Serum levels of osteoprotegerin and RANKL in patients with ST elevation acute myocardial infarction

Alessandra CRISAFULLI*†, Antonio MICARI*, Domenica ALTAVILLA*, Francesco SAVORITO*, Aurora SARDELLA*, Maria PASSANITI*, Santi RAFFA*, Gaspare D’ANNEO‡, Fabiana LUCÀ*, Chiara MIONI*, Francesco ARRIGO* and Francesco SQUADRITO*

*Department of Clinical and Experimental Medicine and Pharmacology, University of Messina, Messina, Sicily, Italy, †Section of Pharmacology, Department of Biomedical Sciences, University of Modena and Reggio Emilia, Italy, and ‡Department of Internal Medicine, Azienda Ospedaliera Papardo, Messina, Sicily, Italy

Abstract

OPG (osteoprotegerin) has been suggested to have an important role in atherogenesis and vascular calcification. In the present study, we have investigated serum OPG and RANKL (receptor activator of nuclear factor κB ligand) concentrations in patients with ST elevation AMI (acute myocardial infarction) and established CAD (coronary artery disease). OPG and RANKL were measured in 58 male patients hospitalized in the coronary care unit with ST elevation AMI, in 52 asymptomatic male patients with an established diagnosis of CAD, and in 52 healthy male controls. These last two groups were matched with the AMI patients for age and body mass index. OPG was significantly \( (P < 0.05) \) higher in patients with AMI at 1 h after AMI \((8.04 \pm 4.86 \text{ pmol/l}) \) than in both patients with established CAD \((4.92 \pm 1.65 \text{ pmol/l}) \) and healthy subjects \((3.15 \pm 1.01 \text{ pmol/l}) \). Subjects with established CAD had significantly \( (P < 0.05) \) increased OPG levels compared with controls. RANKL levels in patients with established CAD \((0.02 \pm 0.05 \text{ pmol/l}) \) and with AMI \((0.11 \pm 0.4 \text{ pmol/l}) \) were significantly \( (P < 0.05) \) lower compared with controls \((0.32 \pm 0.35 \text{ pmol/l}) \). In the AMI group, OPG decreased significantly \( (P < 0.05) \) at 1 and 4 weeks after infarction \((8.04 \pm 4.86 \text{ compared with } 6.38 \pm 3.87 \text{ and } 6.55 \pm 2.6 \text{ pmol/l respectively}) \), but OPG levels, either at 1 h or 1–4 weeks after AMI, remained significantly \( (P < 0.05) \) higher compared with established CAD \((4.92 \pm 1.65 \text{ pmol/l}) \) and controls \((3.15 \pm 1.01 \text{ pmol/l}) \). Our data show for the first time that OPG levels are increased in ST elevation AMI within 1 h of infarction. Whether the increase in OPG is a consequence or a causal factor of plaque destabilization deserves further investigation.

Introduction

Atherosclerosis is a complex multifactorial process resulting from an excessive inflammatory response to various forms of injurious stimuli to the arterial wall [1]. The transition of a stable coronary atherosclerotic lesion into a ruptured and/or eroded plaque results in the clinical manifestation of an acute coronary syndrome [2–5].

Key words: acute myocardial infarction, coronary artery disease, osteoprotegerin (OPG), RANKL (receptor activator of nuclear factor κB ligand).

Abbreviations: ACE, angiotensin-converting enzyme; AMI, acute myocardial infarction; BMI, body mass index; CAD, coronary artery disease; CI, confidence interval; CK, creatine kinase; CRP, C-reactive protein; CV, coefficient of variation; HDL, high-density lipoprotein; IL, interleukin, OPG, osteoprotegerin; OR, odds ratio; PDGF, platelet-derived growth factor; RANK, receptor activator of nuclear factor κB; RANKL, RANK ligand; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; VSMC, vascular smooth muscle cell.

Correspondence: Dr Alessandra Crisafulli, Department of Clinical and Experimental Medicine and Pharmacology, University of Messina, Italy (email crisafulli@jumpy.it).
Plaque instability is the consequence of a complex inflammatory response of the vessel wall ignited by activated macrophages and T-cells, leading to proteolytic degradation of connective tissue matrix, excessive pro-inflammatory cytokine production and apoptosis of vascular wall cells [2].

