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Matrix Concentration of Insulin-like Growth Factor I (IGF-I) is Negatively Associated with Biomechanical Properties of Human Tibial Cancellous Bone Within Individual Subjects

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Abstract. Insulin-like growth factor-I (IGF-I), abundant in bone matrix, is believed to play an important role during bone development and remodeling. To our knowledge, however, few studies have addressed the relationship between the concentration of IGF-I in bone matrix and the biomechanical properties of bone tissue. In this study, forty-five cylindrical specimens of cancellous bone were harvested from six human tibiae and scanned using microcomputed tomography (μCT). The bone volume fraction (BV/TV) was calculated from three-dimensional (3D) μCT images. Mechanical tests were then performed on a servohydraulic testing system to determine the strength and stiffness of cancellous bone. Following mechanical testing, the concentration of IGF-I in bone matrix was measured by using an enzyme-linked immunoabsorbent assay (ELISA). Within each subject, the concentration of IGF-I in bone matrix had significant ($P < 0.01$) negative correlations with the bone volume fraction, strength, and stiffness of cancellous bone. In particular, the anterior quadrant of the proximal tibia was significantly ($P < 0.02$) greater in IGF-I matrix concentration and marginally significantly lower in strength ($P = 0.053$) and stiffness ($P = 0.059$) than the posterior quadrant. The negative correlations between the cancellous bone matrix concentration of IGF-I and cancellous bone biomechanical properties within subjects found in this study may help us understand the variation of the biomechanical properties of cancellous bone in proximal human tibiae.

Key words: Insulin-like growth factor-I — Bone volume fraction — Strength — Tibia — Cancellous bone

Growth factors are believed to be important local regulators of osteoblast and osteoclast activity during bone development and remodeling [1, 2]. Particularly, insulin-like growth factors (e.g., IGF-I) are abundant in bone matrix [2]. An understanding of how these growth factors affect bone remodeling is important in determining the underlying causes of the universal age-related loss of bone mass that can cause osteoporosis, debilitating fracture, and death [3]. It has been demonstrated that the concentration of IGF-I in bone matrix from different sites of the human skeleton correlates significantly correlated with the rate of bone turnover [4]. Furthermore, studies on IGF-I knockout mice and transgenic mice with IGF-I overexpression also indicate that IGF-I plays an important role in the regulation of bone mass [5–7].

It is well known that the biomechanical properties of bone tissue are influenced by bone remodeling in which growth factors play an important role throughout one's lifetime [8]. For example, the variation of biomechanical properties of cancellous bone in proximal human tibiae has been attributed to bone remodeling in response to load of different compartments of human tibiae [9–14]. Therefore, growth factors (e.g., IGF-I) sequestered in bone matrix could play a role in the variation of biomechanical properties of cancellous bone in human tibiae. A few studies have addressed relationships between the growth factors sequestered in bone matrix and the biomechanical properties of bone tissue [4, 15–17]. In particular, the relationship between the matrix concentration of IGF-I and the bone volume fraction of cancellous bone has been studied by using human biopsies from the iliac crest [4]. However, the variation of IGF-I matrix concentration within a subject is not examined because of the limited number of samples from each subject. In the present study, we tested the hypothesis that the concentration of IGF-I sequestered in bone matrix was negatively associated with biomechanical properties of cancellous bone from proximal human tibiae within subjects.

