Association of Osteoprotegerin With Human Abdominal Aortic Aneurysm Progression

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Background—Abdominal aortic aneurysm (AAA) is characterized by destruction of the arterial media associated with loss of vascular smooth muscle cells, infiltration of mononuclear cells, and high concentration of metalloproteinases (MMPs) and cytokines. Osteoprotegerin (OPG) has recently been identified in atherosclerosis. The presence and functional importance of OPG in human AAA was investigated.

Methods and Results—In 146 men with small AAA followed up by ultrasound for 3 years, serum OPG was weakly correlated with aneurysm growth rate. Western analysis showed 3-, 8-, and 12-fold-greater OPG concentrations in human AAA biopsies compared with biopsies of atherosclerotic narrowed aorta (1.4±0.1 versus 0.5±0.1 ng/mg tissue; P=0.002), postmortem nondiseased abdominal aorta (1.4±0.1 versus 0.2±0.1 ng/mg tissue; P<0.001), and nondisease thoracic aorta (1.4±0.1 versus 0.1±0.06 ng/mg tissue; P<0.001). Healthy human aortic vascular smooth muscle cells incubated with recombinant human (rh)OPG (0 to 20 ng rhOPG/10⁶ cells per 1 mL per 24 hours) developed an aneurysmal phenotype defined by impaired cell proliferation (P<0.001), increased apoptosis (P<0.01), and increased MMP-9 (92 kDa) expression (P<0.001). Incubation of monocytic THP-1 cells with 1 ng rhOPG/10⁶ cells per 1 mL per 24 hours induced a 2-fold increase in MMP-9 expression (P<0.001), a 1.5-fold increase in MMP-2 activity (P=0.005), and a 2-fold stimulation of IL-6 production in these cells (P=0.02). Finally, secretion of OPG from human AAA explant was abrogated by treatment with the angiotensin II blocker irbesartan, with the reduction in secreted levels averaging 63.0±0.9 ng/mg tissue per 48-hour period.

Conclusions—These findings support a role for OPG in the growth of human AAA and suggest a potential benefit for angiotensin II blockade in slowing aneurysm expansion. (Circulation. 2005;111:3119-3125.)

Key Words: aneurysm ■ apoptosis ■ osteoprotegerin ■ metalloproteinases ■ muscle, smooth
Methods

Human Samples
Informed consent was obtained for tissue and blood collection in accordance with protocols approved by the relevant ethics bodies. Serum samples were obtained from 146 men, a subset of a total cohort of 545 men with small aortic aneurysms (30 to 50 mm) identified as part of the Western Australia abdominal aortic aneurysm screening study. Patients were followed up by regular ultrasound scans for a minimum of 3 years. The mean aneurysm growth rate for this group of patients over 3 years was 3.7 ± 2.5 mm. Previous analyses of the whole cohort demonstrated that the only clinical determinant of aneurysm expansion (including age, smoking history, hypertension, and C-reactive protein) was initial aortic diameter. Biopsies collected at surgery from male age-matched patients undergoing AAA repair (70 ± 6 years; n = 15) or AOD bypass (67 ± 7 years; n = 10) were obtained from the aeurysm or atherosclerotic aorta opposite the inferior mesenteric artery. “Normal” aortic samples were obtained from postmortem age-matched male abdominal aorta (63 ± 7 years; n = 5), and thoracic aorta (TA) was obtained from male age-matched patients (65 ± 6 years; n = 10) undergoing CABG. Tissue samples of 1.0 mg were retained from several AAA biopsies and VSMCs isolated to estimate cell number per 1 mg tissue. This allowed an estimated comparative standardization of concentration units between in vivo (per mg tissue) and in vitro (per cell number) investigations.

