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Alterations in the growth plate associated with growth modulation by sustained compression or distraction

Ian A.F. Stokes [a,*], Katherine C. Clark [a], Cornelia E. Farnum [b], David D. Aronsson [a]

[a] *University of Vermont, Burlington, VT 05405-0084, USA*
[b] *Cornell University, Ithaca, NY, USA*

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Abstract

Sustained mechanical load is known to modulate endochondral growth in the immature skeleton, but it is not known what causes this mechanical sensitivity. This study aimed to quantify alterations in parameters of growth plate performance associated with mechanically altered growth rate. Vertebral and proximal tibial growth plates of immature rats and cattle, and rabbit (proximal tibia only) were subjected to different magnitudes of sustained loading, which altered growth rates by up to 53%. The numbers of proliferative chondrocytes, their rate of proliferation, and the amount of chondrocytic enlargement occurring in the hypertrophic zone were quantified. It was found that reduced growth rate with compression and increased growth rate with distraction were associated with corresponding changes in the number of proliferative chondrocytes per unit width of growth plate, and in the final (maximum) chondrocytic height in the hypertrophic zone (overall correlation coefficients 0.38 and 0.56 respectively). According to multiple linear regression coefficients for these two variables (0.72 and 1.39 respectively), chondrocytic enlargement made a greater contribution to altered growth rates.

Keywords: Growth plate; Growth modulation; Biomechanics; Proliferative zone; Hypertrophic zone

Introduction

It is known that the rate of growth by endochondral ossification in growth plates is altered in the presence of sustained mechanical compression or tension [12–14] and by cyclic compression [10]. Mechanical modulation of endochondral bone growth has been implicated in the pathomechanics of progressive skeletal deformities including scoliosis [8] and Blount's disease (tibia vara) [3] that are especially liable to progression during rapid skeletal growth.

The mechanism of longitudinal bone growth in growth plates depends on the proliferation of chondrocytes, followed by their enlargement in the hypertrophic zone with associated matrix synthesis, and eventual apoptosis of chondrocytes and mineralization of the synthesized cartilage [1,6]. Thus the rate of growth depends on the rate at which cells are produced in the proliferative zone and the rate at which they hypertrophy and synthesize matrix in the hypertrophic zone. Regulation of bone growth rate likely involves numerous factors, including hormonal levels, nutritional status (blood supply), mechanical loading and soft tissue (e.g. periosteum) constraints [18].

The relative contributions of several parameters of growth plate performance to the differing growth occurring at various anatomical locations of the same animal (rat) were determined by Wilsman et al. [17]. Their histological quantification included bromodeoxyuridine (BrdU) labeling and cell counting in the proliferative zone, and stereological quantification of chondrocytic cell sizes and matrix volume fraction in the hypertrophic zone. Most of the variability in growth rate between different growth plates was explained by differences in chondrocytic enlargement and associated matrix synthesis in the hypertrophic zone, while differences in cellular duplication and matrix synthesis in the proliferative zone made a lesser contribution.

* Corresponding author. Department of Orthopaedics and Rehabilitation, University of Vermont, Burlington, VT 05405-0084, USA. Fax: +1 802 656 4247.
E-mail address: Ian.Stokes@uvm.edu (I.A.F. Stokes).

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Based on the fact that growth plates produce essentially linear (uni-dimensional) growth, a simplification of this approach was presented by Stokes et al. [13]. They reported relative measurements of new cell production in the proliferative zone and chondrocytic height increase in the hypertrophic zone in growth plates subjected to compressive force. This simplified linear analysis considers the contribution of columns of cells to the increase in bone length in the growth direction, rather than a volumetric analysis made by Wilsman et al. [17]. At a steady state, linear growth can then be expressed as:

$$\text{Growth} = N^{*}h_{\max} \quad (1)$$

where:

Growth 24-h growth (μm)
N number of new chondrocytes created per day in the proliferative zone;
$h_{\max}$ the average height (μm) achieved by fully mature chondrocytes in the hypertrophic zone (Fig. 1).

This simplified analysis assumes that there is no matrix space in the growth direction between fully mature hypertrophic chondrocytes, and that all new cells complete their differentiation to fully mature chondrocytes. For a histological section of standard width, the number of new cells per day (N) can be considered as proportional to the number of proliferative chondrocytes present, multiplied by the proportion that is actively proliferating. Proliferation can be determined experimentally by counting cells positive for incorporation of the thymidine analog bromodeoxyuridine (BrdU) i.e. cells in S-phase when exposed to an injection of BrdU [4]. The proportion of proliferative zone cells labelled by BrdU is referred to here as the Labeling Index (LI).

Mechanical load is known to modulate growth rate. A linear relationship between stress (both tensile and compressive) and percentage change in growth rate was reported by Stokes et al. [14] in both vertebral and proximal tibial growth plates of three species (rats, rabbits and cattle). Further, the proportional change in growth per unit applied stress did not vary significantly among the three species and there were only small difference between vertebral and proximal tibial growth plates. These growth plates had very different intrinsic growth rates, varying from 30 μm/day for rat vertebrae to 366 μm/day for rabbit proximal tibia [14]. The mechanical modulation of growth might result from changes in the proliferative and/or hypertrophic zones, but the relative contributions of the various parameters of growth plate performance are not known.

The purpose of this study was to identify alterations in performance parameters of the growth plate that correlate with mechanically-induced alterations in growth rate. The study addressed the hypothesis that the proportional alteration in growth rate associated with mechanical loading is explained quantitatively by the additive effects of the proportional alteration in new cell production and amount of chondrocytic enlargement during hypertrophy. We also compared the contribution of these factors to the locally altered growth caused by mechanical loading to their contribution to different growth rates observed between species and between the two different anatomical locations.

