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20
The Biological and Cellular Role of Fetuin Family Proteins in Biomineralization

Cora Schäfer and Willi Jahnen-Dechent

Abstract

The final step of biomineralization is a chemical precipitation reaction that occurs spontaneously in supersaturated or metastable salt solutions. In physiological bone formation, "osteogenesis", and also in pathological mineralization, "ectopic mineralization or calcification", genetic programs direct precursor cells into a mineralization-competent state. Therefore, all tissues not meant to mineralize must be actively protected against the chance precipitation of mineral. Fetuin-A is a blood protein that acts as a potent inhibitor of ectopic mineralization. Fetuin-A-deficient mice develop severe soft tissue calcification. Fetuin-A combines with calcium and phosphate into transiently soluble colloidal particles termed calciprotein particles. Thus, fetuin-A is a systemic inhibitor of pathological mineralization complementing local inhibitors acting in a cell- or tissue-restricted fashion.

Key words: fetuin, alpha2-HS glycoprotein, calciprotein particles, pathological mineralization, ectopic calcification, knockout mice.

20.1
Osteogenesis and Bone Mineralization versus Calcification

In vertebrates, although mineralization is usually restricted to the bones and teeth, it may also occur outside the skeleton ectopically (out of place) when precursor cells inappropriately receive signals to develop into bone cells. The ectopic activation of osteogenesis also contributes to the calcification of blood vessels, including medial arterial calcification, atherosclerotic aortic calcification (intima calcification), and aortic valve calcification [1]. Calcification is a known major shortcoming of vessel prostheses and bioartificial heart valves [2]. Ectopic calcification may also be considered a primitive response to chronic infection, whereby pathogens that cannot be cleared are encased. Calcification sites in soft tissue organs

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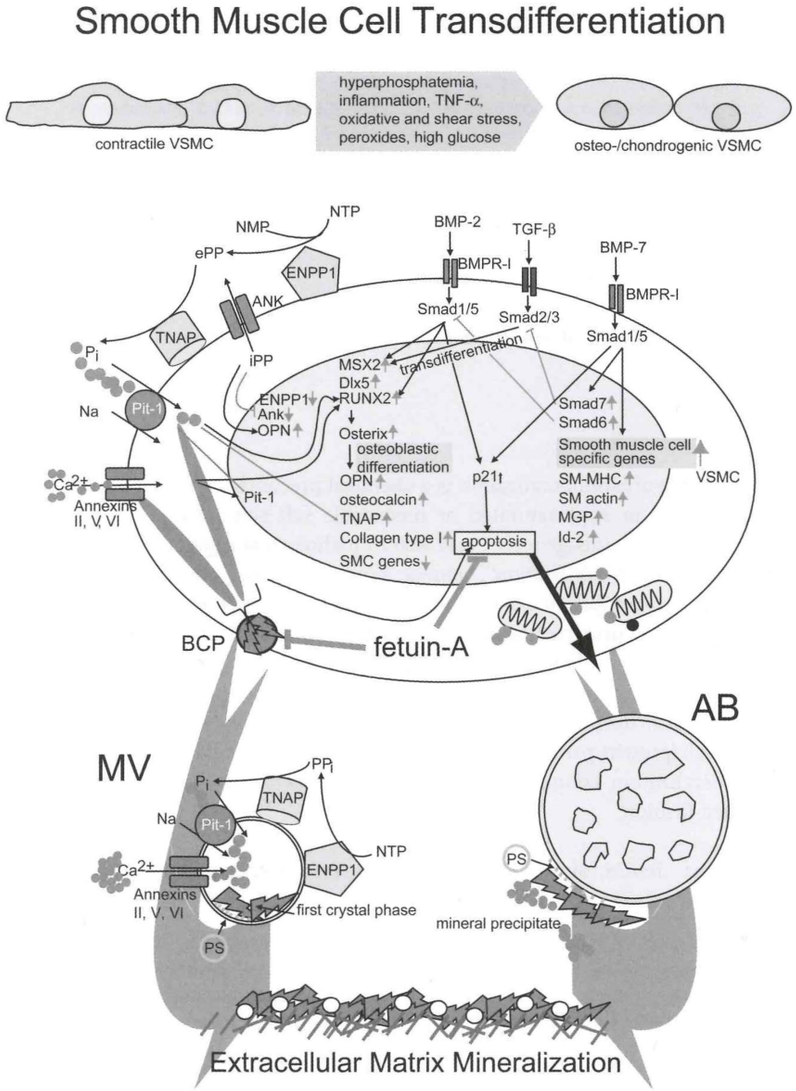

are in fact diagnostic of parasitic infection, for example tuberculoma and breast cancer. The innate immunity response to *Mycobacterium tuberculosis* involves vitamin D-like compounds, which enhance local calcification at the infection sites [3]. Calcification is common in tissue remodeling [4], and most cells are proficient in the shedding of "matrical lipidic debris", which readily calcifies. Following tissue insult and the ensuing remodeling activity, large amounts of cell remnants break off and drift into the extracellular matrix (ECM).

