

Review

The bone matrix protein secreted phosphoprotein 24 kD (Spp24): bone metabolism regulator and starting material for biotherapeutic materials

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Summary. Secreted phosphoprotein 24 kD (Spp24) is a bone matrix protein that appears to be derived primarily from the liver and delivered to other tissues in a protective complex. A significant role in bone growth and turnover is suggested by genetic studies that associate the gene locus (SPP2) with bone mineral density and bone quality. The function of this protein in the normal bone environment is unknown but clues are given by the fact that Spp24, or proteolytic products of Spp24, bind cytokines of the TGF- β superfamily and also activate intracellular signaling pathways. Several potential biotherapeutics have been engineered from this protein including materials that enhance BMP-induced bone healing and, on the other hand, materials that inhibit BMPs in clinical situations where this is called for such as reducing BMP-induced inflammation and inhibiting tumors dependent on BMP autocrine systems. As understanding of the structure and function of this protein increases, more opportunities for rationally developed therapeutics will become apparent.

Key words: Secreted phosphoprotein 24 kD, Spp24, SPP2, Bone morphogenetic protein, Bone matrix proteins

Introduction

Secreted phosphoprotein 24 kD (Spp24) is a protein that has been isolated from bone matrix and that has been used as the starting product for several bone-related biotherapeutic molecules. However, since the production, processing, distribution, and activities of this protein are only now being clarified, the limitations of our knowledge must be kept in mind and premature assumptions pertaining to its primary function avoided. This protein was first recognized thirty years ago but the history of the investigation into it is one of enigmatic and incomplete clues or outright false starts. Of course, the absence of a final definition of its function does not preclude it from being empirically employed to engineer materials with specifically desired properties. On the other hand, discovery of new physiological roles may allow for the rational design of biotherapeutics that intervene in processes far removed from the current applications.

Discovery and history

In retrospect, Spp24 has been “discovered” four times. In 1987 Sen et al. made efforts to purify osteogenic proteins responsible for the activities described by Urist and also Reddi and reported the N-terminal sequence of a 23 kD bovine bone matrix protein that they described as “osteogenic” (Sen et al., 1987).

The results presented for the bone formation assay in the report showed endochondral bone formation. The sequence that they reported (FPVYDYSPARLKEA) was a close (12/14) match to the N-terminal sequence of the protein that would later be called bovine Spp24 (FPVYDYDPASLKEA). However, the amino acid composition of the protein does not match that of Spp24 nor have we been able to convincingly compare it to other known bone morphogenetic proteins (BMPs). We have not found any subsequent reference to this material in the literature.

In 1995 Agarwal examined RNA from the livers of normal and growth hormone (GH) resistant chickens in order to define genes that were responsive to GH stimulation (Agarwal et al., 1995). They reported a material which they described as corresponding to an 120 amino acid protein which they called Growth Hormone Regulated Gene-1 (GHRG-1). They did not assign a function to the protein but hypothesized that it was an intracellular protein because it had no leader sequence. The sequence for this protein corresponds to chicken Spp24. Subsequent investigators convincingly argued that the sequence for this clone was derived from a less than full-length clone (Bennett et al., 2004). Harvey et al. later localized the mRNA for GHRG-1 in various parts of the chicken brain (Harvey et al., 2002) and day 8 chicken embryos (Harvey et al., 2001). We have found no other references to this material though GHRG-1 is listed in some databases and is not described as chicken Spp24.

Also in 1995, Hu et al. published the sequence for bovine Spp24 and promulgated the name, secreted phosphoprotein 24 kD (Hu et al., 1995). They isolated the protein as part of an effort to characterize additional bone matrix proteins. They defined the structural domains of the protein and incorrectly hypothesized that it played a role in bone remodeling through inhibition of proteases because of the presence of a cystatin domain (see below).