Methods

The growth plates of young rats, rabbits and calves were subjected to sustained compression or distraction stress by an external apparatus attached to percutaneous pins that were inserted through adjacent bone. In rats and cattle, growth plates at two locations (proximal tibia, and caudal vertebra) were studied. In rabbits only the proximal tibial growth plate was used. A first set of animals had the loading apparatus installed when they were newly weaned (rats aged 38 days, rabbits 41 days and calves 48 days). In subgroups of rats and rabbits, the intervention was delayed by 20 days (rats) and by 21 days (rabbits) by which time the animals had about 75% of the growth rate of the first group of animals.

Pins were inserted under general anesthesia through the diaphysis and epiphysis of the right proximal tibia, and through tail vertebrae adjacent to the loaded tail vertebra. The pins transfixed the tail or limb (except in the case of calf tibiae where bicortical threaded bone screws were used) and were attached to external loading plates that were linked by passing threaded rods through holes in the loading plates. Details of the external apparatus for each species, and its installation are given in Stokes et al. [14]. Calibrated springs on these rods were tightened to a desired force level to achieve desired stress magnitudes. Each animal's spring lengths were measured and adjusted on days 2, 4 and 7 to maintain the desired load magnitude. The sustained stress magnitudes applied to each loaded growth plate had target values of either 0.1 MPa distraction (Load group −1); 0 MPa (sham) (Load group 0); 0.1 MPa compression (Load group 1); or 0.2 MPa compression (Load group 2). The spring lengths were adjusted to apply forces whose magnitude, divided by the estimated area, produced the desired stress, based on estimates of the area of each animal's growth plates. After the animals were euthanized on day 8, the actual area of each growth plate was measured, to provide corrected values of the imposed stress [14].

Animals were housed in individual cages, provided with standard laboratory animal food and water *ad libitum*. Penicillin G (50 kU/kg) was administered prophylactically starting on the day of surgery to rabbits (for 2 days) and calves (for the duration of the experiment). The fluorochromes Calcein (15 mg/kg) and Xylenol Orange (90 mg/kg) were administered systemically 48 and 24 h prior to death to label the ossifying front under the growth plates. BrdU (25 mg/kg) was administered 30 min prior to euthanasia. All live animal procedures were conducted at approximately the same time of day. Animals were euthanized 8 days after the installation of the loading apparatus. All live animal procedures were reviewed and pre-approved by the University of Vermont Animal Care and Use Committee. After euthanasia, the loaded and within-animal control growth plates were excised and approximately 4 mm cubic blocks were cut from several representative regions of each growth plate.

For vertebrae both of the loaded (Caudal-7) growth plates were examined, and the within-animal control growth plates were the caudal and cephalad growth plates of Caudal-5 and Caudal-9 respectively. For the tibiae, the contralateral (left) proximal growth plate served as control. The contra-lateral tibia and adjacent unloaded vertebrae provided internal controls for each animal, while a group of animals that had the apparatus installed, but without spring forces, provided the sham condition. Provisionally five animals were used in each loading magnitude group, with some exceptions (three, four or six animals per group) resulting from technical difficulties that occurred during the experiments. Thus data were included in this study from 41 rats, 39 rabbits and 18 calves.

Two blocks of each growth plate were prepared by cutting each in its sagittal plane, using a razor blade (rats) or fine-toothed saw (rabbits, calves). Each block was fixed [7], dehydrated first in serial alcohols and then in propylene oxide, and embedded in Epon–Araldite using serial dilutions in propylene oxide and vacuum. Sections of each block were cut (1.5 μm thick) and mounted. Three sets of slides were prepared respectively for growth measurements, BrdU counts and quantification of hypertrophic cell size. Representative images were recorded digitally at 1300×1030 pixel resolution using a Zeiss 'Axioskop' microscope with 10×, 20× or 40× lens. The microscope stage was rotated to align the growth direction with the image frame. Measurements of each recorded image were subsequently made by 'clicking' on image features with a computer 'mouse'.

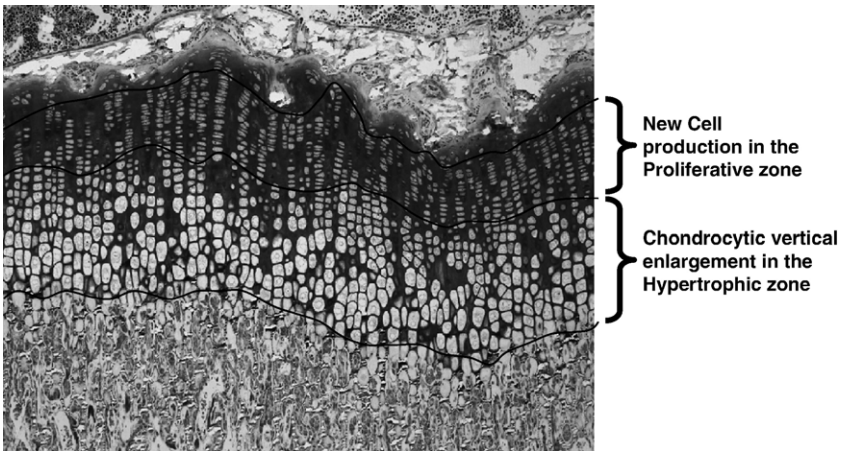

Fig. 1. The rate of endochondral bone growth can be considered as the product of the rate of production of new cells in the proliferative zone, and the final vertical height achieved by hypertrophic chondrocytes.

The recorded points were processed by means of custom computer software that took the image magnification (μm per pixel) into account.

Twenty-four hour growth was measured from the spacing between the two fluorescent labels (Calcein and Xylenol Orange) using a series of points on the labelled bone. The mean spacing between spline curves that were fitted numerically to each set of recorded points represented the growth that occurred during the 24-h period between 2 days and 1 day prior to euthanasia.