RANKL [RANK (receptor activator of nuclear factor κB) ligand], a member of the TNF (tumour necrosis factor) ligand superfamily, its cellular receptor, RANK, and the decoy receptor OPG (osteoprotegerin) represent a novel cytokine triad with pleiotropic effects on bone metabolism, the immune system and endocrine function [6]. Recently it has been found that RANKL/OPG system may have an important role in the regulation of vascular disease (Figure 1); in fact the expression pattern of both OPG and RANKL during atherogenesis suggests a regulatory role of these proteins not only in osteoclastogenesis, but also in atherosclerotic calcification.

Min et al. [7] have shown that OPG is detected in normal arteries, whereas RANKL/RANK is expressed only in calcified arteries. On the other hand, immunohistochemical analyses by Dhore et al. [8] have localized RANKL and OPG in normal and atherosclerotic human vessels. Intriguingly, both RANKL and OPG immunoreactivity can be detected in early atherosclerotic lesions [8]. In contrast, OPG immunoreactivity lined the mineralized lamellar bone-like structures in the fibrocalfic plaque, whereas RANKL immunoreactivity was localized into the extracellular matrix adjacent to calcium mineral deposits [8]. These findings indicate a potential role, as well as a distinct temporal and spatial pattern of expression, for these cytokines in the process of atherogenesis and atherosclerotic calcification.

Consistent with these data, increased OPG serum levels in men and women with CAD (coronary artery disease) and a positive correlation of OPG serum levels with the severity of CAD, as determined by the number of affected vessels, have been found [9,10]. Moreover, serum levels of sRANKL (soluble RANKL), the cytokine that is neutralized by the decoy receptor OPG, were lower in men with CAD than without CAD, and were negatively correlated with OPG serum levels [11].

Thus, in the present study, we examined, for the first time within 1 h of infarction, the serum levels of OPG and RANKL in patients with AMI (acute myocardial infarction) and established CAD to understand whether their serum concentrations may reflect different stages of ischaemic cardiovascular disease.

METHODS

Study subjects
Our study, approved by the Ethical Committee of the University of Messina, was carried out in three groups of patients. Written informed consent was obtained from all subjects.

The first group of subjects consisted of 58 male patients hospitalized in the coronary care unit for ST elevation AMI. AMI diagnosis was made on the basis of typical symptoms consistent with myocardial ischaemia that continued for > 30 min, newly developed ischaemic ST-T changes or Q waves in at least two contiguous ECG leads, and elevation of serum creatine kinase levels to ≥ 2 times the upper limit of the normal range. Standard medication, including aspirin, unfractionated heparin, intravenous nitroglycerine, β-blockers and ACE (angiotensin-converting-enzyme) inhibitors, was usually administered.
following guidelines for the treatment of ST elevation AMI. All patients received thrombolytic therapy with rtPA (recombinant tissue plasminogen activator) following the accelerated protocol. An intravenous glycoprotein IIb/IIIa antagonist was administered at the discretion of the operating physician. Angiography was performed using standard views. Coronary angioplasty and stenting of the infarct-related artery was performed within 1 week of admission and deemed successful in all patients.

The second group consisted of 52 asymptomatic male patients with an established diagnosis of CAD. Patients were eligible if they had angiographically proven CAD, defined as stenosis of 50% or more of the luminal diameter in a major epicardial coronary vessel, and if they were free of symptoms for ≥3 months. Potential participants were excluded from the study if they met the criteria for class IV congestive heart failure according to the New York Heart Association classification, had a pacemaker, atrial fibrillation or other arrhythmia. Participants were instructed to take all medication as usual. A medical history regarding previous myocardial infarction, hypertension, smoking, diabetes mellitus, hypercholesterolaemia and hypertriglyceridaemia was carefully obtained in all patients. Furthermore, all patients with suspected or proven long-term or intercurrent inflammatory diseases likely to be associated with short-term phase response (i.e. patients with infections, malignancies, autoimmune disorders and pulmonary diseases) and patients with musculoskeletal, liver or kidney diseases were excluded. These patients were matched to the AMI patients for age, BMI (body mass index) and major risk factors.

The third group was made up of 52 healthy male control subjects, who were matched with the AMI patients for age and BMI.

