

## Differential Growth by Growth Plates as a Function of Multiple Parameters of Chondrocytic Kinetics

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**Summary:** Differential elongation of growth plates is the process by which growth-plate chondrocytes translate the same sequence of gene regulation into the appropriate timing pattern for a given rate of elongation. While some of the parameters associated with differential growth are known, the purpose of this study was to test the hypothesis that eight independent variables are involved. We tested this hypothesis by considering four different growth plates in 28-day-old Long-Evans rats. Temporal parameters were provided by means of oxytetracycline and bromodeoxyuridine labeling techniques. Stereological parameters were measured with standard techniques. For all four growth plates, the calculated number of new chondrocytes produced per day approximated the number of chondrocytes lost per day at the chondro-osseous junction. This suggests that the proposed equations and associated variables represent a comprehensive set of variables defining differential growth. In absolute numbers, the proximal tibial growth plate produced about four times as many chondrocytes per day as the proximal radial growth plate (16,400 compared with 3,700). In the proximal tibia, 9% of growth is contributed by cellular division; 32%, by matrix synthesis throughout the growth plate; and 59%, by chondrocytic enlargement during hypertrophy. In the more slowly elongating growth plates, the relative contribution to elongation from cellular enlargement decreases from 59 to 44%, with a relative increase in contribution from matrix synthesis ranging from 32% in the proximal tibia to 49% in the proximal radius. This study suggests that differential growth is best depicted as a complex interplay among cellular division, matrix synthesis, and cellular enlargement during hypertrophy. Differential growth is best explained by considering a set of eight independent variables, seven of which vary from growth plate to growth plate. Thus, this study confirms the importance of cellular hypertrophy during elongation and adds to our understanding of the importance of locally mediated regulatory systems controlling growth-plate activity.

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The regulation of long-bone growth must be understood at two levels. Overall, chondrocytes in every growth plate undergo the same differentiation sequence (13,23). Germinal chondrocytes enter the cell cycle and undergo a series of mitotic divisions. After leaving the cell cycle, growth-plate chondrocytes synthesize and secrete unique growth-plate-specific macromolecules, many of which are associated with the mineralization of the cartilaginous matrix adjacent to the metaphyseal chondro-osseous junction. Distal hypertrophic chondrocytes undergo a 4 to 10-fold increase in volume by a water-driven, cellular swelling mechanism. Finally, chondrocytes die by an apoptotic mechanism that is apparently initiated at the same time chondrocytes leave the cell cycle. While we continue to learn about regulatory mechanisms that are

involved as chondrocytes pass from one stage to another, with respect to sequence, all growth plates are the same.

The complicating factor in understanding regulation of long-bone elongation is that each growth plate translates this same differentiation sequence into different rates of elongation. A single growth plate elongates at different rates at different ages in an individual's life. In addition, at any single point in an individual's life, growth plates in different bones are elongating at rates that may vary by a factor of seven or more. Even the two growth plates at opposite ends of the same bone elongate at significantly different rates. For example, while the proximal tibial growth plate elongates more rapidly than the distal tibial growth plate, the distal radial growth plate elongates more rapidly than the proximal radial growth plate. This latter phenomenon is sometimes called differential growth and was discovered by John Hunter more than 200 years ago (13). We are interested in what parameters allow for the rate of elongation to function as a variable.

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Previous studies have attempted to identify variables (height of the proliferative zone, kinetics of chondrocytic hypertrophy, and cell-cycle parameters) responsible for controlling differential growth. When variables are examined one at a time, in general, the result is that the variable under consideration explains a portion but not all of differential growth (4-6,12,22,27,28,30,33,39,41). The purpose of the current experiment was to attempt to conceptually integrate these previous studies by considering four growth plates in the same experimental animals and five variables, which are measurable as eight independent measurements. We made the assumption that over a short time frame (24 hours), with the exception of displacement in space, the growth plates are in steady state. Therefore, the purpose of this experiment was to test the hypothesis that multiple variables are needed to account for differential growth. To test this hypothesis, we (a) proposed two independent equations that describe chondrocytic kinetics during steady-state elongation — one describing chondrocytic production, the other describing chondrocytic loss; (b) measured all parameters in each equation independently; (c) determined whether the calculated number of new chondrocytes produced per day was the same as the calculated number of chondrocytes lost per day in all four growth plates, which were elongating at different rates; and (d) calculated the relative contributions to total elongation from cell division, matrix synthesis, and cellular enlargement during hypertrophy.

### Experimental Framework (Table 1)

The first equation describes the number of new chondrocytes produced per day ( $N_{\text{new}}$ ) as the product of chondrocytic density ( $N_{\text{V proliferative zone}}$ ), total volume of the proliferative zone ( $V_{\text{T proliferative zone}}$ ), growth fraction (GF), and total cell-cycle time:  $N_{\text{new}} = N_{\text{V proliferative zone}} * V_{\text{T proliferative zone}} * \text{GF} * (24_{\text{hours}} / \text{Total cell cycle}_{\text{hours}})$ . So that we can directly compare growth plates with each other, the proliferative zones of each growth plate are modeled as circular cylinders of diameter 1 with appropriate height. Volume is calculated as  $V_{\text{T proliferative zone}} = (\pi * 0.5^2 * \text{Height}_{\text{proliferative zone}})$ . Chondrocytic density is calculated from the stereological relationship (9)  $N_{\text{V proliferative zone}} = (V_{\text{V proliferative zone}} / \bar{v}[c]_{\text{proliferative zone}})$ . Therefore, in expanded form, the number of new chondrocytes produced per day is a function of five variables, each of which can be measured independently:

$$N_{\text{new}} = (V_{\text{V proliferative zone}} / \bar{v}[c]_{\text{proliferative zone}}) * (\pi * 0.5^2 * \text{Height}_{\text{proliferative zone}}) * \text{GF} * (24_{\text{hours}} / \text{Total cell cycle}_{\text{hours}}).$$