In slides processed to label nuclei containing BrdU (Histostain Sp kit, Zymed Labs, San Francisco, CA) the upper and lower boundaries of the proliferative zone were identified by marking a series of points on the zonal boundaries and the proliferative zonal height (Prolif-Z_ht) was recorded as the distance between these lines. The proliferative zone was identified by the region where cells were in columns, as far down as the first cells showing a larger more rounded shape (see Fig. 1). Within this zone, each cell nucleus was counted as either BrdU-positive or BrdU-negative. The total number of proliferative chondrocytes with a visible nucleus (N_{prolif}) was the sum of these two counts. The labeling index (LI) was expressed as the number of chondrocytes with BrdU-positive nuclei divided by the total number of nuclei counted (N_{prolif}). These counts were made in images of a field width equal to 340 μm. Measurements of the proliferative zone were made on all rat growth plates, and on a small number of those from cattle and rabbits to confirm that similar values were present in these larger species.

Hypertrophic chondrocytes were identified in stained sections (Periodic acid, Basic Fuschin, Methylene blue, Azure II) by using a semi-automated process employing Zeiss KS-300 software (Carl Zeiss Vision, München, Germany). First, the upper and lower zonal boundaries were identified manually. The upper boundary was where cells were larger and more rounded than proliferative cells, and the lower boundary was where the cells began to show signs of apoptosis, above where the matrix was mineralized (see Fig. 1). Hypertrophic zonal height (Hyper-Z_ht) was recorded as the mean separation between boundaries. This region of interest was segmented into dark/light pixels, contiguous zones were identified automatically and identified as chondrocytes if they had a 'form factor' (4π*area, divided by squared circumference) greater than 0.3. In a final manual check, regions that represented non-viable or partially sectioned or coalesced cells were eliminated. Chondrocytic enlargement in the growth direction was estimated from the regression relationship of the measured cell height (h) as a function of its vertical position (y) in the hypertrophic zone using the logistic regression curve fit [13]:

$$h = a/(1 + e^{(m-y)/s}) \quad (2)$$

where a, m, and s are parameters for each analyzed image, evaluated by curve-fitting with the Matlab (Mathworks, Natick, MA) least-squares algorithm

Table 1
Mean values of measurements of 'loaded' growth plates

	Load group	Growth (μm/day)	Hyper-Z_ht (μm)	h_{max} (μm)	Prolif-Z_ht (μm)	N_{prolif}	LI
Vertebrae calves	−1	32 (9.6) n=5	129 (70) n=4	15.8 (4.5) n=4	n=0	n=0	n=0
Vertebrae calves	0	35 (7.2) n=4	77 (17) n=4	14.6 (1.5) n=4	n=2	n=2	n=2
Vertebrae calves	1	38 (6.2) n=5	92 (9) n=5	15.6 (1.9) n=5	n=1	n=1	n=1
Vertebrae calves	2	19 (11) n=4	134 (62) n=3	11.1 (2.8) n=3	n=0	n=0	n=0
Vertebrae rats	−1	43 (14) n=10	115 (33) n=10	29.7 (6.6) n=10	56.2 (9.2) n=7	35.9 (8.5) n=7	9.1 (2.9) n=7
Vertebrae rats	0	39 (11) n=10	91 (25) n=10	24.3 (6.4) n=10	52.3 (5.0) n=9	29.4 (6.1) n=9	8.2 (3.2) n=9
Vertebrae rats	1	32 (11) n=9	71 (13) n=9	22.4 (4.8) n=9	47.2 (7.2) n=7	28.3 (6.3) n=8	13.1 (6.3) n=8
Vertebrae rats	2	25 (5) n=12	70 (8) n=12	21.5 (4.1) n=12	48.5 (8.2) n=9	35.3 (14.4) n=9	8.7 (3.8) n=9
Tibia calves	−1	272 (61) n=5	236 (69) n=5	24.9 (7.3) n=5	n=0	n=0	n=0
Tibia calves	0	192 (47) n=4	168 (17) n=4	20.6 (0.8) n=4	n=2	n=2	n=2
Tibia calves	1	112 (38) n=5	158 (23) n=5	17.0 (2.9) n=5	n=1	n=1	n=1
Tibia calves	2	109 (18) n=5	158 (10) n=4	16.5 (1.0) n=4	n=0	n=0	n=0
Tibia rats	−1	217 (30) n=7	224 (71) n=7	28.7 (5.9) n=7	115.0 (26.8) n=5	71.8 (23.2) n=5	11.1 (5.4) n=5
Tibia rats	0	218 (32) n=10	215 (58) n=10	27.0 (5.9) n=10	106.4 (23.2) n=9	65.8 (20.2) n=9	9.0 (4.2) n=9
Tibia rats	1	172 (38) n=8	148 (39) n=8	26.7 (3.7) n=8	98.3 (9.7) n=7	53.5 (12.2) n=8	16.3 (7.5) n=8
Tibia rats	2	181 (52) n=10	151 (34) n=10	27.5 (5.3) n=10	104.9 (21.0) n=9	66.6 (24.5) n=9	7.4 (3.4) n=9
Tibia rabbit	−1	383 (86) n=11	166 (28) n=11	22.7 (2.4) n=11	n=1	n=1	n=1
Tibia rabbit	0	368 (97) n=9	158 (23) n=9	23.3 (2.8) n=9	n=2	n=2	n=2
Tibia rabbit	1	313 (68) n=10	143 (18) n=10	23.0 (3.1) n=10	129.1 (12.9) n=5	96.3 (7.6) n=5	12.1 (3.6) n=5
Tibia rabbit	2	252 (72) n=9	164 (20) n=9	23.2 (2.3) n=9	n=0	n=0	n=0

Load groups: −1: 0.1 MPa distraction; 0: 0 MPa (sham); 1: 0.1 MPa compression; 2: 0.2 MPa compression.

