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 nondiseased 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/10^5 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/10^5 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

Abdominal aortic aneurysm (AAA) is an abnormal focal dilation of the aortic wall that affects 5% to 10% of men >60 years of age.1 The aortic weakening process is believed to be a result of atherosclerosis; however, unlike occlusive arterial disease (AOD), the incidence of AAA is increasing.2 Degradation of the extracellular matrix, depletion of medial vascular smooth muscle cells (VSMCs), and high concentration of matrix-degrading metalloproteinases (MMPs) associated with accumulation of monocyte/macrophages within the adventitia and media of the vessel wall characterize AAA.3

Osteoprotegerin (OPG), a member of the tumor necrosis factor receptor family, was initially identified as a key regulator in bone turnover.4 Recent evidence suggests the involvement of OPG in vascular disease. OPG has been identified in atherosclerotic human vessels,5 and serum levels of OPG have been positively correlated with the onset and severity of atherosclerotic artery disease.6,7 Angiotensin II (Ang II) is considered a causal element in the development of atherosclerosis,8 and it is now clear that Ang II also contributes significantly to the pathology of AAA.9,10 Arterial VSMCs express OPG,11 and it has been shown in vitro that such expression is upregulated by Ang II.12

In view of the association of OPG with atherosclerosis and the potential of this cytokine to influence cellular mechanisms underlying aneurysm expansion,13,14 we were prompted to investigate the expression and functional importance of OPG in human AAA. Specifically, we compared OPG concentration in biopsies of aneurysmal, atherosclerotic, and normal human aorta; assessed the relationship between serum concentration of OPG and AAA growth; and investigated the action of recombinant human (rh)OPG on human aortic VSMCs and monocytic THP-1 cells in vitro. Finally, we studied the effects of Ang II blockade in reducing observed OPG secretion from human aneurysm biopsies.
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 aneurysm 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.

Serum 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%.

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). Results are expressed as mean and standard error of relative density units (RDU).

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 collagenase 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-Hypaque (Amersham) 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 (RJR) 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 rigorous 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 (ImmuKontact; 0 to 20 ng per 1×10⁶ cells per 1 mL per 24 hours). Cultures were pulsed with H-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 Count 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 1×10⁶ 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 Gelatinase Activity
Activated and nonactivated THP-1 cells were incubated with rhOPG (0 to 20 ng per 1×10⁶ 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 gelatinase (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).

Irsbesartan 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. 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 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 $1.1\pm0.1$ compared with $0.9\pm0.03$ ng/mL in 112 patients with slower-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 $0.5\pm0.1$ compared with $0.1\pm0.06$ and $0.2\pm0.1$ 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 ($1.4\pm0.1$ versus $0.5\pm0.1$ 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, $n=5$]; $n=10$ [TA, $n=5$]; $n=10$ [PAA, $n=5$]).

![Figure 1. Weak, positive correlation between serum concentration of OPG and aneurysm growth rate. $n=146; P=0.04; r=0.2$.](http://circ.ahajournals.org/)

