Enhanced T-Cell Expression of RANK Ligand in Acute Coronary Syndrome
Possible Role in Plaque Destabilization

Wiggo J. Sandberg, Arne Yndestad, Erik Øie, Camilla Smith, Thor Ueland, Olga Ovchinnikova, Anna-Karin L. Robertson, Fredrik Müller, Anne G. Semb, Hanne Scholz, Arne K. Andreassen, Lars Gullestad, Jan Kristian Damås, Stig S. Frøland, Göran K. Hansson, Bente Halvorsen, Pål Aukrust

Objective—Based on its role in inflammation and matrix degradation, we hypothesized a role for osteoprotegerin (OPG), RANK, and RANK ligand (RANKL) in coronary artery disease.

Methods and Results—We examined the expression of various members of the OPG/RANKL/RANK axis in patients with stable and unstable angina and in the atherosclerotic lesions of apolipoprotein E–deficient (apoE−/−) mice. Our findings were: (1) Serum levels of OPG were raised in patients with unstable angina (n=40), but not in those with stable angina (n=40), comparing controls (n=20); (2) mRNA levels of RANKL were increased in T-cells in unstable angina patients accompanied by increased expression of RANK in monocytes; (3) strong immunostaining of OPG/RANKL/RANK was seen within thrombus material obtained at the site of plaque rupture during acute myocardial infarction; (4) OPG/RANKL/RANK was expressed in the atherosclerotic plaques of apoE−/− mice, with RANKL located specifically to the plaques; and (5) RANKL enhanced the release of monocyte chemotactic peptide-1 in mononuclear cells from unstable angina patients, and promoted matrix metalloproteinase (MMP) activity in vascular smooth muscle cells.

Conclusions—We show enhanced expression of the OPG/RANKL/RANK system both in clinical and experimental atherosclerosis, with enhanced T-cell expression of RANKL as an important feature of unstable disease. (Arterioscler Thromb Vasc Biol. 2006;26:857-863.)

Key Words: atherosclerosis ▪ inflammation ▪ plaque stability

Numerous inflammatory mediators seem to play a pathogenic role in coronary artery disease (CAD), promoting atherogenesis and plaque destabilization, leading to thrombus formation with development of acute coronary syndromes.1,2 However, although the participation of inflammatory mediators in the atherosclerotic process has become widely recognized, the identification and characterization of the different actors are not fulfilled.

Receptor activator of nuclear factor-kB ligand (RANKL), its membrane-bound receptor RANK and its soluble decoy receptor osteoprotegerin (OPG) are members of the tumor necrosis factor (TNF) receptor superfamily. These factors have been identified as candidate mediators for paracrine signaling in bone metabolism but are also involved in modulation of the immune response through interaction with dendritic cells, T-cell activation, and B-cell maturation.3,4 The pleiotropic effects of the OPG/RANKL/RANK system, such as modulation of cell survival, mineralization and inflammation, make it an interesting candidate mediator in the progression and destabilization of atherosclerotic lesions. mRNA and protein expression of OPG and RANKL have been detected in atherosclerotic plaques in humans.5,6 Moreover, raised serum levels of OPG are reported in CAD patients and have also been shown to predict cardiovascular mortality in elderly women.7-9 Although these findings may suggest the involvement of the OPG/RANKL/RANK system in atherogenesis, our knowledge of the role of this system in human CAD, and particularly in acute coronary syndromes, is still limited.

Based on its involvement in inflammation and matrix degradation, we in the present study attempted to further clarify the potential role of the OPG/RANKL/RANK system in atherogenesis and acute coronary syndromes by different approaches including clinical studies in patients with sta-
ble and unstable angina as well as experimental studies in apolipoprotein E–deficient (apoE−/−) mice.

