

15 Lot's Wife's Problem Revisited: How We Prevent Pathological Calcification

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15.1 A Short History of Calcification Inhibition

Biom mineralization is first documented in the Precambrian invertebrate *Cloudina* [1] and may well have evolved as a protective mechanism against calcification. Considering that the Cambrian sea was extremely mineral-rich, and inarguably caused the build-up of both massive geological and biogenic mineral deposits, it is tempting to speculate that early biomineralizing organisms tried to escape mineralization and hence encrusting by precipitating salts. A basic anti-calcifying strategy of secreting mucoid, charged polymers (carbohydrate, protein) was developed [2]. Together with the mineral this organic matrix comprised a first form of "biomineral". This strategy is conserved in today's shell-forming marine life [3], which may still be struggling not to be immobilized by a mineral crust. Once the encrusting problem was solved by developing a regulated crust called a "shell" this became an evolutionary advantage against predators.

Shells of mollusks and the "exoskeleton" of crustaceans were carried along when these animals left the ocean to colonize the land. Cellular metabolism is critically dependent on calcium as a counter ion, a stabilizer of proteins and nucleic acids, and indeed an important intracellular (second) messenger. In higher organisms, calcium is essential for neuromuscular stimulation, and for the stability of tissues in general and tightness of epithelial cell junctions in particular. Phosphate, on the other hand, is indispensable for making DNA and RNA, i.e. maintaining the genetic code, for energy metabolism, and also for critical switching of cell signals, which rely on phosphate tags attached to sugars, proteins and lipids. Unfortunately, calcium and phosphate tend to form highly insoluble salts, and precipitate at the extracellular concentration required for intracellular function. Thus, most extracellular fluids are "metastable" with regarding to the solubility of these ions. Incomplete as this quick rundown of biomineralization history may be, it illustrates one important point. Cellular metabolism evolved at a time when extracellular mineral abounded. From that time onward cells needed relatively high extracellular calcium and phosphate to function properly. The relative lack of these minerals in the immediate environment of land-dwelling creatures was compensated by mechanisms collecting minerals from the environment and preventing their loss. One important function of the skeleton in vertebrates is to maintain extracellular calcium homeostasis. While this keeps cells "happy", it causes the exact same problem to the, say,

the innards of a typical vertebrate animal that prompted its Cambrian ancestors to “invent” biomineralization in the first place, i.e. prevention of unwanted mineralization by the extracellular fluid. This short history of biomineralization illustrates that the efficient inhibition of unwanted mineralization is a logical consequence of relying on minerals for cell function. A solution to this problem is, therefore, critically important for survival.

Exactly how much of a problem is the supersaturation of extracellular solutions like blood in vertebrates? The stability of a solute system is described by the thermodynamical solubility product. Solubility products are listed in compendia of chemistry, but we will soon learn that the figures are of limited predictive value for our purpose. As an example, we will try to predict the stability of an aqueous calcium phosphate solution in a real-life biological system like blood from the thermodynamical solubility product of synthetic hydroxyapatite [$\text{Ca}_5(\text{PO}_4)_3(\text{OH})$, $\sim 10^{-53} \text{ M}^{-9}$]. To this end we will first set the combine blood serum ion concentrations into the equation:

$$K_{\text{SP}} = [\text{Ca}^{2+}]^5 \times [\text{PO}_4^{3-}]^3 \times [\text{OH}^-] \quad (1)$$

Applying the serum concentrations for $[\text{Ca}^{2+}]$, $[\text{P}_i]$ and $[\text{OH}^-]$ to Eq. (1) we will arrive at a calculated product of 5.47×10^{-31} :

$$K_{\text{SP}} = (1.2 \times 10^{-3})^5 \times (1.3 \times 10^{-3})^3 \times 10^{-7} = 5.47 \times 10^{-31} \text{ M}^9 \quad (2)$$

This suggests that we all live 22 orders of magnitude beyond the chemical equilibrium! In view of this discrepancy of solubility product and actual extracellular concentrations of calcium and phosphate ions, we all suffer what has been aptly called “Lot’s wife’s problem”, the imminent danger of turning into a pillar of salt [4]. However, simply taking serum ion concentrations is incorrect because only a small portion of $[\text{P}_i]$ (less than 0.01 %) will actually exist in the $[\text{PO}_4^{3-}]$ form at neutral pH. Furthermore, the presence in serum of additional electrolytes (NaCl) and ions that destabilize nascent apatite crystals (Mg^{2+} , HCO_3^-) diminishes the difference in solution products. Regardless of the exact contribution of these factors, they cannot fully account for the 22 orders of magnitude gap. For practical purposes, therefore, we resort to a simplified version of the solubility product, the so-called ion product, which is routinely used in clinical chemistry as a predictor of calcification risk. Laboratory experience suggests that at physiological pH, ionic strength (mainly NaCl) and body temperature, solutions with a $[\text{Ca}^{2+}] \times [\text{P}_i]$ ion product equal or greater than $6 \times 10^{-6} \text{ M}^2$ will spontaneously precipitate. Normal serum is a metastable solution in that the ion product ranges from $1.3 \times 10^{-6} \text{ M}^2$ in human adults to $2.6 \times 10^{-6} \text{ M}^2$ in newborns. Small rodent serum ion products have been measured slightly higher at $4 \times 10^{-6} \text{ M}^2$ (adult rat), but still fall within the region of metastability.

