Critical Review

Recent Advances in Research on Human Aortic Valve Calcification

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Abstract. Aortic valve calcification can aggravate aortic stenoses, and it is a significant cause of sudden cardiac death. The increasing number of patients with age-related calcification is a problem in developed nations. However, the only treatment option currently available is highly invasive cardiac valve replacement. Therefore, clarification of the etiology of calcification is urgently needed to develop drug therapies and prevention methods. Recent studies have revealed that calcification is not a simple sedimentation of a mineral through a physicochemical phenomenon; various factors dynamically contribute to the mechanism. Further, we are finally beginning to understand the cellular origins of calcification, which had been unclear for a long time. Based on these findings that help to clarify potential drug targets, we expect to establish drug therapies that reduce the stress on patients. In this paper, I introduce the latest findings on cells that are most likely to contribute to calcification and on calcification-related factors that may lead to the development of drug therapies.

Keywords: aortic valve, aortic stenosis, ectopic calcification, stem cell, drug therapy

1. Introduction

In aortic stenosis, the open cross-sectional area of the aortic valve for blood flow is reduced due to thickening and hardening of the valve, which increases the resistance to blood ejection. This resistance leads to reduced aortic pressure that can lead to loss of consciousness (Adams-Stokes syndrome), pulmonary hypertension and/or pulmonary edema, and left heart failure (1). Further aortic valve calcification may exacerbate these symptoms, considerably deteriorating patient health, and is one cause of sudden cardiac death. Unfortunately, no effective treatment that reduces the stress on patients, other than cardiac valve replacement, has been established for this disease (2, 3). Furthermore, the current rapidly aging population has resulted in an increased number of people affected by this disease. Consequently, the development of effective drug therapies and prevention methods is urgently needed (4). Recent studies have demonstrated that aortic valve calcification, an important process in the progression of stenosis, is not purely

physicochemical, i.e., not simply the precipitation of calcium phosphate, but that genes involved in normal bone metabolism play an important role (5, 6). Moreover, while the cellular origin of the calcification was unclear for a long time, attention has now been drawn to several likely candidates, including osteogenic precursor cells and stem cells. Detailed clarification of the involved cells will allow determination of drug therapy targets. In this paper, I introduce the latest findings on the pathology of ectopic calcification, its molecular mechanism, and the cellular makeup of calcifications associated with aortic stenosis.

2. Ectopic calcification

Unlike in osseous tissue, calcification in visceral and soft tissues is not normally expected; when it does develop, it is called ectopic calcification (7). Ectopic calcification was traditionally thought to be caused by an accumulation of concentrated minerals that exceeded their solubility in blood. Therefore, ectopic calcification was perceived as the end stage of affected tissue and was not considered important (8, 9). Although ectopic calcification is considered benign, it is often a cause of various other diseases and patient prognosis is much worse when calcification is present (10, 11). Recently,

studies have made significant progress on elucidating the biology of normal bone metabolism, which has facilitated research on ectopic calcification at the same time. As a result, it is now known that, similar to the processes of normal ossification and calcification, ectopic calcification involves an extremely dynamic mechanism with various contributing calcification and ossification factors, as well as cellular apoptosis (12). This understanding is supported by the fact that the expression of various osteogenic genes is enhanced by cytokines, which are released during inflammation and are considered to be one cause of stenosis (11). In other words, these findings may already be highlighting candidate molecular targets for drug therapies.

