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SEPARATION OF NEWLY FORMED BONE FROM OLDER COMPACT BONE REVEALS CLEAR COMPOSITIONAL DIFFERENCES IN BONE MATRIX

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Abstract

In long bone diaphyses, woven bone forms first and then transitions into a more mineralized compact bone tissue. Prior evidence suggests that the non-collagenous protein composition of woven bone may be distinct from that of more mature bone tissue, particularly with respect to a diverse group of phosphorylated, extracellular matrix proteins. To critically test this hypothesis, we developed an *in situ* approach to isolate newly formed bone from more mature bone within the same long bone, and combine this anatomical approach with Western blotting to make relative comparisons of 7 phosphorylated matrix proteins important for bone physiology and biomineralization. Interestingly, 75 kDa bone sialoprotein (BSP), 63 kDa osteopontin, and the 75 kDa form of bone acidic glycoprotein-75 (BAG-75) were enriched in primary bone as opposed to more mature cortical bone, while osteonectin, fetuin A, matrix extracellular phosphoglycoprotein (MEPE) and dentin matrix protein-1 (DMP-1) appeared to be equally distributed between these two bone tissue compartments. Analyses also revealed the presence of larger sized forms of osteopontin (and to a lesser degree BSP) mostly in newly formed bone, while larger forms of BAG-75 were mostly detected in more mature cortical bone. Smaller sized forms of DMP-1 and BAG-75 were detected in both newly formed and more mature bone tissue extracts, and they are likely the result of proteolytic processing *in vivo*. Intact DMP-1 (97 kDa) was only detected in unmineralized matrix extracts. These findings indicate that newly formed bone exhibits a non-collagenous matrix protein composition distinct from that of more mature compact bone even within the same long bone, and suggest that the temporal fate of individual non-collagenous proteins is variable in growing bone.

Keywords

Newly formed bone; Extracellular matrix; Bone phosphoglycoproteins; Osteoid; Cortical bone

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Introduction

Primary bone is a woven bone tissue that rapidly forms during development of the intramembranous bones or during fracture healing [1]. This and other sources of newly formed woven bone tissue then rapidly transition into a more mineralized bone tissue. Indeed, values for the degree of mineralization and tissue stiffness of newly formed woven bone (< 4-days old) have been reported to be significantly lower than those values of bone tissue only 1–2 weeks old [2, 3]. Similar to the differences in inorganic content, it has been suggested that the organic composition of newly formed woven bone may be distinct from that of more mature bone tissue [reviewed in 1, 4]. This hypothesis contends that there may be macromolecules unique to primary and other sources of newly formed woven bone, and these organic differences may manifest a direct functional relationship with the formation of nascent mineral crystallites. In this regard, a diverse group of extracellular matrix phosphoproteins in bone has attracted considerable interest because of their affinity for both hydroxyapatite crystals and type I collagen, as well as their accumulation along the mineralization fronts of bone [1].

Support for the hypothesis that differences in non-collagenous protein matrix composition and amount may exist between newly formed woven and more mature bone comes from several areas. First, the calcium to nitrogen (protein) ratios of dry fat-free bone substantially increased with age in several species [5]. Furthermore, the evidence indicates that these ratio differences most likely reflect changes in non-collagenous protein-nitrogen content rather than that for collagen since hydroxyproline content did not change with age [5]. Second, the ratio of organic phosphorus in protein to hydroxyproline content declined by ~60% in cortical bone from mature as compared to juvenile dogs [6]. Third, findings from Northern blot and *in situ* hybridization studies indicated that bone sialoprotein, osteocalcin and alkaline phosphatase mRNA expression levels differ with bone age [7] or bone location [7, 8]. Finally, radioimmunoassays for osteonectin or osteocalcin [9] or immunoblot analyses for bone sialoprotein, osteocalcin, osteopontin or α_2 -HS-glycoprotein (fetuin A) [10] demonstrated differences in the contents of these non-collagenous proteins in different bone tissue compartments.

With the exception of *in situ* hybridization, all of the above mentioned biochemical approaches provided accurate quantitative assessments, but not a determination of tissue or histological location for these non-collagenous proteins. Immunostaining of non-collagenous proteins in bone has indicated specific patterns for their accumulation in osteoid, along the mineralization front of bone, and within more mature bone [11–13]. In particular, these studies showed that bone sialoprotein or osteopontin appeared to be highly accumulated along the mineralization front of bone, which is a location containing newly formed woven bone. Nevertheless, epitope detection by specific antibody binding is technique-dependent and cannot assess the chemical nature of the protein containing the epitope (post-translational modifications such as proteolysis or cross linking). What is needed to provide both protein quality and quantity information, as well as provide protein location within primary bone tissue, is an approach to isolate newly formed bone from more mature bone within the same specimen and combine this anatomical assurance with protein chemistry technology for relative quantitative comparisons.

The diaphyseal cortical bone shaft of rapidly growing juvenile rats provides access to and assurance of a reasonable supply of newly formed bone tissue. The diaphysis increases its girth in a radial outgrowth pattern whereby new bone accumulates principally along the subperiosteal region on top of the pre-existing more mature compact bone [14]. Given that periosteal cell layers can be quantitatively removed from long bones [15], our approach was to isolate the tibial diaphysis from juvenile rats, remove the marrow and periosteal tissues,

and seal the open ends of the cortex to restrict access to newly formed bone tissue along the periosteal surface. In order to address our hypothesis, a chemical extraction approach was developed using increasing concentrations of a calcium chelator, EDTA, to progressively strip mine and separate this younger bone tissue from its underlying older bone.

Several bone matrix proteins (many of which are phosphorylated) have been shown to be important for bone physiology and biomineralization [recently reviewed in 1]. Using this information, we selected seven bone matrix proteins for further investigation in the current study based on their abundance in bone, their interactions with hydroxyapatite crystals, their ability to bind to collagen, and their ability to modulate mineralization reactions: bone sialoprotein [16–19], osteopontin (Secreted Phospho-Protein-I or SPP-I) [11, 20, 21], osteonectin (Secreted Protein Acid-Rich with Cysteines or SPARC) [22], bone acidic glycoprotein-75 (BAG-75) [23–25], fetuin A (α_2 -HS-glycoprotein) [26], matrix extracellular phosphoglycoprotein (MEPE) [27], and dentin matrix protein-1 (DMP-1) [28–30]. In addition, many of these proteins have been immuno-localized in bone tissue sections at both light and electron microscopy levels [11, 13, 24, 31] and we used this prior information as a database to independently corroborate our findings.

We combined a conventional denaturant solvent treatment to quantitatively extract proteins [22] from newly formed bone tissue versus the underlying older bone tissue within the same long bone with a Western blot approach to investigate the temporal-spatial distributions of the above mentioned seven bone matrix proteins in these two bone tissue extracts. This approach provided an assessment of their relative quantities in newly formed versus more mature compact bone tissues within the same bone, providing a direct means to test our hypothesis. Additionally, this approach provided information about the molecular size of each protein and how the nature of post-translational modifications such as proteolysis or cross linking might influence their overall structure-function relationship within younger and older bone tissues.

Experimental Procedures

Materials

All reagents were from Sigma (St. Louis, MO) or Calbiochem (La Jolla, CA), unless otherwise indicated, and were of the highest purity commercially available. Sephadex G 50 fine was purchased from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ).