In the hypertrophic zone, chondrocytes are lost at the chondro-osseous junction. The number of chondrocytes lost can be estimated by a set of parameters that are independent of those used to estimate the number

TABLE 1. Definitions of variables

GF	=	growth fraction, for a defined population of chondrocytes, the ratio of chondrocytes in the cell cycle to total chondrocytes
$V_{\text{V proliferative zone}}$ $V_{\text{V terminal hypertrophic zone}}$	=	volume fraction, the ratio of chondrocytic volume to total volume for the proliferative or hypertrophic zones, respectively
$\bar{v}[c]_{\text{proliferative zone}}$ $\bar{v}[c]_{\text{terminal hypertrophic zone}}$	=	mean cell volume, the average volume of a proliferative or hypertrophic zone chondrocyte (volume weighted) in $\text{mm}^3$
$N_{\text{V proliferative zone}}$ $N_{\text{V terminal hypertrophic zone}}$	=	numerical density, the number of chondrocytes in a unit volume ( $\text{mm}^3$ ) of the proliferative or hypertrophic zones, respectively
$V_{\text{T proliferative zone}}$ $V_{\text{T turned over}}$	=	total or reference volume of the proliferative zone, or total volume of growth plate turned over per day in $\text{mm}^3$
$N_{\text{new}}$ $N_{\text{lost}}$	=	absolute number of chondrocytes produced or lost per day within the respective reference volume

of new chondrocytes. The number of chondrocytes lost at the chondro-osseous junction per day ( $N_{\text{lost}}$ ) is the product of the chondrocytic density and the total volume turned over at the chondro-osseous junction:  $N_{\text{lost}} = N_{\text{V terminal hypertrophic zone}} * V_{\text{T turned over}}$ . Continuing the unit cylinder model, the total volume turned over at the chondro-osseous junction is a function of daily elongation:  $V_{\text{T turned over}} = (\pi * 0.5^2 * \text{elongation})$ ;  $N_{\text{V terminal hypertrophic zone}} = (V_{\text{V terminal hypertrophic zone}} / \bar{v}[c]_{\text{terminal hypertrophic zone}})$ . In expanded form, the number of chondrocytes lost per day is estimated from three independent variables, including  $V_{\text{V terminal hypertrophic zone}}$ ,  $\bar{v}[c]_{\text{terminal hypertrophic zone}}$ , and elongation:  $N_{\text{lost}} = (V_{\text{V terminal hypertrophic zone}} / \bar{v}[c]_{\text{terminal hypertrophic zone}}) * (\pi * 0.5^2) * (\text{elongation})$ .

### METHODS

This study was performed as two separate experiments. In the first experiment, 12 male 28-day-old Long-Evans rats were studied. These animals were used to measure seven parameters ( $\text{Height}_{\text{proliferative zone}}$ ,  $V_{\text{V proliferative zone}}$ ,  $\bar{v}[c]_{\text{proliferative zone}}$ ,  $V_{\text{V terminal hypertrophic zone}}$ ,  $\bar{v}[c]_{\text{terminal hypertrophic zone}}$ , bone elongation rate, and total cell-cycle time). Bone elongation rates and cell-cycle times have been reported previously (43). In the second experiment, eight male 28-day-old Long-Evans rats were used to estimate the growth fraction, which is the percentage of proliferative-zone chondrocytes that are in the cell cycle. In both experiments, four growth plates were studied in each animal. These four growth plates (proximal and distal tibial and proximal and distal radial) represent a range of rates of elongation between about 50 and 400  $\mu\text{m}/\text{day}$ . These experiments were reviewed and approved by our Institutional Animal Care and Use Committee.

#### Experiment 1

Methods for tissue collection, including kinetic labeling with oxytetracycline and bromodeoxyuridine, have been described in detail previously (4,5,12,43). Briefly, for each rat, oxytetracycline

hydrochloride, 1 mg/kg, was injected intraperitoneally 3 days and 1 day prior to death. In addition, 30 minutes prior to death, each animal was injected with bromodeoxyuridine, 25 mg/kg. At the time of tissue collection, each animal was given a lethal overdose (pentobarbital, 300 mg/kg intraperitoneally), and the left and right growth plates were quickly collected. The left growth plates were used to measure the rate of elongation. For each of these growth plates, two thin slabs of growth-plate cartilage, including epiphyseal bone on one end and metaphyseal bone on the opposite end, were cut and were observed under epifluorescence microscopy with an excitation filter of 365-380 nm and a barrier filter of 530-540 nm. To estimate the elongation that occurred over 48 hours, the distance between the leading edges of the two fluorescent bands was measured using an ocular reticle. For each growth plate, elongation was determined by averaging four measurements made on each of two slabs and was subsequently converted to elongation per 24 hours (4,5). Previous studies demonstrated no left-right differences in bone growth rates (19,30).

#### Fixation, Embedment, Sectioning, and Label Detection

The right growth plates were trimmed into  $1 \times 1 \times 2$  mm blocks and fixed in 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.35) with 0.7% ruthenium hexamine trichloride (19,22-24). The tissue blocks were oriented such that growth plates would be sectioned vertically, and the blocks were embedded in Epon-araldite. For each animal and each growth plate, two tissue blocks were selected randomly from all available blocks. From each block, two sets of three serial sections were cut. The sets were spaced 15  $\mu$ m apart. The middle section of each set was stained with a polychrome stain and was used for stereological measurements. The other two sections of each set were immunostained for the bromodeoxyuridine label with a commercially available antibody (Becton Dickinson, Mountain View, CA, U.S.A.) (2,12,45).