Materials and Methods

Patients and Controls

Angina patients undergoing clinically indicated coronary angiography were consecutively recruited into the study (Table I, available online at http://atvb.ahajournals.org). All patients with unstable angina (n=40) had experienced ischemic chest pain at rest within the preceding 48 hours (ie, Braunwald class IIIb), but with no evidence of myocardial necrosis by enzymatic criteria. Transient ST-T segment depression and/or T-wave inversion were present in all cases. All patients with stable angina (n=40) had stable effort angina of >6 months duration and a positive exercise test. Exclusion criteria were myocardial infarction (MI) or thrombolytic therapy in the previous month. ECG abnormalities invalidating ST-segment analyses, concurrent inflammatory disease such as infections and autoimmune disorders, and liver and kidney disease. Coronary angiography was performed by standard techniques, and the diagnosis of CAD was confirmed by at least 1-vessel disease, defined as >50% narrowing of the luminal diameter in all patients. Control subjects in the study were 20 gender- and age-matched healthy individuals (Table I). Informed consent for participation in the study was obtained from all individuals. The study was conducted according to the ethical guidelines at our hospital according to the Helsinki declaration and was approved by the hospitals authorized representative. Serum was collected as previously described.10

Isolation of Cells

Peripheral mononuclear cells (PBMCs) were obtained from heparinized blood by Isopaque-Ficoll (Lymphoprep; Nycosmed, Oslo, Norway) gradient centrifugation. Further separation of CD14+ monocytes (CD14-labeled magnetic beads; MACS, Milteny Biotec, Bergisch Gladbach, Germany) and CD3+ T-cells (negative selection by monodisperse immunomagnetic beads; Dynal, Oslo, Norway) was performed as described elsewhere.11,12 After isolation, cell pellets of monocytes and T-cells were immediately stored in liquid nitrogen (LN2).

Cell Cultures

PBMCs, the human monocyte cell line THP-1 (American Type Culture Collection, Rockville, Md), and the human aortic smooth muscle cells (SMCs) (PromoCell GmbH Heidelberg, Germany) were cultured and stimulated with different concentrations of soluble RANKL (Peprotech, London, UK) and the human aortic smooth muscle cells (PromoCell GmbH Heidelberg, Germany) were cultured and stimulated with different concentrations of soluble RANKL (Peprotech, London, UK). For details, please see the expanded online Methods.

Mice

Female apoE−/− mice on the C57BL/6 background (strain C57BL/6 apoETM1UNC129) were used for experiments.13 The mice were fed standard mouse chow and euthanized at the age of 18 weeks. We also examined the expression of OPG/RANKL/RANK in atherosclerosis-prone apoE−/−knockout mice crossed with transgenic mice carrying a dominant-negative transforming growth factor (TGF)–β receptor-II in T-cells (apoE−/−×CD4dnT2RII mice).13 These mice were also fed standard chow and were euthanized at 12 weeks of age to obtain lesions that were similar in size to those of 18-week-old apoE−/− mice. Tissues were dissected under a microscope and immediately frozen.13 All animal experiments were in accordance with national guidelines and approved by the local ethical committee.

Tissue Sampling of Thrombus Materials

In 10 patients with acute ST-elevation MI (STEMI) undergoing primary percutaneous coronary intervention (PCI), thrombi at the site of the occlusion was aspirated immediately after crossing the lesion with the guide wire. For details, see the expanded online Methods.

Immunohistochemistry

Acetone-fixed sections of the ascending aorta were stained using monoclonal rat anti-mouse CD3 (Southern Biotechnology, Birmingham, Ala) or rat anti-mouse CD68 (Serotec, Oxford, UK) IgG. Formalin-fixed sections of the ascending aorta and paraffin-embedded sections of thrombus material were stained using affinity-purified rabbit anti-human OPG, rabbit anti-human RANKL or goat anti-human RANKL IgG (Santa Cruz Biotechnology, Santa Cruz, Calif). Paraffin-embedded sections of thrombus material were also stained with monoclonal mouse anti-human CD41 IgG (Immuno-tech, Marseille, France) and purified polyclonal mouse anti-human monocytes/macrophages (calprotectin) IgG (MCA874G, Serotec). The primary antibodies were followed by biotinylated anti-rat, anti-rabbit, or anti-goat IgG (Vector Laboratories, Burlingame, Calif). The immunoreactivities were further amplified using avidin-biotin-peroxidase complexes (Vector Laboratories). Diaminobenzidine was used as the chromogen in a commercial metal enhanced DAB kit (Vector Laboratories, Burlingame, Calif). The immunoreactivities were further amplified using avidin-biotin-peroxidase complexes (Vector Laboratories). Diaminobenzidine was used as the chromogen in a commercial metal enhanced DAB kit (Vector Laboratories, Burlingame, Calif).

