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Crystallizing nanoparticles derived from vascular smooth muscle cells contain the calcification inhibitor osteoprotegerin

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ABSTRACT

Osteoprotegerin (OPG), a member of the TNF receptor superfamily, was initially found to modulate bone mass by blocking osteoclast maturation and function. Rodent models have also revealed a role for OPG as an inhibitor of vascular calcification. However, the precise mode of how OPG blocks mineralization is unclear. In this study, OPG was found in an *in vitro* assay to significantly inhibit calcification of vascular smooth muscle cells (VSMC) induced by high calcium/phosphate (Ca/P) treatment ($p = 0.0063$), although this effect was blunted at high OPG concentrations. By confocal microscopy, OPG was detected in VSMC in the Golgi, the same localization seen in osteoblasts, which express OPG in bone. Treatment of VSMC by minerals (Ca, P, or both) induced OPG mRNA expression as assessed by real-time quantitative PCR, and VSMC derived from atherosclerotic plaque material also exhibited higher OPG expression as compared to control cells ($p < 0.05$). Furthermore, OPG was detected by Western blotting in matrix vesicles (MV), nanoparticles that are released by VSMC with the capacity to nucleate mineral. In atherosclerotic arteries, OPG colocalized immunohistochemically with annexin VI, a calcium-dependent membrane and phospholipid binding protein found in MV. Thus, the calcification inhibitor OPG is contained in crystallizing MV and has a biphasic effect on VSMC: physiologic concentrations inhibit calcification, whereas high concentrations commonly seen in patients with vascular disease have no effect. Like other calcification inhibitors, OPG may be specifically loaded into these nanoparticles to be deposited at remote sites, where it acts to inhibit calcification.

1. Introduction

Osteoprotegerin (OPG) is a soluble decoy receptor for receptor activator of NF-κB ligand (RANKL), the principal regulator of osteoclast biology [1]. A number of studies have shown that the RANKL-OPG axis also has a role in the cardiovascular system. Deficiency of OPG in mice resulted in vascular calcification of the aorta and renal arteries [2], whereas RANKL was recently demonstrated to increase vascular smooth muscle cell (VSMC) calcification *in vitro* [3]. Another study using both *in vitro* and *in vivo* approaches mimicking postmenopausal estrogen deficiency demonstrated that RANKL induced osteogenic bone morphogenetic protein (BMP)-2 expression in human endothelial cells and decreased the calcification inhibitor matrix Gla protein (MGP) in human aortic VSMC [4]. By contrast, vascular calcification induced *in vivo* either by (i) challenge of rats with the vitamin K antagonist warfarin or toxic doses of vitamin D, (ii) by feeding ldlr$^{(-/-)}$ mice with an atherogenic diet, or (iii) in apoE$^{(-/-)}$ mice, could be antagonized by treatment with recombinant OPG or by restoring OPG expression [5–7]. The protective role of OPG against vascular calcification observed in rodent models has not been replicated in human trials. Indeed, circulating OPG has been shown to be consistently elevated in patients with coronary artery disease (CAD) [8–10] or chronic kidney disease (CKD) [11], a condition known to be associated with high cardiovascular morbidity and mortality. Thus, the definitive role of OPG in the vascular system is elusive. OPG may act by inhibiting RANKL effects on vascular cells and/or RANKL effects on bone resorption and mineral release. However, direct effects of OPG on VSMC as a survival or proliferation factor and on osteogenic cell protein expression have been described and may also contribute to OPG's role as a calcification inhibitor [7,12,13]. Here, we studied *in vitro* effects of OPG on calcification of VSMC and the subcellular localization of OPG in these cells.

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2. Material and methods

2.1. Cell culture

Explants of human nonatherosclerotic aortic tissue from male and female organ donors of various age were established after informed consent as described previously [14]. VSMC were cultured in M199 medium (Sigma–Aldrich, St. Louis, MO) supplemented with 20% fetal calf serum (FCS) and antibiotics, and used between passages 3 and 12.

2.2. Proteins

Recombinant human full-length OPG was obtained by R&D Systems (Minneapolis, MN), bovine serum albumin (BSA) by Sigma–Aldrich.