Animal Procedures

All animal procedures were approved by the Institutional Animal Care and Use Committee at the Cleveland Clinic. One-month-old male Sprague Dawley weanling rats (45–50 g weight; Harlan Labs, Indianapolis, IN) were used as a model of skeletal growth [14]. Dynamic labeling of bone growth was done by intraperitoneal (*ip*) injection with alizarin red-S at a dose of 10- μ g/g body weight (BW) prepared in phosphate-buffered saline, pH 7.4. One week later, rats were injected *ip* with calcein at a dose of 10- μ g/g BW prepared in phosphate-buffered saline, pH 7.4. After 24 hours, the rats were euthanized by asphyxiation with carbon dioxide from a compressed gas source.

Diaphysis Isolation and Histology

A 7 mm-long segment from the middle diaphysis of each tibia was obtained from the rats using aseptic techniques as previously described [15]. Those diaphyses slated for either decalcified or undecalcified histology were placed into neutral-buffered formalin for 48-h at 4° C and then transferred to and stored in 70% (v/v) ethyl alcohol buffered to pH 7.5 with 10 mM Hepes at 4° C. For undecalcified histology, samples were embedded in methyl

methacrylate plastic after serial dehydration with a graded ethanol series to xylene. Unstained sections of 7- μ m thickness were cut and placed on Superfrost slides for detection of alizarin red and calcein labeling via epifluorescence microscopy using a Leica RXA2 upright wide-field microscope (Heidelberg, Germany), Spot RT CCD digital camera (Diagnostic Instruments, Sterling Heights, MI) and 20 $\times$ and 40 $\times$ objectives. For decalcified histology, the fixed samples were washed in phosphate buffered saline and decalcified in 0.4 M EDTA in neutral-buffered formalin with multiple exchanges over 3-days at 4 $^{\circ}$ C. The decalcified samples were washed in phosphate buffered saline and embedded in paraffin after serial dehydration with a graded ethanol series to xylene. Sections of 7 μ m thickness were cut and were placed on polychloroprene-coated Superfrost slides and subsequently stained with hematoxylin and eosin prior to brightfield microscopy using the aforementioned microscope and camera system.

Cortical Bone Isolation

Segments of tibial diaphyses (7-mm long) were further processed to remove bone marrow and periosteum in order to isolate cortical bone [15]. First, marrow tissue was removed from explants by pressurized irrigation of the diaphyseal medullary cavity using Hank's Balanced Salt Solution expressed through a blunt-end 20-gauge needle. Periosteal tissue layers were then stripped off the bone by dissection leaving the entire osteoid layer bound to the periosteal bone surface [15]. Following this dissection, and only at this time, each bone cylinder was plugged at both open ends using bone wax (Fine Science Tools, Inc.). This sealing was done to assure access of subsequent reagents to the periosteal bone surface only. This bone wax was verified to lack protein contaminants using both MicroBCA protein assay and SDS-PAGE analysis.

Progressive Demineralization and Protein Solubilization

Sealed tibial diaphyses were isolated and placed on ice in storage medium (Dulbecco's Modified Eagle's Medium, 10 mM Hepes, 10 mM Bes, 0.5% bovine serum albumin, 0.25 μ g/ml fungizone, pH 7.2 containing a protease inhibitor cocktail of 0.1 mM Elastinal, 1 mM AEBSF, 0.1 mM Leupeptin, 0.01 mM E-64, 0.01 mM GM 6001, and 0.1 mM Antipain; Calbiochem). Proteins were solubilized from the unmineralized organic matrix of periosteal bone via an 18 h treatment in a 4 M Guanidine-HCl solution containing 1% CHAPS, 1 mM AEBSF, 10 mM N-Ethylmaleimide, 5 mM Benzamidine, 0.01 mM GM6001, and 20 mM HEPES pH 7.5 [GM6001 is a non-chelating metalloproteinase inhibitor]. Each 7-mm long sealed diaphysis (calculated volume of 0.015 mL) was placed into 0.5 mL of the above extraction solution. Specimen mixing was facilitated by rotating on a Roto Torque Heavy duty Rotator (Cole Parmer Instruments, Chicago, IL) at 1 revolution per minute at 4 $^{\circ}$ C. This initial tissue extraction was followed by 10 additional washes (10-min each) using the same solution to quantitatively remove soluble proteins from the unmineralized organic matrix prior to treatments with EDTA. Thereafter, a series of bone mineral and organic matrix extraction treatments occurred at 18 h time intervals at 4 $^{\circ}$ C using progressively higher concentrations of EDTA (20-, 40-, 80-, 200-, and 400-mM) in the aforementioned guanidine-HCl solution (see Supplementary Figure S1). Soluble protein extracts were recovered after each sequential EDTA extraction. All soluble protein extracts were run over 2 mL Sephadex G 50 columns (0.5 cm $\times$ 5 cm) pre-equilibrated and eluted with 10 M Formamide, 0.2 M NaCl, 0.5% (w/v) CHAPS and 0.05 M sodium acetate pH 6 in order to remove guanidine HCl and EDTA. The totally excluded volume from each column was concentrated 10-fold using YM-3 Microcons (Millipore, Billerica, MA) that were pre-blocked with 0.1 mg/ml ovalbumin in 10 M Formamide, 0.2 M NaCl, 0.5% CHAPS, and 0.05 M sodium acetate pH 6. Protein levels in the concentrated retentates were measured using a Pierce MicroBCA Kit (Pierce, Rockford, IL).

Scanning electron microscopy (SEM)

Tibial diaphyses from 5-week old rats were isolated as described above. They were subjected to a cross-sectional cut in the middle of the diaphysis yielding two cut faces juxtaposed within the same bone with identical shape, dimension, and bone quality for direct comparisons. One half of each diaphysis was treated with sequential EDTA treatments as described above. The other half were untreated to be used as controls. Both treated and untreated tibial diaphyses were frozen in liquid nitrogen and carefully fractured so that the fracture surfaces were parallel to the long axis of tibia. The specimens were then lyophilized and the fracture surfaces were coated with carbon, and examined with a JOEL 5310 SEM operating at 5 kV.

Micro-indentation analysis

Ten tibial diaphyses were used for microhardness measurement. Each diaphysis after EDTA decalcification was lyophilized and encased in epoxy resin at room temperature. The cross-section plane of the tibiae were then polished with progressive grades of SiC paper, and finished with alumina slurry (0.5 μm). Series indentations were performed on the polished cross-sectional plane from endosteal to periosteal surface at 5 positions across the thickness of tibia using a Buehler Microhardness instrument at a load of 10 grams. The microhardness of untreated tibia was also measured in the same manner as controls. Methods such as micro- and nano-indentation have been shown to accurately detect 10–15% changes in tissue-level hardness within cortical bone [32].

