RANKL Increases Vascular Smooth Muscle Cell Calcification Through a RANK-BMP4–Dependent Pathway

Sara Panizo, Anna Cardus, Mario Encinas, Eva Parisi, Petya Valcheva, Susana López-Ongil, Blai Coll, Elvira Fernandez,* Jose M. Valdivielso*

Abstract—Vascular calcification commonly associated with several pathologies and it has been suggested to be similar to bone mineralization. The axis RANKL-OPG (receptor activator of nuclear factor κB ligand–osteoprotegerin) finely controls bone turnover. RANKL has been suggested to increase vascular calcification, but direct evidence is missing. Thus, in the present work, we assess the effect of RANKL in vascular smooth muscle cell (VSMC) calcification. VSMCs incubated with RANKL showed a dose-dependent increase in calcification, which was abolished by coincubation with OPG. To test whether the effect was mediated by signaling to its receptor, knockdown of RANK was accomplished by short hairpin (sh)RNA. Indeed, cells lacking RANK showed no increases in vascular calcification when incubated with RANKL. To further elucidate the mechanism by which RANK activation increases calcification, we blocked both nuclear factor (NF)-κB activation pathways. Only IKKα inactivation inhibited calcification, pointing to an involvement of the alternative NF-κB activation pathway. Furthermore, RANKL addition increased bone morphogenetic protein (BMP)4 expression in VSMCs, and that increase disappeared in cells lacking RANK or IKKα. The increase in calcification was also blunted by Noggin, pointing to a mediation of BMP4 in the calcification induced by RANKL. Furthermore, in an in vivo model, the increase in vascular calcium content was parallel to an increase in RANKL and BMP4 expression, which was localized in calcified areas. However, blood levels of the ratio RANKL/OPG did not change. We conclude that RANKL increases vascular smooth muscle cell calcification by binding to RANK and increasing BMP4 production through activation of the alternative NF-κB pathway. (Circ Res. 2009;104:1041-1048.)

Key Words: vascular calcification n RANKL n BMP4 n NF-κB

Vascular calcification is a well recognized and common complication of a variety of pathological conditions like chronic kidney disease (CKD), diabetes mellitus, and atherosclerosis.1 There are 2 main types of vascular calcification, depending on whether the calcium deposits are located in the intima or in the medial layer.2 Intimal calcification is found in atherosclerotic plaques and is associated with a higher likelihood of adverse events such as myocardial infarction and coronary death.3 Atherosclerotic disease involves a complex interplay among several factors like inflammation, thrombosis, and lipid metabolism and different cell types such as endothelial cells, vascular smooth muscle cells (VSMCs), and macrophages. Mediational calcification is usually associated with age and CKD patients. Mediational calcification generates increased vascular stiffness and reduced vascular compliance, which are associated with increases in systolic blood pressure, pulse pressure, and pulse wave velocity. All of these complications lead to altered coronary perfusion and left ventricular hypertrophy.4 Furthermore, mediational calcification of skin arterioles causes calciphylaxis, which is associated with thrombotic cutaneous ischemia, necrotic skin ulceration, and a high mortality rate. In this case, the main cell type involved is the VSMCs.

