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Bone Vol. 30, No. 5
May 2002:733–737

The Effect of Growth/Differentiation Factor-5 Deficiency on Femoral Composition and Mechanical Behavior in Mice

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A subclass of the bone morphogenetic proteins (BMPs), known as growth/differentiation factors (GDFs) 5, 6, and 7, have been shown to affect several skeletal processes, including endochondral ossification, synovial joint formation, and tendon and ligament repair. Mice deficient in GDF-5 have also been shown to exhibit biomechanical abnormalities in tendon that may be associated with altered type I collagen. The purpose of this study was to investigate the effect of GDF-5 deficiency on another type I collagen-rich tissue: cortical bone. Analyses were performed on femora from 8-week-old GDF-5-deficient male brachypodism mice. We hypothesized that GDF-5-deficient bones would exhibit altered geometric, structural, and material properties compared with control littermates. Mutant animals were significantly smaller in body mass than controls (−21%). Geometrically, mutant long bones were significantly shorter (−25%), had a lower polar moment of inertia (−34%), and a lower geometric strength indicator (analogous to the section modulus of a circular section) (−30%). When normalized by body mass, however, geometric differences were no longer significant. Structurally, GDF-5-deficient femora were weaker (−31%) and more compliant (−57%) than controls when tested to failure in torsion. Lower bone structural stiffness in the mutants was not completely explained by the smaller bone geometry, because mutant bones exhibited a significantly lower effective shear modulus (−36%). Although body mass did not fully explain the reduced structural strength in mutant bones, strength differences were adequately explained by bone cross-sectional geometry; maximum effective shear stress was not significantly different between mutants and controls, despite a statistically significant 6% lower ash fraction in mutant femora. No significant difference was detected in collagen content, as indicated by hydroxyproline per dry mass. (Bone 30:733–737; 2002)

Key Words: Bone mechanics; Growth/differentiation factor (GDF); GDF-5; Bone morphogenetic protein (BMP); BMP-14; Mouse; Bone strength.

Introduction

The bone morphogenetic proteins, or BMPs, are intrinsic signaling molecules that have been evolutionarily conserved for over 500 million years.[13] A subclass of the BMPs, known as growth/differentiation factors 5, 6, and 7 (GDF-5, -6, and -7), have been shown to play a role in a variety of skeletal tissues and processes, including joint formation,[12,16,22] endochondral ossification,[25] and tendon and ligament maintenance and repair.[1,7,8,17,27] The discovery of a naturally occurring GDF-5-deficient mouse, *brachypodism* (or *bp*),[22] presents the unique opportunity to investigate the role of this particular GDF family member in the skeleton by performing functional assays at the whole organ, tissue, and molecular levels.

We have recently demonstrated that GDF-5 deficiency affects the composition, ultrastructure, and mechanical behavior of murine Achilles tendon.[17] Mutant Achilles tendons from 8-week-old male mice were weaker and more compliant than those of controls, and they exhibited a significant reduction in collagen content, as indicated by hydroxyproline per microgram of DNA (Hypro/DNA). Mutant Achilles also exhibited a trend toward smaller collagen fibrils, with a qualitatively greater number of irregularly shaped, polymorphic fibrils compared with controls. No differences in glycosaminoglycan (GAG/DNA) were detected.

The effects of GDF-5 deficiency were seen in other tendinous sites as well (again, in 8-week-old male mice). Tail tendon from mutant animals demonstrated increased fibril polymorphism, which was associated with altered time-dependent viscoelastic mechanical behavior, but no differences in quasistatic mechanical properties were detected.[8] In contrast to the Achilles site where significant differences in composition were detected, no differences in GAG/DNA, Hypro/DNA, decorin, lumican, or fibromodulin were found in GDF-5-deficient tail tendons.

Together, these studies suggest that GDF-5 deficiency in mice results in biomechanical abnormalities in two distinct tendinous sites that may be associated with altered type I collagen. Because cortical bone is also rich in type I collagen, the purpose of the present study was to investigate the effect of murine GDF-5 deficiency on long bone composition and mechanical behavior. Analyses were performed on femora from 8-week-old GDF-5-deficient male *brachypodism* mice. We hypothesized that GDF-5-deficient long bones would exhibit compromised structural behavior as a result of altered material properties when compared with control littermates.

