Balanced Mineralization in the Arterial System – Possible Role of Osteoclastogenesis/Osteoblastogenesis in Abdominal Aortic Aneurysm and Stenotic Disease –

Dai Yamanouchi, MD, PhD; Yuichiro Takei, PhD; Kimihiro Komori, MD, PhD

Arterial calcification is the result of the same highly organized processes as seen in bone, which rely on a delicate balance between osteoblasts and osteoclasts. Although previously understood as passive precipitation, evidence has accumulated to suggest that arterial calcification is the result of organized, regulated processes bearing many similarities to osteogenesis in bone, including the presence of subpopulations of arterial wall cells that retain osteoblastic lineage potential. These cells have the potential to form mineralized nodules and express osteoblast markers, including bone morphogenetic protein-2, osteocalcin, osteopontin, and alkaline phosphatase. By contrast, osteoclast-like cells mediate the catabolic process of mineral resorption. Recent data shows that cells positive for tartrate-resistant acid phosphatase, a major marker for osteoclasts, have been histologically identified in atherosclerotic lesions and are referred to as osteoclast-like cells. Evidence has accumulated to suggest that initial arterial calcification through passive precipitation of calcium phosphate initiates balanced mineralization regulated by osteoclast-like and osteoblast-like cells. Subsequently, various pathogenic conditions may trigger an imbalance between osteoblastogenesis and osteoclastogenesis, leading to either calcification in stenotic/occlusive disease or destruction of the extracellular matrix in aneurysmal disease. Further elucidation of these newly emerging concepts could lead to a novel therapeutic approach to arterial stenotic/occlusive disease and/or abdominal aortic aneurysm. (*Circ J* 2012; **76**: 2732–2737)

Key Words: Aneurysms; Aorta; Calcium

Cardiovascular disease is widely recognized as the leading cause of death in the United States. A lesser-known fact is that abdominal aortic aneurysm (AAA) was once the 10th leading cause of death in men over the age of 55.¹ Approximately 15,000 patients die of ruptured AAA or dissections each year,^{2,3} and surgical repair remains the most effective approach to preventing deaths or complications resulting from a ruptured aneurysm. However, it is estimated that 300,000 persons per year die suddenly without receiving medical care,⁴ and studies have shown that the incidence of ruptured AAA in cases of sudden death ranges from 4% to 5%.^{5–7} Thus, the death rate from ruptured AAA could be as high as 30,000 per year, with a significant portion of these patients never reaching the hospital. This reasoning raises the estimated yearly AAA mortality rate to a level comparable to prostate or breast cancer, thus establishing the urgent need for alternative therapeutic strategies. This review will summarize recent advances in our knowledge of balanced mineralization in the arterial system and discuss its possible role in aneurys-

mal degeneration.

Association Between Arterial Calcification, Diabetes Mellitus (DM), Chronic Kidney Diseases (CKD), and Osteoporosis

Ectopic calcification is a phenomenon in which precipitation of calcium (Ca) and inorganic phosphate (Pi) occurs in extracellular tissues such as lung, intestine, kidney, and cardiovascular tissues.⁸ In the cardiovascular system, deposition of Ca and Pi result in the accumulation of calcium phosphate crystals, the loss of elasticity of the vessel, and a decrease in vessel compliance, thus leading to increased cardiovascular morbidity and mortality.⁹ Arterial calcification can be classified into 2 types: (1) calcification of the medial layer, also known as Mönckeberg's sclerosis, and (2) calcification of the atherosclerotic plaque within the intimal layer of the artery.¹⁰ Mineral deposits contribute to plaque destabilization and ex-

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Division of Vascular Surgery, Department of Surgery, University of Wisconsin School of Medicine and Public Health, Madison, WI (D.Y., Y.T.), USA; Division of Vascular Surgery, Department of Surgery, Nagoya University Graduate School of Medicine, Nagoya (K.K.), Japan

Mailing address: Dai Yamanouchi, MD, PhD, Assistant Professor, Division of Vascular Surgery, Department of Surgery, University of Wisconsin School of Medicine and Public Health, 600 Highland Avenue, CSC G5/325, Madison, WI 53792, USA. E-mail: yamano@surgey.wisc.edu

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Table. Upregulated Factors in AAA Related to Osteoclastogenesis