#### Defining the Zones of Proliferating and Hypertrophic Chondrocytes

Through use of electronically captured  $\times 600$  images, a composite map of each growth plate was constructed. All bromodeoxyuridine-labeled nuclear profiles and all mitotic figures were located. Employing the physiological parameters of bromodeoxyuridine labeling and mitotic figures and also considering the contours of the chondro-osseous junctions on both the epiphyseal and metaphyseal sides of the growth plate, two curved parallel lines were drawn, marking the proximal and distal extents of the zone of proliferating chondrocytes. While this zone included all mitotic figures, occasional labeled nuclei were excluded because their position was inconsistent with the general labeling pattern and chondro-osseous contours. After an estimate of the total zone of proliferating chondrocytes was marked, a second zone comprising 80% of the total zone was marked, omitting 10% of the total zonal area from the epiphyseal side and 10% of the zonal area from the metaphyseal side. This latter 80% zone represented the location of the main body of proliferative-zone chondrocytes. Volume fractions and cellular volumes of proliferating chondrocytes were measured in this 80% framing area (43). The hypertrophic zone was defined as the distal 75  $\mu$ m of the growth plate adjacent to the chondro-osseous junction for the proximal tibial, distal radial and distal tibial growth plates, and the distal 40  $\mu$ m for the proximal radial growth plate.

#### Chondrocytic Densities and Volumes

In this experiment, we calculated numerical densities ( $N_V$  proliferative zone and  $N_V$  terminal hypertrophic zone) from volume fractions and mean cellular volumes (9). Volume fractions ( $V_V$  proliferative zone and  $V_V$  terminal hypertrophic zone) were determined using an image analysis system. Accurate thresholding of the system was tested against volume fractions that were determined using standard point-counting

techniques. A paired Student's *t* test showed no significant differences between the results with these two methods. Mean cellular volumes ( $\bar{v}[c]_{\text{proliferative zone}}$  and  $\bar{v}[c]_{\text{terminal hypertrophic zone}}$ ) were determined using the point-sampled mean linear intercept method (9,18), applying eight different angles of intercept. The calculated numerical density of the proliferative zone ( $N_V$  proliferative zone) was tested against the numerical density determined directly using the disector technique (9). No significant differences were found between the results with these two methods. For each of these stereological parameters, four values (two sections per block and two blocks per growth plate) were averaged to represent an independent estimate per growth plate per animal. Analysis of variance and Tukey's multiple comparison techniques were used to make pairwise comparisons between individual growth plates.

#### Calculation of Relative Contributions to Elongation

The calculation of the relative contributions to elongation from cellular division, matrix synthesis, and cellular enlargement requires making two assumptions. First, we continue to assume steady-state kinetics over a short time span (24 hours). Second, we assume that the growth plate is modeled as a two-compartment system (cells and matrix).

In the proliferative zone, the cellular volume component that is renewed daily is the product of the mean cellular volume of proliferative-zone chondrocytes and the  $N_{\text{new}}$  chondrocytes produced per day ( $V_C$  renewed =  $\bar{v}[c]_{\text{proliferative zone}} * N_{\text{new}}$ ). The mean volume of matrix per cell is the product of the matrix volume fraction and the total volume of the proliferative zone divided by the number of cells in the proliferative zone ( $\bar{v}[m]_{\text{proliferative zone}} = [(1 - V_V \text{ proliferative zone} * V_T \text{ proliferative zone}) / N]$ ). The matrix volume component that is renewed daily is the product of the mean volume of matrix per chondrocyte in the proliferative zone and the number of new chondrocytes produced per day ( $V_M$  renewed =  $\bar{v}[m]_{\text{proliferative zone}} * N_{\text{new}}$ ). Therefore, the volume of the proliferative zone that is renewed daily, and therefore contributes to daily elongation, is the sum of the cellular and the matrix volume components that are renewed daily ( $V_T$  renewed =  $V_C$  renewed +  $V_M$  renewed).

The cellular volume component that is lost daily at the chondro-osseous junction is the product of the mean cellular volume of hypertrophic-zone chondrocytes and the number of chondrocytes lost per day ( $V_C$  turned over =  $\bar{v}[c]_{\text{hypertrophic zone}} * N_{\text{lost}}$ ). The mean volume of matrix per hypertrophic chondrocyte is the product of the matrix volume fraction and the total volume turned over divided by the number of cells lost ( $\bar{v}[m]_{\text{hypertrophic zone}} = [(1 - V_V \text{ hypertrophic zone}) * V_T \text{ turned over}] / N_{\text{lost}}$ ). The matrix volume component that is lost daily is the product of the mean volume of matrix per chondrocyte in the hypertrophic zone and the number of chondrocytes lost per day ( $V_M$  turned over =  $\bar{v}[m]_{\text{hypertrophic}} * N_{\text{lost}}$ ). Therefore, the volume lost per day is the sum of the cellular and matrix volume zone components that are lost daily at the chondro-osseous junction ( $V_T$  turned over =  $V_C$  turned over +  $V_M$  turned over).