Methods

Experimental Design

The experimental model used in this study was the *brachypodism* mouse (obtained from the Jackson Laboratory, Bar Harbor, ME), which is known to have a homozygous mutation (−/−) in the

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8756-3282/02/$22.00
PII S8756-3282(02)00699-3

gene coding for GDF-5.[22] In this mouse model, a frameshift mutation leads to a translational stop codon before the mature signaling portion of GDF-5 is formed, thus resulting in a functional null mutation. Mutant animals are clearly identifiable at birth based on the small size of their paws. Control animals consisted of phenotypically normal littermates. Note that the controls were not genotyped and thus represent a combination of wild-type (+/+) and heterozygous (+/−) littermates. Eight-week-old male mice were used to eliminate potentially confounding effects of gender, and animals were killed by CO_2 inhalation in accordance with IACUC guidelines. After recording body weights, the femora from six mutant and eight control mice were isolated and bone length recorded. The bones from these animals were used to determine cross-sectional geometric properties as well as for mechanical testing. A second cohort of six mutant and six control mice of the same age and gender were used to examine femoral cortical bone composition. For all geometric, structural, and compositional analyses, genotype was considered the sole independent variable.

Cross-sectional Geometry

Cross-sectional measurements were made from a transverse section at the middiaphysis of the left femur from mutant and control mice using the third trochanter as an anatomical marker.[18] The middle third of the bone was embedded in polyester resin and sectioned on a circular diamond-blade saw (Model 11-1180 Low Speed Saw, Buehler, Ltd., Evanston, IL). One-hundred-micron-thick sections were obtained and stained using a von Kossa stain. The sections were digitally imaged using a transmission light microscope (Nikon Eclipse E600, Nikon USA, Melville, NY) with a digital camera (Sony DKC 5000, Sony Corp. of America, Park Ridge, NJ) and then analyzed using the program NIH IMAGE (W. Rasbad, National Institutes of Health, Bethesda, MD) to determine cortical bone area (A), medullary area (A_{med}), principal (maximum and minimum) bending moments of inertia (I_1, I_2), and polar moment of inertia (J). In addition, both the torsional constant (K), as well as the normalized geometric maximum shear stress (τ_{norm}), were determined numerically using the FORTRAN program VA-TWIST.[14] (Note that τ_{norm} is purely a geometric property of the cross section being analyzed.) From this parameter, a geometric strength index, S_{cs}, was calculated as $1/(\tau_{norm})$. The geometric strength indicator can be conceptualized as being analogous to the section modulus of a circular cross section. In bones with comparable tissue material strength, S_{cs} would be proportional to the torsional moment to failure and is indicative of the geometric contribution to the strength of the cross section.

Structural Properties

Whole bone torsion tests to failure were performed on the right femur from the same mice used for geometric analysis. Bones were kept moist during dissection and testing with phosphate-buffered saline. Proximal and distal bone ends were potted in polymethylmethacrylate (GC America, Inc., Alsip, IL) and the gage length was measured. All tests were performed under angular displacement control at a rate of 1°/sec using a servo-hydraulic materials-testing system (Bionix Model 810, MTS Corp., Minneapolis, MN) equipped with a 20 in-oz. torque transducer (Model QWFK-8M, Sensotec Corp., Columbus, OH). Testing direction corresponded to internal rotation of the distal end of the long bone with respect to the proximal end. Torque and angular displacement were recorded throughout the test at a rate of 20 Hz. From these data, a plot of torsional moment vs. angle per gage length (i.e., twist) was constructed. The following parameters were calculated: (1) maximum torque (T_{max}); (2) twist, or angle to failure (in radians) normalized by gage length (θ_{fail}/GL); (3) effective torsional rigidity (defined as the slope of the linear portion of the moment-twist curve); and (4) energy to failure.[18]

Effective Material Properties and Normalized Strengths

In addition to the structural properties measured during torsional testing, two effective material properties were also calculated: (1) maximum effective shear stress, τ_{max}; and (2) effective shear modulus, G. These effective material properties were calculated directly from the cross sections without assuming a specific geometry. The effective shear modulus was calculated from the linear region of the torque vs. twist curve by dividing the torsional rigidity (slope) by the torsional constant, K. Maximum effective shear stress was calculated as T_{max}/S_{cs}.[14,19] To determine whether geometric and structural strengths were appropriate for body size, body mass (BM) was used to normalize the geometric strength index (S_{cs}/BM) and maximum torque to failure (T_{max}/BM).

