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BMP signaling in vascular calcification: A study on the probable pathways of inhibition

Abhipsha Ray *

Dept of Life Sciences, Presidency University, Kolkata, India

ABSTRACT

Vascular calcification causes more than 20,000 deaths each year, and the aortic valve is the most commonly affected due to it. It leads to mineral deposition in the aortic side of heart valves which leads to stenosis, - characterized by the narrowing of the vessel lumen, and often requires surgical valve replacement. Initially this condition was attributed to a passive process of "wear and tear" of the heart but recent studies have confirmed the presence of inflammation, infiltration of lipids, and presence of bone related proteins in affected valves. In human-beings, the aortic valve is a trileaflet structure which is generally divided into an outer layer of Valve Endothelial Cells (VECs) and three internal layers of Valve Interstitial Cells (VICs), less than 5 % of smooth muscle cells and myofibroblasts. Bone Morphogenetic Proteins (BMPs) are members of the Transforming Growth Factor (TGFβ) Superfamily, which play inevitable roles in the maintenance and repair of bone and other tissues in an adult. BMPs were first identified to be potential inducers of ectopic bone formation, after being injected subcutaneously in rats. With respect to the calcification of the heart tissue, their fundamental effect is seen in the smooth muscle cells, which gradually change their phenotype from vascular to synthetic, and lay down the ground for subsequent calcification. Common to the variant processes of vascular calcification is a prominent involvement of BMP-2 paracrine signaling. It may also be noted that the processes of bone formation and atherosclerosis are similar to each other in many instances and hence bring about the possibility of common pathways to combat the two similar conditions. This review article focuses on the effects of BMPs in the control and development of vascular calcification, and the probable pathways that may interfere with or augment this BMP-induced vascular calcification process.

Keywords: Bone Morphogenetic Protein, Calcification, Osteogenesis, Vascular Calcification

1. INTRODUCTION

Cardiovascular calcification is the deposition of calcium phosphate salts in the arterial wall, mainly in the form of hydroxyapatite crystals that is a common consequence of processes like ageing, diabetes and hypercholesterolemia. The main contributors to this process are the vascular smooth muscle cells, endothelial cells and immune cells, which produce matrix vesicles . The process of calcification and its

inhibition is regulated by a set of hormones, morphogens and inhibitors. Depending upon the action of inhibitors like Matrix Gla Protein (MGP), these vesicles undergo osteogenesis or bone formation. Calcification also destroys elasticity of the arteries and hampers with the normal cardiovascular blood flow [1]. Alkaline phosphatase is one of the enzymes present in the vesicles derived from the smooth muscle cells which degrades the locally present inorganic pyrophosphates and thus prevents mineralization. It thus serves as a useful marker of the process of vascular calcification [2]. Recent studies suggest that amongst the paracrine signals provided that mediate the process of calcification, the BMPs play the most crucial role.

BMP signaling is induced in calcified valves in a complex way that involves the interplay of various pathways [3]. When Vascular Smooth Muscle Cells (VSMCs) change their phenotype from contractile to synthetic they proliferate into a state where the expression of smooth muscle cell markers is diminished [4]. VSMCs have active roles in atherosclerosis as they undergo increased migration, proliferation, secretion of matrix vesicles, and subsequent de-differentiation from their contractile state to an osteogenic state [1]. Recent research has shown that turbulent flow patterns on the aortic side of the valve maybe a key initiator of BMP 2/4 secretion from the vascular endothelium [5]. BMPs stimulate calcification by activating Smad signaling, in which they phosphorylate Smads (signal transducing molecules of the TGFβ superfamily). Smad1, Smad5, and Smad8 are receptor-regulated Smads (R-Smads) that are phosphorylated by BMP binding, after which they bind to their common mediator, C-Smad. The R-Smad/C-Smad heterodimer travels to the nucleus to induce transcription and gene expression [6-7]. BMPs also induce calcification by upregulating expression of the transcription factor Msx-2. Both of these mentioned signaling pathways ultimately lead to induce expression of the master osteoblast transcription factor Runx2 [3]. Inhibition of Runx2 by using short hairpin RNA has shown to block the calcification of VSMCs, whereas overexpression of Runx2 by adenovirus mediated expression alone was capable to cause VSMC calcification**.** Once Runx2 is expressed, cells are committed to an osteoblast lineage which further upregulate expression of calcification-related proteins like osteopontin, bone sialoprotein II, and osteocalcin .While there is a reduction in smooth muscle marker expression, levels of markers like Smad 1/5/8 and Runx2 have been detected in calcified human aortic valves and they show increased presence before there is evidence of valve leaflet calcification [3].