Total MMP Activity

Total MMP activity was measured by a fluorogenic peptide substrate (R&D Systems) to assess broad-range MMP activity (MMP-1, -2, -7, -8, -9, -12, and -13 can cleave the peptide) as recommended by the manufacturer.

Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction

mRNA levels of OPG, RANKL and RANK were quantified by real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR).14 For details, please see the expanded online Methods.

Enzyme Immunoassays

Serum levels of OPG were measured by an enzyme immunoassay (EIA) using matched antibodies (R&D Systems).15 Levels of macrophage chemoattractant protein (MCP)-1, macrophage inflammatory peptide (MIP)-1a, TNFα, and IL-8 were measured by EIAs (R&D Systems).

Statistical Analysis

When comparing 3 groups of individuals, 1-way ANOVA was followed by Scheffe post hoc test for statistical significance. For comparisons within the same individuals, the Wilcoxon signed-rank test was used. P<0.05 (2-sided) were considered significant.

Results

Serum Levels of OPG

Although serum levels of OPG were only modestly and nonsignificantly increased in stable angina patients (n=40; 2345±96 pg/mL), those with unstable disease (n=40; 2728±211 pg/mL) had significantly raised OPG levels compared with healthy controls (n=20; 2046±94 pg/mL; P<0.05 versus unstable angina) (Figure I, available online at http://atvb.ahajournals.org).

Gene Expression of OPG/RANKL/RANK in Monocytes and T-Cells

We next examined the gene expression of OPG/RANKL/RANK in T-cells and monocytes from 12 of the patients with unstable angina, 10 of those with stable angina and 10 of the healthy controls. T-cells from unstable angina patients, but
not from those with stable angina, showed markedly increased gene expression of RANKL compared with cells from healthy controls (Figure 1A). Notably, this upregulation of RANKL in unstable angina was accompanied by enhanced RANK expression in T-cells and monocytes from these patients comparing cells from healthy controls, although the difference in T-cell expression did not reach statistical significance (Figure 1B and 1C). OPG transcripts were not detected in T-cells and either OPG or RANKL transcripts were detected in monocytes in either patients or controls (data not shown).

The Effect of PCI on RANKL/RANK Expression
To examine the expression pattern of RANKL/RANK on inflammatory stimulation in vivo, we next examined the levels of these genes in PBMC from stable angina patients (n = 8) undergoing PCI. This procedure, representing a mechanically induced plaque rupture, induced a rapid and significant increase in RANKL expression (4 hours), returning to baseline levels after 24 hours (Figure 2). In contrast, PCI did not induce any significant changes in RANK expression in these patients (Figure 2).

Expression of OPG/RANKL/RANK in Arterial Thrombosis
To further characterize the involvement of OPG/RANKL/RANK in plaque destabilization, we examined the expression of these mediators in thrombus material obtained from 6 patients with STEMI undergoing primary PCI. Immunohistochemical staining of thrombus material removed from the site of the ruptured plaque showed fairly strong OPG, RANK, and RANKL immunoreactivities in calprotectin-positive monocytes/macrophages and in area of aggregated CD41-positive platelets (Figure 3). The strong OPG immunostaining in monocytes/macrophages within the thrombus material may seem in contrast with our findings in freshly isolated monocytes showing no detectable OPG mRNA levels. One possible explanation is that OPG immunostaining in areas of monocytes/macrophages reflects binding of RANKL/OPG complexes to RANK that are strongly expressed on activated monocytes/macrophages. Moreover, although we could not detect OPG mRNA in monocytes or in THP-1 macrophages (data not shown), we cannot exclude that OPG can be upregulated during macrophage differentiation within an appropriate microenvironment.16