Genotyping Analysis
Serum samples obtained from whole blood were stored at −70°C before analysis. OPG levels were determined by assaying with an OPG DuoSet ELISA system (R&D Systems). The intra-assay coefficient of variation was <5%. Serum samples were obtained from 146 men, a subset of a total cohort of 545 men with small aortic aneurysms (30 to 50 mm) identified as part of the Western Australia abdominal aortic aneurysm screening study. Patients were followed up by regular ultrasound scans for a minimum of 3 years. The mean aneurysm growth rate for this group of patients over 3 years was 3.7 ± 2.5 mm. Previous analyses of the whole cohort demonstrated that the only clinical determinant of aneurysm expansion (including age, smoking history, hypertension, and C-reactive protein) was initial aortic diameter. Biopsies collected at surgery from male age-matched patients undergoing AAA repair (70 ± 6 years; n = 15) or AOD bypass (67 ± 7 years; n = 10) were obtained from the aeurysm or atherosclerotic aorta opposite the inferior mesenteric artery. “Normal” aortic samples were obtained from postmortem age-matched male abdominal aorta (63 ± 7 years; n = 5), and thoracic aorta (TA) was obtained from male age-matched patients (65 ± 6 years; n = 10) undergoing CABG. Tissue samples of 1.0 mg were retained from several AAA biopsies and VSMCs isolated to estimate cell number per 1 mg tissue. This allowed an estimated comparative standardization of concentration units between in vivo (per mg tissue) and in vitro (per cell number) investigations.

Immunohistochemistry
Immunostaining of formalin-fixed 5-μm paraffin sections was performed with a mouse monoclonal antibody to human OPG (clone 98A1071, Imgenex; 1:1000) and the DAKO EnVision Plus HRP system (DakoCytomation). The primary antibody and horseradish peroxide–labeled polymer were used per kit instructions. Preliminary assessment of OPG staining in sections after microwave/citrate or tris-EDTA buffer antigen epitope retrieval demonstrated no significant difference in immunodetection compared with sections in which epitope retrieval was not used (see the online-only Data Supplement).

Western Blot Analysis
Aortic biopsies were ground under liquid nitrogen, and proteins were extracted and quantified as previously described. Tissue proteins (40 μg) were separated by use of SDS gel electrophoresis (12% SDS; Gradipore), and Western analyses were carried out using mouse monoclonal antibody to human OPG (Imgenex) at 1.5 μg/mL and enhanced chemiluminescence (ECL Advance, Amersham Biosciences). Bands corresponding to OPG (55 kDa) were identified on a ChemiDoc imaging system (Bio-Rad Laboratories) with Quantity-One 1-D Analysis Software (Bio-Rad Laboratories). Quantification of OPG was performed by comparison of band densities with a standard curve of known quantities of rhOPG (ImmunoKontact). Aortic biopsies were ground under liquid nitrogen, and proteins were extracted and quantified as previously described. Tissue proteins (40 μg) were separated by use of SDS gel electrophoresis (12% SDS; Gradipore), and Western analyses were carried out using mouse monoclonal antibody to human OPG (Imgenex) at 1.5 μg/mL and enhanced chemiluminescence (ECL Advance, Amersham Biosciences). Bands corresponding to OPG (55 kDa) were identified on a ChemiDoc imaging system (Bio-Rad Laboratories) with Quantity-One 1-D Analysis Software (Bio-Rad Laboratories). Quantification of OPG was performed by comparison of band densities with a standard curve of known quantities of rhOPG (ImmunoKontact).

Cell Culture
Human VSMCs were obtained from abdominal aorta of healthy individuals without any evidence of atherosclerosis (men; age, 39 and 48 years), or AAA biopsies were isolated by combined collage-nase and elastase digestion and maintained in DMEM containing 10% FCS at 37°C, 5% CO2. Macrophages were obtained from AAA biopsies by allowing enzymically digested slurry to stand for 1 hour before the supernatant was collected, and mononuclear cells were separated by Ficoll-Hyphaque (Amershams) centrifugation at 500g for 20 minutes. Gradient centrifugation was also used to obtain peripheral blood mononuclear cells from human blood samples. Macrophages were isolated after they adhered to the culture dish after 1-hour incubation at 37°C; nonadherent cells were discarded. Cells were verified through fluorescence-activated cell scanning (FACS) analysis with the macrophage-specific marker CD-71 (clone Ber-T9, DakoCytomation). Isolated tissue macrophages and the human monocytic cell line THP-1 were maintained in RPMI 1640 (RJH) containing 10% FCS. Activation of THP-1 cells was induced with 10 μg/mL lipopolysaccharide (S typhimurium, Sigma).

