PKA-induced Receptor Activator of NF-κB Ligand (RANKL) Expression in Vascular Cells Mediates Osteoclastogenesis but Not Matrix Calcification*

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Vascular calcification is a predictor of cardiovascular mortality and is prevalent in patients with atherosclerosis and chronic renal disease. It resembles skeletal osteogenesis, and many bone cells as well as bone-related factors involved in both formation and resorption have been localized in calcified arteries. Previously, we showed that aortic medial cells undergo osteoblastic differentiation and matrix calcification both spontaneously and in response to PKA agonists. The PKA signaling pathway is also involved in regulating bone resorption in skeletal tissue by stimulating osteoblast-production of osteoclast-regulating cytokines, including receptor-activator of nuclear κB ligand (RANKL) and interleukins. Therefore, we investigated whether PKA activators regulate osteoclastogenesis in aortic smooth muscle cells (SMC). Treatment of murine SMC with the PKA agonist forskolin stimulated RANKL expression at both mRNA and protein levels. Forskolin also stimulated expression of interleukin-6 but not osteoprotegerin (OPG), an inhibitor of RANKL. Consistent with these results, osteostatic differentiation was induced when monocytic preosteoclasts (RAW264.7) were cocultured with forskolin-treated aortic SMC. Oxidized phospholipids also slightly induced RANKL expression in T lymphocytes, another potential source of RANKL in the vasculature. Because previous studies have shown that RANKL treatment alone induces matrix calcification of valvular and vascular cells, we next examined whether RANKL mediates forskolin-induced matrix calcification by aortic SMC. RANKL inhibition with OPG had little or no effect on osteoblastic differentiation and matrix calcification of aortic SMC. These findings suggest that, as in skeletal tissues, PKA activation induces bone resorptive factors in the vasculature and that aortic SMC calcification specifically induced by PKA, is not mediated by RANKL.

Vascular calcification is a predictor of cardiovascular mortality and is highly prevalent in patients with atherosclerosis, diabetes, and chronic renal disease. It leads to reduced compliance in the vessels, increased blood pressure, and increased cardiac work (1, 2).

Calcium mineral deposition in atherosclerotic arteries is considered an organized and actively regulated process that resembles endochondral ossification. Factors that regulate both bone formation and resorption have been identified in calcified arteries. We and others (3–7) have found that vascular cells undergo osteoblastic differentiation in response to certain stimuli. Besides osteogenic factor expression, osteoclast-like cells are also present in the calcified artery wall. Multinucleated cells that are positive for acid phosphatase and CD68, which are markers for osteoclasts of hematopoietic lineage, have been found juxtaposed to mineralized bone in human atherosclerotic plaques (8, 9). Jaffer et al. (4) have also demonstrated that cathepsin K activity colocalizes with calcified atherosclerotic lesions in murine carotid arteries. Together, these suggest that a microcosm for the classical bone remodeling unit or “bone multicellular unit,” similar to that observed in remodeling bone, is present in calcific atherosclerosis.

In the skeletal bone-remodeling unit, osteoblasts regulate osteoclast differentiation and activity through receptor activator of nuclear κB ligand (RANKL), osteoprotegerin (OPG), and cytokines, such as interleukin-6 (IL-6). RANKL induces osteoclast maturation by binding to its receptor, RANK, on osteoclast precursors, whereas OPG, a soluble decoy receptor for RANKL, inhibits osteoclast formation by competing with RANK (10).

Several studies suggest that RANKL and OPG also play a role in the vasculature. Serum OPG levels correlate with the severity of coronary disease and rate of atherosclerosis progression (11–16). OPG knock-out mice exhibit both arterial calcification and osteoporosis as well as accelerated progression of atherosclerotic lesions and calcification (17, 18). We previously found that RANKL expression is increased in calcified cartilaginous metaplasia in atherosclerotic lesions of hyperlipidemic mice (19). RANKL expression is also increased by minimally modified low-density lipoprotein in T lymphocytes (20). Kaden et al. (21) have previously shown that treatment of human aortic valvular cells with soluble RANKL promotes osteoblastic differentiation

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§ The abbreviations used are: RANKL, receptor-activator of nuclear κB ligand; SMC, smooth muscle cell; OPG, osteoprotegerin; PAPC, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; TRAP, tartrate-resistant acid phosphatase.