In the past, arterial calcification was regarded as a passive process. Thus, increases in calcium and phosphate levels over its solubility threshold would induce calcium mineral deposition in soft tissues. Nevertheless, accumulating evidence suggests that arterial calcification is the result of organized and regulated processes similar to bone formation.5 Bone remodeling is a lifelong coordinated process of bone formation and resorption that renews and adapts the skeleton.6 Thus, the balance between bone resorption and formation is finely regulated, and imbalances on one side or the other can cause bone disease. The regulation of that balance is achieved by a combination of hormones and the local cytokine milieu within the bone microenvironment.7 Among the hormones that regulate bone remodeling, the discovery of the RANK-RANKL-OPG system (receptor activator of nuclear factor κB ligand–osteoprotegerin) finely controls bone turnover. RANKL has been suggested to increase vascular calcification, but direct evidence is missing. Thus, in the present work, we assess the effect of RANKL in vascular smooth muscle cell (VSMC) calcification. VSMCs incubated with RANKL showed a dose-dependent increase in calcification, which was abolished by coincubation with OPG. To test whether the effect was mediated by signaling to its receptor, knockdown of RANK was accomplished by short hairpin (sh)RNA. Indeed, cells lacking RANK showed no increases in vascular calcification when incubated with RANKL. To further elucidate the mechanism by which RANK activation increases calcification, we blocked both nuclear factor (NF)-κB activation pathways. Only IKKα inactivation inhibited calcification, pointing to an involvement of the alternative NF-κB activation pathway. Furthermore, RANKL addition increased bone morphogenetic protein (BMP)4 expression in VSMCs, and that increase disappeared in cells lacking RANK or IKKα. The increase in calcification was also blunted by Noggin, pointing to a mediation of BMP4 in the calcification induced by RANKL. Furthermore, in an in vivo model, the increase in vascular calcium content was parallel to an increase in RANKL and BMP4 expression, which was localized in calcified areas. However, blood levels of the ratio RANKL/OPG did not change. We conclude that RANKL increases vascular smooth muscle cell calcification by binding to RANK and increasing BMP4 production through activation of the alternative NF-κB pathway. (Circ Res. 2009;104:1041-1048.)

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[RANK–RANK ligand–osteoprotegerin] provided a major breakthrough on the understanding of bone remodeling mechanisms. RANKL is a member of the tumor necrosis factor superfamily and is expressed mainly by osteoblasts and its immature precursors. RANKL activates its receptor (RANK), which is expressed in osteoclasts and its precursors, promoting osteoclast formation and activation and prolonging osteoclast survival by suppressing apoptosis. The final step in RANK activation is the nuclear translocation of nuclear factor (NF-κB), which is controlled by 2 main pathways, the classic and the alternative NF-κB pathways, which are also controlled by different kinases (IKKβ and IKKα, respectively). OPG is a decoy receptor for RANKL, which directly counters all the RANKL-mediated actions. Thus, the RANKL/OPG ratio is critical to determining bone remodeling and bone mass, and imbalances in this ratio or in RANK signaling underlie the pathology of many disorders exhibiting excessive bone loss. In fact, vascular calcification is associated with osteoporotic bone loss, but the reasons for this are unclear. The discovery that mice lacking OPG had severe osteoporosis and arterial calcification provided the first clue that the OPG-RANK-RANKL axis could be an important autocrine/paracrine axis on vascular calcification. Furthermore, the fact that RANKL expression increases in calcified arterial tissue added new evidence to a possible role of RANKL on vascular calcification. However, direct evidence of a role of RANKL on vascular calcification is missing. In the present work, we analyze the role of RANKL in vascular calcification in vitro and in an in vivo model of vascular calcification.

Materials and Methods
All the experiments performed in this study followed the NIH Guide for the Care and Use of Laboratory Animals.

In Vitro Studies

Cell Cultures
Primary rat aortic VSMCs of Sprague–Dawley rats were obtained as described previously and maintained in DMEM (GIBCO) containing 10% FBS.

Cells were plated (10^5 cells/plate) in 100-mm plates. When the cell confluence was ~80%, VSMCs were shifted to calcification media, DMEM containing 15% FBS, 10 mmol/L sodium pyruvate, and 10 mmol/L β-glycerophosphate (Sigma). The effect of RANKL (1, 100, 500, 1000 pmol/L), OPG (100 pmol/L), and noggin (100 pmol/L) (all 3 from Sigma) on the calcification levels was tested. We used cells between passage 2 and 8. All of the experiments were performed in triplicate. In each experiment, 3 plates were used per condition.

Determination of VSMC Calcification
In all of the calcification experiments, the calcium levels were measured 5 days after the addition of the treatments. First, we measured the rate of calcium incorporation of VSMCs incubated with increasing doses of RANKL. Moreover, we tested the effect of OPG and noggin in the calcification level induced by 100 pmol/L RANKL. Quantification of calcium deposits and von Kossa staining were performed as previously described. Alkaline phosphatase (ALP) activity was determined by the para-nitrophenyl phosphate detection (BioAssay Systems).