Table 2
Percent differences (mean of paired (loaded − control)/control * 100 values)

	Load group	D_Growth	D_Hyper-Z_ht	D_h_{max}	D_Prolif-Z_ht	D_N_{prolif}	D_LI
Vertebrae calves	−1	11.3 (37.9) n=5	−9.6 (27.3) n=4	3.8 (9.3) n=4	n=0	n=0	n=0
Vertebrae calves	0	11.2 (19.8) n=4	−12.6 (14.6) n=4	−5.5 (7.6) n=4	n=2	n=1	n=2
Vertebrae calves	1	−21.6 (16.4) n=5	0.9 (9.5) n=5	−5.4 (4.0) n=5	n=1	n=1	n=1
Vertebrae calves	2	−53.3 (10.9) n=4	12.1 (33.1) n=3	−15.6 (15.3) n=3	n=0	n=0	n=0
Vertebrae rats	−1	4.0 (17.8) n=10	1.5 (7.6) n=10	6.2 (7.8) n=10	16.4 (17.1) n=7	9.7 (13.8) n=7	−12.5 (29.4) n=7
Vertebrae rats	0	−4.8 (9.6) n=10	5.3 (16.0) n=10	−4.6 (9.7) n=10	8.0 (14.7) n=9	−1.5 (11.4) n=9	7.4 (55.4) n=9
Vertebrae rats	1	−17.6 (14.1) n=9	−13.5 (7.5) n=9	−6.7 (9.2) n=9	−9.7 (13.1) n=7	−7.7 (11.3) n=8	2.5 (17.1) n=8
Vertebrae rats	2	−39.8 (12.2) n=12	−24.5 (12.4) n=12	−19.9 (11.2) n=12	−9.2 (8.6) n=9	−10.8 (14.8) n=9	8.0 (24.1) n=9
Tibia calves	−1	51.2 (20.0) n=5	17.1 (18.2) n=5	13.6 (5.9) n=5	n=0	n=0	n=0
Tibia calves	0	−0.8 (17.2) n=4	−1.9 (9.0) n=4	−2.1 (5.9) n=4	n=2	n=2	n=2
Tibia calves	1	−31.3 (20.6) n=5	−5.7 (13.1) n=5	−13.0 (17.7) n=5	n=1	n=1	n=1
Tibia calves	2	−50.5 (3.2) n=5	−2.1 (13.6) n=4	−15.7 (6.4) n=3	n=0	n=0	n=0
Tibia rats	−1	−3.7 (9.0) n=7	2.0 (15.5) n=7	−0.7 (6.6) n=7	8.5 (11.4) n=5	1.2 (4.3) n=5	9.2 (35.0) n=5
Tibia rats	0	−9.1 (5.4) n=10	−5.7 (11.7) n=10	−4.2 (7.4) n=10	−0.5 (9.4) n=9	−3.5 (11.3) n=9	14.4 (22.1) n=9
Tibia rats	1	−24.6 (11.1) n=8	−25.9 (9.9) n=8	−1.5 (7.2) n=8	1.3 (15.2) n=7	−4.8 (19.3) n=8	11.6 (22.5) n=8
Tibia rats	2	−33.6 (9.1) n=10	−29.8 (11.5) n=10	−6.2 (6.9) n=10	−9.0 (8.2) n=9	−19.5 (13.8) n=9	6.8 (41.9) n=9
Tibia rabbit	−1	22.4 (14.4) n=11	5.7 (12.0) n=11	−0.1 (7.2) n=11	n=1	n=1	n=1
Tibia rabbit	0	11.1 (8.6) n=9	3.4 (18.7) n=9	3.4 (10.0) n=9	n=2	n=2	n=2
Tibia rabbit	1	−5.9 (12.2) n=10	−7.6 (8.4) n=10	7.3 (6.8) n=10	−3.7 (6.7) n=5	−7.3 (7.6) n=5	−4.3 (19.0) n=5
Tibia rabbit	2	−25.2 (13.6) n=9	0.8 (12.2) n=9	4.4 (4.6) n=9	n=0	n=0	n=0

Load groups: −1: 0.1 MPa distraction; 0: 0 MPa (sham); 1: 0.1 MPa compression; 2: 0.2 MPa compression.

'fminsearch.m'. The maximum hypertrophic chondrocytic height (h_{max}) was estimated as the value of h at the chondro-osseous junction.

Measurements at several spatially separated locations for each growth plate were averaged. Based on initial variance estimates for each measurement, the number of sections averaged was 12 for growth (fluorescent labels); 16 for proliferative zone measurements (BrdU labelled nuclei), and five for the final hypertrophic chondrocytic height measurements. The difference between averaged values of loaded and control growth plates for each animal was calculated and expressed as a percentage of the control value.

Using the notation D_=percent difference in a parameter of growth plate performance, i.e. 100 * (loaded − control)/control, for small changes in the terms in Eq. (1) it was expected that:

$$D\_\text{Growth} = D\_\, N_{prolif} + D\_\, h_{max} \quad (3)$$

Growth and final chondrocytic height h_{max} were measured directly. A relative measure of the new cells per day was obtained from the product of the total number of proliferative chondrocytes (N_{prolif}) and the BrdU labeling index (LI). Hence, Eq. (3) can be expressed as:

$$D\_\text{Growth} = D\_\, N_{prolif} + D\_\, \text{LI} + D\_\, h_{max} \quad (4)$$

(N_{prolif})=Number of proliferative chondrocytes counted in a standard width of growth plate.