Intracellular OPG detection
Isolated AAA VSMCs and macrophages were fixed in 1.5% formalin at room temperature for 10 minutes and pelleted. The cells were permeabilized with vigorous vortexing in ice-cold methanol (~500 μL/106 cells) and incubated at 4°C for 10 minutes. After being washed in PBS containing 1% BSA, the cells were incubated at room temperature with either a mouse monoclonal antibody to human OPG (clone 98A1071, Imgenex) or IgG negative control (X0931, DakoCytomation) for 30 minutes, washed, and then incubated another 30 minutes with a secondary FITC-labeled anti-mouse IgG (F2272, Sigma) before FACS analysis.

VSMC Proliferation
Subconfluent VSMCs from healthy human abdominal aorta were growth arrested for 24 hours in serum-free DMEM before incubation in experimental medium containing FCS (0%, 1%, 10%, or 20%) in the presence or absence of rhOPG (ImmunoKontact; 0 to 20 ng per 1×105 cells per 1 mL per 24 hours). Cultures were pulsed with [3H]-thymidine (1.25 μCi/mL) for 4 hours before termination of the experimental period, and incorporated radioactivity in cell lysates was assessed with a Packard Top Counter microplate scintillation counter (Packard).

VSMC Apoptosis
Subconfluent VSMCs from healthy human abdominal aorta were growth arrested for 24 hours in serum-free DMEM before incubation in experimental medium containing 10% FCS and rhOPG (0 to 20 ng per 1×105 cells per 1 mL per 24 hours). Cells incubated in 10% FCS and in the presence of tumor necrosis factor-α/interferon-γ (400 U/mL each) served as positive apoptosis control. Apoptosis was assessed at 0, 4, 8, and 24 hours with FACS analysis of annexin V/propidium labeling. Apoptosis was assessed in separate rhOPG-treated cells after 24 hours by DNA affinity-mediated nucleosome capture ELISA (Oncogene Research Products).

IL-6 Production and Gelatinate Activity
Activated and nonactivated THP-1 cells were incubated with rhOPG (0 to 20 ng per 1×105 cells per 1 mL per 24 hours). The culture medium was collected after 24 hours and centrifuged (200g), and the supernatant, together with supernatant collected from the above VSMC cultures, either was assayed for IL-6 with the DuoSet ELISA system (R&D Systems) or was examined for gelatinate (MMP-2 and MMP-9) activity with zymography. See the Data Supplement for IL-6 results.

Gelatin Zymography
Proteins in culture supernatant samples were separated at 4°C on a 10% acrylamide-SDS gel impregnated with 1 mg/mL gelatin; the gel was washed in 2.5% (vol/vol) Triton X-100 and then incubated overnight at 37°C in 50 mmol/L Tris (pH 8) containing 5 mmol/L CaCl2. Bands were visualized in a 10% ethanol/10% acetic acid solution after staining with 0.125% Coomassie blue, with enzyme activity semiquantified with densitometric analysis using the ChemiDoc imaging system (Bio-Rad Laboratories) and QuantityOne 1-D Analysis Software (Bio-Rad Laboratories). Results are expressed as mean and standard error of relative density units (RDU).