2. THE BONE MORPHOGENETIC PROTEINS

Bone modeling involves two kinds of cells, 'Osteoblasts' which are involved in bone formation, and 'Osteoclasts', that are involved in the process of bone resorption. These two processes require intricate balance amongst themselves, and BMPs are essential for the differentiation and maintenance of both Osteoblasts and Osteoclasts [6, 8].

Of the fifteen BMPs identified in humans, BMP 2, 4, and 6 are secreted by osteoblasts and are known to be autocrine regulators of bone formation[6]. The osteogenic activities of several BMPs have been analyzed and it was observed that most BMPs (except BMP 3 and 12) induce alkaline phosphatase activity in mature osteoblasts [9]. The most studied BMPs with regard to vascular calcification are BMP-2 ,BMP-4 and BMP-7, and their role in vascular calcification can be characterized as :

BMP 2 - Induction of BMP-2 in the vasculature can be correlated to oxidative stress, inflammation, oxidized lipids and hyper-glycaemia. Enhanced

expression of BMP2 stimulates the osteoregulatory gene MSX-2 [5]. Subsequently, Core-Binding factor-1 (Cbfa 1) also known as Runx2 and Osterix, - which are both transcription factors, stimulate the differentiation of multipotent mesenchymal cells into cells that are capable of bone formation known as "Osteoblasts" [10].

BMP 4 – It is closely related to BMP 2 and along with BMP 2, it has a major role in cardiac arteriopathy, that is characterized by the diseased condition of arteries. It is also suspected to have a pivotal role in the vascular calcification caused in response to the inflammatory cytokine named Receptor activator of nuclear factor kappa-B ligand (RANKL.)

BMP 7 - Whereas BMP-2 is associated with a decrease in smooth muscle cell markers, BMP 7 promotes the VSMC phenotype [10]. Bone formation and phosphate deposition in bone tissue is promoted by BMP-7. Flow cytometric analysis has shown that although treatment of committed osteoblast precursors with BMP-7 causes the formation of bone nodules, the treatment with BMP-7 inhibits it in uncommitted cells [5]. In patients with Chronic Kidney Disease (CKD), large levels of BMP 7 can reverse arterial calcification. But the problem lies in the fact that since BMP 7 is mainly synthesized by the kidney, patients with CKD have low levels of BMP 7. So the inhibitory effect of BMP 7 on valve calcification is lost [10].

The BMPs are a group of almost 30 proteins named for their osteo-inductive properties that have important developmental roles in organogenesis of a variety of tissues. It has been observed that treatment of Human Valve Interstitial Cells (VICs) with BMPs (BMP-2, BMP-4, and BMP-7), and tissue growth factor ([TGF-beta], TGF-1 and TGF-3) for 21 days enhanced the differentiation of VICs into osteoblasts, which is confirmed further by the expression of alkaline phosphatase (ALP) [11].

LDLR-/- (Low density lipoprotein receptor deficient) mice when treated with the small molecule BMP inhibitor LDN-193189, powerfully inhibits development of vascular inflammation, osteogenic activity and calcification. Similar results have been shown by the recombinant BMP antagonist ALK3-FC. Treatment of human aortic endothelial cells with LDN-193189 or ALK3-Fc stopped the production of reactive oxygen species (ROS) induced by oxidized LDL, an event that characterizes narrowing of the vessel walls [12]. This further asserts the role of BMP in vascular calcification.