SDS-PAGE and gel staining

Concentrated protein retentates were mixed with an equal volume of 2X Novex Tris-Glycine Sample Buffer (Invitrogen, Carlsbad CA) containing fresh 5% β -mercaptoethanol (Fisher, Pittsburgh, PA), immediately boiled for 2 minutes, and then placed on ice. Protein samples (2 μg total protein in 20- μL per lane) were separated on Tris-glycine 4–20% gradient gels (Invitrogen, Carlsbad CA) using a Novex X Cell II gel box containing Novex Running Buffer (Invitrogen, Carlsbad CA) at a constant current of 125 mA for 2 h at room temperature. Sypro Tangerine Protein Gel Stain (Molecular Probes - Invitrogen, Carlsbad CA) was used according to manufacturer's directions to detect protein bands in gel. By interacting with the SDS coat around proteins in the gel in a stoichiometric fashion, Sypro Tangerine can assess relative differences in protein band amounts. [It should be noted that the Tangerine Orange staining intensity of highly acidic proteins will not be the same as those that are less acidic, and caution should be applied when making comparisons of band intensities for differing protein species unless it is known that their fixed charge densities are similar.] Gels were stained for 60 min, placed on an Ultra-Lum multiwave Transilluminator under UVB lighting settings, and digitally imaged using a Quantix Photometrics CCD camera. Toluidine blue staining required that protein bands in the gel be fixed for 30 min in 100 ml of 30% (v/v) ethanol/10% (v/v) acetic acid. Following fixation, gels were washed three times for 15 min each with 100 ml MilliQ water (Millipore, Billerica, MA), and then stained for 10 min in 0.2% (w/v) Toluidine Blue O (Fisher, Pittsburgh, PA) in 0.1N acetic acid. To remove excess dye the gel was briefly rinsed in MilliQ water and then destained in 100 ml of 30% (v/v) ethanol/10% (v/v) acetic acid until the background color is removed. Stains-All (3,3'-diethyl-9-methyl-4,5,4',5'-dibenzo-thiacarbocyanine; Sigma Aldrich, St. Louis, MO) staining required the complete removal of SDS from the gel by extensive washing with several exchanges of 25% (v/v) 2-propanol overnight in the dark. The next day, the gel was stained for 3 h in the dark using a freshly prepared 0.005% (w/v) Stains All solution. After staining, bright field pictures were taken immediately to avoid color fading.

Western Blotting

Gel-separated proteins were transferred to Immobilon-P (Millipore, Billerica, MA) using a Novex Tris-glycine Transfer buffer (Invitrogen, Carlsbad CA) containing 5% methanol. The transfer was performed at a constant voltage of 30 V for 5 h with the gel box placed in an ice bath for cooling during transfer. Following transfer, the membrane was blocked overnight in 5% ovalbumin (Sigma) in 20 mM TBS pH 7.2 containing 0.025 % sodium azide at 4° C with gentle rotation. The next day, membrane blots were washed 5 times, 5 minutes each using 20 mM TBS pH 7.2 prior to presenting primary antibodies. Blots were incubated overnight at 4° C with primary antibodies diluted into 20 mM TBS pH 7.2 containing 0.1% ovalbumin. Supplementary Table S1 lists all the primary antibodies used for Western blot analysis; this table includes each antibody type, antigen structure, dilution used, and source of antibody [for additional details refer to 33, 34]. The next morning, all blots were washed 5 times in TBS for 5 minutes each, then incubated at room temperature in horseradish peroxidase-conjugated secondary antibody diluted (1:10,000 – 1:40,000) in 20 mM TBS pH 7.2 containing 0.1% ovalbumin (ECL+ Western Blotting Detection System; GE Healthcare). After 1-h incubation at room temperature with gentle rotation in secondary antibody, the blots were once again washed 5 times with TBS for 5 minutes per wash. Immunoblot signals were detected using ECL+ Western Blotting Detection System (GE Healthcare) as per manufacturer's instructions then exposed to X-ray film (HyBlot, Denville Scientific). Film was developed in an automated Kodak X-Omat 2000A processor (Eastman Kodak, Rochester, NY) and digitally scanned (ScanMaker 8700, Microtek).

Statistical Analyses

A one-way ANOVA test was used to determine the significance between individual data points. Unless indicated otherwise, all data are presented as Mean $\pm$ SD and are considered significant at $p < 0.05$.

Results

Locating newly formed woven bone tissue in the tibial diaphysis of juvenile rats

Figure 1A shows the histological appearance of cortical bone at the tibial mid-diaphysis in 5-week old growing rats. Consistent with its osteogenic potential, the periosteal surface contains a thick cambium layer having numerous pre-osteoblasts and osteoblasts, while a thinner layer of these bone forming cells is observed on the endosteal surface. At this age and location, few osteoclasts were detected and when present they were usually observed on the endosteal as opposed to periosteal surface. The average cortex thickness at this age and location is $275 \pm 20 \mu\text{m}$, and this cortical bone tissue contains large blood vessels and numerous osteocytes. Since the width of the tibia grows by appositional growth wherein the deeper layers are older than the more surface layers [14], we asked whether the molecular composition of mineralized matrix varies with age within the same bone.

To address this question directly, we used *in vivo* dynamic labeling to distinguish the boundaries and spatial locations of newly formed woven bone from that of older cortical bone. In the tibial diaphysis of 5 week old rats, Alizarin red S fluorescence (*red*) demarks the boundaries of the pre-existing 1-month old cortical bone tissue, while the calcein fluorescence (*green*) identifies the boundaries of the newly formed woven bone produced within a subsequent 1-week of growth *in vivo* (Fig. 1B). Alizarin red S labeling is present at both periosteal and endosteal surfaces, but the relative thickness and intensity of the periosteal labeling is greater than that on the endosteal surface. This pattern was also noted with calcein labeling, which appears to be more contiguous on and somewhat thicker at the periosteal versus endosteal surfaces.

In Figure 1B, the distance between the red labeling lines ($253 \pm 15 \mu\text{m}$) represents a direct measure of the amount of mineralized bone produced during the late pre-natal gestation period and during the first four weeks of post-natal age. Likewise, the distance between each pair of green and red labeling lines ($23 \pm 2 \mu\text{m}$ versus $6 \pm 3 \mu\text{m}$ for periosteal versus endosteal, respectively) represents the amount of newly formed bone deposited between 4- and 5-weeks of age. Thus, this radial spatial pattern also defines a temporal pattern whereby newly formed bone tissue is deposited along the outermost stratum of previously formed diaphyseal bone tissue, in effect, resulting in an increased girth of the cortical shaft of long bones [14]. Hereafter, we will use these dynamic histomorphometry values to empirically define the boundaries of “1-week” old as opposed to “1-month” old bone tissue within the same long bone, and periodically refer to these labels as defining bone strata ranging from newly formed to more mature bone matrix.

Matrix deposited by newly formed bone can be decalcified separately from that of older bone within the same long bone

Existing protocols to demineralize and extract proteins from pulverized bone tissue use a high concentration EDTA solution either just before [35] or simultaneous with a 4 M guanidine HCl denaturant solvent [22]. Under these conditions, the extraction solution has complete access to all bone surfaces and thus cannot distinguish periosteal from endosteal bone matrix. In the current study, we sought to gain strict access to the newly formed bone tissue on the periosteal surface by first removing the periosteum by dissection [15, 36] thereby exposing the osteoid layer and the most recently deposited bone tissue. To restrict access of the guanidine HCl/EDTA solution to just the periosteal bone surface, marrow tissue was removed from the medullary cavity and the ends of the diaphyseal bone shaft were sealed with bone wax (Supplementary Figure S1).

