Interleukin-4 differentially regulates osteoprotegerin expression and induces calcification in vascular smooth muscle cells

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Summary
Vascular calcification is characterized by cellular transdifferentiation and expression of bone-related matrix proteins that result in the presence of bone-like structures in the vascular wall. Interleukin (IL)-4, a pleiotropic cytokine, and osteoprotegerin (OPG), an essential regulator of osteoclast biology, have both been linked to vascular disease. Here, we assessed the role of IL-4 and OPG in vascular calcification in vitro. IL-4 induced OPG mRNA levels and protein secretion by 5-fold in a dose- and time-dependent fashion in human coronary artery smooth muscle cells (CASM C). Activation of the transcription factor STAT6 preceded IL-4-induced OPG expression, and blockade of IL-4-induced STAT6 activation by the phospholipase C inhibitor D609 decreased OPG expression. Long-term exposure of IL-4 for 4 weeks resulted in transformation of CASMC towards an osteoblastic phenotype, based on the expression of the transcription factor Cbfa1 and increased mineral deposition. Notably, calcification of CASMC was inhibited by gene silencing of Cbfa1. During osteogenic transformation, IL-4 down-regulated OPG production in CASMC. IL-4 has differential effects in CASMC: While short-term exposure enhances OPG production through a STAT6-dependent mechanism, long-term exposure causes Cbfa1-dependent osteogenic transformation and a decreased production of OPG, an inhibitor of bone resorption.

Keywords
Cbfa1, interleukin-4, osteoprotegerin, smooth muscle cells, vascular calcification

Introduction
Osteoprotegerin (OPG) is a secreted glycoprotein of the tumor necrosis factor (TNF) receptor superfamily that acts as a decoy receptor for receptor activator of NF-κB ligand (RANKL) (1, 2). RANKL activates receptor activator of NF-κB (RANK) which is expressed on osteoclasts and dendritic cells, thus promoting their differentiation and activation and prolonging their survival by suppressing apoptosis (3). The RANKL/RANK/OPG system is essential for bone cell biology (3) and modulates immune functions (4).

Several studies suggest that the RANKL/RANK/OPG system is crucially involved in the homeostasis of the vascular system in rodents and humans. Two thirds of OPG-deficient mice develop medial calcification in the aorta and the renal arteries (in addition to osteoporosis) (5) that can be prevented by transgenic overexpression of OPG from mid-gestation (6). Treatment of rats with OPG prevented arterial calcification provoked by warfarin or vitamin D (7). In humans, immunohistochemical studies demonstrated enhanced OPG expression in areas adjacent to arterial calcification in patients with atherosclerosis or Mönckeberg’s sclerosis (8, 9). Furthermore, elevated OPG serum levels have been detected in patients with coronary artery disease (10, 11) and carotid atherosclerosis (12), and are associated with increased cardiovascular mortality (12, 13). This evidence has led to the hypothesis that OPG may be a potential link between osteoporosis and arterial calcification, both of which coincide in postmenopausal women and the elderly (14).

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Various cellular and humoral mediators of inflammation such as TNF-α and interleukin (IL)-1β have been implicated to promote and maintain vascular diseases (15), whereas the potential role of T-helper (Th) 2 cytokines such as IL-4 and IL-13 in atherosclerosis and arterial calcification remains poorly defined. IL-4 is a pleiotropic cytokine that is produced by T lymphocytes, mast cells, basophils, and natural killer cells (16). Studies on atherosclerosis in IL-4-deficient mice have yielded conflicting results depending on the model used (17–20). Moreover, the role of IL-4 in the process of vascular calcification is unclear, although IL-4 induces mineralization of osteoblastic cells (21, 22).

In this study, we tested the hypothesis that IL-4 regulates OPG expression and osteoblastic transformation of human coronary artery smooth muscle cells (CASMCS) in the process of vascular calcification, and evaluated the underlying mechanisms. We found that IL-4 enhances OPG production through a STAT6-dependent mechanism on short-term exposure, while it promotes Cbfa1-dependent osteogenic transformation with mineral deposition and a decreased production of OPG on long-term exposure.