Materials and Methods

Preparation of Cancellous Bone Specimens

Forty five cylindrical specimens of cancellous bone were obtained from the right tibiae of six male human cadavers that

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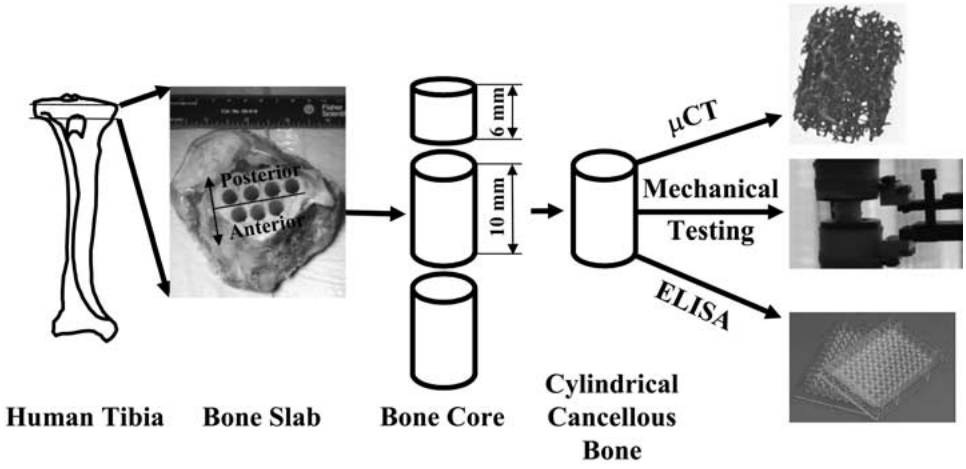

Fig. 1. Specimen preparation of cancellous bone and specimen flow.

were free of bone and joint disease (mean age, 48 ± 14 years; range, 26 to 63 years). The tibiae were stored at −25°C until specimen preparation. During the first cut of the proximal tibia, the subchondral bone plate was completely removed at the center of the condyles to expose cancellous bone using a bandsaw [13]. The second cut was made 35 mm more distal to produce a 35 mm-thick slab of cancellous bone (Fig. 1). The cancellous bone cores (diameter, 8 mm) were taken from the bone slab with a diamond-tipped coring tool (Starlite, Rosemont, PA, USA). A recent study on the bone structure in human knee specimens using microcomputed tomography (μCT) showed that statistically significant depth-dependent changes in the morphologic parameters were observed for the first 6 mm of the bone core from the proximal tibia, but not after the first 6 mm of the bone cylinder [18]. Therefore, the first 6 mm of the bone core was trimmed by using a slow-speed diamond saw (Model 660, South Bay Technology, Inc., Temple City, CA, USA) under constant water irrigation (Fig. 1). Subsequently, a cylindrical specimen of cancellous bone with a length of 10 mm was obtained immediately after the first 6 mm of the bone core (Fig. 1). A special fixture was designed to make sure that the end planes of the cylindrical specimen were parallel during the cutting [19]. Specimens were kept hydrated at all times and stored at −25°C in normal (0.9%) saline solution until use.

Determine Bone Volume Fraction (BV/TV)

The cylindrical specimens of cancellous bone were scanned with a 21-μm voxel size by microcomputed tomography (μCT, Henry Ford Hospital, Detroit, Michigan, USA) using previously developed techniques [20]. The specimens were kept wet during the scanning process. The three-dimensional (3D) μCT images of these specimens were segmented to distinguish bone tissue from marrow by using a global threshold (Fig. 1). The bone volume fraction (BV/TV), a representative of bone mass, of cancellous bone was calculated as the ratio of the number of voxels belonging to the bone to the total number of voxels [21].

Measurement of Strength and Stiffness from Mechanical Testing

Mechanical testing was performed on a servohydraulic testing system (Model 8501, Instron Corp, Canton, MA, USA) at room temperature with a 2000-N load cell and a 12 5-mm gauge length extensometer attached to the brass end caps close to the specimen (Fig. 1). Before mechanical testing, bone marrow was removed from the specimen by using a water jet. Previous studies showed that removal of bone marrow does not influence the mechanical properties of cancellous bone at a strain rate of 0.1%/s [22]. To eliminate the effect of end artifacts on measured stiffness of cancellous bone [23–25], the specimens were glued to brass end caps using a thin layer of cyanoacrylate glue [26, 27]. After preloading at a compressive force of 5 N while the cyanoacrylate glue cured for 2 minutes, the specimens were uniaxially compressed to fracture with a strain rate of 0.1%/s and unloaded to zero force with the same strain rate. The strength and stiffness of cancellous bone were determined from the stress–strain curve as the maximum stress supported by the sample and maximum slope of the linear region of the stress–strain curve, respectively [27].

