

Osteoprotegerin increases leukocyte adhesion to endothelial cells
both *in vitro* and *in vivo*

Running title: OPG promotes leukocyte-endothelial adhesion

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Abstract

Recombinant osteoprotegerin (OPG) promoted the adhesion of both primary polymorphonuclear neutrophils (PMN) and leukemic HL60 cells to endothelial cells. Leukocyte/endothelial cell adhesion was promoted by short (peak at 1 hour) pre-incubation of either endothelial cells or PMN with OPG, and the peak of pro-adhesive activity was observed in the same range of OPG concentrations detected in the sera of patients affected by cardiovascular diseases. While membrane RANKL and TRAIL, the cognate high-affinity ligands for OPG, were detected at significant levels on both PMN and HL60, they were not expressed on the surface of endothelial cells. However, pre-incubation of OPG with heparin abrogated its pro-adhesive activity, while pretreatment of endothelial cells with chondroitinase plus heparinases significantly decreased the pro-adhesive activity of OPG. Taken together, these findings suggest the involvement of both the ligand binding and the N-terminal heparin-binding domains of OPG in mediating its pro-adhesive activity. The relevance of these *in vitro* findings was underscored by *in vivo* experiments, in which the topical administration of recombinant OPG increased leukocyte rolling and adhesion to rat mesenteric post-capillary venules. Our data suggest that a pathological increase of OPG serum levels might play an important role in promoting leukocyte/endothelial cell adhesion.

Introduction

Osteoprotegerin (OPG), a soluble member of the TNF receptor superfamily, has been originally characterized for its ability to suppress osteoclast formation.¹ OPG inhibits osteoclastogenesis by binding to receptor activator of NF- κ B ligand (RANKL), a member of the TNF superfamily of cytokines, and preventing the interaction of RANKL with its high-affinity transmembrane receptor namely receptor activator of NF- κ B (RANK).² It has been shown that in the low nanomolar range, the binding interaction of OPG and RANKL is 1:1 (or some multiple thereof)³ and that dimerization of OPG results from noncovalent interactions mediated by the death domains and to a lesser extent by a C-terminal heparin-binding region. OPG dimer formation is required for the mechanism of inhibition of the RANKL/RANK receptor interaction.³

OPG can also interact with another member of the TNF superfamily, TNF-related apoptosis inducing ligand (TRAIL),⁴ which shows the ability to kill a variety of cancer cell types both *in vivo* and *in vitro*.⁵ A role for OPG as a neutralizing receptor for TRAIL under physiological conditions has been questioned in early studies, but there is now mounting evidence suggesting that the OPG/TRAIL interaction is biologically important, at least in *in vitro* culture systems.⁶ OPG has been shown to act in a paracrine or autocrine manner by binding TRAIL and promoting the survival of prostate cancer cells,⁷ breast cancer cells,⁸ and multiple myeloma cells.⁹ Moreover, when a rationally designed small molecule mimic of OPG was examined for association with TRAIL or RANKL in binding studies, this peptide bound to RANKL at a K_d of 3.89×10^{-6} M and to TRAIL at a K_d of 1.93×10^{-5} M, showing only about 5-fold higher affinity for recombinant RANKL compared to recombinant TRAIL.¹⁰

A number of studies have clearly demonstrated that the serum levels of OPG are elevated in both diabetic and non-diabetic patients affected by coronary artery disease and heart failure after acute myocardial infarction, and increased levels of OPG in these patients represent a risk factor for cardiovascular mortality.¹¹⁻¹⁸ OPG serum levels are also elevated in patients affected by a variety of human malignancies, including hematological disorders^{19,20} and an immuno-histochemical study has demonstrated that OPG is expressed in the tumor-associated endothelium in approximately 60% of malignant tumors.²¹

In spite of the reported findings, the physiopathological role of elevated serum levels of OPG in vascular biology is not well understood. On these bases, we have investigated the effect of OPG on the endothelial-leukocyte interactions both in *in vitro* and *in vivo* experimental models.

Methods

1. All the animal experimental procedures were performed in compliance with the guidelines of European (86/609/EEC) and the Italian (D.L.116/92) laws and approved by the Italian Ministry of University and Research as well as by the Administration of the Animal House of the University of Trieste.

2. Human blood samples were obtained following informed consent and after full explanation of the procedure and its purpose, in accordance with Declaration of Helsinki of 1975, and with approval from the local (University of Ferrara) institutional review boards.

Cells and reagents

Primary human umbilical vascular endothelial cells (HUVEC) and human microvascular endothelial cells (HMVEC) were obtained from BioWittaker (Walkersville, MD). HUVEC and HMVEC were used between the 2nd and 5th passage *in vitro* and were cultured in EGM basal medium supplemented with 2% FBS, 12 mg/ml BBE, 1 mg/ml hydrocortisone and 10 ng/ml ECGF (all from BioWhittaker), as previously described.^{22,23}

EDTA-blood samples for the isolation of primary polymorphonuclear neutrophils (PMN) were drawn from healthy volunteers after obtaining informed consent. Blood samples were diluted 1:2 with PBS and a first separation step was performed by Histopaque ficoll gradient centrifugation (Lymphoprep; Nycomed, Oslo, Norway). PMN in the pellet of the gradient were separated from erythrocytes by further steps consisting of dextran sedimentation and hypotonic lysis, resulting in purity >90% CD15/CD11b⁺ cells, as assessed by flow cytometry using specific FITC- or PE-conjugated monoclonal antibodies (MoAbs; Immunotech, Marseille, France; Miltenyi Biotech GmbH, Bergish Gladbach, Germany). Treatments were performed immediately after cell isolation. The HL60 myeloid cell line (American Tissue Culture Collection, Rockville, MD) was routinely grown in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% FBS.