**Material and methods**

**Material**

The human β-actin cDNA insert and ExpressHyb solution were obtained from Clontech (Palo Alto, CA, USA). IL-4 was from BD PharMingen (San Diego, CA, USA), all other cytokines and soluble IL-4 receptor (sIL-4R) were from R & D Systems (Wiesbaden, Germany). D609 was from Biomol (Hamburg, Germany), G418 was from PAAG Laboratories (Pasching, Austria). DC protein assay and nitrocellulose membranes were from Bio-Rad (Hercules, CA, USA). Antibodies directed against STAT6 and horseradish peroxidase-conjugated anti-rabbit IgG antibody were from Cell Signalling Technology (Frankfurt, Germany). The ECL detection reagents were purchased from Amersham Bioscience (Little Chalfont, UK). All other reagents were obtained from Sigma-Aldrich (Munich, Germany).

**Cell culture**

Human coronary artery smooth muscle cells (CASMCS) were purchased from Cambrex (Verviers, Belgium). These cells have previously been used to study the regulation of OPG by immunosuppressants (23). CASMCS were grown in SmGM-2 medium from Cambrex supplemented with epidermal growth factor (0.5 µg/l), insulin (5 mg/l), fibroblast growth factor-B (2 µg/l), and fetal bovine serum (5%). The cells (plating density: 6,000 cells/cm²) were maintained at 37°C (5% CO₂, 90% humidity) in SmGM-2 medium until reaching 70 to 90% confluence, and were used between passages 4 and 7.

To assess regulation of OPG expression, dose response experiments were conducted, in which CASMCS cells were treated with cytokines (IL-1β, IL-4, IL-13, and TNF-α) at varying concentrations for 48 h. For time course experiments, the CASMCS cells were treated with IL-4 or IL-13 at 10 ng/ml for 0, 1, 2, 6, 24, and 48 h, respectively. To determine the specificity of IL-4-induced effects, CASMCS cells were incubated with sIL-4R, a competitive inhibitor of IL-4, for 30 min followed by IL-4 exposure (10 ng/ml) for further 48 h.

To analyze the STAT6 pathway, CASMCS were exposed to IL-4 or IL-13 (10 ng/ml) for varying duration. D609, a phosphatidylinositol-specific phospholipase C inhibitor which blocks STAT6 phosphorylation, and G418, a phosphatidylinositol-specific phospholipase D inhibitor, which does not block STAT6 phosphorylation, were employed at 50 ng/ml (24). Both agents were used 30 min prior to IL-4 treatment (10 ng/ml) which was continued for 15 min (STAT6 Western blot) or 48 h (OPG Northern blot and protein secretion), respectively.

**Northern blot analysis**

Total RNA was isolated using the RNasy kit from Qiagen (Hilden, Germany). After separation on a denaturing 1.5% (w/v) agarose gel containing 2.2 M of formaldehyde, RNA was transferred onto a nylon membrane by capillary blotting. The human OPG cDNA insert and β-actin cDNA insert were radiolabeled with 5 µl [α-32P]dCTP using random primer DNA labeling. All experiments were repeated at least three times, and representative blots are shown. Control hybridization with human β-actin cDNA verified equal RNA loading.

**Western blot analysis**

CASMCS were lysed in ice-cold buffer (1% Triton X-100, 10% glycerol, 50 mM HEPES pH 7.4, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 5 mM sodium vanadate, 10 µg/ml aprotinin, 5 µg/ml leupeptin, 1.5 mg/ml benzamidine, and 34 µg/ml phenylmethylsulfonyl fluoride) and sonicated. Insoluble cell debris was removed by centrifugation at 10,000 rpm for 10 min. Protein content was quantified by DC protein assay and 5 µg were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes. After blocking in 5% non-fat dry milk for 1 h, membranes were incubated overnight at 4°C with antibodies to phospho-STAT6 and control-STAT6. Signals were detected using horseradish peroxidase-conjugated anti-rabbit IgG antibody and chemiluminescent reaction with ECL detection reagent and visualized on X-Omat films (Kodak, Rochester, NY, USA).

**Osteogenic differentiation and Alizarin red S staining for mineralized matrix formation**

CASMCS were cultured in 12-well plates for up to 28 days in the presence of either regular medium with supplementation (SmGM-2) as described above or osteogenic medium which consisted of SmGM-2 medium supplemented with 10⁻³ M dexamethasone, 10 mM β-glycerolphosphate, and 100µM L-asorbate phosphate. These conditions enhance the differentiation of human marrow stromal cells towards an osteoblastic phenotype (25). Medium was changed three times per week, and the agent of interest replaced. After collecting conditioned medium, mineralized matrix formation was assessed by Alizarin red S staining. Cells were fixed in 70% ethanol for 1 h at room temperature, washed once with PBS, and stained with 40 mL of Alizarin red S (titrated to pH 4.2 with 0.5% ammonium hydroxide) for 10 min. The cells were then washed with deionised water and incubated with PBS at room temperature to remove non-specific staining. The staining was documented with an Olympus E300 digital camera. Alizarin red S was eluted from the cell matrix with 1 mL of cetlylpyridinium chloride for 15 min, and the OD
was quantified by spectrophotometry at 570 nm. Results were normalized to total protein content as determined by the Bradford method.