3. INDUCTION OF VASCULAR MATRIX MINERALIZATION BY BMP-2

Although BMP 2 may have different effects on the Vascular Smooth Muscle Cells (VSMCs) depending upon the state of proliferation, its effect on the decrease of expression of Smooth Muscle Cell Markers in vitro is of prime importance. Once BMP 2 causes cell arrest, further exposure to BMP 2 results in the loss of Smooth Muscle Cell Markers and gain of a bone-like (osteoblastic) gene expression due to the induction of Msx-2,- which is also under the influence of BMP 2. BMP 2 induces both MSX-2 and Runx/Cbfal in VSMCs. The effect of MSX-2 is through upregulation of the transcription factor "Osterix" (Osx), that mediates mineralization and osteoblast differentiation. Its activity is required for induction of alkaline phosphatase and mineralization, downstream to the activities of Cbfa1 and MSX-2,- which are activated by BMP 2 [13]. BMP 7 on the other hand, promotes the VSMC phenotype by stimulating p21 as well as upregulating calcification inhibitory proteins like Smads 6,7 [13].

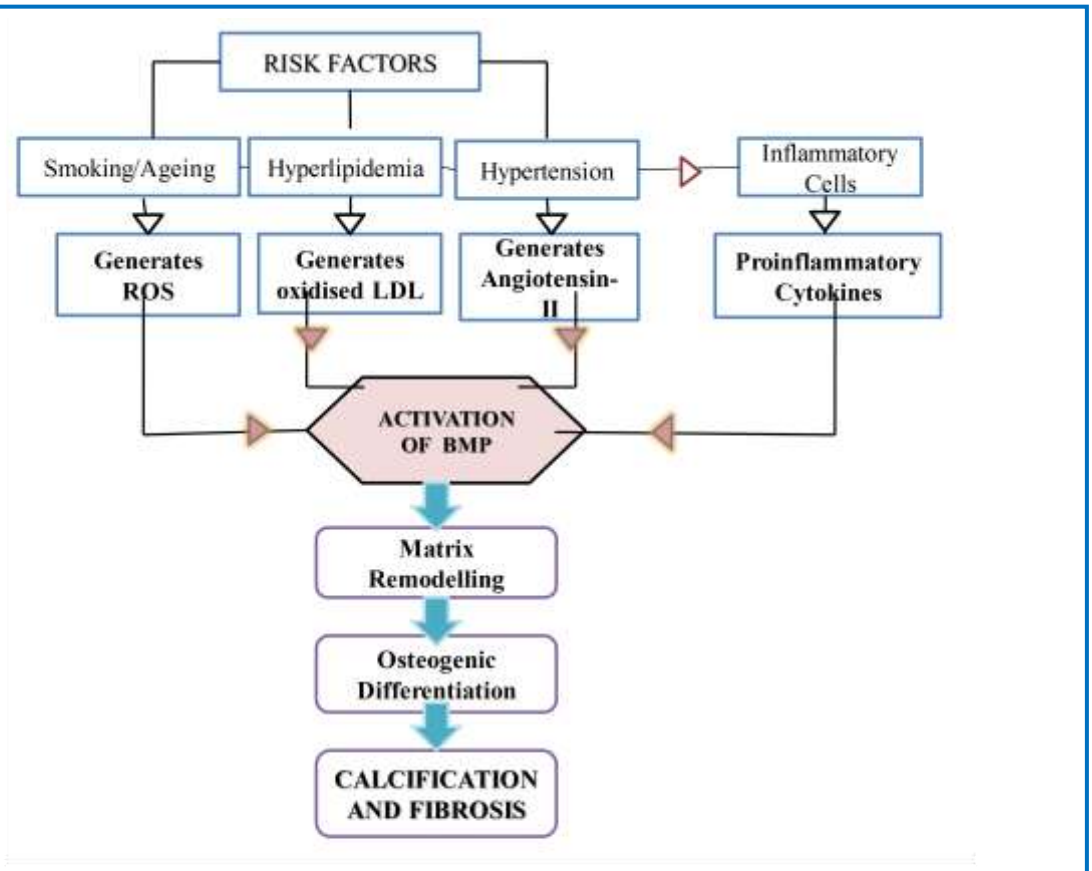

Fig 1. Risk factors that contribute to the calcification and fibrosis of the aortic valve by generating various influential factors that ultimately cause the activation of BMP (Bone Morphogenetic Protein), which is a mediator of the process of calcium deposition. (LDL, low-density lipoproteins; ROS, reactive oxygen species; BMP, bone morphogenetic protein.)