Heparin, heparinases I, II, III, chondroitinase ABC were purchased from Sigma Chemicals (St Louis, MO). Tumor necrosis factor- α (TNF- α) and recombinant OPG were from R&D Systems (Minneapolis, MN). In particular, the recombinant full-length OPG (Met 1-Leu 401) was expressed in the NSO mouse myeloma cell line. After purification, the recombinant OPG, a disulphide-linked homodimeric protein generated after removal of a 21 amino acid signal peptide from each monomer, is lyophilized from a 0.2 μ m filtered solution in PBS ("carrier free"). In selected experiments, we have also used the recombinant OPG-Fc, corresponding to the cysteine-rich region of human OPG (aa 22-202) fused to the Fc portion of human IgG1, therefore lacking the N-terminal heparin-binding domain.

Human OPG levels were measured in HUVEC culture supernatants using sandwich-type enzyme-linked immunosorbent assay (ELISA) kit (purchased from Alexis Biochemicals, Lausen, Switzerland), according to the manufacturers' instructions. The results were read using an Anthos 2010 ELISA reader (Anthos Labtec Instruments Ges.m.b.H, Austria). Measurements were done in duplicates.

Endothelial-leukocyte adhesion assay

Vascular endothelial cells were grown to confluence in 24 or 96-well tissue culture plates and stimulated for 1 min to up 16 hours (overnight) at 37°C with recombinant human OPG or TNF- α , used alone or in combination. After 3 washings with serum-free medium, untreated $1-3.5 \times 10^5$ PMN or HL60 cell suspensions were added to each well and were further incubated at 37°C for 60 min. In some experiments, leukocytes were added simultaneously with recombinant OPG to HUVEC for up to 60 min. After endothelial-leukocyte co-culture, non-adherent PMN or HL60 cells were removed by washing the wells at least twice. Endothelial-leukocyte co-cultures were

photographed under a light microscope (10X magnification). The number of adhered PMN or HL60 cells was evaluated by a colorimetric assay using tetramethyl benzidine (Sigma Chemicals) as a substrate for myeloperoxidase, as previously described,²⁴ and/or by scoring at least 6 random fields for each treatment. In both assays, the viability of both endothelial cells and adherent PMN or HL60 cells was routinely monitored at light microscopy by Trypan blue dye exclusion.

In some experiments, recombinant OPG was pre-incubated with heparin (250 mg/ml) for 1 hour at 37°C before performing adhesion assay. In other experiments, HUVEC cultures were pretreated with heparinases I, II, III (50 U/ml each), a family of enzymes that cleaves highly sulfated regions of heparin-sulfate like glycosaminoglycans (GAGs) at 2-O sulfated uronic acids, and with chondroitinase ABC (50 U/ml), which cleaves chondroitin sulfate side chain of cell surface GAGs,²⁵ for 1 hour at 37°C. After washing, adhesion experiments were performed.

Expression of adhesion molecules

Total RNA was extracted from HUVEC either left untreated or stimulated with recombinant OPG or TNF- α by using the Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) according to the supplier's instructions. The quality of the total RNA preparation was verified by agarose gel and, when necessary, further purification was performed with the RNeasy cleanup system (Qiagen) to remove chromatin DNA. Amplification for adhesion molecules was performed with a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) by using SYBR Green based-technology and the SuperArray Bioscience's RT² Real-TimeTM Gene Expression Assays, that include specific validated primer sets and PCR master mixes (SuperArray Bioscience Corporation, Frederick, MD). All samples were run in triplicate.

In parallel experiments, E-selectin, ICAM-1, and VCAM-1 expression in HUVEC cultures was determined by ELISA. Briefly, cells were washed with PBS, BSA 2%, Ca^{2+} Mg^{2+} 0.7 mM, before incubation with 5 $\mu\text{g}/\text{ml}$ of the following MoAb: anti-ICAM-1 (Dako; Glostrup, Denmark), anti-VCAM-1 and anti-E-selectin (both from Chemicon; Temecula, CA) and then with APC-anti-mouse secondary Ab (Sigma Chemicals). Finally p-Nitrophenyl Phosphate (PNPP; Sigma Chemicals) was applied to cells, and the absorbance was measured at 405 nm by an ELISA reader.

Flow cytometric analyses

Expression of surface cellular antigens on HL60, PMN and/or HUVEC was evaluated by flow cytometric analysis with the FACScan (Becton Dickinson, San José, CA). Surface cell staining was performed at 4°C for 40 minutes by incubating $3\text{-}5 \times 10^5$ cells in 200 μl of PBS (containing 1% BSA and 5% FBS) with 1 μg the indicated Abs. In particular, the expression of surface TRAIL and RANKL was evaluated by indirect staining with primary Abs (both from R&D Systems), followed by PE-conjugated anti-mouse secondary Ab (Immunotech). Non-specific fluorescence was assessed using normal mouse isotype-match IgG followed by secondary Abs. On the other hand, directly FITC- or PE-conjugated mAbs were used for the detection of CD15 (Immunotech) and Mac-1 (Miltenyi) on PMN or HL60, as well as for the detection of E-selectin, ICAM-1 (both MoAbs from Bender Medical System; Wien, Austria), and VCAM-1 (Cymbus Biotechnology; Chandlers Ford, UK). Non-specific fluorescence was assessed by incubation with irrelevant isotype-matched conjugated MoAbs.

In other experiments, PMN, HL-60 or HUVEC were incubated with either recombinant OPG or OPG-Fc for 40 min at 37°C, and after washing, the adhesion of OPG or OPG-Fc to the cell surface was revealed by incubation with MoAb anti-human

OPG (R&D Systems) followed by PE-conjugated anti-mouse secondary Abs.