** This is a preliminary report.
PKA Induction of RANKL in Vascular Cells

and matrix calcification. Panizo et al. (22) have recently demonstrated that NF-κB/BMP4 mediates the matrix calcification in vascular smooth muscle cells (SMC). Whereas these studies indicate a possible role of the RANKL/OPG system in vascular disease, it is not known whether expression of RANKL/OPG in vascular SMC is regulated by the PKA pathway.

We and others (3, 23, 24) have shown that the protein kinase A (PKA) pathway induces vascular calcification both in vitro and in vivo. In this report, we investigated whether RANKL couples vascular osteoblastogenesis to vascular osteoclastogenesis by mediating both PKA-induced osteogenesis and smooth muscle cell-mediated osteoclastogenesis. Our results suggest that PKA induces RANKL expression in vascular SMC, promoting osteoclastic differentiation of vascular monocyte/macrophages, however PKA-induced matrix calcification of vascular SMC is through a RANKL-independent mechanism.

EXPERIMENTAL PROCEDURES

Materials—Forskolin and H89 were from Calbiochem. Y-27632 was obtained from Cayman Chemical, and protein kinase inhibitor (PKI) 14–22 amide, myristoylated was from Tocris Bioscience. Antibodies to GAPDH and tubulin were from Cell Signaling. Human OPG, antibodies to mouse RANKL and OPG were from R&D Systems, and murine TNF-α was from Peprotech. Ox-PAPC was prepared by auto-oxidation of PAPC (1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine; Avanti-Polar Lipids). The oxidation state was verified by liquid chromatography/mass spectroscopy.

Cell Culture—Murine aortic cells (passages 4–8) were isolated from thoracic aortic specimens, as previously described, and the cells were confirmed to be immunoreactive for smooth muscle α-actin (Dako Corp) but not for endothelial markers (von Willebrand factor, Dako Corp) (23). Cells were treated with the indicated reagents in α-minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin, streptomycin, and sodium pyruvate. Media were replaced with fresh agents every 3–4 days. Media were supplemented with β-glycerol phosphate (5 mM) for matrix calcification cultures. Cells were pretreated with inhibitors (H89, PKI, and Y27632) for 1 h, followed by cotreatment with inhibitors and mTNF-α for an additional 6 h.

T lymphocytes were purified from human peripheral blood mononuclear cells (PBMC) by immunomagnetic depletion using a mixture of biotinylated mAbs against CD14, CD16, CD19, CD56, CD36, CD123, CD235a, which were subsequently labeled with anti-biotin microbeads (Miltenyi Biotec). Purity of T lymphocytes (>98%) was confirmed by flow cytometry (Beckton Dickinson) using anti-CD3 antibody (BD Pharmingen). Cell viability following purification was consistently assessed by trypan blue exclusion. Cells were plated at a concentration of 1 × 10⁶/ml in 5% RPMI media (Invitrogen), supplemented with IL-2 (20 units/ml). For experiments testing activated cells, the T lymphocytes were stimulated with 3 μg/ml PHA (Sigma).

Co-culture—Murine aortic SMC were pretreated at confluence with forskolin for 2 days. RAW264.7/C4 (25), a subclone of murine macrophage cell line RAW264.7, were added without forskolin and with or without human OPG. After 2 days, cells were restimulated with forskolin for 2 days. Tartrate-resistant acid phosphatase (TRAP) staining was performed 6–8 days after the addition of RAW264.7 cells. Neither exogenous RANK nor RANKL was added to monocultures or cocultures.