## Experiment 2

#### Experimental Framework for Determination of Growth Fraction

If a population of cells, such as the proliferative-zone chondrocytes, is continuously exposed to the thymidine analog bromodeoxyuridine, initially cells that are in the S-phase of the cell cycle will incorporate bromodeoxyuridine into their DNA. This bromodeoxyuridine label is detectable using immunocytochemistry. Over time, cells will leave the S-phase carrying their bromodeoxyuridine label, and cells originally in the G1-phase will enter the S-phase and label. Thus, over time, the labeling index (labeled cells divided by total cells) will increase. At a time point equivalent to total cell-cycle time minus S-phase time, all cells that are in the cell cycle will have picked up the bromodeoxyuridine label. At this time

TABLE 2. Parameters of the growth plate

Growth plate	Growth rate ( $\mu\text{m}/\text{day}$ )	Growth plate height ( $\mu\text{m}$ )	Cell cycle (hrs)	Proliferative-zone height ( $\mu\text{m}$ )	Growth fraction <sup>a</sup>
Proximal tibial	396 $\pm$ 15 <sup>b</sup>	619 $\pm$ 61 <sup>b</sup>	30.9 <sup>c</sup>	137 $\pm$ 26 <sup>b</sup>	0.99
Distal radial	269 $\pm$ 15 <sup>b</sup>	515 $\pm$ 52 <sup>b</sup>	34.0 <sup>c</sup>	99 $\pm$ 18 <sup>b</sup>	0.99
Distal tibial	138 $\pm$ 10 <sup>b</sup>	326 $\pm$ 47 <sup>b</sup>	48.7 <sup>b</sup>	83 $\pm$ 10 <sup>b</sup>	0.98
Proximal radial	47 $\pm$ 04 <sup>b</sup>	181 $\pm$ 26 <sup>b</sup>	76.3 <sup>b</sup>	43 $\pm$ 04 <sup>b</sup>	0.89

Mean values  $\pm$  SD.

<sup>a</sup>Maximum labeled nuclei/total.

<sup>b</sup>Significantly different from all others.

<sup>c</sup>Significantly different from two others.

point, the labeling index will plateau and will equal the growth fraction, or the percentage of all cells that are actually a part of the proliferating pool. If all chondrocytes in the proliferative zone are in the cell cycle, then eventually, depending on the length of the cell cycle and of the S-phase, 100% of these chondrocytes will be bromodeoxyuridine-positive.

#### Continuous Labeling of Proliferative-Zone Chondrocytes with Bromodeoxyuridine

Eight male 28-day-old Long-Evans rats were used in this experiment. Using methoxyflurane/halothane anesthesia, a 200  $\mu\text{l}$  osmotic pump with a delivery rate of 1  $\mu\text{l}/\text{hour}$  (ALZA, Palo Alto, CA, U.S.A.) was implanted in the superficial fascia in the dorsal interscapular region. Each pump was loaded with 200  $\mu\text{l}$  of bromodeoxyuridine, 20 mg/ml. At 12, 24, 48, and 72 hours after implantation, two rats were killed and four growth plates were collected, fixed, embedded, sectioned, and immunolabeled as described previously. The increase in labeling index over time was measured.

## RESULTS

### Elongation Rate, Cell-Cycle Time, and Height and Total Volume of the Proliferative Zone

The rate of bone elongation ranged from 396  $\mu\text{m}$  per day for the proximal tibial growth plate to 47  $\mu\text{m}$  per day for the proximal radial growth plate (Table 2). The total cell-cycle time, as reported previously (43), was 30.9 hours for the proximal tibial growth plate, 34.0 hours for the distal radial growth plate, 48.7 hours for the distal tibial growth plate, and 76.3 hours for the proximal radial growth plate (Table 2).

The proliferative-zone height was 0.137 mm in the proximal tibial growth plate, 0.099 mm in the distal radial growth plate, 0.083 mm in the distal tibial

growth plate, and 0.043 mm in the proximal radial growth plate (Table 2). The proliferative-zone height correlated positively with rate of elongation,  $p < 0.01$ . Since we modeled each growth plate as a unit cylinder in this experiment, the total volumes of the proliferative zones ( $V_{\text{T proliferative}}$ ) of the four different growth plates were 0.108, 0.078, 0.065, and 0.034  $\text{mm}^3$  for the proximal tibial, distal radial, distal tibial, and proximal radial growth plates, respectively (Table 3).

### Growth Fraction

In the proximal tibial growth plate, the labeling index was 43% at 12 hours, 81% at 24 hours, and 99% at 48 hours. The labeling index in the distal radial growth plate was 30% at 12 hours, 64% at 24 hours, 92% at 48 hours, and 99% at 72 hours. The labeling index in the distal tibial growth plate increased from 33% at 12 hours to 98% at 72 hours. In the proximal radial growth plate, the labeling index was 15% at 12 hours, 37% at 24 hours, 69% at 48 hours, and 89% at 72 hours. For each growth plate, the maximum labeling index was taken as the growth fraction (Table 2).

### Chondrocytic Kinetics and New Chondrocytes per Day in the Proliferative Zone

Both volume fractions and mean cellular volumes for proliferative-zone chondrocytes correlated with rate of elongation. Volume fractions ranged from 0.335 in the proximal tibia to 0.245 in the proximal radius (Table 3). Mean cellular volumes ranged from 1,706

TABLE 3. Parameters of proliferation

Growth plate	$V_{\text{V proliferative}}$	$\bar{v}(c)_{\text{proliferative}}$ ( $\mu\text{m}^3$ )	$N_{\text{V proliferative}}$ (cells/ $\text{mm}^3$ )	$V_{\text{T proliferative}}$ ( $\text{mm}^3$ )	N (total cells/zone)	$N_{\text{new}}$ (new cells/day)
Proximal tibial	0.335 $\pm$ 0.022 <sup>a</sup>	1,706 $\pm$ 360 <sup>a</sup>	196,370 $\pm$ 49,050	0.108 $\pm$ 0.020 <sup>b</sup>	21,100 $\pm$ 7,530 <sup>a</sup>	16,400 $\pm$ 5,850 <sup>b</sup>
Distal radial	0.374 $\pm$ 0.025 <sup>a</sup>	1,622 $\pm$ 380 <sup>a</sup>	228,110 $\pm$ 54,850	0.078 $\pm$ 0.014 <sup>b</sup>	17,700 $\pm$ 6,250	12,500 $\pm$ 4,410 <sup>b</sup>
Distal tibial	0.300 $\pm$ 0.035 <sup>b</sup>	1,266 $\pm$ 300 <sup>b</sup>	236,970 $\pm$ 63,020	0.065 $\pm$ 0.008 <sup>b</sup>	15,400 $\pm$ 4,500	7,600 $\pm$ 2,220 <sup>b</sup>
Proximal radial	0.245 $\pm$ 0.027 <sup>b</sup>	710 $\pm$ 130 <sup>b</sup>	345,070 $\pm$ 106,080 <sup>b</sup>	0.034 $\pm$ 0.003 <sup>b</sup>	11,700 $\pm$ 3,480	3,700 $\pm$ 1,100 <sup>b</sup>

Mean values  $\pm$  SD.