Lentiviral Production and Infection
Lentiviral-based vectors for RNA interference-mediated gene silencing (FSVsi) consisted of a U6 promoter for expression of short hairpin (sh)RNAs and the Venus variant of yellow fluorescent protein under the control of an SV40 promoter for monitoring transduction efficiency. Oligonucleotides to produce shRNA were annealed in buffer (150 mmol/L NaCl; 50 mmol/L Tris, pH 7.6) and cloned into the AgeI-BamHI sites of FSVsi. shRNA target sequence to RANK was TTTCGCTAGGATGCTGGAGGAT. shRNAs to IKKα and IKKβ were a generous gift of Dr X. Dolcet. Negative controls consisted on scrambled sequences. To produce infective lentiviral particles, 293T cells were cotransfected by the polyethylenimine method with the virion packaging elements (VSV-G and Δ8.9) and the shRNA-producing vector (FSVsi-RANK or FSVsi as a control). 293T cells were allowed to produce lentiviral particles during 3 to 4 days in the same culture media used for VSMCs. Culture media was collected and centrifuged for 5 minutes at 1000g, and the supernatant was added to growing VSMCs overnight. After this period, media were replaced with fresh media, and cells were incubated for 4 additional days to allow endogenous gene knockdown. Western blot and/or real-time PCR were performed to check the gene knockdown.

Real-Time PCR
Total cellular RNA was isolated from VSMC control, RANK knockdown, IKKα- and β-knockdown, and tissue samples by the TRIzol method. In the in vitro experiments, isolation of RNA was performed 48 hours after the addition of treatments. Reverse transcription was performed with the first-strand DNA synthesis kit for RT-PCR (Roche Diagnostics). We used TaqMan real-time PCR amplification with gene-specific primer for RANKL, RANK, OPG, bone morphogenetic protein (BMP)2, or BMP4 (Gene Expression Assays from Applied Biosystems), using rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as a reference with an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The relative RNA amount was calculated by standard formulae. Average and standard error from 3 experiments were calculated.

Preparation of Nuclear and Cytoplasmic Protein Extracts
VSMCs were treated with 100 pmol/L RANKL for 0, 10, and 30 minutes and 1, 2, and 4 hours. After each time point, VSMCs were washed with cold PBS. Cytoplasmic and nuclear protein fractions were extracted using NE-PER Nuclear and Cytoplasmic Extraction KIT (Pierce).

Western Blot Analysis
Western blot analysis was performed as described previously, 48 hours after the addition of treatments. After blotting, the membrane was incubated overnight with anti-RANK antibody, anti–active caspase 3 antibody (1:1000, Cell Signaling), anti–IKKα, and anti–IKKβ (1:1000; Calbiochem), anti–REL B (1:1000, Santa Cruz Biotechnology), anti–histone 1 (1:500, Santa Cruz Biotechnology), anti–lactate dehydrogenase (1:1000, Rockland), and anti-tubulin (1:10 000 Sigma). Secondary antibody binding was detected with the ECL Advance Western Blotting Detection Kit (Amersham, Biosciences) and the VersaDoc Imaging system Model 4000 (Bio-Rad).

Enzyme-Linked Immunosorbent Assay
Levels of BMP2, BMP4, RANKL, and OPG were determined in cell culture supernatant, and rat plasma was determined by a commercially available ELISA (Quantikine, R&D Systems, Minneapolis, Minn, and Biomedica, Vienna, Austria).

In Vivo Studies

Experimental Animals
Sprague–Dawley rats (200 to 225 g) underwent 5/6 nephrectomy by previously described procedures and were divided in 2 groups. One group received calcitriol (1 μg/kg 3 times a week for 8 weeks; n=9), whereas the second group received a vehicle injection (n=9). Moreover, 2 more groups of sham-operated rats were used (control, n=9; injected with the same dose of calcitriol, n=9). At euthanasia,
A blood sample was extracted and abdominal aortas were collected and divided into 3 pieces. One was fixed, included in paraffin, and sliced; another piece was used to determine calcium content; and the last was used to isolate RNA.

**Aortic Calcification**

To study the changes in the aortas of the animals, we performed von Kossa staining as described in the section Determination of VSMC Calcification above. Furthermore, we measured total calcium content as described in the same section.

