Physical and Mechanical Characteristics of Tibias from Transgenic Mice Expressing Mutant Bovine Growth Hormone Genes

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Physical and mechanical characteristics of tibia from mice expressing either the M4, M11, or G119K mutant bovine growth hormone (bGH) gene and displaying large, near-normal, or small-size phenotypes, respectively, were compared to those of nontransgenic, control mice (NTC). Three animals of each strain were euthanized at 28, 38, 48, 58, and 68 days of age. Variables were regressed against age to establish the pattern of change throughout the experiment, and the regression results are presented. Tibias from G119K were shorter (13.1 mm) and lighter (37.3 mg) than those from other strains, and M4 tibias were heavier (87.9 mg) and longer (16.6 mm) at 70 days of age. The ratio of tibia length to body weight suggests longitudinal bone growth was not reduced as much as overall growth in G119K mice. The external and internal dimensions of the G119K tibias were smaller than the other strains whereas the M4 tibias were somewhat larger. Differences in physical dimensions between the NTC and M11 mice did not greatly affect bone mechanical characteristics. Tibias from M4 mice resisted more load at both flexure and breaking compared to the other strains. At 50 days of age, stress at flexure was greater at all ages for G119K mice (12.4 kg/mm^2) and was decreased in M4 mice (8.5 kg/mm^2). The bGH mutations produce different effects on bone growth and its mechanical characteristics. There also may be differential tissue responsiveness to the mutant bGH analogs, as longitudinal growth was not as affected as empty body growth in the G119K mice. These transgenic mouse strains provide valuable models to study bone growth, formation, and reformation in response to GH regulation, and more importantly, the M4 and G119K mice may serve as a model in which the priorities for GH action on bone vs muscle may be determined.

[E.B.M. 2001, Vol 226:133–139]

Key words: transgenic; mice; bovine growth hormone; mutation; bone

Supported in part by USDA Hatch funding and the Texas Agricultural Experiment Station to N.D. and F.M.B. J.J.K. received support for this research from a grant from Sensus Corp and the State of Ohio's Eminent Scholar Program, which includes a grant by Milton and Lawrence Goll.

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Received October 12, 1999.
Accepted September 15, 2000.

0037-9727/01/2262-0000$15.00/0

Many of the existing swine and ovine GH transgenic animals have increased long bone growth, but these animals also display extensive joint pathologies and thinner metacarpal cortices that ultimately result in lameness (1, 2). If the potential of GH transgenic animals is ever to be realized, it will be necessary to develop animals capable of supporting increased rates of soft tissue growth by achieving the necessary level of bone growth without developing abnormal structural characteristics or increasing the relative quantity of bone in lean retail product (3).

One of the potential experimental paradigms to study the effects of GH on bone development and structural and mechanical characteristics is to use transgenic animals expressing GH agonists or antagonists. Chen *et al.* (4) have developed several strains that carry and express a single or double-point mutated bGH gene which are phenotypically larger than, nearly similar to, or smaller than their nontransgenic siblings (5). Therefore, this research was conducted using these transgenic mice to determine the effects of the three specific bGH gene mutations on bone growth, development, and mechanical characteristics.

Materials and Methods

Three strains of bGH transgenic male mice displaying large (M4), near-normal (M11), or small (G119K) body size phenotypes were used in this experiment (5). The M4 mice synthesize a bGH analog in which glutamate at position 117 is replaced by leucine (6). Transgenic mice expressing the M11 bGH gene produce bGH containing two mutations; proline is substituted for leucine at position 121 and glutamate at position 126 is replaced by glycine (4). The G119K mice produce bGH with lysine substituted for glycine at position 119 (6). The transgenic and non-transgenic control (NTC) mice were generated by breeding a single transgenic male mouse exhibiting the specific mutation with nontransgenic C57BL/6 female mice. Because a single transgenic male was used for breeding of each line, the number

of insertions was uniform for all offspring carrying the mutated genes. Genotype was confirmed using slot blot analysis or Southern hybridization of genomic DNA from tail clippings (5).