3. What is aortic valve calcification?

Earlier cardiovascular studies revealed that mechanical stress considerably enhances ectopic calcification in vascular smooth muscle cells (13). The heart has four valves, and clinically the left side valves (aortic and mitral valves) are more likely to calcify than the right side valves (tricuspid and pulmonary valves) (14). Therefore, it is typically assumed that the left and right valves undergo different mechanical stresses, such as shear stress and blood pressure due to blood flow. Indeed, in vitro data indicate that the mechanical extension of valve tissue leads to increased expression of bone morphogenetic protein 2 (BMP2), an important calcification-related gene that promotes valve calcification (15). Unfortunately, no solid in vivo data supporting this correlation have been reported to date. However, valve calcification clearly has a large impact on the hemodynamics in the aortic valve. When ectopic calcification or ossification develops along with valve cusp scarring, the opening of the aortic valve becomes extremely small, and the extent of narrowing tends to increase with age (16). As a result, the ventricular wall thickens due to the increased stress on the left ventricle, leading to the deterioration of blood flow in the coronary artery. Furthermore, cardiac failure may occur because of damage to the cardiac tissue. In contrast, the in vitro calcification ability of cells isolated from the different cardiac valves is greater in cells derived from the left valves, and therefore the possibility that cell characteristics may differ according to their site of origin should be considered (17). Advanced age is another important factor (18, 19). As Japanese society is rapidly aging, the number of patients is increasing (20). Although treatment methods are improving, invasive valve replacement is still the only treatment option, and the development of a drug therapy that places little stress on the patient is eagerly awaited (21 – 23).

If we consider the inner structure of the aortic valve, starting from the aorta side, it consists of a fibrous layer, spongy layer, and ventricular layer, all of which are covered with an endothelial cell layer. In rheumatic patients, calcification tends to occur in both the aortic and left ventricle sides of the valve cusp (24). In contrast, in elderly patients without rheumatic disease, valve calcification is typically found in the fibrous layer of the aortic side (25). Ankeny et al. conducted a histochemical examination of aortic valves and found that the important mechanisms involved in calcification included the high expression of calcification promoters bone morphogenetic proteins (BMPs) in the aortic side and the reduced expression of BMP inhibitors (e.g., Smad6), and they thought that these mechanisms were worth examining further (26). My research group also found that cells isolated from human aortic valve notably calcify in the presence of the calcification promoter and inflammatory cytokine tumor necrosis factor α (TNF-α) (Fig. 1), via a BMP2-Dlx5-mediated pathway (27). Moreover, we observed that TNF-α reduces the expression of calcification inhibitors (28).

Figure 2 summarizes the current hypothesis of how aortic valve calcification occurs in aortic valve interstitial cells (AVICs). The mechanism includes two pathways: dystrophic calcification via cell death and osteogenic differentiation via a phenotypic change of AVICs (29). The former pathway is epitomized by the failure of bioprosthetic heart valves prepared from porcine aortic valves that have been devitalized by glutaraldehyde pretreatment. This is considered a passive phenomenon because calcification occurs in dead or damaged cells through the reaction of calcium-containing extracellular fluid with the phosphorus-containing membranes of non-functional cells. Therefore, I will focus on osteogenic differentiation in the following sections.

4. What are the cellular origins in calcification?

Cells isolated from the aortic valve through collagenase digestion are called aortic valve interstitial cells (AVICs) (30) because they uniformly take on the appearance of fibroblast-like cells when cultured. It is unclear whether these cells are a uniform cell group in vivo, and their origins, location, and distribution in valve tissue are also unknown. However, AVICs are a useful model for research on calcification. They have the natural ability to cause calcification in long-term culture. AVICs from elderly individuals seem to be more easily calcified than cells from younger individuals, suggesting an effect of aging on the tendency to calcify (31). It has also been reported that these cells promote in vitro calcification through release of inflammatory cytokines