Cortical bone has an extremely low hydraulic permeability [37, 38], and it has been previously demonstrated that solutions to demineralize ivory dentine yield a relatively sharp front in a slow, diffusion mediated process whereby the distance of the demineralization front is directly proportional to time [39, 40]. We applied these principles in developing our method to progressively solubilize and extract younger periosteal bone matrix from that of older bone matrix. Our procedure incorporated a series of sequential treatments of these sealed tibial diaphyses beginning with 4 M guanidine HCL, 1% CHAPS and protease inhibitors to extract the “unmineralized matrix.” [It should be mentioned that GM6001 was substituted for EDTA as a matrix metalloproteinase inhibitor thereby avoiding any trace chelating agents at this initial step.] After extensive washing with this starting solution to completely remove unmineralized matrix molecules, tibial diaphyses were extracted sequentially with increasing EDTA concentrations (20-, 40-, 80-, 200-, & 400-mM in succession) in the guanidine HCl/CHAPS solution to decalcify & extract upper bone strata from progressively deeper bone strata (Supplementary Figure S1). Validation of the effectiveness of our differential extraction method was obtained via scanning electron microscopic analyses. Specifically, we observed a pattern of decalcification and insoluble matrix collapse which was consistent with a progressive periosteal-to-endosteal pattern of extraction and successive decalcification of ever deeper matrix layers (Supplementary Figure S2).

In order to quantitate the average cortical thickness decalcified by the sequential EDTA treatments we cut individual tibial diaphyseal specimens in half and measured their thickness via morphometry. One segment was left untreated by EDTA to be able to assess the original cortical thickness, while the other segment underwent EDTA decalcification and extraction (Figure 2A). Scanning electron microscopy was then used to calculate the average linear distance from the endosteal surface to the point where evidence of mineral removal was visually clear. This value was then compared to that for the untreated counterpart

(Figure 2A). A plot of mineralized bone cortex thickness values compared to EDTA concentration is shown in Figure 2B. Interestingly, the 20- and 40-mM EDTA concentrations together reproducibly decalcified to a depth of ~20 microns from the periosteal surface (Figure 2B; SEM images), which locates within the 1-week old boundaries of bone growth in the 5-week old tibial diaphyses (compare Figures 1 and 2). As expected, higher concentrations of EDTA resulted in greater depths of decalcification with 400-mM EDTA causing complete decalcification. Comparison of the data in Figures 1 and 2 reveals these greater depths correlate to the 1-month old boundaries of bone growth determined earlier by dynamic labeling.

A potential limitation of our morphometric approach is that the detection of mineral salts is based on visual inspection which does not permit us to determine whether sequential EDTA treatments result in partial alterations in mineral content particularly within the deeper strata of the bone cortex. To address this issue, micro-indentation analysis was also carried out across the cortical span. This method has been shown to accurately detect 10–15% changes in local microhardness within cortical bone [32]. Figure 3 shows plots of microhardness compared to average linear distance from the endosteal surface of tibial diaphyses before and after varying EDTA treatments. Importantly, even though the initial 4 M guanidine HCl/1% CHAPS treatments removed a considerable amount of protein from 5-week old tibial diaphyses (Supplementary Table S1), such treatment did not affect the microhardness values across the cortex (Figure 3, top panel). In contrast, when these bone specimens were subsequently extracted with 20- and 40-mM EDTA in 4 M Guanidine HCl/1% CHAPS, there was a statistically significant drop in microhardness value in the topmost bone layer (~10% & ~20% lower values, respectively). Also, the average cortical thickness was noticeably shorter than their untreated counterparts. Furthermore, the microhardness values for the cortical bone in the deeper and endosteal regions were unchanged by 20- and 40-mM EDTA treatments. In summary, these quantitative data confirm that the 20- and 40-mM EDTA treatments decalcify the outermost periosteal bone matrix strata within the 1-week old bone layer in these 5-week old tibial diaphyses. Additionally, our analyses demonstrate that extraction with 20- and 40-mM EDTA does not affect the bone hardness (or content) of depths corresponding to the 1-month old bone tissue. Finally, the depth of extraction as determined by microhardness analysis correlates very well with that determined by ultrastructural morphological analysis (compare Figs. 2 and 3).

Pre-osteocytes nearer the periosteal surface express higher levels of a cell surface antigen (E11-GP38) [41] and lower levels of another cell surface protein (CD44) [42] as compared to osteocytes within deeper cortical bone layers. Since each of the aforementioned bone strata extracted by EDTA has embedded cells within, we sought to use the differential expression of E11 and CD44 on osteocytes as a function of embedded depth to further validate the microhardness findings mentioned above. Figure 4 shows representative Western blot images for E11 and CD44 in the various EDTA extracts from 5-week old tibial diaphyses. As predicted, relatively higher levels of E11, and relatively lower levels of CD44, were observed in the 20- & 40-mM EDTA extracts as compared to the 80-, 200- & 400-mM EDTA concentrations. We conclude from these analyses that sequentially increasing EDTA treatments of 5-week old, sealed tibial diaphyses progressively decalcify and extract younger bone tissue strata from ever deeper and older bone tissue strata. This provides an ideal opportunity to investigate whether the protein composition of bone matrix changes with age or its woven versus lamellar origins [1, 4].

Bone matrix proteins extracted from newly formed versus older bone tissue strata show differences in prevalence and molecular size

Figure 5 shows Western blot results for several bone matrix proteins in the extracts from the various EDTA concentrations in guanidine HCl/CHAPS. All gel lanes were loaded with

equivalent amounts of protein in order to assess the relative prevalence of each matrix protein within each soluble protein extract (total protein contents are provided in Supplementary Table S1). Notably, bone sialoprotein and osteopontin contents in newly formed bone were substantially greater than those in older cortical bone (Figs. 5A & 5B, respectively). Bone sialoprotein was detected as a predominant band at 75 kDa, a minor band at 65 kDa, and a barely detectable higher molecular sized band of 110–120 kDa (see Supplementary Figure S3). Two forms of osteopontin were detected: a lower amount of a 63 kDa band representing the monomer and a relatively greater amount at 120–150 kDa.

Unlike bone sialoprotein or osteopontin, osteonectin showed a greater prevalence in 40-mM and 80-mM EDTA extracts which locates nearer the boundary between 1-week old and 1-month old bone tissue (Fig. 5C). Intact BAG-75 (75 kDa band indicated by *open arrowhead*) showed a distribution pattern similar to those of 75 kDa bone sialoprotein and 63 kDa osteopontin (Fig. 5D). The 55 kDa fragment of BAG-75 was the most prominent form of this matrix protein in these extracts and it was distributed rather uniformly in both newly formed and more mature bone matrix. The distribution pattern of the 55 kDa form of BAG-75 was similar to that of fetuin A (Fig. 5E).

Interestingly, a higher molecular weight form of BAG-75 (~110 kDa) was also detected and its content appeared greater in more mature as compared to newly formed bone matrix (Fig. 5D, *solid arrowhead*). Though not as evident as that for the 110 kDa form of BAG-75, other bone matrix proteins like MEPE and DMP-1 also exhibited slight trends toward having relatively more in older versus newly formed bone matrix. Another interesting finding was that only 55 kDa BAG-75, 97 kDa DMP-1 and relatively small amounts of osteonectin or fetuin A were detected in unmineralized tissue extracts lacking EDTA (“osteoid” equivalent); none of the other bone matrix proteins were detected in the unmineralized tissue extracts.