Animals and *in vivo* experimental procedure

Male Wistar Kyoto rats, each weighting approximately 250-270 g, were anesthetized intraperitoneally (i.p.) with sodium thiobarbital (100 mg/kg). Details on the surgical procedure, including insertion of intravascular catheters to monitor blood pressure and to inject the fluorescent leukocyte marker, acridine orange (AO), were reported previously.²⁴ AO was diluted in sterile saline and then slowly infused during the experimental procedure at the concentration of 0.025 mg/kg/min and at a rate of 0.5 ml/hour. The rats were placed on an adjustable stage of an upright microscope (model BX50WI; Olympus Optical, Tokyo, Japan) and after a midline incision of the rat abdomen, a loop of ileal mesentery was exteriorized and carefully draped over a transparent pedestal. The exposed tissue was infused throughout the study with sterile buffered saline warmed at 37°C. The AO-labeled leukocytes were made visible by epifluorescence transillumination with a UIF550 filter for excitation light (Olympus Optical). Images were recorded by CCD camera connected through a PCI interface-board (SensiCam PCO, Kelheim, Germany) to a computer device, where they were stored and analyzed off-line using dedicated imaging software (Analytica Lite, Milan, Italy).

After surgery and a baseline evaluation, sterile saline solution containing recombinant OPG was topically applied to the mesentery and after 4 hours image sequences were recorded intermittently at different time intervals up to 90 min (control animals were treated with the vehicle). Segments of 3 to 5 unbranched post-capillary venules (25-40 μ m diameter, 200 μ m length) were selected for analysis, as described.²⁴

Venular diameter and center-line red blood cell velocity were evaluated off-line using a video caliper (Image Research, Ontario, Canada) and customized frame-by-frame analogical image analysis. Red blood cell velocity (VRBC) and venular diameter (D) were used to calculate venular wall shear rate ($\dot{\gamma}$) through the formula $\dot{\gamma}=8(V_{\text{mean}}/D)$, where $V_{\text{mean}}=\text{VRBC}/1.6$. Intravascular circulation of leukocytes in postcapillary venules was analyzed off-line during playback of the digital file sequences. Labeled leukocytes were classified as rolling (if they moved more slowly than red blood cells, thus becoming visible) or adherent (if they remained stationary for more than 30 sec). Rolling flux was expressed as the number of leukocytes seen moving past a reference point per minute, and adherence was measured by counting the number of adherent leukocytes per 200 μm venule length.

Statistical analysis

The results were evaluated by using analysis of variance with comparison by Student's t-test and with the Mann-Whitney rank-sum test. Statistical significance was defined as $p<0.05$.

Results

Recombinant OPG promotes the leukocyte/endothelial cell adhesion

Our initial experiments were designed to investigate whether OPG might affect leukocyte/endothelial interactions. HUVEC were grown to confluence and incubated with increasing concentrations (0.01-10 ng/ml) of recombinant human OPG. In parallel, cells were treated with TNF- α (0.1 ng/ml), used as positive control. An overnight (approximately 16 hour) incubation of HUVEC with recombinant OPG induced a bell-shaped increase in the number of adherent PMN with respect to HUVEC left untreated (**Figure 1A**). Of note, OPG showed maximal pro-adhesive activity at concentrations comprised between 0.1-0.5 ng/ml, which are in the range of concentrations reported to be elevated in the sera of patients affected by cardiovascular diseases.¹¹⁻¹⁸ Similar findings were obtained when experiments were carried out by using HMVEC instead of HUVEC (data not shown), indicating that the OPG pro-adhesive activity was not endothelial cell type specific. In parallel experiments, another cell system was used to test whether the OPG effects are reproducible and could be generalized. For this purpose, we have chosen HL60 myeloid leukemic cell line, taking into account that serum OPG has been reported to be elevated also in the sera of patients affected by hematological malignancies.¹⁹ OPG induced a significant ($p<0.05$) bell-shaped increase of HL60 leukemic cell adhesion to HUVEC, starting from concentrations as low as 0.1 ng/ml (**Figure 1B**).

Pro-inflammatory cytokines, such as TNF- α , are known to increase leukocyte adhesion to endothelial cells through several mechanisms, including the up-regulation of surface adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin, in endothelial cells at both the mRNA level, at early time (**Figure 2A**) and, at later time, at protein level (**Figure 2B-C**). On the contrary, recombinant OPG did not induce any significant

increase of ICAM-1, VCAM-1 or E-selectin mRNA (**Figure 2A**). Consistently with the mRNA data, OPG was unable to modulate ICAM-1, VCAM-1 or E-selectin at the protein level, as evaluated by two independent approaches: ELISA (**Figure 2B**) and flow cytometry (**Figure 2C**), which allowed us to quantify the total and surface expression levels of adhesion molecules, respectively.

As show in **Figure 3A**, while untreated HUVEC released low levels of OPG in the culture supernatants, TNF- α induced a dose-dependent increase of the release of endogenous OPG by HUVEC. Interestingly, the levels of OPG released in response to TNF- α were in the range of OPG concentrations able to promote binding of both PMN and HL60 to endothelial cells (**Figure 1**), suggesting the possibility that OPG endogenously released in response to TNF- α might contribute to TNF- α -mediated pro-adhesive activity. Moreover, the simultaneous overnight treatment of HUVEC with recombinant OPG (0.5 ng/ml) plus TNF- α (0.01-0.1 ng/ml) did not show any additive or synergistic effects with respect to the pro-adhesive activity induced by TNF- α alone (**Figure 3B**).