Behnam et al. (our group) provided the first concrete clues into the physiological functions of Spp24 when they isolated the protein in the course of efforts to characterize the primary osteogenic component of Urist's (who was a collaborator on the project but who died prior to the completion of the work) bone morphogenetic protein/non-collagenous protein (BMP/NCP) (Behnam et al., 2005). Expecting the protein to be osteogenic, this group produced transgenic mice with Spp24 expression under the control of the estrogen-sensitive osteocalcin (OC) promoter (Sintuu et al., 2007). Quite unexpectedly, the bone mineral density of femurs from 3 month and 8 month old female transgenic mice showed reduced bone mineral density (BMD). However, because others (Brown and Dziegielewska, 1997; Demetriou et al., 1996) had noted BMP and TGF- β binding of another protein (fetuin) that contained a cystatin domain, the alternative hypothesis that Spp24 was a BMP-binding protein was tested. Binding to BMP-2 was demonstrated for a cyclic 19

amino acid peptide that was homologous to the BMP binding domain of fetuin (see below) (Behnam et al., 2005). Furthermore, this cyclic 19 amino acid peptide (BMP binding peptide, or BBP) enhanced BMP induced ectopic bone formation and so it was hypothesized that a proteolytic product of Spp24 might account for the osteogenic activity of BMP/NCP.

To date, independent osteogenic activity has not been demonstrated for any form of Spp24. It remains unexplained why two independent investigators (Sen and Urist) attributed osteogenic properties to the protein. We have hypothesized that this could be explained by artifacts of co-purification of Spp24 and a bound BMP.

Structure and phylogeny

Hu et al. defined the major domains of the Spp24 molecule (Hu et al., 1995). Initially, they described the bovine molecule as a 200 amino acid protein. Subsequently, three amino acid residues were added in sequences given in databases. (Accession number Q27967. UniProt database. Swiss Institute of Bioinformatics. www.expasy.org/proteomics.) They described a 20 amino acid signal peptide (aa 1-20), a 107 amino acid cystatin domain (aa 21-127), an 11 amino acid serine-rich domain (aa 128-136), and a C-terminal domain (aa 137-200). They noted similarities between Spp24 and the cystatin domain of kininogen and other members of the cystatin-like protein family including batenecin. With respect to the C-terminal domain, they noted that in the human form of fetuin (α_2 HS glycoprotein) this region is cleaved as part of the process of producing a two chain form. Interestingly, they speculated that the cleaved C-terminus may give rise to a biologically active peptide but they did not indicate what type of function such a peptide might perform.

The first concrete insights into a possible function for Spp24 came as the result of a comparison of the cystatin domain of Spp24 and that of fetuin. Demetriou et al. defined a sub-domain within the cystatin domain of fetuin with sequence similarity to the TGF- β receptor type II which they named the TGF- β Receptor II Homology-1 domain (TRH-1) (Demetriou et al., 1996). While the degree of similarity between the TRH-1 domains in both fetuin and Spp24 and the TGF- β receptor type II is not great (Tian et al., 2015a), the functional significance of these regions was confirmed by studies which demonstrated significant binding between peptides based on these sequences and TGF- β and BMP-2 (Demetriou et al., 1996; Behnam et al., 2005).

As indicated above, Behnam et al. identified a TRH-1 domain in Spp24 (Behnam et al., 2005). This group also defined the proteolytically derived N-terminus truncation products of bovine Spp24 (full-length: aa 24-203; Spp18: aa 24-176; Spp16: aa 24-157; Spp14.5: aa 24-143) (Brochmann Murray et al., 2007) and also characterized the binding of each fragment to BMP-2 and TGF- β (Brochmann et al., 2010; Tian et al., 2013).

In addition, two C-terminal peptides have been examined. These were designated as “complementary to” Spp14.5” and “complementary to” Spp18 (Tian et al., 2015a). That is, c/t Spp14.5 consists of the amino acids that would be on the C-terminal side of the cleavage that produced Spp14.5 (for bovine, c/t Spp14.5 is aa 144-203; for the human protein which was used in some experiments c/t Spp14.5 is amino acids 151 to 211) and c/t Spp18 consists of the amino acids that would be on the C-terminal side of the cleavage that produced Spp18 (for bovine c/t Spp18 is aa 177-203). Neither of these peptides contains any of the TRH-1 domain. However, independent BMP binding was demonstrated for the longer of the two peptides and both displayed biological activity (Tian et al., 2015a) (see below). Spp24 RNA has been reported for several mammalian species (human, cow, pig, rat, and mouse) as well as chicken, trout and salmon (Bennett et al., 2004). The piscine and especially the avian forms have shortened C-termini. Kordis and Turk have opined that the gene is found only in chordates (Kordis and Turk, 2009).