RNA Isolation and Real-time RT-qPCR—Total RNA was isolated using TRIzol reagent (Invitrogen). Real-time PCR was performed using the One-Step qRT-PCR SuperMix Kit (BioChain, Inc.) and Mx3005P (Stratagene).

Western Analysis—Western analysis of whole cell lysates was performed using standard protocols.

ELISA—Confluent aortic SMC were treated for 24 h in serum-free, phenol red-free DMEM (Sigma Aldrich). Secreted interleukin-6 (IL-6) protein levels in cell media were measured using Quantikine mouse IL-6 immunosassay kit (R&D Systems), following the manufacturer’s protocol. Assays were performed in triplicate.

Tartrate-resistant Acid Phosphatase Staining—Cells were washed once with PBS, fixed in 10% formalin for 10 min, permeabilized with 0.1% Triton X-100 for 1 min, and incubated with substrate solution, naphthol AS-BI phosphate (Sigma), in the presence of 50 mM sodium tartrate at 37 °C for 10 min. Resulting mononuclear and multinuclear TRAP-positive cells were visualized under light microscopy and quantified.

Alkaline Phosphatase Activity—Alkaline phosphatase activity was assessed colorimetrically and normalized to total protein (Bradford assay), as described previously (3). The assay is performed in quintuplicate.

Matrix Calcification—Matrix calcium levels (normalized to total protein) were analyzed by the o-cresolphthalein complexone method (Teco Diagnostics) in quintuplicate (23).

Data Analysis—Experiments (>triplicate wells) were performed ≥3 times, and data are expressed as mean ± S.E. Results were compared using a two-tailed, Student’s t test. In comparisons across more than two groups, two-way ANOVA, followed by Fisher’s PLSD, was performed. p < 0.05 was considered statistically significant.

RESULTS

Effects of Forskolin on SMC Expression of Osteoclast Regulatory Cytokines—To examine the effects of forskolin on expression of osteoclast regulatory cytokines, aortic SMC were treated with forskolin for the indicated times, and RANKL, OPG, and IL-6 mRNA expression was assessed by real-time RT-qPCR. As shown in Fig. 1, A–C, forskolin markedly induced expression of the two stimulatory cytokines, RANKL and IL-6, but did not significantly alter expression of OPG, an inhibitor of RANKL. Western analysis and ELISA showed that forskolin also significantly up-regulated protein levels of RANKL and IL-6, but not OPG (Fig. 2, A–C), consistent with the pattern of mRNA induction.

Effects of Forskolin-treated SMC on Osteoclastic Differentiation of RAW264.7 Cells—To test whether induction of osteoclastogenic cytokines in SMC would stimulate differentiation of preosteoclasts, cells from a murine preosteoclast cell line, RAW264.7, were co-cultured with forskolin-pretreated or control-treated SMC and assayed for osteoclastic differentiation by TRAP staining. As shown in Fig. 3, A and B, the number of
TRAP-positive osteoclasts was increased in forskolin-treated versus control SMC. The forskolin-induced increase in TRAP-positive osteoclasts was blocked by treatment with OPG (Fig. 3, C and D). Consistent with our previous findings (26), the control vascular SMC in co-culture significantly reduced the number of TRAP-positive osteoclasts compared with RAW cell monoculture (Fig. 3, C and D). In the RAW cell monoculture in the absence of exogenous RANKL, both forskolin and OPG did not significantly alter the number of basal TRAP-positive cells (Fig. 3, C and D).