A short-term exposure of either endothelial cells or PMN to recombinant OPG is sufficient to promote leukocyte/endothelial adhesion

In the next series of experiments, an optimal concentration of recombinant OPG (0.5 ng/ml) was added simultaneously with PMN to endothelial cells for up to 1 hour (**Figure 4**). The presence of recombinant OPG induced a significant ($p < 0.05$) increase of leukocyte adhesion to endothelial cells as early as 15 min after the beginning of PMN/endothelial cell co-cultures. This rapid induction of pro-adhesive activity suggested that transcriptional-independent mechanisms should account for this biological activity of OPG. To further characterize the adhesive activity of OPG, endothelial cells were pre-

treated with recombinant OPG (0.5 ng/ml) for different time points, washed 3 times to eliminate unbound OPG and then co-incubated with PMN (**Figure 5A**). In parallel, PMN, instead of endothelial cells, were pre-treated with recombinant OPG (0.5 ng/ml) for different time points, abundantly washed and then co-incubated with endothelial cells (**Figure 5B**). As shown in **Figure 5A**, a significant ($p < 0.05$) increase of the pro-adhesive activity of OPG was observed for times of HUVEC pre-treatment as short as 5 min. In addition, also a short pre-treatment of PMN with OPG significantly ($p < 0.05$) increased their adhesiveness to HUVEC (**Figure 5B**), at a comparable extent and kinetics with respect to the pro-adhesive activity observed in experiments in which HUVEC were pre-treated with OPG before co-culture with PMN. Moreover, exposure of PMN to recombinant OPG for up to 4 hours did not induce any modulation of the surface expression levels of Mac-1 (**Figure 5C**) which was investigated taking into account that such leukocyte surface molecule plays a pivotal role for binding to ICAMs and mediating leukocyte extravasation.²⁶

The heparin-binding domain of OPG is involved in mediating the pro-adhesive activity of OPG

We next explored the possibility that OPG might interact with membrane-associated molecules present on the cell surface of leukocytes as well as on endothelial cells. For this purpose, PMN, HL60 as well as endothelial cells were analyzed by flow cytometry for the expression of the cognate high-affinity receptors of OPG, RANKL or TRAIL, which exist not only as soluble but also as transmembrane proteins.^{3,5} Of note, both PMN and HL60 showed a clear-cut expression of both ligands, while endothelial cells did not express either RANKL or TRAIL (**Figure 6A**). In parallel, the ability of the full-length OPG and of the truncated OPG-Fc to bind to the surface of leukocytes or

endothelial cells was assessed by flow cytometry. As shown in **Figure 6B**, a significant ($p < 0.01$) binding of both OPG and OPG-Fc was measured on leukocytes, as expected based on the presence of both its high-affinity receptors. Of note, a modest ($p < 0.05$) binding of full-length recombinant OPG was observed also on endothelial cells, while recombinant truncated OPG-Fc failed to bind to HUVEC (**Figure 6B**). This observation suggests that other domains of OPG beside the ligand-binding domain are involved in the OPG/HUVEC interaction, and therefore in mediating the pro-adhesive activity of OPG. In this respect, previous studies have demonstrated that OPG contains three structural domains specifically influencing its biological activity.^{3,27,28} The first one is a cysteine-rich domain in the N-terminal position which is dispensable for OPG dimerization. The second domain is a heparin-binding domain potentially capable of interacting with numerous proteoglycans^{28,29} and the third one corresponds to a death domain homologous region.²⁷ In order to evaluate whether the heparin-binding domain, which is deleted in the OPG-Fc recombinant molecule, might indeed be involved in mediating the pro-adhesive activity of OPG, recombinant OPG was pre-incubated with heparin for 40 min before either HUVEC or PMN treatment for 1 additional hour. As shown in **Figure 6C**, a significant ($p < 0.05$) decrease in the pro-adhesive activity of OPG was observed when recombinant OPG was pre-incubated with heparin. A similar decrease of the pro-adhesive activity of OPG was observed when HUVEC were pre-treated with heparinases plus chondroitinase before exposure to recombinant OPG (**Figure 6C**). We have also attempted to compare the pro-adhesive activity of full-length recombinant OPG with that of OPG-Fc, to further characterize the involvement of the heparin-binding domain in mediating the pro-adhesive activity of OPG. Unfortunately, however, both OPG-Fc and human IgG, used as control, induced a significant increase of leukocyte adhesion to

endothelial cells through the rapid activation of Mac-1 in PMN due to the Fc-moiety (data not shown).

Leukocytes adhere to endothelium *in vivo* in response to OPG

In the last group of experiments, we sought to determine whether OPG was able to promote leukocyte adhesion to endothelial cells also *in vivo*. Recombinant OPG (1-10 ng/ml) was topically applied to the rat mesentery and after 4 hours the rolling and adhesion of leukocytes was determined by intravital microscopy at different time points (30-60-90 min). This is a previously established *in vivo* procedure, through which single polypeptides as well as big protein complexes easily enter in the blood circulation through the highly permeable post-capillary venules of the rat mesentery.²⁴ As shown in **Figure 7A**, leukocyte rolling was significantly ($p<0.05$) and dose-dependently induced by the treatment with OPG with respect to treatment with the vehicle. Importantly, also the number of leukocytes that adhered to the vessels was significantly ($p<0.05$) higher in rats treated with OPG than in rats receiving saline vehicle (**Figure 7B-C**). The observation that only few cells adhered to endothelial cells in control rats treated with saline excludes the possibility that the pro-adhesive property acquired by the endothelium represents a reaction to the surgical procedure and also ruled out nonspecific effects of the fluorescent dye injected intravenously. Moreover, OPG administration did not induce any modification in the systemic leukocyte and neutrophil counts (data not shown).