2.3. Calcification assay

VSMC were grown in M199 medium supplemented with 20% FCS to subconfluence and then transferred to 24-well plates at 1.8×10^4 cells per well. Subsequently, cells were serum-starved in M199 without FCS containing 0.5% BSA for 24 h. After serum starvation, cells were treated with OPG at different concentrations and/or calcium/phosphate (Ca/P) for another 24 h in M199 plus 0.5% BSA. Calcification was quantified by including ^{45}Ca (50,000 cpm/ml) in test and control media. Afterwards, VSMC were decalcified in 0.1 M HCl, neutralized with 0.1 M NaOH/0.1% SDS, scraped, and ^{45}Ca incorporation was measured by liquid scintillation counting.

2.4. Confocal microscopy

VSMC were plated on coverslips and allowed to adhere in M199 plus 20% FCS for 24 h. Cells were subsequently fixed in 3% formaldehyde for 10 min at room temperature and then permeabilized by adding 0.1% Nonidet P-40 before exposing to blocking solution (3% BSA at room temperature for 2 h). Specimens were exposed to a monoclonal antibody directed against OPG (R&D Systems), detection via donkey anti-mouse Alexa488 (Molecular Probes, Eugene, OR). Treatment of VSMC with brefeldin A (Sigma–Aldrich) at 6 μg/ml was performed for 1 h, which inhibits transport of proteins from the endoplasmatic reticulum (ER) to Golgi areas and induces retrograde protein transport from the Golgi apparatus to the ER. Golgi epitopes were stained with a monoclonal antibody (anti-golgin 97; Molecular Probes), and for costaining with OPG, the antibody was used with a polyclonal anti-OPG antibody (R&D Systems). Bound primary antibodies were detected via donkey anti-mouse Alexa488 and donkey anti-goat Alexa568 antibodies (both Molecular Probes). Nuclei were stained with DAPI. Controls were performed by substituting PBS for the primary antibody.

2.5. Isolation of atherosclerotic plaque derived VSMC

Primary normal and plaque human VSMC were isolated from aortas of cardiac transplant patients or after carotid endarterectomy in patients with recent transient ischemic episodes. In all cases, informed consent and approval of the Local Ethics Committee was obtained.

2.6. Real-time quantitative PCR

Total RNA was isolated as described previously [15]. Expression of OPG and β-actin was analyzed using a Rotor-Gene 3000 (Corbett Research, Mortlake, Australia) and Taqman expression assay systems (Applied Biosystems, Carlsbad, CA) using absolute quantification. Values were normalized to β-actin.

2.7. Preparation of nanoparticles

Matrix vesicles (MV) and apoptotic bodies (AB) were harvested using a modified MV isolation protocol [16,17]. In brief, upon confluence, VSMC were washed three times with PBS and incubated for 24 h in serum-free M199 plus 0.5% BSA. Cells were washed thoroughly with PBS and subsequently incubated in phenol red free DMEM supplemented with 0.1% BSA for 12 to 18 h. The medium was decanted and spun at 2500 rpm in a Sorvall RF7 centrifuge to remove AB. MV were harvested from the supernatant by centrifugation at 45,000 rpm for 40 min at 4 °C in a Beckman Ultracentrifuge. The pellet was centrifuged at 14,000 rpm for 10 min at 4 °C to collect AB. Protein content of MV and AB was assessed by a BioRad protein assay (Munich, Germany).

2.8. Western blotting

Samples for protein analysis were prepared either from MV or AB. Each sample of total protein (10 μg/lane) was dissolved in sample buffer containing 100 mmol/l β-mercaptoethanol, boiled, and applied to a polyacrylamide gel (10% (wt/vol)) run under denaturing conditions in 8 M urea. Afterwards, gels were transferred to a nitrocellulose membrane (Immobilon-P; Millipore Billerica, MA) using a semi-dry electroblotting system (BioRad). Nitrocellulose membranes were blocked in 5% milk in PBS for 1 h and incubated at 4 °C overnight with the respective primary antibodies (R&D Systems). After stringent washing with PBS, incubation with the respective secondary horseradish peroxidase-conjugated antibodies (Dako, Glostrup, Denmark) and chemiluminescence (ECL; Amersham, Little Chalfont, UK) followed.

2.9. Immunohistochemistry

Aortic tissue sections were stained with a monoconal antibody against OPG (R&D Systems) and a polyclonal antibody against annexin VI (BD Bioscience, Oxford, UK). For detection, a peroxidase conjugated secondary antibody (VECTASTAIN; Vector Laboratories, Burlingame, CA) was used. Controls were performed with the primary antibody substituted for PBS.