Extreme cases of vascular calcification are associated with typical bone structures, including trabeculae, lacunae, and areas resembling marrow [5, 6]. Several chapters in this book have detailed clear parallels of bone formation and ectopic calcification (see Chapters 1, 21, and 24). The roles of mineralization regulators and bone-related genes in the context of smooth muscle cell mineralization are illustrated schematically in Figure 20.1. Both osteogenesis and ectopic calcification invariably terminate in matrix mineralization as the final step. The process of matrix mineralization requires a calcifiable substrate such as collagen, the activity of alkaline phosphatase to provide high local phosphate concentrations, and an absence of soluble inhibitors (e.g., pyrophosphate) or tissue-bound inhibitors (e.g., matrix Gla protein) [7].

Most of the calcium available for precipitation circulates in the blood. Historically, the classic investigations of Blumenthal and colleagues demonstrated that the blood serum contains potent inhibitors of spontaneous calcium salt precipitation [8], preventing mineralization of the blood itself. In the absence of circulat-

Fig. 20.1 Calcification-related genes "at work" in vascular smooth muscle cells (VSMCs) undergoing metaplasia. Following "transdifferentiation" into mineralizing VSMCs, the cells elaborate markers of the osteo/chondrogenic lineage. The Na/phosphate co-transporter Pit-1 mediates phosphate transport into the cell. Elevated phosphate levels in the cytoplasm up-regulate expression of Runx2/Cbfa-1, an osteogenic transcription factor. In addition, hyperphosphatemia enhances the production of apoptotic bodies and matrix vesicles that nucleate vascular mineral deposition. The TGF-β-like cytokine bone morphogenetic protein-7 (BMP-7) maintains the contractile phenotype (via similar to mothers against decapentaplegic 6 (Smad 6) and Smad 7 signaling), BMP-2 and TGF-β_1 enhance the osteogenic phenotype. Extracellular calcium is transported into matrix vesicles (MV) by Ca^{2+}-channel-forming annexins II, V, and VI. Calcium enhances the phosphate-dependent osteogenic differentiation by up-regulation of Pit-1 expression. Pyrophosphate (PP) acts as an inhibitor of basic calcium phosphate (BCP) crystal growth. The concentration of PP is controlled by nucleotide pyrophosphatase/phosphotransferase1 (ENPP1) which generates PP, the PP transporter ANK, and tissue non-specific alkaline phosphatase (TNAP), which cleaves PP. Unlike MV-mediated mineralization, apoptotic body (AB)-mediated mineralization does not require alkaline phosphatase and annexins. In addition, phosphatidylserine (PS) is localized on opposite sides of the plasma membrane of MV (inside) and AB (outside). PS is externalized to the outer membrane leaflet during apoptosis. Fetuin-A prevents intravesicular BCP growth in MV, and thus reduces calcium-induced apoptosis in VSMCs. BMPR-I = BMP receptor-I; MGP = matrix GLA protein; NTP = NMP, nucleotide tri (mono) phosphate; OPN = osteopontin; SM-MHC = smooth muscle myosin heavy chain; TNF-α = tumor necrosis factor-α.

ing "systemic" inhibitors, an organism would run a high risk of mineralizing the extracellular fluid, which itself is typically a metastable solution with regards to calcium and phosphate solubility. This phenomenon was aptly termed "Lot's wife's problem", and has been addressed previously [9]. Candidate systemic inhibitor proteins included bulk serum proteins such as albumin [10, 11]. Apart from their affinity towards calcium apatite, these proteins also bind several more ligands, including lipids, proteases, growth factors and ECM. It was, therefore, difficult to decide if the inhibition of calcium salt precipitation *in vitro* was fortuitous and due to bulk binding, or whether it represented a true physiological function of a given protein. With the advent of gene targeting technology however, this function could be tested *in vivo*, in mutant mice. Subsequently, studies conducted in our laboratory have shown that α_2-HS glycoprotein/fetuin-A (genetic symbol *Ahsg* or *Fetua*), a serum protein, is a *bona fide* systemic inhibitor of calcification.