**Cbfα1 gene silencing and transfection procedures**

Small interfering RNA (siRNA) was used for gene silencing of human Cbfα1, an essential osteoblastic transcription factor (Gene Bank # NM_004348) (26). Two Cbfα1 siRNAs and a non-specific control siRNA were purchased from Qiagen (Hilden, Germany). The first targeted the sequence CAC CTT GAC CAT AAC CGT CTT with the sense oligonucleotides r(CCU UGA CCA UAA CCG UCU)TdTT and the antisense oligonucleotides r(AAG ACG GUU AUG GUC AAG G)dTdT. The second siRNA targeted the sequence CAG AAG CTT GAT GAC TCT AAA using sense oligonucleotides r(GAA GCC GUA UGA CUC UAA A)dTdT and antisense oligonucleotides r(UUU AGA GUC AUC AAG CUU)CdTdT. Both siRNAs were mixed 1:1 and used at a final concentration of 100 nM. Control siRNA was also used at a concentration of 100 nM. Transfection was repeatedly performed with SiLentFect lipid reagent from Bio-Rad (Hercules, CA, USA) on days 0, 4, 10, 17, and 24 for experiments that were assayed for mineral formation or gene expression, and the medium changed after 24 h.

**PCR analysis of bone-related genes**

Total RNA was prepared using the RNeasy total RNA extraction kit from Qiagen. The cDNA was synthesized from 1 μg of total RNA in a total volume of 40 μl containing 3 mM MgCl₂, 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 10 mM DTT, dCTP, dGTP, dATP, and dTTP each at 0.4 mM, RNAse inhibitor (40 U), M-MLV reverse transcriptase (400 U), and poly-dT15 primer (80 pM) from Roche Molecular Biochemicals (Mannheim, Germany). Reaction times were 1 h at 38°C and 10 min at 72°C. Aliquots of the total cDNA were amplified in each PCR reaction in a 15 μl reaction mixture containing 20 pmol of 5’ and 3’ primer each, 50 mM KCl (pH 8.3), 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, and dCTP, dGTP, dATP, and dTTP each at 0.02 mM and Taq polymerase (0.5 U) from Roche Molecular Biochemicals (as reported (26, 27)). Each cDNA sample was run in triplicate for each PCR reaction. Competitive RT-PCR was performed using exogenous DNA competitors ("mimics") as internal control that were synthesized with the PCR mimic construction kit from Clontech (Palo Alto, CA, USA). PCR reactions were carried out in 15 μl reactions at a cycle number ensuring a linear amplification profile (L7, 2 min at 94°C, 20 cycles [of 1 min at 94°C, 1 min at 54°C, 2 min at 72°C], 10 min at 94°C, 24 cycles [of 30 sec at 94°C, 60 sec at 54°C, 90 sec at 72°C], 7 min at 72°C). The oligonucleotides for L7 (sense: 5’-AGA TGT ACA GAA CTG AAA TTC-3’; antisense: 5’-ATT TAC CAA GAG ATC GAG CAA-3’); OPG (sense: 5’-GAA CCC CAG ACG GAA ATA CA-3’; antisense: 5’-CGT TGT TTT CAC AGA GGT CA-3’); Cbfα1 (sense: 5’-CCACCTCGACTTGCCTC-3’; antisense: 5’-GAC TGG CGG GTG ATA AGT AA-3’) (Gen-Bank Accession #AH 005498) were synthesized at MWG (Ebersberg, Germany).

PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining under UV light. In all experiments, the expression of each gene was quantified as target to mimic ratio and normalized to the ribosomal housekeeping gene, L7. To ensure specificity of the PCR products, the amplification product was sequenced with the ABI Prism system from Perkin Elmer (Weiterstadt, Germany).

