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REVIEW ARTICLE

Bone matrix proteins: their function, regulation, and relationship to osteoporosis

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Abstract Bone is a unique tissue composed of numerous cell types entombed within a mineralized matrix each with its own unique functions. While the majority of the matrix is composed of inorganic materials, study of the organic components has yielded most of the insights into the roles and regulation of cell and tissue specific functions. The goal of this review will be to describe some of the major known organic components of the bone matrix and discuss their functions as currently perceived. The potential usefulness of bone matrix protein assays for diagnosing the status of bone diseases and our current understanding of how these proteins could be related to diseases such as osteoporosis will also be reviewed.

Keywords Bone matrix proteins · Collagens · Noncollagenous proteins · Osteoporosis

Collagens

Bone differs from all other tissues in the body tissue in that it is largely mineral (70–90%) and because a uniquely large proportion of its total organic material is collagen (90% versus 10% in most soft tissues). On the basis of this skewed composition, it is generally agreed that collagen plays critical roles in the structure and function of bone tissue. The first part of this article will summarize the structure and synthesis of collagen using type I collagen, the most dominant collagen in bone, as a model. The fact that many of the markers currently used to measure bone formation and turnover are related to the structure and production of collagen will be emphasized (for a more detailed review of these serum and urine assays used for bone formation and turnover see [1, 2, 3]). Evidence linking abnormal collagen expression to osteoporosis will be summarized. The study of animal models and cultured cells derived from skeletal tissues shows that, in addition to having structural roles outside the cell, collagens can directly affect signaling and activity inside bone cells.

Collagens comprise a large family of multimeric proteins with up to 38 genes giving rise to 20 different collagens (for review see [4]). After these large polypeptide chains are synthesized, they become intertwined with two other chains in a triple helical structure. Each triplex can contain three identical chains or two or three different chains depending on the collagen triplex type. The major collagen triplex in mineralized tissues is type I and is composed of two α1 chains (also called COL1A1 or α1[I]) and one α2 chain (COL2A1 or α2[I]). Each of the 38 different collagen chains has a unique repeating structure of Gly-X-Y (where Y is often proline). After synthesis, the chains are extensively modified inside the cell by enzymes that add a variety of additional molecules to the chain, further increasing the complexity of the triplex structure. An important modification to collagen is the addition of hydroxyl groups (OH) to the amino acids proline or lysine. Based on the fact that bone has so much collagen and, further, that this collagen has so many proline residues, one can easily see how release of small fragments containing hydroxyproline from the bone collagen could be used to indirectly measure bone resorption. Osteoclasts degrading the matrix would release the collagen fragments that eventually end up in body fluids that can be assayed. After hydroxylation, the chains form a ''nucleus'' at the carboxy terminus where they assemble into a triple helix in a zipper-like fashion. This nucleation and ''zippering'' process must be highly orchestrated and depends on precise alignment of all the chains. Considering these structural constraints, it is easy to understand why

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mutations in the genes encoding the individual collagen chains have such profound effects on bone tissue. There are hundreds of examples of single-base mutations in collagen genes [5] that appear to disrupt assembly of the chains into triple helices and fibrils and, consequently, have profound effects on the bones of afflicted individuals. Most mutations leading to type I collagen assembly defects are lumped together as the genetic disease osteogenesis imperfecta (''brittle bone'') [6]. Since collagen's role as a structural template for mineral deposition is widely accepted [7, 8], some researchers further theorize that certain forms of osteoporosis might arise from structural mutations in the type I collagen chain genes [9].