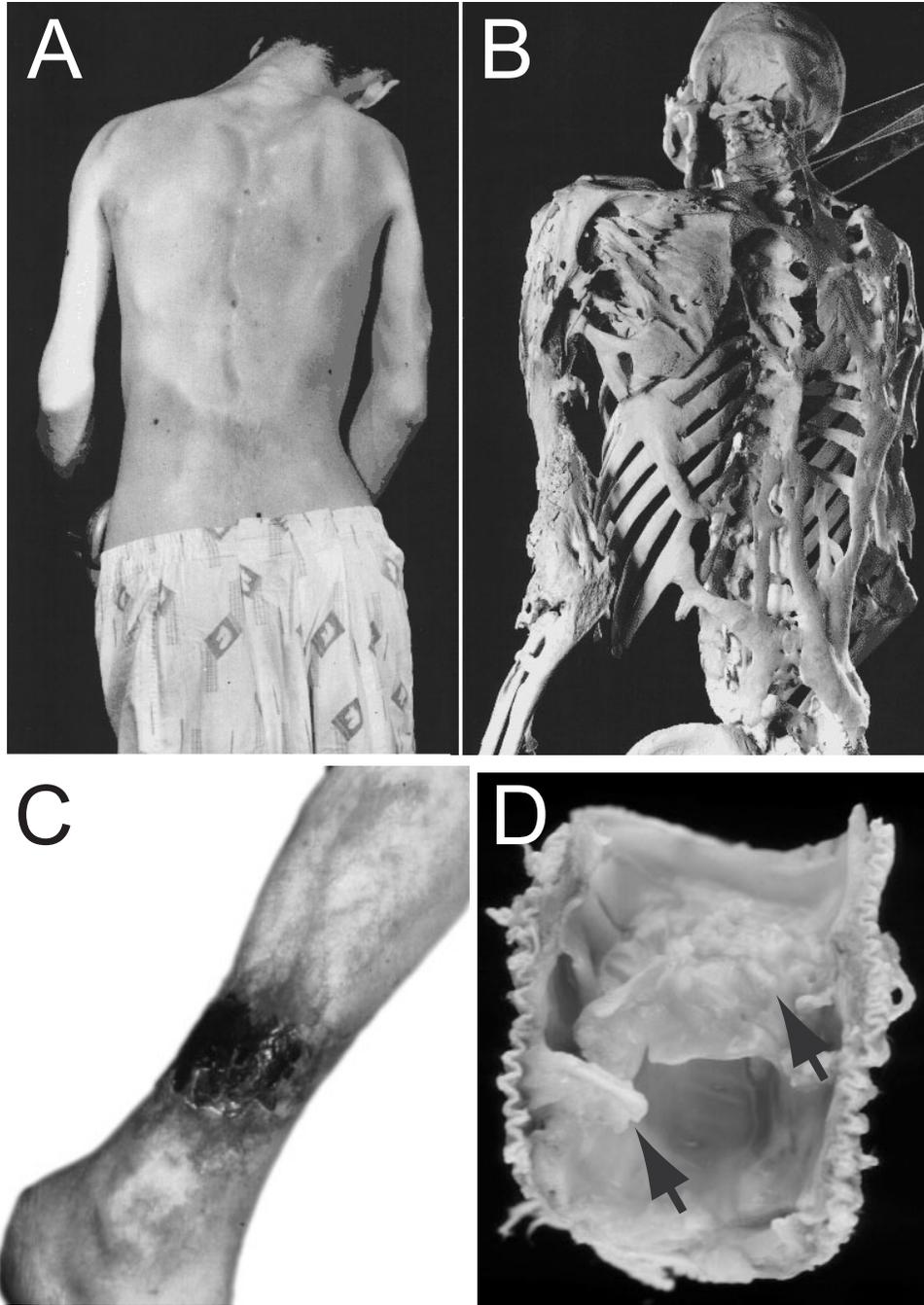
Carbonate-substituted apatite of variable stoichiometry and morphology will eventually form at most calcification sites in vertebrates, because at neutral pH and body temperature it is the thermodynamically most stable of all possible

$\text{Ca}^{2+} \times \text{P}_i$ compounds. Even chemically pure hydroxyapatite (HAP) has a fairly complex crystal unit cell of 18 ions $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. It is almost inconceivable that these ions will spontaneously assemble into a correct crystallographic unit merely through diffusion and collision in solution. Biogenic apatite is formed through a series of increasingly complex $[\text{Ca}^{2+}] \times [\text{P}_i]$ compounds that assemble on their respective precursor. These precursors include dicalcium phosphate dihydrate (DCPD), $\text{Ca}(\text{HPO}_4) \cdot 2\text{H}_2\text{O}$; octacalcium phosphate (OCP), $\text{Ca}_8\text{H}_2(\text{PO}_4)_6$; amorphous calcium phosphate (ACP), $\text{Ca}_9(\text{PO}_4)_6(\text{var.})$; and HAP $\text{Ca}_5(\text{PO}_4)_3\text{OH}$. In addition, the biogenic formation of apatite is greatly facilitated by a process called heterogeneous nucleation – the formation of crystals on an organic template. Purified collagen serves as an excellent template and will calcify at $[\text{Ca}^{2+}] \times [\text{P}_i]$ products as low as $1.3 \times 10^{-6} \text{ M}^2$. This is well below $6 \times 10^{-6} \text{ M}^2$, the level required for spontaneous, homogeneous nucleation. This brings us back to the question why not all collagen-containing tissues calcify and, indeed, why the extracellular fluid does not calcify. Obviously potent inhibitors of spontaneous calcification must exist.

15.2 Osteogenesis and Bone Mineralization versus Calcification

Mineralization is usually restricted to bones and teeth in vertebrates. However, bone formation (osteogenesis) can also occur outside the skeleton. This so-called ectopic (out of place) ossification happens when precursor cells inappropriately receive signals to develop into mature bone cells, to synthesize extracellular matrix (ECM) and to create a specialized environment favoring mineralization. In one of the most extreme examples of ectopic bone formation, *fibrodysplasia ossificans progressiva*, inflammatory cells produce bone morphogenetic protein-4 (BMP-4) and thus stimulate their immediate tissue-resident stem cells to transform muscle into bone [5]. Ultimately this results in a “skeleton outside the skeleton” as depicted in Figure 15.1(A and B).

Ectopic activation of osteogenesis also contributes to the calcification of blood vessels, calcifying atherosclerosis [6], the leading cause of death and disability in developed countries [7]. This link of bone biology and atherosclerosis has grown so strong that many researchers hypothesize that calcification in the body may always be preceded by osteogenesis [8–10]. The calcifying entities in bone mineralization called matrix vesicles [11] (spherical structures in bone and cartilage where mineralization is believed to start) and the calcifying apoptotic bodies (blebs of dying smooth muscle cells in atherosclerosis) are strikingly similar, thus corroborating this view [12]. However, there are differences [13]. In addition, calcification disease usually progresses slowly. In contrast, osteogenesis has evolved to form complex mineralized tissues – bones and teeth – in a relatively short period of time. Osteogenesis requires that bone progenitor cell migrate to the sites of bone formation,



proliferate and differentiate into chondrocytes, osteoblasts and osteoclasts. Blueprints of the bones are first laid down by cartilage-forming cells (chondrocytes) that are subsequently replaced by bone-forming cells (osteoblasts). The process is called endochondral (out of cartilage) ossification and allows growth of the skeleton. Osteoid is then formed as the bone ECM, which will eventually mineralize. It contains collagen and non-collagen proteins, presumably regulating the mineralization of the organic matrix [14, 15]. Matrix mineralization is the very final step of osteogenesis. This entire developmental sequence including mineralization is completed within less than 2 weeks in mice. In contrast, a widely adopted model of atherosclerosis in mice, the apolipoprotein E-deficient mouse, takes months to develop atherosclerosis [16]. Bone formation continues throughout adolescence. Several rounds of remodeling of primary bone by osteoblasts and osteoclasts (bone-resorbing cells) are required before a skeleton reaches its final size. Even in the adult state, bone is continuously remodeled by osteoblasts and osteoclasts. Every step in this orchestrated organ development requires that complex genetic programs are started, regulated and eventually stopped by interdependent genes [17]. The observation that several bone marker proteins are also expressed in cells near calcification sites outside the skeleton [8, 9, 18], especially in calcifying smooth muscle cells, has prompted the view that unwanted calcification is essentially a consequence of the fact that the entire bone formation program is inappropriately started in cells not meant to calcify [19, 20]. It is, however, controversial whether calcification is the cause or a consequence of the ectopic expression of an osteoblastic phenotype. *In vitro* evidence from studies with cultured cells shows that high extracellular calcium [21], high extracellular phosphate [10] and an elastin-deficient ECM [22] can induce cellular phenomena reminiscent of extracellular calcification in fibroblasts, smooth muscle cells and retinal pigmented epithelial cells. The differentiation of precursor cells into atherosclerotic plaque-forming and calcifying cells is indeed considered an important step in the pathology of atherosclerosis and vascular calcification [9, 23]. Local factors present in inflamed tissue, matrical lipidic debris (see below) and initially small calcium phosphate deposits may drive the differentiation of precursor cells into osteoblastic cells, which will stabilize this bone-like compartment and thus establish a vicious circle.