<sup>a</sup>Significantly different from two other growth plates.

<sup>b</sup>Significantly different from all other growth plates.

**TABLE 4.** Parameters of chondrocytic loss

Growth plate	$V_V$ hypertrophic	$\bar{v}(c)$ hypertrophic ( $\mu\text{m}^3$ )	$N_V$ hypertrophic (cells/mm <sup>3</sup> )	$V_T$ turned over (mm <sup>3</sup> )	$N_{\text{lost}}$ (lost cells/day)
Proximal tibial	0.685 ± 0.021 <sup>a</sup>	14,997 ± 1,970 <sup>b</sup>	45,680 ± 5,560 <sup>a</sup>	0.311 ± 0.011 <sup>b</sup>	14,200 ± 1,650 <sup>b</sup>
Distal radial	0.675 ± 0.036 <sup>a</sup>	12,452 ± 2,220 <sup>b</sup>	54,210 ± 7,430 <sup>a</sup>	0.211 ± 0.012 <sup>b</sup>	11,450 ± 1,620 <sup>b</sup>
Distal tibial	0.595 ± 0.034 <sup>b</sup>	8,572 ± 1,570 <sup>b</sup>	69,410 ± 14,400 <sup>b</sup>	0.108 ± 0.008 <sup>b</sup>	7,520 ± 1,350 <sup>b</sup>
Proximal radial	0.504 ± 0.042 <sup>b</sup>	4,135 ± 510 <sup>b</sup>	121,890 ± 15,570 <sup>b</sup>	0.037 ± 0.003 <sup>b</sup>	4,500 ± 800 <sup>b</sup>

Mean values ± SD.

<sup>a</sup>Significantly different from two other growth plates.

<sup>b</sup>Significantly different from all other growth plates.

$\mu\text{m}^3$  in the proximal tibia to 710  $\mu\text{m}^3$  in the proximal radius (Table 3). However, the density of proliferative-zone chondrocytes was inversely correlated with the rate of elongation. The numerical density of proliferative-zone chondrocytes was 196,370, 228,110, 236,970, and 345,070 chondrocytes/mm<sup>3</sup> in the proximal tibial, distal radial, distal tibial, and proximal radial growth plates, respectively (Table 3). Taking into account the volume of the proliferative zones in different growth plates, the total number of chondrocytes in the proliferative zone was 21,100, 17,700, 15,400, and 11,700 chondrocytes for the proximal tibial, distal radial, distal tibial, and proximal radial growth plates, respectively (Table 3). Finally, considering different cell-cycle times for different growth plates, the calculated number of new chondrocytes per day ranged from 16,400 chondrocytes per day in the proximal tibia to 3,700 chondrocytes per day in the proximal radius (Table 3). The number of new chondrocytes produced per day varied significantly from growth plate to growth plate and correlated positively with the rate of elongation ( $r^2 = 0.82$ ).

**Chondrocytic Kinetics and Chondrocytes Lost per Day**

In the hypertrophic zone, both volume fractions and mean cellular volumes for the four different growth plates also correlated with the rate of elongation. Volume fractions ranged from 0.685 in the proximal tibia

to 0.504 in the proximal radius (Table 4). Mean cellular volumes ranged from 14,997  $\mu\text{m}^3$  in the proximal tibia to 4,135  $\mu\text{m}^3$  in the proximal radius (Table 4). Again, density correlated inversely with rate of elongation. The numerical density of hypertrophic-zone chondrocytes was 45,680, 54,210, 69,410, and 121,890 chondrocytes/mm<sup>3</sup> in the proximal tibial, distal radial, distal tibial, and proximal radial growth plates, respectively. Taking into account the volume of the hypertrophic zone that was turned over, the number of chondrocytes lost per day at the chondro-osseous junction ranged from 14,200 chondrocytes in the proximal tibia to 4,500 chondrocytes in the proximal radius (Table 4). As was the case in the proliferative zone, in the hypertrophic zone the number of chondrocytes lost per day varied significantly from growth plate to growth plate and correlated positively with the rate of elongation ( $r^2 = 0.92$ ).

**Contributions to Elongation from Cell Division, Enlargement, and Matrix Synthesis**

*Proximal Tibial Growth Plate*

The proximal tibial growth plate elongates at a rate of 396  $\mu\text{m}/\text{day}$  (Table 2). Assuming the unit cylinder model, this growth plate turns over 0.311 mm<sup>3</sup> per day at the chondro-osseous junction (Tables 4 and 6). The contribution to this volume from the proliferative zone is 0.084 mm<sup>3</sup> per day (Table 5), or 27% of the total. In the proliferative zone, the volume contribu-

**TABLE 5.** Calculated daily volumes renewed in proliferative zone

Growth plate	Cellular volume renewed ( $V_C$ renewed) <sup>a</sup>	Matrix per cell ( $\bar{v}(m)$ proliferative) <sup>b</sup> ( $\mu\text{m}^3$ )	Matrix volume renewed ( $V_M$ renewed) <sup>c</sup>	Total volume renewed ( $V_T$ renewed) <sup>d</sup>
Proximal tibial	0.028 ± 0.004	3,400 ± 800	0.056 ± 0.006	0.084 ± 0.016
Distal radial	0.020 ± 0.003	2,780 ± 640	0.035 ± 0.008	0.055 ± 0.010
Distal tibial	0.010 ± 0.002	2,940 ± 800	0.022 ± 0.002	0.032 ± 0.004
Proximal radial	0.003 ± 0.000	2,190 ± 500	0.008 ± 0.001	0.011 ± 0.001

Units are in mm<sup>3</sup> unless otherwise specified.