**Immunohistochemistry**

Sequential slides were used for immunohistochemistry and von Kossa staining as previously described. Sections were incubated in 1:50 anti-RANKL (Imgenex), anti-BMP4 (Abcam), anti-TRAP (Santa Cruz Biotechnology) polyclonal antibodies or nonimmune serum (negative controls) overnight at 4°C. After washing, the sections were incubated with 1:200 biotinylated secondary antibody and Vectastain ABC and DAB substrate kits (Vector Labs).

**Biochemistry Data**

Blood obtained at the end of the experiment was analyzed for calcium and phosphate using a multichannel autoanalyzer (Roche/Hitachi Modular Analytics).

**Statistical Analysis**

Differences between groups were assessed by ANOVA followed by Dunnett’s post hoc test. A value of \( P<0.05 \) was considered statistically significant.

**Results**

In Figure 1, we show the effect of adding RANKL to VSMCs cultured with calcification media. RANKL increased VSMC calcification measured as VSMC calcium levels (Figure 1A) and ALP activity (Figure 1B) in a dose-dependent manner and starting at concentrations of 100 pmol/L. The calcification induced by RANKL was also visualized by von Kossa staining, as we show in Figure 2B. In this case, we can see that incubation of VSMCs with RANKL increased the brown staining that marks calcified areas.

In Figure 2, we show the effect of coincubation of RANKL with OPG in the calcification of VSMCs. The increase in calcium levels induced by RANKL was inhibited by OPG (Figure 2B, 2D, and 2E).

To determine whether the effect of RANKL was mediated by activation of RANK, we designed shRNA to decrease RANK protein levels. In Figure 3, we can see that levels RANK (Figure 3A) were decreased in the cells infected with FSVsi-RANK. The incubation of those cells with 100 pmol/L RANKL showed that the elimination of RANK blunted the increase in calcification (Figure 3B), suggesting that the effect of RANKL in calcification is mediated by binding to RANK. To determine whether or not part of the effect of OPG was mediated by inhibition of TRAIL (tumor necrosis factor–related apoptosis-inducing ligand), and thus inhibiting apoptosis, we also checked the effect of RANKL and coincubation of RANKL and OPG on the levels of active caspase 3. Incubation of VSMCs with RANKL or RANKL plus OPG for 48 hours did not modify the levels of active caspase 3 (data not shown). To further elucidate the pathway, we infected cells with shRNA for both kinases involved in the classic and the alternative pathways of NF-κB activation (Figure 3A). The disruption of the alternative pathway (but not the classic pathway) blunted the increase in calcification (Figure 3B). Accordingly, incubation of VSMCs with
RANKL increased the nuclear levels of RelB (Figure 3C), confirming the activation of the alternative pathway of NF-κB activation.

In Figure 4 we show the effect of adding RANKL in the mRNA (Figure 4A) and protein (Figure 4B) levels of BMP4. RANKL increased BMP4 levels in VSMCs. No effect of RANKL on BMP2 levels was detected either by real-time PCR or ELISA (data not shown). Elimination of RANK or IKKα (but not IKKβ) blunted the effect of RANKL on BMP4, pointing again to the alternative pathway of activation of NF-κB as the responsible of the increase in BMP4 induced by RANKL. In Figure 5A and 5B, we show the effect of adding Noggin (a BMP4 inhibitor) in the calcification induced by RANKL. The addition of 100 pmol/L Noggin blunted the increase in calcification induced by RANKL, suggesting that this increase is mediated by a BMP.

Figure 6 shows the results obtained in the in vivo model of vascular calcification. Arteries obtained from animals with 5/6 nephrectomy showed a significant increase in vascular calcification, measured as calcium content, which was exacerbated by treatment with calcitriol for 8 weeks (Figure 6A).

Blood levels of RANKL were not modified by the treatment but OPG levels increased in uremic animals, leading to a tendency to decrease the RANKL/OPG ratio (Figure 6B). However, vascular expression of RANKL in those animals increased with no changes in OPG (Figure 6C) and was colocalized with the calcified areas (Figure 6E and 6F). In addition, BMP4 expression (Figure 6D) also increased in arteries from uremic rats and uremic rats treated with calcitriol and was immunolocalized in heavily calcified areas (Figure 6E and 6G). No staining for TRAP was detected in areas expressing BMP4 or RANKL (Figure 6H).