Localization of OPG/RANKL/RANK in Plaques from ApoE−/− Mice
To further examine the OPG/RANKL/RANK system in atherosclerotic disorders, we went on to analyze its expression within atherosclerotic lesions of aorta by immunohistochemistry in a murine model of atherosclerosis, the apoE−/− mice. Several significant findings were revealed (Figure 4). First, although strong RANK and OPG immunoreactivity was observed in SMCs and endothelium of the nonatherosclerotic vessel wall, more intense staining was seen for both OPG and particularly for RANK within the atherosclerotic lesions. Second, in the plaques, strongest OPG staining was located adjacent to apparently foam cells, whereas RANK was distributed more evenly through the lesion, except in areas with debris and in foam cells. Third, whereas no RANKL immunostaining was detected in the nonatherosclerotic vessel wall, significant immunoreactivity was seen within the atherosclerotic lesions. Cell specific staining showed strong RANKL staining in areas rich in CD3+ T-cells, but also in areas with predominantly CD68+ macrophages. When apoE-knockout mice were crossed with transgenic mice carrying a dominant-negative TGF-β receptor II in T-cells (apoE−/− × CD4dntβRII mice), the offspring develops markedly increased atherosclerosis with larger and more unstable lesions, possibly at least partly involving increased interferon (IFN)-γ production in T-cells from these mice.13 Thus, lesions of
apoE<sup>−/−</sup>×CD4<sup>dnTβRII</sup> mice displayed increased T-cell activation, activated macrophages, increased local and systemic expression of inflammatory cytokines, and also reduced lesion collagen, consistent with a more vulnerable plaque phenotype. Spontaneous MI has also been observed in these mice from 15 weeks of age, further supporting their unstable phenotype (Robertson AKL, Hansson GK, unpublished observations). Although immunostaining showed the same pattern in apoE<sup>−/−</sup> and apoE<sup>−/−</sup>×CD4<sup>dnTβRII</sup> mice, the staining for OPG, RANKL, and RANK was in general markedly increased in the more vulnerable lesions in the apoE<sup>−/−</sup>×CD4<sup>dnTβRII</sup> mice, suggesting a “dose-response”–like pattern for these mediators along with the enhanced plaque vulnerability in mice lacking TGF-β–mediated inhibition of T-cell activation (Figure 4).

**Effects of RANKL Stimulation of PBMCs From Angina Patients and Healthy Controls**

Our findings so far suggest increased OPG/RANKL/RANK expression in both clinical and experimental atherosclerosis, with increased expression of RANKL in T-cells as a prominent feature of unstable disease. To elucidate any functional consequences of the enhanced RANKL/RANK expression in mononuclear cells in unstable angina, we examined the in vitro effect of RANKL on various inflammatory cytokines in PBMC supernatants from six of the patients with unstable angina, six of the patients with stable angina and six of the healthy controls. While RANKL had no significant effect on MCP-1 levels in PBMC from healthy controls and stable angina patients, RANKL dose-dependently increased the release of MCP-1 in PBMC from unstable angina patients suggesting that the enhanced RANK expression in cells from these patients influence their functional responses (Figure 5). Also when comparing differences in changes (ie, MCP-1 levels in PBMC supernatants after stimulation with the highest RANKL concentration minus levels in un-stimulated cells), the increase in unstable angina patients was significantly higher than the RANKL-induced increase in both stable angina patients (P<0.005) and healthy controls (P<0.001). In contrast to the effect on MCP-1, RANKL had no effect on IL-8, TNFα and MIP-1α levels in either patients or controls (data not shown).

**Effects of RANKL and OPG on MMP activity**

The vulnerability of the plaque is to a large degree defined by the integrity of the intercellular matrix of the vessel wall, which is compromised by actions of MMPs. Because strong RANK expression was seen in macrophages and SMCs within the atherosclerotic plaques, we next examined the effect of RANKL on MMP activity in supernatants from THP-1 macrophages and SMCs after culturing for 24 hours. As shown in Figure 6A, RANKL significantly increased total MMP activity in vascular SMC, but not in THP-1 macrophages (data not shown). Functionally, OPG binds to RANKL thereby inhibiting RANKL binding to its cognate receptor RANK. Whereas such inhibitory effects seem to be true under high OPG/RANKL ratios, stabilization of the trimeric RANKL molecule could theoretically also be seen, in particular under lower OPG/RANKL ratios, and interestingly, whereas the molar ratio between OPG and RANKL in serum is ~100:1, suggesting neutralizing effect, we found that OPG markedly enhanced the RANKL-stimulated effect on MMP activity in SMC at molar ratios of 0.5 and 3 (Figure 6B). Moreover, at very high concentrations (ie, 12 mg/mL), OPG induced MMP activity also when given alone (Figure 6B).