**Effect of Atherogenic Agents on Vascular Cell Expression of Osteoclast Regulatory Cytokines**—Because osteoclast-like cells have been observed in atherosclerotic lesions (8, 9), we next examined whether atherogenic agents, TNF-α and oxidized phospholipids, regulate expression of osteoclastogenic cytokines in SMC. TNF-α significantly induced expression of RANKL and IL-6 mRNA by 4.6 ± 0.8- (p < 0.05) and 3.2 ± 2-fold (p < 0.005), respectively, in SMC. Interestingly, TNF-α also significantly induced mRNA expression of OPG by 1.6 ± 0.2-fold (p < 0.05). Oxidized phospholipids did not induce RANKL expression (data not shown). H89, a PKA inhibitor, partially attenuated TNF-induced RANKL and OPG but not IL-6 (Table 1). However, a more specific PKA, PKI, did not attenuate TNF-induced RANKL expression (Table 2). A Rho-associated kinase II (ROCK-II) inhibitor, Y27632, also failed to
attenuate TNF-induced RANKL expression (data not shown), suggesting it is governed by an H89-sensitive protein kinase other than PKA or ROCK-II, such as mitogen- and stress-activated protein kinase-1 or S6 kinase 1 (27).

Because RANKL-positive T lymphocytes are also present in atherosclerotic lesions (19), we next examined whether human T-lymphocyte RANKL expression is affected by bioactive oxidized phospholipids found in atherosclerotic lesions. As shown in Fig. 4A, oxidized phospholipids (ox-PAPC) had a statistically significant but small induction on RANKL expression in T lymphocytes. Interestingly, pretreatment with H89 did not attenuate ox-PAPC-induced RANKL expression by T lymphocytes (data not shown). Ox-PAPC had a non-statistically significant trend toward a decrease in OPG expression (Fig. 4B).

**Effect of RANKL Inhibition on PKA-induced Osteoblastic Differentiation**—Because PKA activation induces osteoblastic differentiation and matrix calcification by aortic smooth muscle cells, and, as others have reported that RANKL in-

FIGURE 3. Effects of forskolin on osteoclastic differentiation of RAW264.7 cells. A, TRAP cytochemical staining of murine aortic SMC that were treated with control vehicle or forskolin (Fsk; 25 μM), and co-cultured with RAW 264.7 cells for 8 days. Magnification ×400. B, average number of TRAP-positive cells/well from co-cultures treated with control vehicle or forskolin (Fsk, 25 μM) for the indicated times. C, average number of TRAP-positive cells/well (arrow) from RAW264.7 monoculture or RAW264.7 and murine aortic cell cocultures that were treated with control vehicle, forskolin (25 μM), OPG (200 ng/ml), as indicated, for 8 days. D, TRAP cytochemical staining of murine aortic SMC that were treated with control vehicle (a and e), forskolin (b and f; 25 μM), forskolin + OPG (c and g; 200 ng/ml), OPG (d and h; 200 ng/ml), and co-cultured with RAW 264.7 cells for 8 days. Arrows indicate TRAP-positive cells. Magnification ×400. *, p ≤ 0.0001; **, p < 0.005; N.S., statistically nonsignificant. Refer to “Experimental Procedures” for detailed treatment protocol.
duces matrix calcification in aortic valvular cells, we tested whether the osteogenic effects of forskolin are mediated through RANKL in these smooth muscle cells. As shown in Fig. 5, RANKL inhibition with OPG minimally attenuated forskolin-induced alkaline phosphatase activity, a pro-calcification factor. To test whether the small inhibitory effect on alkaline phosphatase activity affects matrix calcification induced by forskolin, matrix calcium was assessed. As shown in Fig. 5C, OPG failed to attenuate forskolin-induced matrix calcification.

DISCUSSION

This study demonstrates that PKA activation in murine vascular SMC induces expression of osteoclastogenic cytokines, including RANKL, and that PKA-treated vascular cells upregulate osteoclastogenesis in adjacent preosteoclasts (Fig. 6A). Because monocyte/macrophages are known precursors of...
osteoclasts, and they are present in large numbers in atherosclerotic plaque (19), the results suggest that PKA activation may promote remodeling of ectopic bone in the vasculature, as it does in skeletal bone.