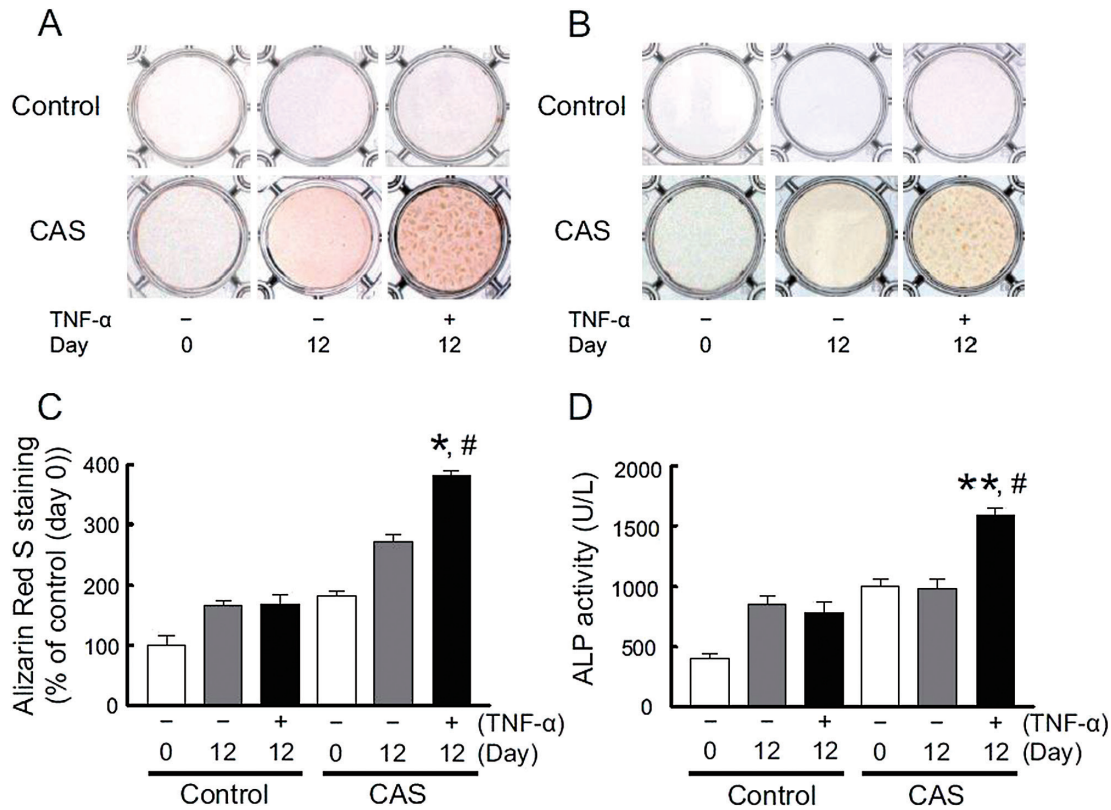**Fig. 1.** TNF-*α* accelerates calcification of AVICs obtained from patients with CAS. AVICs were cultured in medium containing 10% FBS. After reaching confluence (day 0), AVICs were further cultured for 12 days (day 12). A and B: typical images of Alizarin Red S staining (A) and Von Kossa staining (B) of AVICs from a patient with CAS (67-year-old woman) and a control patient (70-year-old man, aortic dissection) in the presence or absence of TNF-*α* (30 ng/ml). C: quantification of Alizarin Red S staining at day 12 via extraction with cetyl-pyridinium chloride. The amount of released dye was quantified by spectrophotometry at 550 nm. All ratios were calculated versus the control group at day 0 as 100%. White bars, day 0; gray bars, TNF-*α* (−) at day 12; black bars, TNF-*α* (+) at day 12. Bars represent the mean ± S.E.M. (control group: n = 4, CAS group: n = 6). Significant differences: *$P < 0.05$ vs. CAS group in the absence of TNF-*α* at day 12; #$P < 0.01$ vs. control group in the presence of TNF-*α* at day 12. D: ALP activity at day 12. The cells were solubilized with 1% Triton X-100 in 0.9% NaCl, and the supernatants were assayed for ALP activity using a commercially available kit (Laboassay ALP). One unit was defined as the activity producing 1 nmol of *p*-nitrophenol for 30 min. All ratios were calculated versus the control group at day 0. White bars, day 0; gray bars, TNF-*α* (−) at day 12; black bars, TNF-*α* (+) at day 12. Bars represent the mean ± S.E.M. (control group: n = 4, CAS group: n = 6). Significant differences: **$P < 0.001$ vs. CAS group in the absence of TNF-*α* at day 12; #$P < 0.01$ vs. control group in the presence of TNF-*α* at day 12. (The figure was from Ref. 27 with permission). ALP: alkaline phosphatase, AVICs: aortic valve interstitial cells, CAS: calcified aortic valve, TNF-*α*: tumor necrosis factor-*α*.