For statistical analysis, each measurement appearing in Eq. (4) and the two zonal heights was expressed as a percentage difference between the loaded and control growth plate. It was assumed that the differences between the loaded and internal control growth plates were due to the effect of mechanical load intervention.

The experimental data were examined by correlation analyses (Pearson correlation coefficients) to determine whether there were significant associations between proportional differences in growth and the histological measurements thought to be responsible for the growth differences. Subsequently, for variables that correlated most significantly with altered growth, a multiple regression analysis was performed to quantify the relative contributions of alterations in the proliferative and hypertrophic zones to altered growth. Since all measurements in these analyses were experimental variables, Reduced Major Axis regression analyses were used. These analyses are based on the shortest (perpendicular) distances from the regression line to observed data points. Statistic analyses were performed with use of SPSS Version 12 (Chicago, IL, USA) and custom programs written in Matlab (Mathworks, Natick MA). Correlations were considered significant if $p<0.05$.

Results

When compared by load Group (0.1 MPa Distraction, Sham, 0.1 MPa or 0.2 MPa compression), generally the distracted growth plates had the greatest averaged growth rates, with progressive reduction in growth rate in the sham and the two compressed groups (Table 1). The independent variables mostly followed a similar trend. These effects are clearer in Table 2,

Table 3
Pearson correlation coefficients between percent alteration in growth (D_Growth) and percent alterations in five histologically derived parameters of growth plate

Correlation of D_Growth with	Vertebral growth plates		Proximal tibial growth plates			All growth plates
	Rat	Calf	Rat	Rabbit	Calf	
D_N_{prolif}	0.47** (33)	(3)	0.46** (31)	0.70 (8)	(3)	0.38** (77)
D_LI	0.15 (33)	(3)	−0.16 (31)	0.70 (8)	(3)	−0.05 (78)
D_Prolif-Z_ht	0.44* (32)	(3)	0.36* (30)	0.61* (30)	(3)	0.37** (76)
D_h_{max}	0.75** (41)	0.41 (16)	0.36* (16)	−0.14 (39)	0.83** (17)	0.56** (148)
D_Hyper-Z_ht	0.7** (41)	−0.33 (16)	0.78** (35)	0.27 (39)	0.5* (18)	0.43** (149)

The number of growth plates is given in parentheses.
*Significant correlation ($p<0.05$); **significant correlation ($p<0.01$).

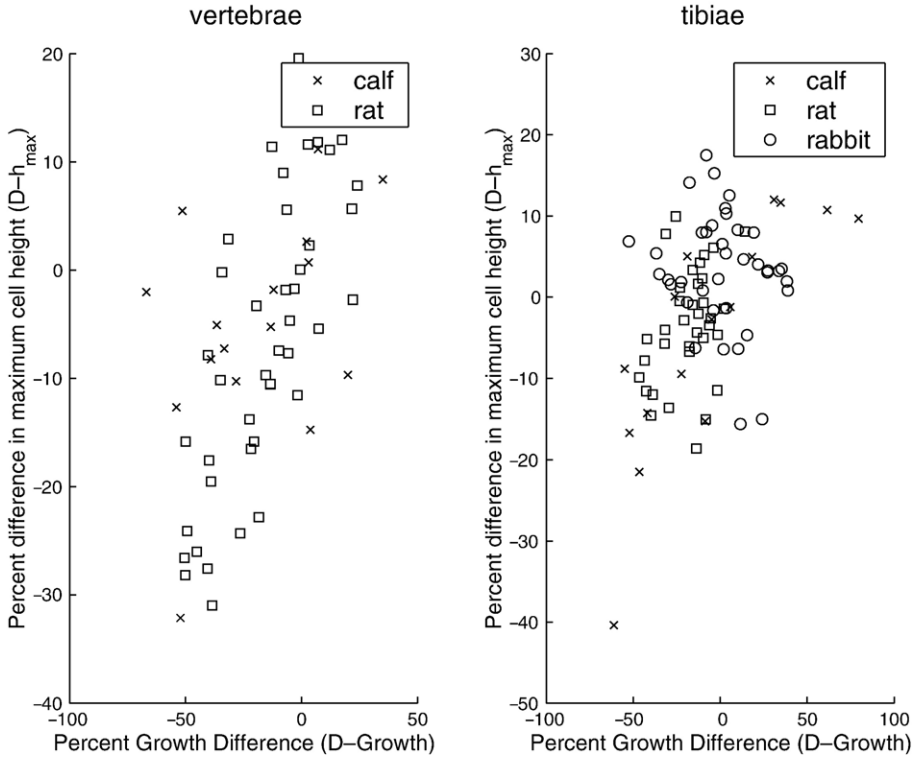

Fig. 2. Scatterplots of D_h_{max} versus D_Growth. Pearson correlation coefficients = 0.62 (vertebrae) and 0.50 (tibiae).

where values for loaded growth plates are expressed as percentage differences from the internal control values. Again, the independent variables followed similar trends, except for Hypertrophic Zonal height (Hyper-Z_ht) and Labeling index (LI).

The relationships between the alterations in growth plate parameters and those in growth rate were evident in the correlations between altered growth and alterations in the independent variables (Table 3 and Figs. 2–4).