Upregulated factors	Method	Author
IL-6, NFκB, AP-1	AAA vs. control aorta; qPCR, ELISA, western blotting	Abdul-Hussien H et al ⁷⁸
Sclerostin	AAA vs. control aorta; Microarray, qPCR	Lenk GM et al ⁷⁹
IL-6, IL-8, MCP-1, MCP-2, CCL5/RANTES TNF-α, oncostatin M	AAA vs. control aorta; Antibody-based protein array	Middleton RK et al ⁸⁰
IL-6, IL-8, NFκB, AP-1, STAT3	AAA vs. stenotic aorta; ELISA, PCR, western blotting, IHC	Lindeman et al ⁸²
Vitronectin, vitamin D binding protein	AAA expansion rate; 2D-PAGE, mass spectrometry	Urbonavicius S et al ⁸¹
IL-6, IL-8	Ruptured AAA vs. AAA; Microarray, qPCR	Choke E et al ⁸²
IL-8, VEGF, oncostatin M receptor	Ruptured AAA vs. AAA; Microarray, qPCR	Choke E et al ⁸³

AAA, abdominal aortic aneurysm; ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemistry; IL, interleukin; MCP, monocyte chemotactic protein; NFκB, nuclear factor κ B; qPCR, quantitative polymerase chain reaction; VEGF, vascular endothelial growth factor.

acerbate occlusion. To date, it has been reported that both atherosclerotic and medial calcification are frequently observed in patients with DM,^{11,12} CKD,^{13–15} and osteoporosis,¹⁶ and possibly correlated among these diseases.

Many basic and clinical studies have found a possible association between DM, CKD, and osteoporosis. It is well known that DM induces diabetic nephropathy, which eventually proceeds to CKD.¹⁷ As implied by the term “chronic kidney disease-mineral bone disorder (CKD-MBD)”, there is a strong correlation between CKD and osteoporosis.¹⁸ In DM, both type I and type II are reported to be associated with increased risks of osteoporosis and bone fracture, although some clinical data suggest that bone mass increases in patients with type II DM.^{19,20} It is also a well-known fact that one of the common pathogenic presentations in patients with DM, CKD, or osteoporosis is arterial calcification, and several reports have elucidated the molecular mechanisms underlying such calcification. Serum levels of advanced glycation endproducts (AGEs), known pathogenic molecules caused by hyperglycemia in DM, are reported to be upregulated in nondiabetic CKD patients and osteoporosis patients in comparison with age-matched healthy subjects.^{21,22} A recent study has also shown that carboxymethyl-lysine, a major immunogen of AGEs, accelerates atherosclerotic calcification under high-lipid conditions through increased osteogenic differentiation of aortic smooth muscle cells (SMCs) with upregulation of bone-related genes.²³ Furthermore, AGEs induce the phosphorylation of p38 MAPK through the receptor for AGE and accelerate the calcification of vascular SMCs (VSMCs).²⁴ A receptor activator of the nuclear factor κ B ligand (RANKL)-RANK/osteoprotegerin (OPG) signaling has also been shown to play a major role in arterial calcification. Although RANKL is a key molecule in osteoclastogenesis, but not in osteoblastogenesis, several recent studies have found that RANKL induces VSMC calcification.^{25–27} Deficiency of OPG, a decoy receptor of RANKL, leads to medial calcification in the aorta and renal arteries, accompanied by osteoporosis, in OPG-null mice.²⁸ The serum RANKL/OPG ratio is increased in type I DM patients and diabetic Charcot neuroarthropathy patients.^{26,29}

The underlying mechanism of aortic valve calcification shares a similar pathogenesis with atherosclerotic artery calcification, and aortic valve calcification is also recognized as a manifestation of systemic atherosclerosis.³⁰ In the early stages of aortic valve calcification, pro-inflammatory monocytes and macrophages are recruited and infiltrate via activated endothelial cells into the intimal layer. Proteolytic enzymes, such as matrix metalloproteinases (MMPs) and cathepsins derived from activated macrophages, subsequently degrade the elastin matrix. This matrix remodeling causes the proliferation of valvular

myofibroblasts. These activated macrophages also release cytokines, tumor necrosis factor (TNF)-α, transforming growth factor-β, and insulin-like growth factor 1, resulting in the activation of osteoblast-like differentiation and mineralization of myofibroblast-like SMCs.^{31–33}