20.2
α_2-HS Glycoprotein/Fetuin-A is a Systemic Inhibitor of Ectopic Calcification

The name α_2-HS glycoprotein refers to the fact that this protein migrates with the alpha-2 fraction of serum proteins upon traditional cellulose acetate paper-based electrophoresis. Furthermore, it is reminiscent of the two co-discoverers of this protein in humans [12], namely Heremans [13] and Schmid [14]. Bovine fetuin-A was described in 1944 by Pedersen as fetuin (from the Latin, *fetus*), the most abundant globular serum protein in fetal calf serum [15]. Following the discovery of a second fetuin, fetuin-B [16, 17], the protein originally named fetuin was renamed fetuin-A [16].

Fetuins belong to the cystatin superfamily of cysteine protease inhibitors, which encompass a series of closely related liver-derived serum proteins. Further members of this superfamily sharing cystatin-like domains are the kininogens and histidine-rich glycoproteins [18, 19].

Fetuin-A has been implicated in several diverse functions, including osteogenesis and bone resorption [20], regulation of insulin activity [21], hepatocyte growth factor activity [22], response to systemic inflammation [23], and inhibition of unwanted mineralization [24–26]. These seemingly diverse functions show that fetuins are multi-ligand binding proteins that potentially interfere with any biochemical pathway the components of which they can bind and sequester. Using gene knockout technology in mice, the inhibition of ectopic calcification was shown to be the major biological function of fetuin-A [24, 27].

Fetuin-A-deficient mice on a mixed C57BL/6-129 genetic background displayed only a mild calcification phenotype [27]. The lack of generalized ectopic mineralization in fetuin-A-deficient mice was somewhat anticipated, because fetuin-A accounted for only a fraction of the inhibition of apatite precipitation observed with total serum of normal mice [25]. Against the calcification-prone genetic background DBA/2 [28], severe, systemic calcification occurred (see Fig. 20.2) [24].

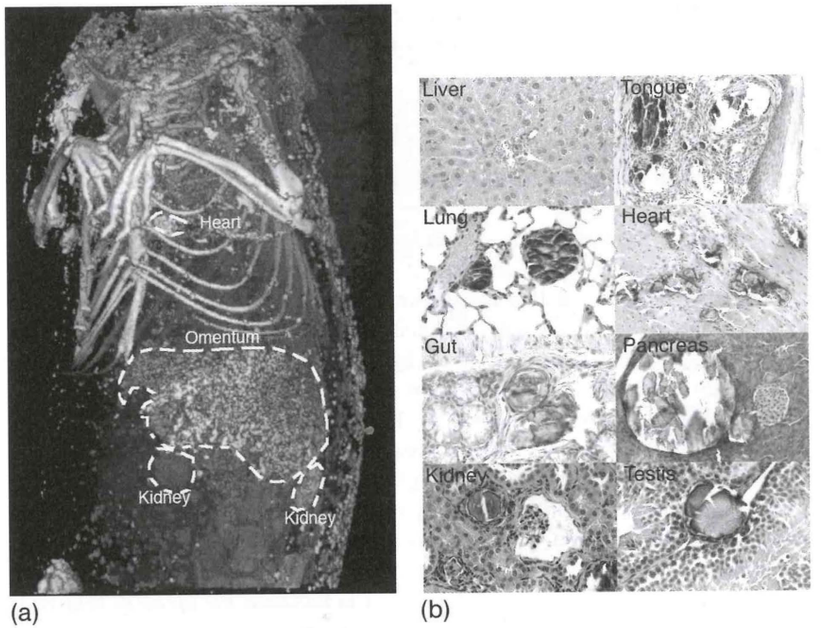

Fig. 20.2 Extensive soft tissue calcification in Ahsg/fetuin-A-deficient DBA/2 mice. (A) Microcomputed tomography (μCT) of a fetuin-A knockout mouse. Usually, only the bones and teeth should be visible, but the bright spots in the mouse are calcified lesions in the skin, heart, omentum, and kidneys. (B) Histological sections show scale-like calcified lesions in almost all organs, including tongue, lung, heart, gut, pancreas, and urogenital tract. The liver is mostly spared from calcification. This phenotype is associated with a reduced life-span and fertility; these mice may live well into adulthood, but stop breeding at an age of about 6 months.