Extraction of IGF-I from Bone Matrix

IGF-I was extracted from bone matrix by using methods similar to those previously published for bone [4, 17].

First, bone powder was obtained from cylindrical specimens of cancellous bone. After mechanical testing, the central part of cancellous bone was sectioned from the glued end caps by using the slow-speed diamond saw. The glued ends were not included in the extraction. Subsequently, each bone sample was immersed in liquid nitrogen and mechanically crushed into a fine powder by using a biopulverizer (BioSpec Products, Inc., Bartlesville, OK, USA).

The IGF-I extraction process was then conducted in a mini dialysis unit by removing bone mineral with ethylene diamine tetraacetic acid (EDTA) and extracting proteins from bone matrix by using Guanidine-HCl. After being weighed in an electronic balance the bone powder, ranging from 20 mg to 40 mg, was placed in a mini dialysis unit that had a regenerated cellulose membrane with 3.5 K Molecular Weight Cut-Off (Slide-A-Lyzer®, Pierce, Rockford, IL, USA). Next, 0.5 mL of extraction solution (4 M Guanidine-HCl, 0.05 M EDTA, 30 mM Tris and 1 mg/mL bovine serum albumin (BSA) at the pH value of 7.4) was added in the mini dialysis unit to decalcify bone tissue and extract proteins from bone matrix. In addition, protease inhibitors (1 mM phenylmethyl-sulfonyl fluoride, 5 mM benzamidine-HCl, 0.1 M E-aminocaproic-acid, 2 μg/mL leupeptin) were added to the extraction solution to prevent the degradation of proteins during the extraction. The mini dialysis tube was covered with a cap and placed in a float such that the unit's membrane was in contact with the extraction solution in a beaker (300 mL for 15 mini dialysis units). The extraction procedure was conducted at 4°C on a stir plate with a low-speed setting for 48 hours. Following the extraction process, the bone sample was re-dialyzed against phosphate-buffered saline (PBS) solution for 72 hours. Finally, the extract was stored at −20°C until assayed for growth factor activity.

Measurement of the Concentration of IGF-L

The matrix concentration of IGF-I was determined, by an enzyme-linked immunoabsorbent assay (ELISA) (R & D Systems, Minneapolis, MN, USA). In order to avoid the IGF-binding protein (IGFBP) artifacts, the extract was first pretreated to release IGF-I from binding proteins with an acidic dissociation solution. The second pretreatment solution (buffered protein with blue dye and preservatives) was added to prevent further association between IGF-I and IGFBP. Previous studies indicated that ELISA was not strongly susceptible to IGFBP effects [28, 29]. The assay method has been previously validated and shows good correlations with measurements obtained by radioimmunoassay after acid chromatography [29]. Duplicate assays were performed for each bone sample, and the results were averaged.

For the IGF-I immunoassay, as performed in our laboratory, the intra-assay coefficient of variation was 4.0%. The inter-assay coefficient of variation was 8.0%. The average recovery of IGF-I was 99%, with a range from 97% to 102%. The detection limit of the assay was 26 pg/mL.

Normalization of IGF-I Measurements

In previous studies, the concentration of IGF-I has been normalized against mineralized tissue or bone powder, extract residual bone matrix, hydroxyproline content of bone samples, or total protein content [4, 17, 30]. It has been demonstrated that the concentration normalized against mineralized bone tissue weight is highly correlated with the concentration normalized against residual bone matrix [4], hydroxyproline content [30], and total protein content [30].

In the present study, therefore, the concentration of IGF-I in bone matrix was expressed per gram of dry weight of bone powder. The normalized values of duplicate assays were then averaged.

