Transforming Growth Factor-β Expression and Serum Concentration of Osteoprotegerin in Rheumatoid Arthritis Patients with Incidence of Hypertension


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ABSTRACT

Patients with long term rheumatoid arthritis (RA) suffered from a lot of complications. The most important complications are atherosclerosis which represents itself as hypertension. In this research we assessed and studied the correlation between Transforming Growth Factor-β (TGF-β) expression and serum osteoprotegerin (OPG) in RA patients with and without hypertension. Forty five patients with rheumatoid arthritis were enrolled in this study; 25 patients with normal blood pressure and 20 patients with hypertension. Twenty volunteers apparently healthy were selected as a control group. All patients and controls were subjected to physical examination including measuring body mass index (BMI) and blood pressure. Laboratory investigation; C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), lipid profile, serum osteoprotegrin and TGF-β expression by flowcytometry were performed. CRP was positive in 80% of rheumatoid hypertensive group while it was positive in 56% of rheumatoid normotensive patients. There was a significant increase in cholesterol, triglyceride and LDL in rheumatoid hypertensive patients compared to control and rheumatoid normotensive patients (P < 0.001). While HDL showed a significant decrease in rheumatoid hypertensive patients compared to control (P < 0.01) and rheumatoid normotensive patients (P ≤ 0.05). There was a significant increase in both OPG level and TGF-β expression in both rheumatoid patient's group compared to control group. OPG and TGF-β were highly significantly increased in rheumatoid hypertensive group compared to normotensive group. Excluding BMI as risk factor of atherosclerosis, this study provided evidence that OPG and TGF-β could be used as biomarkers for incidence of hypertension in rheumatoid patients.

Key words: Rheumatoid arthritis, hypertension, osteoprotegrin and TGF-β.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory progressive bone disease. RA is characterized by growth of the synovium with articular manifestations (Chung et al., 2005). Bone destruction with osteopenia and reduced bone mass are still the main unresolved problem in patients with RA (Romas et al., 2002).

There is an increasing evidence that patients with RA have accelerated atherosclerosis manifested as hypertension (Roman et al., 2006). The rheumatoid patients are considered to have hypertension if they are taking antihypertensive agents or they have a systolic blood pressure ≥ 140 mmHg and/or a diastolic blood pressure ≥ 90 mmHg (Asanuma et al., 2007).

The relationship between atherosclerosis and osteoporosis is still debated. Arterial calcification is associated with low bone mass. Among the potential pathogenic mechanisms that linked osteoporosis to atherosclerosis, a dysregulation of the OPG/RANK/RANKL system (Anagnostis et al., 2009).

Osteoprotegerin (OPG) is a glycoprotein, produced mainly by osteoblast lineag cells, endothelial cells and arterial smooth muscle cells (Van Campenhout and Golledge, 2009). Serum concentrations of OPG are elevated with inflammatory changes in RA as well as correlated with systemic atherosclerosis (Jono et al., 2002; Ali et al., 2009). OPG is expressed in the vascular endothelium and may play a role in developing atherosclerosis.
Osteoprotegrin interferes with the receptor activator of nuclear factor-kappa B (RANK) for binding to its ligand (RANKL). Binding of the receptor to its ligand will stimulate osteoclastogenesis promoting bone resorption in patients with RA (Poulsen et al., 2008). Dysregulation of the RANKL/OPG system with increased expression of RANKL gene has been implicated in pathophysiology of bone remodeling in RA (Vega et al., 2007; Kim et al., 2007).

The strategy of new therapy of RA is directed towards decreasing the RANKL/OPG ratio by either decreasing the expression of RANKL as by antioxidants or anti-inflammatory agents (Kolahi et al., 2010) or by increasing local OPG concentration by using gene therapy with human recombinant OPG gene (Hamerman, 2005).

Transforming growth factor-β1 (TGF-β1) is similar to OPG being cytokines closely associated with bone metabolism (Janssens et al., 2005). TGF-β1 is expressed by osteoblasts and stimulates the differentiation and survival of osteoclasts and hence regulates bone formation and resorption. Moreover, TGF-β1 significantly induces OPG expression (Chenu et al., 1988).

In this paper, the correlation between OPG and TGF-β1 had been studied in rheumatoid patients with or without hypertension.