The mutated bGH genes are linked to the mouse metallothionein promoter and are expressed primarily in the liver and small intestine (6). This produces elevated basal expression without activation by an exogenous heavy-metal inducer. Thus elevated levels of zinc were not required in the diet or drinking water to activate the gene. This becomes very important for studying bone characteristics, as high levels of zinc are detrimental to bone formation (7). Relatively constant levels of the bGH analog and IGF-I are maintained in the transgenic mice (5).

The mice were housed in a temperature- and light-controlled room using standard laboratory-approved procedures (under an Ohio University AUP); 25°C and a 14:10-hr light-dark cycle. Individually housed mice had *ad libitum* access to water and Purina Mouse Chow® No. 5020 after weaning at 28 days of age (5). Non-transgenic controls were from the same litters as the transgenic mice. The mice were randomly assigned to be sacrificed at 28, 38, 48, 58, or 68 days of age. At the appropriate age, animals were decapitated, and the eviscerated bodies were frozen. Both tibias were removed, cleaned of adherent material, measured for length, dorso-ventral (D/V) and latero-medial (L/M) widths, and weighed.

Tibias taken from other mice not on the study were used to establish the testing conditions on an Instron Universal Testing Machine. The conditions used were as follows: 5 kg maximum load, 10 mm/min crosshead speed, and a 20:1 strip chart to crosshead speed recording ratio. Output from the test included the load and deformation measured at flexure and at breaking. Calculations of stress, strain and modulus of elasticity were performed using the procedures of Crenshaw *et al.* (8).

Data were initially subjected to allometric analysis in which data were transformed logarithmically and fitted to a linear equation ($\log Y = \log a + b \log X$) using SAS (9). Allometric analysis would facilitate comparisons of transgene effects on allometric growth coefficients as well as comparisons of various bone measurements at equal body mass endpoints. However, results from the allometric analyses were not able to adequately describe the results obtained from these very different transgenic mouse lines and, thus, are not presented. One reason behind our lack of success was that the G119K and M4 mice were still growing and had not completely matured by the end of the experiment, as suggested by the continued increase in body weight in these groups (10). It is critical that stage of maturity be known if the results of allometric analyses are to be interpretable and meaningful inferences are to be drawn concerning the effects of physiological state or genotype (11). Even though the G119K and M4 mice were still growing at the end of the experiment, body weight of the G119K mice did not overlap body weights from the other groups at any age (10). The combination of not reaching maturity, not having overlapping body weights, and the relatively short time period over which the experiment was conducted prevented us from being able to generate exponents capable of distinguishing between the effect of body weight and transgene status on tibia growth and mechanical characteristics. Further studies will need to be conducted in order to acquire the data necessary for this analysis to be successful.

In addition to allometric analyses, standard regressions using the main effects of transgene line and age were used in the model along with an interaction of the two main effects. Growth was analyzed using a continuous quadratic effect of age and its interaction with transgene status. Results from the regression models are presented in figures along with the variability in the predicted values ($S\hat{y}$) calculated from the model mean squares error so differences among genetic lines can be determined at any age throughout the experimental time line.

Results

Physical Characteristics. Tibia weight and length paralleled changes in body weight of these mice (10). In general, the NTC and M11 strains had similar gross external tibia characteristics until 60 days of age when the M11 mice began exhibiting heavier ($P < 0.05$) and longer ($P < 0.05$) tibias than the NTC mice (Fig. 1). However, tibias from G119K mice were not only lighter and shorter ($P < 0.05$)