Statistical Analysis

Because there were multiple specimens of cancellous bone from each subject (i.e., pseudoreplication), it could be misleading to perform statistical analyses by combining all cancellous bone specimens from several subjects and then calculating the correlation as if the data were a simple sample [31, 32]. The appropriate analysis depends on the research question.

Following the methods described by Bland and Altman [31, 32], statistical analyses (SYSTAT, Systat Software Inc., Point Richmond, CA, USA) were performed to assess the relationships between the concentration of IGF-I in bone matrix and the biomechanical properties of cancellous bone. The relationship was considered statistically significant at $P < 0.05$.

The first question tested was whether a change in the concentration of IGF-I in bone matrix was associated with a change in the biomechanical properties of human tibial cancellous bone within an individual subject. In order to remove the differences between subjects and examine only changes within subjects, multiple regression models with dummy variables (see Appendix, Equation 2) were used [32].

The second question tested was whether subjects with high average matrix concentration of IGF-I also tended to have high average values of biomechanical properties. Both Pearson and Spearman correlation coefficients were calculated for relationships between average IGF-I matrix concentration and average biomechanical properties of cancellous bone (see Appendix).

In addition, we wanted to determine whether there was any particular site in the proximal tibia that was greater in IGF-I matrix concentration and lower in strength and stiffness consistently across all the subjects. Therefore, the proximal tibia was divided into anterior and posterior sites (Fig. 1). The effect of site and subject on the matrix concentration of IGF-I and the biomechanical properties of cancellous bone in human tibiae was examined using a two-way analysis of variance (ANOVA).

Table 1. Relationships between the insulin-like growth factor-I (IGF-I) concentration in bone matrix and the biomechanical properties of cancellous bone within subjects

	BV/TV	Strength	Stiffness
IGF-I	$R^2 = 0.64$; $P = 0.005$[a]	$R^2 = 0.50$; $P = 0.001$[a]	$R^2 = 0.45$; $P = 0.001$[a]

[a] Significant relationship
R^2 is the coefficient determination for multiple regression models with dummy variables; and P is the significance level

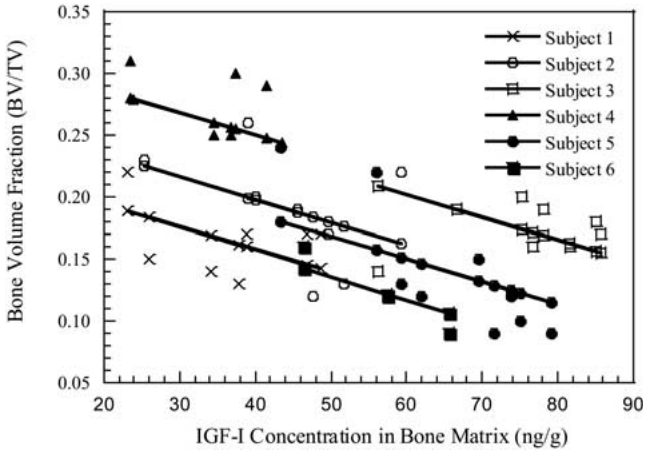

Fig. 2. The matrix concentration of insulin-like growth factor-I (IGF-I) versus the bone volume fraction of cancellous bone for six subjects, with parallel lines fitted for each subject: $BV/TV = 0.271 - 0.002\ \text{IGF-I} - 0.044\ D_1 - 0.040\ D_2 - 0.011\ D_3 - 0.000\ D_4 + 0.042\ D_5$.

Results

Significant negative correlations ($P = 0.005$) were observed between the concentration of IGF-I in bone matrix and the bone volume fraction of cancellous bone within subjects (Table 1, Fig. 2). A significant negative relationship ($P = 0.001$) was also observed between the strength of cancellous bone and the concentration of IGF-I in bone matrix (Table 1, Fig. 3). In addition, the stiffness of cancellous bone was significantly ($P = 0.001$) correlated with the concentration of IGF-I (Table 1, Fig. 4).