Subjects and Methods:

Patients and control:

This study included 45 patients; they were selected from The Rheumatology Out patients' clinic of Tanta University Hospital, diagnosed as RA according to the 2010 American College of Rheumatology (ACR) /European League against Rheumatism (EULAR) classification criteria for rheumatoid arthritis and fulfilled the classification criteria for RA (Aletaha et al., 2010). The age of the patients ranged from 47 to 68 years. All patients were treated with methotrexate as a monotherapy disease modifying antirheumatic drugs (DMARDs) in a dose of 12.5 mg/week. Patients suffered from acute infection, renal disease, diabetes mellitus, or malignancies have been excluded from this study. Patients were divided in two groups according to their blood pressure; twenty-five of the patients were normotensive and twenty of the patients were hypertensive. Twenty apparently healthy normotensive volunteers with matched age (48 – 62 years) have been participated as control subjects.

A written consent was taken from all patients and healthy volunteers before starting any of the procedures of the research.

All cases included in this study were subjected to the following clinical and laboratory investigations:

- Complete history taking and clinical examination with special stress on cardiac symptoms, presence of cardiovascular risk factors such as smoking, hypertension, dyslipidemia, family history of premature chronic artery disease (CAD), body mass index (BMI) and the duration of rheumatoid illness.

- Laboratory investigations for all the patients and controls including serum CRP, lipid profile, serum OPG and TGF-β gene expression were measured.

Sample collection:

Four ml of peripheral venous blood fasting samples (12 hours) were taken from the patients and healthy subjects. Samples were collected and divided in 2 different sterile test tubes; heparinized tube for mononuclear cells isolation by ficoll-hypaque and plain tube to separate serum for all biochemical investigations.

OPG estimation:

Osteoprotegerin (OPG) levels were estimated by DuoSet ELISA kit (Catalog Number: DY805) which is a quantitative sandwich enzyme immunoassay technique purchased from R&D Systems, Inc.

Flow cytometry analysis of TGF-β:

Flow cytometric analysis of total TGF-β proteins was performed on the mononuclear cells after ficoll sedimentation. Immuno-staining was carried out using the mouse monoclonal antibody conjugated with allophycocyanine against TGF-β1, -β2, -β3 (R&D Systems “UK & Europe”). A FACSCalibur (BECTON DICKINSON) flow cytometer was used for analysis and the data were collected in the list mode. TGF-β1, -β2, -β3 labeling, measured in the fluorescence detector (FL) forward scatter (FSC) and side scatter (SSC) were collected using linear scales. The fluorescence signals were collected using logarithmic scales. Data acquisition and analysis by Cell Quest program (the magnitude of the signal was measured by using cell TM DNA
experiment document user's guide '02-61539-00' were performed on $10^4$ viable cells. Expression was evaluated as Cell percent (The number of stained cells minus the number of cells stained by irrelevant negative control as shown in figure 1).

![Flowcytometry data analysis of TGF-β expression in peripheral blood](image)

**Fig. 1:** Diagram of flowcytometry data analysis of TGF-β expression in peripheral blood.

Data were statistically analyzed using SPSS program, standard version 16. Quantitative data were presented as mean ± standard deviation, Student’s t-test and ANOVA were used to compare between the means. Correlation between variables was done using Pearson’s correlation study. $P \leq 0.05$ was considered to be statistically significant.

**Results:**

The general demographic and clinical data of control, rheumatoid normotensive and rheumatoid hypertensive groups were explored and presented in table 1. The control group included 8 male and 12 female with mean age 56.0 ± 4.2 years, their body mass index (BMI) 26.8 ± 2.4, two of them were positive for serological CRP test, and with mean ESR level 5.7 ± 2.7 mm/h and the mean blood pressure level were normal. Regarding rheumatoid normotensive group they were 3 male and 22 female with mean age 60.2 ± 4.7 years, with mean BMI 27.1 ± 2.5, fourteen of them were positive for serological CRP test, and the mean ESR level for all rheumatoid normotensive group 33.0 ± 10.0 mm/h and approximately normal mean of blood pressure. The rheumatoid hypersensitive group included 2 male and 18 female with mean age 59.0 ± 6.0 years, with mean BMI 26.3 ± 2.6, sixteen of them were positive for serological hypersensitive CRP test, and with mean ESR level 39.6 ± 10.60 mm/h and blood pressure (diastolic and systolic 101.5 ± 6.1 & 160.7 ± 9.2, respectively).