**Discussion**

Herein we show increased RANKL expression in T-cells from unstable angina patients accompanied by enhanced expression of its corresponding receptor in macrophages. A similar pattern of increased RANKL expression was also seen after PCI, representing a mechanically induced plaque rupture. The relationship between increased activity in the OPG/RANKL/RANK system and unstable disease was further supported by our immunohistochemical analyses showing strong immunostaining of these mediators within thrombus material obtained at the site of plaque rupture in patients with STEMI. We also found that RANKL enhanced the release of MCP-1 in PBMC from unstable angina patients and promoted MMP activity in SMC. If such activities also exist within the atherosclerotic plaques, the increased RANKL levels could contribute to inflammation, leukocyte recruitment, and matrix degradation within these lesions. Thus, although the raised RANKL/RANK/OPG levels in angina
patients may be secondary to enhanced immune activation, being part of the inflammatory cytokine network in CAD, these mediators seem not only to be markers of inflammation, but also contributors to atherogenesis and plaque destabilization in these patients.

Previously, RANKL and OPG expression has been detected within atherosclerotic plaques from abdominal aortas and carotid arteries in humans.5,6,21 Moreover, raised serum levels of OPG are reported to be associated with the presence and severity of CAD.7 Herein we extend these findings in several ways, particularly focusing on RANKL expression in unstable angina. Thus, the upregulation of RANKL on T-cells in unstable, but not in stable angina, as well as the induction of RANKL expression after mechanically induced plaque

Figure 4. Representative photomicrographs demonstrating OPG, RANK, and RANKL immunoreactivity in 18-week-old atherosclerotic apoE−/− mice with abrogated TGF-β signaling in T-cells (dominant-negative TGF-β receptor II; apoE−/− × CD4dnTβRII). Mean atherosclerotic lesion size was markedly larger in the apoE−/− × CD4dnTβRII mice with thicker intima (I) and a necrotic core with cellular debris (*) indicating more advanced atherosclerotic lesions. In the apoE−/− mice, scattered CD3+ T-cells were seen in the atherosclerotic areas, whereas increased number of CD3+ T-cells was found in the lesions in apoE−/− × CD4dnTβRII mice, especially adjacent to the lumen. In contrast, CD68 staining showed macrophages throughout the lesions inside the cap. Substantial OPG, RANK, and RANKL immunoreactivities were seen in the atherosclerotic lesions with strongest staining in the advanced lesions outside the necrotic acellular areas. Strongest OPG staining was observed adjacent to apparently foam cells, whereas RANK and RANKL were distributed more evenly through the lesion, except in areas with debris and foam cells. In the advanced lesions, particularly strong RANK and RANKL immunostaining was located in areas rich in T-cells. Fairly strong OPG and RANK immunoreactivities were also found in smooth muscles cells of the nonatherosclerotic vessel wall, whereas little or no RANKL immunostaining was observed (left panels). M, media. Magnification ×400.

Figure 5. The effect of different concentrations of soluble RANKL on the release of MCP-1 in PBMC supernatants after culturing for 24 hours in 6 patients with unstable angina pectoris (AP), 6 patients with stable AP, and in 6 healthy controls. Data are mean±SEM. *P<0.05 vs unstimulated cells.

