation rate</td>
<td>8.48</td>
<td>&lt;0.01</td>
<td>HS</td>
</tr>
<tr>
<td>disease activity score</td>
<td>0.542</td>
<td>&lt;0.5</td>
<td>S</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>0.493</td>
<td>&lt;0.5</td>
<td>S</td>
</tr>
</tbody>
</table>

DISCUSSION

Rheumatoid arthritis is a classical example of inflammatory angiogenic diseases, which is mediated by pro-inflammatory and pro-angiogenic cytokines (Yin et al., 2002). Pathological angiogenesis is a crucial aspect of RA, exuberant proliferation of new blood vessels was observed in RA synovium. Varieties of angiogenic mediators, including cytokines and growth factors, have been identified in RA joints (Matsumo et al., 2002).

Vascular endothelial growth factor
VEGF has been recognized as a key factor in the induction of angiogenesis, and it is involved in several steps of both physiological and pathological angiogenesis including endothelial survival, proliferation, and migration (Ferrara & Bunting, 1996). VEGF expression is up regulated in macrophages and fibroblasts of RA synovium and it is detectable in synovial fluid and serum of RA patients (Ballara et al., 2001). On the other hand, endostatin is angiogenic inhibitor, it inhibits the endothelial cell proliferation and migration in vitro, and it can produce apoptosis on RA synovia (Matsuno et al., 2002). The persistence of inflammation and the disorganized angiogenesis in rheumatoid joints are consequences of an imbalance between the inducers and inhibitors of angiogenesis (Maruotti et al., 2006).

This study was designed to measure the serum levels of the main angiogenic inducer factor (VEGF) and the main angiogenic inhibitor factor (endostatin) in rheumatoid arthritis patients and to study their correlation to the clinical and laboratory variables of the disease in an attempt to provide more insight regarding their possible role in the angiogenesis imbalance and pathogenesis of RA.

The significantly higher mean values of serum VEGF in RA patients compared to control subjects in our study (510±104 versus 160±82, p=.000) support the postulation that VEGF is one of the most specific and critical regulators of angiogenesis in RA and other inflammatory - angiogenic diseases and that its receptors are the best characterized system in the angiogenesis regulation of rheumatoid joints (Maruotti et al., 2006). The increased VEGF level in RA patients is an attempt to maintain and nourish the growing synovial tissue mass through neovascularization (that is unfortunately defective and disorganized one) and to facilitate recruitment of inflammatory cells to the synovium (Ballara et al., 2001). Recent clinical trials are testing the hypothesis that inhibition of VEGF may have therapeutic implication in RA.

Our results are in agreement with (Harada et al., 1998, Lee et al., 2001, Klimiuk et al., 2002 and Strunck et al., 2004) who detected higher serum VEGF levels in RA patients compared to control and postulated that VEGF is involved in the pathogenesis of RA.

In our study serum VEGF was positively correlated with ESR (r =8.48, p<0.01), DAS (r = 0.542, p<0.5) and CRP (r = 0.493, p<0.5). Our results are in agreement with Harada et al. (1998) who demonstrated positive correlation between serum concentration of VEGF and serum level of CRP. In their study the serum level of VEGF before treatment was significantly higher than after treatment (in patients who experienced clinical remission) and they concluded that measurement of serum VEGF is a noninvasive useful method for monitoring disease activity in RA.

In addition, our results are in agreement with Lee and his colleagues in 2001 who reported positive correlation between serum VEGF and ESR, rheumatoid factor, and number of tender and swollen joints. Matching with our results, Klimiuk et al. (2004) reported that serum VEGF was positively correlated with ESR, and CRP and they concluded that VEGF might be useful in prediction of RA activity.

Kuryliszyn & his coworkers (2006) recently reported that serum VEGF was significant positively correlated with DAS, ESR and CRP.

In our study RA patients with extra articular manifestations showed significantly higher mean values of VEGF in contrast to patients without extra articular manifestations (t=2.98, p<0.01) and this may signify a relation between the
extra articular manifestations of RA (that might have an inflammatory/angiogenic origin) and VEGF as an angiogenic inducer.

To the best of our knowledge, only one published study (up to the date of submission of our article) had evaluated serum VEGF in RA patients with extra articular involvement. Kuryliszyn et al. in their study published in 2006, had demonstrated that the elevated serum level of VEGF was associated with systemic organ involvement in RA patients. They concluded that elevated serum levels of VEGF in these patients might play a role in the pathogenesis of extra-articular manifestation of RA.