4. EXPERIMENTAL EVIDENCES ON PROBABLE PATHWAYS OF INHIBITION

Inhibition by Noggin

Noggin is an extracellular protein and is one of the primary antagonists of BMP . It regulates its activity in vivo by binding to it [14]. It has a vital role in controlling the BMP-mediated differentiation of mesenchymal precursor cells into osteoblasts [15].

The Noggin/BMP interaction prevents the binding of BMPs to their cell surface receptors, thus disrupting the initiation of BMP signal transduction in target cells, which lead to the pathway of vascular calcification [15]. Noggin binds with BMPs with increased affinity towards BMP-2, BMP-4 than BMP-7. Moreover, addition of Noggin prevents the BMP-induced osteoblastic differentiation in mesenchymal cells, further supporting the inhibition imposed by Noggin on BMP activities.

Upregulation of Matrix Gla Protein

Matrix Gla Protein (MGP) is a small, Vitamin-K dependent regulatory protein of BMP-2 ,and has an intense inhibitory effect on calcification when present in intermediate amounts [16]. It achieves this function by directly binding to BMP-2 and calcium crystals [16-17]. It has been speculated that MGP binds to Phospatidyl Serine Residues on matrix vesicles and apoptotic bodies [18]. Mice genetically engineered to be deficient in MGP develop extensive

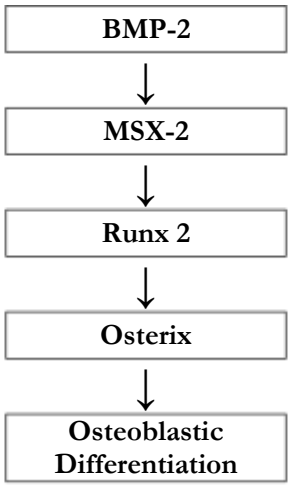

Fig 2 (a). Proposed pathway of gene activation in BMP 2 /MSX2 signaling involved in aortic valve calcification. BMP-2 (a growth factor) activates the osteoregulatory gene MSX-2 that induces the transcription factor Runx2 to activate another transcription factor downstream to it,- Osterix. Osterix mediates the formation of osteoclasts.

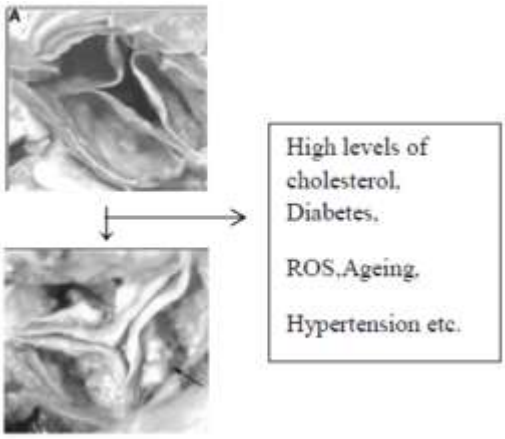

Fig 2 (b). Shows the physical effect that can be correlated with the proposed pathway, which is marked by the gradual calcification of the aortic valve due to various causal factors.

medial calcification, even in the absence of atherosclerosis. Furthermore, VSMCs obtained from the aorta of MGP-deficient mice have shown to undergo differentiation into bone and cartilage when treated with BMP2, whereas the control VSMCs do not [19]. It may also be noted that MGP has a dose-dependent effect on BMP-2. When MGP is present in slightly less amount as compared to BMP-2, mild enhancement of BMP-2 activity can be seen. Intermediate levels of MGP(1–15-fold)result in inhibition of BMP-2, and high levels (above 15-fold) result in prominent upregulation of BMP-2 activity [16].