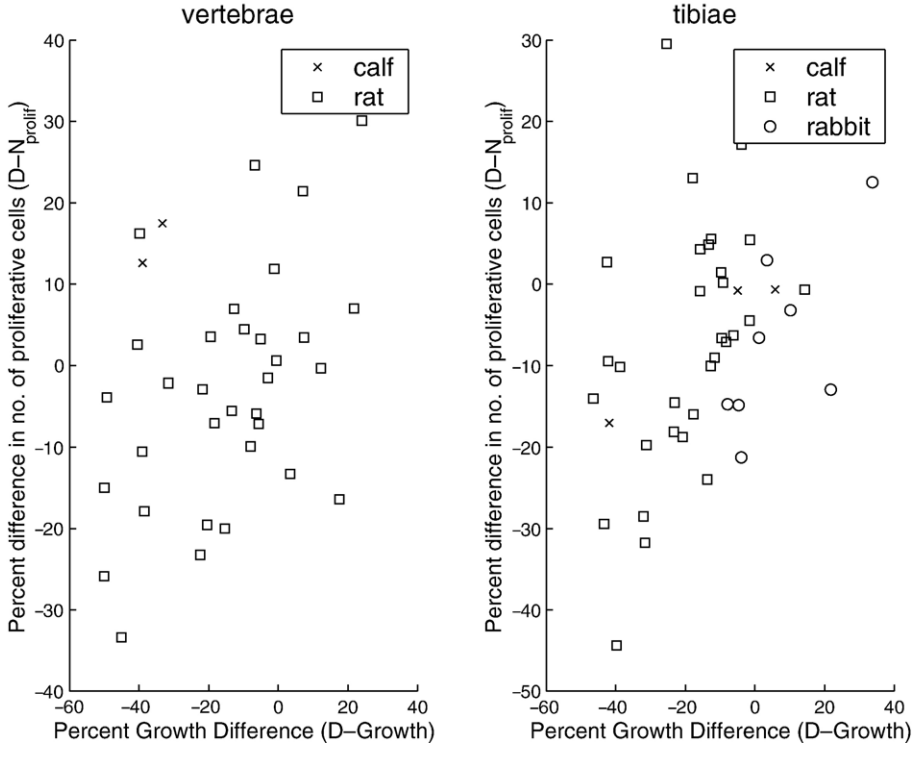

Fig. 3. Scatterplots of D_N_{prolif} versus D_Growth. Pearson correlation coefficients = 0.37 (vertebrae) and 0.42 (tibiae).

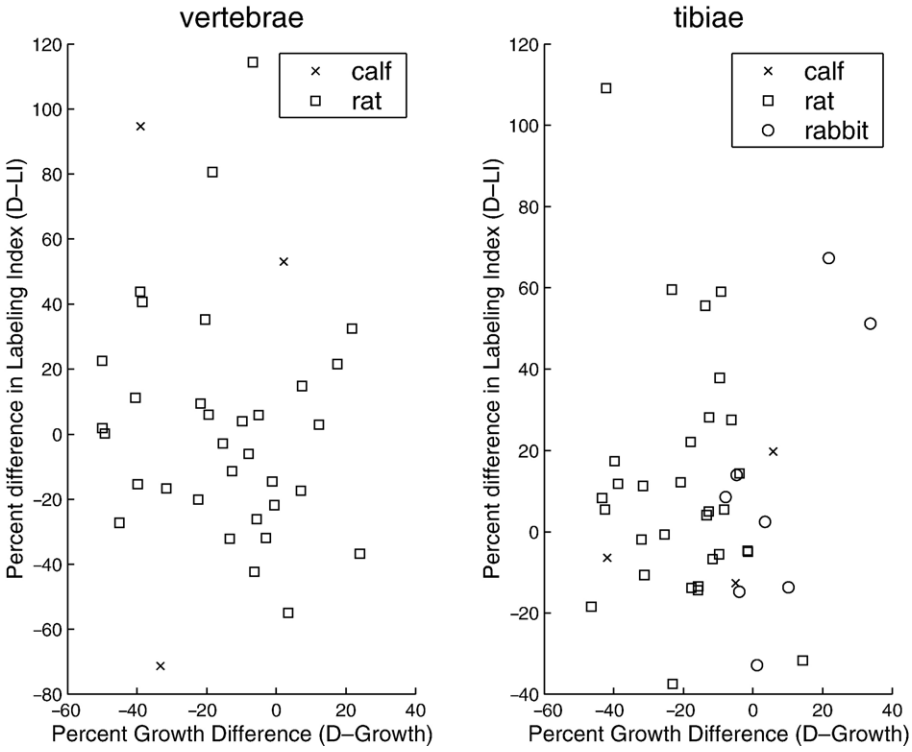

Fig. 4. Scatterplots of D_LI versus D_Growth. Pearson correlation coefficients were not significant: $r=-0.11$ (vertebrae) and $r=0.03$ (tibiae).

The strongest correlations with D_Growth (largest values of Pearson correlation coefficient) were for the two parameters N_{prolif} and $h_{\max}$. These correlations were expected, according to Eq. (4), though LI did not correlate significantly with altered growth. Therefore, the two parameters N_{prolif} and $h_{\max}$ were entered into a multiple linear regression analysis using the model:

$$D\_\text{Growth} = B_1{*}D\_N_{\text{prolif}} + B_2{*}D\_h_{\max} + B_3{*}D\_N_{\text{prolif}}{*}D\_h_{\max} \quad (5)$$

The interaction term $D\_N_{\text{prolif}}{*}D\_h_{\max}$ was included initially, because there was a moderate correlation ($r=0.36$) between the two independent variables D_N_{prolif} and $D\_h_{\max}$. However, this interaction term was not significant in any of the regression relationships that were examined, so it was subsequently excluded.

The data from all three species were pooled in the regression analyses, since there was no evidence of different regression relationship by species (Figs. 2–4). Both separate and combined analyses for tibiae and vertebrae were made because of the large difference in growth rate between these two anatomical locations. Regression relationships (Table 4) indicated that a one percentage change in D_N_{prolif} was associated with 0.77 percentage change in growth. A one percentage change in $D\_h_{\max}$ had a larger effect on growth — between 1.28% and 1.63% according to the regression coefficients for vertebrae and tibiae respectively.