(27, 32). Interestingly, clear differences have been found between AVICs isolated from patients with and without calcification in terms of natural calcification ability and the calcification-promoter reaction against the inflammatory cytokine TNF-α. In patient-derived cells, there was considerable calcification (27). This suggests that isolated AVICs retain their (patho)physiological state from the original tissue, indicating that a genetic or epigenetic mechanism is involved in calcification (33). However, for a more rigorous argument, the cellular origin of AVICs and what type of cell actually calcifies need to be clarified.

Vascular smooth muscle cells and multipotent cells in blood vessel walls may also be involved in calcification via transformation or differentiation into osteoblast-like cells and proliferation (34, 35). Attention has also been drawn to inflammation associated with hyperlipidemia and arteriosclerosis that trigger this transformation. This hypothesis is supported by the fact that statins, lipid-lowering agents, reduce the ossification ability of vascular cells, as well as inflammation (36). However, reports from the latest randomized controlled trial indicate that this effect is not very significant and further studies are needed (2). In addition, hyperphosphatemia is also thought to be a risk factor for valve calcification and should be considered as a trigger for this directed
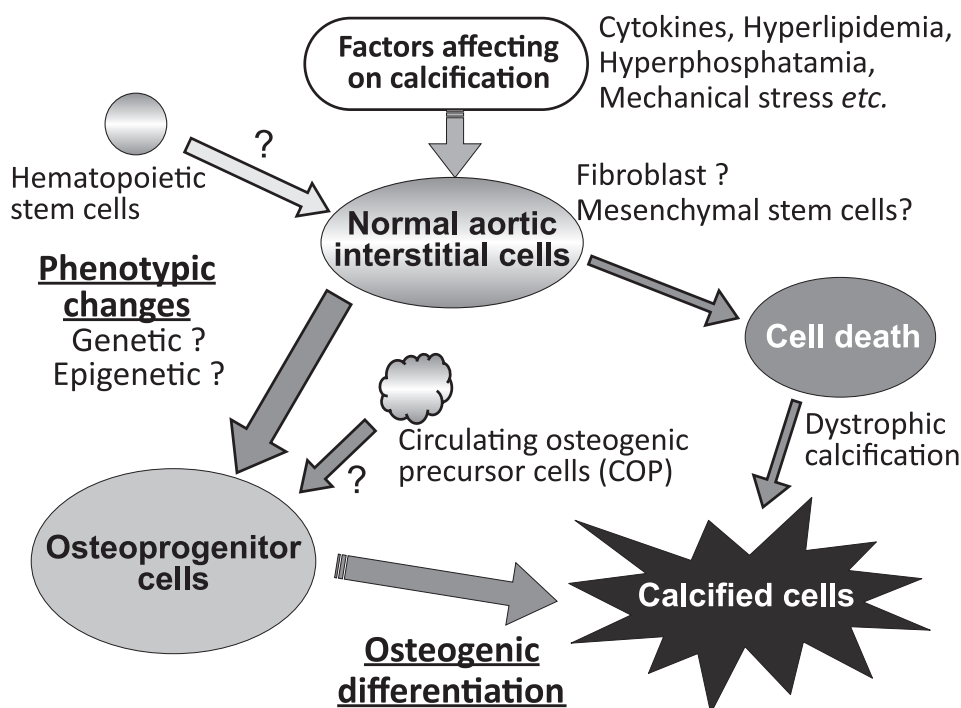
Fig. 2. Schematic diagram of the transformation and calcification of aortic interstitial cells. Aortic valves are thought to be composed of various types of cells including fibroblasts, mesenchymal stem cells, and hematopoietic stem cells. Aortic valve calcification seems to be a multifactorial disease, suggesting that aortic valve interstitial cells (AVICs) could take two different pathways. One is a phenotypic change into osteoprogenitor cells, which may be caused by several environmental and local factors, such as cytokines, mechanical stress, hyperphosphatemia, and hyperlipidemia. Alternatively, circulating osteogenic precursor cells (COP) residing in the aortic valve may play a role as osteoprogenitor cells. Osteoprogenitor cells are calcified through osteogenic differentiation. The factors mentioned above may also evoke cell death in AVICs followed by dystrophic calcification via the other pathway. Such calcification occurs in dead or damaged cells through the reaction of calcium-containing extracellular fluid with the phosphorus-containing membranes of non-functional cells.