The comparison between all groups regarding sex, age and BMI using Fisher test resulted in no significant difference. On the other hand the t-test resulted in a significant difference in CRP, ESR and blood pressure.

**Table 1:** General parameters of both rheumatoid arthritis patients and control

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 20)</th>
<th>Rheumatoid normotensive (n = 25)</th>
<th>Rheumatoid hypertensive (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>8/12</td>
<td>3/22</td>
<td>2/18</td>
</tr>
<tr>
<td>Age (Years)</td>
<td>56.0 ± 4.2</td>
<td>60.2 ± 4.7</td>
<td>59.0 ± 6.0</td>
</tr>
<tr>
<td>BMI</td>
<td>26.8 ± 2.4</td>
<td>27.1 ± 2.5</td>
<td>26.3 ± 2.6</td>
</tr>
<tr>
<td>CRP (+/-)</td>
<td>2/18</td>
<td>14/11</td>
<td>16/4</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>5.7 ± 2.7</td>
<td>33.0 ± 10.0*</td>
<td>39.6 ± 10.6*</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>77.0 ± 6.4</td>
<td>80.8 ± 6.7</td>
<td>101.5 ± 6.1*</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>118.7 ± 6.5</td>
<td>122.6 ± 9.0</td>
<td>160.7 ± 9.2*</td>
</tr>
</tbody>
</table>

**NOTE:** BMI (body mass index); CRP (C-Reactive protein); ESR (Erythrocytes sedimentation rate)

Table 2 showed the laboratory data of all studied groups. The comparison between rheumatoid normotensive patients and control group regarding lipid profile was not significant. On the other hand, rheumatoid hypertensive group showed a significant increase in cholesterol, triglycerides and LDL compared to control group ($P < 0.001$) and compared to rheumatoid normotensive group ($P < 0.001$). HDL showed a significant decrease in rheumatoid hypertensive compared to control ($P < 0.01$) and compared to rheumatoid normotensive ($P \leq 0.05$). Regarding OPG level, the comparison between rheumatoid normotensive and control group was significantly increased ($P < 0.001$) and also the comparison between rheumatoid hypertensive and rheumatoid normotensive patients were significantly increased ($P \leq 0.05$). Also, TGF-β showed a significant increase in both rheumatoid normotensive and rheumatoid hypertensive compared to control group ($P < 0.001$). In addition, rheumatoid hypertensive showed a significant increase in TGF-β compared to rheumatoid normotensive ($P \leq 0.05$).
The increase in OPG levels was not related to severity studied groups.

The extent of rheumatoid activity under control of antirheumatic; methotrexate therapy was evaluated by measuring ESR and hypersensitive CRP which showed a significant increase in RA as related to control group with a significant difference in rheumatoid hypertensive patients than normotensive group. The incidence of positive CRP was more in rheumatoid hypertensive group (80 %) than in normotensive group (56 %).

Table 3 explores the correlation between different prognostic and diagnostic parameters in all rheumatoid arthritis patients. From this table it is clear that there was a positive correlation between duration of illness and inflammatory marker (hypersensitive CRP and ESR). Also there were positive significant correlations between hypersensitive CRP and TGF-β, blood pressure and ESR. Regarding OPG, there is no correlation with any other studied parameters. On the other hand, there were positive correlations between TGF-β and both inflammatory markers (hypersensitive CRP and ESR) and also blood pressure.

Table 2: Lipid profile, OPG and TGF-β cell percent in rheumatoid arthritis patients and control.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 20)</th>
<th>Rheumatoid normotensive (n = 25)</th>
<th>Rheumatoid hypertensive (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>117.8 ± 10.8</td>
<td>121.2 ± 15.8</td>
<td>195.2 ± 20.1**</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>85.9 ± 9.3</td>
<td>90.5 ± 10.7</td>
<td>115.9 ± 11.0**</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>58.9 ± 3.8</td>
<td>56.4 ± 3.3</td>
<td>53.7 ± 3.9**</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>41.7 ± 14.1</td>
<td>46.7 ± 16.1</td>
<td>118.4 ± 22.4**</td>
</tr>
<tr>
<td>OPG (ng/ml)</td>
<td>2.2 ± 0.3</td>
<td>2.9 ± 0.5*</td>
<td>3.2 ± 0.5**</td>
</tr>
<tr>
<td>TGF-β (Cell %)</td>
<td>5.7 ± 1.2</td>
<td>8.4 ± 1.9*</td>
<td>10.0 ± 1.5**</td>
</tr>
</tbody>
</table>