Discussion

OPG has been shown to be abundantly expressed in vascular cells and in particular in vascular smooth muscle cells both *in vitro* and *in vivo*.³⁰⁻³² Moreover, several studies have clearly demonstrated that OPG plays a role in promoting *in vitro* endothelial and vascular smooth muscle cells (VSMC) survival and preventing vessel wall calcification.³³⁻³⁶ In fact, mice lacking OPG show calcified arteries,^{34,35} while an elegant study has recently demonstrated that OPG is strongly expressed in and around the ventricular-side endothelium but it shows a little expression on the aortic side, which is more prone to calcification³⁶. In spite of these findings which clearly suggest a protective role of physiological concentrations of OPG in the vascular system, other studies have demonstrated that the expression and release of OPG by endothelial cells and VSMC is markedly upregulated in response to inflammatory cytokines, such as TNF- α ³¹ and platelet-derived growth factor (PDGF),³⁰ two important regulators of vascular pathogenesis. Conversely, OPG production is inhibited by PPAR- γ ligands in VSMCs,³² agents associated with anti-inflammatory and anti-atherogenic effects *in vitro* and *in vivo*. Thus, the picture emerging from these previous studies is quite complex: while a basal constitutive production/release of OPG by vascular smooth muscle cells and endothelial cells is essential to protect the vessel wall against calcification, an increased release of OPG correlates to increased cardiovascular risk.¹¹⁻¹⁸

In this study, we have demonstrated for the first time that the treatment of vascular endothelial cells with recombinant OPG promotes the adhesion of both primary PMN and leukemic HL60 cells to the endothelial cell surface. Interestingly, the greatest effect was observed at concentrations of OPG comprised in the same range of those described to be elevated in the serum of patients affected by cardiovascular diseases.¹¹⁻¹⁸

The ability of OPG to promote leukocyte adhesion to endothelial cells was very rapid (5-15 min). A similar increase of pro-adhesion activity was observed when PMN rather than HUVEC were pre-treated with OPG before the co-culture incubation. In this respect, it is noteworthy that both PMN and HL60 exhibit significant surface levels of both RANKL and TRAIL while endothelial cells, obtained by different sources, did not show surface expression of the high-affinity transmembrane ligands of OPG. Therefore, OPG likely interacts through its ligand-binding domain with RANKL and/or TRAIL expressed on the surface of leukocytes. On the other hand, the data obtained pre-treating OPG with heparin before the adhesion assays strongly suggest that OPG interacts with endothelial cell membrane through its heparin-binding domain. In this respect, it should be mentioned that the heparan sulfates expressed on the cell surface are important participants in cell-surface signaling and have been involved in actin cytoskeleton regulation, cell adhesion and migration, and modulation of specific receptor interactions.^{37,38} It should also be underlined that the interaction between OPG and glycosaminoglycans was confirmed in a recent study by surface plasmon resonance, which demonstrated that OPG binds to heparin with a high-affinity (KD: 0.28 nM).²⁸

By using intravital microscopy, which provides a useful tool for observing leukocyte-endothelium interactions *in vivo*, we have also demonstrated that the findings obtained *in vitro* are relevant *in vivo* since leukocytes circulating in the rat mesentery under physiological flow conditions firmly adhered to the endothelium of the post-capillary venules after the topical administration of recombinant OPG. Since an abnormal increase of leukocyte adhesion to endothelial cells is considered an early step in endothelial cell dysfunction,³⁹ the results illustrated in our study suggest a potential mechanism to explain why pathologically elevated serum OPG levels are linked to the development or status of vascular disease. In fact, a number of reports have shown that

increased circulating OPG levels often occur in cardiovascular diseases.¹¹⁻¹⁸ Moreover, linkage of two OPG genetic polymorphisms was associated with an increased risk of coronary artery disease in Caucasian men, and serum OPG levels correlated with one of these polymorphisms.⁴⁰

Studies of other authors have shown that OPG is an NF- κ B-inducible gene, whose expression and release in culture of vascular cells is significantly increased by inflammatory cytokines.³⁰⁻³³ In this respect, we have confirmed that TNF- α potently induced the release in HUVEC culture of endogenous OPG. Although it cannot be excluded that the increased release of OPG in response to inflammatory cytokines might merely represent a bystander effect or even an attempt to counteract the endothelial damage induced by inflammatory cytokines, our present data rather suggest that elevated levels of OPG might substantially contribute to establish a vicious inflammatory circle by enhancing the pro-adhesive activity of TNF- α . Since we have recently demonstrated that the systemic administration or recombinant TRAIL shows anti-atherosclerotic activity in apolipoprotein E-null diabetic mice,⁴¹ an alternative not mutually exclusive mechanism by which elevated levels of serum OPG might contribute to cardiovascular risk is by inhibiting the anti-atherosclerotic activity of circulating TRAIL.

Our present findings are particularly noteworthy also considering the enormous surface area of the endothelium throughout the body, which suggests that endothelial cells are a key cell type involved in the production/release of circulating OPG in human serum.^{33,42} Although previous studies have reported the possibility that OPG may affect relevant cell parameters, such as release of matrix metalloproteinase-9 (MMP-9) activity,³⁴ this is the first study to the best of our knowledge that demonstrates the ability of OPG to markedly increase the leukocyte-endothelial interactions both *in vitro* and *in vivo*. Overall, our study suggests how the enhanced OPG production and release

associated to pathological conditions may contribute to the inflammatory status of endothelium characterizing cardiovascular disease and tumor angiogenesis. Therefore, a suggestion deriving from our study is that therapeutic strategies aimed to decrease the OPG serum levels may be suitable for improving the vascular function in vascular pathologies characterized by a chronic inflammation state.

Acknowledgments

The specific contribution of the authors is as follow: Giorgio Zauli, Paola Secchiero and Francesco Tedesco have designed, analyzed the data and wrote the paper; Federica Corallini, Fleur Bossi, Paolo Durigutto, Fabio Fischietti, Claudio Celeghini have performed research and contributed analytical tools.

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Figure Legends

Figure 1. Dose-response effect of OPG on leukocyte adhesion. HUVEC cells were either left untreated or exposed for 16 hours to the indicated concentrations of OPG. TNF- α (0.1 ng/ml) was used as positive control. After HUVEC washing, PMN (**A**) or HL-60 (**B**) were added to the endothelial monolayer, and the percentage of adherent cells was determined after a co-culture of 60 minutes at 37°C. Cell adherence on HUVEC is reported as fold of increase respect to cell adhesion in the absence of treatment. Results are expressed as mean \pm SD of seven experiments, each performed in triplicate. *, $p < 0.05$.