Localization and distribution

It is critical to keep in mind that Spp24 is a delivered protein and, therefore, studies employing detection of RNA will provide information about where it is synthesized but not where it resides. No comprehensive survey of Spp24 protein localization in embryonic or adult animals has been published. Obviously, several groups have isolated the protein from bovine bone (Sen et al., 1987; Hu et al., 1995; Behnam et al., 2005). Agarwal reported the presence of RNA specific for GHRG-1 (that is, chicken Spp24) in normal embryonic chicken liver and the absence in fat, muscle, thymus, bursa (presumably bursa of Fabricius but not specified), and pituitary (Agarwal et al., 1995). Harvey et al. used *in situ* hybridization to localize GHRG-1 in several areas of adult chicken brain (Harvey et al., 2002) and a variety of tissues in day 8 chicken embryos including epidermis, spinal cord, lung, esophagus, crop, liver, heart, skeletal muscle, and developing bone (Harvey et al., 2001). Hu detected the presence of Spp24-specific RNA in bovine periosteum and liver but not in heart, lung, kidney or spleen (Hu et al., 1995). Spp24 has been detected in arthritic joint tissue (see below). Using analytical 2D gel electrophoresis, Bos et al. demonstrated that Spp24 was three times more abundant in adult mouse long bone (endochondral bone) than in calvariae (membranous bone) (Bos et al., 2008). The GeneCards database (www.genecards.org; Weizmann Institute of Science, Rehovot, Israel) lists estimated levels of protein expression for humans in serum, platelets, kidney (HEK-293), and liver only. (It should be kept in mind that data deposited in databases have not been subjected to the same review process as data published in journals.)

A limited amount of information relating to production of Spp24 by cell lines is available. Several databases allow access to information from gene array

screening studies for RNA from a very large selection of cell lines. The absolute levels of expression are not presented and comparisons between studies are not reliable. The absence of expression is obviously easier to interpret. Searches of Geo Profiles (www.ncbi.nlm.nih.gov/geo) and Protein Atlas (www.proteinatlas.org) found what appeared to be significant levels of expression only in HEK293 (human embryonic kidney). On the other hand, Mashima reported that as rat pancreatic AR42J cells are differentiated towards insulin secreting cells, increased levels of Spp24 RNA were detected (Mashima et al., 1999).

Currently, it is unknown if the Spp24 present in bone (or other tissues) is synthesized at the site or produced in the liver and delivered to bone, or, a combination of both processes. However, the case for significant delivery became more tenable with the discovery by Zhao et al. that Spp24 is transported in the serum in a protective complex with α_2 macroglobulin (Zhao et al., 2013).

Others have reported Spp24 to be present in complexes in serum. Price et al. have reported on the presence of fetuin mineral complexes (FMC) in the serum of rats (Price et al., 2003). These complexes are composed of calcium phosphate mineral, fetuin, matrix Gla protein, and several minor components, including Spp24. They hypothesize that the function of the FMC is to prevent ectopic calcification and that Spp24 contributes to this activity. However, the FMC has not been demonstrated to exist in normal physiological serum. The complex is induced by administering the bisphosphonate etidronate at a dose 30 times the equivalent human dose and inducing extreme hypercalcemia of up to 40 mg/dl (10 mM). Furthermore, the complex is described as “cleared” after 24 hours which would appear to imply that even post-treatment serum is devoid of the FMC.

Role in the genetics of bone properties and pathological conditions

Some studies have indicated that variations in the gene locus (SPP2) for Spp24 are correlated with variations in bone mineral density (BMD) and bone strength. It should be noted that these results do not distinguish between a positive effect and a negative effect. That is, does enhanced Spp24 activity (whatever that may be) result in increased or decreased BMD? Wilson et al. reported on a study relating Qualitative Trait Loci (QTL) and broadband ultrasound attenuation (BUA) in dizygous twins in Great Britain (Wilson et al., 2004). Two chromosomal regions were identified as the locations for candidate genes. One of these regions was 2q33-37 which includes the locus for SPP2 which is 2q37 (Bennett et al., 2004). However, it should be noted that the entire region (2q33-37) contains more than 260 genes and several of these, such as the PTH receptor and IGF binding protein are very relevant to bone metabolism (Wilson et al., 2004). Hsu et al. employed a complex strategy combining genome-wide association

study (GWAS) and analyses of single nucleotide polymorphisms (SNP) applied to BMD in three population groups comprised of mostly Caucasians subjects (Hsu et al., 2010). In that study, the SNP for 2q37.1 (given as SPP2) gave a positive correlation with femoral neck BMD in females.