In our study, there was no significant difference between RA patients and control group regarding mean values of serum endostatin (t=0.06, p>0.05) and this finding can lead us to postulate that the disorganized angiogenesis in RA that is due to the imbalance between the angiogenic inducers (represented by the VEGF) and the angiogenic inhibitors (represented by Endostatin) is mainly due to the over expression of VEGF rather than the decrease in the formation of Endostatin. To the best of our knowledge, only one published study (up to the date of submission of our article) had evaluated the serum levels of Endostatin in RA patients. Nagashima et al. (2000) evaluated serum level of endostatin in RA patients and found that, in agreement with our results, there is no difference in serum endostatin levels between RA patients and control. In addition, they had also found that serum VEGF level was markedly elevated in RA patients in comparison to control.

Angiogenesis inhibition, which has been extensively studied for the treatment of various malignancies, is an emerging new potential therapy for proliferative synovitis, particularly RA (Lainer & Brahn 2005). There are numerous, published and ongoing, clinical trials testing the hypothesis that inhibition of VEGF may have therapeutic implications in management of RA (Pandya et al., 2006). Other studies suggested the anti-angiogenic therapy using human recombinant endostatin as a potential new therapeutic strategy for RA (Matsuno et al., 2002).

Currently there are more than 30 angiogenesis inhibitors under study in several clinical trials depending on the idea of suppression of activity and signaling pathways of VEGF. These new angiogenesis inhibitors include IL-6 blockade, which effectively suppress VEGF production in RA synovial fibroblasts (Nakahara et al., 2003), IL-Ira, which might be beneficial as a protector from VEGF in pathologic conditions (Inove et al., 2004 & Honorati et al., 2004) reported that IL-1 beta and TNF-alpha can inhibit the spontaneous secretion of VEGF. Moreover, Yoo et al. (2005) suggested that anti-VEGF peptide RRKRR (dRK6) may be an effective strategy in treatment of RA. In addition, Haas et al. (2006) reported that IL4 had reduced synovial tissue neovascularization via its angiostatic effects.

Yin et al. (2002) reported that endostatin gene transfer inhibits joint angiogenesis and pannus formation in inflammatory arthritis.

Conclusions:

We suggest that there is an imbalance between the production of angiogenic growth factors and inhibitors in RA, which may play an important role in the angiogenesis imbalance and pathogenesis of RA. In addition we conclude that VEGF level is related to disease activity and extra-articular manifestation of RA. So it can be considered a good indicator for evaluation of disease activity, systemic organ involvement and treatment planning strategies.
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دراسة مستوى عامل النمو البطاني والاندوستاتين في الريثمان المفصلي: الدور المحتمل في عدم توازن نشوء الأوعية الدموية

نجلاء يوسف عساب ومحمد عيدالباسط القرماوي وناهد علوان

قسم الروماتزم والتاليم كلية الطب جامعة عين شمس والباثولوجيا الإكلينيكية

المريض بالمقارنة بالمجموعة الضابطة في حين ليس هناك اختلاف بين مستوى الاندوستاتين في مصل المريض بالمقارنة بالمجموعة الضابطة. وقد أظهرت النتائج أيضاً ارتفاعاً ذو دالة إحصائية في مستوى عامل النمو البطاني بين المرضى الذين يعانون من ظواهر الإكلينيكية الخارجية عن إثبات المفصل والمرضى الذين لم يعانون منها، كما وجد علاقة إيجابية ذو دالة إحصائية عالية في مستوى عامل النمو البطاني وعمل نشاط المرض.

الهدف من البحث: هو قياس مستوى أهم عامل لنشوء الأوعية الدموية وهو عامل النمو البطاني وقياس مستوى أهم عامل لتلبية نشوء الأوعية الدموية وهو الإندوستاتين في مصل مرضى الريثمان المفصلي مع دراسة ارتباطهما بالحالات الإكلينيكية والفحوصات المعملية للمرض في محاولة للتوصيل لآهميتهما في عدم توازن نشوء الأوعية الدموية وتولد المرض.

الطريقة: أجري هذا البحث على عشرين مريضاً بالريثمان المفصلي وكذلك خمسة عشر من الأصحاء كمجموعة ضابطة، وقد تم تقييم الإكلينيكية وعمل الفحوصات المعملية لكل من المريض والمجموعة الضابطة. وتم أيضاً قياس عامل النمو البطاني الواعي والاندوستاتين في مصل الدم من المقارنة بين المرضى والمجموعة الضابطة. وتم أيضاً المقارنة بين مرضى الريثمان المفصلي ذو الطوارئ الإكلينيكية الخارجية عن التهاب المفصل والمريض الذين لا يعانون منها.

النتائج: أظهرت النتائج زيادة ذهالة إحصائية عالية في مستوى عامل النمو البطاني في مصل

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