Figure 2. Lack of effect of OPG on the expression of adhesion molecules. HUVEC were exposed to OPG or TNF- α (used as positive control) for 16 hours and expression levels of adhesion molecules (ICAM-1, VCAM-1 and E-selectin) were evaluated at both mRNA (**A**) and protein levels by ELISA (**B**) and flow cytometry (**C**). In **A**, RNA from HUVEC either left untreated or exposed to OPG or TNF- α were quantitatively analyzed by ICAM-1, VCAM-1 and E-selectin RT-PCR after normalization to the level of GAPDH mRNA. Each sample was determined in duplicate. In **B**, adhesion molecules expression was determined by ELISA and data are shown as average OD \pm SD of three independent experiments. In **C**, the control (unshadowed) histograms represent the background fluorescence obtained from the staining of the same cultures with isotype-matched control antibodies. One of six experiments with similar results is shown.

Figure 3. Endogenous OPG release by endothelial cells. **A**, HUVEC were either left untreated or stimulated with the indicated concentration of TNF- α . After 24 hours, the levels of OPG released in culture supernatant were measured by ELISA. Results are expressed as means \pm SD of four independent experiments, each performed in triplicate. **B**, HUVEC were either left untreated or stimulated with OPG (0.5 ng/ml) in the presence or absence of TNF- α . After washing, leukocytes were added to endothelial monolayer, and the number of adherent leukocytes was scored. Values are mean \pm SD of triplicate determinations of 3 separate experiments.

Figure 4. Effect of short-term OPG treatment on leukocyte/endothelial cell adhesion. Endothelial monolayers were exposed to PMN in the absence or presence of recombinant OPG (0.5 ng/ml) for the indicated times before scoring the number of adherent PMN. Cell adhesion in untreated control culture was set as unity. Values are mean \pm SD of triplicate determinations of 3 separate experiments. *, $p < 0.05$ versus control.

Figure 5. Effect of OPG pre-treatment on leukocyte/endothelial cell adhesion and determination of Mac-1 expression in PMN. Endothelial monolayers (**A**) or PMN (**B**) were either left untreated or exposed to OPG (0.5 ng/ml) for the indicated times. After washings, PMN and endothelial cells were co-cultured for 1 hour, and the number of adherent PMN was scored. Cell adhesion in untreated control culture was set as unity. Values are mean \pm SD of triplicate determinations of 5 separate experiments. *, $p < 0.05$ versus control. (**C**) PMN were either left untreated or exposed to OPG (0.5 ng/ml), and surface expression levels of Mac-1 on CD15⁺ cells was evaluated by double staining in flow cytometry. Irr. Abs: staining of the PMN culture

with isotype-matched control antibodies. One of three experiments with similar results is shown.

Figure 6. Cell surface binding of recombinant OPG. **A**, surface expression of TRAIL and RANKL in PMN, HL60 and HUVEC was evaluated by flow cytometry. The control (unshadowed) histograms represent the background fluorescence obtained from the staining of the same cultures with isotype-matched control antibodies. One of four experiments with similar results is shown. **B**, HL60 and HUVEC were incubated with either recombinant OPG or OPG-Fc, and binding to cell surface was revealed by flow cytometry. Results are expressed as mean fluorescence intensity and are mean \pm SD of determinations of three separate experiments. **C**, the specific contribution of heparan sulfate proteoglycans in OPG-mediated leukocyte adhesion was evaluated by pretreatment of OPG with heparin or pretreatment of HUVEC with Heparinase I, II and III (Hep.) plus Chondroitinase (Chond.) before adhesion assays. Values are mean \pm SD of triplicate determinations of 3 separate experiments. *, $p < 0.05$.

Figure 7. Effect of OPG on leukocyte trafficking in rat mesenteric postcapillary venules. Traffic of leukocytes was monitored at various time intervals after 4 hours of i.p. administration of 1-10 ng/ml of OPG, and of the control vehicle. Measurements were performed on 4 to 6 different segments of unbranched venules (25- μ m to 40- μ m diameter, 200- μ m length). **A**, Values of rolling leukocyte flux defined as number of cells that become visible as bright spheres if they travel inside the venules more slowly than red blood cells. **B**, Numbers of leukocytes stably adherent to the same site of postcapillary vascular endothelium of rat mesentery. **C**, Leukocytes labeled *in vivo*

with AO were made visible by fluorescence epi-illumination and appeared as bright spheres. One of four experiments with similar results is shown. Magnification 100x.