Mice suffered calcification affecting the kidney, myocardium, lungs, and skin. The phenotype of these animals closely resembled uremia-associated arteriolopathy/calciphylaxis, with its clinical hallmarks [24]. Secondary hyperparathyroidism was observed in older mice (aged >5 months) due to kidney damage. A recent study of the cardiovascular system in these mice showed extensive cardiac calcification and fibrosis with impaired cardiac function [29], reminiscent of models of dystrophic cardiac calcification [30, 31]. Interestingly, the large arteries were spared from calcification. Taken together, it was demonstrated by using reverse genetics that the serum protein α_2-HS glycoprotein/fetuin-A is a systemic inhibitor of ectopic calcification.

The question remained, however, as to whether Ahsg deficiency is also important in human pathology. To this end, a clinical study was conducted in uremic and healthy subjects, the results of which showed that a lack of Ahsg correlated with the severity of calcification and, indeed, was (statistically) a highly significant

predictor of short-term morbidity and mortality in uremic patients [32]. This finding has subsequently been confirmed by two other groups [33, 34].

20.3 The Mechanism of Fetuin-A Inhibition of Calcification

Ahsg/fetuin-A is easily purified and can be obtained in large quantities for structure–function analyses. Important parts of the three-dimensional (3-D) structure can be modeled after the known structure of chicken egg white cystatin [35]. Taken together, these data provided an excellent opportunity to study the mechanism of calcification inhibition by a mammalian protein. By using dynamic light scattering and transmission electron microscopy, it was shown that Ahsg solubilizes apatite as a colloid [26]. This was reminiscent of how apolipoproteins ensheath and thereby solubilize insoluble lipids such as cholesterol. In analogy to the lipoprotein particles of varying buoyant density (HDL, LDL, VLDL, etc.) comprising apolipoproteins and lipids, the calcium- and phosphate-containing Ahsg colloid was referred to as the "calciprotein particle" (CPP), the molecular structure of which is described in detail in Volume I, Chapter 24.

Importantly, although the inhibitory effect is transient for up to 36 h at body temperature, within 24 h the CPPs undergo a marked morphological transformation from non-diffractive nanospheres with a diameter of ~50 nm to larger, more crystalline irregular spheres of up to several hundred nanometers in size. It is important to remember that Ahsg binds calcium phosphate, though bovine fetuin-A calcium binding is rather poor (K_d 0.95×10^{-4} M) [36]. Even if three calcium-binding sites were to exist, Ahsg/fetuin-A (10 μM serum concentration) would cause only a minute change in the serum calcium concentration of 2.5 mM. Therefore, albumin (1 mM serum concentration) should be considered the major binding protein for ionized calcium, whilst Ahsg/fetuin-A is a highly effective scavenger of basic calcium phosphate (BCP), which precipitates in the absence of this protein [24].

20.4 The Fate of Calciprotein Particles

The route and mechanism of CPP elimination, and the clearance of calcified lipidic debris from the body by endothelial cells and by macrophages is shown schematically in Figure 20.3.

There are three equally important mechanisms which prevent extracellular calcification: (i) hormone-regulated calcium homeostasis to prevent excessive fluctuations in extracellular calcium; (ii) the stabilization of calcium phosphate, which will form spontaneously as a soluble colloid (CPP) to prevent mineral from precipitating and clogging the small vessels; and (iii) an efficient clearing of CPPs by phagocytic cells such that excessive CPP – and hence precipitable mineral – is cleared from the circulation. Whilst the main established mechanisms for the removal of calcium phosphate crystals are phagocytosis and acidification [37–39], the reticuloendothelial system (RES) is also capable of removing particulate matter (e.g., cell remnants, molecular aggregates, and "mineral dirt") from the circulation. This network of phagocytic cells encompasses endothelial cells and macrophages in the liver, spleen, and bone marrow, and it is likely that CPPs are phagocytosed and recycled in the RES. Annexin binding may mediate fetuin uptake by pinocytosis, as both annexin-II and -VI have been identified as putative cell-surface receptors for fetuin-A in the presence of Ca^{2+} ions [40]. Cell culture