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Figure 15.1 (A and B) Clinical appearance and skeleton of a man with *fibrodysplasia ossificans progressiva*. The rigid posture in this 25-year-old man was due to calcification of the spine, shoulders and elbows (ankylosis). He died of pneumonia at the age of 40 years. Major muscles were turned into plates of bone contouring the skin over the back and arms (A), and can be seen directly on the skeleton (B). Courtesy of the Mütter Museum, College of Physicians of Philadelphia. (C) Ulcerous skin lesion in calciphylaxis. In this disease, patients calcify small subdermal vessels to the point of complete obstruction. As a result, the surrounding tissue dies and disintegrates. Secondary infections of the skin lesions are associated with a mortality of up to 80 %. (D) Calcified prosthetic aorta made of Dacron®. Calcification of this vascular prosthesis completely obstructed the vessel lumen and required revision operation and replacement. (C and D) Photographs courtesy of the Institute of Pathology, University of Aachen.

15.3 Calcification Disease

Pathologic calcification can thus occur before and after osteogenesis. Old pathology wisdom reminds us of the prominent role electron microscopy has played in the elucidation of calcification mechanisms. Feroce Ghadially brings back to all of us who are “blissfully ignorant of past history” some very basic facts of cellular calcification [24]. In particular, he points out that most cells shed of “matrical lipidic debris”, which are processes breaking off and drifting into the ECM. Large amounts of these cell remnants are formed following tissue insult and the ensuing increased remodeling activity. Matrical lipidic debris readily calcifies. Figure 15.1(C and D) illustrates two examples of this so-called dystrophic calcification in the human body. Figure 15.1(C) shows the leg of a patient suffering from calciphylaxis (nephropathy associated arteriolopathy). In this rare, but serious, calcification disease [25] calcified concrements precipitate inside the lumen of small blood vessels. Surrounding tissues starve and die. The necrotic tissue is prone to serious bacterial infections, which are lethal in 80 % of the calciphylaxis patients. Figure 15.1(D) illustrates yet another example of “boneless calcification” – calcification of a synthetic blood vessel prosthesis made of Dacron®. In general, calcification is a major problem of implants like bioartificial heart valves or vessel prostheses [26]. Material surfaces of artificial implants tend to calcify because the synthetic materials they are made of have no secretory epithelia and, hence, no natural protection against calcification. Calcification is known to be a major shortcoming of bioartificial heart valves [27].

15.4 Regulation of Calcification

Figure 15.2 summarizes recognized pro- and anti-calcifying principles. High extracellular calcium and phosphate concentrations drive calcification along following the chemical equilibrium. So do lipid-containing matrical remnants [24] or apoptotic or matrix vesicles, or other calcifiable cell remnants present in necrotic tissue (dead cells). The ECM itself is readily calcifiable in bone osteoid, but inhibits calcification in any other body location, presumably because of the presence of highly charged matrix components. Low-molecular-weight inhibitors of calcification include magnesium, inorganic pyrophosphate [28] and its synthetic derivatives bisphosphonates [29]. High-molecular-weight inhibitors include glycoproteins, phosphoproteins, γ -carboxyl glutamic acid containing proteins (GLA proteins) as well as ECM proteoglycans and hyaluronans.

Extracellular calcium is tightly regulated in blood serum in mammals through a concerted effort of the gut, kidney and bone, with the parathyroid gland as the master regulator. Mild forms of calcification can be caused by continuously feeding a diet rich in lactose and calcium (milk alkaline diet), thus over-exaggerating the nutritional composition of mothers' milk, a natural diet with the highest calcium

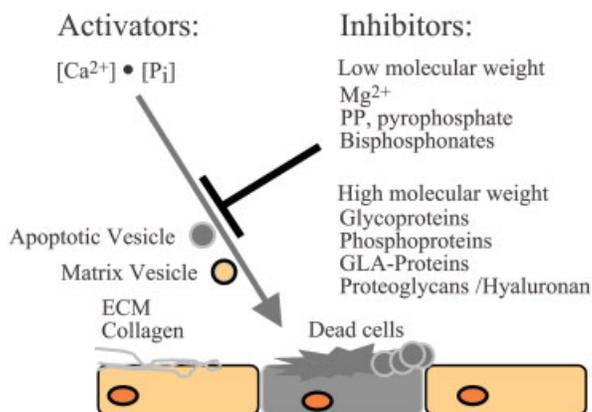


Figure 15.2 Activating and inhibiting principles in mammalian calcification. High calcium and phosphate serum concentration caused by metabolic disease or by kidney disease form a high $Ca \times P$ product facilitating calcification. Matrical lipidic vesicles of remodeling cells [24], matrix vesicles of cartilage and bone-forming cells, and apoptotic vesicles of dying cells strongly enhance calcification [13]. Hence, both bone osteoid and apoptotic vesicles of damaged and dying cells readily calcify. Low-molecular-weight inhibitors (Mg^{2+} , intracellular and extracellular PP and their synthetic derivatives, bisphosphonates) interfere with mineral formation at the level of crystal morphology. High-molecular-weight inhibitors can interfere with calcification at the level of mineral formation, stability, dissolution and removal by phagocytosis (remodeling). Depending on their expression pattern in the body, inhibitors can be tissue-restricted or systemic.

content. A more drastic form of calcification can be induced in certain strains of mice by withholding dietary magnesium, damaging tissue, e.g. with a cold probe [30], or poisoning animals with exceedingly high doses of the calcitropic hormone, vitamin D [31]. A “natural” version of this latter treatment occurs in cattle feeding on yellow oat grass (*Trisetum flavescens*), a foodstock rich in vitamin D metabolites [32]. In the 1970s, the disease enzootic calcinosis was described as an endemic condition in cattle of Southern Bavaria causing calcification of the vasculature, lung, kidney, knee joints and reproductive organs. Similar diseases are known around the world as “Enteque seco” in Argentina and Brazil, “Manchester wasting disease” in Jamaica or “Naalehu disease” in Hawaii. This form of generalized soft tissue calcification illustrates the pivotal role of $1,25(OH)_2$ -vitamin D_3 (calcitriol) in extracellular calcium homeostasis of mammals which is illustrated in Figure 15.3.

Parathyroid hormone (PTH) and calcitriol [$1,25(OH)_2D_3$] are key regulators of extracellular calcium transport and mobilization. A calcium-sensing receptor senses the concentration of extracellular calcium and triggers a regulating cascade involved in extracellular calcium homeostasis [33]. Serum usually contains 2.5 mM total calcium. Only free ionic calcium (around 1.25 mM) is biologically active and signals back to the calcium sensing receptor in the parathyroid and the kidney tubules. Calcium complexed to proteins (1 mM) or phosphate or hydrogen carbonate (0.25 mM total) serves as a buffer reservoir. Blood pH changes greatly affect the buffering capacity of albumin the major ionized calcium binding protein in blood.