Figure 1A

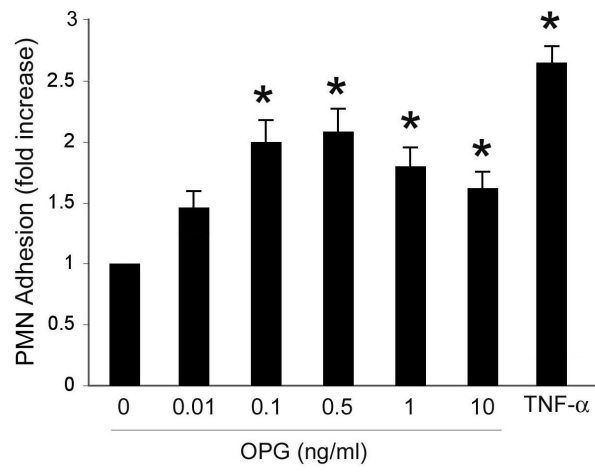


Figure 1B

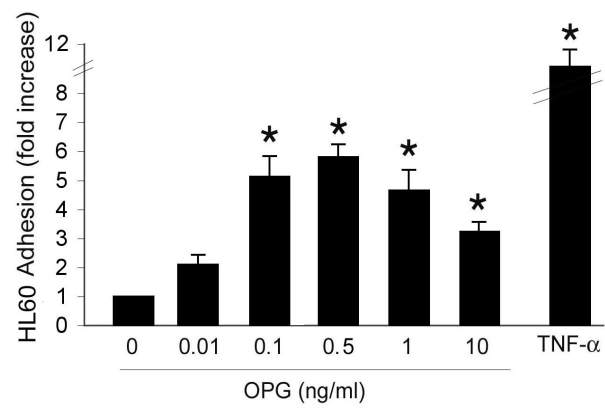


Figure 2A

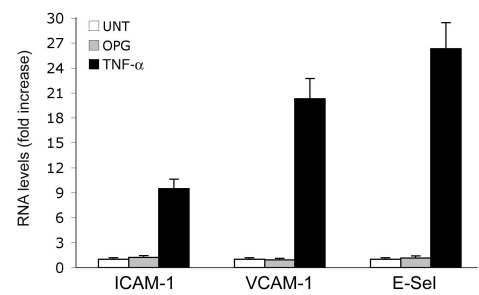


Figure 2B

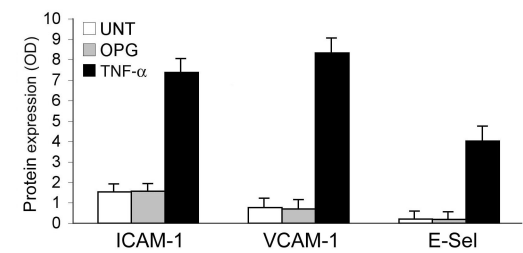


Figure 2C

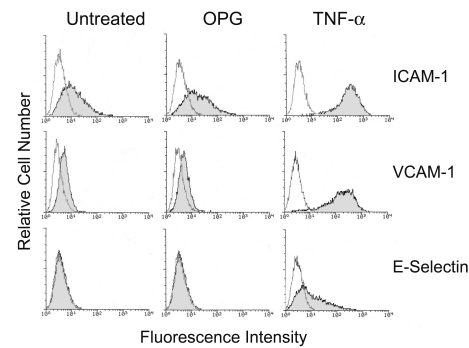


Figure 3A

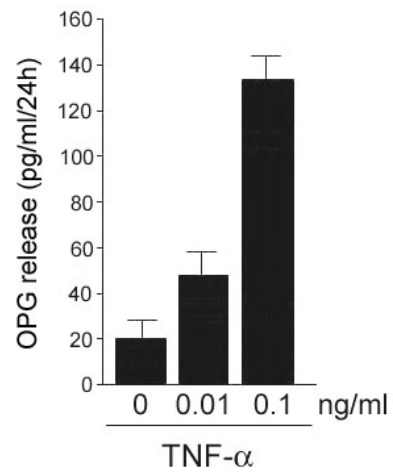


Figure 3B

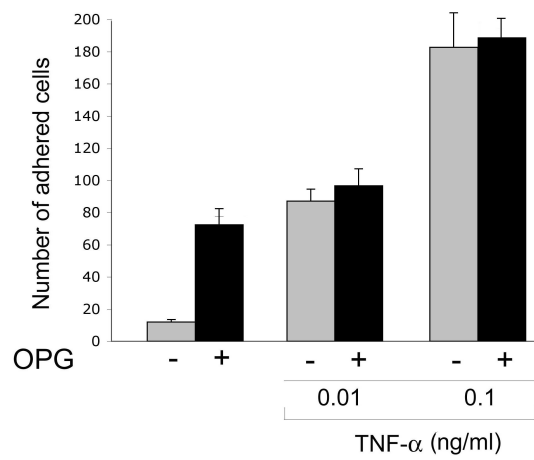


Figure 4

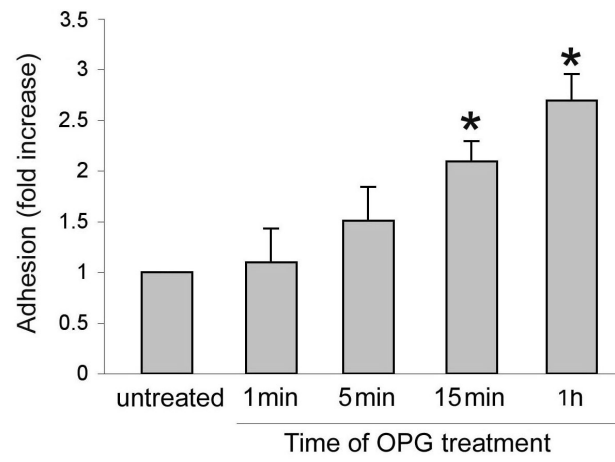


Figure 5A

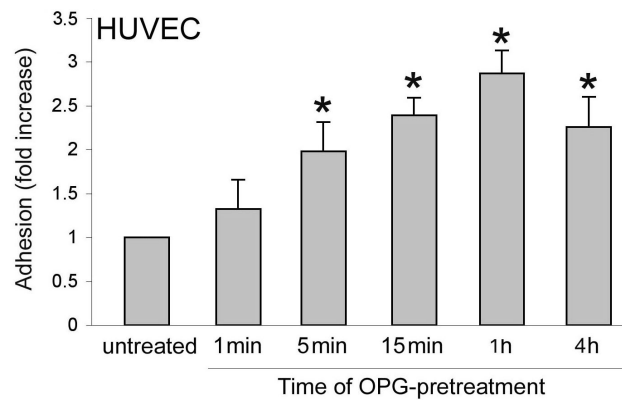


Figure 5B

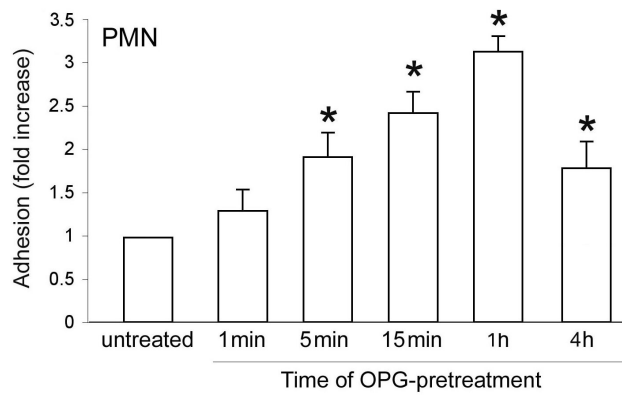


Figure 5C