Between subjects, however, no significant correlation ($P > 0.1$) was found between the average matrix concentration of IGF-I and the average biomechanical properties of cancellous bone (Table 2).

The two-way ANOVA indicated that the IGF-I matrix concentration, strength, and stiffness of cancellous bone differed significantly between anterior and posterior sites in human tibiae (Table 3). However, the bone volume fraction of cancellous bone in human tibiae did

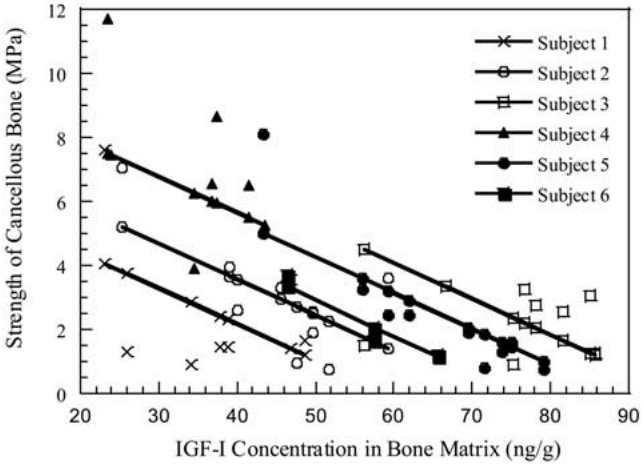

Fig. 3. The matrix concentration of IGF-I versus the strength of cancellous bone for six subjects, with parallel lines fitted for each subject: Strength = 9.001 – 0.112 IGF-I – 0.478 D_1 – 2.356 D_2 + 0.858 D_3 – 0.960 D_4 + 1.807 D_5.

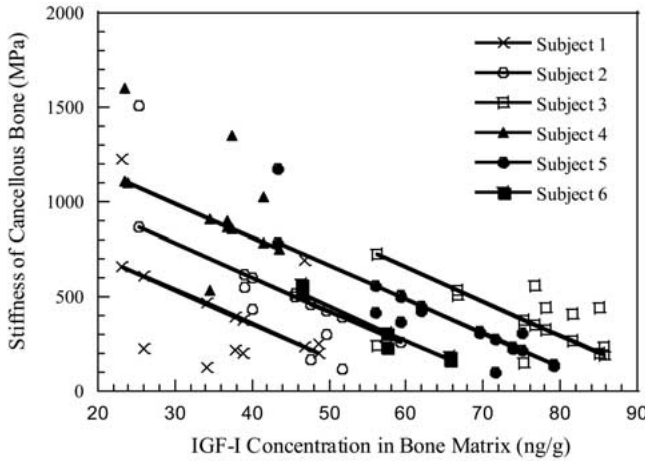

Fig. 4. The matrix concentration of IGF-I versus the stiffness of cancellous bone for six subjects, with parallel lines fitted for each subject: Stiffness = 1427.864 – 17.976 IGF-I – 80.898 D_1 – 352.843 D_2 + 135.505 D_3 – 108.572 D_4 + 304.092 D_5.

Table 2. Relationships between average insulin-like growth factor-I (IGF-I) concentration and average biomechanical properties between subjects

	BV/TV	Strength	Stiffness
IGF-I (Pearson)	$r = -0.57$; $P = 0.24$	$r = -0.61$; $P = 0.20$	$r = -0.65$; $P = 0.16$
IGF-I (Spearman)	$r_s = -0.54$; $P = 0.30$	$r_s = -0.71$; $P = 0.14$	$r_s = -0.77$; $P = 0.10$

r is the Pearson correlation coefficient; r_s is the Spearman rank correlation coefficient

not show significance difference ($P = 0.266$) between anterior and posterior sites. The IGF-I matrix concentration and biomechanical properties of cancellous bone were significantly different ($P < 0.03$) across all subjects (Table 3). The interaction of site and subject on the IGF-I matrix concentration and biomechanical properties of cancellous bone was not significant ($P > 0.43$).