Ash Content

One femur per animal from an additional cohort of six mutant and six control mice was processed for measurement of ash fraction. Dry mass was obtained by defatting bones in acetone for 72 h, drying in air for 24 h, and 6 h at 60°C.[18] Ash weight was determined after 18 h at 600°C, and ash content was calculated as 100 × (ash mass/dry mass).

Hydroxyproline Content

To determine collagen content, as indicated by hydroxyproline (Hypro), cortical bone samples from the remaining femora of the second cohort of mutant and control mice (n = 6/group) were cleaned of marrow and soft tissue, defatted in acetone for 72 h, air-dried for 24 h, and dry weight recorded. After acid hydrolysis in 6N HCl for 18 h at 110°C, the dimethylaminobenzaldehyde (DMBA) colorimetric assay was performed to determine Hypro content.[9] All samples and standards were processed in duplicate and average values were used for analysis. Hypro measures were expressed as a percentage of dry mass.

Statistical Analysis

All geometric, structural, material, and compositional parameters were analyzed statistically using a one-factor analysis of variance (ANOVA) with genotype (mutant vs. control) as the independent variable. Statistical significance was set at $p < 0.05$.

Results

GDF-5-deficient mice were significantly smaller than controls, exhibiting a 21% lower body mass as well as shorter femora (−25%) ($p < 0.05$; **Table 1**).

Table 1. Morphometric parameters from control and *brachypod* mice [mean (SD)]

Parameter	Control (n = 8)	GDF-5 (−/−) (n = 6)	Difference between genotypes (%)
Body mass (g)	23.2 (2.1)	18.4 (1.6)	−21%[a]
Femur length (mm)	14.2 (0.32)	10.7 (0.32)	−25%[a]

[a] $p < 0.05$.

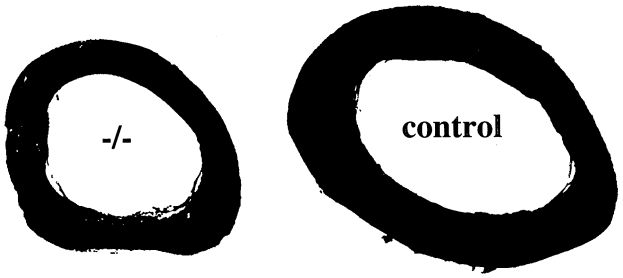

Figure 1. Representative femoral cross sections taken immediately distal to the third trochanter from GDF-5-deficient (left) and control (right) mice. Both sections are shown at the same magnification.

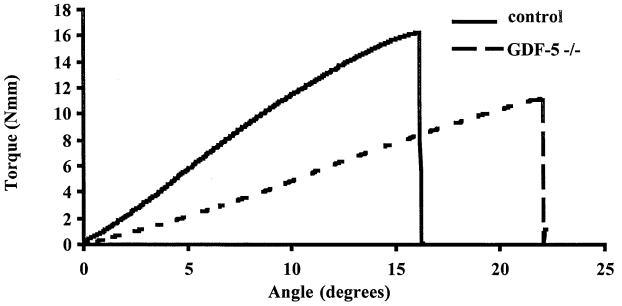

Figure 2. Representative torque vs. angle mechanical testing curves for control (solid line) and GDF-5-deficient (dashed line) femora.

Cross-sectional Geometry

Compared with control bones, GDF-5-deficient femora had significantly lower values of all cross-sectional geometric parameters that were measured, including medullary area, cortical area, polar moment of inertia, minimum and maximum bending moments of inertia, torsional constant, and geometric strength index (−17% to −38%, $p < 0.05$; **Figure 1** and **Table 2**). When the geometric strength index was normalized by body mass, no significant difference was found between mutants and controls (Table 2). Thus, although mutant femora were significantly smaller than control femora, these geometric differences appear to be appropriate for animal body size.

