JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE **12** (2001) 699–702

The distribution of osteocalcin, degree of mineralization, and mechanical properties along the length of *Cyprinus carpio* rib bone

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This study examined the spatial distribution of selected biochemical and mechanical properties along the length of carp rib bone. Carp rib bone was chosen because of its unusually high osteocalcin content relative to other extractable proteins. The amount of osteocalcin was significantly lower ($p < 0.01$) at the most distal section, relative to all other sections. The amount of phosphate ($p < 0.05$) and the elastic modulus in the longitudinal plane ($p < 0.0001$) were found to be significantly higher in the most distal section, relative to the most proximal section. There was no significant difference in the calcium distribution, molar Ca/P ratio, or elastic modulus in the transverse plane. It was speculated that the distal section contains less mature bone. The methods illustrate the potential usefulness of nanoindentation to characterize the mechanical properties of bone, relative to its biochemical composition.

1. Introduction

Osteocalcin (OC), also known as γ-carboxyglutamic acid-containing protein or BGP, is an abundant and extensively studied noncollagenous protein of bone. It has a high affinity for hydroxyapatite (HA) relative to calcium phosphate, and is a potent inhibitor of HA formation [1]. OC is a biochemical marker of bone metabolism or turnover [2] that is commonly used to assess bone metabolic disease [3]. An increase in OC concentration closely follows mineralization, suggesting a potential mechanistic role in the regulation of bone turnover [4]. Osteocalcin deficiency has long been associated with bone mineralization [5, 6], but OC has little effect on morphology or structure [7]. OC expression is highest in growing bone, implicating its developmental role in mineralization [8]. OC-deficient transgenic mice have increased bone mass and strength with normal mineral content, suggesting that osteocalcin prevents excessive bone formation [9]. The relationship of OC to bone mineral has led to the current hypotheses that differences in osteocalcin concentration along the length of a bone will be reflected in the Ca/P ratio, an index of the degree of mineralization, and the microstructural elastic modulus along the length of the bone. The results of this study should help characterize the relationship between osteocalcin, the type of mineral, and the microstructural mechanical properties of bone, and establish methods to link chemical and mechanical properties at the microstructural level.

2. Materials and methods

The first through fifth pairs of ribs were harvested from a fresh frozen 9 lb. carp (*Cyprinus carpio*). A carp rib bone model was chosen for study primarily because of its unusually high (~ 60%) content of OC relative to other extractable proteins, like bluegill rib bone [10]. The first, third, and fifth pairs of ribs were cut into 14 pieces of approximately 5 mm each, producing segments of bone each representing about 6% of the overall length. Specimens were divided into two groups such that alternating pieces were used for different tests. The end result was seven levels of discretization along the length of the rib for every test.

Specimens used for assays were prepared by extracting their mineral and soluble proteins with 10% formic acid over a period of 24 h, following an established method [11]. Supernatants of the specimen

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extracts were used in all subsequent assays. The OC content was determined in triplicate by a radioimmunoassay (RIA) for carp osteocalcin (manuscript in preparation). Raw data were normalized to the initial sample weight and corrected for dilution as described previously [12].

The degree of mineralization of the bone specimens was determined spectrophotometrically. Phosphate assays were performed in duplicate following Sigma Diagnostics procedure 360-UV. Ultra-violet absorbance was read on a SPECTRAmax® *PLUS* (Molecular Devices Corporation, Sunnyvale, CA) spectrophotometer at 340 nm. Calcium was assayed by flame atomic absorption spectroscopy (AAS) using a Varian AA-775 (Varian Techtron Pty. Ltd., Springvale, Australia), at a wavelength of 422.7 nm [13]. The Ca/P ratio, an index of the degree of mineralization, was determined upon adjustment for dilution factor and normalization to the initial sample weight.

Microstructural mechanical properties were determined by nanoindentation, allowing the determination of a material's microstructural mechanical properties at the microstructural level with an ultimate resolution of better than 1 µm [14, 15]. Specimens were prepared by mounting transverse and longitudinal sections of bone from ribs 1, 3 and 5 in epoxy resin and polishing to a 0.05 µm finish. Specimens were tested using the Nano Indenter® II at the Oak Ridge National Laboratory, with each specimen programd for at least three indentations with target depths of about 1000 nm. The indentation moduli (E, a measure of elasticity; in GPa) were determined via indentations in both the transverse (T) and longitudinal (L) planes, with Poisson's ratio assumed to be 0.3. Fig. 1 illustrates the transverse and longitudinal planes examined in this study. Data from each rib section were measured at its distal end (E_T), or from a region of bone (E_L; assumed to be representative of the midpoint of the bone section; all other data). Because plotted data will cluster by bone section, it is reasonable to compare data from different ribs by section rather than by percent length.