Figure 6 shows images of all the protein extracts from newly formed versus older bone matrix resolved on SDS-PAGE and detected using several different staining procedures. In this one dimensional SDS-PAGE analysis it should be emphasized that apparent “bands” likely reflect multiple protein species in differing relative proportions. Nevertheless, it is apparent that the staining profiles for the newly formed and older bone tissue extracts display some protein accumulation differences. Close inspection of Tangerine Orange stained gels revealed protein signals at 64 kDa and 50 kDa in 0 mM, 20 mM and 40 mM EDTA extracts that were less visible in 80–400 mM EDTA extracts (Fig. 6, Tangerine Orange, *solid arrowheads*). In addition, the 0 mM EDTA extract also contained a ~40 kDa band not readily apparent in 80–400 mM EDTA extracts (Fig. 6, Tangerine Orange, *open arrowhead*). Conversely, 80–400 mM EDTA extracts contained a prominent band at 57 kDa that was in relatively lower proportion in 20–40 mM extracts (Fig. 6, Tangerine Orange). Additionally, all extracts contained similar amounts of Tangerine Orange stained bands at 100–120 kDa (Fig. 6, Tangerine Orange; verified as soluble collagen by Western blots not shown) and Toluidine blue stained proteoglycans [43] as broad bands at 250–300 kDa and 100–150 kDa that are likely to be biglycan and decorin, respectively [44].

Stains-All staining of separate gels reflects the acidic and phosphoprotein content in all bone extracts. Interestingly, a major phosphorylated protein at 57 kDa and a minor one at ~45 kDa were observed in 80–400 mM EDTA extracts that were much less apparent in 20–40 mM EDTA extracts. When the 57 kDa protein band in the 200 mM EDTA extract (Fig. 6, Stains All, *box*) was cut out of the gel and re-run on 4–12% Nu-PAGE gels, this sample resolved into two bands, one at ~63 kDa and another at ~57 kDa (Supplementary Figure S4). When both bands were cut out of the gel and submitted to trypsin digestion and mass spectroscopic peptide analysis, they were identified as serum albumin and fetuin A,

respectively. Consistent with the findings of previous studies [22, 45], these data and those from the Tangerine Orange stained gel indicate that fetuin A is a major non-collagenous phosphoprotein in cortical bone.

Discussion

There is a longstanding interest in the matrix composition of newly formed woven bone particularly with respect to the function of its non-collagenous matrix phosphoproteins. Previous studies have queried whether any of these matrix proteins are unique to newly formed bone and would exhibit a functional role in the formation of nascent mineral crystallites. The current study sought to rigorously address this central but as yet unanswered question in bone biology. To this end, a system was developed that recovered non-collagenous matrix proteins from newly formed bone tissue and compared them directly to extracts of older compact bone tissue from the same long bone. Such a system should be able to control for all physiological variables within a specific bone except for the time of deposition. In this way, younger woven bone tissue could be examined to determine whether its non-collagenous matrix protein composition is distinct from that of older compact bone.

Anatomically, the spatial region predominantly accumulating newly formed bone tissue in rapidly growing diaphyseal long bones is located along the periosteal surface [14]. Thus, a highly predictable temporal-spatial bone maturation relationship exists within diaphyseal long bones, and equally important, these subperiosteal regions containing newly formed bone are readily accessible for isolation and study. The temporal-spatial boundary between newly formed bone tissue (≤ 1 -week-old) and that of the pre-existing more mature compact bone (~ 1 -month-old) was readily identified using dynamic fluorochrome labeling. Thereafter, a means to access and separate this newly formed bone tissue from the more mature compact bone was developed. Our approach took advantage of the low hydraulic permeability properties of compact bone, which severely retards the permeation of aqueous solutions and their dissolved solutes into the extracellular matrix space of cortical bone [37–40]. Additionally, by sealing the ends of the bone shaft with bone wax, our approach restricted exposure of denaturing extraction solutions containing low concentrations of a calcium mineral chelator to just that of the periosteal bone surface. This restriction permitted EDTA mediated decalcification of a 20–25 micron deep layer of sub-periosteal bone, which is well within the temporal-spatial boundary of newly formed bone tissue in this location. The simultaneous presence of 4 M guanidine HCl and 1% CHAPS during this decalcification process facilitated solubilization of the extracellular matrix of this newly formed bone layer without removing the deeper layers of more mature bone. Finally, Western blot analysis was used to assess the relative proportions of bone matrix phosphoproteins in extracts of newly formed bone in comparison to those from more mature cortical bone within the same long bone.

Bone sialoprotein (75 kDa), osteopontin (63 kDa), and the 75 kDa form of BAG-75 were present in all of the EDTA extracts, but were found to be enriched in newly formed bone matrix as opposed to more mature compact bone matrix. In contrast, osteonectin, fetuin A, MEPE and DMP-1 appeared to be similarly distributed and relatively unchanged between these two bone tissue compartments. Most notable, the enrichment of bone sialoprotein in newly formed bone was nearly an order of magnitude greater than its prevalence in more mature compact bone tissue. Thus, newly formed bone is enriched more than 10-fold in bone sialoprotein, a nucleator of mineralization [16], as compared to older cortical bone. These results imply that bone sialoprotein may be subject to changes in expression and/or proteolytic turnover particularly after mineralization is initiated. In this light, we have observed that bone sialoprotein is cleaved at the time when mineralization occurs in osteoblastic culture models [46].

Consistent with the findings of Kaartinen et al. [47], both bone sialoprotein and osteopontin epitopes were also detected in larger sized forms ranging from 120–150 kDa. It was noted that the proportion of the larger sized form of bone sialoprotein was relatively low in comparison to that for osteopontin. Indeed, assessments of integrated optical densities for osteopontin bands in Figure 5B suggest that nearly 4 times more of the epitope reactivity resides in the 150 kDa as opposed to the 63 kDa bands. Given previous reports of osteopontin's ability to bind to type I collagen fibers, and that transglutaminase-cross linked forms of osteopontin exhibit much higher collagen binding affinity than its monomer [20], a plausible explanation for the 150 kDa form of osteopontin would suggest that it may be a dimer resulting from transglutaminase cross linking [47]. In light of the above hypothesis, it would be worthwhile to further investigate the effects of these larger forms of osteopontin with respect to collagen matrix assembly and transglutaminase stabilization.

An even higher molecular sized form of osteopontin was noted around 250 kDa though an explanation for its presence is unclear. In addition, DMP-1 Western blots repeatedly exhibited a high molecular weight “smear” mostly in the 200 mM EDTA extract, though relatively lower amounts were also detected in the 400 mM EDTA extract. Its appearance resembles that of proteoglycan migration patterns on SDS-PAGE. In light of previous reports that some of DMP-1 exists in a proteoglycan form [29], this observation suggests the presence of a DMP-1 proteoglycan form in these higher EDTA concentration extracts implying a temporal expression pattern during juvenile long bone growth.

Similar to osteopontin, BAG-75 immuno-reactivity was also noted in larger forms (mostly at 110 kDa and 150 kDa) though their distributions were unlike that of osteopontin's 150 kDa form. Whereas more of the 150 kDa form of osteopontin was apparent in newly formed bone as compared to older bone, the higher molecular weight forms of BAG-75 were more evident in more mature as compared to newly formed bone tissue extracts. The fact that monomeric BAG-75 is more abundant in newly formed bone while the 110 kDa form is more abundant in older bone suggests a temporal precursor-product relationship during the transition from woven bone to more mature compact bone. We previously identified similar 116 kDa and 180 kDa forms of BAG-75 in calvarial bone extracts and in rat growth plate primary spongiosa, and also showed that BAG-75 monomer can form aggregates *in vitro* which have the capacity to sequester substantial quantities of phosphate [48]. Thus, we speculate that the differential distribution of larger sized forms of BAG-75 in more mature versus newly formed bone matrix could also reflect distinctive physiochemical properties in these two bone tissue compartments.