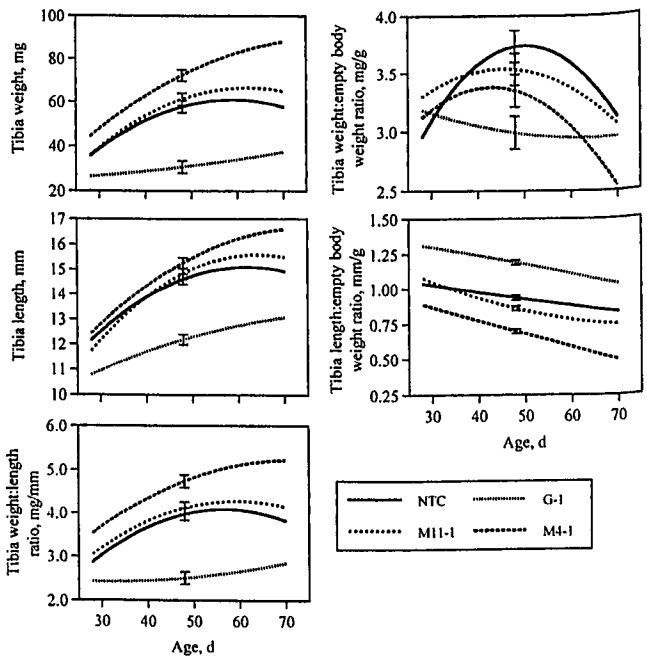

Figure 1. Weight, length, and relationships of tibia to empty body weight of 28- to 68-day-old mice expressing one of three mutant bGH genes (G-119K, M11, M4) or non-transgenic control mice (NTC). Values were calculated from regression equations which included age × transgene and age^2 × transgene interactions. The number of observations was 15 for NTC and G119K, and 12 for M11 and M4 mice. The $S\hat{y}$ = 2.7, 0.21, 0.14, 0.14, and 0.017 for tibia weight, length, weight:length ratio, weight:empty body weight ratio, and length:empty body weight ratio, respectively.

but also exhibited a different pattern of growth than the NTC (Fig. 1). Increases in G119K tibia length and weight were approximately linear throughout the experiment, whereas the curves for both the NTC and M11 mice were curvilinear and reached a plateau around 55 days of age. The M4 mice had tibias that weighed more ($P < 0.05$) than tibias of the other strains, even though tibia length was not different ($P < 0.05$) from NTC until the M4 mice were 40 days of age. The plateau in tibia weight and length growth curves of NTC and M11 mice indicates that these mice had reached maturity (Fig. 1). Tibia weight in the M4 group was still increasing at 70 days of age although at a decreased rate (Fig. 2). Tibias from G119K mice were still increasing in weight and length, albeit at a very slow rate, when the experiment was terminated (Fig. 2).

Using the ratio of tibia weight to length as a predictor of bone density, the NTC and the M11 group were similar until 68 days of age when the density of M11 tibias was greater ($P < 0.05$) than those of NTC (Fig. 1). The M4 group had greater ($P < 0.05$) tibia densities than the other lines, and the ratio continued to increase. The density estimate for G119K mice was less ($P < 0.05$) than all other groups at all ages tested, yet the ratio for this group continued to increase throughout the experiment (Fig. 2).

The ratio of tibia weight to empty body weight of NTC, M11, and M4 mice increased until 40–50 days of age and then decreased (Fig. 1). Typical animal tissue growth patterns predict that bone accretion occurs earlier and slows sooner than growth of muscle and adipose tissue in most animals (12). Therefore bone weight gain of the NTC, M11, and M4 mice fit a fairly normal pattern. In contrast, the pattern of bone growth in G119K mice was markedly different. In these mice, the ratio of tibia weight to empty body weight changed little over the course of the experiment as a consequence of the low, but constant, increases in both variables (Fig. 2). The initial ratio in the M4 group was similar to the other strains. However, the age-related decrease in the ratio in the M4 mice was greater, resulting in a ratio of 2.54 by 70 days of age, which was less than the ratio for the smaller, G119K mice (Fig. 1). These data indicate that tibias in the M4 mice were carrying a larger ($P < 0.05$) relative load than tibias from NTC, M11, or G119K mice.