![Figure 2. Immunodetection of OPG (brown stain) in healthy human aorta (A) and human aneurysmal aorta (B). Upregulation of OPG in AAA vs AOD and control tissue confirmed with Western analysis (C, D). Data are expressed as mean±SEM; $n=10$ (PAA, $n=5$); $*P=0.002$, $**P<0.001$, $***P<0.05$ (Mann-Whitney U test).](http://circ.ahajournals.org/)
The level of OPG present in AAA biopsies correlated approximately to 311 ± 0.02 pg/10^5 cells when standardized against cell number per 1 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 ± 20 and 17 ± 5 pg per 1 × 10^5 cells per 1 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 ± 10 and 98 ± 6 pg per 1 × 10^5 cells per 1 mL per 24 hours, respectively) and significantly higher levels compared with peripheral blood monocyte controls (107 ± 10 versus 0.43 ± 0.02 pg per 1 × 10^5 cells per 1 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 (³H-thymidine incorporation) compared with VSMCs from healthy aorta (225 ± 40 versus 11 300 ± 937 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 pg rhOPG per 1 × 10^5 cells per 1 mL per 24 hours halved the rate of ³H-thymidine incorporation by these normal VSMCs. A higher dose of rhOPG (20 pg per 1 × 10^5 cells per 1 mL per 24 hours) induced an 8-fold reduction in healthy VSMC proliferation. Annexin V/propidium iodide FACS analysis demonstrated dose-dependent induction of
apoptosis in normal VSMCs after 4 hours of incubation with rhOPG (0 to 10 pg per 1×10⁵ cells per 1 mL per 24 hours). Annexin V staining in these cells was significant after 8 hours of incubation and maximal with 10 pg rhOPG per 1×10⁵ cells per 1 mL per 24 hours, with the percentage of apoptotic cells >2-fold higher than untreated cells (n=3; P=0.02; Figure 4B and 4C). No significant difference in annexin V staining was observed between the control and treatment groups at termination of the 24-hour experimental period. Analysis of VSMC lysate for internucleosomal fragmentation by DNA affinity-mediated capture ELISA at 24 hours confirmed a significantly higher apoptotic index in cells treated with rhOPG (0.9±0.05, 1.4±0.01, 2.7±0.09, 2.3±0.07, and 1.8±0.07; 0 to 10 ng per 1×10⁵ cells per 1 mL, respectively; P=0.01).

rhOPG Induces Gelatinase Activity in VSMCs and THP-1 Cells
Gelatinase activity in culture supernatant from healthy human abdominal aortic VSMCs showed dose-dependent upregulation of MMP-9 proform (92 kDa) expression in these cells when incubated with 0 to 20 pg rhOPG per 1×10⁵ cells per 1 mL per 24 hours (0.9±0.08, 1.2±0.1, 2.4±0.1, 4.0±0.07, and 5.2±0.04 RDU, respectively; P=0.01; Figure 5A). OPG had no significant influence on MMP-2 expression in VSMCs. Incubation of resting THP-1 cells with rhOPG also induced gelatinase activity in these cells. Unlike in VSMCs, this effect was not dose dependent. Stimulation of activity in treated cells was significant only in the presence of 1 pg rhOPG per 1×10⁵ cells per 1 mL per 24 hours compared with untreated controls (Figure 5B). MMP-2 activity induced in THP-1 cells by rhOPG was increased by >30% compared with control cells (1.1±0.02 versus 0.7±0.01 RDU; n=3; P=0.01). Expression of the 92-kDa proform and 83-kDa active form of MMP-9 was concurrently upregulated (0.4±0.05 versus 0.2±0.01 RDU, P=0.03; and 0.9±0.03 versus 0.7±0.03 RDU, P=0.01, respectively).

Irbesartan Attenuates OPG Secretion From AAA Explants
Treatment of human AAA explants with the Ang II receptor blocker irbesartan (1 mg/mL) induced a time-dependent decrease in OPG secretion from the tissue (P<0.001; 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 rate¹⁶ 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.² 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 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-\(\alpha\).\(^{12}\) 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.\(^{21,22}\) 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 abdominal aortic 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), a known mitogen for these cells.\(^{23}\) A pathophysiological feature of AAA is extensive apoptotic loss of medial VSMCs.\(^{3}\) Given the sensitive influence of rhOPG on VSMC proliferation, we hypothesized that OPG may also induce apoptosis in these cells.

Earlier studies have shown that OPG influences cell survival in a variable way, depending on cell type and experimental conditions. For example, OPG promotes osteoclast apoptosis\(^{24}\) but protects other cell lines from induced apoptosis.\(^{21,22}\) The present study demonstrates that rhOPG induces dose-dependent apoptosis in healthy aortic VSMCs. We used 2 methods to assess the proapoptotic effect of rhOPG on these cells: FACS analysis with annexin V/pro-
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 MTP-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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