Smaller sized forms of both BAG-75 and DMP-1 were observed in newly formed and older bone extracts. In contrast, fragments of MEPE were observed only in the 400 mM EDTA extract, which includes the endocortical bone surface that slowly remodels even at this young age. Stable breakdown products for these matrix phosphoproteins have been reported in bone tissue, and it is presumed that these smaller forms are the result of such proteolytic processing. Consistent with this notion, both the 57 kDa and 37 kDa breakdown products of DMP-1 [49] were observed in both newly formed and more mature compact bone tissue. Recently, it has been reported that BAG-75 is cleaved by a serine protease activity, in part representing the actions of a pro-protein convertase (SKI-1) [50], and the 55 kDa BAG-75 breakdown product observed in the current study may result from this proteolytic activity.

On a final note, unmineralized “osteoid” matrix extracts did not contain appreciable amounts of bone sialoprotein, osteopontin, and MEPE even though the amount of total soluble protein in the 0 mM EDTA extract was ~2 times greater than any of the EDTA extracts (Supplementary Table S1). Only trace amounts of osteonectin and relatively small amounts of fetuin A and the 55 kDa fragment of BAG-75 were detected in these unmineralized

matrix extracts. Interestingly, full length DMP-1 (97 kDa) was only present in the unmineralized matrix extracts suggesting that DMP-1 proteolytic processing is associated with the mineral deposition process. This relatively low abundance of phosphoproteins in the unmineralized matrix may reflect a combination of (1) the nature of their secretion and assembly into the extracellular matrix, (2) high binding affinity for mineral crystallites in the mineralization front, or (3) proximity of synthesis to the mineralization front given that surface osteoblasts or “osteoid osteocytes” are the predominant cell type producing these matrix proteins. Prior studies reporting the presence of bone sialoprotein or osteopontin in “unmineralized” matrix extracts were based on procedures using 10 mM EDTA as a matrix metalloproteinase inhibitor. Our findings in the current study suggest that these observations are likely to be a result of a small amount of decalcification of mineralized bone tissue by the low concentration of EDTA used.

This study design provided the localization assurance of past immunohistochemistry studies combined with the relative quantitative assurance of past biochemistry studies. Its findings indicate that newly formed bone exhibits a non-collagenous matrix phosphoprotein composition distinct from that of more mature compact bone even within the same long bone. At this juvenile age, the time frame over which the phosphoprotein composition of newly formed bone converts to that of a more mature bone tissue is relatively short and consistent with the timeframe for changes in the mineral content and stiffness measured in young growing bone [2, 3]. These findings represent a major step towards answering the question of whether there exists a matrix protein unique to newly formed bone that may manifest a functional relationship with the formation of nascent mineral crystallites. At present, none of the noncollagenous proteins analyzed was exclusively localized to newly formed bone. Yet, given Western blotting’s limited epitope screening capabilities, the current study could not determine whether newly formed bone contains unique matrix proteins not present in more mature compact bone tissue. Such a conclusion could be only addressed via further investigations using a broader proteomics approach comparing matrix protein extracts from newly formed versus more mature compact bone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Gorski JP. Biomineralization of bone: a fresh view of the roles of non-collagenous proteins. *Front Biosci.* 2011; 17:2598–2621. [PubMed: 21622198]
2. Miller LM, Little W, Schirmer A, Sheik F, Busa B, Judex S. Accretion of bone quantity and quality in the developing mouse skeleton. *J Bone Miner Res.* 2007; 22:1037–1045. [PubMed: 17402847]
3. Donnelly E, Boskey AL, Baker SP, van der Meulen MC. Effects of tissue age on bone tissue material composition and nanomechanical properties in the rat cortex. *J Biomed Mater Res.* 2010; 92A:1048–1056.
4. Gorski JP. Is all bone the same? Distinctive distributions and properties of non-collagenous matrix proteins in lamellar vs. woven bone imply the existence of different underlying osteogenic mechanisms. *Crit Rev Oral Biol Med.* 1998; 9:201–223. [PubMed: 9603236]
5. Field RA, Riley ML, Mello FC, Corbridge JH, Kotula AW. Bone composition in cattle, pigs, sheep and poultry. *J Anim Sci.* 1974; 39:493–499. [PubMed: 4412232]

6. Pinto MR, Gorski JP, Penniston JT, Kelly PJ. Age-related changes in composition and Ca²⁺-binding capacity of canine cortical bone extracts. *Am J Physiol.* 1988; 255:H101–H110. [PubMed: 3134822]
7. Chen J, Shapiro HS, Sodek J. Developmental expression of bone sialoprotein mRNA in rat mineralized connective tissues. *J Bone Min Res.* 1992; 7:987–997.
8. Turner RT, Kapelner SN, Spelsberg TC. Tissue-specific expression of bone proteins in femora of growing rats. *Am J Physiol.* 1992; 263:E724–E729. [PubMed: 1415691]
9. Ninomiya JT, Tracy RP, Calore JD, Gendreau MA, Kelm RJ, Mann KG. Heterogeneity of human bone. *J Bone Min Res.* 1990; 5:933–938.
10. Sodek KL, Tupy JH, Sodek J, Grynblas MD. Relationships between bone protein and mineral in developing porcine long bone and calvaria. *Bone.* 2000; 26:189–198. [PubMed: 10678415]
11. McKee MD, Farach-Carson MC, Butler WT, Hauschka PV, Nanci A. Ultrastructural immunolocalization of noncollagenous (osteopontin and osteocalcin) and plasma (albumin and α_2 -HS-glycoprotein) proteins in rat bone. *J Bone Min Res.* 1993; 8:485–496.
12. Pinero GJ, Farach-Carson MC, Devoll RE, Aubin JE, Brunn JC, Butler WT. Bone matrix proteins in osteogenesis and remodeling in the neonatal rat mandible as studied by immunolocalization of osteopontin, bone sialoprotein, α_2 -HS-glycoprotein and alkaline phosphatase. *Archs Oral Biol.* 1995; 40:145–155.
13. Ramstad VE, Franzen A, Heinegard D, Wendel M, Reinholt FP. Ultrastructural distribution of osteoadherin in rat bone shows a pattern similar to that of bone sialoprotein. *Calcif Tissue Int.* 2003; 72:57–64. [PubMed: 12384815]
14. Leblond CP, Wilkinson GW, Belanger LF, Robichon J. Radio-autographic visualization of bone formation in the rat. *Am J Anat.* 1950; 86:289–299. [PubMed: 15410672]
15. Midura RJ, Su X, Morcuende JA, Tammi M, Tammi R. Parathyroid hormone rapidly stimulates hyaluronan synthesis by periosteal osteoblasts in the tibial diaphysis of the growing rat. *J Biol Chem.* 2003; 278:51462–51468. [PubMed: 14514685]
16. Hunter GK, Goldberg HA. Nucleation of hydroxyapatite by bone sialoprotein. *Proc Natl Acad Sci USA.* 1993; 90:8562–8565. [PubMed: 8397409]
17. Malaval L, Wade-Gueye NM, Boudiffa M, Fei J, Zirngibl R, Chen F, Laroche N, Roux JP, Burt-Pichat B, Duboeuf F, Boivin G, Jurdic P, Lafage-Proust MH, Amedee J, Vico L, Rossant J, Aubin JE. Bone sialoprotein plays a functional role in bone formation and osteoclastogenesis. *J Exp Med.* 2008; 205:1145–1153. [PubMed: 18458111]
18. Malaval L, Monfoulet L, Fabre T, Pothuaud L, Bareille R, Miraux S, Thiaudiere E, Raffard G, Franconi JM, Lafage-Proust MH, Aubin JE, Vico L, Amedee J. Absence of bone sialoprotein (BSP) impairs cortical defect repair in mouse long bone. *Bone.* 2009; 45:853–861. [PubMed: 19524706]
19. Fujisawa R, Kuboki Y. Affinity of bone sialoprotein and several other bone and dentin acidic proteins to collagen fibrils. *Calcif Tissue Int.* 1992; 51:438–442. [PubMed: 1451011]
20. Kaartinen MT, Pirhonen A, Linnala-Kankkunen A, Maenpaa PH. Cross-linking of osteopontin by tissue transglutaminase increases its collagen binding properties. *J Biol Chem.* 1999; 274:1729–1735. [PubMed: 9880554]
21. Gericke A, Qin C, Spevak L, Fujimoto Y, Butler WT, Esben S, Sørensen ES, Boskey AL. Importance of phosphorylation for osteopontin regulation of biomineralization. *Calcif Tissue Int.* 2005; 77:45–54. [PubMed: 16007483]
22. Termine JD, Kleinman HK, Whitson SW, Conn KM, McGarvey ML, Martin GR. Osteonectin, a bone-specific protein linking mineral to collagen. *Cell.* 1981; 26:99–105. [PubMed: 7034958]
23. Chen Y, Bal BS, Gorski JP. Calcium and collagen binding properties of osteopontin, bone sialoprotein, and bone acidic glycoprotein-75 from bone. *J Biol Chem.* 1992; 267:24871–24878. [PubMed: 1447223]
24. Gorski JP, Wang A, Lovitch D, Law D, Powell K, Midura RJ. Extracellular bone acidic glycoprotein-75 defines condensed mesenchyme regions to be mineralized and localizes with bone sialoprotein during intramembranous bone formation. *J Biol Chem.* 2004; 279:25455–25463. [PubMed: 15004029]