Table 4
Regression coefficients the relationship D_Growth$=B_1{*}D_N_{\text{prolif}}+B_2{*}D_h_{\max}+C$ and the coefficient of determination R^2

	B_1	B_2	C	R^2
All data	0.72	1.39	−6.81	0.43
All vertebrae	0.63	1.28	−7.91	0.54
All tibiae	0.77	1.63	−5.82	0.31

The heights of the two principal zones of the growth plates (the proliferative and hypertrophic zones) were generally increased in distracted growth plates and decreased in compressed growth plates (Table 2). The percent alterations correlated with percentage change in growth — the correlation of D_Growth with height of the proliferative zone was $r=0.23$ ($p<0.05$) and with height of the hypertrophic zone $r=0.43$ ($p<0.01$). The heights of the two zones correlated with the number of proliferative chondrocytes and the final chondrocytic height $h_{\max}$ respectively — the correlation was $r=0.44$ ($N=75$) between D_N_{prolif} and D_Prolif-Z_ht and it was $r=0.41$ ($N=146$) between $D\_h_{\max}$ and D_Hyper-Z_ht.

It was hypothesized that the percent difference in growth rate (D_Growth) attributed to mechanical modulation would be equal to the sum of the percentage differences in the three parameters of the growth plates, as stated in Eq. (4). The observed relationship (Fig. 5, left) indicated a qualitative agreement with this hypothesis, with a non-significant Pearson correlation coefficient ($r=0.3$). Since D_LI had been found not to be significantly associated with growth differences, this variable was subsequently excluded from the analyses (Fig. 5, right). Then there was a significant correlation ($r=0.6$) between D_Growth and the sum of $D\_h_{\max}$ and D_N_{prolif}.

The large differences in actual growth rate (in μm/day) between anatomical locations and between species were most strongly associated with differences in the number of proliferative

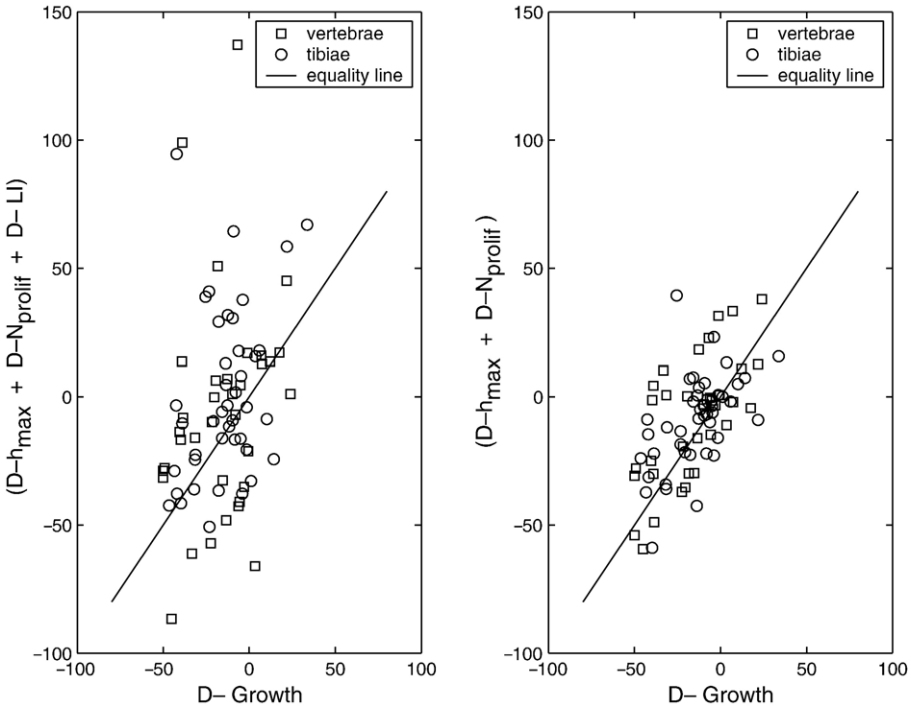

Fig. 5. Left: scatterplot of the sum of percentage differences in the three growth plate parameters versus *D*_Growth. The Pearson correlation coefficient $r=0.3$ was not significant. After exclusion of the percent differences in labeling index (*D*_LI), there was a significant correlation ($r=0.6$) with *D*_Growth.

cells (N_{prolif}), according to multiple regression analyses. For each anatomical location and species, variations in the actual growth rates (in μm) correlated with the values of the independent variables (Table 5), with coefficients similar to those for the percentage differences (Table 3). This suggests that the mechanical modulation of growth is associated with alterations in the same growth plate histological parameters that are associated with the differences in growth at different anatomical locations and in different species.

Discussion

The increased growth rates in distracted growth plates and the reduced rates in compressed growth plates correlated with corresponding alterations in the number of proliferative chondrocytes present, and with the estimated final height of the chondrocytes in the hypertrophic zone, prior to apoptosis.

Table 5
Pearson correlation coefficients between growth (in μm) and five histologically derived parameters of growth plate

Correlation of growth with	Vertebral growth plates		Proximal tibial growth plates		
	Rat	Calf	Rat	Rabbit	Calf
N_{prolif}	0.52** (66)	(6)	0.49** (62)	−0.33 (16)	(6)
LI	0.19 (66)	(6)	0.04 (62)	−0.34 (16)	(6)
Prolif-*Z*_ht	0.62** (64)	(6)	0.08 (60)	0.62* (16)	0.88* (6)
h_{max}	0.67** (82)	0.30 (32)	0.46* (70)	0.45** (78)	0.49** (36)
Hyper-*Z*_ht	0.68** (82)	−0.29 (32)	0.61** (70)	0.20 (18)	0.42** (36)

The number of growth plates is given in parentheses.
*Significant correlation ($p<0.05$); **significant correlation ($p<0.01$).