<sup>a</sup> $(\bar{v}(c)_{\text{proliferative}} * N_{\text{new}})$ .

<sup>b</sup> $([1 - V_{\text{proliferative}}] * V_{\text{Tproliferative}} / N)$ .

<sup>c</sup> $(\bar{v}(m)_{\text{proliferative}} * N_{\text{new}})$ .

<sup>d</sup> $(V_C \text{ renewed} + V_M \text{ renewed})$ .

**TABLE 6.** Calculated daily volumes turned over at the chondro-osseous junction

Growth plate	Cellular volume turned over ( $V_C$ turned over) <sup>a</sup>	Matrix per cell ( $\bar{v}[m]_{\text{hypertrophic}}$ ) <sup>b</sup> ( $\mu\text{m}^3$ )	Matrix volume turned over ( $V_M$ turned over) <sup>c</sup>	Total volume turned over ( $V_T$ turned over) <sup>d</sup>
Proximal tibial	0.213 ± 0.010	6,900 ± 980	0.098 ± 0.007	0.311 ± 0.011
Distal radial	0.143 ± 0.013	5,970 ± 1,100	0.068 ± 0.008	0.211 ± 0.012
Distal tibial	0.064 ± 0.004	5,780 ± 1,270	0.044 ± 0.006	0.108 ± 0.008
Proximal radial	0.019 ± 0.002	4,090 ± 780	0.018 ± 0.002	0.037 ± 0.003

Units are in  $\text{mm}^3$  unless otherwise specified.

<sup>a</sup>  $(\bar{v}[c]_{\text{hypertrophic}} * N_{\text{lost}})$ .

<sup>b</sup>  $([1 - V_V^{\text{hypertrophic}}] * V_T \text{ turned over} / N_{\text{lost}})$ .

<sup>c</sup>  $(\bar{v}[m]_{\text{hypertrophic}} * N_{\text{lost}})$ .

<sup>d</sup>  $(V_C \text{ turned over} + V_M \text{ turned over})$ .

tion from cell duplication is 0.028  $\text{mm}^3$  per day (Table 5), or 9% of the total, and the volume contribution from matrix synthesis is 0.056  $\text{mm}^3$  per day (Table 5), or 18% of the total.

The relative contributions from the hypertrophic zone are determined by subtracting the contributions from the proliferative zone (Table 5) from the total contributions (Table 6). Thus, in the hypertrophic zone, the volume contribution from cell enlargement is 0.185  $\text{mm}^3$  per day (0.213-0.028  $\text{mm}^3$ ), or 59% of the total. The volume contribution from matrix synthesis in the hypertrophic zone is 0.042  $\text{mm}^3$  per day (0.098-0.056  $\text{mm}^3$ ), or 14% of total. Thus, in the proximal tibia, cell duplication contributes 9% of the total, matrix production contributes 32% of the total (summing the matrix contributions in the proliferative and hypertrophic zones), and cellular enlargement in the hypertrophic zone contributes 59% of the total (Fig. 1).

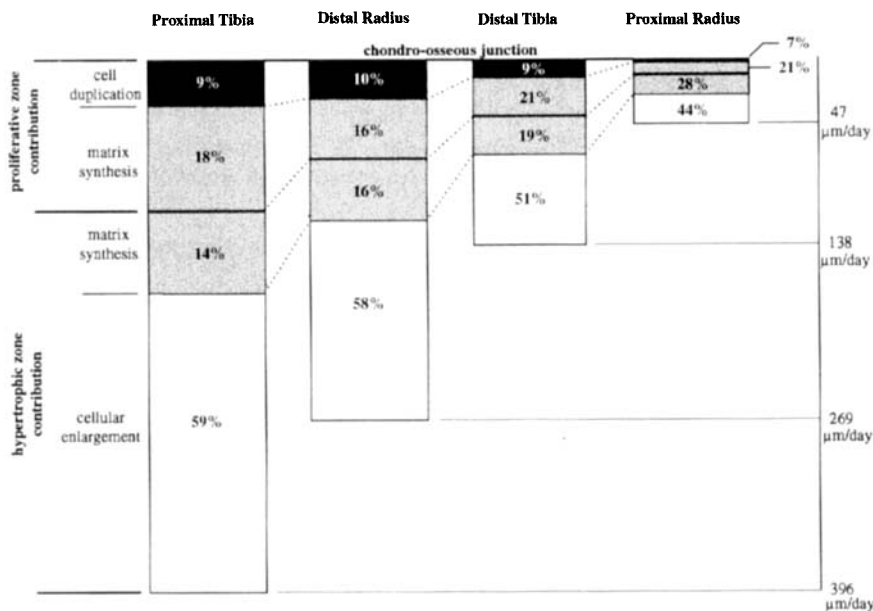
*Three Other Growth Plates*

In the three other growth plates, as the rate of elon-

gation slows, there is a relative shift away from the cellular contribution toward the contribution from matrix production. For example, in the slowly elongating proximal radial growth plate, cell duplication contributes 7% of the total, whereas matrix production contributes 49% of the total and cellular enlargement in the hypertrophic zone contributes 44% of the total (Tables 5 and 6, Fig. 1).