*P < 0.001 (in comparison to control group)  
** P < 0.01 (in comparison to rheumatoid normotensive group)  
* P ≤ 0.05 (in comparison to rheumatoid normotensive group)

Table 3: Correlations between different parameters in all rheumatoid arthritis patients.

<table>
<thead>
<tr>
<th></th>
<th>Duration</th>
<th>CRP*</th>
<th>OPG</th>
<th>TGF-β</th>
<th>Diastolic</th>
<th>Systolic</th>
<th>ESR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration</td>
<td>P≤</td>
<td>P ≥</td>
<td>r=</td>
<td>r=</td>
<td>r=</td>
<td>r=</td>
<td>r=</td>
</tr>
<tr>
<td>Duration</td>
<td>crp</td>
<td>—</td>
<td>0.344*</td>
<td>-0.1</td>
<td>0.142</td>
<td>0.105</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>P≤</td>
<td>0.021</td>
<td>0.25</td>
<td>0.56**</td>
<td>0.001</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>P≤</td>
<td>—</td>
<td>0.25</td>
<td>0.56**</td>
<td>0.001</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>P≤</td>
<td>0.021</td>
<td>0.25</td>
<td>0.097</td>
<td>0.001</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>r=</td>
<td>-0.1</td>
<td>0.25</td>
<td>0.215</td>
<td>0.156</td>
<td>0.149</td>
<td>0.056</td>
</tr>
<tr>
<td></td>
<td>P=</td>
<td>0.51</td>
<td>0.09</td>
<td>0.215</td>
<td>0.001</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>r=</td>
<td>0.352</td>
<td>0.000</td>
<td>0.156</td>
<td>0.001</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>P=</td>
<td>0.352</td>
<td>0.000</td>
<td>0.156</td>
<td>0.001</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>r=</td>
<td>0.105</td>
<td>0.468*</td>
<td>0.219</td>
<td>0.42**</td>
<td>0.049</td>
<td>0.94**</td>
</tr>
<tr>
<td></td>
<td>P=</td>
<td>0.493</td>
<td>0.001</td>
<td>0.149</td>
<td>0.004</td>
<td>0.000</td>
<td>0.000</td>
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<tr>
<td></td>
<td>r=</td>
<td>0.352</td>
<td>0.000</td>
<td>0.156</td>
<td>0.001</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>P=</td>
<td>0.352</td>
<td>0.000</td>
<td>0.156</td>
<td>0.001</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>r=</td>
<td>0.049</td>
<td>0.498*</td>
<td>0.287</td>
<td>0.43**</td>
<td>0.94**</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>P=</td>
<td>0.751</td>
<td>0.000</td>
<td>0.056</td>
<td>0.003</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>r=</td>
<td>0.308*</td>
<td>0.826*</td>
<td>0.269</td>
<td>0.54**</td>
<td>0.42**</td>
<td>0.41**</td>
</tr>
<tr>
<td></td>
<td>P=</td>
<td>0.04</td>
<td>0.000</td>
<td>0.074</td>
<td>0.000</td>
<td>0.004</td>
<td>0.005</td>
</tr>
</tbody>
</table>

*Correlation is significant at the 0.05 level  
** Correlation is significant at the 0.01 level  
* Hypersensitive

Discussion:

This paper studied the correlation of serum OPG and TGF-β signaling expression associating rheumatoid arthritis with or without vascular atherosclerosis as manifested by hypertension. Patients were divided into two subgroups; rheumatoid normotensive patients (n = 25) and rheumatoid hypertensive patients (n = 20) according to all criteria of either the systolic and/or the diastolic blood pressures as reported by Asanuma et al. (2007).

Obesity as an underlying risk factor of atherosclerosis was excluded by measuring BMI of different patients' groups.