T lymphocytes, which are found in close proximity to calcium mineral deposits in atherosclerotic lesions (19), are a known source of RANKL (20, 28–30). In this study, we found that T lymphocytes were induced to express increased RANKL in response to specific oxidized phospholipids that are associated with atherosclerotic plaques. This RANKL induction appears to be modest but it suggests an additional source of RANKL in the vasculature. The findings are also consistent with our previous study that shows induction of RANKL in T lymphocytes by minimally modified lipoprotein particles (20), of which ox-PAPC is a biologically active, phospholipid component (31, 32). These findings suggest that ox-PAPC is a molecular component that is largely responsible for biological activities of minimally modified lipoproteins.

We also investigated whether RANKL induction mediates PKA-induced matrix calcification since previous studies have shown that RANKL promotes matrix calcification of SMC and human cardiac valvular cells (21, 22). Our data showed that RANKL inhibition had a minimal inhibitory effect (<10%) on PKA induction of alkaline phosphatase activity, which promotes matrix calcification. Although statistically significant, it appears to be too small to have a biologically significant effect on calcification. Indeed, RANKL inhibition failed to attenuate PKA-induced matrix calcification. Thus, RANKL has a limited role, if any, in PKA-induced matrix calcification in vascular cells (Fig. 6B).

It has been shown that vascular smooth muscle cells and skeletal osteoblasts respond oppositely to the same stimuli, such as oxidized phospholipids and continuous activation of the PKA pathway (as in hyperparathyroidism) (3, 24, 33–37), i.e. these factors promote osteoblastic differentiation of vascular cells but inhibit osteoblastic differentiation of bone cells. Interestingly, our results now show that PKA agonists may promote osteoclastogenic factor expression in both vascular and bone stromal cells (38). The increase in osteoclast differentiation by PKA activation suggests that a microcosm of bone exists in vascular ossification with cells responsible for bone formation and resorption similar to the bone-remodeling unit, as evidenced by the presence of both osteoblast and osteoclast-like cells in calcific vasculopathy (4, 8, 9, 19, 39). Yet, vascular ossification appears to consistently progress despite abundance of monocyte-macrophages, precursors of osteoclasts, in atherosclerotic lesions, indicating an imbalance of bone remodeling favoring formation over resorption in the vascular milieu.

Several factors could account for this imbalance. Aortic smooth muscle cells secrete high levels of osteoclast inhibitory factors and low levels of osteopontin, an osteoclast adhesion factor (26, 40). While in smooth muscle cells, atherogenic agents promote osteoblastic differentiation and matrix calcification (33, 41), they may have limited impact on smooth muscle for cytokine secretion. For example, oxidized phospholipids did not induce RANKL expression in vascular smooth muscle cells. Non-atherogenic factors also inhibit osteoclasts in the vasculature; Mozar et al. (42) found that inorganic phosphate inhibits osteoclastic activity by inhibiting JNK and Akt activation. Combined with low expression of osteoclast activators, and secretion of osteoclast inhibitory factors, vascular cells may limit osteoclast formation thereby promoting a milieu favoring progression of ossification.

At present, there are no known therapies for vascular calcification, which contributes substantially to worldwide morbidity and mortality, through clinical cardiovascular disease, including heart failure, hypertension, coronary insufficiency, left ventricular hypertrophy, and myocardial infarction. In the absence of successful preventative measures, the only alternative is to find a way to reverse vascular calcification, most likely through cell-mediated resorption. Although bone marrow-derived osteoclasts have been reported to resorb mineral from calcified elastin (43), the ability of osteoclasts to differentiate and function within the vascular milieu are not thoroughly ascertained. Up to now, it has not been clear which cell types near vascular mineral deposits express RANKL and/or whether its expression in vascular cells is inducible.

In this report, we are the first to show that vascular SMC can be induced to express RANKL, opening the possibility of therapeutic cell-mediated regression of vascular calcification through RANKL induction in vascular SMC. If osteoclastic activity is inducible in human arteries, it raises the potential for therapeutic regression of human vascular calcification, a cellular therapy that could reduce vascular stiffness and heart failure. Further studies are necessary to develop cell-based therapies for calcified and ossified arteries.

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