**Measurement of OPG and other bone-related proteins**

Conditioned medium was harvested from cultured cells before RNA analysis or Alizarin red S staining, and centrifuged to remove debris. Samples were stored at –80°C until analysis. OPG protein secretion was determined in triplicate measurements with an immunoassay from Immundagnostik (Bensheim, Germany) (27). Osteocalcin secretion was assessed with an assay (lower limit of detection: 2.0 ng/ml) from Brahms (Berlin, Germany), matrix GLA protein was measured with an assay (lower limit of detection: 0.3 nmol/l) from Biomedica (Vienna, Austria).

**Statistical analysis**

Each of the experiments was reproduced at least three times. Values are expressed as the mean ± SD of triplicate measurements. Student's paired t-test was used to analyze differences between the sample of interest and its control. Time courses and dose responses were compared by multiple measurement ANOVA and corrected by Student-Newman-Keul’s test for differences between groups. A P value of less than 0.05 was considered statistically significant. Standard software from StatView 5.0 (SAS Institute, Cary, NC, USA) was used.

**Results**

Unstimulated CASMC expressed only low OPG mRNA levels (Fig. 1A, B). Baseline OPG protein secretion in conditioned medium harvested from untreated sub-confluent CASMC was between 8 and 24 pmol/l. We then assessed whether IL-4 and the closely related IL-13 modulate OPG gene expression of CASMC. Both IL-4 and IL-13 dose-dependently enhanced OPG mRNA levels with marked effects at 1 ng/ml and a maximal effect at a dose of 10 ng/ml after exposure for 48 h (Fig. 1A). TNF-α also induced OPG mRNA levels at a dose of 10 ng/ml, whereas IL-1β had no effect at this dose (Fig. 1A). Consistent with these results, OPG secretion increased in a dose-dependent fashion in CASMC treated with IL-4, IL-13, and TNF-α by 5-fold (P <0.001), 4-fold (P <0.0005), and 6-fold (P <0.005), respectively, at a dose of 10 ng/ml each. Of note, TNF-α synergistically enhanced the stimulatory effect of IL-4 and IL-13 on OPG mRNA levels (Fig. 1B) and protein secretion (by 40-fold, P <0.01 and 29-fold, P <0.0001, respectively).

Both IL-4 and IL-13 (at a dose of 10 ng/ml) induced OPG mRNA steady state levels in a time-dependent manner with a maximum effect after 48 h (Fig. 1C). OPG secretion time-dependently increased by 5-fold after IL-4 (P <0.0001) and by 6-fold after IL-13 exposure (P <0.0001) after 48 h. To demonstrate specificity of IL-4-induced effects on OPG production, sil-4R, a competitive inhibitor of IL-4 effects, was evaluated. Pretreatment of CASMC with sil-4R at various concentrations for 30 min dose-dependently abrogated IL-4-induced increase of OPG mRNA levels (data not shown) and protein secretion from 39.4 ± 2.55 pmol/l with IL-4 alone (10 ng/ml) to 24.8 ± 1.10
pmol/l with IL-4 (10 ng/ml) and sIL-4R (50 ng/ml) (P < 0.05). When sIL-4R was given simultaneously with or after IL-4, it did not significantly mitigate the IL-4 effects (data not shown), possibly because of the rapid STAT6 phosphorylation by IL-4 as demonstrated in Figure 2A.

In order to analyze the intracellular pathway whereby IL-4 and the closely related IL-13 may induce OPG production in CASMC, we evaluated the STAT6 signaling pathway, because STAT6 activation is induced by IL-4 (24, 28). At a dose of 10 ng/ml, STAT6 phosphorylation was induced after 5 min (IL-4) and 5 to 15 min (IL-13) of cytokine exposure (Fig. 2A). Western blot analysis demonstrated maximal STAT6 phosphorylation by IL-4 after 15 min and by IL-13 after 1 h (Fig. 2A). STAT6 phosphorylation after IL-4 exposure (10 ng/ml) was detected for 48 h (Fig. 2B). Next, we studied the effect of D609, a phosphatidylincholine-specific phospholipase C inhibitor which blocks STAT6 phosphorylation (22). Pretreatment of CASMC cells with D609 (50 ng/ml) inhibited IL-4-induced STAT6 phosphorylation (Fig. 2C) or OPG mRNA expression (Fig. 2D). IL-4-induced OPG protein secretion (IL-4 at 10 ng/ml) was reduced by D609 pretreatment (50 ng/ml) from 160.7 ± 14.5 pmol/l to 47.7 ± 2.81 pmol/l (P < 0.01). By contrast, pretreatment with G418, a phosphatidylincholine-specific phospholipase D inhibitor, did not prevent IL-4-induced STAT6 phosphorylation (Fig. 2C) and OPG mRNA levels (Fig. 2D). These findings indicate that IL-4-induced OPG production in CASMC is dependent on phosphatidylincholine-specific phospholipase C and STAT6 phosphorylation.