When the individual collagen chains are assembled into fibrils, they exit the cell, and globular regions at both ends of the triplex are cleaved by specific proteases leaving a shortened triple helical chain. The two ends of the type I collagen fibril that are released are the amino-terminal propeptide (PINP) and the carboxy-terminal propeptide (PICP) and are both used in the clinic as a way to access collagen synthesis and bone formation. Once outside the cell, the triplex undergoes further modification: covalent cross-linking within and between collagen molecules takes place. Since the cross-linking patterns are different between bone and soft tissues [10], the ability to detect the different kinds of cross-links provides a means of measuring bone resorption and includes the clinically used markers that assay free and total deoxypyridinolines (DpD). From a functional standpoint, new theories suggest that this differential cross-linking may be responsible for the unique ''toughness'' of bone [11] and that certain of these cross-linking intra-collagen covalent bonds may be ''sacrificed'' to release energy when subject to mechanical stress (so-called sacrificial bonds [12]). It is tempting to predict that other unique cross-links will eventually be identified that represent both mature and immature fragments of collagen released during bone tissue development and aging [11]. Bone also contains many other noncollagenous proteins (NCPs) that are being used as markers of bone health, including osteocalcin (for bone formation), which will be discussed later. As we learn more about the biochemical nature of bone matrix proteins such as collagen it is likely more markers will be developed that could be used as indicators for osteoporosis. While some controversy still surrounds the value of measuring bone matrix protein markers to evaluate bone remodeling [2, 13], they are still widely used to monitor bone heath status. Further, some NCPs are being evaluated as markers for other important skeletal diseases, including gum destruction [14], particulate osteolysis [15] or bone metastasis [16, 17].

There is now genetic evidence linking variation in the collagen genes to osteoporosis [18]. Genetic polymorphisms in the regulatory regions (an SP1 site) of the COL1A1 gene are associated with abnormal bone mineral density (BMD) and body mass [19]. The affected alleles are called ''S'' and ''s'', where individuals with ''s'' have lower BMD and higher incidence of fracture. Analysis of gene activation from these alleles shows that the ''s'' allele has more binding of the transcription factor SP1 leading to increased transcription of the gene. The result is increased COL1A1 mRNA and protein production. This increase in the COL1A1 gene further leads to an imbalance in the ratio of $\alpha 1$ to $\alpha 2$ chains in the triplex with the presumed production of triple helical collagens made of all $\alpha 1$ chains [I] [20]. The clinical outcome is reduced integrity of bone and increased fracture occurrence [21].

Cell and molecular evidence indicate that collagen also has direct effects on important bone cell functions including apoptosis [22], cell proliferation [23] and differentiation [24] under complex control by signaling from the cell surface to the nucleus [25]. These research findings about collagen control and function could be used as a foundation to design novel treatments for osteoporosis. For example, one can imagine simple assays for rapid testing of large numbers of new compounds for their ability to induce collagen or any of the other numerous molecules in and outside bone cells that are affected by collagen and whose increased synthesis might be a means of increasing bone mass and strength.

Noncollagenous matrix proteins (NCPs)

Because type I collagen is also made in other non-bone tissues, researchers have looked for other organic components (NCPs) that occur only exclusively in bone tissue and cells and which might be used as markers for bone function. Some NCPs were indeed found to have highly restricted patterns of expression in bone, including osteocalcin. Assays for this protein are currently used clinically to monitor the levels of bone formation. Increases in osteocalcin levels are presumed to reflect increases in bone formation and bone turnover. Most NCPs in bone, however, are like type I collagen and are also made in soft tissues. Nevertheless, research reports have shown that at least some NCPs have multiple functions in bone cells including regulation of collagen fibril mineralization [26] and modulation of cell division, migration, differentiation and maturation. Some of these functions will be described below because they help clarify the ''specificity problem.'' That is, although it may be useful to have bone ''markers'' that are specific indicators for bone formation or breakdown, it is important to realize that essential bone activities may be controlled at many levels. Ultimately, specificity may come from an overall balance of factors, including collagen, NCPs, and the repertoire of growth factors and hormones that are harbored within this mix of mineral, protein and cells.

Many excellent reviews on the structure, expression and function of NCPs [27, 28] are available. They are recommended for readers interested in a broad, in-depth survey of this subject. In the present article, selected studies will be described to illustrate certain

developments that are currently unfolding in bone research, with the focus on the main NCPs in the field. The article will emphasize how research on NCPs may increase our understanding of osteoporosis.