From the plot of least square means for IGF-I matrix concentration, we observed that the anterior quadrant had a significantly higher IGF-I concentration than the posterior quadrant did (Fig. 5). Similarly, the strength and stiffness of the anterior quadrant were significantly lower than those of the posterior quadrant (Fig. 5).

Table 3. The effect of subject and site on the matrix concentration of insulin-like growth factor-I (IGF-I) and biomechanical properties of cancellous bone

	Subject	Site	Subject*Site
IGF-I	$P = 0.000$[a]	$P = 0.016$[a]	$P = 0.720$
BV/TV	$P = 0.000$[a]	$P = 0.266$	$P = 0.433$
Strength	$P = 0.006$[a]	$P = 0.053$[a]	$P = 0.941$
Stiffness	$P = 0.026$[a]	$P = 0.059$[a]	$P = 0.879$

[a] Significant relationship
P is the significance level

Discussion

A significant negative correlation between the concentration of IGF-I in bone matrix and the bone volume fraction of cancellous bone is consistent with a previous study on IGF-I deficient mice (gene knockout). Although smaller bones from the IGF-I-deficient mice were observed, the bone volume fraction of cancellous bone in the tibiae of the IGF-I-deficient mice was significantly greater than that of the wild type [6]. The increase in the bone volume fraction of proximal tibiae of the IGF-I-deficient mice was associated with a significant increase in connectivity and trabeculae number [6].

We have demonstrated that there is a statistically significant negative correlation of variation of biomechanical properties of cancellous bone in human tibiae with the variation of the concentration of IGF-I in bone matrix within individual subjects. Spatial variation of the biomechanical properties of cancellous bone in the proximal human tibiae has been well documented in the literature [9–13, 33–35]. Particularly, a recent study on the morphologic analysis of trabecular bone also demonstrates that there are strong within-individual associations in the relation between bone volume fraction and the measured morphologic parameters (e.g., trabecular