The ratio of tibia length:empty body weight indicates the NTC and M11 mice had similar patterns of longitudinal growth in relation to their increases in body weight (Fig. 1). Initially and throughout the experiment, longitudinal growth in G119K mice was not as depressed as was body weight. The M4 mice had ratios in the range of 0.87–0.49, indicating that their body mass was always increasing at a greater rate than the growth in tibia length. These data indicate that there may have been differential tissue responses (skeletal versus muscle and adipose) to the G119K and M4 mutant bGH analogs. The reason for this difference is not immediately evident.

External D/V diameters did not change much in the NTC, G119K, and M11 mice between 30 and 70 days of age; 0.01–0.08 mm. Tibias from G119K mice had smaller ($P < 0.05$) D/V diameters at all ages in comparison to the other groups of mice (Fig. 3). In the M4 mice, external D/V diameters were not larger than the NTC at 30 days of age, yet the D/V diameter had increased by 0.24 mm by 70 days of age, and as a result, the M4 mice had the largest ($P < 0.05$) D/V diameters of all the strains. The external L/M diameter was smaller ($P < 0.05$) for the G119K mice than all other strains throughout the experiment (Fig. 3). The NTC and the M11 mice had similar external L/M dimensions until 60 days of age, when the L/M diameter exceeded ($P < 0.05$) that of the NTC mice. In comparison, the M4 line had external L/M diameters that were larger ($P < 0.05$) than the NTC line at all ages measured in this experiment. Similar responses were observed for the internal D/V and L/M measurements.

Starting at 32 days of age, the D/V cortical widths observed in the G119K mice were smaller ($P < 0.05$) than those of the NTC tibias; a difference that was retained throughout the period studied (Fig. 3). The D/V cortical width was greater for the M4 than the NTC between 28 and 34 days of age, and again between 66 and 70 days of age. The L/M cortical width was not different between the G119K and NTC mice. Although the LM cortical width of M11 mice was greater initially, the rate of increase was less for these mice such that this measurement was not different

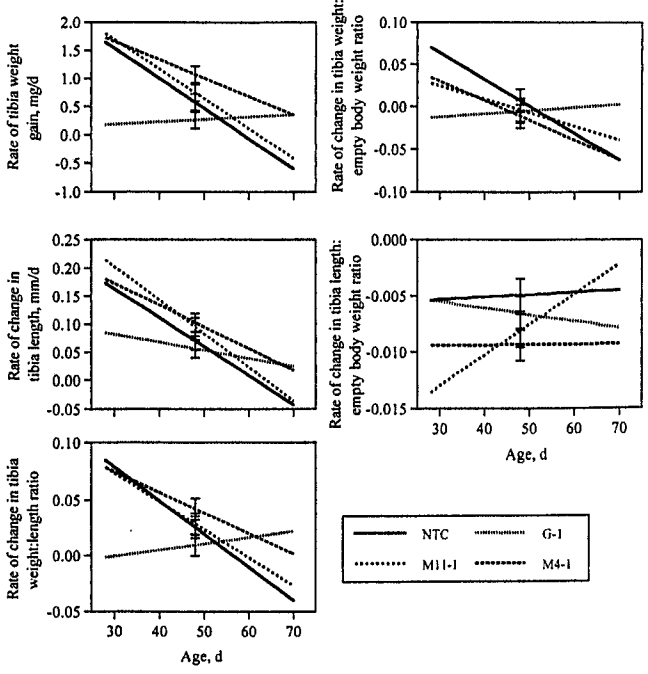

Figure 2. Daily rates of change in tibia weight, length, weight:length ratio, weight:empty body weight ratio, and length:empty body weight ratio in 28- to 68-day-old mice expressing one of three mutant bGH genes (G-119K, M11, M4) or non-transgenic control mice (NTC). Values were calculated as the first derivative of the variable regressions which included age × transgene and age^2 × transgene interactions. The number of observations was 15 for NTC and G119K, and 12 for M11 and M4 mice. The $S\hat{y}$ = 0.15, 0.016, 0.010, 0.014, and 0.001 for the respective variables.