Figure 6. A, Total MMP activity, measured as relative fluorescence units (r.f.u.), in vascular SMCs after culturing for 48 hours with and without soluble RANKL (1.5 μg/mL). Data are mean±SEM of 6 independent experiments. B, Total MMP activity at different molar ratios of OPG:RANKL where 1.5:1.5 mg/mL and 12:1.5 mg/mL represents a OPG:RANKL molar ratio of 0.5 and 3, respectively. Data are given as percentage of MMP activity when RANKL was given alone (mean±SEM of 6 independent experiments). *P<0.05 vs MMP activity when RANKL was given alone (B) or vs controls (A).
rupture, suggest that enhanced RANKL expression in T-cells could be a characteristic of unstable disease. These patients also had increased RANK expression in monocytes, affecting their functional responses by showing enhanced RANKL-mediated MCP-1 response in PBMC from unstable angina patients. Furthermore, although RANK and OPG were detected in the plaque and the healthy vessel wall of apoE−/− mice, we found RANKL to be located specifically to the plaques. Dhore et al have previously reported that RANKL immunostaining in atherosclerotic lesions was restricted to the extracellular matrix surrounding calcium deposits. In the apoE−/− mice model, we show strong RANKL immunostaining toward the lumen, colocating with macrophages and in particular with T-cells, accompanied by strong OPG staining in areas of foam cells and enhanced RANK staining distributed evenly throughout the lesion. Notably, these features, including increased RANKL immunostaining on T-cells, were particularly enhanced in the more vulnerable plaque phenotype in the apoE−/−×CD4drT/BII mice. The relationship between increased activity in the OPG/RANKL/RANK axis and plaque destabilization was further supported by our findings of strong immunostaining of all these mediators in thrombus material obtained from the site of plaque rupture in patients undergoing STEMI. Taken together, these findings suggest that increased expression of the OPG/RANKL/RANK system and particularly enhanced RANKL expression in T-cells, may be a feature of unstable atherosclerotic disease.

In the present study we found significantly raised serum levels of OPG in unstable but not in stable angina. Because OPG is produced in many tissues including the lung, kidney, intestine, and bone, we can only speculate about the source of the increased serum levels of OPG in these patients. However, recent studies suggest that also the cardiovascular system may be an important contributor to the circulating OPG levels. Thus, we have recently showed OPG immunoreactivity in the human myocardium with particularly strong staining in patients with chronic heart failure. Moreover, OPG expression has been demonstrated in different vascular cell types, and both coronary SMCs and endothelial cells have been implicated as cellular sources of OPG production. In the present study we show strong immunostaining within the vulnerable atherosclerotic lesions, and it is therefore tempting to hypothesize that the atherosclerotic lesion in itself could contribute to the raised OPG levels in unstable angina patients.

In the present study we show that soluble RANKL induced MMP activity in vascular SMC. Moreover, whereas OPG is thought to neutralize RANKL activity by inhibiting RANKL binding to RANK, we found that OPG, at least under lower OPG/RANKL ratios, enhanced the MMP inducing effect of RANKL. Such findings have also been reported for other soluble receptors in the TNF receptor superfamily. Thus, although soluble TNF receptors (sTNF-Rs) may have neutralizing effects on TNFα in situation with high sTNF-Rs/TNFα ratios such as in serum, these receptors could augment TNFα stimulation by stabilizing and prolonging its effect in situation with low sTNF-Rs/TNFα ratios such as in inflamed tissues. If a similar pattern exist for the interaction between OPG and soluble RANKL, one could hypothesize an enhancing effect of OPG on RANKL within the atherosclerotic plaque showing strong and colocalized immunostaining of both OPG and RANKL. However, recent data suggest that OPG is not only a modulator of RANKL but also may have RANKL-independent effects such as induction of monocyte chemotaxis. Herein we found that OPG in addition to its enhancing effects on RANKL also had MMP-inducing effects on its own, suggesting that such mechanisms also could be operating in SMC. However, high concentrations of both OPG and RANKL were used in the present in vitro experiments and if similar concentrations exist within the inflammatory microenvironment are unclear. Nevertheless, our data suggest a potential for RANKL-enhancing effects of OPG and challenge the notion that high OPG levels within the atherosclerotic lesion may be only protective. Thus, the recent finding of Golledge et al showing that OPG is expressed at higher concentrations in symptomatic than in asymptomatic plaques of carotid arteries could potentially reflect its ability to enhance MMP activity with and without RANKL costimulation.

We show increased expression of the OPG/RANKL/RANK system in clinical and experimental atherosclerosis, with enhanced T-cell expression of RANKL as an important feature of unstable disease. The OPG/RANKL/RANK system has been demonstrated to play a pivotal role in bone metabolism. Our findings further support the notion that this system also is involved in atherogenesis and plaque destabilization.