differentiation (37).

Mesenchymal stem cells are multipotent, can self-renew, and demonstrate long-term survival. They can be isolated from various tissues (38 – 42). When restoring damaged tissue, it is believed that they essentially provide the precursor cells that reconstitute the tissue. They have thus gained attention as a cellular source for regenerative medicine applications (43). However, it has been suggested that these cells may work in undesirable ways in pathological conditions, such as in aortic valve calcification (44, 45), fibrodysplasia ossificans progressiva (FOP) (46), ectopic ossification in spinal ligaments (47, 48), and ectopic calcification following burn injury (49). Mesenchymal stem cells can cause improper calcification and ossification after differentiating into chondrocyte- or osteoblast-like cells according to environmental and local factors (48 – 50). In recent years, my research group showed that mesenchymal stem-like cells (MSLCs) with the same cell surface markers (CD70$^+$, CD90$^+$, CD105$^+$) as mesenchymal stem cells are present among AVICs and in human aortic valve tissue

(28). We also found that CD34-negative MSLCs are more likely to cause hyperphosphate-induced calcification than CD34-positive MSLCs and were common in calcified valve tissue. This suggests that these cells may influence calcification in that tissue.

Conversely, the presence of hematopoietic stem cells among the interstitial cell population has also been demonstrated (51). If hematopoietic stem cells initiate calcification, then even after the causal relationship between those cells and calcification is clarified, we will need to determine whether these cells are normally present in the valve tissue or if they migrate there from the blood stream around the time of a pathological change. In fact, it has been observed that calcification tends to occur in the valve annulus where there are relatively more blood vessels (52). To this, Suda et al. reported that osteoblast precursor cells, circulating osteogenic precursor (COP), are present in circulating blood, and that they are found in ectopic ossification that develops in tissues throughout the body (53). COP cells are derived from bone marrow and are either

osteocalcin or collagen type-1–positive (OCN^{+}/Col1^{+}). CD45^{+} Col1^{+}, and CD45^{+} OCN^{+} cells were also present in calcified aortic valve tissue (54, 55). Therefore, COP cells may arrive in the aortic valve and contribute to calcification. However, as mentioned above, the MSLCs that we found in the calcified aortic valve were CD45-negative (CD45^{−}) and therefore clearly different cells from COPs. Thus, the interstitial cell populations in the cardiac valve are not uniform and we need to examine whether there is a single cell type in the calcifications. The putative cellular origins of calcification and their roles are summarized in Fig. 2.