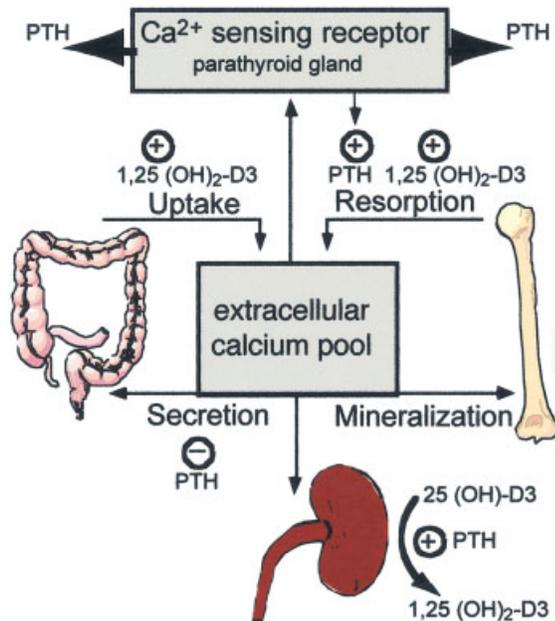


Figure 15.3 Simplified scheme of mammalian extracellular calcium homeostasis. In the gut, calcium intake is effected by calcium-binding proteins, calbindins, mediating epithelial transport to the blood. Excess calcium is secreted into feces (gut) or urine (kidney). PTH inhibits calcium secretion. In healthy subjects, high serum calcium (and phosphate) enhances physiological bone mineralization. The extracellular calcium pool (blood and extracellular fluids) calcium concentration is monitored by a calcium-sensing receptor in the parathyroid gland. Low serum calcium triggers the release of PTH, which stimulates bone resorption, renal calcium re-uptake in the kidney tubules and the synthesis of calcitriol [$1,25(\text{OH})_2\text{D}_3$] in the kidney. $1,25(\text{OH})_2$ stimulates both bone resorption and synthesis of calbindins, and hence calcium uptake in the gut. Note that the kidney is involved at almost every step of this regulatory loop. Therefore, patients with renal failure (no functioning kidneys or no kidneys at all) run an extremely high risk of vascular and soft tissue calcification. In addition, their bone tissue becomes unresponsive to bone-forming stimuli (adynamic bone disease) associated with excess soft tissue calcification.

Low serum calcium triggers the release of PTH, which stimulates mobilization of calcium from the bone, retention in the kidney and increased uptake through the gut. This is mediated by the PTH-stimulated synthesis of $1,25(\text{OH})_2\text{D}_3$ in the kidney. Subsequently $1,25(\text{OH})_2\text{D}_3$ stimulates the synthesis of calcium binding and intracellular transport proteins, calbindins, in the gut [34]. Phosphate uptake in the gut is likewise increased by vitamin $1,25(\text{OH})_2\text{D}_3$. In addition, low serum phosphate stimulates the synthesis of $1,25(\text{OH})_2\text{D}_3$ in the kidney through “phosphatonins”, a group of signaling proteins including fibroblast growth factor, FGF23 and secreted Frizzled-related peptide, sFRP [35]. Concomitantly, phosphatonins activate a sodium-dependent phosphate transporter in kidney epithelial cells mediating the re-uptake of phosphate in the kidney tubuli. Dysfunction of this transporter

is associated with low serum calcium, hypophosphatemia, and defective bone mineralization, osteomalacia.

Disturbance of every single key regulator of the extracellular calcium regulatory network can cause severe pathological calcification. Dialysis patients are particularly vulnerable to calcification because they have no functioning kidneys. Kidneys are however, critically involved in the control of mineral homeostasis at the level of PTH, vitamin D₃, phosphatonins and calcium, as well as phosphate transporters [36].

Most of the calcium available for precipitation circulates in the blood. Classic experiments of Blumenthal et al. have shown that blood serum contains potent inhibitors of spontaneous calcium salt precipitation [37]. Serum proteins mediating this precipitation inhibition were identified by testing *in vitro* their ability to inhibit the spontaneous formation of calcium salt precipitates from supersaturated ion solutions or by binding to solid-phase calcium apatite. This research revealed candidate inhibitor proteins including bulk serum proteins like albumin [38] and apatite-binding proteins in serum [39]. The apatite-binding proteins are often generic carrier proteins. Apart from their affinity to calcium apatite, they also bind several more ligands including lipids, proteases, growth factors and ECM. Therefore, it is hard to decide if the inhibition of calcium salt precipitation *in vitro* is fortuitous and due to bulk binding or whether it represents a true physiological function of a given protein. Whether or not a protein fulfills a protective role against unwanted calcification *in vivo* was for a long time impossible to determine due to the lack of genetic mutants. With the advent of gene-targeting technology, however, this function can be tested in mutant mice. Gene knockout models with pathological calcification are listed in Table 15.1. We tentatively grouped genes according to their point of interference with calcification.

The first group of gene products affects cell stability and survival. Deletion of the gene for desmin is associated with severe cardiomyopathy and cardiac calcification. Mice lacking the glycosidase I protein family member *klotho* suffer from premature ageing, pulmonary emphysema with increased cell death with associated alveolar calcification [40]. Both mouse models illustrate the importance of necrosis in calcification. In the *klotho* knockout mice a 5-fold increase in serum calcitriol was recently reported. Therefore *klotho* may also function as a negative regulator of vitamin D production [41]. We would predict that genes involved in cell survival or apoptosis could generally be involved in the development of dystrophic calcification when their activity or lack thereof causes necrosis.

As stated before, several bone-related genes play a role in calcification. Examples of this second group of genes are the genes *Smad6*, mother against decapentaplegic homolog 6, and *Opg*, osteoprotegerin, which are involved in both osteoblast and osteoclast signaling, and therefore in bone remodeling. *Smad6* is an intracellular inhibitor of BMP signaling. In the absence of *Smad6*, BMP signaling in the heart leads to aortic and cardiac calcification. *Opg* is a decoy receptor for OPGL, osteoprotegerin ligand/RANKL, receptor activator of NF- κ B ligand. RANKL is a potent differentiation and survival factor for osteoclasts [42] and a disturbance of the *Opg*/RANKL balance is associated with osteoporosis and vascular calcification [42]. Once again it should be pointed out that bone-related genes cause calcification

Table 15.1 Genes which have been associated with calcification in mutant mice.

Group	Gene	Level of interference	Mouse mutant	Calcification phenotype	Reference
1	Klotho	cell integrity, age promoting	<i>klotho</i> ^{-/-}	lung (accelerated ageing in general)	40
	Desmin	cytoskeleton stability	<i>desmin</i> ^{-/-}	cardiac, septum and right ventricle wall	99
2	Osteoprotegerin	osteogenesis, remodeling	<i>Opg</i> ^{-/-}	vascular, associated with osteoporosis	100
3	Smad6/Madh6 MGP	osteogenesis mineral, remodeling	Madh6-mutant <i>Mgp</i> ^{-/-}	aorta aorta media, arterial, valves, cartilage	101 57, 102
	Osteopontin	mineral, remodeling	<i>Opn</i> ^{-/-}	implant calcification, compound phenotype with <i>Mgp</i> ^{-/-}	46, 103
4	Carbonic anhydrase II	mineral, pH and ion activity	<i>Car2</i> ^{-/-}	small arteries, osteopetrosis, renal tubular	53
5	ENPP1, PC-1, nucleotide PP Ank TNAP, TNSAP; tissue non-specific alkaline phosphatase	mineral, PP production mineral, PP transport mineral, failure to cleave/inactivate PP	<i>tnv</i> , <i>tip-toe walking</i> <i>Ank</i> , <i>ankylosis spondylitis</i> <i>Tnap</i> ^{-/-}	articular cartilage, arteries articular joints CPPD deposition in articular cartilage, bone hypomineralization	63 62 64, 66
6	α_2 -HS glycoprotein/fetuin-A	mineral, solubilization transport (remodeling?)	<i>Ahsy</i> ^{-/-}	generalized and systemic, interstitial and intravascular	56, 79

by recapitulating osteogenic development. The question is still pending as to what activates these genes in precursor cells and turns the affected tissues into bone-like calcified tissues. For a more comprehensive listing of bone-related genes and their putative role in calcification, the reader is referred to a recent review [6].