To study whether CASMC acquire an osteoblastic phenotype under appropriate culture conditions, formation of mineralized matrix was assessed using an Alizarin red S assay. Culture of CASMC in regular medium over 28 days enhanced formation of mineralized matrix by 3.3-fold compared to the control at day 0 in a time-dependent fashion (P < 0.0001 by ANOVA) (Fig. 3A). Exposure of CASMC to IL-4 (10 ng/ml) further enhanced this process at each time point, and resulted in 47% higher mineralized matrix formation after 28 days (P < 0.0005) (Fig. 3A). For comparison, culture of CASMC for 28 days in osteogenic medium (with dexamethasone, β-glycerolphosphate, and vitamin C) resulted in pronounced stimulation of mineralized matrix formation as evident from a 15-fold (compared to CASMC on day 0) or 4.7-fold increase (compared to CASMC cultured for 28 days in regular medium, P < 0.0001) of Alizarin red S staining on spectrophotometry (Fig. 3A) and photography (Fig. 3B, single panel). In order to analyze the role of the osteoblastic transcription factor Cbfa1 in the process of CASMC calcification, we used siRNA directed against Cbfa1 and assessed the effects of Cbfa1 blockade on mineralized matrix formation in CASMC cultured for 28 days. IL-4 promoted mineralized matrix formation (P < 0.0005), and the IL-4 effect was completely prevented by Cbfa1 siRNA (P < 0.0005), but not by non-specific
control siRNA (P = 0.56) (Fig. 3B,C). Of note, Cbfa1 siRNA resulted in even lower mineralized matrix formation as compared to control (P < 0.0001). As expected, administration of Cbfa1 siRNA inhibited Cbfa1 expression in unstimulated CASMC by up to 91% and in IL-4-stimulated CASMC by up to 63%, whereas no inhibitory effect of control siRNA was observed.

Next, we evaluated osteogenic differentiation of CASMC by analyzing gene expression of Cbfa1. RT-PCR analysis revealed that IL-4 (10 ng/ml) transiently up-regulated Cbfa1 gene expression in normal medium after 7 and 14 days (Fig. 4A and B). Osteogenic medium caused strong and sustained up-regulation of Cbfa1 gene expression over a period of 3 weeks (Fig. 4A, B). Concurrently, OPG gene expression (measured by RT-PCR) and protein secretion (measured by an immunoassay from conditioned medium) were inhibited by IL-4 (10 ng/ml) by 84% (P < 0.0001) (Fig. 4C, D). By contrast, osteogenic medium markedly enhanced OPG protein secretion by CASMC (measured in the culture medium) (Fig. 4D), which is similar to the effects observed in human marrow stromal cells (25). Since osteogenic medium promoted excessive calcification, we were unable to isolate mRNA in order to assess OPG gene expression.

Osteocalcin secretion was < 2 ng/ml in CASMC on day 0 and increased to 6.8 ng/ml (normal medium) and 8.5 ng/ml (osteogenic medium) on day 21, respectively, but was not regulated by IL-4. Matrix GLA protein secretion which was < 0.3 nmol/l in CASMC on day 0 increased to levels of 1.5 nmol/l over time in culture and was not regulated by IL-4.

**Discussion**

IL-4 represents a pleiotropic cytokine that is required for an adequate immune response against parasitic infections (29), is linked to airway hyperreactivity in asthma, and confers protection against bone loss in an animal model of arthritis (30). The biological role of IL-4 in the vascular system and its involvement in vascular disease is unclear. While studies on atherosclerosis in IL-4−/− mice yielded conflicting results (17–20), the role of IL-4 in vascular calcification has not been studied. In bone cells, IL-4 has been shown to promote mineralization in human osteoblasts (21, 22) and to directly inhibit osteoclasts (31, 32). OPG and RANKL are essential regulators of bone metabolism (1, 2), and may also play an important role in vascular calcification (14).