25. Midura RJ, Wang A, Lovitch D, Law D, Powell K, Gorski JP. Bone acidic glycoprotein-75 delineates the extracellular sites of future bone sialoprotein accumulation and apatite nucleation in osteoblastic cultures. *J Biol Chem.* 2004; 279:25464–2543. [PubMed: 15004030]
26. Jahnen-Dechent W, Schäfer C, Ketteler M, McKee MD. REVIEW: Mineral chaperones: a role for fetuin-A and osteopontin in the inhibition and regression of pathologic calcification. *J Mol Med.* 2008; 86:379–389. [PubMed: 18080808]
27. Boskey AL, Chiang P, Fermanis A, Brown J, Taleb H, David V, Rowe PSN. MEPE's Diverse Effects on Mineralization. *Calcif Tissue Int.* 2010; 86:42–46. [PubMed: 19998030]
28. Ye L, Mishina Y, Chen D, Huang H, Dallas SL, Dallas MR, Sivakumar P, Kunieda T, Tsutsui TW, Adele Boskey A, Bonewald LF, Feng JQ. Dmp1-deficient Mice Display Severe Defects in Cartilage Formation Responsible for a Chondrodysplasia-like Phenotype. *J Biol Chem.* 2005; 280:6197–6203. [PubMed: 15590631]
29. Qin C, Huang B, Wygant JN, McIntyre BW, McDonald CH, Cook RG, Butler WT. A Chondroitin Sulfate Chain Attached to the Bone Dentin Matrix Protein 1 NH₂-Terminal Fragment. *J Biol Chem.* 2006; 281:8034–8040. [PubMed: 16421105]
30. Huang B, Sun Y, Maciejewska I, Qin D, Peng T, McIntyre B, Wygant J, Butler WT, Qin C. Distribution of SIBLING proteins in the organic and inorganic phases of rat dentin and bone. *Eur J Oral Sci.* 2008; 116:104–112. [PubMed: 18353003]
31. Ingram RT, Clarke BL, Fisher LW, Fitzpatrick LA. Distribution of noncollagenous proteins in the matrix of adult human bone: evidence of anatomic and functional heterogeneity. *J Bone Mineral Res.* 1993; 8:1019–1029.
32. Su XW, Feng QL, Cui FZ, Zhu XD. Microstructure and micromechanical properties of the diaphysis of human fetal femurs. *Conn Tissue Res.* 1997; 36:271–286.
33. Fisher LW, Stubbs JT 3rd, Young MF. Antisera and cDNA probes to human and certain animal model bone matrix noncollagenous proteins. *Acta Orthop Scand Suppl.* 1995; 266:61–65. [PubMed: 8553864]
34. Ogbureke KUE, Fisher LW. Expression of SIBLINGs and Their Partner MMPs in Salivary Glands. *J Dent Res.* 2004; 83:664–670. [PubMed: 15329369]
35. Sampath TK, Reddi AH. Distribution of bone inductive proteins in mineralized and demineralized extracellular matrix. *Biochem Biophys Res Comm.* 1984; 119:949–54. [PubMed: 6712678]
36. Midura RJ, Vasanthi A, Su X, Wang A, Midura SB, Gorski JP. Calcospherulites isolated from the mineralization front of bone induce the mineralization of type I collagen. *Bone.* 2007; 41:1005–1016. [PubMed: 17936099]
37. Li GP, Bronk JT, An KN, Kelly PJ. Permeability of cortical bone of canine tibia. *Microvasc Res.* 1987; 34:302–310. [PubMed: 2448591]
38. Wen D, Androjna C, Vasanthi A, Belovich J, Midura RJ. Lipids and collagen matrix restrict the hydraulic permeability within the porous compartment of adult cortical bone. *Ann Biomed Eng.* 2010; 38:558–569. [PubMed: 19967451]
39. Birkedal-Hansen H. Diffusion of H³⁶Cl in decalcified ivory dentine. *J Histochem Cytochem.* 1974; 22:428–433. [PubMed: 4407514]
40. Birkedal-Hansen H. Kinetics of acid demineralization in histologic technique. *J Histochem Cytochem.* 1974; 22:434–441. [PubMed: 4601636]
41. Zhang K, Barragan-Adjemian C, Ye L, Kotha S, Dallas M, Lu Y, Zhao S, Harris M, Harris SE, Feng JQ, Bonewald LF. E11/gp38 Selective Expression in Osteocytes: Regulation by Mechanical Strain and Role in Dendrite Elongation. *Mol Cell Biol.* 2006; 26:4539–4552. [PubMed: 16738320]
42. Jamal HH, Aubin JE. CD44 Expression in Fetal Rat Bone: In Vivo and in Vitro Analysis. *Exp Cell Res.* 1996; 223:467–477. [PubMed: 8601425]
43. Moller HJ, Heinegard D, Poulsen JH. Combined alcian blue and silver staining of subnanogram quantities of proteoglycans and glycosaminoglycans in sodium dodecyl sulfate-polyacrylamide gels. *Anal Biochem.* 1993; 209:169–75. [PubMed: 8465952]
44. Fisher LW, Hawkins GR, Tuross N, Termine JD. Purification and partial characterization of small proteoglycans I and II, bone sialoproteins I and II, and osteonectin from the mineral compartment of developing human bone. *J Biol Chem.* 1987; 262:9702–9708. [PubMed: 3597437]