Group 3 genes code for non-collagenous bone proteins presumably involved in bone remodeling [14]. A role in bone turnover was confirmed in knockout mice lacking *On*, osteonectin/SPARC/BM-40 [43]. Osteonectin-deficient mice developed profound osteopenia, but no ectopic calcification. Mice lacking the non-collagenous bone Gla protein *Oc*, osteocalcin, had slightly increased bone density, but also no ectopic calcification. However, a third non-collagenous bone protein *Opn*, osteopontin, seems to form a link between bone biology and immunology [44]. Osteopontin inhibits calcification *in vitro*, but unchallenged *Opn*^{-/-} mice do not calcify [45]. However, implanted biomaterials calcify more readily in *Opn*^{-/-} mice than in wild-type mice. Interestingly, *Opn* promotes regression of calcification by inducing expression of carbonic anhydrase II (*Car2*) in monocytic cells [46], suggesting a role for *Car2* in calcification as well (see below). Osteopontin is a pro-inflammatory cytokine and macrophage chemoattractant [47, 48] involved in many inflammatory diseases including atherosclerosis [16], PTH-induced osteoporosis [49], collagen-induced arthritis [50] and multiple sclerosis [48]. These results were obtained with *Opn*-deficient mice maintained on a hybrid genetic background. Some of the result were called into question by a recent study using a new *Opn*-deficient mouse strain on a defined genetic background [51]. Nevertheless, *Opn* has a clear and important role in bone formation and inflammatory processes [44]. In atherosclerosis, *Opn* proved pro- [52] or anti-atherosclerotic [16] depending on the mouse model employed. In both instances, however, macrophages were activated by osteopontin. Osteopontin's role in calcification may therefore be both at the level of direct interference with mineral formation and in stimulating phagocytic cells to clear calcified remnants.

Group 4 genes code for transporters or ion channels involved in pH and mineral homeostasis. As stated above, carbonic anhydrase II is involved in the *Opn*-stimulated dissolution of calcified deposits [46]. *Car2* participates in proton secretion by phagocytic cells, which promotes acidification of the extracellular milieu and hence apatite dissolution. Unsurprisingly, the lack of carbonic anhydrase II in *Car2*^{-/-} mice by itself is associated with calcification of small arteries, nephrocalcinosis and osteoporosis [53]. *Car2* is critically important for the regulation of local pH and mineral ion activity [54], and its total lack cannot be compensated for in all tissues. Both conditions greatly influence mineralization and mineral dissolution. Theoretically, any major disturbance in calcium or phosphate transport leading to elevated Ca × P_i products in cells and tissues could cause calcification. Idiopathic hypercalciuria is the most common kidney stone risk factor, and evidence in humans and in a rat model indicates that hypercalciuria is a complex, polygenic trait. Candidate genes for idiopathic hypercalciuria include the renal sodium dependent phosphate transporter (NPC) and chloride channels [55]. The sodium dependent phosphate co-transporter Pit-1 (*Glv-1*) was already shown to contribute to phosphate induced calcification of vascular smooth muscle cells and calcification could be prevented by the specific NPC inhibitor, phosphonoformic acid [10].

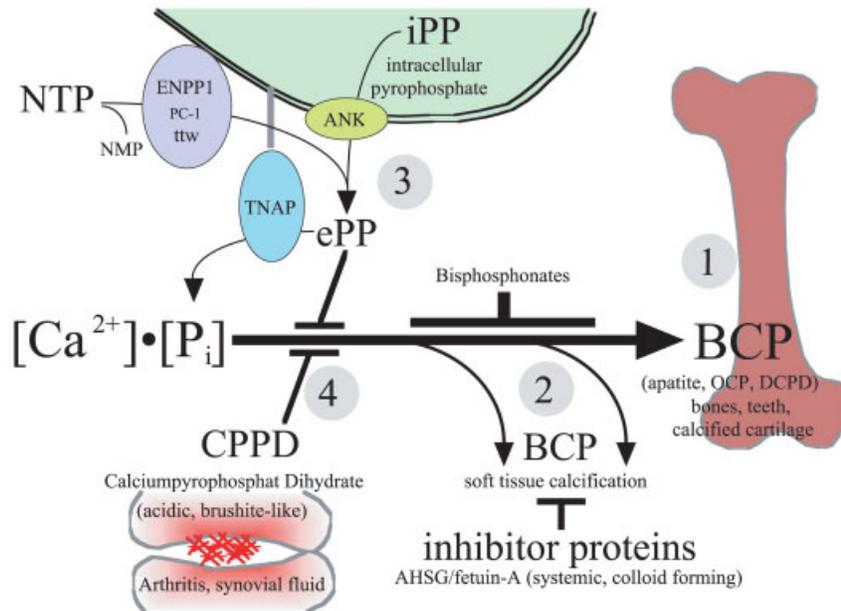


Figure 15.4 Calcium phosphates in mammals. Physiological mineralization of bones and teeth (reaction 1) yields mainly BCPs including carbonated, substituted apatite, OCP, DCPD and amorphous calcium phosphates. Soft tissue calcification including renal calcification and abdominal (peritoneal) calcification as well as atherosclerotic plaque calcification likewise contain BCP. Deposition of calcified remnants is inhibited by the systemic inhibitor protein Ahsg/fetuin-A and possible other systemic or tissue restricted inhibitors. ePP is an extremely efficient inhibitor of BCP formation in articular joints and in the spinal canal, and possibly also in the general circulation. ePP originates from intracellular organic phosphate metabolites and is transported outside the cell by the PP transporter, ANK. The ectoenzyme nucleotide pyrophosphatase/phosphotransferase 1 (ENPP1, PC-1) catalyzes the formation of ePP from nucleotides. Both ENPP1 and ANK thus maintain ePP levels, and mutations or deletions of these genes cause BCP calcification due to the lack of ePP as an inhibitor. TNAP cleaves ePP into phosphate. TNAP deficiency is associated with hypophosphatasia and osteomalacia, a bone mineralization defect syndrome. Excess ePP in articular joints can cause CPPD disease, the deposition of an acidic brushite-like calcium phosphate, calcium PP dihydrate, in the articular cartilage.

Group 5 gene products directly interfere with mineral formation and stability. The reactions of these chemical inhibitors of calcification are illustrated in Figure 15.4. As shown in reaction 1, physiological bone minerals form from Ca^{2+} and inorganic phosphate, P_i . They comprise mainly basic calcium phosphates (BCP), apatite, OCP, DCPD and ACP, because these are most stable at physiological pH and body temperature. A similar reaction can take place in the general circulation and in the extracellular fluid of soft tissues. In healthy animals reaction 2 is strongly inhibited by circulating inhibitors like Ahsg [56] or by tissue-specific inhibitors like matrix Gla protein (MGP) [57] – two glycoproteins that will be discussed below in more detail.