Structural Properties

GDF-5-deficient femora withstood significantly lower torque to failure during torsion tests than did controls (−31%, $p < 0.0001$; **Table 3** and **Figure 2**). These differences in strength were not explained by animal body mass, because body mass normalized torque to failure was significantly lower in mutants compared with controls. In addition to being structurally weaker, mutant bones were also more compliant; effective torsional rigidity was 57% lower in mutant femora ($p < 0.01$; Table 3), and twist to failure (angle/gage length) was significantly higher (+53%). Energy to failure was not significantly different, however.

Effective Material Properties

Two effective material properties, shear modulus and maximum shear stress, were calculated using the combined geometric and mechanical testing data. Mutants exhibited a statistically significant lower effective shear modulus (−36%, $p < 0.01$; Table 3), although no significant difference was detected in maximum effective shear stress.

Tissue Composition

Mean ash fraction (as a percentage of control ash) was 6% lower in mutant femora ($p < 0.05$; **Table 4**). By contrast, no significant difference in collagen content was detected (as indicated by Hypro/dry weight).

Discussion

In this study, we demonstrated that GDF-5 deficiency in 8-week-old male mice results in significantly altered femoral cortical bone composition and geometric, structural, and material properties when compared with bones of control littermates. Mutant animals were significantly smaller than controls, with correspondingly lower values of all bone size indicators, including femoral length and cross-sectional geometric parameters. In addition to being smaller, GDF-5-deficient bones were also structurally weaker and less stiff than control bones. Although GDF-5-deficient femora were significantly weaker in torsional tests to failure, differences in structural strength were due to smaller bone cross-sectional geometry, as no significant difference in the material property of maximum effective shear stress was detected. By contrast, smaller bone size was not sufficient to explain the greater compliance of mutant bones (significantly lower structural stiffness, and higher twist [angle/gage length] to failure), because the material property of effective shear modulus was significantly lower compared with that of control bones.

In the present study, GDF-5-deficient femora exhibited a statistically significant reduction in ash fraction, but no significant difference in collagen content, as indicated by hydroxyproline per dry mass. When expressed as a percentage of wet weight, there was still no significant difference in hydroxyproline content, nor was water content significantly different between mutants and controls (data not shown).

Although the reduction in ash fraction could have contributed

Table 2. Cross-sectional properties of femora from control and *brachypod* mice [mean (SD)]

Parameter	Control (n = 8)	GDF-5 (−/−) (n = 6)	Difference between genotypes (%)
Medullary area (mm^2)	0.594 (0.060)	0.492 (0.092)	−17%[a]
Cortical bone area (mm^2)	0.706 (0.067)	0.573 (0.037)	−19%[a]
I_1 (mm^4)	0.148 (0.029)	0.092 (0.015)	−38%[a]
I_2 (mm^4)	0.077 (0.013)	0.056 (0.009)	−27%[a]
Polar moment of inertia J (mm^4)	0.225 (0.040)	0.148 (0.023)	−34%[a]
Torsional constant K (mm^4)	0.195 (0.034)	0.131 (0.020)	−33%[a]
Geometric strength index S_{cs} (mm^3)	0.228 (0.028)	0.159 (0.037)	−30%[a]
S_{cs}/body mass (mm^3/g)	9.83E-3 (4.49E-3)	8.69E-3 (2.18E-3)	−12%

[a] $p < 0.05$.

Table 3. Structural and material properties of femora from control and *brachypod* mice [mean (SD)]

Parameter	Control (n = 8)	GDF-5 (−/−) (n = 6)	Difference between genotypes (%)
Maximum torque T_{max} (N mm)	16.7 (1.6)	11.6 (1.4)	−31%[a]
T_{max}/body mass (N mm/g)	0.720 (0.030)	0.634 (0.087)	−12%[a]
Angle to failure/gage length (rad/mm)	0.043 (0.01)	0.066 (0.01)	+53%[a]
Effective torsional rigidity (N mm^2/rad)	464 (130)	201 (39)	−57%[a]
Energy to failure (N mm · rad/mm)	0.370 (0.10)	0.416 (0.12)	+12%
Effective shear modulus G (MPa)	2399 (659)	1543 (219)	−36%[a]
Maximum effective shear stress τ_{max} (MPa)	73 (3)	76 (20)	+4%