**DISCUSSION**

The elongation of a bone achieved by a given growth plate in any one 24-hour period is determined by a complex interplay of proliferative kinetics, matrix synthesis throughout the growth plate with controlled matrix degradation, and chondrocytic enlargement during hypertrophy that is accompanied by a disproportionate increase in height (in the direction of elongation) relative to width as the volume of the cell expands (4-6,22,28,33). Previously, we and others had demonstrated that both rate and extent of hypertrophic chondrocytic enlargement, translated into appro-



**FIG. 1.** Relative contributions to daily total elongation at the chondro-osseous junction.

appropriate cell-shape changes, are actively regulated and function as critical variables in controlling differential growth. In other words, growth plates in which hypertrophic chondrocytes became larger, grew faster. However, we and others have also demonstrated that, by itself, this idea is insufficient to explain all the complexities of differential growth. Specifically, the effect of hypertrophic chondrocytic enlargement varies among species and varies at different times in an individual's life (4-6,22,24,30,31). With respect to parameters associated with proliferation, it has been known for some time that the height of the growth plate and, more specifically, the height of the proliferative zone have positive correlations with differential growth (6,22,27,39,41). In addition, we recently demonstrated that cell-cycle times were different in growth plates elongating at different rates (43).

The results of the current study are in agreement with those of previous studies with regard to the positive correlations of hypertrophic chondrocytic volume, height of the proliferative zone, and cell-cycle time with rate of elongation (22,23,39). The present study, however, places these and other variables in a new (and three-dimensional) context. The height of the proliferative zone is important in the context of determining the total volume of the proliferative zone. The volume of the proliferative zone and the cell-cycle time are two of four parameters needed to calculate the total number of new chondrocytes produced. The other two parameters required in this calculation are growth fraction and the density of proliferative-zone chondrocytes. A result that was both unexpected and counterintuitive was that the density of proliferative-zone chondrocytes varied inversely with the rate of elongation. Therefore, proliferation kinetics can be calculated only when all four parameters (volume of the proliferative zone, density of the proliferative-zone chondrocytes, growth fraction, and cell-cycle time) are known. Similarly, this study confirms the importance of hypertrophic-cell volume (4,6,22,30); however, that parameter is placed in a new context with rate of elongation and density of hypertrophic chondrocytes, all of which are required to determine the number of chondrocytes lost per day at the chondro-osseous junction.

One parameter that did not vary with rate of elongation was growth fraction. We interpret the results of the growth-fraction experiment (Experiment 2) to mean that in all growth plates essentially 100% of the chondrocytes in the proliferative zone, as defined in this study, are in the cell cycle. Similar results were demonstrated in a previous study of the proximal tibial growth plate using repeated pulse labeling with bromodeoxyuridine (12). Therefore, we assumed a growth fraction of 1.0 for all four growth plates and ignored this parameter in subsequent calculations of

chondrocytic kinetics. We recognize that for the proximal tibial, distal radial, and distal tibial growth plates, the actual measured growth fraction was 0.98-0.99, and these may reflect our best estimates of growth fractions for these growth plates. We interpret our result of 0.89 for the growth fraction for the proximal radial growth plate as an underestimate of the true growth fraction. Our last sampling point in this experiment was at 72 hours, and by this time point the slope of the regression of increasing labeling index relative to time had not reached a plateau. Had we sampled at a later time point, the measured growth fraction also would have continued to increase and approach 1.0. We believe that assuming the growth fraction of all four growth plates at 1.0 is conservative, because if growth fractions of 0.89-0.98 were applied to the calculations of  $N_{\text{new}}$ , the calculated number of new chondrocytes produced would generally be slightly smaller, and for three growth plates it would be in even closer agreement with the calculated number of chondrocytes lost per day.

For all four growth plates, the difference between  $N_{\text{new}}$  and  $N_{\text{lost}}$  ( $d = |N_{\text{new}} - N_{\text{lost}}|$ ) was not significantly different from zero. We suggest several possible interpretations of this observation. The first interpretation is that the two independent equations are reasonable estimates of these processes. The additional fact that, in three of four cases, the calculated number of chondrocytes produced exceeded the calculated number lost is either a random phenomenon (remembering the relatively large variations associated with the measured parameters in this study) or a failure to include growth fraction in the calculation. A second possible interpretation is that the number of cells produced actually does slightly exceed the number lost at the chondro-osseous junction. If, for example, some chondrocytes in the upper hypertrophic zone are associated with the process of creating matrix vesicles from apoptotic cell death, then more chondrocytes would be produced than would be turned over at the chondro-osseous junction (1,11,17,21,25,26,37,44). Also, it has been suggested that while most hypertrophic chondrocytes die at the chondro-osseous junction, a subset of these hypertrophic chondrocytes survive and differentiate into osteoblasts (7,14,16,36). This idea could also explain why more chondrocytes are produced than are turned over at the chondro-osseous junction.

A third possible interpretation is that the number of cells produced does equal the number turned over, but that in the current study the equations are in error. In calculating  $V_{\text{T terminal hypertrophic zone lost}}$  the equations presented in this study do not allow for an increase in the diameter of the model cylinder. This is a minor error. As bones elongate, they also grow in diameter. Although some of this increase in diameter is associ-

ated with chondrocytic activities in the groove of Ranvier, some of the increase in diameter is interstitial and associated with an intrinsic increase in the diameter of chondrocytes in the hypertrophic region (5,8). If we assume (based on previous studies of the tibia) that for every unit increase in elongation there is a concomitant 7% interstitial increase in diameter, then when calculating  $V_{T \text{ terminal hypertrophic zone lost}}$ , the diameter of the circular model cylinder would be 1.07, not 1.0. In the proximal tibial growth plate,  $V_{T \text{ terminal hypertrophic zone lost}}$  would be 0.356 mm<sup>3</sup>, not 0.311 mm<sup>3</sup>;  $N_{\text{lost}}$  would be 16,300 cells, not 14,206, and this number would be in almost perfect agreement with the number of new cells produced per day (16,400). Similar calculations can be made for the three other growth plates.