Uremia induced a significant increase in serum calcium (Ca) but not in phosphorus (P) levels (control Ca: 10.42±0.24 mg/dL; control P: 5.81±0.24 mg/dL; uremia Ca: 11.02±0.14 mg/dL; uremia P: 6.06±0.27 mg/dL; P<0.01). Administration of vitamin D further increased both calcium and phosphorus levels in uremic animals (uremic+vitamin D Ca: 12.14±0.37 mg/dL; uremic+vitamin D P: 6.68±0.34 mg/dL; P<0.01), whereas treatment of control animals with vitamin D did not change control Ca nor P blood levels (control+vitamin D Ca: 10.78±0.07 mg/dL; control+vitamin D P: 5.7±0.08 mg/dL).

**Discussion**

To our knowledge, this is the first report showing a direct effect of RANKL increasing VSMC calcification. The possible effect of RANKL on VSMC calcification has been suggested by several authors mainly based on indirect experimental results found in the literature. On the one hand,
Furthermore, Price et al. showed that RANKL inducing VSMC-mediated matrix mineralization. On the other hand, the expression of OPG as a transgene on an OPG-null background prevented the appearance of vascular calcification. Thus, it has been proposed that an imbalance in calcium allocation allows its movement from bone to vascular wall via mechanisms that involve OPG. This hypothesis was supported by results that link arterial calcification with diseases with a high bone resorption rate and also because treatments that inhibit bone resorption can inhibit vascular calcification in experimental models. However, in patients with chronic renal failure, high levels of OPG seem to be unable to protect against vascular calcification. Nonetheless the significance of those results is limited because of the fact that in those studies, RANKL levels were not measured, and, thus, RANKL/OPG ratios were not determined. The other mechanism is by acting directly on cells in the artery inhibiting the effect of calcification stimulators. OPG is a decoy receptor for RANKL and also for TRAIL. VSMCs express both OPG and TRAIL. The binding of TRAIL to its receptor induced apoptosis, which has also been related to vascular calcification. In vitro models of calcifying VSMCs have shown that a mineral imbalance induces VSMC apoptosis and vesicle release and that these apoptotic bodies and vesicles form a nidus for the deposition of calcium phosphate. Thus, inhibition of TRAIL could inhibit also vascular calcification. However, no direct evidence of apoptosis has been found in models of vascular calcification in which OPG treatment was able to inhibit it. There is a third possibility, which is that OPG inhibits vascular calcification by directly inhibiting RANKL. Our results clearly show that coinubation with OPG inhibits RANKL-induced VSMC calcification. However, it could be hypothesized that incubation with RANKL will increase VSMC calcification by depleting endogenously produced OPG and, thus, leaving TRAIL free to induce apoptosis. Our experimental results show that incubation of VSMCs with RANKL did not increase the level of active caspase 3, which is involved in TRAIL-induced apoptosis. Furthermore, we performed experiments in which we inhibited RANK expression by shRNA. In those cells, incubation with RANKL did not increase VSMC calcification, pointing to a direct role of RANKL increasing VSMC calcification by binding to RANK.

The binding of RANKL to its receptor RANK activates both the canonical and the alternative NF-κB pathways. We further investigated how activation of RANK induced vascular calcification by inhibiting either pathway in the NF-κB cascade. The results showed that inhibition of the canonical pathway did not affect vascular calcification, whereas the use of shRNA for the main kinase involved in the alternative pathway totally blunted RANKL-induced VSMC calcification. Accordingly, incubation with RANKL induced an increase in nuclear translocation of RelB, proving an activation of the alternative NF-κB pathway. Furthermore, activation of RANK increased the production of BMP4. BMP4 is a member of the BMP family, a group of signaling molecules that belong to the transforming growth factor β superfamily and were initially identified by their capacity to induce endochondral bone formation. In addition, BMP4 has been involved in the osteogenic transition of VSMCs, leading to vascular calcification. It has also been described that BMP4 increases in vitro VSMC calcification and is upregulated in calcified atherosclerotic lesions. Our results