Upregulation of Smad-6 Gene

Disruption of the BMP inhibitor Smad-6 gene increases vascular calcification and has been shown to produce aortic ossification and hypertension in mice aged only two weeks. Results have shown that expression of Smad-6 gets reduced in aortic and valve tissues of hyperlipidemia compared to mice with normal lipid levels.Smad-6 is suspected to have a role in inflammation- induced calcification, which is mainly mediated by the pro-inflammatory cytokine TNF- α.This has been proved by testing for markers in aortic valve leaflets have been isolated from mice with normal lipid levels and cultured to form VICs. These mVICs test strongly positive for vimentin,- which is a mesenchymal cell marker, weakly positive for smooth muscle alpha actin, and negative for an endothelial cell marker. Gene expression analysis have also shown that, on treatment of these VICs with TNF- α, BMP-2 expression was significantly upregulated while Smad6 expression was downregulated.

In cells transfected with Smad6 shRNA (Smad6 gene knocked down by lentiviral transfection), TNF- α further augmented ALP activity, and expression of BMP-2 and matrix mineralization suggested that TNF- α induces vascular cell calcification by specifically reducing the expression of a BMP-2 signaling inhibitor, Smad6 [20].

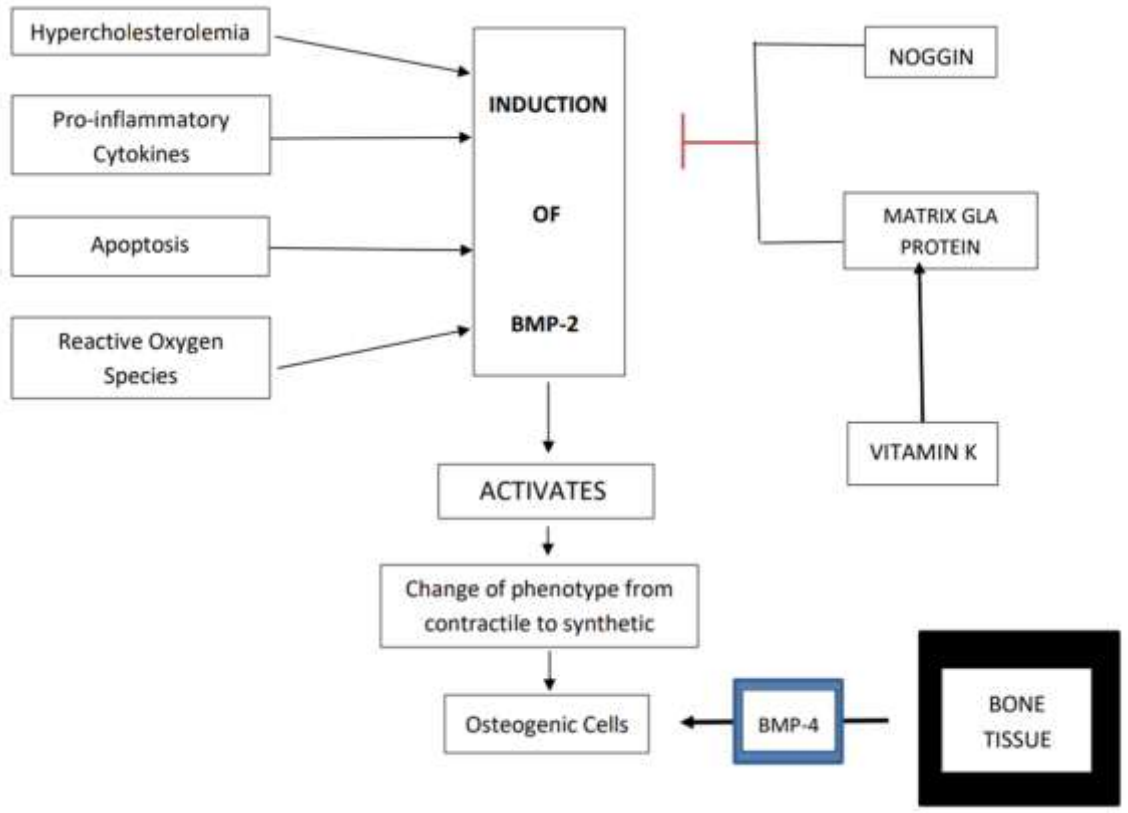

Fig 3. The process of vascular calcification- Cellular processes like apoptosis, high level of cholesterol, generation of pro-inflammatory cytokines and Reactive Oxygen Species contribute to the stimulation of BMP (mainly BMP-2) which further catalyzes the transition of contractile cells into synthetic cells. This stimulation is blocked by inhibitors such as Noggin and Matrix Gla Protein (MGP),- which is ultimately activated by adequate levels of vitamin K. BMP-4 on the other hand has been shown to promote osteogenesis in bone tissue.

Inhibition of Apoptosis

Apoptosis appears to be critical to the initiation of valve calcification[1].Apoptosis is assumed to have an active role in production of matrix vesicles from VSMCs,-which are thought to be the sites of vascular calcification .In plaque formation, apoptotic remnants of such matrix vesicles cannot be phagocytosed by macrophages , due to the presence of oxidized lipids in the surroundings which compete with the apoptotic bodies in binding with the macrophages [18, 21]. Such unphagocytosed apoptotic bodies either undergo secondary necrosis, or undergo calcification.