Thus, a new and integrating idea emerging from this study is that in steady-state differential growth, parameters of proliferation initiate differential growth, but parameters of hypertrophy amplify these initial differences. The combination of initiation in the proliferative zone and amplification in the hypertrophic zone explains differential growth. In the proximal tibial growth plate, about 27% of elongation is contributed by the proliferative zone and 73%, by the hypertrophic zone; however, the balance between proliferation and hypertrophy also varies with the rate of elongation. On the basis of this and other studies, as well as studies of the avian growth plate (3,29), we predict that it is this amplification signal and process that will vary among species and vary at different ages in an individual. It is likely that a clearer understanding of this amplification process and signal will provide clues to important regulatory mechanisms.

The present study, including the measurement of eight independent parameters in each of four different growth plates, may be more comprehensive than previous investigations; however, in previous studies some of the parameters of interest in the current study were measured, with similar techniques, in some growth plates in adolescent rats (22,30,32). All of those measurements are consistent with values estimated in the current study. However, in going beyond individual measurements to extrapolate to chondrocytic kinetics, there are difficulties in comparing the present study with previous attempts to define chondrocytic kinetics of the growth plate. First, the present study uses physiological markers for the determination of a key reference volume,  $V_{T \text{ proliferative zone}}$ . Previous studies using morphological criteria may have overestimated the size of the proliferative zone and, therefore, the number of new chondrocytes produced per day. Second, total cell-cycle time cannot be estimated from labeling indices created with a single pulse label of [<sup>3</sup>H]thymidine or a single pulse label of bromodeoxyuridine. A labeling index based on a single pulse label is a ratio of S-phase time and total cell-cycle time. Without an

independent measure of S-phase time, total cell-cycle time cannot be determined. In the past, this has led to an overestimation of the total cell-cycle time, which would lead to an underestimation of the number of new chondrocytes produced per day (28,45).

A recent study by Luder (32) is particularly noteworthy because its overall conclusion of relative contributions from cellular duplication, matrix synthesis, and chondrocytic hypertrophy is similar to the conclusion of the present study, in spite of two major differences in experimental design. The two major differences include focusing on a growth plate in the axial skeleton (elongation of the mandibular condyle) and a difference in incorporating temporal kinetics. While the study by Luder did not measure the rate of elongation directly, it introduced temporal kinetics into the study by measuring the rate of progression of maximum labeling index passing through the condyle. This study concluded that in the mandibular condyle of the weanling rat, the relative ratio of contributions from cellular duplication, matrix synthesis, and chondrocytic hypertrophy was 1:3:6. Using data from the present study, we would conclude that in the proximal tibia similar ratios would be 1:3.5:6.6.

A methodological concern in the design of this study and in comparison with previous studies is the issue of circadian rhythms and their significance during endochondral ossification. There are two broad concerns: (a) the extent to which circadian factors influence each of the multiple parameters of importance in this study, and (b) the extent to which these circadian factors track throughout the 24-hour cycle (20,40). Since the extent of neither of these two concerns is known, and there is conflicting evidence about some parameters, this experiment was designed to collect two animals at each of six different time periods throughout the 24-hour cycle. In this way, whatever circadian factors were present would cancel by the multiple temporal collection design. On the other hand, such a collection design raises the intrinsic variability of each measured parameter by whatever circadian effect is present.

This study supports the concept of locally mediated regulatory controls of growth-plate elongation. With respect to this concept, an important result of the study is the demonstration that in the rapidly elongating growth plate, the number of chondrocytes produced and turned over is about four times that in the slowly elongating growth plate (16,400 compared with 3,700). Although overall regulation of chondrocytic performance is under a set of systemic control systems such as hormones, growth factors, and nutrient status (13,23,35), this study again demonstrates the presence and importance of locally mediated control systems. Under steady-state conditions, the number of chondrocytes produced must equal the number lost. Since

this number differs widely in different growth plates, this argues not only for locally mediated regulatory systems but also for the concept that the controls for proliferation and the controls for hypertrophy must be tightly coupled (32,42). We can think of no better illustration of the concept of locally mediated regulatory mechanisms in the growth plate than that of patients exhibiting tibial overgrowth (38). These patients, after sustaining an injury to the distal femoral growth plate resulting, as expected, in shortening of the femur, exhibit a compensatory lengthening of the ipsilateral tibia. The spontaneous regulatory mechanisms signaling one or both growth plates in this ipsilateral tibia to compensate for the femoral shortening with tibial lengthening remain unexplained. Perhaps there is a regional acceleratory phenomenon (15), a systemic acceleratory phenomenon (34), or another systemic response to skeletal injury (10); however, the nature of the modulator or modulators and the interaction in the cellular or molecular biology of growth-plate chondrocytes is unknown.

It is assumed that all growth-plate chondrocytes undergo the same pattern of gene activation and repression, resulting in a characteristic sequential pattern of phenotypical expression. However, the rate at which this pattern is displayed varies greatly from growth plate to growth plate. Even growth plates at different ends of the same long bone are known to elongate at rates that differ by a factor of two or three. In addition, any single growth plate functions over a defined period of growth, during which chondrocytic numbers and chondrocytic performance are in a steady state over a short period of time but change through time with changing rates of growth. Therefore, the understanding of this dynamic cellular regulation and control must include not only the generation of the correct pattern but also the correct timing of the pattern appropriate for a given rate of elongation.

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