45. Ashton BA, Hohling HJ, Triffitt JT. Plasma proteins present in human cortical bone: Enrichment of the α_2 HS-glycoprotein. *Calcif Tissue Res.* 1976; 22:27–33. [PubMed: 1000340]
46. Huffman NT, Keightley JA, Chaoying C, Midura RJ, Lovitch D, Veno PA, Dallas SL, Gorski JP. Association of specific proteolytic processing of bone sialoprotein and bone acidic glycoprotein-75 with mineralization within biomineralization foci. *J Biol Chem.* 2007; 282:26002–13. [PubMed: 17613519]
47. Kaartinen MT, El-Maadawy S, Rasanen NH, McKee MD. Tissue transglutaminase and its substrates in bone. *J Bone Mineral Res.* 2002; 17:2161–2173.
48. Gorski JP, Kremer EA, Chen Y, Ryan S, Fullenkamp C, Delviscio J, Jensen K, McKee MD. Bone Acidic Glycoprotein-75 Self-Associates to Form Macromolecular Complexes In Vitro and In Vivo with the Potential to Sequester Phosphate Ions. *J Cell Biochem.* 1997; 64:547–564. [PubMed: 9093904]
49. Qin C, Brunn JC, Cook RG, Orkiszewski RS, Malone JP, Veis A, Butler WT. Evidence for the Proteolytic Processing of Dentin Matrix Protein 1 – Identification and characterization of processed fragments and cleavage sites. *J Biol Chem.* 2003; 278:34700–34708. [PubMed: 12813042]
50. Gorski JP, Huffman NT, Chittur S, Midura RJ, Black C, Oxford J, Seidah NG. Inhibition of Proprotein Convertase SKI-1 Blocks Transcription of Key Extracellular Matrix Genes Regulating Osteoblastic Mineralization. *J Biol Chem.* 2011; 286:1836–1849. [PubMed: 21075843]

Highlights

1. Newly formed bone's matrix composition is distinct from that of more mature bone;
2. Select matrix proteins in younger versus older bone exhibit differences in post-translational modifications;
3. These modifications involve proteolysis associated with mineralization or remodeling;
4. These modifications also involve cross-linking into higher molecular sized forms;
5. Newly formed bone rapidly transitions into mature bone involving temporal-spatial changes in matrix proteins.

Figure 1. Newly formed bone tissue is predominantly deposited along the periosteal surface at mid-diaphysis

Panel A shows a brightfield image of hematoxylin & eosin stained cortical bone tissue section at mid-diaphysis of a 5-week old rat tibia. Locations of *Periosteum*, *Bone* and *Marrow* tissues are indicated in the image. Panel B shows a similar cortical bone region at mid-tibial diaphysis from a juvenile rat injected *in vivo* with alizarin red S at 4-weeks of age followed by an injection of calcein at 5-weeks of age. Tibiae were recovered one day after calcein injection and processed for hard tissue sectioning and epifluorescent microscopy to detect the locations of alizarin (*red*) and calcein (*green*) labeling. Note the greater amounts and extents of labeling along the periosteal (*p*) relative to the endosteal (*e*) cortical bone surfaces; boundaries of *1-month old* pre-existing cortical bone and *1-week old* newly formed bone are denoted. Scale bar shown (100 microns) is a reference for both panels.

Figure 2. Treatment with progressively increasing concentrations of EDTA decalcifies bone tissue strata separating bone matrix of different ages

Panel A illustrates the morphometric approach of cutting the same tibial diaphysis in half in order to more accurately assess the depth of decalcification as measured from the periosteal (P) surface via SEM after lining up the endosteal (E) surfaces (*top right image*, scale bar is 50 microns). *Double-arrow white lines* depict triplicate measurements made for each specimen. **Panel B** plots the mineralized cortical thickness in microns against the concentration of EDTA in extraction buffer using the successive solution exchanges as described in the text (see also Supplementary Figure S1). A total of 10 tibial diaphyses were included in these analyses and the values shown represent mean cortical thickness measures ($\pm$ SD; $p < 0.05$ between 20–40 mM versus 20–40–80 mM EDTA concentrations). *Dashed lines* in the graph depict the 25-micron depth of 1-week old newly formed bone (as shown in Figure 1) as opposed to the thickness of the 1-month old more mature cortical bone tissue. High resolution SEM images shown to the right of panel B demonstrate the presence of decalcified tissue matrix at the periosteal surface (to the right of the *arrowhead*, scale bars are 25 microns) resulting from 20 mM + 40 mM EDTA successive treatments (see also Supplementary Figure S2).

Figure 3. Microhardness of periosteal newly formed bone layers is reduced by successive 20 mM and 40 mM EDTA treatments while not affecting more mature cortical bone strata
 Except for *untreated* specimens, all sealed diaphyses were exposed to the GuHCl/CHAPS denaturants as well as the indicated EDTA concentrations. The microhardness values for untreated fully mineralized cortical bone (60–65 kg/mm²) were always greater than those for fully decalcified bone (15–20 kg/mm²) tissue at this age and bone location (n = 10 tibial diaphyses; mean ± SD).

Figure 4. Successive 20 mM and 40 mM EDTA treatments of sealed tibial diaphyses solubilize most of E11 cell surface receptor (pre-osteocyte) and relatively little of CD44 cell surface receptor (mature osteocyte)

All sealed diaphyses were exposed to the GuHCl/CHAPS denaturants as well as the indicated EDTA concentrations. The following antibodies were used for the Western blots shown: GP38-E11 detected using 9C11 monoclonal, and CD44 detected using OX-49 polyclonal. Results shown are representative from 3 independent tissue recoveries and 2 repeat Western blots for each of the antibodies.

Figure 5. Western blot analyses of bone matrix proteins in primary versus more mature cortical bone within the same tibial diaphysis

"Osteoid" refers to the GuHCl/CHAPS extraction without EDTA, "1-wk old" refers to GuHCl/CHAPS extraction with 20 mM & 40 mM EDTA, and "1-mo old" refers to GuHCl/CHAPS extraction with 80 mM, 200 mM & 400 mM EDTA. The following antibodies were used for the Western blots shown: bone sialoprotein detected using LF-100 polyclonal, osteopontin detected using MPIIB10 monoclonal, osteonectin/SPARC detected using BON-1 polyclonal, BAG-75 detected using 5O4 polyclonal, Fetuin A detected using AB 871 polyclonal, MEPE detected using LF-154 polyclonal, and DMP-1 detected using a mixture of 784 (C-terminus) + 783 (N-terminus) polyclonals. Results shown are

representative from 3 independent tissue recoveries and 2 repeat Western blots for each of the antibodies.

Figure 6. SDS-PAGE analyses of bone matrix proteins in primary versus more mature cortical bone within the same tibial diaphysis

“*Osteoid*” refers to the GuHCl/CHAPS extraction without EDTA, “*1-wk old*” refers to GuHCl/CHAPS extraction with 20 mM & 40 mM EDTA, and “*1-mo old*” refers to GuHCl/CHAPS extraction with 80 mM, 200 mM & 400 mM EDTA. *Closed arrowheads* to the left of the Tangerine Orange panel denote the positions of 64 kDa and 50 kDa bands in osteoid and 1-wk old bone extracts which are less apparent in the 1-mo old bone extracts. *Open arrowhead* to the left of the Tangerine Orange panel denotes the position of a 40 kDa band in osteoid (and less so in 1-wk old bone) extracts which is not readily apparent in 1-mo old bone extracts. *Box* in the Stains-All panel denotes the location of the 57 kDa band that was cut out of the gel for further analysis (see Supplementary Figures S4 & S5 for additional details).