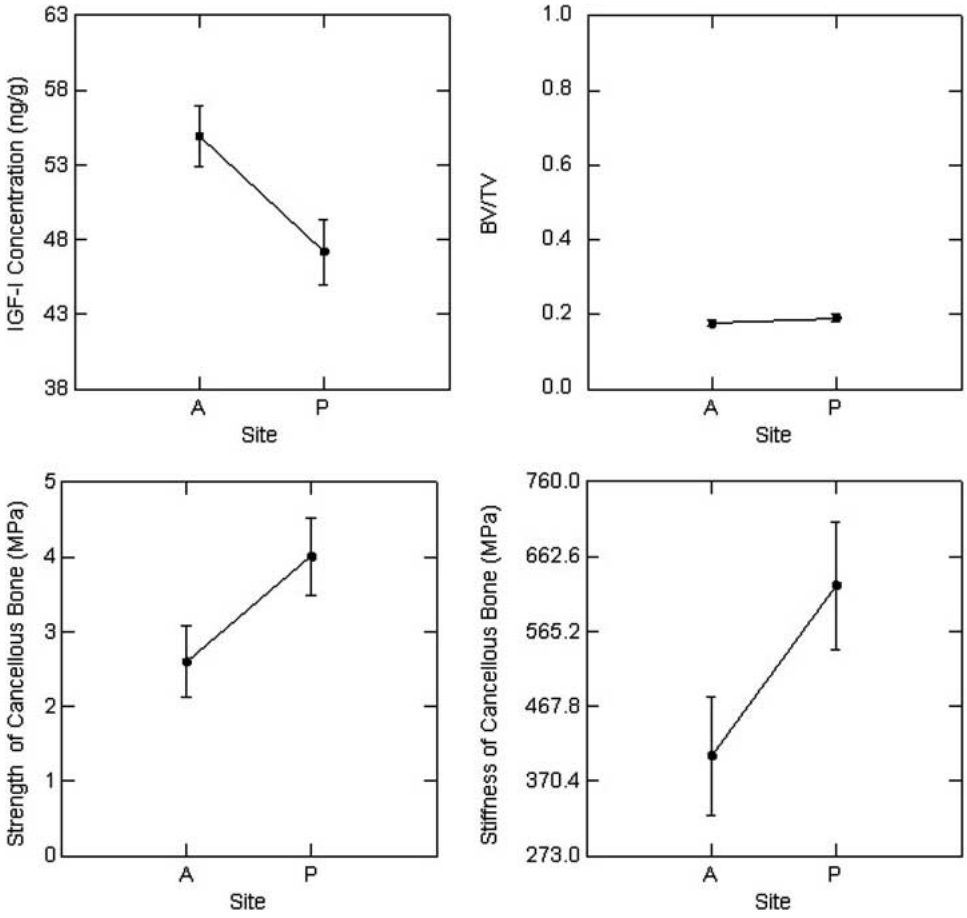

Fig. 5. Plot of least square means for IGF-I matrix concentration and biomechanical properties of cancellous bone. **A** is anterior quadrant; **P** is posterior quadrant.

number, trabecular thickness, and trabecular spacing) for each donor [36]. The authors attribute these within-individual associations to the within-individual remodeling strategies and loading histories. It has been well described in the literature that bone is adapted to its usage and is mechanically optimized [8]. Therefore, we speculate that the apparent optimization of mechanical properties to load might be related to the concentration of IGF-I in bone matrix.

Our results are in contrast with those of a previous study indicating a positive correlation between the matrix concentration of IGF-I and the bone volume fraction of cancellous bone from the iliac crest of human subjects [4]. However, this inconsistency may indicate that the impact of the local IGF-I components on the variability of human bone mass may differ markedly between various skeletal sites. For example, the same extraction method used in the previous study on iliac crest was also employed to examine the relationship between IGF-I concentration and bone mass of cortical bone from human femoral shaft [37]. The investigators found that there was no significant correlation between IGF-I concentration and cortical bone mass. In addition, a recent study examined the hypotheses that the effect of IGF-I on bone in men can be site-specific [38]. The serum IGF-I concentration and the bone mineral density of lumbar spine, hip, and distal forearm in 721 men were measured. In men aged 19–60 years, IGF-I was correlated with the bone mineral density of the hip. However, IGF-I did not correlate with the bone mineral density of the lumbar spine and distal forearm.

Lack of associations between the average matrix concentration of IGF-I and the average biomechanical properties of human tibial cancellous bone were observed between subjects. However, the between-subject analysis was not conclusive because the number of subjects ($n = 6$) in the present study was too small. To fully understand the between-subject relationship of average matrix IGF-I concentration and average biomechanical properties, more subjects should be included in a future study to address this issue.

There are some limitations to our current study. One limitation is that trabecular level hard-tissue properties and architecture were not assessed. The structural mechanical properties, which were measured in this study, are affected both by architecture and bone tissue material properties.

In addition, the present study could not differentiate IGF-I that was resident on the bone surface from that contained in the interior of bone matrix. Growth factors may exist in various compartments of bone tissue. A sequential extraction of mineralized bone matrix was

employed in a previous study that demonstrated that about 25% of total growth factors was associated with bone cells and unmineralized bone matrix (osteoid); 15% was associated with mineral phase; and 60% was associated with collagenous matrix [39]. However, the extraction method employed in the present study on the proximal tibia and the previous study on iliac crest did not differentiate the growth factors from bone cells or osteoid, bone mineral, and bone matrix. In particular, the previous study on iliac crest used biopsies from patients while the present study used the specimens from cadavers. We speculate that growth factors from bone cells and osteoid is the leading role in the previous study on iliac crest while growth factors from bone mineral and bone matrix in the present study on the proximal tibia is the dominating feature. It is possible that the inconsistency between our results on the proximal tibia and the previous study on iliac crest comes from the differences of growth factors in different compartments of bone tissue.