Apoptotic activity of BMP-2 occurs in an 'Noggin' – dependent manner and it also depends on the maturation state of the osteoblasts [22]. While mesenchyme cells show no effect to BMP-2 treatment, immature and mature osteoblasts show profound apoptosis on being treated by BMP-2. BMP-2 also stops the proliferation of both normal and malignant gastric epithelial cells, by down-regulating CDK4 expression in gastric cancer cells and by causing arrest in G1-phase in cell cycle [23]. Thus it can be seen that not only does apoptosis appear to precede nodule formation in Cardiovascular cells (CVCs), but inhibition of apoptosis appears to reduce calcification in vitro. Thus, in this aspect, anti-apoptotic drugs with or without combination with BMP inhibitors may be looked upon as possible mechanisms to combat calcification.

Table 1. Effect of various cellular pathways on BMP signaling affecting vascular calcification

Cellular Pathways/ Potent Molecules	Effect on BMP Activity
Noggin	Inhibition[14,15]
MGP	Inhibition[16,17,18,19]
Smad-6 Gene Disruption	Activation[20]
Apoptosis	Activation[18,21,22]
HMG CoA reductase activity	Inhibition[12,24]
Notch Signaling Inactivation	Activation[2,25,26]
Pro-inflammatory cytokines	Activation[28,30]
ROS	Activation[35]
Vitamin K	Inhibition[17,32,33]
Gallic Acid	Inhibition[36]

Prevention of oxidation of LDL

Low Density Lipoprotein (LDL) is susceptible to oxidative damage and oxidized LDL contributes to Atherosclerosis by increasing adherence of circulating monocytes to arterial endothelial cells ,as well as increased entry of LDL into intima of arterial wall. This further leads to the migration of smooth muscle cells which then cause lesions in the arteries [24]. The osteoblast cell markers Alkaline Phosphatase and Runx2 have been shown to decrease upon lipid-lowering that is caused by HMG-CoA reductase inhibitors, to decrease valve calcification in rabbit models of experimental hypercholesterolemia.

Experiments have also shown that treatment with LDN-193189 (a BMP inhibitor) reduces total cholesterol level, LDL levels, alkaline phosphatase (ALK) levels in LDLR -/- mice fed on a High Fat Diet (HFD) for 20 weeks. Similar results have been reported on treatment of human valve ICs with atorvastatin (which is a lipid-lowering agent) [12].

Bone morphogenetic protein (BMP) inhibition lowers hepatic cholesterol biosynthesis as serum LDL cholesterol levels were reduced by 35% in LDN-193189-treated mice whereas HDL cholesterol levels were not altered . In the absence (Control) or presence of LDN-193189 or pravastatin (a cholesterol-lowering drug), Pravastatin but not LDN-193189, have been shown to inhibit HMG-CoA reductase activity [12].