![Figure 5. Effect of the incubation of rat VSMCs with RANKL (100 pmol/L) and Noggin (100 pmol/L). A, Quantification of calcium incorporation. Data are expressed in nanograms of calcium per milligram of protein. B, Effect on ALP activity. Data are expressed in international units of ALP per milligram of protein. Data are means±SEM. *P<0.05 vs RANKL 0 pmol/L, Noggin 0 pmol/L; #P<0.05 vs RANKL 100 pmol/L, Noggin 0 pmol/L.](http://circres.ahajournals.org/figure5.html)
further show that parallel to a decrease in vascular calcification, inhibition of the alternative NF-κB activation pathway also decreased BMP4. Furthermore, the addition of noggin (a pharmacological inhibitor of the BMPs) to the incubation media also inhibited the RANKL-induced VSMC calcification, suggesting that it is mediated by an increase in BMP4 expression.

We also tested our results in an in vivo model of vascular calcification. In that model, subtotally nephrectomized rats were treated with high doses of vitamin D to intensify vascular calcification. This model has been used before, and it has been shown that administration of OPG was able to decrease the vascular calcification.23 The results show that administration of calcitriol to normal animals does not increase either the expression of RANKL/OPG or BMP4 in arteries or in blood. Those animals showed no increases in vascular calcification. In addition, uremia induces an increase in the RANKL/OPG ratio and in BMP4 expression in arteries.

Consistently, the levels of vascular calcification are increased in those animals. The increase in vascular calcification is higher in the uremic animals treated with calcitriol, together with a tendency to increase arterial RANKL and BMP4 expression. This finding suggests that there are other factors that have an effect on the degree of vascular calcification in vivo that can be influenced by calcitriol treatment. For instance, uremic animals treated with calcitriol show higher levels of phosphorus in blood, a parameter that has been shown to increase vascular calcification.37 In addition, the expression of RANKL and BMP4 were localized in areas of medial calcification, supporting the role of RANKL in promoting VSMC calcification. The staining for TRAP was negative in those areas and agrees with previous reports suggesting that, contrary to atherosclerotic plaque calcification, in medial calcification macrophage infiltration is not involved.38 The clinical implications of our in vivo model to
human pathology are relative, because vitamin D intoxication in uremic patients is currently a rare phenomenon.

We also tested the role of circulating levels of RANKL as a possible marker for vascular calcification in our model. However, whereas the vascular expression of RANKL increased, the circulating levels of RANKL did not change. The association of circulating OPG levels with traditional vascular risk factors has been extensively reported, but baseline RANKL levels were shown to be a predictor of vascular risk.44 Thus, the role of serum RANKL levels as predictors of cardiovascular risk is unclear. In our experimental model, we have shown that although vascular levels of RANKL are increased, circulating levels did not change. Thus, changes in circulating levels of RANKL could not reflect changes in vascular levels. Furthermore, and in agreement with previous reports, OPG levels did not change. Thus, changes in circulating levels of RANKL levels appear unaltered, although they have sometimes declined as serum OPG levels increased.20,21,43 Furthermore, in a recent study, Kiechl et al showed in a large scale epidemiological study that serum RANKL levels did not correlate with atherosclerosis, but baseline RANKL levels were shown to be a predictor of vascular risk.44 Thus, the role of serum RANKL levels as predictors of cardiovascular risk is unclear. In our experimental model, we have shown that although vascular levels of RANKL are increased, circulating levels did not change. Thus, changes in circulating levels of RANKL levels as predictors of cardiovascular risk is unclear. In our experimental model, we have shown that although vascular levels of RANKL are increased, circulating levels did not change. Thus, changes in circulating levels of RANKL could not reflect changes in vascular levels. Furthermore, and in agreement with previous reports, OPG levels did not change. Thus, changes in circulating levels of RANKL levels appear unaltered, although they have sometimes declined as serum OPG levels increased.20,21,43

In summary, we have shown that RANKL is able to induce VSMC calcification in vitro by binding to RANK. The activation of RANK will increase BMP4 expression by launching the alternative NF-κB pathway. These results add new evidence to the role of the OPG-RANK-RANKL system in vascular calcification and confirm RANKL inhibition as a possible target to treat vascular calcification.

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Disclosures
None.

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