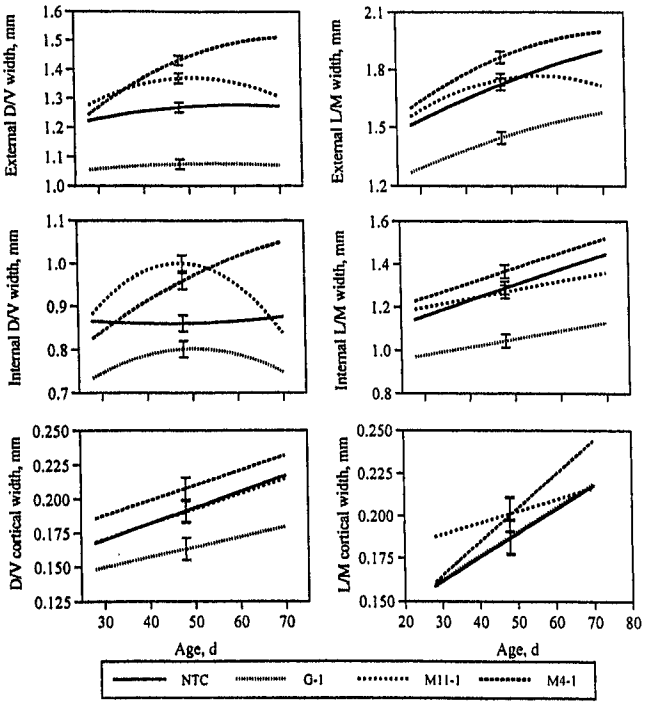

Figure 3. Dorso-ventral (D/V) and latero-medial (L/M) dimensions of tibias from 28- to 68-day-old mice expressing one of three mutant bGH genes (G-119K, M11, M4) or non-transgenic control mice (NTC). Values were calculated from regression equations which included age × transgene and age^2 × transgene (external D/V and L/M, and internal D/V) or age × transgene (internal L/M, and D/V and L/M cortical widths) interactions. The number of observations was 15 for NTC and G119K and 12 for M11 and M4 mice. The $S\hat{y}$ = 0.017, 0.031, 0.019, 0.030, 0.008, and 0.010 for external D/V and L/M, internal D/V and L/M, and D/V and L/M cortical widths, respectively.

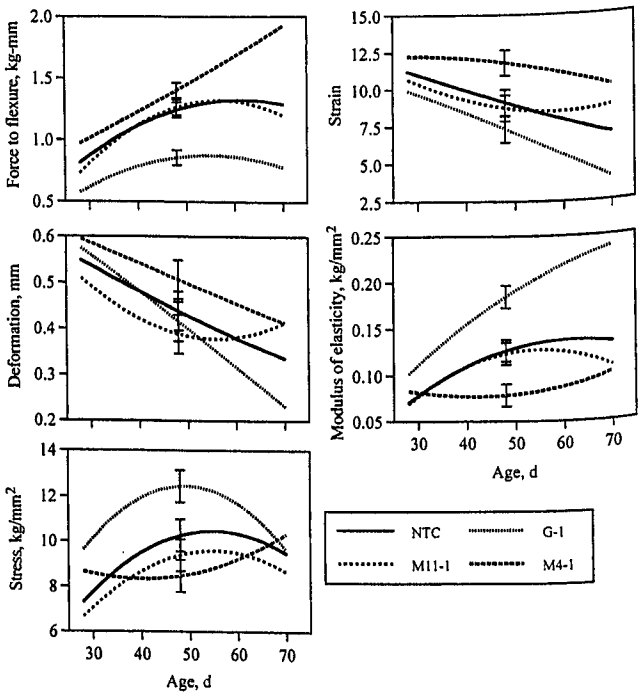

Figure 4. Mechanical characteristics tested to flexure of tibias from 28- to 68-day-old mice expressing one of three mutant bGH genes (G-119K, M11, M4) or non-transgenic control mice (NTC). Values were calculated from regression equations which included age × transgene and age^2 × transgene interactions. The number of observations was 15 for NTC and G119K and 12 for M11 and M4 mice. The $S\hat{y}$ = 0.06, 0.04, 0.71, 0.87, and 0.01 for force, deformation, stress, strain, and modulus of elasticity, respectively.