Upregulation of NOTCH Signaling

Loss-of-function mutations of Notch-1 gene are strongly associated with early calcifications of bicuspid aortic valves in humans [25]. Mice that are haplo-insufficient in Notch-1 develop calcific aortic valve disease due to inhibition of repression of BMP 2 [5].

A marked reduction in active NOTCH1 has been found by measuring with an antibody specific to the NOTCH1 intracellular domain (NICD), in regions of patients having calcified valves. Overall expression of NICD show increase in the fibrosa of diseased valves compared to the acellular fibrosa of controls. These findings prove that the loss of NOTCH1 expression in areas of calcification is one of the major parameters of CAVD [26].

OPG Mediated inhibition of RANKL (Receptor activator of nuclear factor ligand) Induced Differentiation of osteoclasts

RANKL and OPG (Osteoprotegerin) are proteins expressed by osteoblasts that regulate the maturation of osteoclasts. Osteoclasts require RANKL to differentiate, mature and survive, whereas OPG is a RANKL inhibitor that inhibits inflammation and

blocks differentiation and function of osteoclasts [6, 27].

RANKL increases vascular smooth muscle cell calcification by binding to RANK and increasing BMP4 production through activation of the NF-kB pathway [27-28]. BMP-2 also boosts RANKL-mediated osteoclast differentiation by increasing the size and number of osteoclasts [29]. VSMCs incubated with RANKL show a dose-dependent increase in calcification, which can be abolished by co-incubation with OPG [27].

RANKL induces differentiation of osteoclast precursors by binding to Receptor activator of nuclear factor (NF)-kB (RANK) on osteoclasts, thus initiating bone resorption. In an in vivo model, the increase in calcium content of VSMCs has been shown to be parallel to the increase in RANKL and BMP 4 levels. This calcification induced by RANKL can be negated by 'Noggin' [27]. It may also be noted that 'Chordin' protein from *Xenopus* sp. shows high affinity towards BMP-4 and may be considered as a potential molecule to block RANKL-mediated VSMC Calcification [29].

Decrease in Proinflammatory Cytokines

Osteoclast formation and function are influenced by many other pro-inflammatory cytokines, have also been shown to play a role in bone modeling. TNF-α activates osteoclasts directly and also appears to accentuate the effects of RANKL signaling. Three key cytokines that are involved in osteoclast development and function and have pro-inflammatory properties (RANKL, CSF-1, and TNF-α) are present in atherosclerotic plaque [28]. TNF – α may be a critical mediator of inflammation-induced-calcification [5]. In cell culture, IL-1beta and TNF- α increased BMP-2 mRNA and protein levels by eightfold and fifteenfold [30]. Inflammatory macrophages co-localize with calcium phosphate deposits in developing atherosclerotic lesions and can promote calcification through the release of TNF α. Administration of "Infliximab" (a TNF –α neutralizing antibody) inhibits BMP 2 – Msx- Wnt signaling in aorta [31].

Uptake of Vitamin K

Matrix Gla Protein (MGP), an extracellular protein, is a Vitamin K dependent protein, that plays a vital role in the inhibition of BMP-2. Inactive MGP requires Vitamin K to carboxylate it for its activation. Thus, Vitamin K can be a future candidate for trials on inhibition of BMP-2, as it apparently has indirect but crucial role [32]. Increased calcium salt deposition has been found in cells treated with the vitamin K antagonist warfarin as compared to controls [17]. High vitamin K intake has been shown to be associated with low aorta calcification [33] and has beneficial effects on the elastic properties of the vessel wall [34].

Reduction of Oxidative Stress

Oxidative stress plays a critical role in vascular calcification including the formation of lipid laden macrophages and the development of inflammation. H_2O_2 has been shown to promote a phenotypic switch of VSMC from contractile to osteogenic phenotype. This was also associated with an increased expression and activity of Runx2, the key transcription factor for osteogenic differentiation. The production of reactive oxygen species (ROS) and nitrogen species by vascular cells generate an oxidative environment.