One of the most abundant NCPs in bone is the glycoprotein fibronectin. It accumulates extracellularly at sites of osteogenesis. It is synthesized at distal sites and delivered by the serum, or is synthesized in cells close to where it accumulates in bone. Recent studies using high-throughput DNA sequencing of thousands of genes expressed in cultured human marrow stromal fibroblasts (the osteoblast precursor cell) showed that fibronectin was the most abundant mRNA species (4.65%), followed by the type I collagen mRNA (1A1 and 1A2 together accounted for 4.26%) [29]. An even higher level of fibronectin mRNA was found in cells cultured from human trabecular bone biopsies (L. Jia, unpublished observations, 2002) under conditions that allowed them to develop into more "mature" or differentiated bone-forming cells [30]. The high level of fibronectin expression in both types of cells implies that it is important in bone. While it must be cautioned that mRNA expression data comes from cells cultured in vitro, many additional lines of evidence point to critical roles for fibronectin in bone development and function. An important structural-function discovery about fibronectin is that it contains a short amino acid sequence (Arg-Gly-Asp or RGD) that is critical for binding to integrin receptors located at the cell surface and for subsequent osteoblast differentiation [31, 32] and survival [33]. Control of cell proliferation and differentiation by fibronectin (and collagen, which also has an RGD site) appears to involve protein kinase signaling pathways that ultimately affect the transcription factors c-*jun* and c-*fos* at AP1 sites within the promoter region of genomic DNA. Fibronectin may also regulate mineralization in bone [34, 35] by binding to other matrix proteins, modifying their activities [36, 37]. Studies using cancer cells indicate that fibronectin can facilitate release of the cell-bound protease matrix metalloproteinase 2 (MMP-2) [38], implying it could have roles in regulating bone turnover. Genetically engineered mice unable to make fibronectin die at a very early age, showing its importance in tissue formation in vivo [39]. The presence of high levels of fibronectin and its fragments in intervertebral discs is correlated with morphologic degeneration, suggesting that fibronectin fragments are derived from altered protease activity, which could in turn alter cell signaling functions [40]. Production of fibronectin , like that of most NCPs [41], is regulated by numerous hormones including dexamethasone, estrogen and parathyroid hormone (PTH). The stimulatory effect of PTH on fibronectin in vivo [42] is particularly intriguing given that it may, at the same time, regulate the integrins it depends on for cell signaling [43]. One wonders whether the intermittent PTH administration that has been shown to increase bone formation in vivo works by increasing fibronectin and its corresponding receptors to elicit combined, maximum effectiveness in vivo.

One of the first NCPs postulated to have bone-specific functions was discovered over 20 years ago by John Termine and his colleagues and was named osteonectin ("bone connector") [26]. It was called this because it has a strong affinity for both collagen and mineral and was theorized to be a bone-specific nucleator of mineralization. Since that time, osteonectin has been found throughout the body, particularly at sites of tissue remodeling and matrix assembly. Overexpression of the nematode *Caenorhabditis elegans* homolog (also called SPARC, for Secreted Protein Acidic and Rich in Cysteine) caused reduced mobility and paralysis, further indicating that it may have general roles in cell and tissue function [44]. Recent experiments in mice showed that deletion of osteonectin (by gene knockout) caused osteopenia resulting from low bone turnover, with defects apparent in both osteoblast and osteoclast activity [45]. The cellular basis for this osteopenia may be caused by any number of "matricellular" functions controlling either other NCP functions [46] or inhibition of the cell cycle [47]. The osteonectin-deficient mouse should be an excellent model to determine which of the in vitro ascribed functions for the protein hold true in vivo. It could also be used to test new therapies designed to cure low-turnover-induced osteoporosis.

Thrombospondin-2, like osteonectin, is an abundant NCP in bone [27, 48] that may also affect multiple cellular functions [49]. It should receive renewed attention in skeletal research because the bones of the knockout mice unable to make it have increased cortical thickness and increased total density that appears to be correlated with increased endosteal bone formation [50]. The cellular basis for the cortical thickness increase appears to be an increased number of bone cell precursors, whose numbers normally are negatively controlled by thrombospondin-2 [51]. Future research in this area might be designed to find ways to "inhibit the inhibitor" resulting, ultimately, in increased bone precursors that would develop into cells that increase bone mass.