To determine trends in the distribution of a property along the length of a bone, data from each test of each rib were pooled together in groups by section. Significant differences between groups of data within each test were revealed using one-way analysis of variance (ANOVA) and Scheffé's method. By pooling together data from corresponding sections of different ribs, a more conservative estimate of the trends along the length of the rib was obtained. A significance level of 95% was used for all tests.

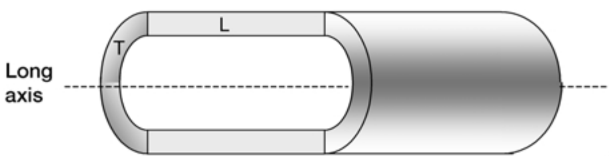

Figure 1 Sketch of a typical long bone specimen, illustrating the orientations of interest: axial direction, transverse (T) and longitudinal (L) planes.

3. Results

Osteocalcin data summarized by bone section are given in Table I. The concentration of osteocalcin was significantly lower at the most distal end (Section 14) relative to the other sections (Table II). Calcium and phosphate data, along with the molar Ca/P ratio, are summarized by bone section in Table III. Statistically significant differences by section among each variable were determined by ANOVA and Scheffé's method (Table IV). The most distal section (Section 14) had the lowest OC concentration of all sections and a high phosphate concentration. The calcium distribution was relatively flat, with the only significant difference occurring between the proximal end and the midpoint.

The mean indentation elastic moduli (in GPa) and anisotropy ratios (the ratio E_T/E_L) by section are summarized in Table V. The elastic modulus distribution is relatively flat in the transverse plane, but generally increases with percent length in the longitudinal plane. The ANOVA results of the nanoindentation data are

TABLE I Osteocalcin distribution of carp rib bone, summarized by section (section 2 = proximal end (0% length), section 14 = distal end (100% length)).

Rib section	µg OC/mg bone, mean ± S.D. (n)
2	0.538 ± 0.051 (9)
4	0.551 ± 0.045 (8)
6	0.596 ± 0.027 (9)
8	0.539 ± 0.076 (9)
10	0.489 ± 0.117 (8)
12	0.441 ± 0.082 (9)
14	0.297 ± 0.033 (9)

TABLE II Statistically significant results of ANOVA and Scheffé's test ($p = 0.05$) for osteocalcin differences, by section. The distal section had the lowest concentration of OC of all sections.

Comparison	Significance
2, 14	$p < 0.0001$
4, 14	$p < 0.0001$
6, 12	$p < 0.01$
6, 14	$p < 0.0001$
8, 14	$p < 0.0001$
10, 14	$p < 0.001$
12, 14	$p < 0.01$

TABLE III Phosphate, calcium, and molar Ca/P distribution of carp rib bone, summarized by section (section 2 = proximal end (0% length), section 14 = distal end (100% length)).

Rib section	mg PO_4/mg bone mean ± S.D. (n)	mg Ca/mg bone mean ± S.D. (n)	Molar Ca/P
2	0.227 ± 0.023 (6)	0.179 ± 0.0018 (6)	1.88
4	0.242 ± 0.016 (4)	0.191 ± 0.0043 (6)	1.93
6	0.233 ± 0.018 (6)	0.187 ± 0.0006 (6)	1.91
8	0.267 ± 0.006 (6)	0.194 ± 0.0031 (6)	1.72
10	0.265 ± 0.009 (6)	0.192 ± 0.0024 (4)	1.70
12	0.257 ± 0.022 (6)	0.191 ± 0.0023 (6)	1.77
14	0.262 ± 0.003 (6)	0.185 ± 0.0022 (6)	1.67

TABLE IV Statistically significant results of ANOVA and Scheffé's test ($p = 0.05$) for phosphate and calcium distribution differences, summarized by section. The calcium distribution was relatively flat, although the most proximal section had a lower concentration of calcium than the middle section. Generally speaking, the most distal section had a higher concentration of phosphate than the most proximal section.

Variable	Comparison	Significance
PO_4	2, 8	$p < 0.01$
PO_4	2, 10	$p < 0.05$
PO_4	2, 14	$p < 0.05$
PO_4	6, 8	$p < 0.05$
PO_4	6, 10	$p < 0.05$
Ca	2, 8	$p < 0.05$

TABLE V Indentation elastic modulus results of carp rib bone, summarized by plane and section (section 1 = proximal end (0% length), section 13 = distal end (100% length)).