3. Results

3.1. Inhibition of VSMC calcification by OPG

High mineral concentrations of Ca and P (2.7 mM/2.5 mM) have been demonstrated to induce calcification of VSMC. As evidenced by radioactive-labeled Ca incorporation, this treatment induced robust calcification of VSMC compared to control media containing 1.8 mM Ca/1.0 mM P (Fig. 1). OPG added at different concentrations within a range of 0.1–100 ng/ml was able to significantly reduce this calcification at low (physiologic) concentrations ($p = 0.0063$ by Student's t-test), whereas OPG at higher concentrations failed to exhibit an effect. OPG at 1 ng/ml was the most effective concentration. Thus, the calcification inhibitor OPG was able to dose-dependently block Ca/P-induced mineralization of VSMC *in vitro*.

3.2. OPG expression is induced by minerals and in atherosclerotic plaque-derived VSMC

VSMC were treated with high concentrations of Ca (5.4 mM), P (5.0 mM), or both (2.7 mM/2.5 mM) for 24 h (Fig. 2A). Ca increased

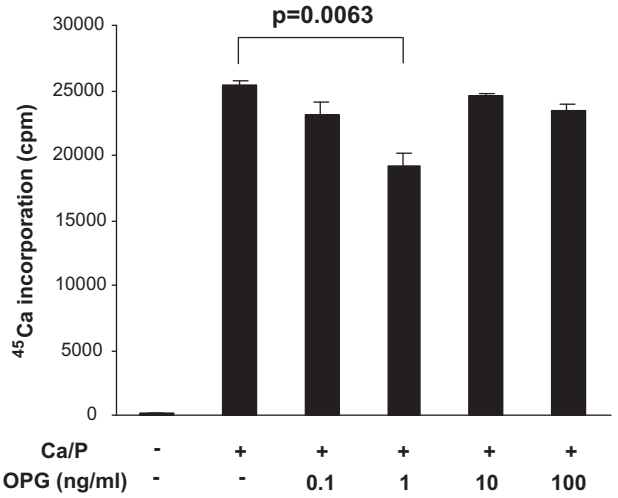

Fig. 1. OPG inhibits calcification of VSMC *in vitro*. VSMC were treated with high Ca/P concentrations (2.7 mM/2.5 mM) to induce calcification as assessed by radioactive labeled Ca. OPG was added at a range of 0.1–100 ng/ml, and OPG was able to reduce calcification with an optimum concentration of 1 ng/ml but was ineffective at concentrations above 10 ng/ml. BSA, bovine serum albumin; Ca, calcium; OPG, osteoprotegerin; P, phosphate; VSMC, vascular smooth muscle cells.

OPG mRNA expression in normal human VSMC to levels greater than the control TNF-α-stimulated cells (Fig 2A). Exposure to P and Ca/P both at low supraphysiologic concentrations also increased OPG expression, although not to the same level as Ca (Fig 2A). Interestingly, VSMC derived from atherosclerotic plaques of human carotid arteries exhibited increased OPG expression compared to VSMC from the normal aorta (Fig. 2B).

3.3. Distribution of OPG in VSMC assessed by confocal microscopy

Confocal microscopy revealed OPG to be localized to Golgi areas (Fig. 3), shown by costaining with antibodies against Golgi epitopes. Its distribution in Golgi areas was lost upon treatment with brefeldin A, an antibiotic which interferes with anterograde protein transport from the ER to the Golgi apparatus leading to accumulation of protein within the ER, confirming its Golgi localization.

3.4. Detection of proteins contained in MV and AB

In response to elevated extracellular Ca and/or P ions, VSMC shed an increased number of membrane-bound nanoparticles, either MV released from viable cells, or AB released from dying cells. Both have the capacity to nucleate mineral, and their accumulation in the VSMC extracellular matrix results in widespread calcification. Proteomic studies (data not shown) have identified a number of proteins in these MV. Nanoparticles were isolated by ultracentrifugation from VSMC and further analyzed by Western blotting (Fig. 4A). OPG is a protein of 55–60 kDa in its monomeric form [18], and AB show bands at the size of approximately 60 and 50 kDa, i.e. monomeric OPG and a fragment thereof. MV predominantly contained a band of approximately 180 kDa, indicating that these nanoparticles contain multimers (trimers) of OPG, which have been reported for other proteins in shed microparticles [19]. Staining of atherosclerotic tissue specimens by immunohistochemistry revealed the presence of OPG in areas of calcification, and OPG colocalized with annexin VI, a component of MV, suggesting that OPG may be specifically loaded into these particles to be deposited at remote sites, where it acts to inhibit calcification.