Several bone matrix proteins are induced by transforming growth factor beta (TGF-β), including type I collagen, fibronectin and βig-h3, another major matrix protein (the twelfth most abundant mRNA in human stroma cells) [29]. βig-h3 is downregulated in cells isolated from patients with melorheostosis [52], a rare bone disease characterized by hyperostosis. βig-h3 can also be downregulated in cultured skeletal cells treated with dexamethasone [53], leading some to speculate that βig-h3 could mediate the actions of this hormone in bone tissue. Animal knockout models or cases of genetic aberration in humans will be needed to clarify the in vivo roles of βig-h3 in calcified tissues. There are numerous other NCPs in bone, such as vitronectin and tetranectin, that are structurally and not surprisingly functionally distinct from each other. As more and more of the genes expressed in skeletal cells are characterized [29], it is certain they will be used to develop new lines of experimentation for basic research into NCP function and new bone markers. New animal models with dele-

tions or alterations in these NCPs may also provide new models of skeletal disease that would be useful for testing new therapies for bone diseases such as osteoporosis.

If most of the matrix proteins in bone are also found in other connective tissues, what makes bone unique? Clearly, the answer is that no other tissue in the body contains as much mineral as bone. Thus, a better question would be to ask: do the matrix proteins play roles in bone that they do not play in other connective tissues and, if so, what are these roles and how do they relate to the mechanisms that control the mineralization process (for review see [54, 55])? We now know that mineralization may be controlled in part by the small NCP bone gla protein (BGP, OCN or osteocalcin) and by the related but distinct matrix gla protein (or MGP). These small gla-containing proteins have post-translational modifications dependent on vitamin K (see [56] for review). OCN has a very narrow expression pattern being made only by the osteoblasts and osteocytes in bone while MGP (also found in bone) is also highly expressed in cartilage and arteries. It has been known for many years that when rats are treated with warfarin, an antagonist to vitamin K that blocks gamma carboxylation, they have greatly reduced levels of OCN in bone (2% of normal levels) but have, surprisingly, apparently normal bones. Since the warfarin-treated rats also had excessive mineralization of the growth plate it was concluded that OCN levels are not critical for bone development and that warfarin might act as an "inhibitor of an inhibitor." That is, gamma-carboxylated modified MGP could normally act as an inhibitor of mineralization [57]. Recently, studies with knockout mice unable to make either MGP or OCN revealed the precise roles of the two gla-containing NCPs. Mice unable to make OCN were grossly normal [58]. MGP-deficient mice, on the other hand, had normal bones but spontaneous calcification of arteries and cartilage leading to death by 2 months of age [59]. These observations indicate that MGP is a powerful inhibitor of mineralization in arteries and cartilage and can block hydroxyapatite accumulation in tissues where it is expressed. Treatments designed to selectively inhibit MGP in bone and not in tissues where its expression is needed could be useful in bone diseases with severe osteomalacia. One caveat to this approach might be that even if mineralization could be induced locally only in bone, the quality of the mineral and bone may not be normal and would need careful testing using animal models. Inhibition of the inhibitor in soft tissues could also very well have deleterious effects leading to atherosclerotic lesions [60].

As investigators probe more deeply using the MGP and OCN gene knockout models described above it is likely that regional or other subtle changes in bone structure or mineralization will be uncovered [61]. Indeed, a recent report revealed a significant correlation between the carboxylated form of OCN and bone quality judged by ultrasound. Considering that gamma-carboxylation of these small NCPs may help bone structure, it has been further suggested that vitamin K could be used as a "neutraceutical" therapy to prevent bone fracture [62].