Several lines of evidence suggest that a simple inorganic compound, pyrophos-

phate (PP) is an extremely efficient inhibitor of BCP formation in the way depicted in reaction 3. PP is an established inhibitor of biological calcification [28]. Its chemical structure inspired the development of synthetic calcification inhibitors, bisphosphonates [58], which have been developed into potent drugs preventing mineral loss in osteoporosis [29]. Work reviewed in detail elsewhere [20, 59] demonstrates that reduced production of extracellular (ePP) or insufficient transport of intracellular (iPP) into the extracellular milieu are both associated with excessive calcification of articular joints or spinal canal. More specifically, deletion of the gene for ENPP1 (ecto nucleotide PP/phosphodiesterase), also known as PC-1 (plasma cell protein 1) [60] is associated with a phenotype similar to the mouse mutant *ttw* (*tip-toe walking*). These mice suffer from arthritis, calcification and stiffening of articular cartilage, which prevents articulation of the joints and normal walking. In humans, mutations in ENPP1 are associated with idiopathic infantile arterial calcification [61]. The importance of ePP is further illustrated by the mouse mutant *ank/ank* [62]. The *ank* mice have a truncation mutation in the gene for a membrane-associated PP transporter. This genetic defect is associated with myopathy and calcification similar to the ENPP/PC-1-deficient phenotype [63].

Further evidence for the importance of ePP in the prevention of calcification comes from knockout mice lacking tissue non-specific alkaline phosphatase (TNAP). This enzyme cleaves and inactivates ePP in the bone. TNAP deficiency is associated with a complex phenotype. As would be expected in the presence of excess ePP, the bones of TNAP-deficient mice suffer from osteomalacia, a hypomineralization defect, [64] as well as from hypophosphatasia [65]. In addition, lack of TNAP promotes the deposition in articular and meniscal cartilage of excess ePP as an acidic calcium phosphate, calcium PP dihydrate (CPPD) depicted in reaction 4. Thus, TNAP deficiency in humans, like in mice [66], is associated with CPPD disease, a syndrome of osteomalacia, pseudo gout and articular chondrocalcinosis [59, 67].

One mutant mouse strain mentioned earlier carries a targeted deletion of the gene for MGP [57], a protein originally isolated from bone organic matrix [68]. Mice lacking MGP die within the first 2 months post-natal due to calcification of the main aortic trunk and surrounding connective tissue. The same tissues also produce most MGP in early post-natal life [57]. Therefore, MGP is considered a *bona fide* inhibitor of calcification in these tissues. MGP is poorly soluble and is a prototypic matrix-bound or tissue-restricted protein inhibitor of unwanted calcification. None of the proteins mentioned so far are expressed throughout the body like a systemic inhibitor of calcification should be. However, as stated earlier, free soluble inhibitors must exist on theoretical grounds to prevent calcification of the extracellular fluid itself.

15.5 α_2 -HS Glycoprotein/Fetuin-A is a Systemic Inhibitor of Unwanted Calcification

Work from our laboratory has shown that α_2 -HS glycoprotein/fetuin-A (genetic symbol *Ahsg* or *Fetua*), a serum protein, lends itself to this function. The name α_2 -

HS glycoprotein refers to the fact that this protein migrates with the α_2 fraction of serum proteins upon traditional cellulose acetate paper based electrophoresis. Furthermore, it reminds us of the two co-discoverers of this protein in humans [69] – Heremans [70] and Schmid [71]. In the 1980s, a relationship was noticed between human α_2 -HS glycoprotein and bovine fetuin, the major globulin of fetal calf serum [72]. It was soon established that α_2 -HS/fetuin proteins are liver-derived, highly soluble and abundant serum glycoproteins present in all extracellular fluids, including blood, lymph and cerebrospinal fluid [73]. Bone tissue fractionation with guanidine hydrochloride and EDTA [15, 74] and immunochemistry [75] showed that fetuin is highly concentrated in the mineralized bone matrix. This seems paradoxical considering that Ahsg is an efficient inhibitor of calcification both *in vitro* and *in vivo*. We will see in a detailed description of the inhibitory mechanism that the inhibition of calcification is transient and that Ahsg remains tightly associated with the mineral phase after precipitation has eventually occurred.

Bovine fetuin-A was described in 1944 by Pedersen as fetuin (derived from the latin word *fetus*), the most abundant globular serum protein in fetal calf serum [72]. After the discovery of a second fetuin, fetuin-B, the protein originally named fetuin was renamed fetuin-A [76]. Fetuin-A and the human species homolog α_2 -Heremans and Schmid glycoprotein (α_2 -HS glycoprotein/Ahsg) are major serum proteins in mammals, including cattle, sheep, pig and goat, as well as in humans and rodents [77]. 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 kininogens and histidine-rich glycoproteins [78]. Ahsg has been implicated in several diverse functions, including osteogenesis and bone resorption [79], regulation of insulin activity [80], hepatocyte growth factor activity [81], response to systemic inflammation [82], and inhibition of unwanted mineralization [56, 83, 84]. These seemingly diverse functions may well be redundant or interdependent. They bear witness of the fact that fetuins are multi-ligand binding proteins that potentially interfere with any biochemical pathway whose components they can bind and sequester. Whether or not this is physiologically relevant can be tested in a mouse animal model that we have generated [56, 85]. This animal model unambiguously demonstrated that the inhibition of unwanted calcification is one major biological function of Ahsg.

Affinity depletion experiments demonstrated that in human serum Ahsg contributed about half to one-third of a 10-fold redundancy of total serum to inhibit the spontaneous precipitation of apatite from supersaturated solutions of calcium and phosphate [83]. These findings were fully confirmed when sera from Ahsg-deficient mice were analyzed in a precipitation inhibition assay [85]. Apart from this partial lack of precipitation inhibition, the Ahsg-deficient mice on a mixed C57Bl/6-129 genetic background displayed only a mild calcification phenotype. The lack of generalized ectopic mineralization in Ahsg-deficient mice was somewhat anticipated, because Ahsg only accounted for a fraction of the inhibition of apatite precipitation observed with total serum of normal mice. Reversing this argument, combining the Ahsg knockout with genetic strains of mice which are naturally prone to calcification like the strain DBA/2 [86] should exacerbate the mild calcification phenotype

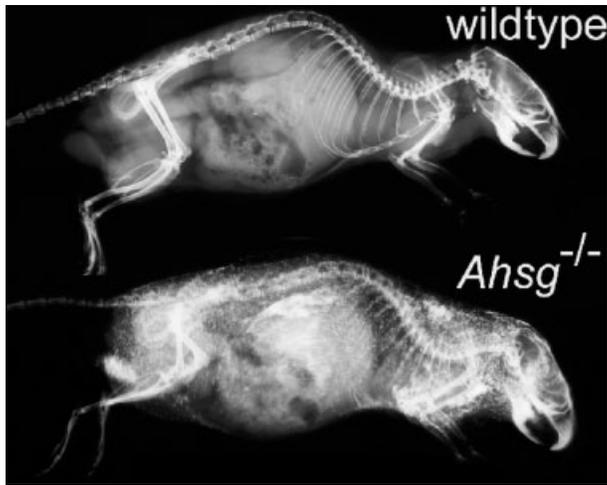


Figure 15.5 Whole-body radiographs of wild-type and Ahsg/fetuin-A deficient male DBA/2 mice. The bright spots in the *Ahsg*^{-/-} mouse are calcified lesions present in subcutaneous fat, and especially in the tongue, lung, myocardium, kidney and reproductive organs. The lesions are generally non-inflammatory. This phenotype is associated with a reduced lifespan and fertility. The mice can live well into adulthood, but stop breeding at an age of about 6 months.

observed in the original Ahsg-deficient mice. Furthermore, severe induced hypercalcemia in these animals should overwhelm the residual inhibition of BCP precipitation and should therefore result in severe calcification. Both experiments were met with the expected outcome, i.e. severe, systemic calcification in Ahsg-deficient mice [56], as depicted in Figure 15.5.