Increased levels of expression of Spp24 have been noted in association with osteoarthritis. The role of the protein in this situation and even whether it is deleterious or beneficial is completely unknown. Meng et al. used micro-array studies to examine gene expression in experimental osteoarthritis (OA) of the temporomandibular joint (TMJ) in rats (Meng et al., 2005). Of the materials tested, Spp24 showed the greatest increase in expression with a 19-fold increase in "early OA" compared to normal and a 32-fold increase in "late OA" compared to normal. Balakrishnan et al. surveyed synovial fluid from OA patients with chromatographic separation followed by high resolution mass spectroscopy (Balakrishnan et al., 2014). This group identified 677 proteins in synovial fluid, one of which was Spp24. Francis, in a dissertation reported that Spp24 bound to progranulin (PGRN) (Francis, 2005). PGRN is a protein with a number of proposed functions, including a TNF agonist that is hypothesized to play a role in the pathogenesis of inflammatory arthritis (Liu, 2011).

Possible functions in the skeletal environment

Before describing what Spp24 might do in the skeletal environment, it is critical to clarify and emphasize what it does not do. Because of the presence of the cystatin domain, Hu et al. speculated that Spp24 functioned as a cysteine protease inhibitor (Hu et al., 1995). This conjecture was clearly stated as a hypothesis. However, many papers and databases still cite this reference as establishing this function as a fact. Subsequent studies have not borne out this hypothesis. The cystatin domain in Spp24 does not contain the entire consensus sequence that is required for cystatin activity (Kordis and Turk, 2009). Experimentally, Bos et al. demonstrated that Spp24 did not inhibit the activity of cathepsin B and K (Bos et al., 2008).

The first concrete clue pertaining to the function of Spp24 came with the demonstration by Behnam et al. in 2005 that a peptide, the sequence of which was based on the TRH-1 domain of Spp24, bound BMP-2 (Behnam et al., 2005). Subsequent studies examined the association between the four primary forms of Spp24 and several members of the TGF- β superfamily of growth factors. Full-length Spp24 binds to BMP-2 with a K_D of approximately 18 nM (Brochmann et al., 2010). The reported affinity was slightly less for Spp18 and Spp16 but considerably less (236 nM) for Spp14.5. (The authors indicated that the very poor solubility of Spp24-related proteins could compromise the precision of the estimates of the kinetic constants which were determined by surface plasmon resonance.) These studies also

demonstrated that Spp24 proteins inhibit BMP-induced bone formation and that full-length Spp24 inhibits BMP activity to a greater degree than the other forms (Brochmann et al., 2010; Sintuu et al., 2007, 2011). How this relates to the normal bone environment is not entirely clear, however, female transgenic mice that expressed full-length Spp24 under the control of the OC promoter, exhibited reduced femoral BMD at 3 and 8 months (Sintuu et al., 2007).

The association of the different forms of Spp24 and TGF- β 1 and TGF- β 2 have also been examined (Tian et al., 2013). The affinities between the TGF- β molecules and full-length Spp24 were less than that for BMP-2 (K_D for TGF- β 1 was 650 nM and K_D for TGF- β 2 was 194 nM). Similar to the case of BMP-2, the affinities for the smaller forms of Spp24 were less than that of the full-length protein. In a model of periosteum-based bone formation induced by TGF- β , full-length Spp24 inhibited the bone formation whereas Spp14.5 did not. Instead, co-administration of TGF- β 2 and Spp14.5 resulted in improved bone quality in terms of BV/TV as well as an increase in the ratio of bone/cartilage seen. This effect may be a concentration effect since in the original description of the assay, "low dose" TGF- β was associated with a predominance of bone over cartilage when compare to "high dose" TGF- β (Joyce et al., 1990).