As hypothesized (Eq. (4)), the alteration in growth was explained by the sum of the alterations in the number of proliferative cells (N_{prolif}) and in the final height achieved (h_{max}) but with residual unexplained variability (Fig. 5). According to the pair-wise correlation coefficients (Table 3) and the multiple regression analyses (Table 4) the mechanically mediated alteration in growth was associated with differences in the amount of chondrocytic enlargement (final chondrocytic height h_{max}), and to a lesser extent with changes the number of chondrocytes in the proliferative zone (N_{prolif}). These are both parameters that have also been associated with regulation of differential growth, and were therefore expected to be involved in the locally altered growth associated with imposed sustained mechanical loading. Labeling index (LI) was the other putative parameter controlling growth rate (Eq. (4)), but it was not influenced significantly by the mechanical loading. In a study of stapled growth plates, in which there was a severe irreversible reduction in growth rate, Farnum et al. [5] found reductions in all measured parameters of growth plate performance, including the BrdU Labeling Index.

Regression coefficients (Table 4) implied that growth is more sensitive to a change in chondrocytic enlargement than it is to a change in the number of new cells produced. Conversely one could say that mechanical loading has a larger effect on chondrocytes in the hypertrophic zone than it does on the proliferative zone. In the proliferative zone the BrdU labeling index was not associated with different magnitudes of mechanical loading, but the variability in the labeling index (LI) measurements was very large. At the end of the experiment there were differences in the number of proliferative cells associated with mechanical loading, despite no evidence of altered

labeling index. There are several possible explanations for this: there may have been an early but not sustained change in LI and chondrocytic cycle time, a small but undetectable sustained alteration in LI, or a slightly delayed differentiation of chondrocytes to the hypertrophic phenotype. The LI was only about 0.1 in these growth plates so a large number of cells (in 16 sections of each growth plate) were counted to obtain reliable measurements. The large variability in the Labelling Index indicated by the standard deviation in Tables 1 and 2 likely reflected large inter-animal variations. The overall differences in growth rates between loaded and unloaded growth plates was relatively small in most cases, with greatest mean (by group) differences of +51% and −53%, so in most cases only small alterations in growth plate parameters were expected.

In a previous study of rat vertebral and proximal tibial growth plates subjected to nominally 0.1 MPa compression [13], reduced amount of hypertrophic chondrocytic enlargement explained about half of the growth suppression. It is not known which growth regulating pathways are altered by the mechanical loads. Villemure [15] demonstrated by in situ hybridization that the expression of mRNA for Collagen II and Collagen X was restricted to a narrower band of compressed growth plates, whereas that for parathyroid hormone receptor was not noticeably affected. These findings point to likely roles of chondrocytic hypertrophy and matrix synthesis in the hypertrophic zone in the alteration of growth by mechanical loading.

The growth plate parameters that were found to be most strongly associated with mechanically altered growth were similar to those also found to have the greatest contributions to differential growth (in normally loaded growth plates) [9,17]. Thus it appears that the normal systemic regulation of growth, as well as alterations in growth produced locally by sustained mechanical load are both controlled more by regulation of hypertrophy and matrix synthesis than by alterations in rate of chondrocytic proliferation. However, the regulatory mechanisms are probably different between the response to sustained altered mechanical loading and the genetic or systemic factors controlling differential growth under normal circumstances.

The observed reduced final chondrocytic height of hypertrophic chondrocytes may have been caused by a combination of physical and biological factors. The applied stresses would be expected to deform growth plate and its cells visco-elastically on the order of 10%, based on the published equilibrium elastic modulus of growth plate [2,11]. These deformations may have been still present (although some visco-elastic recovery may have occurred) in the fixed tissue, since the growth plates were unloaded after dissection and immediately placed in a fixative that is specifically designed to maintain cellular dimensions. Whether the histologically observed alterations in the growth plate were physico-chemical effects, as opposed to results of biological processes within the cells could not be determined within the constraints of the measurements actually made in the reported investigation.

It should be noted that the experimental design assumed that the differences between 'loaded' and 'control' growth plates were all biomechanically mediated. In fact, there was evidence of a sham effect with altered growth associated with application of apparatus alone, without compression of the springs. Both increased and decreased growth was observed in the sham growth plates, without any evident association with species or anatomical site. The reasons for this are unclear. Possible explanations for increased growth include altered loading (e.g. 'favoring' of the instrumented limb or vertebrae) or increased blood flow associated with surgery. Both would be expected as greater effects in tibiae than tail vertebrae, but no such difference by anatomical location was observed.

The duration of the experiment (7 days) was expected to allow for a complete turn-over of the growth plate, so this was the basis for the assumption that it had reached a new equilibrium state [4,16].

The selection of the three species and the two growth plate anatomical locations was intended to provide a diverse set of growth rates, and sizes of growth plates. The growth rates of the vertebrae were an order of magnitude less than that of the proximal tibia, while there was a lesser (factor of two) difference between species in growth rates at each of the two anatomical locations. Data from all three species and both anatomical locations were pooled in the regression analyses. This was considered to be justified based on (1) only small differences seen in growth sensitivity to stress between anatomical location [14]; (2) the absence of any evident species differences in the data in Figs. 2–4.

Alterations of mechanical loading have been associated with growth-related deformities of the immature skeleton. Therefore, quantification of the mechanical and cell-kinetic factors responsible for the altered growth can help in understanding the causes of these deformities, and help to guide therapeutic interventions in patients having residual growth. Such interventions could be directed towards local alteration of the growth plate loading, or could focus on local alteration in the growth plate kinetics.

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