In conclusion, our study has assessed the relationship between the matrix concentration of IGF-I and the biomechanical properties using cancellous bone from human tibiae. Despite a failure to find an association of average IGF-I concentration and average biomechanical properties between subjects, our results conclusively demonstrate the negative association of local matrix concentrations of growth factors with biomechanical properties within an individual.

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Appendix

A.1 Correlation coefficients with repeated observations

Because there were multiple specimens of cancellous bone from each subject (i.e., pseudoreplication), it could be misleading to perform statistical analyses by combining all cancellous bone specimens from several subjects and then calculating the correlation as if the data were a simple sample. Although forty-five cylindrical specimens of cancellous bone were harvested, there were only six actual independent observations, which was equal to the number of subjects used in the present study. This is similar to the problem of multiple physiological measurements from individual patients as discussed by Altman and Bland [40].

A.2 Correlation within subjects

It is sometimes useful to introduce additional variables into a regression model, to account for the effects of nominal scale variables (i.e., categorical variables such as subject) on the response variable [41].

For example, we might be considering fitting the model

$$\mathrm{BV/TV} = \mathrm{a} + \mathrm{b\ IGF - I} \tag{1}$$

where BV/TV is the bone volume fraction of cancellous bone, IGF-I is the concentration of IGF-I in bone matrix, a is the intercept, and b is the slope. However, this model does not take account of the variability between subjects.

Therefore, we might be interested in determining the effect of subject variations on BV/TV. There are six levels of the nominal scale variable (i.e., six subjects). Therefore, five dummies are required for the regression model [41–43]. Our regression model could then be expanded to

$$\begin{aligned}\mathrm{BV/TV} =& \mathrm{a} + \mathrm{bIGF - I} + \mathrm{d_1D_1} + \mathrm{d_2D_2} + \mathrm{d_3D_3} \\ &+ \mathrm{d_4D_4} + \mathrm{d_5D_5}\end{aligned} \tag{2}$$

where D_1, D_2, D_3, D_4, and D_5 are dummy variables that account for average inter-individual variation. The five dummy variables can be used to represent six levels of a categorical variable (subject). The use of these dummy variables accounts for pseudoreplication and will yield significantly more accurate predictions of the dependent variable than will the preceding model without dummy variables (Equation 1). The method is also known as analysis of covariance and is equivalent to fitting parallel lines through each subject's data [43].

We can make use of the analysis of variance (ANOVA) table associated with the multiple regression model [32]. The residual sum of squares in the table represents the variations about those lines fitted to each subject. We remove the variation due to the subjects and express the variation in the bone volume fraction to the matrix concentration of IGF-I as a proportion of what remains (Sum of squares for IGF-I)/(Sum of square for IGF-I + Residual sum of squares). This proportion is also called the coefficient of determination (R^2) for a multiple regression. The P value is calculated from the F test in the associated analysis of variance table.

A.3 Correlation between subjects

If we want to know whether subjects with high average values of IGF-I concentration also tend to have high average values of biomechanical properties, we can use the correlation between the subject means of the independent and dependent variables. We can calculate the mean values of IGF-I concentration and the biomechanical properties for each of six subjects. The 45 pairs of measurements from which these means were calculated were given in the Figures 2–4. Here we are interested in whether the average IGF-I concentration for a

subject is related to the subject's average biomechanical properties.

We can calculate the Pearson correlation coefficient and the Spearman correlation coefficient for the mean concentration of IGF-I in bone matrix and the average biomechanical properties by using simple linear correlation and rank correlation analysis, respectively [41].

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