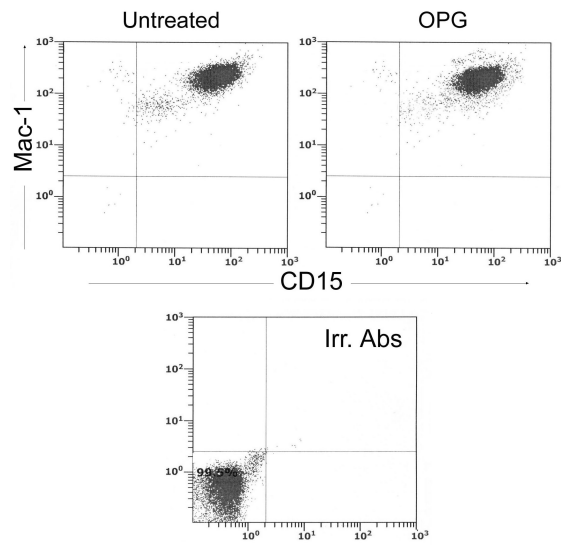


Figure 6A

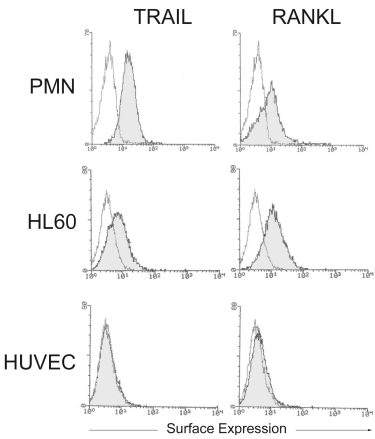


Figure 6B

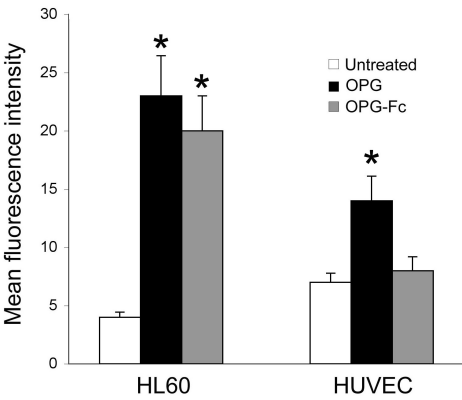


Figure 6C

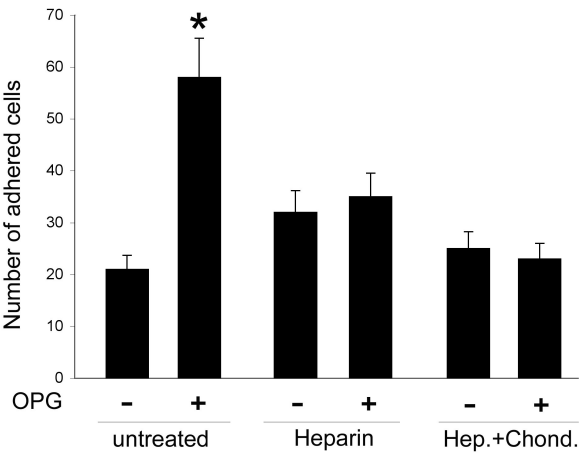


Figure 7A

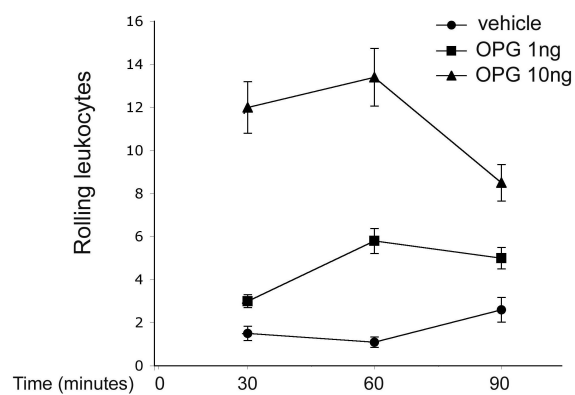


Figure 7B

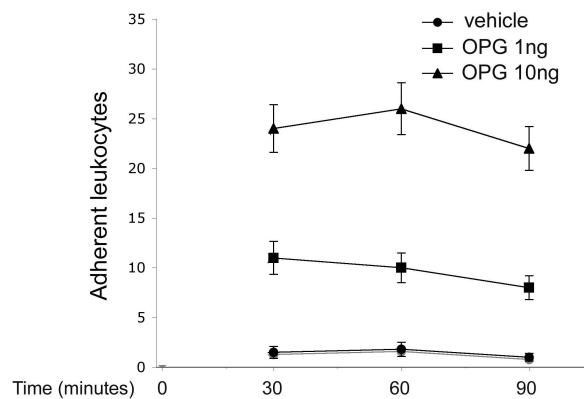
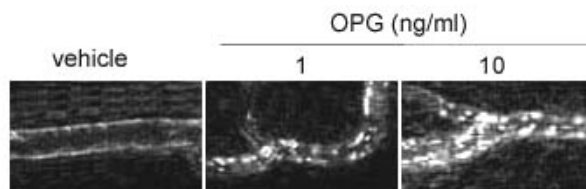


Figure 7C





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Osteoprotegerin increases leukocyte adhesion to endothelial cells both *in vitro* and *in vivo*

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