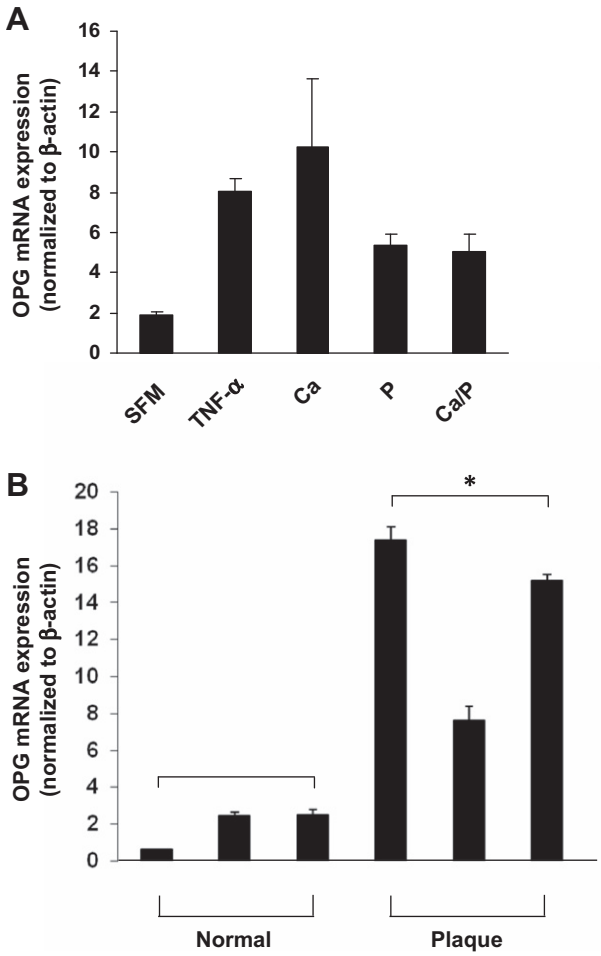

Fig. 2. (A) VSMC were exposed to Ca, P, or both, followed by assessment of OPG mRNA expression (real-time quantitative PCR). TNF-α was used as positive control. (B) OPG expression is higher in plaque-derived VSMC from human carotids compared to VSMC derived from the normal aorta. OPG mRNA expression was assayed using Taqman probes and the Corbett real-time PCR using absolute quantification. Error bars represent SEM; $n = 3$, $p < 0.05$ compared to normal samples. Ca, calcium; OPG, osteoprotegerin; P, phosphate; SFM, serum-free medium; VSMC, vascular smooth muscle cells.

4. Discussion

Although rodent models of vascular calcification attribute OPG a function as an inhibitor of vascular calcification, the exact role of OPG in this pathogenic process is elusive. Three different modes of action are conceivable: (i) OPG blocks bone resorption by inhibiting RANKL, (ii) OPG modulates vascular calcification by inhibiting the calcification inducing effect of RANKL on VSMC, or (iii) OPG has direct effects on cells of the vascular wall distinct from its role as a decoy receptor for RANKL.

An important observation in this study was that, when OPG was added to VSMC at a concentration of 0.1–1 ng/ml, calcification of VSMC induced by high concentrations of Ca/P was significantly reduced. In contrast, higher OPG concentrations (equivalent to serum levels in CAD or CKD patients) had no inhibitory effect on calcification. On the other hand, OPG was upregulated in primary VSMC by calcific stimuli such as Ca or P, and high OPG expression was detected in VSMC derived from atherosclerotic plaques of human carotids compared to VSMC from the normal aorta, a finding which is also seen in specimens of vessels with calcification either of the intimal or the medial layer [20].