from that of the NTC or G119K mice by the end of the experiment. The L/M cortical width of the M4 mice was not different from that of NTC mice until 50 days of age, a difference that was retained through 70 days of age (Fig. 3).

Mechanical Characteristics. Loads withstood by the bones while in flexure were similar for NTC and M11 mice at all ages tested (Fig. 4). However, the effect of the G119K transgene was evident at the initiation of the study as the load withstood by tibias from these mice (0.57 kg-mm) was 29% and 41% smaller ($P < 0.05$) than tibias from NTC and M4. The load at flexure increased for the NTC, G119K, and M11 groups until between 50 and 60 days of age, whereas the load increased for tibias from M4 mice at all ages (Fig. 4). The greatest ($P < 0.05$) load at flexure (1.9 kg-mm) was observed with tibias from M4 mice at 70 days of age. As expected, the flexibility of tibias was greatest at the younger ages (Fig. 4). This is demonstrated by the amount of deformation the bone underwent before starting plastic deformation. Although there was a significant age × transgene effect in the analysis of deformation, only results for the G119K mice (from 66 to 70 days of age) were different from those of the NTC mice. The G119K tibias also deformed less ($P < 0.05$) than the M11 tibias from 62 to 70 days of age, and less than the M4 mice from 46 to 70 days of age.

Stress at flexure is the maximum stress developed in a bone just before it undergoes plastic deformation, and it takes into consideration the amount of force applied per unit area of the bone. Between 30 and about 60 days of age, the amount of stress endured by tibias from G119K mice was greater than the stress for NTC, M11, and M4 mice (Fig. 4). These data indicate that tibias from the G119K mice were able to resist a greater ($P < 0.05$) amount of force per unit area (12.4 kg/mm^2 at 50 days of age) than most other groups (mean = 9.5 kg/mm^2 at 50 days of age). Analysis of the strain data, a function of change in unit length in relation to the original length, indicated that there was no quadratic relationship with age. However, there was a linear effect of age × transgene ($P < 0.0006$). There was no difference in strain between the NTC and M11 mice. Yet by 36 days of age, tibias from M4 mice withstood more ($P < 0.05$) strain than tibias from NTC. The G119K tibias exhibited lower ($P < 0.05$) strain than NTC tibias from 48 to 70 days of age (Fig. 4). These results indicate that M4 tibias were more able to bend under the force applied to flexure than the controls, and that tibias from G119K mice were more rigid.

Young's modulus of elasticity is the rate of change in strain as a function of stress, which is equal to the slope of the straight line region of a stress–strain diagram. The data for all mice demonstrate that as the animal ages, the amount

of stress per unit change in strain increases (slope is positive for all strains), and thus the bone becomes more able to withstand forces without physical deformation as the animal ages (Fig. 4). This is true until the bone becomes mature (or extensively mineralized), at which time the modulus of elasticity begins to decrease (8), which was observed for the NTC and M11 mice starting around 50–60 days of age. The modulus of elasticity for the M4 mice indicated that their tibias were either not as mineralized as the other lines, or the tibias were less mature, because a plateau in the curve was not observed. The highest modulus ($P < 0.05$) was observed in the G119K mice at all ages.