Further attempts to discover NCPs with "bone-specific" functions revealed a family of NCPs that is now called the SIBLINGS (Small Integrin-Binding LIgand, N-linked Glycoprotein) [63]. Gene structure-function studies showed that all the family members are clustered in tandem on human chromosome 4 and that they all have type 0 intron-exon structures. This means that each exon is always interrupted (by an intron) at an amino acid junction (unlike most genes, which are interrupted within an amino acid triplet). The SIBLINGS all contain an RGD (Arg-Gly-Asp) cell attachment sequence, always encoded in the last gene exon, which binds to integrin receptors at the cell surface. The family is also characterized by extensive post-translational modifications including both *N*- and *O*-linked oligosaccharides, many of them rich in sialic acid. They can also be phosphorylated and, for the most part, are acidic. Members of the family include: bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein (DMP), dentin sialophosphoprotein (DSPP) and matrix extracellular protein (MEPE). A protein with many structural characteristics of the SIBLINGS called Bag 75 [64] has so far eluded genetic cloning and verification that it may be part of the family.

A mouse model in which the osteopontin gene has been deleted has been the subject of several studies. The adult bones of these mice are grossly normal but resist the bone resorption that is normally induced by ovariectomy [65], reduced mechanical stress [66] or continuous PTH treatment [67]. These and other results have suggested that osteopontin is required for the resorption induced by these treatments because it increases the angiogenesis and vascularization that is induced by these treatments [68]. Since OPN mRNA and protein are normally found in both the osteoblast and osteoclast [69], it is tempting to speculate that it could also be a key component in coupling the normal bone turnover process. Expression of several SIBLINGS proteins is upregulated in the primary tumor sites in many osteotropic cancers such as breast [70] and prostate cancer [71], leading to the theory that they can assist cancer cells to either metastasize or, more likely, to colonize bone. Biochemical evidence using purified BSP and OPN shows they bind to and inhibit factors in the immune complement pathway of cell lysis, suggesting that they provide cancer cells with a method to evade attack and destruction by host defenses [72]. Evidence to support this theory in vivo comes from OPN-deficient mice, which acquire fewer metastatic tumors when injected with B16 melanoma cells [73].

Only one of the SIBLINGS genes, DSPP, has been found to have mutations in humans. Four different pedigrees from two separate areas of China [74, 75] have either missense or nonsense mutations in the DSPP gene; afflicted patients have abnormal teeth (the protein is

expressed in dentin) and, surprisingly in some cases, hearing loss in the high-frequency range. Considering the fact that there are multiple SIBLINGS and that they are all made by bone, it is possible that some have overlapping, redundant functions with an evolutionary advantage when expressed in the proper context. When expressed out of context, dire consequences may occur. For example, ectopic expression of the SIBLING protein MEPE is associated with oncogenic hypophosphatemic osteomalacia (OHO) [76]. Because the SIBLINGS are linked closely together on the same chromosome, the single knockout mice lines generated so far will not be useful to create double knockouts (the level of recombination in linked genes is very low making the generation of double knockouts unreasonably challenging). Serum assays for individual NCPs have been developed and could easily be used for a combined evaluation of individuals with metastatic cancer [17, 77] as well as bone disease [78].