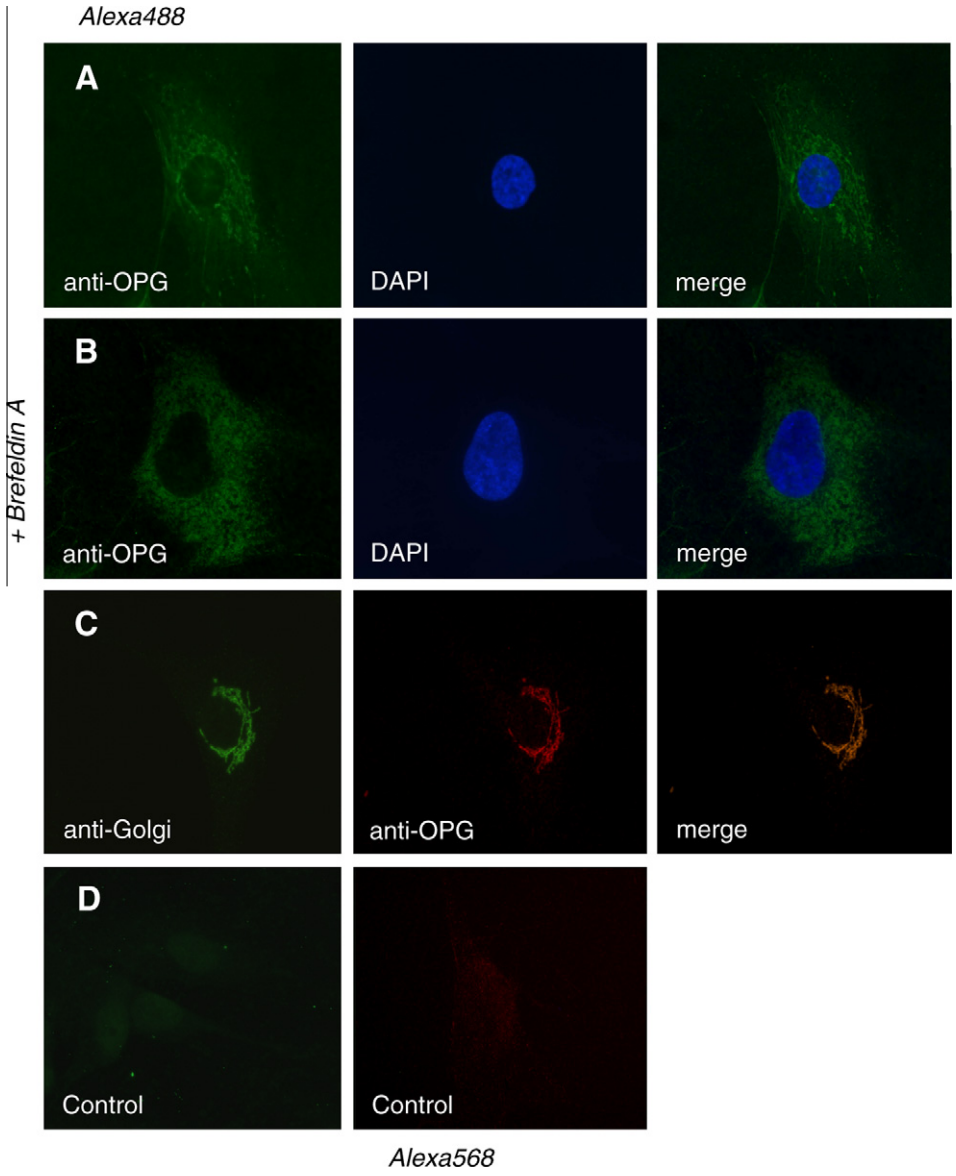

Fig. 3. OPG localizes to the Golgi apparatus in VSMC. By confocal microscopy, OPG is localized in Golgi structures of VSMC, as evidenced by costaining with a monoclonal antibody against Golgi epitopes (anti-golgin 97). This colocalization is lost upon treatment with brefeldin A (B). (A and B) Monoclonal anti-OPG antibody; (C) monoclonal anti-Golgi antibody and polyclonal anti-OPG antibody, detection via donkey anti-mouse Alexa488 (*green fluorescence*) and donkey anti-goat Alexa568 (*red fluorescence*). (A and B) Nuclei stained with DAPI. (D) Control with the primary antibody substituted for PBS. OPG, osteoprotegerin; VSMC, vascular smooth muscle cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

This may indicate a feed-back mechanism such that expression of the calcification inhibitor OPG is induced by calcification inducing agents. However, in the presence of constant calcification stimuli, as seen in CKD, this feedback is overwhelmed and leads to increased serum concentrations of OPG. Importantly, our studies show that inhibition of calcification by OPG is biphasic with inhibition of vascular calcification at physiologic concentrations but loss of this inhibitory capacity and a resistance effect in diseased arteries at the high circulating levels that are commonly seen in patients with vascular diseases [8–11].