Molecular Mechanism of Vascular Calcification

Although previously understood to be passive precipitation, accumulating evidence now suggests that arterial calcification is the result of organized, regulated processes bearing many similarities to osteogenesis in bone, including the presence of subpopulations of arterial wall cells that retain osteoblastic lineage potential. These cells are referred to as osteoblast-like cells and, under selective stimuli, have the potential to form mineralized nodules and express osteoblast markers, including bone morphogenetic protein (BMP)-2, osteocalcin, osteopontin, and alkaline phosphatase.^{34,35} Osteoblast-like cells are sparse, if not nonexistent, in the normal intimal and medial layers of the artery, but become more numerous in atherosclerotic areas.^{31,36} Among many other factors associated with vascular calcification, the most notable is runt-related transcription factor (Runx) 2, also known as core binding factor-α1,^{37,38} which was first identified as an essential transcriptional factor in osteoblast differentiation.^{39,40} During the early stages of this process, Runx2 regulates major bone matrix protein gene expression (ie, type I collagens, osteocalcin, and fibronectin) and induces osteoblast differentiation of bone marrow stromal cells.⁴¹ Several reports have demonstrated that this transcription factor is expressed in atherosclerotic calcified plaques and medial calcific lesions in uremic mice^{29,42,43} and also plays a key role in the development of human VSMC calcification.^{38,44,45} It has been documented that several factors, such as BMPs, oxidative stress, and a high phosphate concentration, exacerbate VSMC calcification via induction of Runx2 gene expression.^{46–49} In addition, VSMC-specific, Runx2-deficient mice exhibit inhibition of atherosclerotic calcification under high-fat, diet-fed, apoE-deficient conditions.⁵⁰ Recently, it was reported that BMP-2 enhances Pi uptake via upregulation of expression of the type III sodium-dependent phosphate cotransporter Pit-1 and calcification via increasing Runx2 gene expression in VSMCs.⁴⁶ Matrix GLA protein (MGP), which inhibits the activity of BMPs,^{51,52} has also been recognized as a potent inhibitor of arterial calcification. Indeed, patients with Keutel syndrome, which is caused by mutations in the gene encoding human MGP, exhibit medial arterial calcification.⁵³ Moreover, MGP-deficient mice show accelerated arterial calcification and die from rupture of the aorta.⁵⁴

Figure 1. Calcium chloride-induced aneurysm created through the application of 0.5 mol/L CaCl_2 solution to the mouse infrarenal abdominal aorta peri-adventitially in the CaCl_2 experimental group. Calcium phosphate (CaPO_4) crystals were manually precipitated by the additional application of phosphate-buffered saline after conventional CaCl_2 treatment in the CaPO_4 experimental group. Sodium chloride (NaCl , 0.5 mol/L) served as a control. **(A, C)** Accelerated aneurysmal dilation identified in the CaPO_4 -induced aneurysm compared with a conventional CaCl_2 -induced aneurysm with accelerated calcification. **(B)** Elastica van Gieson staining of the aorta from both groups of mice. Accelerated fragmentation of the elastin in the media was identified in CaPO_4 -induced aneurysm. Scale bar=100 μm ; fold change=maximum diameter/pre-surgery diameter, $n=6$. (Modified from Yamanouchi et al with permission.⁷⁶)

Osteoclastogenesis and Arterial Calcification

In most regulated biological systems, anabolic processes are accompanied by counteracting catabolic processes. In vascular calcification, osteoclasts mediate the catabolic process of mineral resorption. Osteoclasts are large, multinucleated cells that degrade bone tissue by absorbing its mineralized matrix and have been reported to be derived from the monocyte lineage of hematopoietic cells largely by the stimulation of macrophage colony-stimulating factor and RANKL.^{55,56} Recent studies report show cells positive for tartrate-resistant acid phosphatase, a major marker for osteoclasts, have been histologically identified at the edges of bone-like structures in atherosclerotic lesions.^{57,58} The presence of mature osteoclasts in the arterial wall is still controversial, and thus cells with osteoclast features have been labeled as osteoclast-like cells.⁵⁹ Interestingly, macrophages exposed to particulate calcium mineral have been reported to undergo osteoclastic differentiation. Thus, atherosclerotic lesions, being rich in monocytes and macrophages, are an abundant source of potential pre-osteoclasts or osteoclast-like cells. Although their exact role in the arterial system is not fully understood, studies have already suggested that the osteoclast is a source of proteases, including MMPs.^{60,61}

AAA and Osteoclastogenesis

Although severe calcifications are abundant in the aortas of patients with occlusive disease, a thin layer of calcification is also seen in the arterial wall in AAA. By utilizing cDNA microarrays, quantitative polymerase chain reaction, and protein analysis (antibody-based protein array, western blotting, enzyme-linked immunosorbent assay, and immunohistochemistry), many investigators have reported possible factors differentiating aneurysmal aorta from non-aneurysmal aorta or from occlusive disease. Proteins are mainly categorized as pro-inflammatory cytokines/chemokines, growth factors, intracellular signaling proteins, or matrix proteins. Although never before summarized, many factors reported to be upregulated in AAA have also been shown to act as differentiation factors in osteoclastogenesis (Table). Interleukin (IL)-6, an inflammatory cytokine secreted by SMCs in the arterial wall of aneurysms,⁶² is also secreted by osteoblasts and is a potent stimulator of osteoclast-like cell formation and activation.⁵⁶ Sclerostin, produced downstream of IL-6, downregulates osteoblastogenesis.⁶³ TNF- α , a multifunctional cytokine mainly produced by macrophages, has been shown to drive the differentiation of monocyte/macrophage lineage cells to osteoclast-like cells and to stimulate maturation of these cells with the help of IL-1.⁶⁴ Among the growth factors upregulated in AAA, vascular endothelial growth factor (VEGF) has been

reported to enhance the survival, differentiation, maturation, and resorptive activity of osteoclasts.⁶⁵