Irbesartan Treatment
Full-thickness AAA explants (n=6) were maintained in DMEM+10% FCS and incubated at 37°C, 5% CO2, in the presence or absence of the Ang II blocker irbesartan for 6 days. Biopsies from
patients receiving Ang II blockers or ACE inhibitors were excluded. An irbesartan concentration of 1 mg/mL was based on safe serum levels measured in patients receiving this medication.20 Culture supernatant was collected and refreshed at 48-hour intervals. Secretion of OPG by explants was determined by assaying the conditioned medium with an OPG DuoSet ELISA system (R&D Systems). Tissue viability was assessed with histology, and assaying for tissue ATP before and after culture was done with a bioluminescent ATP assay kit (Sigma).

Data Analysis

The association between serum OPG concentration and aneurysm growth rate was investigated with Spearman’s correlation coefficient. Multiple regression analysis was performed with aneurysm growth rate as the dependent variable and patient age, diabetic status, smoking history, initial aortic diameter, serum cholesterol, OPG, HDL, LDL, and C-reactive protein as the independent variables. The concentration of OPG in 40 aortic biopsies is expressed as mean±SEM and compared between AAA and AOD, postmortem abdominal aorta (PAA), or TA with the Mann-Whitney U test. VSMC and THP-1 function in 3 repeated experiments is expressed as mean±SEM and compared statistically with the Kruskal-Wallis test. When appropriate, Bonferroni’s adjustment was applied to post hoc Mann-Whitney multiple comparisons to prevent type I error inflation. Significance was assumed at a value of \( P<0.05 \).

Results

Serum OPG Correlates With AAA Growth Rate

Serum OPG correlated weakly with aneurysm growth rate (\( P=0.04; r=0.20 \); Figure 1). In 34 patients with rapidly expanding aneurysms (>5 mm over a 36-month period), mean serum OPG at the start of surveillance was 1.1±0.1 compared with 0.9±0.03 ng/mL in 112 patients with slow-growing aneurysms (\( P=0.04 \), unpaired \( t \) test). Serum OPG remained a predictor of aneurysm expansion on multiple regression analysis (\( P=0.02 \); coefficient, 1.33; SE, 0.51) in a model consisting of patient age, diabetic status, smoking history, initial aortic diameter, serum cholesterol, HDL, LDL, and C-reactive protein.

OPG Is Upregulated in AAA Tissue

Immunostaining for OPG in healthy aorta was minimal (Figure 2A) in contrast to marked staining within the media of AAA biopsies (Figure 2B). There was no significant difference in OPG concentration measured in control thoracic and abdominal aortic biopsies (\( P=0.57 \)). OPG levels were higher in AOD tissue by Western analysis compared with age-matched TA or PAA tissue, measuring 0.5±0.1 compared with 0.1±0.06 and 0.2±0.1 ng/mg tissue, respectively (\( n=5; P=0.02 \), \( P=0.07 \); Figure 2C and 2D). The concentration of OPG in age-matched AAA biopsies was significantly greater: 3-fold higher than in AOD (1.4±0.1 versus 0.5±0.1 ng/mg tissue; \( n=10; P=0.002 \)) and 8- to 12-fold higher levels than in PAA and TA tissue, respectively (\( n=10 \) [PAA, \( n=5 \);
The level of OPG present in AAA biopsies correlated approximately to $311 \pm 0.02 \text{ pg}/10^5 \text{ cells}$ when standardized against cell number per $1 \text{ mg}$ tissue.

**OPG Secretion From AAA-Derived VSMCs and Macrophages**

FACS analysis detected intracellular OPG in both macrophages and VSMCs derived from human AAA (Figure 3A and 3B). VSMCs isolated from AAA tissue secreted significantly higher levels of OPG in vitro than VSMCs from healthy abdominal aorta, measuring $267 \pm 20$ and $17 \pm 5 \text{ pg}$ per $1 \times 10^5 \text{ cells}$ per $1 \text{ mL}$ per 24 hours, respectively ($n=3; P=0.001$). Macrophages isolated from AAA tissue secreted levels of OPG similar to those observed from LPS-activated THP-1 cells ($107 \pm 10$ and $98 \pm 6 \text{ pg}$ per $1 \times 10^5 \text{ cells}$ per $1 \text{ mL}$ per 24 hours, respectively) and significantly higher levels compared with peripheral blood monocyte controls ($107 \pm 10$ versus $0.43 \pm 0.02 \text{ pg}$ per $1 \times 10^5 \text{ cells}$ per $1 \text{ mL}$ per 24 hours; $n=3; P<0.001$).