When proteins are modified with so many sugar residues that most of their mass is carbohydrate they are referred to as proteoglycans (PGs). The enzymatic reactions that control these modifications (glycosaminoglycans/GAGs) of the "core proteins" are numerous and complex [79]. Large PGs can be embedded into the extracellular matrix or intercalated into cell surface membranes. One example of a PG in this latter category with apparent relationships to bone function is perlecan [79]. There are also "small" PGs, a growing family that now constitutes at least 12 members that are characterized by a repeating amino acid motif rich in leucine and which are called SLRPs (small leucine-rich proteoglycans) [80, 81]. They are considered "small" because they have a core protein that ranges from 40 to 60 kDa, depending on the PG, and can contain either keratan sulfate (KS) or dermatan/chondroitin sulfate (DS/CS) depending on the tissue. Several SLRPs have been shown to bind to collagen (for review see [81]) and to TGF-β [82, 83] using different binding sites on the PG molecule. The best-characterized SLRP in bone is called biglycan because it has two GAG chains attached to a leucine-rich repeat (LRR) core protein. Mice deficient in biglycan develop lower "peak bone mass" as they age due to defects in bone formation in a phenotype resembling osteoporosis [84]. The cellular basis for the defect appears to arise, in part, from defective osteoblast precursors, which are fewer in number and are less responsive to TGF-β [85] than normal osteoblast precursors. Mice made doubly deficient in biglycan and the closely related molecule decorin have bones that are even more defective than those deficient in biglycan alone (and decorin-knockout mice have no gross phenotype), indicating that decorin can compensate for biglycan when it is absent [86]. Severe defects in the shape and size of collagen fibrils in the doubly deficient biglycan- and decorin-deficient bone were detected using transmission electron microscopy, providing further evidence that these SLRPs have a role (perhaps indirect) in collagen assembly and function [86]. The abnormal structure and composition of the collagen matrix in the biglycan/decorin knockout mice could be the molecular basis for the decreased bone mineralization observed in such mice. The concept of functional redundancy and compensation is further demonstrated in mice deficient in biglycan and the more distantly related fibromodulin. The single knockout mice each have substantial ectopic tendon calcification. However, this ectopic calcification is more severe in the double knockout than would be expected simply by adding the phenotypes together [87]. These studies provide an additional example of how matrix proteins, when expressed in a different tissue context (in this case the tendon), can be negative inhibitors of mineralization (the other example discussed is MGP). To date, 9 of 12 known SLRPs are found in skeletal tissue. There is still much more to be learned about their physical properties and their unique and overlapping biomechanical and biological roles in bone at many levels, including maintenance of matrix integrity, growth factor modulation, control of cell division, and signaling from the cell surface to the nucleus.

If we assume that matrix proteins are vital to bone, a critical question to ask is what regulates their production? Like the search for bone-specific matrix proteins, there has been a parallel search for "master genes" that could control and coordinate the expression of the many matrix genes. One candidate in this category is the gene encoding the transcription factor Cbfa/Runx2. Genetically engineered mice (knockout) unable to make this factor do not form any bone [88] and die just after birth. Cell culture experiments have shown that the promoters for two NCPs, osteocalcin and osteopontin, require this transcription factor [89]. It is unlikely, however, that one factor can, by itself, control the expression of all bone matrix genes in all cell types. Indeed, other transcription factors have also been shown to be able to regulate the NCPs, including DLX5, MSX-1 and 2, fra1, fos and Ets (reviewed in [90, 91, 92]). A new master gene was recently described called osterix [93]. The factor was named osterix by modifying the name of the cartoon character Asterix (who is smart and powerful) with "os", the prefix for bone (B. de Crombrugghe, personal communication). Osterix, like Cbfa, is critical for bone development but acts "downstream" (later in the pathway) compared with Cbfa/Runx2 to direct the fate of stem cells toward osteoblast formation. Like Cbfa/Runx2, this activity leads to the induction of bone matrix proteins and bone tissue formation. While these studies have so far been mostly limited to embryos, new experiments designed to examine the functions of these master genes in older animals must be performed to know what role they play in aging bone tissue. Questions that remain unanswered are how precisely these master genes work and how they themselves are controlled. Will these genes be subject to environmental cues such as fluctuation in hormones, nutrition or exercise? Eventually one may be able to know how the responses of genes encoding transcription factors that control NCPs will be affected by an individual's genetic background.

In addition to "master genes," one should not ignore the importance of nuclear factors made by bone and non-bone tissues that could also control matrix production. Coming back to the aforementioned "specificity problem," there are several more broadly expressed transcription factors such as vitamin D receptor and steroid receptors, or nearly ubiquitously expressed ones like SP1 and c-*fos*, that are known to be important in bone cell gene activation. The balance of their expression, activity and localization in the nucleus with associating factors will likely control the specific pattern and level of NCPs in normal and diseased bone.

In summary, bone matrix contains a multitude of proteins that have distinct and overlapping roles in controlling the structure and function of bone. Many play important roles in mineralization, having both positive and negative effects and in maintaining bone strength, stability and integrity. Additional understanding of the unique biochemical nature of bone matrix proteins will continue to allow the development of new and better serum and blood assays to monitor and diagnose bone health status and, eventually, therapy. Continued research on the biochemical nature of bone matrix proteins and on the elements that regulate their expression and function should greatly improve our ability to design better treatments for osteoporosis and other disorders of the skeleton.

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