The lipid profile was parallel to hypertension. A significant increase in cholesterol, LDL-cholesterol and triglyceride levels were detected in rheumatoid hypertensive patients as compared with the control group or even the rheumatoid normotensive patients. HDL-cholesterol showed significant decrease in rheumatoid hypertensive patients in comparison with other groups. Moreover, the lipid profile was not related to BMI of the studied groups.

Asanuma et al. (2007) studied the correlation of OPG with RA. They reported an elevated serum OPG levels in patients with either early or long standing RA. The increase in OPG levels was not related to severity of coronary artery calcium score. However, a previous study by Jono et al. (2002) showed the presence of strong
correlation between the increase in OPG levels and the severity of coronary artery disease that correlated to accelerated atherosclerosis.

Van Campenhout and Golledge (2009) reported that the increase in OPG level represented a risk factor for developing atherosclerosis which could explain the strong association between OPG and the severity of coronary artery calcification in patients with long standing RA which is characterized by accelerated atherosclerosis.

The present study showed a significant increase in both OPG level and TGF-β expression in rheumatoid patients' groups in comparison to control group. Mean while, OPG and TGF-β were more significantly higher in rheumatoid hypertensive group than the normotensive group.

There was a parallel correlation between the OPG levels and TGF-β expression. Toffoli et al. (2011) suggested the existence of a vicious circle between TGF-β and OPG. Recombinant OPG in experimental mice is able to initiate TGF-β dependent changes in vascular smooth muscle cells (VSMC) stimulating proliferation and inflammation. On the other hand, TGF-β significantly induces OPG expression in VSMC.

Wu et al. (2010) reported that in perimenopausal period, there is a strong correlation between OPG and TGF-β levels that indicate their influence in post-menopausal bone changes. This might explain the present result as postmenopausal females represented (88%) of rheumatoid normotensive group and (90%) of rheumatoid hypertensive group. Pennisi et al. (2010) showed a supportive evidence of an association between post-menopausal osteoporosis and atherosclerosis. There are many researchers that reported that elevated circulating OPG levels are detected in post-menopausal patients (Sandberg et al., 2006; Crisafulli et al., 2005).

TGF-β is a cytokine involved in the regulation of the production and release of the neuroendocrine hormone, gonadotropin releasing hormone GnRH (Bouret et al., 2004). Sun et al. (2006) reported that there is a close relationship between the levels of gonadotropins and cytokines including OPG and TGF-β that influence bone metabolism. TGF-β induces the expression of OPG in osteoblasts (Thirunavukkarasu et al., 2001). Low concentrations of TGF-β increased the RANKL/OPG ratio and increased the differentiation of osteoclasts with increasing bone resorption (Karst et al., 2004). In TGF-β knockout mice, bone resorption lead to a decrease of bone mineral content by about 30% (Geiser et al., 1998).

The present study showed a positive correlation between OPG and the inflammatory process as detected by ESR and hypersensitive CRP. These results were parallel to those reported by Kiechl et al. (2004).

Ziolkowska et al. (2002) and Kubota et al. (2004) documented the role of preinflammatory cytokines to enhance OPG production. The association of OPG expression and the inflammatory process in RA might attribute the increase in OPG level to either activation of the immune system or through a counter-regulatory mechanism that compensates for increased production of RANKL (Ziolkowska et al., 2002).

The role of OPG in atherosclerosis is not clear, but the link between atherosclerosis and osteoporosis suggests that OPG plays a fundamental role for their co-incidence. A potential mechanism may involve the immune system, however, OPG seems to play a role in the shift of calcium from bones to the vessels (Hofbauer and Schoppet, 2001). Presence of atherosclerosis and arterial calcification in osteoporotic patients is the commonest pathogenesis (Hak et al., 2000).

Stepien et al. (2011) found that the bone remodeling biomarker, osteoprotegrin is significantly elevated in hypertensive subjects. Association between hypertension, OPG and CRP levels documented the role of inflammatory processes in endothelial dysfunction.

Conclusion:

The present study provided evidence that OPG and TGF-β could be used as biomarkers for incidence of hypertension in rheumatoid patients. However, further studies with statistically convenient numbers of patients and control are mandatory to show the specificity and sensitivity of these biomarkers.

References