VSMC are one of the main sources for OPG in the vascular wall [21]. In these cells, OPG was found to localize to Golgi areas, a distribution that is reminiscent of that in osteoblasts [22]. Like calcifying osteoblasts, VSMC shed an increased number of membrane-bound nanoparticles in response to elevated extracellular Ca and/or P ions. These nanoparticles consist of MV released from viable cells and AB released from dying cells. Both have the capacity to nucleate mineral, and their accumulation in the VSMC extracellular matrix results in widespread calcification. Thus, these nanoparticles seem to have similar functions in the vasculature as in bone, where matrix vesicles derived from osteoblasts or chondrocytes act as a nidus for calcification [23–25]. In VSMC, calcification inhibiting proteins such as MGP and fetuin-A have been reported to be incorporated in these matrix vesicles, limiting their ability to accumulate minerals [23]. As a potential calcification inhibiting protein, OPG was detected in MV and AB similar to MGP and fetuin-A, which may indicate, that the calcification inhibiting role of OPG is related to its presence in these nanoparticles. The subcellular distribution of OPG with its presence in the Golgi suggests an endosomal origin of the nanoparticles, which are loaded with OPG via trafficking from the ER, and this is consistent with intracellular loading of the vesicles with inhibitors.

Taken together, these findings support the hypothesis that OPG at physiologic concentrations directly inhibits calcification of

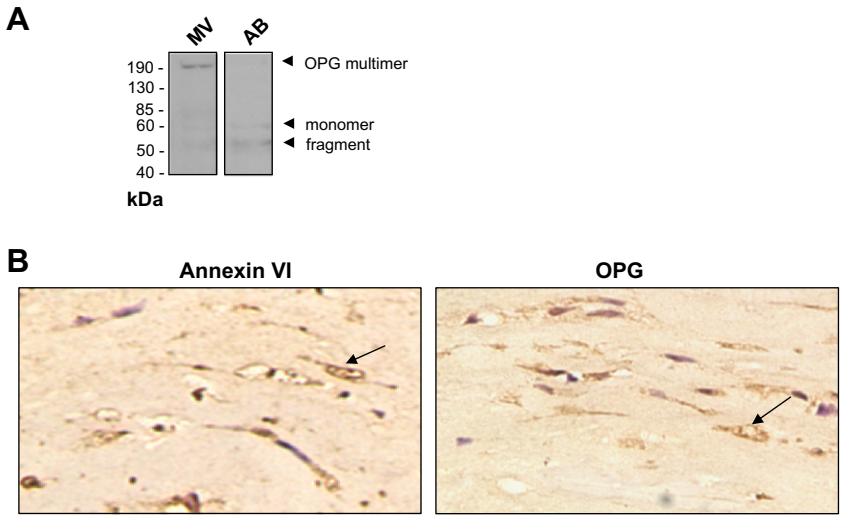

Fig. 4. OPG is contained in nanoparticles. (A) VSMC were cultured to subconfluence, serum-starved for 24 h before MV and AB were isolated by differential ultracentrifugation. Western blotting used 10 μg of nanoparticle protein. Membranes were incubated with the respective primary antibodies, visualization was via the respective secondary horseradish peroxidase-conjugated antibodies and development with ECL Western blotting detection reagents. Note the presence of high molecular aggregates of OPG in MV, whereas AB contain the monomeric form of OPG and a fragment thereof. (B) OPG colocalizes with annexin VI in areas of calcification (arrows) in atherosclerotic tissue specimens. AB, apoptotic body; MV, matrix vesicle; OPG, Osteoprotegerin; VSMC, vascular smooth muscle cells.

VSMC, potentially by a mechanism whereby OPG is secreted via vesicle release from viable or apoptotic VSMC and potentially limiting the MV-driven mineral nucleation and deposition of hydroxyapatite in the vascular wall. The resistance effect at high concentrations of OPG requires further investigation and insight into a potential cell surface OPG receptor.

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