Associations with other members of the TGF- β superfamily have been tested but with BBP (the cyclic 19 amino acid peptide the sequence of which is derived from the TRH-1 domain of Spp24) rather than Spp24 and significant binding was demonstrated for BMP-7 (also called OP-1) and GDF-5 (growth and differentiation factor-5) (Taghavi et al., 2010). As discussed above, an association between Spp24 and proteins not within the TGF- β superfamily, notably progranulin, has been described (Francis, 2005).

It is also possible that the C-terminal fragments of the protein have different functions from the N-terminal forms which are truncated with respect to the C-terminus. The C-terminal fragment that is complimentary to Spp14.5 has been shown to bind BMP-2 as well as TGF- β 1 and TGF- β 2 with K_D values in the low μ M range and to inhibit BMP-2 induced bone formation (Tian et al., 2015a). The C-terminal fragment complementary to Spp18 did not avidly bind BMP nor did it inhibit BMP-induced bone formation. However, it appeared to stimulate proteoglycan deposition and non-ossseus calcification in a process that is not understood. Also, it appeared to have a synergistic effect with BMP-2 and improved the quality of bone induced by BMP-2.

The overall hypothesis derived from the studies of the relationships of Spp24 with TGF- β s and BMPs is that Spp24 influences bone growth, remodeling, and repair by affecting the economy of available BMP and TGF- β in the active areas of the skeletal environment. In this regard, it is important to keep in mind that most TGF- β exists in bone mineral in a highly sequestered latent form whereas no such form has been described for

the BMPs.

While the protein binding properties of Spp24 provide a clue to the possible function of the protein in bone and other tissues, other, unrelated functions certainly remain possible. Binding to progranulin and the induction of proteoglycan deposition are examples of processes unrelated to BMP/TGF- β binding. Intriguingly, Spp18, but not full-length Spp24, has been demonstrated to activate G $_i$ protein-related signaling pathways, stimulate MEK/ERK kinase pathways, and increase expression of osteoblastic differentiation related transcription factors (Zhao et al., 2015). Thus, Spp24 appears to have both growth factor binding properties and cytokine receptor agonist properties. This situation is reminiscent of the case of sclerostin that binds and inhibits BMPs and inhibits the Wnt pathway and for which an inhibitory monoclonal antibody has been developed and employed as a treatment for osteoporosis (McClung et al., 2014). The agonist properties of Spp24 require much further investigation.

Potential therapeutic applications

Bone healing

Bone morphogenetic protein binding peptide (BBP) is a cyclic 19 amino acid peptide the sequence of which is based on the TRH-1 domain of bovine Spp24. (Reduction of the terminal cysteine-cysteine bond extinguished BMP binding.) As described above, it avidly binds a number of members of the TGF- β superfamily of cytokines. When tested alone in the mouse hindquarter ectopic bone formation assay, BBP did not have osteogenic activity. (Behnam et al., 2005). Some dystrophic calcification, primarily localized to the intramuscular adipose, was observed. However, when BBP was co-implanted with BMP-2, there was a 2.8 fold increase in BMP-induced bone formation as manifest by increased bone mineral content (BMC) with histological confirmation of osteogenesis. This material was subsequently tested in a number of pre-clinical models of bone healing including spinal fusion with BMP-2 (Alanay et al., 2008) and with BMP-7 (Taghavi et al., 2010), as well as femoral defect repair with BMP-2 (Morishita et al., 2010) and with BMP-7 (Liao et al., 2011). The hypothesized mechanism of action is increased residence time (RT) of BMP at the site of action since the amount of BMP retained in collagen sponge implants that contained both BMP and BBP was approximately twice that of implants onto which BMP alone was loaded out to one week (the last time point tested) (Taghavi et al., 2010). This proposed mechanism of action also explains the observed variation in the efficacy of BBP synthesized by different manufacturers. Freely soluble BBP is much less efficacious than is BBP that is granular and must be delivered in suspension (unpublished observation). Thus, properties, such as aggregation, in addition to BMP-binding contribute to

the action of BBP.

Inhibition of inflammation

The induction of an inflammatory response with edema in association with the use of BMP in spinal fusion surgery has become an issue of considerable concern. Lee et al. observed that when BMP was delivered in association with BBP, the amount of edema, as measured by MRI and grossly, was reduced (Lee et al., 2011a). Efforts were then undertaken to employ Spp24 impregnated collagen sponges as barriers ("molecular sponges") to protect vulnerable structures (such as nerve roots) by specifically sequestering and neutralizing BMP (Tian et al., 2015b).