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References


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SUPPLEMENTAL DATA

Methods

Cell cultures

PBMC were incubated in flat-bottomed 96-well trays (2x10^6/mL, 200 µL/well; Costar, Cambridge, MA), in medium alone (RPMI 1640 with 2 mmol/L L-glutamine and 25 mmol/L HEPES buffer [Gibco, Paisley, UK] supplemented with 10% fetal calf serum [FCS; Sigma, St. Louis, MO]) or stimulated with different concentrations of soluble RANKL (Peprotech, London, UK). The human monocytic cell line THP-1 (American Type Culture Collection, Rockville, MD) was cultured in RPMI-1640 with 10% FCS, supplemented with 50 U/mL penicillin and 50 µg/mL streptomycin (Gibco) and 2 mmol/L L-glutamine (Sigma) in 6-wells trays (1.5x10^6 cells/mL, 1 mL/well; Costar). At experimental start, the cell medium was changed to serum-free medium (OptiMEM; Gibco), and the cells were further incubated with and without different concentrations of soluble RANKL (Peprotech). Prior to all experiments the THP-1 cells were differentiated into macrophages by incubating for 72 hours with 100 nM phorbol myristate acetate (Sigma). Human aortic smooth muscle cells (SMC) were obtained from PromoCell GmbH (Heidelberg, Germany) and grown in Smooth Muscle Cell Growth Medium 2 with complete supplement mix (PromoCell; 5% FCS, 0.5 ng/mL epidermal growth factor, 2.0 ng/mL basic fibroblast growth factor, 5.0 mg/mL insulin and gentamicin/amphotericin B). At 90% confluence, the culture was trypsinated and replated. Two days prior to experiments, cells were seeded in 24-well plates (1.5x10^5 cells/mL, 0.5 mL/well; Costar) and grown in the same medium. At experimental start, the cell medium was changed to serum-free medium (OptiMEM, Gibco) and incubated with and without different concentrations of soluble RANKL (Peprotech), OPG (R&D Systems, Minneapolis, MN) or a combination thereof. At different time points cell-free supernatants were harvested from PBMC, THP-1 cells and SMC and stored at -80°C until analysis. The endotoxin levels of all
stimulants and culture media were <10 pg/mL (Limulus Amebocyte Assay; BioWhittaker, Walkersville, MD).

**Tissue sampling of thrombus materials**

In 10 patients with acute ST-elevation MI (STEMI), undergoing primary percutaneous coronary intervention (PCI), thrombi at the site of the occlusion was aspirated immediately after crossing the lesion with the guide-wire. A monorail aspiration catheter (Pronto, Vascular Solutions, Minneapolis, MN) was advanced over the wire, and a 20 mL air-filled syringe was used to aspirate during advancing the catheter through the occluded segment. The catheter was pushed and pulled through the segment during continuous aspiration until the syringe was filled with no vacuum left. The aspiration catheter was removed and solid plaque material was separated from liquid blood by means of a sieve (pore filter size, 40 µm), which is part of the Pronto set. The solid material was fixed in 4% paraformaldehyde and embedded in paraffin.

**Real-time quantitative RT-PCR**

Total RNA was extracted from monocytes, T-cells and PBMC using RNeasy columns (Qiagen, Hilden, Germany), subjected to DNase I treatment (RQI DNase; Promega, Madison, WI) and stored in RNA storage solution (Ambion, Huston, TX) at -80°C. Primers were designed using the Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA) for OPG (forward primer [FP]: 5'-AATCAACTCAAAAATGTGGAATAGATGT-3', reverse primer [RP]: 5'-GCGTAAACTTTGTAGGAACAGCAA-3'), RANKL (FP: 5'-GTGCAAAAGGAATTACAACATATCGT-3', RP: 5'-AACCATGAGCCATCCACCAGC-3') and RANK (FP: 5'-CCCCTGACTCCAAGAGGAA-3', RP: 5'-GCATTTCGCTGGAGGAA-3'). Quantification of mRNA was performed using the ABI Prism 7000 (Applied Biosystems). Gene expression of the housekeeping gene β-actin was used for normalization.
Table I. Characteristics of the Study Group

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Data are presented as mean±SD. Statins, hydroxymethylglutaryl coenzyme A reductase inhibitors.
Figure I. Serum levels of OPG in 40 patients with unstable angina pectoris (AP), 40 patients with stable AP and 20 healthy controls. Data are mean±SEM. *p<0.05 versus healthy controls.