In order to study the role of IL-4 and OPG, both regulators of bone metabolism, in the vascular system, we employed smooth muscle cells from coronary arteries of human origin, since OPG is expressed within the arterial media by smooth muscle cells (1,
9). In this study, we observed stimulation of OPG production by IL-4 and IL-13 which have common biological effects due to binding to a shared cell surface receptor complex (28). Both cytokines stimulated OPG at the mRNA and protein level up to 5–6-fold in a dose- and time-dependent manner. Their stimulatory effect was synergistically enhanced by TNF-α, and pretreatment with sIL-4R abrogated the stimulatory effect of IL-4, indicating that the stimulatory effects are physiologically relevant and specific.

Recently, a gene array approach identified STAT6 as a gene that is up-regulated in coronary arteries of patients with coronary artery disease as compared to healthy controls, and immunohistochemistry of STAT6 showed increased expression in vascular smooth muscle cells of atherosclerotic specimen (33). Biological effects of IL-4 are mediated by STAT6 activation (28) as evident from blockade of STAT6 activation by the phosphoprotein C inhibitor D609 (24) or analysis of STAT6-/- mice (32). In our study, rapid STAT6 activation preceded the stimulatory effect of IL-4 on OPG mRNA steady state levels and protein secretion, and was required for this effect, since blockade of STAT6 activation by D609 prevented IL-4-induced OPG expression. Interestingly, IL-4 suppresses osteoclastogenesis by a STAT6-dependent mechanism that included down-regulation of RANK (34). In addition, RANKL-induced osteoclastogenesis was blocked by IL-4-induced inhibition of NF-κB activation, an important downstream signal following RANK activation by RANKL (31, 32). Moreover, local IL-4 overexpression inhibited RANKL expression and prevented bone erosions in collagen-induced arthritis (30). Our data suggest that, in addition to RANKL and RANK, OPG is also an IL-4-responsive and STAT6-dependent gene. In fact, STAT6 activation has been shown to precede smooth muscle cell proliferation in an artery injury model (35).

Arterial calcification involves a complex and regulated process that is distinct from atherosclerosis and is characterized by the expression of bone-related proteins and formation of a mineralized matrix (36, 37). We report first evidence that, as in human osteoblasts (30), IL-4 induces calcification of CASMC which is associated with up-regulation of the osteoblastic transcription factor Cbfa1, an essential inducer of osteoblastic differentiation and bone formation (26, 37). This IL-4 effect can be specifically abrogated by Cbfa1 gene silencing, indicating that Cbfa1-dependent cellular transdifferentiation and gene expression may be involved in osteogenic differentiation of CASMC in the process of vascular calcification. In contrast to short-term exposure, IL-4 along with enhancing osteogenic differentiation of CASMC decreased OPG production at the mRNA and protein level. Consistent with these in vitro findings, OPG expression in the arterial media was lower in calcified arteries compared to normal specimen (38).

The transcription factor Cbfa1 serves as a master gene regulating osteoblast-specific gene expression. Consistently, Cbfa1-deficient mice display lack of osteoblastic bone formation (40). Cbfa1 is expressed in osteoblastic lineage cells, but when expressed and up-regulated in non-skeletal cells such as smooth muscle cells under calcifying conditions, the cells lose their lineage markers but gain an osteogenic phenotype as indicated by an increase in expression and DNA-binding activity of Cbfa1 (41, 42). In the vascular system, histological specimens with calcifications exhibit expression of Cbfa1 and several bone-associated proteins (osteopontin, osteocalcin, alkaline phosphatase, type I collagen) in both the intima and medial layers, demonstrating the association of vascular calcification with phenotypic transition of smooth muscle cells (43, 44). Therefore, we hypothesized that the transcription factor Cbfa1 may also be involved in regulating IL-4 driven calcification. While osteocalcin and matrix GLA protein increased with osteogenic differentiation, they were not regulated by IL-4. Taken together, this suggests that osteogenic differentiation of CASMC resembles, but is not identical with, osteogenic differentiation of osteoblasts with respect to the expression of transcription factors and secretion of bone-related proteins.

In conclusion, IL-4 has differential effects in CASMC. While short-term exposure specifically enhanced OPG production through a STAT6-dependent mechanism, long-term exposure causes Cbfa1-dependent osteogenic transformation with formation of mineralized matrix and a decreased production of OPG, an inhibitor of bone resorption. The question whether OPG has a causative role in vascular calcification in vivo remains open. Further cellular and molecular characterization of the similarities and differences between vascular calcification and osteogenesis may result in novel therapeutic strategies that may benefit patients with vascular and bone diseases.

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