5. Factors involved in calcification

Inflammation of the endocardium is assumed to be one cause of aortic stenosis, and it has been suggested that this inflammation promotes activation of osteoblast-like cells (36). We found that TNF-α promotes the calcification of AVICs isolated from calcified valve tissue and then analyzed the signaling pathways responsible (27). We demonstrated that TNF-α causes nuclear translocation of the transcription factor nuclear factor-κB (NF-κB) and, consequently, accelerates production of BMP2, which activates alkaline phosphatase through the Runx5/Dlx5 pathway, resulting in calcification. In contrast, other signaling pathways downstream of TNF-α, such as the MAP kinase pathways, and the Wnt/β-catenin pathway downstream of BMP2 have little involvement. Additionally, the Notch signaling pathway is downstream of TNF-α (35). When the Notch 1 pathway is inactivated, aortic valve stenosis and calcification is enhanced and, therefore, may play a role in inhibiting calcification (56). Alfieri et al. (57) reported that in interstitial cells exposed to culture medium for ossification, Wnt3a provoked nuclear translocation of β-catenin and caused a change in the expression of ossification-related genes. Furthermore, oxidative stress causes AVICs to transform into osteoblasts via the Wnt3a/Lrg5 pathway (58). This demonstrates that downstream signaling may differ according to the type of stimulus for calcification and that we can expect drugs targeting calcification-related signaling to have various effects.

One of the other calcification promoters that have drawn attention is serum phosphate (37). In patients receiving dialysis for renal failure, calcification tends to accelerate as renal functioning decreases (59), and elevated levels of serum phosphate are correlated with the degree of calcification (37, 60, 61). In addition, hyperlipidemia has been observed to correlate with stenosis and valve calcification (62). It has also been pointed out that serum low-density lipoprotein (LDL), especially oxidized LDL, is a risk factor for valve

Table 1. Currently identified calcification promoters and inhibitors

	promoter/inhibitor	Ref.
Promoter	inflammation (TNF-α)	11, 27, 35, 79
	hyperphosphatemia (PiT-1)	36, 62, 63, 76
	hyperlipidemia (oxidized LDL)	62, 63
	mechanical stress (sheer stress, BMP2)	14, 15, 65
Inhibitor	matrix Gla protein	67 – 72
	Notch 1	35, 56, 75
	osteoprotegerin	73 – 75
	NO	76, 77

PiT-1: inorganic phosphate transporter-1, NO: nitric monoxide.

calcification (63). Interestingly, oxidized LDL increases expression of BMP2 (an ossification factor) and PiT-1 (the sodium-phosphate co-transporter). PiT-1 is involved in BMP2-induced mineral decomposition (64), while the PiT-1 inhibitor suppresses the increased expression of BMP2 (64). Therefore, the calcification-inducing action of oxidized LDL is believed to be closely associated with phosphate metabolism. As mentioned above, the phosphate transporter PiT-1 is thought to be involved in both hyperphosphatemia and hyperlipidemia, and has attracted attention as a potential therapeutic target.

Mechanical stress, such as sheer stress associated with blood flow, is thought to play an important role in aortic stenosis by inducing calcium accumulation in AVICs. TGF-β is assumed to be a key player in this phenomenon (65). TGF-β–evoked apoptosis is followed by caspase-independent nodule formation and caspase-dependent calcification of the nodule. Extracellular matrix stiffness modulates the reactivity of AVICs against TGF-β, resulting in the differentiation of AVICs to osteoprogenitor cells and calcification (66). The cause of matrix stiffness remains unclear and should be investigated in order to understand and prevent these pathophysiological changes in AVICs.

A reduction in the activity of intrinsic calcification inhibitors is equally important as enhanced activities of calcification inducers. Matrix Gla protein (MGP) binds to the calcification promoter BMP2 and inhibits its effect (67, 68). The activation of MGP via carboxylation is carried out by vitamin K–dependent enzymes (69). In calcified tissues, the ratio of inactive-to-active (non-carboxylated to carboxylated) forms of MGP and the degree of calcification were reported to be strongly inversely correlated with the vitamin K level (70). The active form of MGP, i.e., carboxylated-MGP, is thought to inhibit calcification in valve tissue. Furthermore, TNF-α, which induces calcification, strongly inhibits the expression of MGP (Seya et al., unpublished observation). Clinical findings that support this idea include