Biochemical analysis

Whole blood was obtained by venipuncture of the peripheral vein in all subjects. In patients with AMI, blood samples were drawn within 1 h and at 1 and 4 weeks after infarction. In patients with established CAD, venous blood samples were collected following an overnight fasting between 08.00 and 09.00 hours. In all patients, fibrinogen, total cholesterol, HDL (high-density lipoprotein)-cholesterol, LDL (low-density lipoprotein)-cholesterol, triacylglycerides (triglycerides), CRP (C-reactive protein), white cell count, CK (creatinine kinase), CK-MB, troponin I and myoglobin levels were measured immediately after drawing the venous blood by semi- or automated routine procedures (Azienda Poli-clinico Universitario Immunochemistry Laboratory). To measure OPG and sRANKL, serum was separated from the blood corpuscles by centrifugation at 5000 g for 10 min and kept frozen at −80°C until analysis.

OPG and sRANKL serum concentrations were analysed in a blinded manner with respect to any clinical information. OPG was measured with a commercially available ELISA kit according to the manufacturer’s protocol (Immundiagnostik). A monoclonal IgG antibody was used as the capture antibody, and a biotin-labelled polyclonal anti-(human OPG) antibody was used as the detection antibody. This assay detects monomeric, dimeric and ligand-bound forms of OPG [intra-assay CV (coefficient of variation), 7%; interassay CV, 8%; lower detection limit, 0.14 pmol/l]. Serum levels of sRANKL were measured by an ELISA system (Immundiagnostik) that detects free sRANKL, but not sRANKL complexed with OPG (intra-assay CV, 4%; interassay CV, 6%; lower detection limit, 0.08 pmol/l).

Statistical analysis

Values are presented as means ± S.D. The clinical and biochemical parameters of the three study groups described were compared by χ² test, Mann–Whitney U-test and ANOVA, followed by post-hoc evaluation. The values of biochemical parameters obtained at different time periods in the AMI group were compared by paired Student’s t test or Wilcoxon signed-rank test. Logistic regression models and linear regression models were used to estimate possible associations between serum OPG levels with dichotomous and continuous variables. Differences were considered statistically significant at P < 0.05.

RESULTS

The clinical characteristics of the three groups are shown in Table 1. Age and BMI were similar in the three groups. Patients with AMI and established CAD were similar for all of the major cardiovascular risk factors, such as hypertension and diabetes mellitus, and for a family history of CAD. A history of previous myocardial infarction was significantly lower in the patients in the AMI group compared with those in the established CAD group. The angiographic characteristics were similar between the AMI and CAD groups. Patients with AMI and established CAD differed in the use of β-blockers, long-acting nitrates, HMG-CoA reductase inhibitors, ACE-inhibitors and aspirin (Table 1). There were significant differences in the frequency of smoking between patients with AMI and established CAD (Table 1).

In the AMI group, baseline white blood count, CRP, fibrinogen, CPK, troponin I and myoglobin concentrations were significantly higher than in patients with CAD or the control group (Table 2).

White cells (results not shown), CPK, troponin I and myoglobin returned to normal levels in patients with AMI after 1–4 weeks, whereas CRP remained significantly elevated during this time compared with patients with established CAD and controls (Figure 2). The fibrinogen concentration at 1 week was significantly
Table 1  Characteristics of the study groups  
Values are means ± S.D. *P < 0.05 compared with controls; †P < 0.05 compared with established CAD.  

<table>
<thead>
<tr>
<th></th>
<th>AMI</th>
<th>Established CAD</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>58</td>
<td>52</td>
<td>52</td>
</tr>
<tr>
<td>Age (years)</td>
<td>65 ± 10</td>
<td>63 ± 9</td>
<td>62 ± 11</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26 ± 2.3</td>
<td>26 ± 4</td>
<td>25 ± 4.4</td>
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<tr>
<td>Family history of CAD (%)</td>
<td>27*</td>
<td>38*</td>
<td>11</td>
</tr>
<tr>
<td>Previous myocardial infarction (%)</td>
<td>27†</td>
<td>62</td>
<td>0</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>75</td>
<td>77</td>
<td>0</td>
</tr>
<tr>
<td>Current smokers (%)</td>
<td>48†</td>
<td>23</td>
<td>35</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>41</td>
<td>54</td>
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</tr>
<tr>
<td>Hypercholesterolaemic (%)</td>
<td>65</td>
<td>77</td>
<td>0</td>
</tr>
<tr>
<td>Hypertriglyceridaemic (%)</td>
<td>26</td>
<td>38</td>
<td>10</td>
</tr>
<tr>
<td>≥ 50% stenosed vessels (n)</td>
<td>2.2 ± 0.83</td>
<td>1.9 ± 0.79</td>
<td>0</td>
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<tr>
<td>Affected coronary arteries (n)</td>
<td>One</td>
<td>24</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Two</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Three</td>
<td>45</td>
<td>38</td>
</tr>
<tr>
<td>Medication used (%)</td>
<td></td>
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<tr>
<td>β-Blockers</td>
<td>10†</td>
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<tr>
<td>Long-acting nitrates</td>
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</tr>
<tr>
<td>ACE-inhibitors</td>
<td>10†</td>
<td>85</td>
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</tr>
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</table>