In general, the force required to break the tibias increased as age increased (Fig. 5). However, in the G119K mouse line, the force started declining as the animals reached 56 days of age. The highest ($P < 0.05$) breaking loads were withstood by tibias from M4 mice that were between 36 and 70 days of age. At all ages tested, the breaking force withstood by tibias from G119K mice was less than tibias from NTC mice. The amount of deformation before breaking was greatest in young mice (Fig. 5). Thirty-six- to 58-day-old G119K mice tibias deformed less prior to breaking than tibias from NTC mice, a pattern similar to that displayed by the data from M11 mice. Ultimate strength, or stress at breaking, was similar among the NTC, M11, and M4 mice until about 50 days of age when the stress withstood by NTC tibias began to plateau. Interestingly, stress at breaking was elevated above NTC from 36 to 52 days of age in the G119K mice. These data indicate that the G119K tibias were able to withstand more force per unit area than the other lines, indicating a possible difference in bone mineralization. The strain at breaking decreased as age increased, except in the M4 mice from 30 to 40 days of age (Fig. 5). The ability of tibias from G119K mice to deform in response to added forces without breaking declined rapidly with age until around 50 days of age when the ability to resist additional forces by bending (deforming) increased.

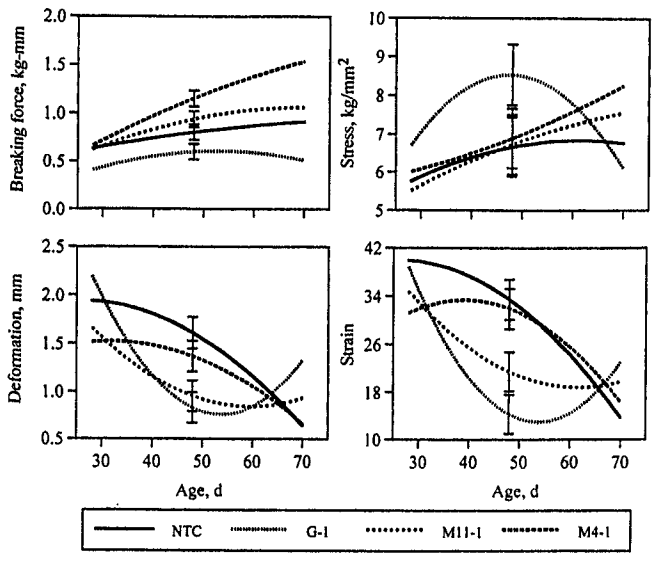

Figure 5. Mechanical characteristics at breaking of tibias from 28- to 68- day-old mice expressing one of three mutant bGH genes (G-119K, M11, M4) or non-transgenic control mice (NTC). Values were calculated from regression equations which included age × transgene and age^2 × transgene interactions. The number of observations was 15 for NTC and G119K and 12 for M11 and M4 mice. The $S\hat{y}$ = 0.08, 0.16, 0.78, and 3.3 for force, deformation, stress, and strain, respectively.

Discussion

Even though body weight and degree of locomotion can both affect bone size and mechanical characteristics, the design of this experiment did not permit us to assess the independent impact of these possible factors. However, growth hormone has been demonstrated to regulate bone physical characteristics such as length, diameter, and cortical width both through its direct effects and indirectly through IGF-I (13–18). The transgenic mice utilized in these experiments provide models of altered growth hormone activity on the processes of bone growth and development ranging from a dwarf animal to a giant animal. Not only did the growth hormone mutations expressed by the transgenic mice modify the bone characteristics at maturity, but patterns of bone growth and development were also changed during the period from post-weaning to maturity. Patterns of growth in other tissues and organs were also altered by the GH mutations (5, 10). Continued increases in length and weight of G119K and M4 tibias suggest that these lines had not reached maturity by 70 days of age, as compared to the tibias of NTC and M11 mice that had plateaued by 60 days. However, tibias of M11 mice were different from NTC tibias.