Mice treated in the ways described above suffered systemic calcification affecting major organs, including the kidney, myocardium, lung and skin. The animals closely resembled uremia-associated arteriolopathy/calciphylaxis with its clinical hallmarks [56]. This drastic phenotypic expression of Ahsg deficiency also reduced breeding performance and life expectancy. Taken together, we demonstrated by reverse genetics in mice that the serum protein α_2 -HS glycoprotein/fetuin-A is a systemic inhibitor of ectopic calcification.

Is Ahsg deficiency also important in human pathology? To this end we performed a clinical study in uremic and healthy subjects, and showed that lack of Ahsg correlates with the severity of calcification, and indeed is a statistically highly significant predictor of short-term morbidity and mortality in uremic patients [87]. Regardless of the precise molecular mechanism, it is important to state that, unlike established inhibitors of ectopic calcification, Ahsg acts systemically, not locally. This suggests that raising Ahsg concentrations in the circulation may be one reasonable approach to prevent ectopic calcification accompanying various diseases.

15.6 How does Inhibition of Calcification Work?

Ahsg/fetuin-A is easily purified and can be obtained in large quantities for structure function analyses. Important parts of the three-dimensional structure can be modeled after the known structure of chicken egg white cystatin [88]. Taken together, this offered an excellent opportunity to study the mechanism of calcification inhibition by a mammalian protein. Using dynamic light scattering and transmission electron microscopy we showed that Ahsg solubilizes apatite as a colloid [84]. This was reminiscent of how apolipoproteins ensheath and thereby solubilize insoluble lipids like cholesterol. In analogy to the lipoprotein particles of varying buoyant density (high-density, low-density, very-low density lipoproteins, etc.) formed by apolipoproteins and lipids, we called the calcium and phosphate-containing Ahsg colloid a *calciprotein particle* (CPP). An important feature of the inhibition is the fact that CPPs are only transiently soluble for up to 36 h at body temperature. The mineral phase of CPPs is initially amorphous and non-diffractive. Within 24 h the CPPs undergo a marked morphological transformation from rounded nanospheres with a diameter of around 50 nm to larger, irregular and progressively more crystalline appearance and sizes up to several hundred nanometers (Figure 15.6). It is important to remember that Ahsg binds calcium phosphate. Bovine fetuin-A calcium binding is rather poor with a K_d of 0.95×10^{-4} M [89]. Even if three calcium-binding sites exist, Ahsg/fetuin-A (10 μ M serum concentration) would only cause a minute change in serum calcium concentration (2.5 mM). Therefore, albumin (1 mM serum

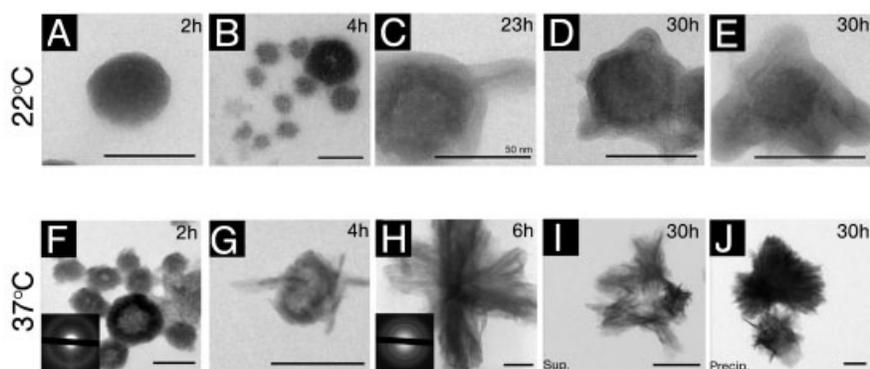


Figure 15.6 Electron micrographs of CPPs – colloidal spheres containing calcium, phosphate and Ahsg/fetuin-A. Growth and transformation of soluble CPPs. The inhibition of BCP precipitation by Ahsg is achieved by the formation of soluble colloidal complexes containing calcium, phosphate and Ahsg. The CPPs have a diameter of 30–150 nm (A, B and F). Diffraction analysis indicated the temperature- and time-dependent transition of amorphous into crystalline BCP precipitate (inserts in F and H). Radial growth of crystalline needles on the surface of the particles after 23 h at 22 °C (C) or after 4 h at 37 °C (G). After 30 h at 37 °C, crystalline but soluble needles were found in the supernatant. (I) Electron-dense crystals accumulated in the precipitate (J). Scale bar = 100 nm, if not depicted otherwise. Reproduced from [84] with permission.

concentration) should be considered the major binding protein for ionized calcium and Ahsg/fetuin-A is a highly effective scavenger of BCP, which precipitates in the absence of this protein [56].

Can we watch this inhibitory mechanism in action in living animals? So far we failed to isolate CPP-like structures from the blood of mice, probably because the removal of CPPs from the circulation is extremely fast and efficient. Experiments conducted in rats come to the rescue, however. Treatment of baby rats with high doses of the bisphosphonate etidronate, with overdoses of calcitriol or with RANKL, all caused acute and severe hypercalcemia, raising total serum calcium from a normal of around 2.5 up to 10 mM [90]. Almost all of the surplus calcium was sequestered in a high-molecular-weight complex of calcium, phosphate and fetuin-A. Small amounts of MGP and prothrombin were also present in this complex. The complex originated in bone and the amount of complex circulating in serum correlated with arterial calcification [91]. These studies corroborated that Ahsg/fetuin-A is the major protein component in a high-molecular-weight complex, which is very likely identical to CPP. Therefore, Ahsg may stabilize CPP and hence insoluble calcium phosphates as colloids *in vivo* similar to what we observed *in vitro* [83, 84].

