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Refinement of collagen–mineral interaction: A possible role for osteocalcin in apatite crystal nucleation, growth and development

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ABSTRACT

Mineralization of vertebrate tissues such as bone, dentin, cementum, and calcifying tendon involves type I collagen, which has been proposed as a template for calcium and phosphate ion binding and subsequent nucleation of apatite crystals. Type I collagen thereby has been suggested to be responsible for the deposition of apatite mineral without the need for non-collagenous proteins or other extracellular matrix molecules. Based on studies in vitro, non-collagenous proteins, including osteocalcin and bone sialoprotein, are thought to mediate vertebrate mineralization associated with type I collagen. These proteins, as possibly related to mineral deposition, have not been definitively localized in vivo. The present study has reexamined their localization in the leg tendons of avian turkeys, a representative model of vertebrate mineralization. Immunocytochemistry of osteocalcin demonstrates its presence at the surface of, outside and within type I collagen while that of bone sialoprotein appears to be localized at the surface of or outside type I collagen. The association between osteocalcin and type I collagen structure is revealed optimally when calcium ions are added to the antibody solution in the methodology. In this manner, osteocalcin is found specifically located along the a_{4-1}, b_1, c_2 and d bands defining in part the hole and overlap zones within type I collagen. From these data, while type I collagen itself may be considered a stereochemical guide for intrafibrillar mineral nucleation and subsequent deposition, osteocalcin bound to type I collagen may also possibly mediate nucleation, growth and development of platelet-shaped apatite crystals. Bone sialoprotein and osteocalcin as well, each immunolocalized at the surface of or outside type I collagen, may affect mineral deposition in these portions of the avian tendon.

Introduction

Mineralization of normal vertebrate tissues such as bone, cementum, dentin, enamel and calcified cartilage and tendon has been studied for several decades. Typically, the mineral phase in vertebrate tissues serves many purposes, including, for example, as a reservoir for ion homeostasis of extracellular fluid [1] as well as a means by which to strengthen the tissues in order to bear high loads [2–4]. Presently there remain many questions about the mechanism(s) of formation of vertebrate mineralization. A major uncertainty, in particular, is whether differences occur in mineral deposition between intrafibrillar or interfibrillar collagen regions of all such tissues except enamel (which has no collagen) [5–7].

Collagen fibrils are considered to be a template for the nucleation and growth of apatite, the mature form of vertebrate mineral [7–9]. In this regard, electron microscopic studies reveal that the initial deposition of apatite crystals associated with collagen fibrils occurs principally within their so-called hole zones [9–12]. Other studies [13–17] suggest that certain small, charged non-collagenous proteins (NCPs) also play an essential role in mediating the mineralization process, and there is considerable discussion as to precisely the action of either or both collagen and NCPs in vertebrate mineralization events. A recent report, for instance, suggests that oriented apatite crystals can be formed on tightly packed collagen fibrils without the need of NCPs [18]. On the other hand, findings concerning loosely packed collagen fibrils in fish bones would indicate other or additional mechanism(s) of the mineralization process [9].

A hypothetical concept describing intrafibrillar collagen mineralization has been proposed lately in which charged NCPs are suggested to be incorporated initially in the vicinity of the hole zones of collagen fibrils [6]. Together with other collagen molecules and fibrils, these charged NCPs would facilitate apatite crystal nucleation by binding calcium and phosphate ions present in the fluid that normally bathes the mineralizing tissue. The controlled growth and development of apatite crystals may be a result of steric hindrance from neighboring collagen molecules surrounding their hole zones as well as preferential adsorption of NCPs on certain apatite crystallographic planes [6].

Work in vitro using a special polymer-induced liquid-precursor (PILP) process successfully demonstrated mineralized collagen fibrils

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with apatite crystal platelets orientated along the fibrils [19–22]. However, accompanying transmission electron microscopy (TEM) images [19–22] did not provide clear localization of the apatite crystals and whether they were specifically associated with the hole zones of intrafibrillar collagen, as otherwise demonstrated by Nudelman et al. [23]. The possible action of NCPs in these studies in vitro [19–22] was not a direct consideration and the specific association of these small molecules with intrafibrillar or interfibrillar collagen and vertebrate mineral formation remains a fundamental question.

Among the NCPs, bone sialoprotein (BSP) and osteocalcin (OC) have been widely studied because their nature, presence and localization are thought to be correlated with the mineralization process in vivo [13–17,24–26]. Mammalian BSPs are phosphoproteins that are heavily glycosylated and sulfated and have a molecular weight in the range of 33–34 kDa [13–15]. BSP is also characterized by an open flexible structure with two polyglutamic acid domains that interact with apatite [15]. OC consists of 49–50 amino acids, depending on mammalian species, with a molecular weight of ~5.7 kDa [16,17]. Again depending on species, it contains 3–5 residues of γ-carboxyglutamic acid responsible for binding calcium ions (Ca^{2+}) and adsorption to apatite crystal surfaces [16,17].

The respective functions of BSP and OC associated with mineralization have been effectively deduced by studying the character of the individual proteins in vitro, that is, whether these NCPs promoted or inhibited apatite nucleation in various systems mimicking physiological conditions [13,14,16,17]. It remains conjectural as to how they might mediate collagen mineralization either in intrafibrillar or interfibrillar collagen regions. A size-exclusion model [27] suggests that BSP is too large to be accommodated within intrafibrillar collagen and must therefore be a component of the interfibrillar collagen regions of mineralizing vertebrate tissues. On the other hand, because of its smaller size, OC can be accommodated in intrafibrillar collagen [27].