[a] $p < 0.05$.

to the lower effective shear modulus in mutant femora, it is surprising that the smaller ash fraction was not also associated with a reduction in the material strength of the tissue, given the extensive literature data supporting the relationship between bone mineral and bone strength.[3,10,11,15] For example, based on Currey's work,[10] one would expect that a reduction in ash of 4% (absolute) would be associated with a reduction in material strength of approximately 20%. Although Currey's data on the relationship between ash fraction and breaking stress were obtained from three point bending tests, and were based on wild-shot rabbits with ash contents greater than those of the mice used in the present study, his results would lead one to expect a significant reduction in maximum effective shear stress in GDF-5-deficient femora. Thus, it seems likely that other compositional and/or microstructural changes could be masking the effects of decreased ash fraction in the mutant bone tissue. For example, changes in collagen orientation (without changes in collagen content) could contribute to the maintenance of bone strength despite a lower ash fraction.[20] Alternatively, some other noncollagenous organic component of the bone matrix could be elevated in GDF-5-deficient mice, thereby masking the effects of changes in mineral content.[2] It is important to acknowledge that, as with most studies of murine bones, the effective material properties of shear modulus and shear strength are derived rather than directly measured quantities. Although not likely, it is possible that this could contribute to the somewhat inconsistent results obtained regarding ash fraction, effective shear modulus, and effective shear strength. It is also important to note that the results of this study were obtained from a single timepoint (8 weeks) and may not be indicative of bone properties from older or younger mice.

The lower ash content observed in *bp* femora raises the intriguing question of why GDF-5 deficiency would lead to a reduction in bone mineral. We have previously shown that GDF-5-deficient tendon exhibits abnormalities in type I collagen.[8,17] Achilles tendons from *bp* mice contain less collagen per microgram of DNA than do control tendons, and GDF-5-deficient tail tendons exhibit a marked irregularity in collagen fibril morphology. Although a detailed analysis of type I collagen in GDF-5-deficient bone has not yet been performed (beyond bulk content measures), it is possible that the observed reduction in mineral content may be associated with some abnormality in the type I collagen found in the long bones of these animals. Indeed, such a mechanistic link is not without precedent; based on the extensive literature on osteogenesis imperfecta (OI),[5,6,21,24,26,28] it has been proposed that a disruption in the collagen of the organic matrix of bone can provide an abnormal template for mineralization, thereby leading to altered mineral properties as well as mechanical properties of bone tissue.[4] Thus, to fully explain the reduction in mineral content and effective material properties (shear modulus) observed in the present study, it is clear that a more detailed characterization of matrix constituents in GDF-5-deficient long bones is required.

Table 4. Femoral composition from control and *brachypod* mice [mean (SD)]

Parameter	Control (n = 6)	GDF-5 (−/−) (n = 6)	Difference between genotypes (%)
Femoral ash content (%)	57.0 (3.0)	53.8 (1.9)	−6%[a]
Hydroxyproline/dry mass (%)	1.71 (0.19)	1.65 (0.20)	−4%

[a] $p < 0.05$.

In conclusion, this study has demonstrated that GDF-5 deficiency in mice results in altered long bone geometry, composition, material properties, and structural behavior. Mutant femora were structurally weaker than control bones, but bone strength was adequate for bone size. In addition, mutant femora were more compliant than controls, although the reduction in stiffness was due to both reduced material properties (effective shear modulus) as well as smaller bone cross-sectional geometric properties. The altered material properties observed in this study may have been due, at least in part, to a small but statistically significant reduction in ash fraction in mutant femora. These data suggest that GDF-5 deficiency affects the composition and material properties of cortical bone tissue in the femur, but the detailed mechanisms by which this occurs remain to be determined.

Acknowledgments: The authors thank Jane Maxwell and Michael Fisher for their technical assistance. This work was funded in part by grants from the NIH (AR45828 and AR47097) to B.M.

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Date Received: August 29, 2001
Date Revised: January 9, 2002
Date Accepted: January 24, 2002