15.7 What Happens to the CPPs?

So far, we have discussed the evidence that Ahsg is an important inhibitor of unwanted calcification *in vivo*. In addition, we have presented functional data suggesting that the precipitation of calcium phosphates is transiently prevented by the formation of soluble colloids consisting of Ahsg, calcium and phosphate. In analogy to the well-established lipoprotein particles we termed these colloids CPP. This leaves us with the question of where and how the CPPs are normally eliminated, and indeed how mineral is removed in the body. The efficient recycling and cellular catabolism of calcified remnants and CPPs is equally important as extracellular calcium homeostasis (Figure 15.3) and the stabilization of calcium phosphate as a colloid (Figure 15.6), because this process completes the extracellular calcium phosphate cycle and thus prevents build-up of unwanted calcification. Established mechanisms capable of removing calcium phosphate crystals are phagocytosis and acidification [92]. An obvious candidate organ for the removal from the circulation of particulate matter like cell remnants, molecular aggregates and likely also of “mineral dirt” is the so-called reticuloendothelial system (RES). This network of phagocytic cells encompasses endothelial cells and macrophages in the liver, spleen and bone marrow. It is likely that CPPs are phagocytosed and thereby recycled in this compartment. Do fetuins play a role in this context as well? Several reports throughout the literature indeed suggest that Ahsg has opsonizing properties, and promotes the phagocytosis of DNA and latex particles [93]. It is striking that the removal of calcified remnants like bone turnover is usually non-inflammatory. Even the large calcareous deposits of *Ahsg* knockout mice show no signs of inflammatory cell infiltrates. Is

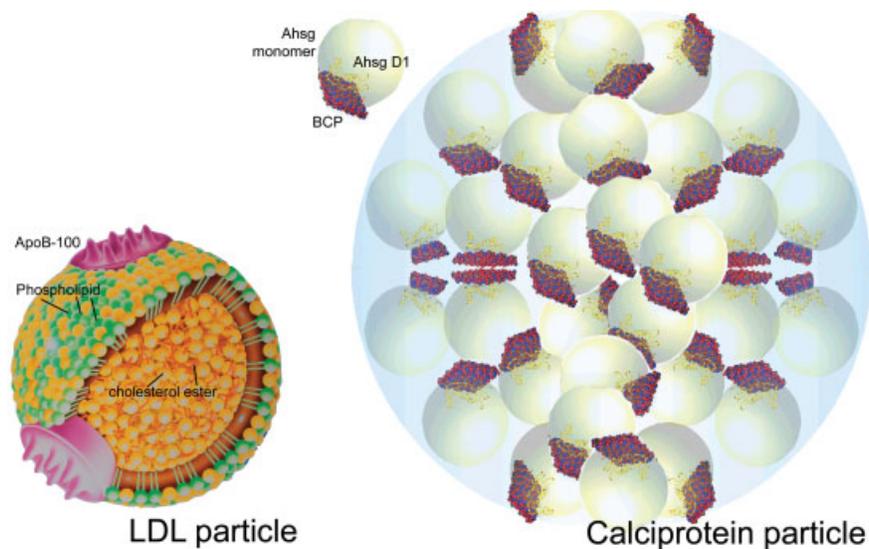


Figure 15.7 Hypothetical models of a low-density lipoprotein particle (LDL) and a CPP. LDL is about 22 nm in diameter, and contains many esterified cholesterol molecules in the hydrophobic core, cholesterol, phospholipids and few apolipoprotein B-100 molecules (513 kDa) in the hydrophilic coat. A CPP contains up to 100 globular Ahsg molecules (52 kDa) with the cystatin-like domain D1 juxtaposed to nine apatite unit cells (BSP). Note that nine apatite unit cells arranged in a lattice of 3×3 closely match this number as well as the surface area of the extended β -sheet in Ahsg domain D1, which is drawn to scale. Modified after [84]. LDL particle modified after [104].

this suppression of inflammation possibly also mediated by fetuin and possibly other mineral binding proteins? In the case of fetuin the answer may be yes. Fetuin coating may well render phagocytosed material non-inflammatory by carrying along the anti-inflammatory polyanions like spermine [82, 94] and the anti-inflammatory cytokine transforming growth factor- β [79, 95]. The combination of tight mineral binding and stabilization in calciprotein particles, the opsonizing and phagocytosis-promoting properties, and the anti-inflammatory activity make fetuin an ideal bridging molecule for the efficient and harmless uptake of mineral debris from tissue and circulation. Such debris will form due to short-term fluctuations in mineral homeostasis or in the course of normal tissue remodeling during development or following a tissue insult. *Ahsg* knockout mice will be a valuable tool in identifying the pathways of this mineral debridement. Indeed we expect the revelation of basic regulatory mechanisms of mineralization, and their integration into the general tissue and organ formation and remodeling landscape which is effective throughout life [96]. Basis biological phenomena like this are best studied in simple model organisms. Recently, an Ahsg/fetuin-A like molecule was shown in the carp to inhibit nephrosin, a matrix metalloproteinase belonging to the astacin protein family [97]. The presence of fetuin-like molecules in the zebrafish genome database holds promise that fetuin biology may be studied in this model organism. This little fish has already helped biomineralization researchers to identify molecules involved in gravitation perception by otoliths [98]. An entire chapter dealing with zebrafish

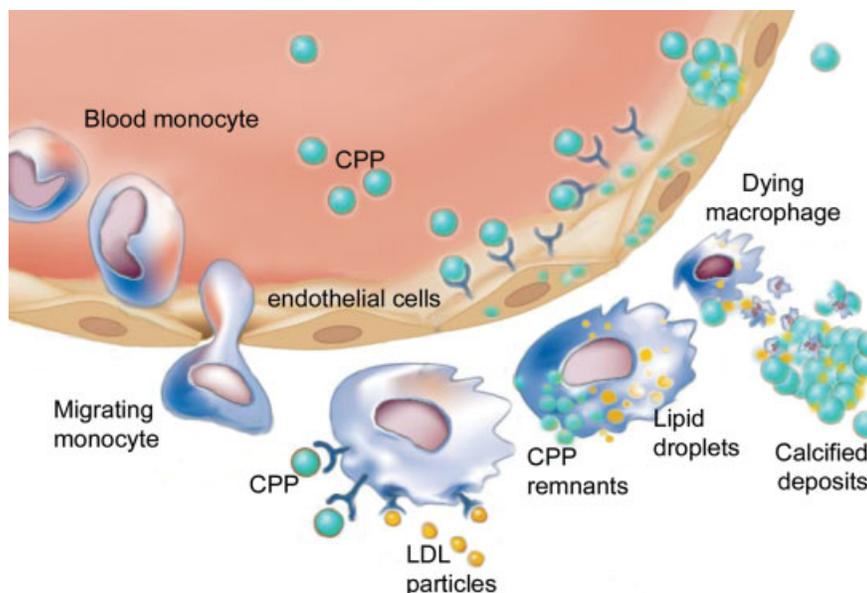


Figure 15.8 Hypothetical pathway of removal of CPP from circulation by endothelial cells and tissues resident phagocytes. In healthy individuals, CPP may form spontaneously in small numbers or may occur as a spill-over into blood of bone catabolism. When mineral homeostasis is severely disturbed, e.g. in dialysis patients, bouts of hypercalcemia and hyperphosphatemia will result in the formation of high numbers of CPP. Ahsg/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 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 RES system phagocytes and perhaps the lymphatic system, and may result in apoptosis and deposition of calcified apoptotic cell remnants also rich in lipid. Ahsg/fetuin-A stabilizes CPP in circulation and mediates their efficient uptake by phagocytes. This latter function remains to be experimentally verified. Note that many “inhibitors of calcification” activate monocytes/macrophages and stimulate phagocytosis, and therefore stimulation of calcified remnant removal may be equally important like inhibition of precipitation. Modified after [105].

genetics and mineralization in this book on biomineralization is therefore both timely and rewarding (see Chapter 14). It will be interesting to learn what the fish teach us about the role of fetuins and other calcification inhibitors in vertebrate mineral formation and turnover.

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