The literature regarding precise localization of these two NCPs in normally mineralizing vertebrate tissues in vivo is sparse. It is known that a complex between OC and type I collagen forms in vitro (one OC binding site for each collagen molecule) [28], but only two studies, one utilizing immunohistochemistry to investigate calf bone [29] and one applying immunocytochemistry to examine rat tooth germ [30], have shown OC labeling of intrafibrillar collagen. No additional evidence of specific binding or localization of OC with collagen in vivo has been reported for other mineralized tissues. Meanwhile, further work in vitro proposes that OC binds to apatite through a close match between the positions of several negatively charged groups on the α-helical structure of OC and those of positively charged calcium cations of the specific (001) crystallographic plane of apatite [16]. However, inconsistencies remain about potential preferential binding of OC to apatite crystallographic planes [16,31,32], and, even though structural relations between apatite platelets and collagen fibrils have been examined [33,34], the possible localization of OC associated with these orientated apatite platelets is uncertain. Moreover, while these structural considerations and studies in vitro suggest a critical role for OC with regard to mineralization, the skeleton of OC-knockout mice does not show apparent changes [35]. Instead, bone mineral maturation is affected by the loss of OC in these animals [35]. To summarize exact spatial interaction between OC and type I collagen in vivo, the association between these proteins is incompletely documented and difficult to determine specifically in either of the two published immunolocalization reports noted above [29,30].

With regard to BSP, previous immunolocalization results for normal bone and cementum show BSP distribution throughout respective tissue matrices with accumulations at mineralization fronts or cement lines [24,36]. However, for examples of pathological mineralization such as in urolithiasis, BSP appears to be absent in rat or human renal calculi [37]. In detailed immunocytochemistry, BSP is apparently associated with tissue regions that lie between collagen fibrils, that is, in the interfibrillar collagen spaces of the tissues [36], but precise immunolocalization of BSP to collagen and extracellular matrix mineralization has been investigated in only the few instances cited [24,36]. The BSP knock-out mouse model shows a phenotype that is characterized by thinner bone in fetuses and young adults but without specification as to collagen–mineral relations [38,39]. Parenthetically, because of the critical importance of OC in mineralization and other biological processes or functions, if OC is reduced or absent (as in OC knock-out mice), it is very likely that some other molecule is redundant to OC to mitigate an overt change in bone phenotype. In the case of the BSP knock-out model, replacement of one SIBLING family member protein by another can lead to normal bone function and phenotype [40,41].

In addition to the involvement of OC and BSP in aspects of vertebrate mineralization, these major non-collagenous extracellular matrix proteins are important for regulating cell behavior. Interactions between cells of mineralizing tissues, OC, BSP and other NCPs have been discussed [15,42,43].

The present paper focuses on OC and BSP, with an emphasis on OC, with respect to their immunolocalization in the calcifying avian tendon, a model of vertebrate mineralization previously studied in part with respect to collagen–mineral interaction [8]. In this report, the association of these NCPs has now been examined and, from the immunocytochemical results observed, possible roles for the proteins in mineralization of intrafibrillar and interfibrillar collagen have been proposed. The study here uses modifications of current technical methods and post-embedding immunocytochemistry [24,44] to gain further insight into the mineralization process of vertebrates. Summary data suggest that OC is localized to both intrafibrillar and interfibrillar collagen. In the former, OC binds to type I collagen and may mediate nucleation, growth and development of platelet-shaped apatite crystals within structural units of collagen such as microfibrils, fibrils and fibers. BSP, as well as OC, is immunolocalized in interfibrillar collagen regions at the surface of or outside type I fibrils and fibers and may mediate mineral deposition in these regions of the avian tendon.

Materials and methods

Fifteen-week-old male turkeys (*Meleagris gallopavo*) were obtained from a local farm (Hawk Farms, East Rochester, OH) and freshly sacrificed. Gastrocnemius tendons were dissected from their legs and the attached muscles were carefully removed [45]. Tendon tissues were immediately pinned onto paraffin wax in a flat plastic container and immersed at 4 °C for 10–12 h in a modified Karnovsky's fixative solution containing formaldehyde (4% final concentration) and glutaraldehyde (either 1% or 2.5% final concentration) in 0.1 M cacodylate buffer (pH 7.4). Fixed tendons were examined and documented for mineralization by X-ray radiography and then further cut into smaller specimens having dimensions of ~5 × 7 mm in width and length, respectively. Thickness varied between 3 and 7 mm depending on location of the specimen within the tendon as a whole. Orientation of the tissues for embedding was aided by the cut specimen shape so that proximal and distal sample regions were known and distinguished following embedment. After fixation, specimens were washed in 0.1 M cacodylate buffer and then dehydrated through graded (70–100%) ethanol solutions. Infiltration in LR White resin (hard grade; Electron Microscopy Sciences, Hatfield, PA) followed dehydration [46]. A Junior Orbit Shaker (Lab-line Instruments, Inc., Melrose Park, IL) was used to facilitate the infiltration process. Once blocks were embedded in 100% LR White resin using gelatin capsules (Electron Microscopy Sciences), specimens were polymerized at 55 °C for 24–48 h without the addition of accelerator. From X-ray radiographs, tendon zones of unmineralized, intermediate and heavily mineralized regions were identified and intermediate zones were carefully selected for subsequent immunocytochemistry.

Tissue sections ~80 nm thick were cut at a speed of 0.2–1 mm/s with an ultramicrotome (Reichert Ultracut S, Leica Microsystems, Inc., Buffalo Grove, IL). Ni grids (Electron Microscopy Sciences) that were