higher than at baseline in the AMI group, and also significantly higher when compared with patients with CAD and controls (Figure 2).

There were no significant differences in the lipid profile among the three experimental groups (Table 2).

OPG levels were significantly (P < 0.05) higher in patients with AMI at 1 h after infarction than in patients with established CAD and healthy controls (Table 2). Furthermore, the level of OPG in patients with established CAD was also significantly (P < 0.05) higher compared with the controls. In the AMI group, OPG decreased significantly at weeks 1 and 4 after infarction (Figure 2), but OPG levels at baseline and at 1–4 weeks of follow-up remained significantly higher compared with the levels in patients with established CAD and controls (Figure 2).

Serum sRANKL did not change during the study period in the AMI group (Figure 2). Serum sRANKL levels in patients with established CAD and in the AMI group, at baseline, and 1 and 4 weeks, were significantly lower compared with healthy controls (Table 2 and Figure 2).

RANKL levels were undetectable in 34 out of 52 patients in the group with established CAD. In the AMI group, RANKL levels were undetectable in 36 out of 58.

Sixteen patients out of 52 also had undetectable RANKL levels in the control group.

OPG levels did not correlate with CRP in patients with AMI (r = 0.03, P = 0.78) and established CAD (r = 0.01, P = 0.66) or in the control group (r = 0.23, P = 0.17).

In AMI patients, the small percentage reduction in OPG levels from baseline to 1 week was a statistically significant predictor of elevated cardiovascular events at 4 weeks [OR (odds ratio) = 1.10 (95 % CI, 0.98–1.23; P = 0.03), whereas the association between OPG levels at 1 week and cardiovascular events at 4 weeks had an OR of 1.58 (95 % CI, 1.26–1.99; P < 0.001).

The medication used by CAD patients may influence the serum level of OPG; however, we found no association between the concentration of OPG and the use of the actual medication. Furthermore, no association was found between OPG levels and smoking status, cardiovascular risk factors, necrosis markers or lipid status in patients with AMI or established CAD (results not shown).

DISCUSSION

The present study shows, for the first time, that AMI patients have significantly higher levels of serum OPG compared with patients with established CAD and controls. Moreover, OPG levels declined during follow-up in AMI patients, suggesting a specific link between the cytokine and the acute phase of instability.
The presence of an elevated OPG concentration in patients with CAD has been reported previously. Browner et al. [12] showed that, in a cohort of postmenopausal women over 65 years of age, serum OPG levels were greater in women with diabetes than those without and were positively correlated with overall cardiovascular mortality. Moreover, patients with CAD have higher serum OPG levels than healthy patients and enhanced levels of the cytokine were found to be positively correlated with the severity of CAD [9,10]. Another recent prospective large population-based survey study has shown that the relative risk of cardiovascular mortality is increased by 3-fold in patients with high serum OPG levels [13].

Our new finding in the present study of higher serum OPG concentrations in patients with AMI compared with those with established CAD might be related to an increase in the secretion of inflammatory cytokines that occurs in subjects with acute coronary syndromes [14,15]. In fact, OPG produced by VSMCs (vascular smooth muscle cells) and endothelial cells has been implicated in inflammation [15]. In vitro studies have shown that inflammatory cytokines, such as IL (interleukin)-1, TNF-α, IL-6 and PDGF (platelet-derived growth factor), up-regulate OPG expression in those cell types [16,17] and that OPG is distributed around areas of calcification and associated with inflammatory cells and VSMCs [18]. However, we did not find any association between inflammation markers and OPG. This is consistent with data by Browner et al. [12], but it is in disagreement with other studies in which OPG was related to systemic inflammatory markers [13,19].