**rhOPG Inhibits Proliferation and Induces Apoptosis in VSMCs**

Human aneurysm VSMCs exhibited a marked inability to carry out DNA synthesis ($^3\text{H}$-thymidine incorporation) compared with VSMCs from healthy aorta ($225 \pm 40$ versus $11300 \pm 937 \text{ cpm}; n=3; P<0.001$). Increasing concentrations of rhOPG induced a dose-dependent reduction in DNA synthesis and cell proliferation ($n=3; P<0.01$; Figure 4A). A dose of only $1 \text{ pg}$ rhOPG per $1 \times 10^5 \text{ cells}$ per $1 \text{ mL}$ per 24 hours halved the rate of $^3\text{H}$-thymidine incorporation by these normal VSMCs. A higher dose of rhOPG ($20 \text{ pg}$ per $1 \times 10^5 \text{ cells}$ per $1 \text{ mL}$ per 24 hours) induced an 8-fold reduction in healthy VSMC proliferation. Annexin V/propidium iodide FACS analysis demonstrated dose-dependent induction of...
blocker irbesartan (1 mg/mL) induced a time-dependent decrease in OPG secretion from the tissue \( (P<0.001; \text{Figure } 6) \), with production of the cytokine reduced to half by day 4 of culture. Viability of treated \( (n=6) \) or untreated \( (n=6) \) specimens was assessed at day 6 by comparison of tissue ATP with that in specimens frozen before culture \( (n=6) \). No significant difference between samples was detected (before culture, 0.47±0.03 mg ATP/mg tissue; untreated, 0.54±0.03 mg ATP/mg tissue; treated, 0.44±0.03 mg ATP/mg tissue; \( P>0.5 \)).

**Discussion**

The findings of our study support an association between OPG and aneurysm development in the human abdominal aorta. We observed a correlation between serum level of OPG in patients with AAA and aneurysm growth rate. Aneurysms 30 to 50 mm in diameter were followed up. Sixty percent of aortic aneurysms measured 30 to 40 mm, resulting in an observed average growth rate\(^{16} \) slower than previously reported by other investigators.\(^1 \) Despite a cohort of 146 patients, the observed range of OPG serum concentrations was relatively small, measuring 0.1 to 3.6 ng/mL. The correlation coefficient associating serum OPG with aneurysm growth rate was weak at 0.2. Allowing for other known determinants of AAA expansion on multiple regression analysis, a significant association between serum OPG and aneurysm expansion was identified. Interestingly, serum OPG has been identified as a risk factor for the progression of atherosclerosis and onset of cardiovascular disease.\(^7 \) However, analysis of a larger cohort is required to confirm the association of OPG with AAA progression.

More convincing data of OPG upregulation in human AAA were demonstrated by assessing tissue levels of the protein. We investigated OPG expression in biopsies of 4 types of human aortic tissue: AAA, AOD, nondiseased PAA, and nondiseased TA. The highest levels of OPG were found in human aortic tissue: AAA, up to 12-fold higher than levels observed in control tissue (PAA/TA) and 3-fold higher than seen in AOD samples.