Inhibition of skeletal tumor growth

The growth of primary (osteosarcoma) and secondary (lung, prostate, breast) tumors in bone is a complex process. However, one critical element that allows these tumors to thrive in the skeletal environment is the presence of autocrine growth factor systems, especially those involving BMPs. Studies were undertaken, therefore, in an effort to use Spp24 to sequester and neutralize BMPs (and other growth factors) and reduce tumor growth. Lee et al. co-implanted various combinations of Spp24, BMP-2, and A549 human non-small cell lung cancer cells into subcutaneous and intra-osseous sites in SCID mice (Lee et al., 2011b). In both sites, Spp24 inhibited BMP-2 induced tumor growth and, when co-injected only with cells, dramatically reduced tumor growth for up to 8 weeks (the last time point). Similar studies were undertaken employing 143B human osteosarcoma cells (Tian et al., 2014). A similar dramatic reduction in tumor growth was observed.

Other potential applications

A number of additional applications for the use of Spp24 can be envisioned. In any situation where excessive BMP or TGF- β activity is contributing to pathology, Spp24-based therapeutics could be used to reduce that activity. For example, ossification of the posterior longitudinal ligament (OPLL) is a pathological condition, more common in Asia than elsewhere, which results in spinal stenosis and which almost certainly involves unregulated BMP activity (Hoshi, 2006). Inhibition of BMP activity before or after surgery would contribute to management. Ectopic bone formation is seen in spinal cord injury and even in vascular pathology. Such conditions, however, would not be amenable to local administration of BMP-sequestering therapeutics but systemic agents are under development. As described above, Spp24 may play a role in the pathology of OA or the response to it. A role for an Spp24-based therapeutic for this condition must await

further definition of the pathogenesis of the disorder. TGF- β is involved in the pathophysiology of a vast spectrum of human disorders including tumors, pathological fibrosis, vascular disease, and others. Since Spp24 also sequesters and neutralizes TGF- β , a therapeutic target for a variety of conditions including tumors and fibrosis (Akhurst and Hata, 2012), a large number of potential applications for insoluble and soluble forms of Spp24 await exploration.

Conclusion

Spp24 is an important subject for skeletal biology research because of its potential role in the genetics of osteoporosis, its role in normal bone growth and turnover, and because it can serve as the starting material for a number of therapeutics with applications in the management of skeletal disorders. The issues that most need to be clarified are: 1. What is the origin of the Spp24 protein that is found in bone? Is it made locally in the bone or is it produced in the liver and transported to the bone? If it is produced mostly in the liver and delivered to bone, this would indicate some similarity to insulin-like growth factors (IGFs) in that both are produced in the liver, the production of both is induced by growth hormone, and both regulate bone metabolism in some way. 2. What is the significance of the cytokine binding activity? Does this function to control the economy of available TGF- β and BMPs? Or does this serve a more passive function such as allowing for latency or, is it adaptive and, therefore, does it function to inhibit osteogenesis and calcification in situations where these processes are not advantageous? 3. Do different forms of the protein perform different functions in bone? This is suggested by the fact that different forms of Spp24 have been demonstrated to inhibit BMPs to different degrees, affect the activity of TGF- β differently, affect proteoglycan synthesis differently, and activate intracellular signaling differently. 4. What is the net outcome of the intracellular signaling that is activated by Spp18? Does this signaling affect osteoblast proliferation and differentiation like another BMP-binding protein, sclerostin? 5. In what pathophysiological situations can Spp24-derived materials be used to achieve an effective outcome? BMPs and TGF- β have many roles in normal physiology and in pathological processes. The cytokine binding properties of Spp24-derived materials have already been demonstrated to produce effects on a spectrum from enhancement (through slow release) to neutralization (through sequestration). Thus, existing materials can be tested in many more pre-clinical models. The roles of Spp24 products as cytokine agonists are only beginning to be investigated. Spp24 was first described about 30 years ago. We can engineer many materials from Spp24 and test them empirically but we must be cautious about thinking that we know the natural function of this enigmatic protein.

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