In agreement with our present data, Zhang et al. [17] have shown that OPG expression in the rat aorta is increased after balloon injury, and Golledge et al. [18] have demonstrated that OPG is expressed and secreted at higher concentrations in symptomatic carotid plaques than in asymptomatic ones. Thus it may be hypothesized that increased OPG levels in AMI patients at baseline may result from rupture or splitting of the plaque.

OPG serves as a decoy receptor for RANKL, but it can also bind TRAIL (TNF-related apoptosis-inducing ligand), a potent activator of apoptosis [20]. Therefore another possibility is that OPG influences vascular
disease by inhibiting TRAIL-induced apoptosis in vascular cells. In fact, an in vitro study [21] has shown that OPG prevents apoptosis induced by growth factor deprivation in endothelial cells in a dose-dependent manner.

Therefore we hypothesize that OPG may be expressed by VSMCs, endothelium and macrophages in response to pro-apoptotic stimuli and suggest a protective role for OPG, as VSMC apoptosis can weaken the cap tissue and favour plaque rupture [2].

Matrix-degrading enzymes probably play a crucial role in determining the integrity of the tissue in an atherosclerotic plaque and then in the plaque rupture process [23,24]. Interestingly, it has been demonstrated that OPG modulates the release of matrix-degrading enzymes such as cathepsins within bone and thus might influence plaque vulnerability by modulating these enzymes directly or by RANKL binding [25,26].

OPG-deficient mice also exhibit medial calcification of the aorta and renal arteries [27]; OPG, which inhibits bone resorption, is able to potently halt calcification of arteries induced by warfarin and vitamin D treatment [28]. Therefore OPG counteracts calcification by its well-established capacity to inhibit bone resorption and is considered to be a candidate vascular calcification inhibitor [7,8,30]. The increase in OPG observed in our patients with AMI may have two interpretations, either beneficial or injurious, depending on whether [32] or not [33] we consider coronary calcification responsible for reducing stresses in the plaque.

The decrease in OPG levels at 1–4 weeks could be due to the effects of therapy post-AMI. In agreement with this, it has been shown [18] that irbesartan reduces OPG secretion by carotid atherosclerotic explants removed from symptomatic patients, probably by reducing potential plaque destabilizing effects, such as decalcification and matrix degradation. Moreover, OPG levels remained elevated for 1–4 weeks compared with those seen in patients with established CAD, confirming the possibility that OPG might reflect an unstable plaque phenotype. Furthermore, the reduction in OPG levels observed in AMI patients following therapy for 1 week was associated with an improved outcome at 4 weeks of follow-up.

Circulating OPG may not fully reflect the activity of OPG within the plaque; in fact this cytokine is synthesized by various tissues and is regulated by a variety of hormones and cytokines [34]. Moreover, it is also likely that its biological activity is dependent on the relative levels of both OPG and RANKL [35]. For this reason, we measured the serum levels of sRANKL and found no change in the AMI group, and we confirmed previous data of lower RANKL levels in men with CAD when compared with controls [17].

A limitation of the present study is that the methodology for detection of OPG and RANKL in serum is still under development; however, the assay used in the present study was designed to detect all circulating forms of OPG: monomeric, dimeric and ligand-bound forms.

The marked rise in serum OPG levels observed in AMI may reflect a counter-regulatory mechanism aimed at balancing plaque instability in an attempt to regulate this process. Alternatively, increased OPG levels in an acute coronary event might contribute to the progression and aggravation of the disease, leading to enhanced destabilization of the coronary plaque. However, the possibility that the enhanced circulating concentration of OPG represents a simple epiphenomenon of the disease cannot be ruled out at this time.

In conclusion, OPG might represent a novel marker of plaque instability as the OPG levels are increased in ST elevation AMI within 1 h after the onset of pain and, although decreased after 1–4 weeks of follow-up, remained higher than those observed in patients with established CAD.

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REFERENCES

7 Min, H., Morony, S., Sarosi, I. et al. (2000) Osteoprotegerin reverses osteoporosis by inhibiting endosteal osteoclasts and prevents vascular calcification by blocking a process resembling osteoclastogenesis. J. Exp. Med. 192, 463–474
10 Jono, S., Ikari, Y., Shioi, A. et al. (2002) Serum osteoprotegerin levels are associated with the presence and severity of coronary artery disease. Circulation 106, 1192–1194
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