Rib section	E_T (GPa) mean ± S.D. (n)	E_L (GPa) mean ± S.D. (n)	Anisotropy ratio
1	15.4 ± 1.24 (11)	8.25 ± 0.27 (9)	1.87
3	16.2 ± 1.17 (9)	7.95 ± 0.63 (8)	2.04
5	16.0 ± 0.84 (8)	8.60 ± 0.51 (8)	1.86
7	16.1 ± 0.98 (9)	9.19 ± 0.68 (8)	1.76
9	16.0 ± 0.84 (10)	9.43 ± 0.42 (7)	1.70
11	15.8 ± 1.90 (11)	8.99 ± 0.31 (9)	1.76
13	16.0 ± 0.76 (10)	9.64 ± 0.39 (9)	1.67

TABLE VI Statistically significant results of ANOVA and Scheffé's test ($p = 0.05$) for indentation elastic modulus differences, summarized by plane and section. All E_T data were statistically significantly higher than E_L data. No moduli differences were found among sections in the transverse plane. In the longitudinal plane, generally speaking the most distal sections had a higher elastic modulus than the most proximal section.

Variable	Comparison	Significance
E_L	1, 7	$p < 0.05$
E_L	1, 9	$p < 0.001$
E_L	1, 13	$p < 0.0001$
E_L	3, 7	$p < 0.01$
E_L	3, 9	$p < 0.0001$
E_L	3, 11	$p < 0.01$
E_L	3, 13	$p < 0.0001$
E_L	5, 13	$p < 0.01$

summarized in Table VI. In the longitudinal plane, the proximal sections (1, 3) had a lower elastic modulus than the central and distal sections (7, 9, 11, 13). In the transverse plane, E_T did not vary significantly by section.

4. Discussion

In this study, the amount of osteocalcin was found to be significantly less at the distal end of the bone relative to the other sections of bone. Combined with the results of previous studies [4–6, 16–18], this implies that the distal section contains more growing bone, or younger bone. It has been postulated that osteocalcin delays bone crystal nucleation and stimulates mineral maturation [19, 20]. It would thus be expected that younger, growing bone would have a lower concentration of osteocalcin.

The degree of mineralization results were mixed. While the calcium distribution was relatively flat, the concentration of phosphate was significantly higher at the distal end relative to the most proximal section. In addition, larger than expected molar Ca/P ratios were found. If mature bone was predominantly HA, it would have a molar Ca/P ratio of 1.67. Ca/P ratios in this study varied from 1.88 at the most proximal section, reached a peak of 1.93 at the second section, and thereafter generally decreased to 1.67 by the most distal section. The higher Ca/P ratio may be due to the limited substitution of carbonate for phosphate, as calcium carbonate [21]. This provides additional support to the notion that bone is a combination of different mineral phases rather than just a single apatite phase [22]. Given that the carbonate : phosphate ratio has been shown to be lower in OC-deficient bone [20], it is not surprising that the distal section had both the lowest concentration of OC and the lowest molar Ca/P ratio.

No significant differences were found in E_T along the length of the bone, but E_L increased with percent length. Assuming a transversely isotropic model of bone, one would expect that the elastic modulus and hardness of bone would be highest in the load-bearing direction. In all cases, the elastic modulus data in the transverse plane were significantly higher than that in the longitudinal plane (calculation not shown), which agrees with the transversely isotropic model of bone. In addition, this study found the ratio of transverse to longitudinal elastic modulus (E_T/E_L) of carp rib bone to vary from 2.04 to 1.67. Without correcting for the effects of drying, this range correlates well with other studies (1.77 for wet whole human bone [23]), although the current study focuses on bone from a different species and is tested at a much more basic level of organization. The trend observed for the elastic modulus data in the longitudinal plane is similar to the trend observed for the phosphate data. Previously we have shown that there is indeed a significant positive correlation [24]. The absence of a similar distribution in the transverse plane implies that the phosphate concentration, rather than the calcium or molar Ca/P ratio, is a better predictor of microstructural mechanical property data. Finally, it is interesting to note that upon simple inspection, the carp elastic modulus anisotropy ratio appears to correlate well with the molar Ca/P ratio (and in fact it can be shown there is a mild correlation, with $p = 0.0679$).

This research illustrates that the concentration of osteocalcin is lower at the distal end, with a higher amount of phosphate and E_L, relative to the proximal end. This implies that the distal end is composed of less mature bone, and graphically reinforces previous findings [25], illustrating the distribution of osteocalcin, degree of mineralization, and elastic modulus along the length of carp rib bone.

Acknowledgments

Portions of this research were sponsored by the Whitaker Foundation, a junior faculty research fellowship from the Oak Ridge Institute for Science and Technology, and NIH grant #AR45297 (JYR). The AAS facilities at the Surgical Research Laboratories of The University of

Tennessee, Memphis was supported in part by grants to SKB from NIH (#R01-AR-38540) and the UT-CAN-DO Laboratory research fund. Analytical instrumentation for nanoindentation testing was provided by the Division of Materials Sciences, U.S. Department of Energy, under contract DE-AC05–96OR22464 with Lockheed Martin Energy Research Corp. and through the SHaRE Program under contract DE-AC05–76OR00033 between the U.S. Department of Energy and Oak Ridge Associated Universities.

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Received 7 February
and accepted 26 December 2000