Immunodetection of OPG in AAA biopsies localized the protein primarily within the fragmented arterial media. This region comprises a reduced VSMC population, together with a dense infiltration of inflammatory cells, including mono-
cyte/macrophages. We observed sustained OPG secretion from AAA biopsies in explant culture and demonstrated that both AAA-derived VSMCs and macrophages contribute to OPG production. VSMCs isolated from AAA biopsies secreted 16-fold-higher levels of OPG than VSMCs isolated from healthy abdominal aorta. Macrophages isolated from AAA tissue secreted significantly greater levels of OPG in vitro compared with human peripheral blood monocytes, mirroring quantities produced by LPS-activated THP-1 cells. The level of OPG secreted by AAA-derived macrophages per $1 \times 10^5$ cells/mL was less in direct comparison with aneurysm VSMCs. However, the difference in cell number between these 2 populations at late-stage aneurysm should be taken into account. With a decreased VSMC density typically observed within biopsies taken at surgery, it is probable that most of OPG present at this stage is inflammatory cell derived.

OPG is known to be upregulated by proinflammatory cytokines such as tumor necrosis factor-$\alpha$. It is possible that the observed high concentration of this protein simply represents an expected result of the inflammatory process central to atherosclerosis. As such, we assessed the functional consequence of elevated concentrations of OPG on the 2 main cell types important in AAA progression, VSMCs and macrophages. OPG concentrations used in previous in vitro studies examining the biological effect of the protein range between 1 and 5000 ng/mL. We estimated the concentration of OPG to which cells within the human aortic aneurysm are exposed to determine a suitable concentration range of rhOPG for use in culture. This was achieved by quantifying the level of OPG protein in AAA tissue and levels produced in vitro by cells isolated from AAA. The level of OPG quantified from these sources ranged from 0.11 to 0.31 ng per $1 \times 10^5$ cells per 1 mL over 24 hours. From this, we exposed healthy human VSMCs and monocytes to an in vitro concentration range of 0 to 20 ng rhOPG per $1 \times 10^5$ cells per 1 mL. The high end of the range was chosen by taking into account the probable accumulation of OPG within the aneurysmal aortic wall associated with de novo synthesis by VSMCs and inflammatory cells over years of aneurysm development.

Incubation of healthy human abdominal aortic VSMCs with increasing concentrations of rhOPG over 24 hours induced a sensitive dose-dependent reduction in DNA synthesis. The inhibition of VSMC proliferation by rhOPG coincided with an additional effect of the cytokine in suppressing VSMC production of IL-6 (see the Data Supplement), an effect attenuated in the presence of receptor activator of nuclear factor-$\kappa$B ligand (RANKL), a physiologic ligand for OPG. The ability of OPG to induce MMP-9 expression in VSMCs and IL-6 release and MMP-2/9 activity in monocytes suggests a proactive role of this cytokine in AAA expansion.

The expression of OPG in VSMCs in vitro has been shown to be upregulated by Ang II, which is involved in the development and progression of arterial disease as a mediator of inflammation, is instrumental in the pathogenesis of AAA. We investigated the effect of Ang II blockade on OPG secretion by AIA explants in culture and observed that biopsies incubated with the Ang II receptor blocker irbesartan displayed a time-dependent reduction in OPG secretion, averaging 1.3-fold over 48 hours. Unlike AOD, the medical management of aortic aneurysm is in its infancy; no pharmacological treatment has been shown to limit aneurysm growth. These findings support a role for Ang II in the induction of OPG synthesis within the aneurysm wall and
highlight the potential of Ang II blockade in slowing aneurysm expansion through targeting of OPG production.

In summary, the present study demonstrates, for the first time, upregulation of OPG in human AAA. We show in vitro that rhOPG stimulates MMP-9 activity while limiting proliferation and survival of VSMCs and promotes IL-6 secretion and gelatinase activity in monocytes, demonstrating a feasible active role for OPG in the progression of AAA. Moreover, our findings indicate Ang II blockade as a potential therapeutic avenue by which to target arterial expression of OPG and limit aneurysm expansion. However, despite these collective results, the exact involvement of OPG in the pathogenesis of human AAA cannot yet be defined. The issue of whether the observed high concentration of OPG in AAA biopsies is a causative factor in aneurysm development or simply a consequence of the disease process requires further investigation. Studies using an animal model to assess the effects of OPG on both aortic aneurysm formation and development in vivo are currently underway.

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References

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