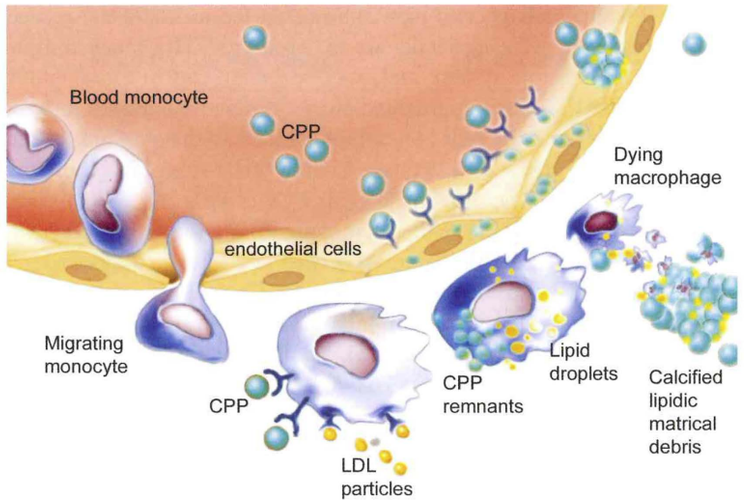

Fig. 20.3 Hypothetical pathway of removal of calciprotein particles (CPP) from circulation by endothelial cells and tissue-resident phagocytes. In healthy individuals CPP may form spontaneously in small numbers, or may occur as a spill-over into the blood of bone catabolism. When mineral homeostasis is severely disturbed (e.g., in renal dialysis patients), bouts of hypercalcemia and hyperphosphatemia will result in the formation of high numbers of CPP. Fetuin-A will be consumed in the process. CPP in the interstitial space will be phagocytosed by macrophages (as depicted), or by any other phagocytosing tissue-resident cell type. In hyperlipidemia, excess low-density lipoprotein (LDL) will concur and be cleared through similar pathways. Too much CPP remnants, in combination with lipid droplets, may overwhelm the clearing capacity of the reticuloendothelial system phagocytes, and perhaps also the lymphatic system; this may result in apoptosis and the deposition of calcified apoptotic cell remnants also rich in lipid. Fetuin-A appears to stabilize CPP in circulation and mediates their efficient uptake by phagocytes (this latter function remains to be verified experimentally). Note that many "inhibitors of calcification" activate monocytes/macrophages and stimulate phagocytosis, and therefore the stimulation of calcified remnant removal may be equally important as the inhibition of precipitation. Figure modified after Ref. [62].

assays with "professional" phagocytes have shown that fetuin-A and the related histidine-rich glycoprotein (Hrg) generally act as "opsonins". Hrg binds components of the humoral immune system, and is an opsonizing agent for apoptotic and necrotic cells [41–43]. Fetuins augmented phagocytosis in monocytes, macrophages, osteoclasts and dendritic cells [44–50], in addition to the shuffling of calcified vesicles in smooth muscle cells [51] (see Chapter 21). Fetuins also inhibited the inflammatory response of neutrophils towards BCP crystals [11].

It is striking that the removal of calcified remnants from bone turnover is usually non-inflammatory, and even the large calcareous deposits of *Ahsg* knockout mice showed no signs of inflammatory cell infiltrates [24, 29]. Fetuin-A coating may well render phagocytosed material non-inflammatory by carrying along anti-inflammatory polyanions such as spermine [23, 52] and the immunosuppressive cytokine transforming growth factor beta (TGF-β) [20, 53].

In conclusion, the plasma protein fetuin-A protects the body from unwanted calcification in various ways:

- Chemically, by binding to calcium phosphate nuclei and inhibiting further mineral growth [25].
- Biochemically, by stabilizing and opsonizing CPPs to be cleared from the circulation before the growing crystals reach a critical size at which they begin to precipitate [26].
- On the cellular level, by alleviating the detrimental effects of calcium overload during the shuffling of calcifying vesicles, thus indirectly inhibiting apoptosis [54] (see also Chapter 21).
- On a systemic level, by binding and antagonizing TGF-β and BMP, thereby regulating their osteogenic activity [20, 55].

In summary, these roles suggest that fetuin-like proteins have a more general function in the prevention of calcification and innate immunity, and it is likely that anti-calcification mechanisms and general tissue remodeling may become merged [56, 57]. Today, a dual role is emerging for fetuin-A and osteopontin (see Chapter 24) as mineral-binding proteins in the inhibition of calcification and immunomodulation, especially as both agents are present in calcified atherosclerotic plaques [58, 59]. Whilst fetuin-A is derived from serum, osteopontin may be expressed by macrophages resident in the plaques. It might well be that these key components of the anti-calcifying machinery, which originally evolved to fend off excessive mineral deposition, are now utilized in a more elaborate manner to discriminate self from non-self. Therefore, we should perhaps begin to consider ectopic calcification not only as a lack of inhibitors of precipitation (the chemical viewpoint) but also as the evolution of molecules that promote opsonization and the timely removal of calcified remnants (the immunological viewpoint). Investigations into apoptotic cell clearing [60], plaque deposition diseases and atherosclerosis [61] have already led to immunology being embraced as the main focus of research in the harnessing of potential therapies. Likewise, the reversal of calcification by immunological means will undoubtedly soon become a "hot topic" of research.

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