the induction of calcification in cardiovascular tissue due to warfarin, an anticoagulant. Warfarin may be described as a double-edged sword because it inhibits the activation (carboxylation) of vitamin K–dependent blood coagulation factor on the one hand, but also inhibits the activation of MGP at the same time, ultimately enhancing calcification (71, 72). This phenomenon in itself is a major problem than cannot be overlooked in medical treatment. The role of osteoprotegerin as an intrinsic calcification inhibitor has also drawn attention (73) and, more importantly, as an inhibitor of calcification caused by hyperlipidemia (74). Investigations of the calcification mechanism of vascular smooth muscle have revealed that osteoprotegerin inhibits the Notch 1-RBP-JK pathway and, as a result, considerably inhibits the expression of Msx2, a downstream calcification-related transcription factor, ultimately inhibiting vascular calcification (75). We need to examine whether the same mechanism occurs in cardiac valves. In any case, these calcification-related factors are worthy of attention as candidates for drug targeting (Fig. 2).

6. The key to developing drug treatments and prevention of valve calcification

Of existing therapeutic drugs, those that can donate nitric monoxide (NO) are expected to be effective in inhibiting calcification. NO is produced by endothelial cells and has an intrinsic inhibitory action on calcification (76). Kennedy et al. (77) reported that transforming growth factor-β–induced calcification is notably suppressed by NO donors. One group of antianginal drugs works as NO donors, which are designed for coronary vasodilation and may inhibit cardiovascular calcification, making them worth examining as potential analogs for calcification inhibitors. Furthermore, given the close relation between phosphate metabolism and valve calcification, one of the key players is likely the sodium-phosphate cotransporter PiT-1, which may be a promising target for therapeutic drugs (63). However, a known inhibitor of PiT-1, phosphonoformate, has relatively low PiT-1 selectivity (77), but is still expected to be applied in the clinical setting after improvements are made to its specificity through further studies on the structure-activity relationship. TNF-α, an inflammatory cytokine, induces accumulation of calcium not only in the aortic valve (27), but also in the aorta (79). Infliximab, a TNF-α neutralizing antibody, attenuated the accumulation of aortic calcium and decreased the expression of several osteogenic genes in rat aorta. It may be productive to test its relevance to valvular calcification. Furthermore, several signaling pathways downstream of TNF-α may be important. The NF-κB pathway, stimulated by TNF-α,

plays an important role in calcification of cells from human aortic valve (27, 28, 73). Resveratrol (3, 5, 4′-trihydroxystilbene) is a naturally occurring compound that can modulate the risk of cardiovascular degenerative diseases (atherosclerosis) (80) and effectively suppresses NF-κB signaling by inhibiting the activities of NF-κB and IκB kinase (81). Resveratrol and other related compounds with inhibitory effects on NF-κB may become drug candidates for the treatment of valvular calcification.

Studies on a novel drug candidate with anti-calcification effects have drawn attention to tissue non-specific alkaline phosphatases as targets for therapeutic drugs (82, 83). A strong correlation was found between the degree of calcification and alkaline phosphatase activity in a high phosphate medium of isolated AVICs. Narisawa et al. (84) also noticed the importance of this enzyme as a key enzyme in calcification. They built a high throughput system that screened for substances that simultaneously inhibit alkaline phosphatase and calcification in vascular smooth muscle cells and found several very interesting substances. The specific inhibitor of this enzyme has also been considered a promising drug candidate. By expanding the use of this system to AVICs and screening many small molecule substances produced by microbes, we can expect to find new pharmacological agents.

7. Conclusion

Over the past 10 years, the pathophysiological significance of cardiac valve calcification has greatly increased. The inhibition and prevention of the progression of calcification has become indispensable in improving the disease prognosis. It has also been pointed out that osteoblast precursor cells or stem cells may participate in calcification. Clarifying which cells contribute to calcification, as well as the detailed molecular mechanism of calcification, may help new drugs to emerge that target such cells (Fig. 2).

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