Several experimental models of aneurysm have been utilized, including chemically induced aneurysms induced by intraluminal infusion of elastase and the periaortic application of calcium chloride (CaCl_2). Aneurysmal changes in the carotid artery of hyperlipidemic rabbits resulting from periaortic application of CaCl_2 was first described by Gertz et al.⁶⁶ This method was further expanded and applied to the abdominal aortas of hyperlipidemic rabbits in combination with thioglycollate, as reported by Freestone et al.⁶⁷ and subsequently adapted to mice.^{68,69} Similar to elastase-induced mouse aneurysms, CaCl_2 -induced aneurysmal dilation is accompanied by the degeneration of medial SMCs with elastin degradation, infiltration of lymphocytes and macrophages, elevation of pro-inflammatory cytokines, and the increased activation of MMPs.^{68–70} We have also identified significant calcification in CaCl_2 -treated arteries, mainly in the medial layer. We hypothesized that this calcification, which mainly consists of calcium phosphate (CaPO_4) crystals, also contributes to aneurysm formation and have explored its possible role. CaPO_4 has been identified as the major component of calcification found in atherosclerosis^{71–73} and has been suggested as having significant pro-inflammatory effects, inducing apoptosis of various cell types and stimulating production of pro-inflammatory cytokines in monocytes and macrophages and SMCs.^{74,75} We tested whether CaPO_4 is a more potent than CaCl_2 in stimulating apoptosis and inducing inflammatory cytokines. Our data suggested that CaPO_4 formation through sequential application of CaCl_2 and phosphate-buffered saline (PBS), induced apoptosis of VSMCs. A detailed time course study in a mouse model showed a relationship between calcification and monocyte and macrophage accumulation by histological and im-

munochemical analyses. We found similar spatial and temporal patterns between CaPO_4 deposition and monocyte and macrophage accumulation after CaPO_4 injury. Finally, we have shown a rapid and robust acceleration of this aneurysmal degeneration by precipitating CaPO_4 compared with the conventional CaCl_2 model (Figure 1).⁷⁶ Furthermore, we have preliminarily shown a possible association between osteoclastogenesis and AAA through histological study of human AAA tissue and the mouse AAA model.⁷⁷ That association was further validated by the inhibition of osteoclastogenesis using bisphosphonate, which resulted in the suppression of AAA in mice.⁷⁷ Although these findings require further validation, the possible mechanism of arteriosclerosis, stenotic/occlusive disease, and aneurysmal disease through a pathogenic imbalance between osteoblastogenesis and osteoclastogenesis was indicated. As shown in Figure 2, we believe that the pathogenic reaction of osteoblastogenesis or osteoclastogenesis to ectopic calcification in the arteriosclerotic artery may lead to stenotic/occlusive disease or aneurysmal disease, respectively. Although not fully understood nor evidenced yet, we currently speculate that the abundant inflammation found in the aneurysmal artery compared with the stenotic artery initiates macrophage infiltration/activation, leading to the initiation of osteoclastogenesis in the aneurysm. Further studies focusing on the differentiating factors of osteoclastogenesis/osteoblastogenesis and characterization of those cells in the arterial system are warranted.

Conclusions

We have provided an overview of the current knowledge of ectopic calcification and the molecular mechanisms of balanced mineralization and pathogenic acceleration of osteo-

blastogenesis and osteoclastogenesis in arteries. We have also described our preliminary reports, which indicate a possible role of osteoclastogenesis in the pathogenesis of aneurysms. Although careful validation has yet to be performed, we believe that ectopic calcification through the passive precipitation of CaPO_4 crystals initiates balanced mineralization in the arterial wall, regulated by osteoclast-like and osteoblast-like cells. Subsequently, various pathogenic conditions trigger an imbalance between osteoblastogenesis and osteoclastogenesis, leading to either manifestation of calcification in stenotic/occlusive disease or destruction of the extracellular matrix in aneurysmal disease. Further evaluation of this concept could lead to a novel therapeutic approach to arterial stenotic/occlusive disease and AAAs.

Disclosures

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