H_2O_2 regulates VSMC calcification through modulation of the expression of Runx2. It has been found that H_2O_2 at non-toxic concentrations can induce VSMC calcification as indicated by the black

granule formation after von Kossa staining indicating total calcium levels. The effect of oxidative stress on osteogenic differentiation of VSMCs and their markers showing transition from contractile to synthetic phenotype have been examined [35]. Real-time PCR analysis have confirmed that expression of bone/calcification markers shows increase ,and those of VSMC Markers decreases after VSMC were exposed to 0.4mM H_2O_2 for 10 days. This proves that H_2O_2 caused transition of VSMCs from smooth muscle cells to bone cells. Treatment by H_2O_2 for 10 days in the same experiment has revealed a 4.5-fold increase in expression of Runx2. Western Blot Analysis has further confirmed this at the mRNA level [35].

Upregulation of Twsg1

Twsg1 is a BMP-binding protein that prevents BMP from binding to its receptors, and thus serves as a BMP-inhibitor. Genetic Deletion of Twsg1 in mice has also shown increased maturation of osteoclasts, and an increase in the number of phosphorylated Smads 1/5/8 which downregulate the process of Vascular Calcification [6, 29]

Gallic Acid

Gallic acid has been identified as a potential phytochemical that inhibits the phosphate induced calcification of VSMCs that is regulated by a BMP-2 dependent pathway. BMPs are known to activate BMP activators, which cause the phosphorylation of Smad 5/6/8, which ultimately causes differentiation into osteoblasts. Both Western Blot and Immunocytochemistry have shown that expression of phospho-Smad 5/6/8 was diminished when cell cultures were pre-treated with Gallic Acid [36].

5. DISCUSSION

The interplay of various regulatory factors and their effect on the function of BMP protein has been summarized in the following table (Table 1)

6. CONCLUSION

The BMPs are one of the most important regulators of vascular calcification. Although their roles in bone formation are well defined, their role in vascular calcification is more complex, and is an area where more light needs to be thrown upon. The expression of bone morphogenetic proteins (BMPs) is shown to be enhanced in human atherosclerotic and calcific vascular lesions. Whereas BMP 2 acts as a strong activator of the process, BMP 7 inhibits it. The role of BMPs in the interplay of different pathways of vascular calcification needs to be correlated and looked upon. The pathways of hypercholesteromia, oxidative stress and many other signaling pathways may have unexplored cross-talks between them, and BMPs may act as a common thread that unites these processes to ultimately lead to vascular calcification. Although genetic gain- and loss-of-function experiments in mice have supported a causal role of BMP signaling in atherosclerosis and vascular calcification, it fails to explain the exact mechanisms that direct the growth and development of the disease. Thus, a clearer understanding of the BMP-regulated-calcification pathway is a prerequisite to deal with the disease.

With the present understanding, drugs that can act as antagonists to the various mediators of vascular calcification, like Apoptosis, Reactive Oxygen Species and pro-inflammatory cytokines may be designed to combat the whole process. In this regard, anti-apoptotic drugs or processes of utilizing free Reactive Oxygen Species may be designed or explored to check their competency to inhibit calcification. More of antibodies like 'Infliximab', or phytochemicals like 'Gallic Acid' which can neutralize inflammatory

cytokines need to be searched for and designed. In patients suffering from CAVD ,the effects of antagonistic processes,- like knock-down of Smad1/5/8 genes, as well as agonistic ones like uptake of Vitamin-K, and upregulation of Smad 6 gene need to be routinely tested to prove our conceptions, and put them to pharmaceutical use.

It is also evident that the whole focus of research has predominantly been channeled towards BMP-2,since its pathways of involvement in the calcification process is most conspicuous. More attention needs to be drawn to the other members of the BMP family as well,- like BMP4 and BMP 7, and their roles need to be elucidated to get the complete picture of the BMP-induced vascular calcification process.

7. CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

8. SOURCE/S OF FUNDING

No source of funding

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