The functional antagonism reported by Chen *et al.* (4, 6) between bGH-G119 analogs and endogenous GH reduces circulating IGF-I in the G119K line (5). The M11 and M4 mutations act as a partial agonist and full agonist, respectively, and increase circulating IGF-I accordingly (5). During normal growth, IGF-I concentrations within bone matrix have been shown to rise during the period of rapid growth post-weaning (19). Because localized expression of IGF-I in bone is dependent on GH, it would be expected that altered IGF-I expression in bone may be part of the mechanism underlying the changes observed in tibia growth in these transgenic mice. However, a comparison of the tibia length-:empty body weight ratios indicate that longitudinal growth was not depressed as much as body weight gain in the G119K mice, suggesting that localized growth factors in bone may not have been affected as much as circulating factors. Tibia length:empty body weight was less in the M11 and M4 mice, indicating that body weight gain was affected more than bone growth in these lines and further substantiating this hypothesis. Changes in levels of other growth factors and gonadal hormones may also be contributing to the altered patterns of bone growth relative to whole animal growth (17, 20).

Treatment of swine with exogenous pST has been

shown to increase femur cortical width and decrease stress and modulus of elasticity (21). Similar responses were observed in the tibias of M4 mice, while tibias of M11 mice did not differ from those of NTC mice. In the G119K mice, cortical width was moderately smaller (10%), while the external diameters were substantially affected (20%). The change in cortical bone contributes to an explanation of the greater stress per cross-sectional area resisted by the tibias of G119K mice, even though the total force resisted was less than tibias from the other mouse lines. The mechanical properties of tibias were significantly changed in M4 mice as compared to NTC mice, while M11 mice were not different. Thus, alterations in the biological activity of GH caused by point mutations affects the mechanical properties of bone such as flexure, deformation, and ability to resist strain and stress.

Adequate bone mineralization may not occur in animals in which rapid rates of growth occurs (13). This may be the result of increased mineral demands for higher priority body functions or by differences in physiological maturity caused by growth regulatory agents (13). Young's modulus of elasticity serves as an indicator of mineralization, with higher values reflecting greater mineralization (22). The reduction in modulus of elasticity for M4 mice indicates either that tibias from these mice were still rapidly growing and/or that the mice were not able to supply sufficient calcium and phosphorus to meet the mineralization demands of the bone. Using a glucocorticoid model of osteopenia, Ørtoft *et al.* (23) found that although GH overcame the depression in bone growth and volume, GH was not able to reverse the decrease in mineralizing surface that occurred with glucocorticoid administration. Turner *et al.* (3) also found that implanting steers with zeranol resulted in a sufficiently increased demand for minerals by other tissues such that calcium and phosphorus concentrations in bone ash were reduced. Using a transgenic mouse model that continuously expresses GHRH, Tseng and Goldstein (24) found an increase in bone mass, but that quality of the deposited bone was compromised. They concluded that although the mice could maintain "normal" structural integrity of bone, they had to sacrifice mechanical integrity to meet the growth stimulus provided by continual GH release. In contrast to the response observed with the M4 mice, the modulus of elasticity for the G119K mice was increased above that of the NTC mice. The conclusions drawn by Tseng and Goldstein (24) are supported by the data from the M4 and G119K mice, in that the rapid growth in the M4 mice caused a reduction in mechanical quality of the bone, but the slow rate of growth in the G119K mice allowed both near-normal structural and mechanical qualities to be maintained.

These data lead to the following possible conclusions: (i) expression of the GH analogs alters the response of bone to ligand binding to the GH receptor, and/or (ii) individual tissues (e.g., bone, muscle, adipose) may have responded differently to the changes in systemic IGF-I reported by Knapp *et al.* (5). Pell and Bates (25) put forth the hypothesis that a priority for GH action on bone vs muscle may exist, but that there was no direct evidence in the literature to document this effect. Their hypothesis is supported by the incongruous patterns of bone growth relative to body weight in the G119K and M4 mice. These transgenic mice are effective tools in discovering how GH directly and indirectly affects bone at the cellular and tissue levels. The G119K and M4 lines may be useful in experiments designed to determine the priorities for GH action on bone versus muscle.

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